Investigation of the impact of CMV reactivation and GVHD prophylaxis on alloreactivity after hematopoietic cell transplantation by data-driven analysis

Inaugural-Dissertation

zur

Erlangung des Doktorgrades

Dr. rer. nat.

der Fakultät für

Biologie an der

Universität Duisburg-Essen

vorgelegt von

Saskia Leserer

aus Bochum

November 2021

Die der vorliegenden Arbeit zugrundeliegenden Arbeiten wurden an der Klinik für Hämatologie und Stammzelltransplantation (bis 2020: Klinik für Knochenmarktransplantation) des Universitätsklinikums Essen durchgeführt.

1. Gutachter: Prof. Dr. med. Dietrich W. Beelen

2. Gutachter: Prof. Dr. rer. nat. Ulf Dittmer

Vorsitzender des Prüfungsausschusses: Univ.-Prof. Dr. Ralf Küppers

Tag der mündlichen Prüfung: 28.03.2022

Diese Dissertation wird via DuEPublico, dem Dokumenten- und Publikationsserver der Universität Duisburg-Essen, zur Verfügung gestellt und liegt auch als Print-Version vor.

DOI: 10.17185/duepublico/77457
URN: urn:nbn:de:hbz:465-20230411-121940-8

Dieses Werk kann unter einer Creative Commons Namensnennung - Nicht kommerziell 4.0 Lizenz (CC BY-NC 4.0) genutzt werden.

Table of Contents

1 Preface 5

2 Zusammenfassung 6

3 Summary 8

4 Introduction 10

4.1 Hematopoietic Stem Cell Transplantation 10

4.1.1 Background 10

4.1.2 Alloreactivity after allogeneic HCT 12

4.1.3 Impairment of clinical outcome after allogeneic HCT and its relation to alloreactivity 13

4.1.4 Importance of immune reconstitution 15

4.2 Cytomegalovirus 17

4.2.1 Background 17

4.2.2 Relevance of CMV reactivation in allogeneic HCT 19

4.3 Acute Graft-versus-host disease 21

4.3.1 Background and pathophysiology of acute GVHD 21

4.3.2 Classification and risk factors 22

4.4 Considerations on statistical approaches in HCT studies 23

5 Objective of this thesis 25

6 Articles 26

6.1 Article I 27

6.2 Article II 57

6.3 Article III 81

6.4 Article IV 107

7 Discussion 155

8 References 166

9 Appendix 181
9.1 Abbreviations 181
9.2 List of Figures 184
9.3 Danksagung 185
9.4 Conference Contributions 186
9.4.1 Talks 186
9.4.2 Posters 187
9.5 Curriculum vitae 189
9.6 Stellungnahmen 190
9.7 Eidesstattliche Erklärung 191

1 Preface

The herein presented thesis comprises a selection of three published original articles and one manuscript in preparation.

The work presented in this thesis was performed in the time between April 2018 and November 2021 under the supervision of Prof. Dr. med. Dietrich W. Beelen and Dr. med. Dr. phil. Amin T. Turki at the Department of Bone Marrow Transplantation (as of May 2020: Department of Hematology and Stem Cell Transplantation), University Hospital Essen.

2 Zusammenfassung

Die T-Zell Alloreaktivität nach allogener hämatopoetischer Stammzelltransplantation (HSZT) ist durch den damit verbundenen Transplantat-gegen-Leukämie (GVL) Effekt, vermittelt durch alloreaktive T-Zellen, ein wesentliches Element der antineoplastischen Wirkung bei Patienten mit malignen hämatologischen Erkrankungen. Allerdings sind alloreaktive T-Zellen auch für die Transplantat-gegen-Wirt-Reaktion (GVHD) verantwortlich, welche die häufigste und bedrohlichste Immun-vermittelte Komplikation nach allogener HSZT darstellt. Gegenwärtig befassen sich zahlreiche Arbeiten mit der Aufklärung potentieller Mechanismen und daraus resultierenden zielgerichteten Interventionen, welche den GVL Effekt verstärken und gleichzeitig die GVHD vermindern sollen. Aufgrund der großen Komplexität dieser Effekte, die unter anderem durch zahlreiche Transplantations-assoziierte Faktoren beeinflusst werden können, ist dieses Ziel aber bislang unerreicht. Vor diesem Hintergrund wurde in dieser Promotionsarbeit der Einfluss von Transplantations-assoziierten Faktoren - die Zytomegalie-Virus (CMV) Reaktivierung und die Wahl der immunpharmakologischen GVHD-Prophylaxe - insbesondere auf die T-zelluläre Immunrekonstitution und auf die alloreaktiven klinischen Manifestationen der GVHD und GVL anhand folgender Zielsetzungen untersucht:

1) Entwicklung von Kinetik-Modellen der CMV Reaktivierung und Analyse des Einflusses der Viruskinetik auf die Alloreaktivität

2) Aufklärung der Beziehung zwischen der CMV Reaktivierung und dem Rezidivrisiko in Abhängigkeit vom Krankheitsstadium und der Verwendung einer *in vivo* T-Zell-Depletion mit Anti-T-Lymphozyten-Globulin (ATG)

3) Analysen zur Dosisabhängigkeit der *in vivo* T-Zell-Depletion auf die zelluläre Immunrekonstitution und zur potenziellen ATG-Dosisoptimierung

4) Vergleich der zellulären Immunrekonstitution nach GVHD Prophylaxe mit ATG oder post-Transplant Cyclophosphamid (PTCy)

Diese Ziele wurden mithilfe datenbasierter Modelle von klinischen Faktoren und Parametern der Immunrekonstitution realisiert. Die erzielten Ergebnisse bestätigen einen Zusammenhang zwischen der CMV Reaktivierung und der Reduktion des leukämischen Rezidivrisikos in Abhängigkeit von der CMV Viruslast **(Leserer, 2021),** dem leukämischen Krankheitsstadium sowie der Verwendung der *in vivo* T-Zell-

Depletion mit ATG **(Turki, 2021)**. Basierend auf Analysen zur T-Zell Immunrekonstitution in CMV "Peak Titer"-Subgruppen sowie zum leukämischen Rezidivrisikos in Patienten mit oder ohne ATG deuten beide Arbeiten darauf hin, dass die durch eine CMV Reaktivierung potentiell verstärkte T-Zell-Alloreaktivität, an der Kontrolle der Leukämierezidive beteiligt ist. Des Weiteren, unterstützt die sequentielle Analyse von CMV Reaktivierung und akuter GVHD die Hypothese, dass in Patienten ohne ATG die akute GVHD als Auslöser für die CMV Reaktivierung relevant werden kann während die CMV Reaktivierung in Patienten mit ATG eher auf die T-Zell-Suppression zurückzuführen ist. Zusätzlich bestätigen die hier gezeigten Ergebnisse frühere Arbeiten, dass die immunpharmakologische Prophylaxe mit ATG oder PTCy das Risiko einer schweren akuten GVHD effektiv minimiert, aber gleichzeitig auch eine Verminderung des GVL Effektes bewirkt. In verschiedenen Arbeiten konnte mittels Analyse der Immunrekonstitution gezeigt werden, dass ATG einen dosisabhängigen Effekt auf die Rekonstitution von Helfer T-Zellen ausübt **(Turki, 2020)** und das die GVHD-protektiven Effekte von ATG und PTCy durch verschiedene Zell-Subtypen vermittelt werden **(Leserer, Manuskript in Bearbeitung)**. Zudem unterstreichen die Ergebnisse dieser Forschungsarbeit die Notwendigkeit von Methoden zur gezielten Regulation der Alloreaktivität. Abschließend ist es mir über die Entwicklung eines meines Wissens nach neuen Tools gelungen, multidimensionale kontinuierliche Immunrekonstitutionsdaten zu analysieren und dadurch die Heterogenität der Rekonstitution von Patienten mit gleicher Behandlung zu charakterisieren. Die Ergebnisse dieser Promotionsarbeit setzen die T-Zell Immunrekonstitution mit Transplantations-assoziierten Faktoren wie der CMV Reaktivierung und der immunpharmakologischen GVHD-Prophylaxe in Bezug und geben damit Hinweise über den Einfluss dieser Faktoren auf die Alloreaktivität. Insbesondere die Erkenntnisse über einen verstärkten GVL Effekt durch CMV und die differentielle Beeinflussung der T-Zell Rekonstitution nach ATG oder PTCy könnten zur Verbesserung der Behandlung von HSZT-Patienten beitragen.

3 Summary

T cell alloreactivity after allogeneic hematopoietic stem cell transplantation (HCT) is by its associated graft-versus-leukemia (GVL) effect, mediated by alloreactive T cells, an essential element of the antineoplastic outcome in patients with malignant hematologic diseases. However, alloreactive T cells are also responsible for the graft-versus-host disease (GVHD), which is the most frequent and threatening immune-mediated complication after allogeneic HCT. Consequently, current research focuses on the elucidation of potential mechanisms and as a result on target-oriented interventions, which should augment the GVL effect while simultaneously reduce GVHD. Based on the high complexity of both effects, which is amongst others affected by numerous transplant-related factors, this is still an unmet goal. Before this background this thesis investigated the impact of transplant-related factors – Cytomegalovirus (CMV) reactivation and the choice of immune pharmacological GVHD prophylaxis – in particular on T cell immune reconstitution and on the alloreactive clinical manifestations of GVHD and GVL by the analysis of the following aspects:

1) Development of CMV reactivation kinetics models and the analysis of their impact on alloreactivity

2) Elucidation of the relationship between CMV reactivation and relapse risk as a function of the disease status and the application of *in vivo* T cell depletion with anti-T-lymphocyte globulin (ATG)

3) Analysis of dose-dependency of *in vivo* T cell depletion on cellular immune reconstitution and for a potential ATG dose optimization

4) The comparison of cellular immune reconstitution after GVHD prophylaxis with ATG or post-transplant cyclophosphamide (PTCy)

These aims were realized by data-driven models of clinical factors and parameters of immune reconstitution. The obtained results validate the relationship of CMV reactivation and a reduction of the leukemic relapse risk, which is dependent on the CMV viral load **(Leserer, 2021)**, the leukemic disease stage at transplantation as well as the use of *in vivo* T cell depletion with ATG **(Turki, 2021)**. Based on analyses of the T cell reconstitution in CMV peak titer subgroups as well as of the leukemic relapse risk in patients with or without ATG, both articles show evidence for a CMV-augmented T cell alloreactivity which contributes to the abatement of residual disease. In addition,

the sequential analysis of CMV reactivation and acute GVHD support the hypothesis that in patients without ATG acute GVHD might be the trigger for CMV reactivation while in patients with ATG CMV reactivation might be based on the profound T cell suppression. Furthermore, the presented results confirm previous data, that the immune pharmacological prophylaxis with ATG or PTCy effectively minimizes the risk of severe GVHD but concomitantly attenuates the GVL effect. In several approaches it was shown, via the analysis of immune reconstitution, that ATG has a dose-dependent effect on the reconstitution of helper T cells **(Turki, 2020)** and that the GVHD-protective effect of ATG and PTCy is mediated by different cell subsets **(Leserer, manuscript in preparation)**. Additionally, the results of this thesis emphasize the necessity of approaches for the selective regulation of alloreactivity. Finally, I developed to my knowledge a new tool, which was capable to analyze multi-dimensional continuous cellular immune reconstitution data and to characterize the heterogeneity of cellular reconstitution in patients with the same treatment. The results of this thesis relate the reconstitution of T cells to transplant-associated factors such as CMV reactivation and the immune pharmacological GVHD prophylaxis and shed light onto the impact of these factors on alloreactivity. In particular, the findings of an CMV-augmented GVL effect and the differential influence of ATG and PTCy on T cell reconstitution could contribute to improvements in HCT patient treatment.

4 Introduction

4.1 Hematopoietic Stem Cell Transplantation

4.1.1 Background

Hematopoietic stem cell transplantation (HCT) is a well-established approach in the field of cellular therapy of life-threatening hematological malignancies and non-malignant disorders. Since its first description in humans in 1957[1] more than one million HCTs[2] have been performed with an increasing frequency over the past decades[3] (**Figure 1A**). It is the most frequently used cellular therapy procedure at large scale[4]. HCT requires the use of a conditioning regimen and the subsequent transplantation of hematopoietic stem cells (HSCs). The conditioning regimen can be a combination of high-dose radiation and/or chemotherapeutics and causes the eradication of the diseased marrow, leading to a suppression of patient's immunity and the acceptance of the donor graft[5]. HSC infusion leads to the recovery of the damaged hematopoietic tissue and immunity, utilizing the ability of HSCs of self-renewal and differentiation into progenitor and mature cell types - the basis for HCT's regenerative capacity[6]. The stem cell source can be either derived from bone marrow (BM), umbilical cord blood (CB) or peripheral blood (PB), of which the latter is nowadays the predominantly used one[3] (**Figure 1B**). Peripheral blood stem cells (PBSCs) are more frequently used in malignant diseases, especially in advanced disease stages[7], as there is evidence for a more pronounced graft-versus-leukemia (GVL) effect in transplantations with PBSCs compared with BM[8]. However, HCTs from PBSCs show an increased risk for graft-versus-host disease (GVHD)[9], which associates to higher non-relapse mortality (NRM)[10]. PBSCs are also known to associate with faster hematopoietic recovery compared to BM-derived stem cells[11].

HCT is mainly performed in two different settings namely autologous (59%) and allogeneic (41%) stem cell transplantation[3]. While in the autologous transplantation the HSCs are derived from the patient him-/herself[12], allogeneic transplantation with PBSC for example uses granulocyte-colony stimulating factor (G-CSF)-enriched $CD34^+$ HSCs from volunteer donors[13]. This thesis focuses on allogeneic HCT. The identification of a suitable donor is highly relevant for allogeneic HCT[14], in which the gold standard is the use of a human leukocyte antigen (HLA)-identical sibling donors

or matched unrelated donors[15]. This is exemplified by the prevailing HCT number using these sources in Europe (**Figure 1C**).

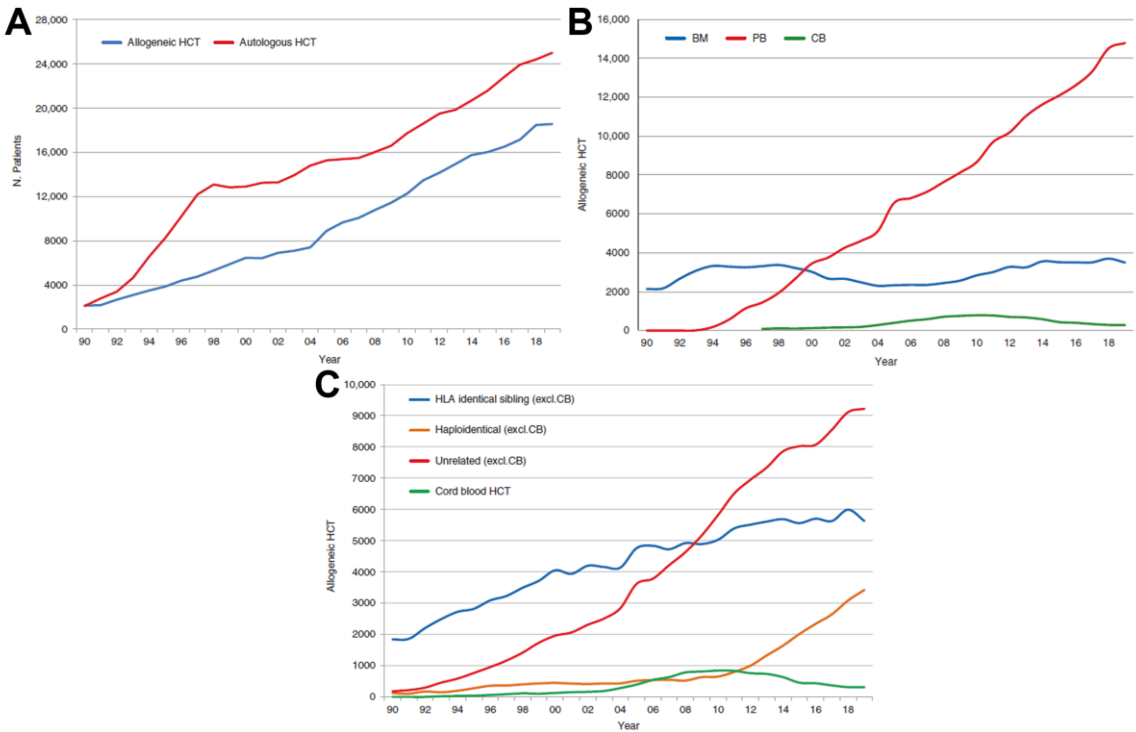


Figure 1: Developments in HCT from 1990-2018. Development of **(A)** patient numbers receiving either autologous or allogeneic HCT, **(B)** the use of different stem cell sources and **(C)** donor source frequency in allogeneic HCT from 1990-2008. (from Passweg *et al.*[3]).

Unfortunately, the identification of an HLA-identical sibling donor, further stated as matched-related donor (MRD), is only successful in ~30% of cases[16]. The best alternative to HCT from MRD is the identification of matched-unrelated donors (MUD) in donor registries, with >39 million volunteer donors available in 2020[17]. In HCT from unrelated donors (UD) histocompatibility, the HLA-concordance between recipient and donor, is ensured by high-resolution typing of the loci HLA-A, -B, -C, -DRB1 and -DQB1[18]. The first choice are 10/10-but also 9/10-matches are considered[19]. In cases of multiple 9- or 10/10-matches for one recipient further aspects beyond the HLA matching are evaluated for the prioritization of donors: 1) donor age, where younger adults should be considered over older donors[20,21]; 2) sex matching, where evidently male donors should be preferred for male recipients as the transplantation from female donors bear higher risks of GVHD[22]; 3) Cytomegalovirus (CMV) serostatus, in which CMV- donors should be used for CMV- recipients and CMV+ donors for CMV+ recipients[23] and 4) AB0 matching, as incompatibilities are reported to modestly influence outcomes[24]. Recent studies also recommend the additional typing of HLA-

DPB1, which can identify non-permissive mismatches in MUD associated with increased risks of NRM and severe acute GVHD[25]. Following improvements, such as high-resolution HLA-typing techniques[14], HCTs from MUD exceeded the number of transplantations from related donors since the 2000s[3] (**Figure 1C**). This common use of MUD-HCT is justified by comparable outcomes of recipients transplanted with MRD or MUD allografts[26,27]. Tertiary donor options, including mismatched-unrelated donors (MMUD), haploidentical family donors and cord blood transplantation[14], are also relevant in allogeneic HCT as the likelihood to find an available MUD is about ~75% for white European patients and much lower for other ethnic groups[28]. MMUD transplantations display a disadvantageous risk profile as they associate with an increased risk of GVHD resulting in higher overall mortality, whilst HLA-incompatibilities also bear the chance to promote the beneficial GVL effect[29]. Haploidentical transplantation, albeit associated with increased risks of infections and disease relapse[29], have the advantage of the availability of family donor in the range of 90%[30]. Since the introduction of post-transplant cyclophosphamide as GVHD-prophylaxis[31] and further improvements concerning reduced-intensity conditioning (RIC), allo-HCT with haploidentical donors is considered a safe and promising alternative[32] with increasing numbers over the last decade[3] (**Figure 1C**). Cord blood HSCs are readily available from established banks and can be used for a high diversity of patients[29]. Even though it is associated with decreased GVHD its usage declined over the past years[3] (**Figure 1C**) due to delayed engraftment and immune reconstitution[33]. Compared to HCT from MRD or MUD, tertiary donor options associate to increased NRM through different disease-risk strata[34].

4.1.2 Alloreactivity after allogeneic HCT

Alloreactivity is the central mechanism of allogeneic HCT and originates in histocompatibility barriers between donor and recipient[30]. The term alloreactivity describes the recognition of non-self, allogeneic antigens on healthy but also malignant cells by alloreactive cells and their subsequent immune-mediated attack[35]. HLAs are the most relevant antigens[30], which are encoded on a polymorphic locus called Major Histocompatibility complex (MHC), located at the short arm of chromosome 6 (6p21.3)[36]. The MHC locus, which accounts for 30522 distinct HLA alleles[37], can be divided into three different regions knows as MHC class I, -II, and -III[38]. In allogeneic transplantation HLAs from MHC class I & II are the most relevant, due to their

ubiquitous expression and their roles in antigen processing and presentation[39]. While MHC class I is expressed by all nucleated cells, MHC class II are constitutively expressed only on so called professional antigen-presenting cells[40]. Peptides presented by MHC class I or II molecules are mainly recognized by cytotoxic and helper T cells, respectively[39]. Furthermore, MHC class I antigens can also be noticed by killer-cell immunoglobulin-like receptors on NK cells[36,41]. The nine classical HLA genes studied in the HCT context originate from HLA-A, -B, and -C (MHC class I) and HLA-DR, -DQ, and -DP (MHC class II)[38]. HLA-disparities, for example in mismatched HCT, highly favor the occurrence of alloreactivity in manifestations such as GVHD or GVL[29]. Additional to HLA-mismatches, alloreactivity can also be induced by differences in minor histocompatibility antigens (mHAgs), for example in MRD transplantation[42]. mHAgs are foreign peptides, derived from polymorphic genes other than HLA and presented by self HLA molecules to T cell receptors only[42]. Here, the mediation of T cell alloreactivity follows an indirect pathway[40]. In short, alloreactivity has twofold effects on potential HCT outcomes: While it drives detrimental complications, such as graft rejection[43] and GVHD[44], its induction of the beneficial GVL against malignant cells is the central aim of HCT[45].

4.1.3 Impairment of clinical outcome after allogeneic HCT and its relation to alloreactivity

After allogeneic HCT, recipients are exposed to several impairments of clinical outcome that can also heavily affect quality of life. Among the most relevant early adverse events are infectious complications, GVHD as well as the relapse from original disease, all increasing the hazard of death after HCT[46] (**Figure 2A**). Further problems such as organ- or graft failures[46] are not discussed in this chapter. A high number of risk factors (i.e. donor and stem cell source, HLA matching, disease status, recipient age, conditioning) contribute to the occurrence and intensity of such issues[47-50].

After HCT, a frequent cause of early mortality are infectious complications (14%, see **Figure 2A**)[46]. Suppressed immunity, damaged anatomical barriers caused by the conditioning regimen and immunosuppressive agents modulating alloreactivity favor the incidence of infections post-transplant[47]. Three types of infections, bacterial-, fungal-, and viral infections[47] can be observed throughout different phases of immune reconstitution post-HCT: The pre-engraftment phase (~2-4 weeks post-HCT), the early post-engraftment phase (~2-3 months post-HCT) and the late phase (>3 months post-

HCT)[51]. While bacterial infections predominantly occur in the neutropenic and early post-engraftment phase, fungal infections are prevalent throughout all three phases[47] (**Figure 2B**). The occurrence of viral infections is also connected to patients' immune reconstitution[47] and associates to several risk factors, such as older age or the use of T cell depleting agents[52]. The most common contributor to viral infections is the human *Cytomegalovirus*[53] (CMV, see chapter 4.2), which is predominantly present in the early post-engraftment phase until day +100[54] and has been shown to heavily interact with the reconstituting immune system[55]. In general, viral infectious complications may manifest with a broad range of symptoms from mild to life-threatening diseases[56], hence predicting its severity is essential to optimal anti-infective patient management.

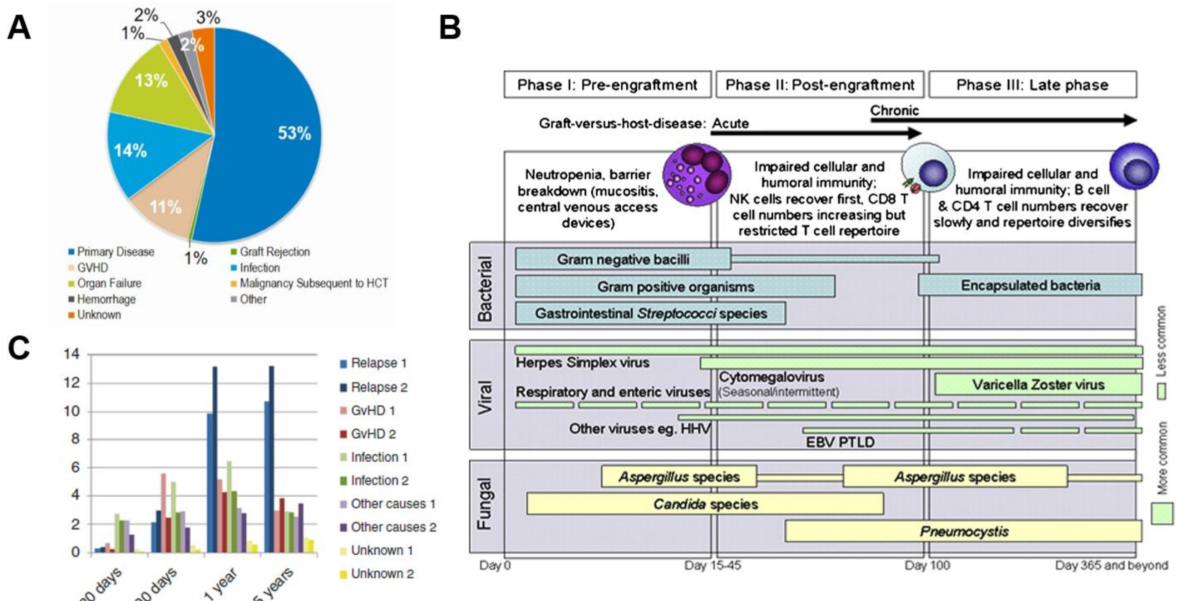


Figure 2: Frequent impairments of clinical outcome after HCT. (A) Contribution of different complications after HCT to the 3-year mortality after unrelated donor HCT between 2018-2019 (from Phelan *et al.*[46]). **(B)** Occurrence of infectious diseases and GVHD with respect to post-transplant phases (from Tomblyn *et al.*[47]) **(C)** Main causes of deaths for allo-HCT patients at different timepoints after HCT compared between 1980-2001 (cohort 1) and 2002-2015 (cohort 2) (from Styczyński *et al.*[57]).

Still, the most frequent early complication after allo-HCT is GVHD, which associates to 11% of deaths within 3 years post-HCT[46]. GVHD is predominantly mediated by alloreactive donor T cells recognizing host cells as foreign and attacking the healthy tissues of a recipient[58]. This process is provoked by HLA-incompatibilities and differences in the mHAgs[29,42]. GVHD can be differentiated into an acute and chronic form[59], with a cumulative incidence of acute GVHD between 35-80%[58] and chronic GVHD in 30-70% of HCT recipients[60]. The immune-mediated attack by alloreactive

donor T cells (detailed in chapter 4.3.1) can lead to critical or even lethal impairment in different organs of transplant recipients. While in acute GVHD the organ involvement is restricted to the skin, liver, and GI tract, chronic GVHD also affects others such as lungs, and kidneys[58]. Several approaches for instance the application of T cell depleting agents for GVHD prophylaxis or the improvement of GVHD treatment protocols reduced GVHD-associated mortality up to 1-year post-HCT[57] (see **Figure 2C**). Additionally, the increasing use of high-resolution HLA-typing participated in this effect by decreasing the number of HLA-mismatches, which trigger alloreactivity[57].

With the predominance of hematologic malignancies as indication for allo-HCT, disease relapse remains the most frequent cause of death accounting for ~50% of 3-year mortality reported by the CIBMTR registry[46]. Risk factors for relapse are often disease-related e.g. remission status at HCT, minimal residual disease, cytogenetic disease risk or treatment-related e.g. RIC or T cell depletion[50], but also relate to alloreactivity. Besides the response to the conditioning regimen the immune-mediated GVL effect by alloreactive donor T and NK cells is the central tool in limiting relapse. Relapse originates from residual malignant (leukemic) cells, which escaped from conditioning regimen and GVL[61]. While early relapse occurs following insufficient response to HCT therapy, late relapse happens if the immune system develops a tolerance or the disease undergoes immune escape[62]. In the absence of HLA escape, modulation of alloreactivity by immunosuppressive drugs or donor lymphocyte infusion (DLIs) may re-induce remission via alloreactive mechanisms[63].

Over the past decades, the incidence of infectious complications and GVHD has decreased substantially[57] (**Figure 2C**), resulting in decreased NRM[34]. Yet, it still contributes to significant NRM. Beyond NRM, disease relapse remains the major challenge after HCT with constantly high frequencies over the years[10]. The induction of a sufficient GVL effect parallel to minimizing or ultimately abrogating GVHD[45] therefore remains critical.

4.1.4 Importance of immune reconstitution

Immune reconstitution after HCT is one of the key factors for protection against various pathogens and disease relapse and consequently long-term patient survival[51]. HCT is followed by a phase of pancytopenia[47] as the conditioning regimen not only eliminates malignant cells but also diminishes the immune system of the patient[64] urging the need

for immune recovery. Immune reconstitution is dependent on several transplant-, patient-, and therapy-related factors including conditioning regimen, stem cell source and purity, recipient age, post-transplant immunosuppression and GVHD[65]. Generally, immune reconstitution can be differentiated into the phases of innate and adaptive immune recovery[51] (see **Figure 3A+B**).

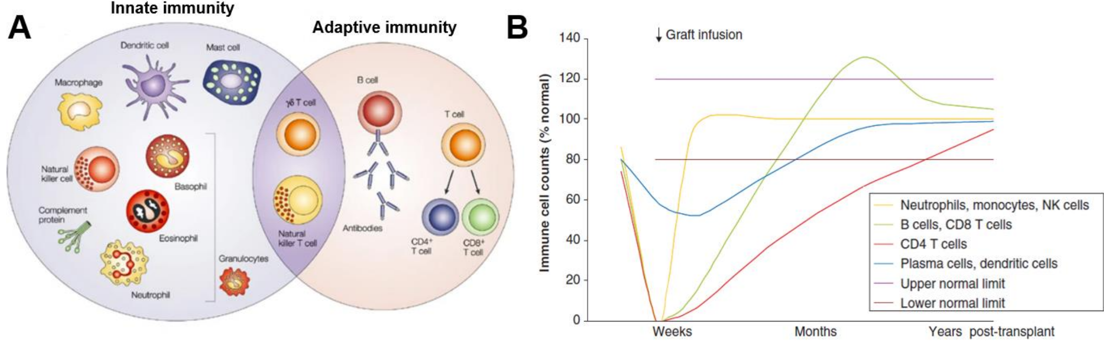


Figure 3: Reconstitution of innate and adaptive immunity after HCT. (A) Overview of innate and adaptive immunity comprising different immune cell types. (adapted from Dranoff[66]) **(B)** Approximation of immune cell counts (in percentages of normal counts) after HCT following myeloablative conditioning. (adapted from Storek[67])

Neutrophils are the first cells to recover, highly dependent on the used stem cell source. Neutrophil recovery to ≥0.5 x10^9/L[68] from PBSC is known to be the fastest, followed by BM and CB within ~14, ~21 and ~30 days, respectively[51]. Functional recovery occurs in parallel to quantitative recovery if patients do not suffer from GVHD[69]. Neutrophil reconstitution is followed by monocytes and NK cells from the innate compartment, reaching normal levels in the first weeks post-transplant[67]. Lymphocyte recovery, i.e. of NK cells or T cells, is mainly driven by two different pathways[47]. The first pathway includes the reconstitution from lymphoid progenitors (thymopoiesis)[70], typically from graft origin, while the second describes a thymic-independent process called homeostatic peripheral expansion (HPE)[71] via cytokines (interleukin (IL)-7 and IL-15) and allogeneic antigens[51]. NK cells recover exclusively from the ontogenic pathway[47] within ~3-4 weeks after HCT both in cell counts and in function[67]. This cell subset plays a role in pathogen defense as NK cells show for instance a faster reconstitution in CMV-positive recipients[72]. Specific NK cell subsets, i.e CD56dimCD57$^+$NKG2C$^+$ cells, which expand during CMV reactivation, might also be relevant in relapse protection[73]. NK cell reconstitution is followed by regeneration of adaptive immune cell subsets such as B cells and T cells[67]. Recovery of B cells is primarily accomplished via thymopoesis[74] and is highly prone to damage caused by

conditioning, GVHD and its' treatment, causing a delay in recovery[47]. Although B cell numbers recover within 6 months after HCT in patients without GVHD, they are not fully functional as antibody production is also dependent on sufficient $CD4^+$ T cell reconstitution[47]. Consequently, full B cell regeneration can last ~1-2 years post-HCT[51]. Impaired levels and diversity of immunoglobulins due to a lack of memory B cells ($CD19^+CD27^+$) make HCT patients prone for example to bacterial infections[75]. In the first months post-transplant, $CD8^+$ T cells levels are decreased reaching (supra-)normal levels around +12 months[67]. The recovery through the HPE pathway follows a rapid expansion from mature T cells of the donor graft or host cells which survived the conditioning[76], with memory T cells to recover first[47]. Via HPE, $CD4^+$ T cells can also expand but in much lesser extent than their $CD8^+$ counterparts, resulting in a reduced CD4/CD8 ratio[77]. T cells expanded within the HPE pathway have a skewed T cell receptor (TCR) repertoire with limited diversity whereas T cells generated by thymic-dependent reconstitution show diverse TCRs[78]. The ontogenic pathway is highly used for naïve $CD4^+$ T cell recovery[79] but also reconstitutes naïve $CD8^+$ T cells. However, this pathway is highly dependent on thymic function which is known to decrease with advanced age[79]. Together with $CD8^+$ T cells, $CD4^+$ T cells build the defense against various pathogens and play a major role in eradicating malignant cells (GVL effect)[51]. $CD4^+$ T cells counts might provide a good predictive marker of recovered immunity correlating with improved transplant outcomes[47]. Taken together, insufficient reconstitution of innate and adaptive immunity contributes importantly to different detrimental HCT outcomes.

4.2 Cytomegalovirus

4.2.1 Background

The human Cytomegalovirus (hCMV, further referred as CMV), is a double-stranded linear DNA virus with a genome of ~250 kilobases and the biggest member from the human viral family of *Herpesviridae*[80]. Its genome is enveloped by a proteinaceous nucleocapsid, a viral tegument composed of viral phosphoproteins, and an outer lipid bilayer with viral glycoproteins[81]. Primary infection with CMV in immunocompetent individuals is mostly asymptomatic[82] and mainly regulated by innate immune responses, especially by NK cells and type I interferons[83,84]. Primary infection drives the development of CMV-specific cellular and humoral immunity, essential for the control of subsequent reactivation episodes (see **Figure 4A**)[85]. After CMV infection

which primarily occurs in lung, liver, spleen and the gastrointestinal (GI) tract[86] the virus establishes a lifelong latency[55]. This is ensured through several immune evasion mechanisms[87], for instance by the inhibition of the viral lytic gene expression[88]. During latency, where CMV is localized in $CD34^+$ progenitor cells, $CD14^+$ monocytes[86] and endothelial cells[89], CMV-specific T cells against various CMV peptides, especially pp65, pp50, IE-1 and IE-2, gB and gH are crucial for infection control[80]. Th1 cells appear ~1 week after peak infection with the ability to secrete cytokines such as interferon (IFN)-γ or tumor necrosis factor (TNF)-α[89]. $CD8^+$ T cells arise subsequently which can establish an effector memory type post-infection, capable to lyse CMV peptide-presenting cells[89]. Besides Th1 cells, γδ T cell levels are increased during active infection[90] and might be relevant in CMV control[85]. The role of humoral responses is under debate, and small compared to cellular immunity[91], but seems to contribute i.e. to restricted dissemination[92]. In mice, a limiting effect of humoral immunity was shown in the presence of GVHD[93]. Major targets for B cell immunity against CMV are the glycoproteins gB and gH[94].

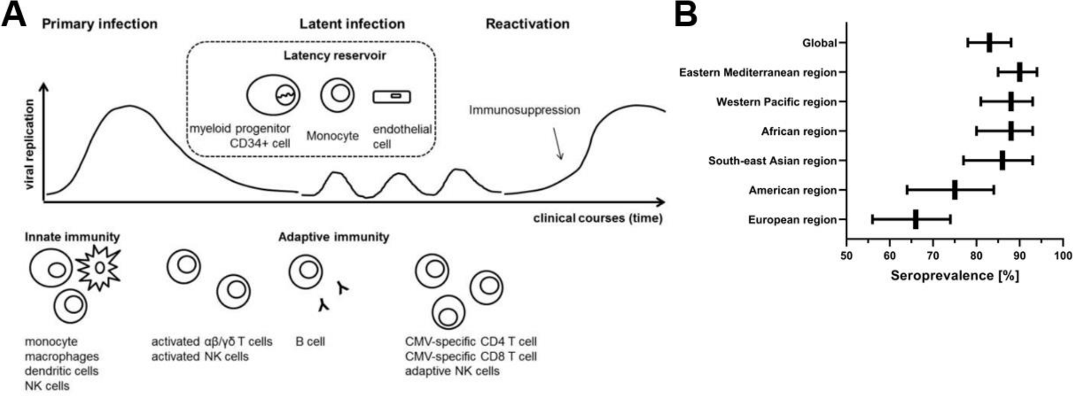


Figure 4: Phases of CMV infection and worldwide distribution. (A) Infection with CMV shows three distinct phases (primary infection, latent infection, and reactivation) controlled by host's innate and adaptive immunity (from Cho *et al.*[89]). **(B)** Estimated seroprevalence of CMV in different geographic regions of the world depicted by mean and 95% confidence interval (adapted from Zuhair *et al.*[95]).

Interestingly, host immunity is effective to lyse CMV infected cells but is not able to fully eliminate the virus facilitating latency[82]. The proportion of circulating CMV-specific T cells during latency might be up to 10%[96] in healthy seropositive individuals showing higher ratios with increasing age[91]. CMV-specific cytotoxic $CD8^+$ T cells might insufficiently control CMV while CMV-specific $CD4^+$ T cells are absent, as those take part in activation of the $CD8^+$ ones[97]. Furthermore, CMV-specific $CD4^+$ T cells might also play a role in killing virus-infected cells mainly restricted gB and gH antigens[98].

CMV is ubiquitously distributed showing 83% seroprevalence all over the globe[95] (**Figure 4B**), highly depending on different factors such as age[88], socioeconomic status, sex or ethnicity[86]. Although the infection with CMV in immunocompetent persons is generally associated with low disease incidences, it leads to clinical symptoms and severe disease courses in immunocompromised individuals such as newborns, HIV patients, or recipients of solid organ transplants or HCT[80].

4.2.2 Relevance of CMV reactivation in allogeneic HCT

CMV reactivation after allo-HCT is the most frequent viral infectious complications[99,100], which associates to poor post-transplant outcomes such as increased NRM[54] and increased risks of GVHD and secondary bacterial and fungal infections[101,102]. Severe infection can lead to the occurrence of CMV end-organ disease such as pneumonia, hepatitis, colitis or retinitis[102], with mortality rates of up to 60%[103]. CMV reactivation is highly frequent in HCT recipients as those are immunocompromised and the conditioning regimen often eradicates potential CMV-specific immunity. Clinically significant CMV reactivation in HCT patients relates to poor immune reconstitution after transplantation or a lack of transferred CMV immunity when using grafts from seronegative donors[55]. While CMV reactivation is a consequence to eliminated immunity it may also induce immune recovery[55]. Exemplarily, CMV reactivation stimulates a rapid reconstitution of IFN-γ producing $NKG2C^+$ NK cells necessary for disease control[104] or the expansion of memory-like NK cells ($NKG2C^+CD57^+$)[105]. Besides, also T cell immunity is stimulated as CMV-specific $CD4^+$ and $CD8^+$ cells are indispensable for infection control and subsequent protection. HCT patients with CMV reactivation feature an accelerated recovery of $CD8^+$ T cells due to clonal expansion of CMV-specific effector-memory αβ $CD8^+$ T cells[106]. Further, CMV reactivation leads to changes in the T cell compartment such as reduced TCR diversity, and lower ratios of naïve T cells up to one year post-transplant[106]. As T cells are a key inducer of alloreactivity, CMV affects this mechanism via its impact on the T cell repertoire.

Without medical prophylaxis, the first CMV reactivation episode occurs in the early post-engraftment phase before d+100[54] but also later episodes can arise, which might be of recurrent nature or the consequence due to CMV antiviral treatment[107]. The incidence of CMV reactivation varies between 20-70% primarily dependent on the serostatus constellation of recipient (R) and donor (D)[100]. Generally, the incidence is highest in the R+/D-, due to a lack of CMV immunity in the donor graft, followed by

R+/D+, R-/D+ and R-/D-[102]. Based on the high seroprevalence, varying reactivation rates, and the association to NRM the pre-transplant serostatus is used as standard risk indicator in donor selection algorithms. Recipient seropositivity alone is a prognostic factor for decreased OS[54]. Further risk factors for CMV reactivation include the administration of corticosteroids[108] and post-transplant cyclophosphamide (PTCy)[109], T cell depleted grafts[110], acute GVHD[111], increasing recipient age, and the use of unrelated donors[112]. Additionally, also immunologic and virologic parameters such as lymphopenia, low CD4+ T cell counts (<50 cells/mm^3) and CMV viral load kinetics were associated to CMV reactivation, especially to late CMV disease[103,113]. With the introduction of preemptive therapy, comprising an active CMV monitoring and treatment in case of reactivation, the incidence of CMV disease was effectively reduced[114], but reactivation rates are still high and associate to survival detriments[54]. Albeit this strategy is established as standard of care in most centers it also presents disadvantages due to myelosuppressive or nephrotoxic effects of anti-CMV agents such as ganciclovir[89]. Furthermore, preemptive therapy with ganciclovir is also known to delay the recovery of CMV-specific T cells and to bear risks for late reactivation and disease[103,107]. Recent advances in CMV prophylaxis involve the development of the viral terminase inhibitor letermovir showing reduced incidences of clinically significant CMV reactivations[115]. Consequently, it is approved for the usage in adult CMV(+)-recipients and is typically administered until d+100 after HCT[115]. Although letermovir shows a high efficacy[116] and favorable toxicity[115], also delayed CMV reactivation after discontinuation of prophylaxis and break through reactivations are observed[117]. Recently, its' potential influence on immune recovery is controversially discussed[118].

Another matter of debate is the ambivalent picture of CMV reactivation on HCT outcomes. While it has been well established, that CMV reactivation associates to increased NRM, and consequently a significant decrease in OS[54,119] across different hematologic malignancies, recent studies presented contradicting findings. Those reports either showed no association to NRM[120,121] or comparable outcomes in overall survival to patients without reactivation[120,122,123] challenging the above-mentioned studies. Further controversial findings associate CMV reactivation with disease recurrence. On the one hand, there are several studies reporting reduced relapse incidences in recipients with CMV reactivation across acute leukemia and further hematologic malignancies[121,123-125]. On the other hand, studies question those data

with comparable relapse rates in patients with and without reactivation[54,126] or restricted the association with reduced relapse to the absence of the *in vivo* T cell depleting agent anti-thymocyte globulin[127].

4.3 Acute Graft-versus-host disease

4.3.1 Background and pathophysiology of acute GVHD

GVHD was first described by Barnes and Loutit in 1956 using a murine model[128] and later taken up by Billingham who postulated three requirements for the pathogenesis of GVHD: 1) the transplanted graft must contain immunocompetent cells, 2) the transplant recipient must be incapable of eliminating or rejecting the transplanted cells and 3) the recipient has to express tissue antigens which are not present in the donor graft, thus recipient antigens are recognized as foreign by donor cells[129]. These three postulates still hold true as we nowadays know for example that those immunocompetent are alloreactive T cells, that the inability of rejecting the transplant is evoked by the conditioning regimen and that the tissue antigens which provoke the evolvement of GVHD are mostly HLA but also mHAgs[58].

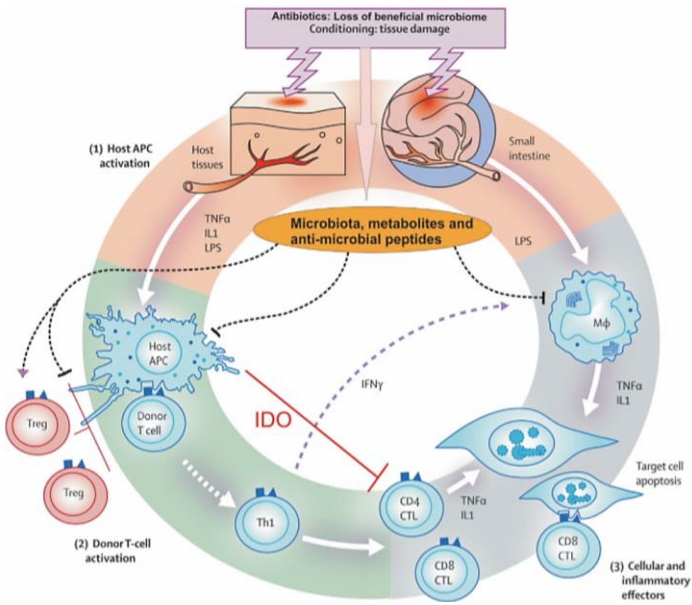


Figure 5: Pathophysiology of acute GVHD (aGVHD). The development of aGVHD is a complex process involving cellular interactions and inflammatory cascades in three consecutive phases: **1)** Activation of host antigen-presenting cells (APCs), **2)** Donor T cell activation followed by proliferation, differentiation, and migration and **3)** the cellular and inflammatory effector phase in which the target tissue destruction takes place (from Ghimire *et al.*[130]).

This thesis focuses on acute GVHD (aGVHD) after HCT. Its pathogenesis (**Figure 5**) is initiated by damage due to the underlying hematological disease and the

conditioning regimen[58]. The latter is required for HSC engraftment[5] but also damages e.g. epithelial cells[130]. Following this damage, pro-inflammatory cytokines, such as TNF-α or IL-1 are released and activate host antigen-presenting cells (APCs)[131]. Specifically looking at aGVHD in the GI tract, the pathogenesis includes the systemic translocation of microbial products, for example lipopolysaccharide (LPS), amplifying the activation of host APCs[132]. In a second step, mature donor T cells get activated through recognition of alloantigens on host APCs, followed by proliferation and differentiation into Th1 or Th17 phenotypes, which regulate the activation of $CD4^+$ and $CD8^+$ cytotoxic T lymphocytes (CTLs), later causing tissue damage[130]. Induction of aGVHD by $CD4^+$ and $CD8^+$ CTLs is mediated by differences in HLA class II & I, respectively[133]. The proliferation of donor T cells can be inhibited by regulatory T cells[134], which present a good target for limiting aGVHD. The last phase in aGVHD pathogenesis is the phase of target cell apoptosis via cellular and inflammatory effectors[130]. At this point, CTLs and NK cells – the cellular effectors – lyse target cells using the Fas/FasL or perforin/granzyme pathways[135]. The migration to the respective organ sites is mediated by chemokines such as the macrophage inflammatory protein-1alpha (MIP-1α) or CXCL2[136]. Additionally, tissue damage is also observed through the secretion of inflammatory cytokines such as TNF-α or IL-1, initiated by signals stemming from phase I, for instance LPS[130].

Acute GVHD can be reduced by modifications in the conditioning regimen, for example reduced-intensity conditioning, which is known to cause less initial tissue damage[58]. Classical pharmacological strategies in GVHD prophylaxis include, immunosuppressive agents, e.g. calcineurin inhibitors or corticosteroids[58] and the use of *in vivo* T cell depleting agents. Agents often used for *in vivo* T cell depletion are anti-T-lymphocyte globulin (ATG), the CD52 antibody Alemtuzumab, and more recently also post-transplant cyclophosphamide (PTCy)[31,58].

4.3.2 Classification and risk factors

The actual definition for aGVHD from the National Institute of Health (NIH) includes two categories, in fact the classical manifestation until d+100 post-HCT and a persistent, recurrent, or late onset aGVHD[59]. Acute GVHD can be assessed by different grading systems, such as the consensus[137], MAGIC[138] or Minnesota[139] grading, according to the number of involved organs and the respective disease severity[48,59]. The different grading systems for aGVHD differentiate between grades 0-

IV and vary in their definitions for GVHD grades as shown by a consensus statement focusing on the terminology of GVHD assessment[140]. Prominent risk factors for aGVHD comprise the grade of HLA-matching, gender mismatch (female D/male R), stem cell source (PBSC>BM>CB), the amount of T-lymphocytes in the graft, increasing donor and recipient age, the use of total body irradiation, or infections[48].

4.4 Considerations on statistical approaches in HCT studies

Biostatistical methods are fundamental features throughout all fields of biomedical research, including studies in the field of HCT. They are applied in clinical trials for the approval of new medical devices, drugs or treatment protocols as well as in the assessment of patient prognosis in prospective and retrospective studies[141]. Generally, biostatistical analyses are applied to test hypotheses related to open research questions. Besides mere data analysis, biostatistics comprises research design, data collection and processing, statistical analysis, data interpretation as well as data presentation[142]. Information, e.g. about a study population, are displayed in variables differentiated into: 1) categorical or qualitative, describing the assignment of individuals to particular groups, classes or categories (e.g. sex or disease) and 2) quantitative or measurable, which take numerical values into account (e.g. age, height, weight)[142]. Commonly used tools in HCT research for the analysis of such variables are descriptive analysis, Kaplan-Meier survival analysis[143], the Cox proportional hazard model[144] and competing risk analysis by Fine and Gray[145]. More advanced tools comprise multistate models, propensity score matching or the development of risk-scoring systems[146]. Descriptive analyses are utilized for data summary using tables or diagrams[142]. The examination of time-to-event data by Kaplan-Meier survival analysis, yields to nonparametric estimates of the survival function[143]. The resulting survival curves are necessary for the comparison of survival patterns of different patient subgroups depending on the research question[147]. Cox regression analysis also focuses on the investigation of time-to-event data. This model is defined by its' non-parametric hazard function and follows the intention to test for effects of *n* variables, called covariates, on times to a specified event[147]. The Cox model utilizes regression parameters for covariates to estimate hazard ratios (HR), describing the likelihood for the occurrence of an event to happen in a predefined time interval[144]. Those hazard ratios can either show beneficial (HR<1) or detrimental (HR>1) contributions of covariates to the event of interest, e.g. OS or NRM[148]. Cox regression is adequate as

long as no competing event to the event of interest exists as these would be otherwise regarded as censored observations[149]. In case of competing events, for example on relapse and NRM in HCT studies, the competing risk analysis by Fine and Gray can be one method of choice[149]. This analysis describes the effects of covariates on the cumulative incidence function by subdistribution hazards[145,149]. Integral parts of all these methods are the calculation of p-values and confidence intervals. The computing of p-values is applied to test the null hypothesis and indicate the likelihood that assumed probability distributions adequately account for the observed results[150]. The commonly used level in biomedical research for statistical significance is $p<0.05$[150]. Also very frequently employed are confidence intervals (CIs) where it is believed that the true parameter value lies, for instance with 95% certainty, in the calculated interval[150].

The forecasting of clinical events via machine learning (ML)-based approaches is of increasing interest for the biomedical community[151]. ML differentiates between unsupervised- and supervised learning[152]. The main difference between these two approaches is that the training process in supervised learning involves the use of fixed input- and corresponding target variables (e.g. event of interest) whereas unsupervised learning misses the knowledge of target variables[152]. Unsupervised learning involves clustering approaches for the identification of similarity patterns and therefore homogeneity within heterogenous data (e.g. laboratory parameters)[152]. *Supervised* machine learning algorithms on the other hand are trained to extract information and patterns from complex and heterogeneous medical datasets that are mandatory for a data-driven prediction for the event of interest[153]. Trained ML-models should be able to predict outcomes of patients from training-independent datasets[153]. Commonly used supervised learning approaches are artificial neural networks, support vector machines or decision trees[154,155]. In the field of leukemia and HCT, machine learning studies primarily utilized decision tree algorithms for predictions[156],[157] and dimensionality reduction and clustering algorithms for single cell- and experimental data[158,159].

While biostatistical methods are useful in clinical decision-making or the identification of risk factors machine learning approaches have the ability of forecasting clinical courses or events. Thus, the combination of both approaches may respond most adequately to the challenge of predicting an event of interest most appropriately.

5 Objective of this thesis

To date it is still an unmet goal to create HCT-transplant settings, which prevent GVHD while preserving GVL activity. Due to the central role of T cells in the induction of alloreactivity the analysis of the T cell subset reconstitution, which is strongly influenced by several transplant-related factors, can potentially inform about later HCT outcomes. Increasing availability and detailed information from cellular immune reconstitution may be integrated into complex data-driven models, which have the potential to delineate patient groups concerning the predominant alloreactive effect and could provide new approaches for preventing GVHD and harnessing GVL. The aim of this thesis is to shed light on the complexity of these alloreactive processes the data-driven analysis and investigation of immune reconstitution influenced by the transplant-related factors of (1) CMV reactivation and (2) GVHD-prophylaxis by:

1) In-depth characterization of CMV reactivation kinetics and the analysis of its' impact on transplant outcomes and immune reconstitution
2) Analysis of the effect of CMV reactivation on leukemia relapse in AML patients and the potential impact of disease stages and T cell depletion with ATG
3) Clarification of conflicting results concerning *in vivo* T cell depletion using ATG in addition to calcineurin inhibitor and methotrexate standard prophylaxis of aGVHD by analyzing ATG dosage dependency in MUD recipients
4) A comparative study on the immune reconstitution following HCT with ATG or PTCy as GVHD-prophylaxis agents together with the identification of clinically relevant patient subgroups within ATG and PTCy-exposed cohorts using time-series clustering of immune reconstitution data

6 Articles

I. Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution

Saskia Leserer, Evren Bayraktar, Mirko Trilling, Rashit Bogdanov, Esteban Arrieta-Bolaños, Nikolaos Tsachakis-Mück, Pietro Crivello, Michael Koldehoff, Fabienne Maaßen, Rudolf Stefan Ross, Katharina Fleischhauer, Dietrich W. Beelen and Amin T. Turki

Published in: American Journal of Hematology, **Online:** January 2021; **Print:** April 2021; **DOI:** 10.1002/ajh.26094

II. Impact of CMV reactivation on relapse of acute myeloid leukemia HCT is dependent on disease stage and ATG

Amin T. Turki, Nikolaos Tsachakis-Mück, **Saskia Leserer**, Pietro Crivello, Tobias Liebregts, Luisa Betke, Ferras Alashkar, Nils B. Leimkühler, Mirko Trilling, Katharina Fleischhauer and Dietrich W. Beelen

Published in: Blood Advances, **Online:** October 2021; **DOI:** 10.1182/bloodadvances.2021005509

III. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies

Amin T. Turki, Vesna Klisanin, Evren Bayraktar, Lambros Kordelas, Rudolf Trenschel, Hellmut Ottinger, Nina K. Steckel, Nikolaos Tsachakis-Mück, **Saskia Leserer**, Markus Ditschkowski, Tobias Liebregts, Michael Koldehoff, Katharina Fleischhauer and Dietrich W. Beelen

Published in: American Journal of Transplantation, **Online:** October 2019; **Print:** March 2020; **DOI:** 10.1111/ajt.15642

IV. Cellular immune reconstitution analysis reveals distinct phenotypes and clinically relevant heterogeneity among patient cohorts receiving either anti-T-lymphocyte globulin or post-transplant cyclophosphamide in HCT

Saskia Leserer, Theresa Graf, Rashit Bogdanov, Martina Franke, Ulrike Buttkereit, Nils Leimkühler, Katharina Fleischhauer, H. Christian Reinhardt, Dietrich W. Beelen and Amin T. Turki

Manuscript in preparation

6.1 Article I

Author contributions

Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution

Saskia Leserer, Evren Bayraktar, Mirko Trilling, Rashit Bogdanov, Esteban Arrieta-Bolaños, Nikolaos Tsachakis-Mück, Pietro Crivello, Michael Koldehoff, Fabienne Maaßen, Rudolf Stefan Ross, Katharina Fleischhauer, Dietrich W. Beelen and Amin T. Turki

Research Article in **American Journal of Hematology**[160]

Impact factor: 10.047 (2020)

Status: published online on January 13th, 2021, American Journal of Hematology, licensed under CC BY-NC 4.0, article included without any changes

DOI: 10.1002/ajh.26094; PubMed-ID: 33439488

Complete citation of the original source:

Leserer, S, Bayraktar, E, Trilling, M, et al. Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution. Am J Hematol. 2021; 96: 436– 445. https://doi.org/10.1002/ajh.26094

Contributions:

Conception:	25 %	- study design, cohort definition
Experimental work:	70 %	- data collection, data validation
Data analysis:	70 %	- data processing, data filtering, data matching, establishment of patient subgroups
Statistical analysis:	60 %	- clinical analysis, analysis of immune reconstitution, data interpretation
Writing the manuscript:	30 %	- visualization of the results, literature research, writing the manuscript, preparation of the supplemental material
Revising the manuscript:	30 %	- Revision of the reviewed manuscript, upload of manuscript

____________________ ____________________

Prof. Dr. med. Dietrich W. Beelen Saskia Leserer

Received: 14 October 2020 | Revised: 23 December 2020 | Accepted: 8 January 2021

DOI: 10.1002/ajh.26094

RESEARCH ARTICLE

Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution

Saskia Leserer[1,2,3] | Evren Bayraktar[1,2,4] | Mirko Trilling[5] | Rashit Bogdanov[1,2] | Esteban Arrieta-Bolaños[3,6] | Nikolaos Tsachakis-Mück[1] | Pietro Crivello[3] | Michael Koldehoff[1] | Fabienne Maaßen[3,5] | Rudolf Stefan Ross[5] | Katharina Fleischhauer[3,6] | Dietrich W. Beelen[1] | Amin T. Turki[1,2,3]

[1]Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

[2]Computational Hematology Workgroup, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

[3]Institute for Experimental Cellular Therapy, West-German Cancer Center, University Hospital Essen, Essen, Germany

[4]Chair of Applied Mathematics and Numerics III, TU Dortmund University, Dortmund, Germany

[5]Institute for Virology, University Hospital Essen, University Duisburg-Essen, Essen, Germany

[6]German Cancer Consortium (DKTK), Heidelberg, Germany

Correspondence
Amin T. Turki, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany.
Email: amin.turki@uk-essen.de

Funding information
Bundesministerium fur Bildung und Forschung, Grant/Award Number: 031L0027; Deutsche Forschungsgemeinschaft, Grant/Award Numbers: #1208/1-1, #FL843/1-1, UDE-UMEA; Deutsche Knochenmarkspendedatei, Grant/Award Number: #DKMS-SLS-MHG_2018-01; Jose Carreras Leukamie-Stiftung, Grant/Award Number: 20R/2019; University Duisburg-Essen IFORES grant

Abstract

Even in the era of PCR-based monitoring, prophylaxis, and preemptive therapy, Cytomegalovirus (CMV) viremia remains a relevant cause of non-relapse mortality (NRM) after allogeneic hematopoietic cell transplantation (HCT). However, studies using binary analysis (presence/absence of CMV) reported contradicting data for NRM, overall survival and leukemia relapse. Here, we analyzed CMV replication kinetics in 11 508 whole blood PCR samples of 705 patients with HCT between 2012 and 2017. Using two independent models based on CMV peak titers and on the time point of first CMV reactivation, we stratified patients into risk cohorts. Each cohort had distinct cellular immune reconstitution profiles and differentiated for relevant clinical outcomes. Patients with high CMV peak titers had significantly reduced overall survival (HR 2.13, 95% CI 1.53–2.96; $p < .0001$), due to high NRM. Early impaired T cell reconstitution was a risk factor for high CMV peak titers, however relevant CMV viremia also related to boosted T cell reconstitution. Importantly, intermediate CMV peak titers associated with a significantly reduced relapse probability (HR 0.53, 95% CI 0.31–0.91; $p = .022$). In short, CMV kinetics models distinguished relevant clinical outcome cohorts beyond the R+ serostatus with distinct immune reconstitution patterns and resolve in part contradicting results of previous studies exclusively focused on the presence or absence of CMV.

Abbreviations: aGVHD, acute graft-versus-host disease; AML, acute myeloid leukemia; ATG, anti-T-lymphocyte-globulin; AUC, area under the curve; BMT, bone marrow transplantation; cGVHD, chronic graft-versus-host disease; CI, confidence interval; CIBMTR, Center for International Blood and Marrow Transplant Research; CMV, cytomegalovirus; D, donor; DNA, deoxyribonucleic acid; EBMT, European Society for Blood and Marrow Transplantation; GVHD, graft-versus-host disease; HCT, allogeneic hematopoietic cell transplantation; HLA, human leukocyte antigen; HR, hazard ratio; IU, international units; NRM, non-relapse mortality; OS, overall survival; PCR, polymerase chain reaction; qPCR, quantitative polymerase chain reaction; R, recipient.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2021 The Authors. *American Journal of Hematology* published by Wiley Periodicals LLC.

1 | INTRODUCTION

Cytomegalovirus (CMV) reactivation is a frequent complication after allogeneic hematopoietic cell transplantation (HCT)[1-3] that remains incompletely understood, as revealed by an ambivalent picture of its clinical impact in previous studies. Most reports associated CMV viremia and in particular the development of CMV end-organ disease with an increase in non-relapse mortality (NRM) across different hematologic malignancies.[4-8] Moreover, large studies from both the CIBMTR and EBMT have shown a significant decrease in overall survival (OS) for patients with CMV reactivation.[6,7] Yet, others reported comparable OS outcomes to patients without CMV reactivation,[5,9-11] or did not significantly associate CMV reactivation with NRM.[11-13] Recently, an NRM risk model using specific baseline characteristics has been reported.[14] Donor (D) and recipient (R) CMV serostatus constellations are currently used as standard risk indicators of CMV reactivation and OS after HCT.[15] The D−/R− serostatus is associated with improved OS,[6,7] while the R+ serostatus associates with an increased probability of CMV viremia and increased NRM.[6] Consequently, the CMV serostatus constellation is part of the donor selection algorithm of many HCT centers.

The past discussion on CMV viremia and reduced relapse has been controversial. While the first report of reduced relapse in patients with CMV dates from the 1980s,[16] the effect of CMV seropositivity on relapse independent of chronic graft-versus-host disease (GVHD) was investigated more carefully during the 2000s.[17] Independent of this serostatus effect, CMV pp65 antigen detection was also associated with reduced relapse.[12] Several studies confirmed this finding for acute leukemia[5,18] and other hematologic malignancies.[9,10,19] Other studies, however, challenged these data reporting that relapse rates were comparable to patients without reactivation,[6,20] or that the effect on relapse was only observed in the absence of anti-thymocyte globulin conditioning in patients with acute myeloid leukemia.[21]

Based on these long-standing controversies, we hypothesized that binary analyses (presence or absence of CMV viremia) may suffer from important limitations, which may be overcome by more elaborate kinetics models. Quantitative virus models have been previously applied as a trigger to preemptive CMV treatment,[22] associated with OS[23,24] and persistent viral infections.[25] The present report addresses both the question of NRM and relapse taking different viral load kinetics, such as peak titers and time points into account and may be better suited to predict the clinical outcome of CMV viremia after HCT and support clinical decision making.

2 | PATIENTS AND METHODS

2.1 | Patients

From a total of 1043 consecutive patients who underwent allogeneic HCT between January 2012 and December 2017 at the Department of Bone Marrow Transplantation of the West-German Cancer Center at University Hospital Essen, 705 patients were included into this retrospective analysis (CONSORT diagram, Suppl. Figure S1). Applied inclusion criteria were a minimum of five whole blood quantitative CMV-PCR samples during the first 200 days after HCT, required for kinetics model development. Quantitative PCR (qPCR) was performed between February 2012 and March 2018. Patients were followed up until last clinical assessment or death by any cause. Follow-up data was closed on January 31st 2020, and surviving patients were censored. HLA mismatch between patients and related/unrelated donors was limited to one antigen/allele difference at the HLA loci A, B, C, DRB1, and DQB1. HLA-DPB1 was not considered for HLA matching. Early supportive and follow-up care was identical for all patients. Further details are provided in the supplementary methods sections.

2.2 | CMV monitoring and CMV kinetics models

CMV titers were measured at the Institute for Virology at the University Hospital Essen. Patients underwent weekly CMV surveillance until day +100 and later in extended intervals at every outpatient visit until day +200. Between January 2012 and August 2013, whole blood CMV DNA load was monitored using the Artus CMV Real-time PCR Kit (Qiagen GmbH, Hilden, Germany; detection limit 150 copies/mL; n = 142). From August 2013 to March 2018, CMV monitoring was performed with the CMV Real-time PCR Kit (Abbott Molecular, Des Plaines, IL, USA; detection limit 40 copies/mL; n = 504). During the transition of CMV qPCR assays in 2013, 59 HCT patients were monitored with both assays. Data obtained by both CMV qPCR kits (Qiagen and Abbott Molecular) were converted into a logarithmic scale and observed to be comparable ($y = 0.978x + 0.212$, 95% confidence interval [CI] for α −0.011 – 0.485 and for β 0.913–1.048, $r = 0.939$, $n = 83$, Suppl. Figure S2) in validation assays by nonparametric regression analysis according to Passing and Bablok.[26] Excellent concordance for both assays was also previously shown by other groups and datasets.[27] Technicians performing CMV qPCR were blinded for the patients' clinical status. Relevant CMV reactivation events were defined by a cut-off of >500 genome copies/mL.

An initial CMV peak titer model was developed adopting a logarithmic scaling of peak viral loads (>1000 -, >10 000 -, and >100 000 copies/mL). Following published data that indicated a higher rate of CMV end-organ disease in patients with >20 000 CMV copies/mL [28] as well as data on the impact of low level CMV reactivation,[23] the cutoffs of the first 2 log-cohorts were adjusted to 500 copies/mL and 20 000 copies/mL, respectively. Peak titer cohorts were defined as follows: low (500–20 000 copies/mL), intermediate (20 000–100 000 copies/mL) and high (>100 000 copies/mL). Using conversion factors provided by the manufacturers (1 copy of CMV DNA correspond to 0.61 IU/mL (Qiagen) or 0.64 IU/mL (Abbott), respectively) these peak titer cutoff values corresponded to 305 IU/mL, 12 200 IU/mL, and 61 000 IU/mL for the Qiagen kit and 320 IU/mL, 12 800 IU/mL, and 64 000 IU/mL for the Abbott kit. For comparison of different time point models, cohorts were stratified according to different time

points of first CMV reactivation (≤d + 10 versus [vs] d > 10; ≤d + 20 vs. d > 20, ≤d + 30 vs. d > 30, ≤d + 40 vs. d > 40, ≤d + 50 vs. d > 50, ≤d + 60 vs. d > 60). One outlier patient with an atypically late first CMV reactivation (d + 154) was only excluded from the time point analysis. A visualization of the methodological workflow of this study is provided as Suppl. Figure S3.

2.3 | Flow cytometry

Details on sampling, sample preparation, markers used and gating strategy for immune reconstitution analyses can be found in the supplementary methods.

2.4 | Statistical analysis

Patients' OS was analyzed using the Kaplan–Meier method obtaining event probabilities of time-to-event intervals. The heterogeneity of survival distributions of different cohorts was compared using the log-rank test. In Kaplan–Meier and Cox regression analysis, *p*-values <0.05 were accepted for indication of statistical significance. Beside peak titer and time point cohorts further clinical factors were tested in univariate Cox analysis: serostatus (all combinations, R– vs. R+), aGVHD, cGVHD, age (<50 vs. >50), age intervals (<30, 30–39, 40–49, 50–59, 60–69, >70), sex match and HLA match. Peak titer cohorts and significant co-variables (R– vs. R + serostatus, acute- and chronic GVHD, Age (<50 vs. >50) for OS and NRM; relapse: only acute- and chronic GVHD) from univariate Cox analysis (*p* < .05) were tested in a multivariate Cox regression model. Significant co-variables were further tested for their interaction terms. Kaplan–Meier and Cox regression analyses were performed using Statistical Package for the Social Science (SPSS 25.0; SPSS Inc., Chicago Illinois). Flow cytometry data for all patients at a given time point after transplantation were pooled and median values of different cohorts were compared using Mann–Whitney U test using GraphPad Prism software (GraphPad Prism 8.3.0, GraphPad Software, LLC, San Diego California). Patient baseline characteristics were also analyzed in GraphPad Prism using Mann–Whitney U, one-way ANOVA, Fisher's exact test, and Chi-square test, where appropriate.

2.5 | Ethics

This study was conducted in accordance with German legislation and the revised Helsinki Declaration. The performance of this study and data acquisition was evaluated by the institutional review board of the University Duisburg-Essen (Protocol N° 18-8496-BO). All patients have given written consent to collection, electronic storage, and scientific analysis of anonymized transplant-specific patient data. We confirm that no patient can be identified by use of anonymized patient data.

3 | RESULTS

3.1 | Patients' baseline characteristics and binary analysis of CMV viremia after HCT

In total, 11 508 whole blood samples obtained from 705 patients between d + 0 and d + 200 after HCT were analyzed. CMV viremia (>500 copies/mL) was detected in 351 patients (50%, Suppl. Table S1). AML was the predominant disease irrespective of CMV viremia (both 43%). Significant differences between patients with or without CMV reactivation included age (58 vs. 51 years, *p* < .0001) and CMV serostatus (*p* < .0001). In the AML subgroup, conditioning (*p* < .0001), the use of total body irradiation (*p* < .0001) and of ATG (*p* = .035) differed between CMV reactivation and no-reactivation cohorts. The great majority of patients with CMV reactivation (n = 351) received preemptive therapy (92%).

In a binary analysis (presence/absence of CMV viremia >500 copies/mL), NRM (hazard ratio [HR] 1.50, 95% CI 1.14–1.99, *p* = .004) and overall mortality (HR 1.24, 95% CI 1.01–1.53, *p* = .038) were significantly higher in patients with CMV reactivation (Supplementary Figure S4A). However, CMV reactivation did not significantly associate with reduced relapse (HR 0.88, 95% CI 0.67–1.17, *p* = .384). As in previous reports, the R-/D- donor-recipient serostatus associated with a very low incidence (1%) of CMV viremia after HCT, whereas it was much higher for R–/D+ patients (18%; Suppl. Figure S4B). Patients with R+ serostatus had a 79% probability of early CMV viremia, which translated into significant differences in 5-year OS and NRM as compared to R– patients (*p* = .007, Suppl. Figure S4D and *p* = .006, Suppl. Table S2). Next, we wanted to better understand the differential impact of CMV viremia on clinical outcome (Supplement Figure S3). Therefore patients with CMV viremia >500 copies/mL were stratified into different kinetics-based risk models according to their (a) CMV peak titers: Low, intermediate or high, and (b) the time point of first CMV viremia detection (≤d + 30 or ≥d + 31 after HCT). These cohorts' characteristics are compared in Suppl. Tables S3 and S4.

3.2 | High CMV peak titers associate with increased NRM and reduced OS, while intermediate peak titers associate with reduced relapse

In this study, CMV peak viral loads after HCT ranged from 40 to 7 251 216 copies/mL. Using a modified logarithmic model, we stratified patients' longitudinal CMV viremia into low-, intermediate-, and high peak titer cohorts, resulting in a differentiated clinical outcome (Figure 1(A)). The 5-year OS was significantly reduced for patients with high CMV peak titers compared to intermediate and low peak titers (median OS: 10-, 59-, and 42 months respectively, *p* < .0001, Figure 1(B)). Cox regression analyses (Table 1) confirmed the reduced OS for patients with high peak titers with a HR of 2.13 (95% CI 1.53–2.96, *p* < .0001) as well as significantly increased NRM (HR 2.90, 95% CI 1.92–4.36, *p* < .0001). All other CMV peak titer cohorts showed no significant difference in NRM compared to

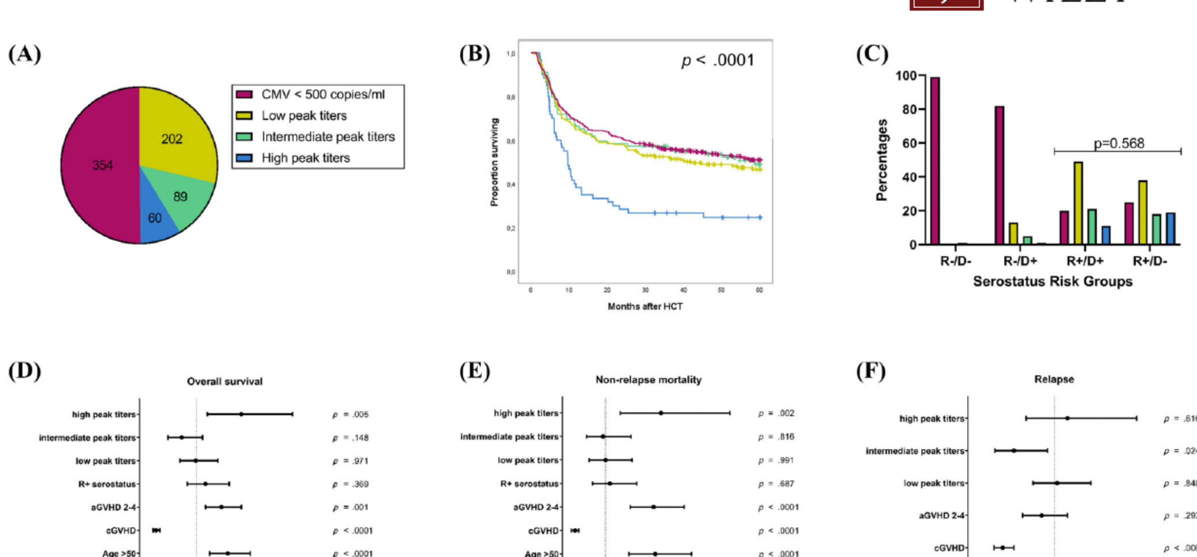


FIGURE 1 Peak titers of CMV reactivation reveal differential impact on patient outcome. (A) Patients' distribution in the peak titer cohorts. (B) Kaplan–Meier analysis of overall survival according to CMV peak titers from HCT until death by any cause. Data were censored at 60 months. (C) Distribution of peak titer cohorts within CMV serostatus risk groups. Statistical significance was tested with the ***two-sample t test*** as $p < 0.0001$ for all combinations, except for R+/D+ and R+/D− with $p = 0.568$. (D–F) Multivariate analysis including all significant co-variates from univariate Cox-regression analysis confirms independent impact of peak titer cohorts. Forest plots show results for (D) overall survival, (E) non-relapse mortality and (F) relapse. Significant factors from this analysis were further tested in a multivariate analysis with interaction terms (Suppl. Table S5) [Color figure can be viewed at wileyonlinelibrary.com]

patients without CMV reactivation. Besides high CMV peak titers, multivariate Cox models also identified severe aGVHD, cGVHD and age >50 as significant variables impacting OS and NRM (Figure 1(D), (E)). A multivariate Cox model including interaction terms also confirmed high CMV peak titers as an independent predictor of NRM and OS (Suppl. Table S5).

Interestingly, patients with intermediate peak titers had a significantly lower relapse rate (HR 0.53, 95% CI 0.31–0.91, $p = .022$; Table 1) as compared to patients without CMV reactivation. This was confirmed by multivariate analysis, which also identified cGVHD as significant factor (Figure 1(F)). However, cGVHD did not correlate with intermediate peak titers in the multivariate Cox model including interaction terms, which confirmed intermediate peak titers as a factor for reduced relapse risk independent from other co-variates (Suppl. Table S5). The combined analysis of CMV peak titers and CMV serostatus revealed that the CMV serostatus did not assign patients to a specific CMV peak titer cohort of clinical relevance, which were evenly distributed among R+/D+ and R+/D− patients ($p = .568$, Figure 1(C)).

3.3 | Very early detection of CMV viremia associates with increased mortality

All patients in this analysis had their initial CMV reactivation prior to d + 100 (early CMV reactivation) post HCT and interestingly this data adopted a Gaussian normal distribution (Suppl. Figure S5A). Median first CMV reactivation was detected on d + 33 (Suppl. Figure S5A) and the median peak of CMV viremia on d + 47 after HCT. Kaplan–Meier analysis revealed lower 5-year OS in patients with CMV reactivation ≤d + 30 compared to patients with later reactivation (median OS 17 vs. 45 months, $p = .057$, Suppl. Figure S5C), which was also corroborated by Cox regression analysis (HR 1.38, 95% CI 1.06–1.79, $p = .019$, Table 1). NRM was significantly increased in both groups (≤d + 30: HR 1.61, 95% CI 1.13–2.30, $p = .008$ and ≥d + 31: HR 1.42, 95% CI 1.04–1.96, $p = .03$). Earlier CMV reactivation time points (≤d + 20, $p = .045$ and ≤d + 10, $p < .0001$) also revealed significant differences, however its relevance may be questioned by the small sample size of comparators (Suppl. Table S6).

3.4 | Specific cellular immune reconstitution profiles characterize CMV peak titer cohorts, while early CMV viremia associates with impaired reconstitution

Beyond its relevance for clinical outcome, we investigated immunological impacts of the CMV kinetics-based risk models and found that patients of each CMV viremia peak titer cohort had distinct cellular immune reconstitution profiles. Those with high CMV peak titers revealed an immunophenotype constellation of $CD3^{+}$ T cells, cytotoxic T cells, and T helper cells, which was significantly distinct from all other

TABLE 1 Univariate Cox regression analysis of OS, NRM and relapse considering CMV peak titer and time point cohorts

	Cox regression analysis		
Risk factor	HR	95% CI	*p*
Overall survival			
CMV ≤d + 30	**1.38**	**1.06–1.79**	**.019**
CMV ≥d + 31	1.16	0.91–1.47	.226
CMV <500 copies/mL	–	–	–
CMV high peak titers	**2.13**	**1.53–2.96**	**<.0001**
CMV intermediate peak titers	1.05	0.75–1.46	.774
CMV low peak titers	1.13	0.89–1.44	.319
CMV <500 copies/mL	–	–	–
Non-relapse mortality			
CMV ≤d + 30	**1.61**	**1.13–2.30**	**.008**
CMV ≥d + 31	**1.42**	**1.04–1.96**	**.030**
CMV <500 copies/mL	–	–	–
CMV high peak titers	**2.90**	**1.92–4.36**	**<.0001**
CMV intermediate peak titers	1.49	0.98–2.24	.060
CMV low peak titers	1.19	0.84–1.66	.329
CMV <500 copies/mL	–	–	–
Relapse			
CMV ≤d + 30	0.91	0.62–1.32	.612
CMV ≥d + 31	0.87	0.63–1.21	.412
CMV <500 copies/mL	–	–	–
CMV high peak titers	1.09	0.65–1.85	.737
CMV intermediate peak titers	**0.53**	**0.31–0.91**	**.022**
CMV low peak titers	1.00	0.73–1.37	.982
CMV <500 copies/mL	–	–	–

Note: Bold values indicate significant values.
Abbreviations: CI, Confidence interval; CMV, Cytomegalovirus; HR, hazard ratio; –, reference.

peak titer cohorts (Figure 2(A)–(C)). Their signature was characterized by delayed early immune reconstitution with significant T cell paucity at months 1 and 3 after HCT, and significantly increased T cell levels at later time points after HCT. In part, this pattern was also observed for naïve and memory helper T cells, while naïve- and memory cytotoxic T cells significantly expanded from month 6 after HCT (Suppl. Figure S6A,B,E,F). These observations were consistent with the increased NRM in this cohort and indicative of immune modulation due to CMV exposure. Interestingly, patients without relevant CMV reactivation (CMV <500 copies/mL) also had a significantly distinct cellular immunophenotype. From 6 months after HCT, these patients were characterized by significantly lower median T cell numbers throughout different T cell subsets (Figure 2, Suppl. Figure S6) as compared to patients with different levels of CMV reactivation. This signature was not observed for NK cells and B cells (Figure 2(D), Suppl. Figure S6D) and is indicative of immune modulatory effects of CMV, in particular on T cells and its subsets. Patients with intermediate peak titers had significantly higher T cell numbers than patients without CMV reactivation, indicating adequate reconstitution of anti-leukemia activity. Furthermore, higher $CD3^+$ T cell and cytotoxic T cell numbers distinguished these patients from others with lower peak titers (Figure 2(A),(B)). The $CD3^+$ T cell reconstitution level of distinct CMV peak titer cohorts appeared to be proportional to the magnitude of CMV viremia after HCT. Finally, patients with very early CMV reactivation (≤d + 30) had significantly lower naïve helper T cell counts at 1 month after HCT as compared to both patients without CMV reactivation or later reactivation time points (median 2 cells/μL vs. 5 cells/μl, with $p = .0005$ and $p = .0002$ respectively; Figure 3(A),(B)). Memory helper T cells were also significantly suppressed at months 1 and 3 after HCT (Figure 3(C),(D)). Consequently, these findings suggest that CMV kinetics cohorts, its clinical outcome and cellular immune reconstitution are intimately connected.

4 | DISCUSSION

CMV viremia is of extraordinary relevance in allogeneic HCT, due to its high incidence with significant morbidity, its profound effects on the reconstituting donor immune system and its interaction with both malignant and healthy patient tissues. These complex processes are incompletely understood and subject to conflicting results of previous studies. Here, we show in a large monocentric patient cohort, that different CMV kinetics (both peak titer and time point) significantly associated with mortality risks after HCT and correlated with specific patterns of immune reconstitution. Early impaired T cell reconstitution after HCT is a risk factor for severe high titer CMV reactivation, while in turn CMV viremia may boost subsequent T cell reconstitution, compared to patients without relevant CMV viremia. These findings are well in line with the profound imprinting of CMV on the transplanted immune system[29] and they set the stage for using this innovative platform for CMV-related risk assessment after allogeneic HCT.

The discussion concerning the relationship of CMV reactivation and clinical outcomes has been ongoing for decades and has revealed the complexity of interactions between CMV and its host.[30] At the same time, contradicting findings between studies prevailed.[6,12] Methodological differences between the analysis of pp65 antigens and quantitative PCR, and relating to the definition of CMV reactivation cutoffs have been blamed for such discordances.[13,31] Based on our data, such inconsistent study results were at least in part related to the binary understanding of CMV reactivation (i.e. CMV reactivation vs. no CMV reactivation), disregarding the relevant impact of cellular immune reconstitution and viral disease burden.

In previous analyses of CMV viral load kinetics in HCT recipients, the combination of $\log_{10}$ initial and peak viral load predicted CMV end-organ disease,[32,33] or was used as criterion for the start of preemptive CMV treatment.[22,34] Significant CMV viremia with varying cutoffs (between >250 IU/mL and >1000 IU/mL) was found to be associated with significantly higher NRM, but resulted in similar HR values for early mortality.[23] Similarly, a recent publication from Europe considered CMV peak titers but utilized a low cutoff putting all patients with >500 IU/mL into the same risk level.[11] Based on our data, NRM and OS differed significantly in patients with high CMV

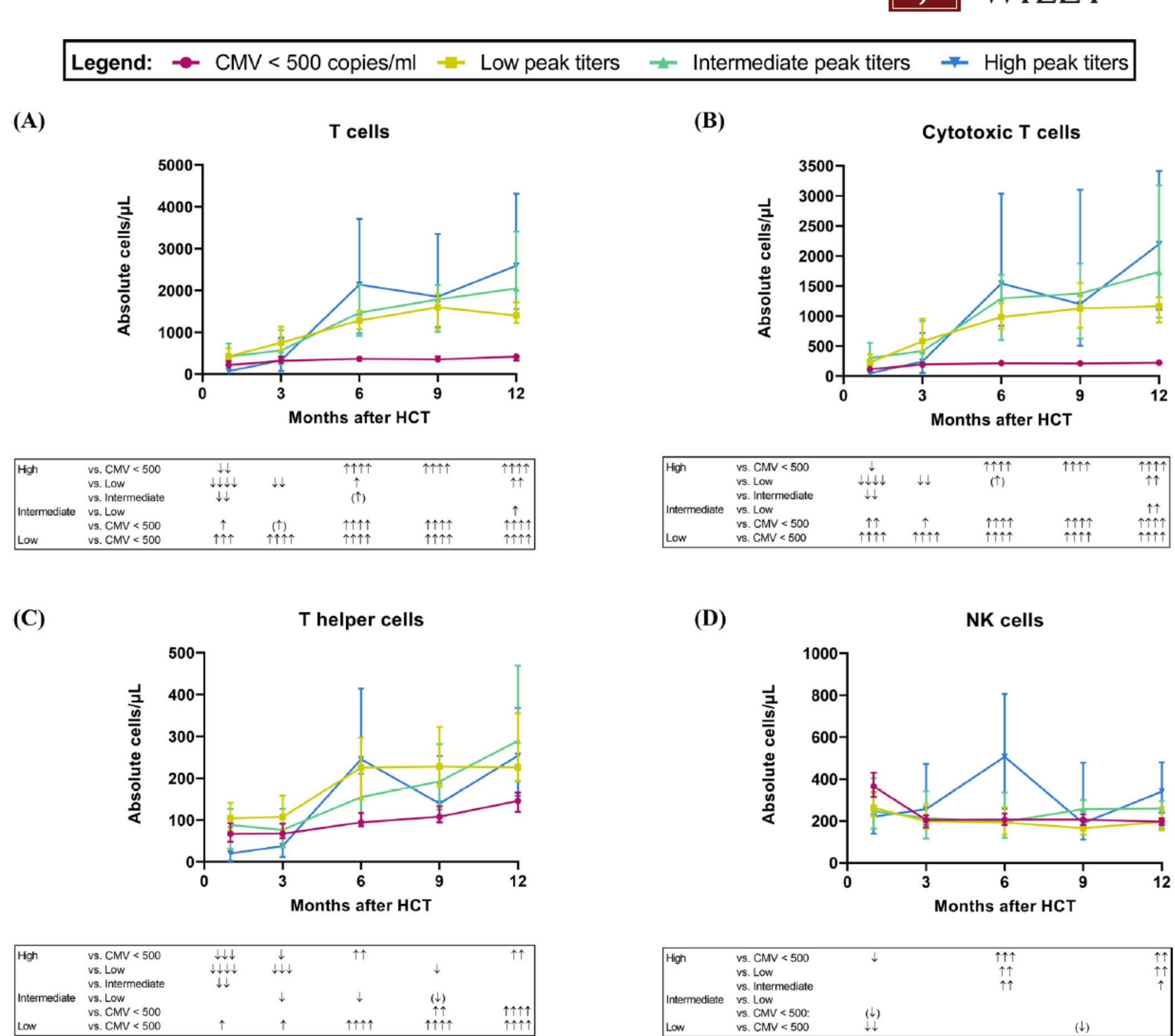


FIGURE 2 Distinct cellular immune reconstitution profiles characterized CMV peak titer cohorts. Lymphocyte subsets in the peripheral blood were measured by flow cytometry after HCT. Cell subsets within the CD45^{+} gate were characterized as follows: (A) T cells, CD3^{+}; (B) cytotoxic T cells, CD3^{+}/CD8^{+}; (C) T helper cells, CD3^{+}/CD4^{+}; (D) NK cells, CD16/56^{+}. Median absolute cell numbers and the 95% CI were analyzed by the Mann–Whitney-U-test. A *p*-value <0.05 was considered statistically significant and indicated in the figure with an arrow instead of an asterisk, *p* < .01 was indicated with two arrows, *p* < .001 was indicated with three arrows and *p* < .0001 was indicated with four arrows. An arrow shown in brackets refers to *p*-values <.05–.10. Arrow direction illustrates significantly higher or lower levels. Cohorts and sample numbers: CMV < 500 copies/mL (purple line) around 1 month (n = 135), 3- (n = 110), 6- (n = 140), 9- (n = 121) and 12 months (n = 107); low CMV peak titers (yellow line) around 1 month (n = 78), 3- (n = 60), 6- (n = 76), 9- (n = 64), and 12 months (n = 63); intermediate CMV peak titers around 1 month (n = 35), 3- (n = 34), 6- (n = 35), 9- (n = 36), and 12 months (n = 26); high peak titers around 1 month (n = 21), 3- (n = 19), 6- (n = 12), 9- (n = 10), and 12 months (n = 18) [Color figure can be viewed at wileyonlinelibrary.com]

peak titers. Our results are complementary to a previously published approach that assessed the cumulative infectious burden of different viral infections after HCT.[24] Beyond the clinical association of kinetics with outcome, correlative studies of cellular immune reconstitution revealed distinct signatures of the respective cohorts, which could serve as early risk screening indicators. In particular, the significantly reduced helper T cell levels 1 month after HCT, which were observed in association with high titer CMV viremia, might serve as an appropriate marker to identify patients at risk for life-threatening CMV reactivations. Productive CMV replication necessarily causes tissue damage since it is a cytopathic virus.[35] It is therefore plausible that high titer viremia directly associated with profound pathology, culminating in decreased OS and increased NRM.

With respect to established CMV risk factors from previous registry studies, the present study is consistent and in addition identifies new factors. Both, CMV serostatus distribution and the proportion of

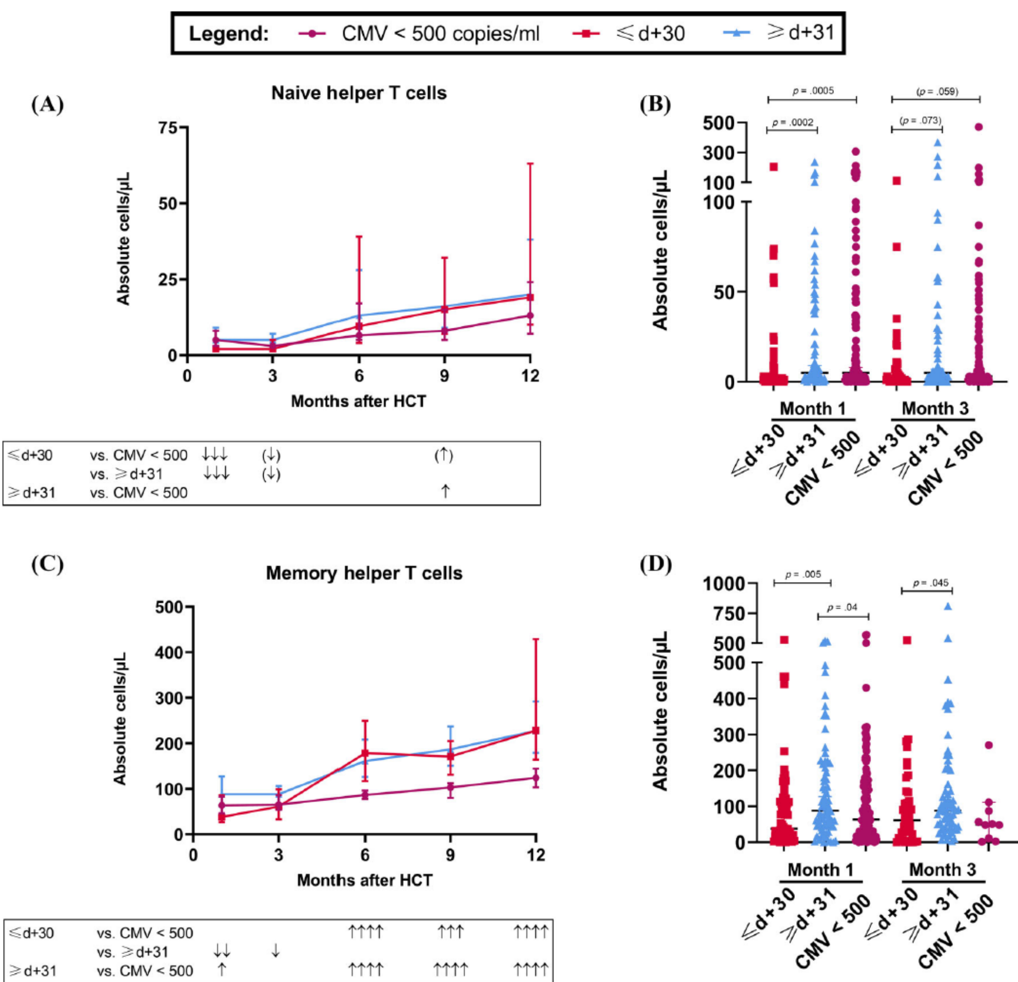


FIGURE 3 Patients with CMV reactivation ≤d + 30 are characterized by a delayed reconstitution of naïve and memory helper T cells. Lymphocyte subsets in the peripheral blood were measured by flow cytometry after HCT. Cell subsets within the $CD45^+$ gate were characterized as follows: (A,B) naïve helper T cells, $CD3^+/CD4^+/CD45RA^+$; (C,D) memory helper T cells, $CD3^+/CD4^+/CD45RO^+$. Descriptions of statistical analysis and significance levels are the same as detailed under Figure 2. Cohort and sample numbers: CMV <500 copies/mL (purple line) around 1 month after HCT (n = 134), 3- (n = 110), 6- (n = 140), 9- (n = 121) and 12 months (n = 107); ≤d + 30 cohort (red line) around 1 month (n = 61), 3- (n = 45), 6- (n = 50), 9- (n = 42) and 12 months (n = 41); ≥d + 31 cohort (blue line) around 1 month (n = 73), 3- (n = 66), 6- (n = 73), 9- (n = 68) and 12 months (n = 66) [Color figure can be viewed at wileyonlinelibrary.com]

R+ patients in our data were comparable to a large CIBMTR study.[6] However, while our data confirmed that R+ serostatus predicted overall CMV reactivation, R+ serostatus did not assign patients to a specific CMV peak titer cohort of clinical relevance. Consistent with other studies,[6,21] the binary model of CMV reactivation did not associate with relapse. Increased use of ATG as additional GVHD prophylaxis leading to a higher incidence of CMV viremia after HCT together with improved sensitivity of quantitative PCR over pp65 antigenemia resulted in higher detection rates than in a previous report from our department, which was the first to correlate CMV viremia and relapse.[12] While the exact biological mechanisms of the association between CMV replication and reduced leukemic relapse[12,36,37] are not entirely understood, the role of T cells appears to be crucial, as this effect is impaired in patients with in vivo T cell depletion using ATG.[13,21,38] Interestingly, our data within an ATG exposed cohort showed that intermediate CMV peak titers after HCT still associated with significantly reduced relapse, while lower virus titers did not. This observation may relate to adequate alloreactivity levels, stimulated by CMV-responsive T cells that might directly mediate anti-leukemic effects via heterologous immunity, or might boost other T cells with alloreactive potential. It is likely that in some patients, ATG-mediated low T cell numbers early after HCT favored the presence of CMV viremia. Dosage optimizing strategies of ATG[39,40] may prevent excessive depletion and subsequent CMV reactivation. Other risk models have associated CMV area under the curve (AUC)[25] or cumulative infections of different

viruses to OS and NRM but did not identify correlations with relapse.[24]

In order to understand the impact of reactivation timing, we also analyzed different time points of initial CMV reactivation before d + 100. Intriguingly, the d + 30 cut-off date, which identified statistically relevant subgroups with distinct clinical outcome, coincides with the reconstitution of cytotoxic effector functions of NK cells.[41,42] Consequently, CMV viremia prior to its reconstitution is likely to cause life-threatening disease, as indicated by decreased OS in the early time point cohorts. However, their distinct immune reconstitution profiles become comparable with time, which is consistent with data showing CMV reactivation to drive $CD8^+$ T cell activation[29] and associating CMV with a narrowing of the T-cell receptor repertoire.[43] Current surveillance practices after HCT recommend a close, weekly monitoring of CMV reactivation until d + 100. Indeed, a large CIBMTR study[6] and our data agree that >99% of first time CMV reactivations occurred before d + 100. Our data showed a median time of first CMV reactivation at d + 33 and a Gaussian normal distribution, which is in agreement with findings of recent PCR-based studies with increasing sensitivity (41 days[6] or 27 days[10]). This finding suggests an early beginning and close monitoring of CMV events, especially in the absence of prophylaxis, since these early events significantly impact OS. Detection of first CMV events after day +60 on the other hand appears less relevant with respect to OS. The observed severely depleted T cell subset numbers at month 1 after HCT in the patient subgroup with reactivation ≤d + 30 (naïve helper T cells) and in patients with high peak titers (helper T cells) indicate that these might be applicable as early biomarkers of unfavorable clinical outcome in the presence of CMV after HCT. Apart from the suggested partial resolution of previous conflicting results relating to the binary understanding of CMV reactivation through the peak titer model and the provided biological insights into the interaction between CMV and its host, these new risk factors should be prospectively validated as an additional criterion, when to hospitalize HCT patients for pre-emptive CMV treatment (e.g. patients with low CMV peak titers might be eligible for outpatient preemptive treatment, while patients with high peak titers should better be hospitalized). This study has some limitations due to its retrospective character and the use of two different PCR assays (Qiagen and Abbott), despite comparable results in validation assays. Clinical data were collected before the approval of Letermovir for the prophylaxis of CMV infection and disease in patients with R+ serostatus,[44] which has reduced the overall incidence of CMV reactivations after HCT and may impact the time point of reactivations. Yet, CMV replication is still observed in patients under CMV prophylaxis. In addition, changing GVHD prophylaxis strategies with increasingly used post-transplant cyclophosphamide[43,45] will likely have an impact on CMV incidence and prevention strategies.

5 | CONCLUSION

This longitudinal CMV kinetics analysis provides important new insights into the complex relationship between CMV viremia, NRM, and relapse. Instead of evaluating the impact of CMV viremia with a binary perspective (presence/absence), the burden of viremia is of importance and not every single episode of CMV reactivation is a threat to patient's survival. The herein developed model suggests considering CMV kinetics in order to distinguish patients with increased NRM from patients with a manageable risk under preemptive therapy and refines risk cohorts beyond R+ serostatus. Furthermore, our data contributes to clarifying the ongoing discussion on the interaction between CMV and relapse and provided new insights into immune reconstitution after HCT. Future studies evaluating the clinical impact of CMV should adopt kinetics based models.

SUPPORTING INFORMATION STATEMENT

Additional supporting information may be found online in the Supporting Information section at the end of the article.

ACKNOWLEDGMENT

This study was in part supported by Bundesministerium für Bildung und Forschung through grant 031L0027 (DWB), the University Duisburg-Essen IFORES grant (ATT), the Deutsche Forschungsgemeinschaft through a UDE-UMEA grant (ATT), grant #FL843/1-1 (KF), grant #1208/1-1 (MT), the Deutsche José Carreras Leukämiestiftung, grant 20R/2019 (KF) and the Deutsche Knochenmarkspendedatei, grant #DKMS-SLS-MHG-2018-01 (PC). We would like to thank all technicians of the BMT Flow Cytometry Laboratory and the PCR laboratory section of the Institute for Virology for assistance. Open Access funding enabled and organized by ProjektDEAL.

CONFLICT OF INTEREST

The authors of this manuscript have potential conflicts of interest to disclose. ATT: Consultancy for MSD, JAZZ, CSL. Travel subsidies from Neovii Biotech outside the submitted work. DWB received travel subsidies from Medac, all outside the submitted work. The other authors declare no competing financial interests within the submitted work.

ETHICS APPROVAL STATEMENT

This study was conducted in accordance with German legislation and the revised Helsinki Declaration. The performance of this study and data acquisition was evaluated by the institutional review board of the University Duisburg-Essen (Protocol N° 18-8496-BO).

PATIENT CONSENT STATEMENT

All patients have given written consent to collection, electronic storage, and scientific analysis of anonymized transplant-specific patient data. We confirm that no patient can be identified by use of anonymized patient data.

PERMISSION TO REPRODUCE MATERIAL FROM OTHER SOURCES

Not applicable.

CLINICAL TRIAL REGISTRATION

Not applicable.

AUTHOR CONTRIBUTIONS

Amin T. Turki, Saskia Leserer, Katharina Fleischhauer and Dietrich W. Beelen designed the study. Saskia Leserer, Rashit Bogdanov and Nikolaos Tsachakis-Mück performed data collection; R. Stefan Ross and Fabienne Maaßen participated in data acquisition. Saskia Leserer, Evren Bayraktar and Amin T. Turki performed statistical analysis, Amin T. Turki, Saskia Leserer, Katharina Fleischhauer, Mirko Trilling and Dietrich W. Beelen interpreted the data. Esteban Arrieta-Bolaños, and Pietro Crivello participated in data analysis. Amin T. Turki, Dietrich W. Beelen and Michael Koldehoff provided clinical expertise. Amin T. Turki and Saskia Leserer wrote the manuscript. Dietrich W. Beelen, Mirko Trilling, Esteban Arrieta-Bolaños and Katharina Fleischhauer contributed to write the manuscript. All authors had access to primary clinical trial data, read and approved the final manuscript.

DATA AVAILABILITY STATEMENT

On reasonable request, primary data is available from the corresponding author in accordance with ethical restrictions.

ORCID

Pietro Crivello https://orcid.org/0000-0001-9668-5013
Amin T. Turki https://orcid.org/0000-0003-1347-3360

REFERENCES

1. Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol Blood Marrow Transplant*. 2003;9(9):543-558.
2. George B, Kerridge IH, Gilroy N, et al. A risk score for early cytomegalovirus reactivation after allogeneic stem cell transplantation identifies low-, intermediate-, and high-risk groups: reactivation risk is increased by graft-versus-host disease only in the intermediate-risk group. *Transpl Infect Dis*. 2012;14(2):141-148.
3. Ljungman P. The role of cytomegalovirus serostatus on outcome of hematopoietic stem cell transplantation. *Curr Opin Hematol*. 2014;21(6):466-469.
4. Broers AE, van Der Holt R, van Esser JW, et al. Increased transplant-related morbidity and mortality in CMV-seropositive patients despite highly effective prevention of CMV disease after allogeneic T-cell-depleted stem cell transplantation. *Blood*. 2000;95(7):2240-2245.
5. Green ML, Leisenring WM, Xie H, et al. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood*. 2013;122(7):1316-1324.
6. Teira P, Battiwalla M, Ramanathan M, et al. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood*. 2016;127(20):2427-2438.
7. Schmidt-Hieber M, Labopin M, Beelen D, et al. CMV serostatus still has an important prognostic impact in de novo acute leukemia patients after allogeneic stem cell transplantation: a report from the acute leukemia working party of EBMT. *Blood*. 2013;122(19):3359-3364.
8. Camargo JF, Kimble E, Rosa R, et al. Impact of cytomegalovirus viral load on probability of spontaneous clearance and response to preemptive therapy in allogeneic stem cell transplantation recipients. *Biol Blood Marrow Transplant*. 2018;24(4):806-814.
9. Ito S, Pophali P, Co W, et al. CMV reactivation is associated with a lower incidence of relapse after Allo-SCT for CML. *Bone Marrow Transplant*. 2013;48(10):1313-1316.
10. Peric Z, Wilson J, Durakovic N, et al. Early human cytomegalovirus reactivation is associated with lower incidence of relapse of myeloproliferative disorders after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant*. 2018;53(11):1450-1456.
11. Solano C, Vázquez L, Giménez E, et al. Cytomegalovirus DNAemia and risk of mortality in allogeneic hematopoietic stem cell transplantation: analysis from the Spanish hematopoietic transplantation and cell therapy group. *Am J Transplant*. 2020;00:1-14. https://doi.org/10.1111/ajt.16147.
12. Elmaagacli AH, Steckel NK, Koldehoff M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood*. 2011;118(5):1402-1412.
13. Manjappa S, Bhamidipati PK, Stokerl-Goldstein KE, et al. Protective effect of cytomegalovirus reactivation on relapse after allogeneic hematopoietic cell transplantation in acute myeloid leukemia patients is influenced by conditioning regimen. *Biol Blood Marrow Transplant*. 2014;20(1):46-52.
14. Kaito S, Nakajima Y, Hara K, et al. Heterogeneous impact of cytomegalovirus reactivation on nonrelapse mortality in hematopoietic stem cell transplantation. *Blood Adv*. 2020;4(6):1051-1061.
15. Ayuk F, Balduzzi A. Donor selection for adults and pediatrics. In: Carreras E, Dufour C, Mohty M, Kröger N, eds. *The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies*. Cham: Springer International Publishing; 2019:87-97.
16. Löunnqvist B, Ringdegn O, Ljungman P, Wahren B, Gahrton G. Reduced risk of recurrent leukaemia in bone marrow transplant recipients after cytomegalovirus infection. *Br J Haematol*. 1986;63(4):671-679.
17. Nachbaur D, Bonatti H, Oberaigner W, et al. Survival after bone marrow transplantation from cytomegalovirus seropositive sibling donors. *Lancet*. 2001;358(9288):1157-1159.
18. Takenaka K, Nishida T, Asano-Mori Y, et al. Cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation is associated with a reduced risk of relapse in patients with acute myeloid leukemia who survived to day 100 after transplantation: the Japan Society for Hematopoietic Cell Transplantation Transplantation-related Complication Working Group. *Biol Blood Marrow Transplant*. 2015;21(11):2008-2016.
19. Koldehoff M, Ross SR, Dührsen U, Beelen DW, Elmaagacli AH. Early CMV-replication after allogeneic stem cell transplantation is associated with a reduced relapse risk in lymphoma. *Leuk Lymphoma*. 2017;58(4):822-833.
20. Remberger M, Ringden O. Survival after bone-marrow transplantation. *Lancet*. 2002;359(9309):888.
21. Busca A, Passera R, Pini M, et al. The use of ATG abrogates the antileukemic effect of cytomegalovirus reactivation in patients with acute myeloid leukemia receiving grafts from unrelated donors. *Am J Hematol*. 2015;90(6):E117-E121.
22. Green ML, Leisenring W, Stachel D, et al. Efficacy of a viral load-based, risk-adapted, preemptive treatment strategy for prevention of cytomegalovirus disease after hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2012;18(11):1687-1699.
23. Green ML, Leisenring W, Xie H, et al. CMV viral load and mortality after hematopoietic cell transplantation: a cohort study in the era of preemptive therapy. *Lancet Haematol*. 2016;3(3):e119-e127.
24. Hill JA, Mayer BT, Xie H, et al. The cumulative burden of double-stranded DNA virus detection after allogeneic HCT is associated with increased mortality. *Blood*. 2017;129(16):2316.
25. Hill JA, Mayer BT, Xie H, et al. Kinetics of double-stranded DNA viremia after allogeneic hematopoietic cell transplantation. *Clin Infect Dis*. 2017;66(3):368-375.
26. Passing H, Bablok W. A new biometrical procedure for testing the equality of measurements from two different analytical methods.

Application of linear regression procedures for method comparison studies in clinical chemistry, Part I. *J Clin Chem Clin Biochem*. 1983;21(11):709-720.
27. Schnepf N, Scieux C, Resche-Riggon M, et al. Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard. *J Clin Microbiol*. 2013;51(7):2096-2102.
28. Jang JE, Hyun SY, Kim YD, et al. Risk factors for progression from cytomegalovirus viremia to cytomegalovirus disease after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2012;18(6):881-886.
29. Suessmuth Y, Mukherjee R, Watkins B, et al. CMV reactivation drives post-transplant T cell reconstitution and results in defects in the underlying TCRβ repertoire. *Blood*. 2015;125(25):3835-3850.
30. Stern L, Withers B, Avdic S, et al. Human cytomegalovirus latency and reactivation in allogeneic hematopoietic stem cell transplant recipients. *Front Microbiol*. 2019;10:1186.
31. Elmaagacli AH, Koldehoff M. Cytomegalovirus replication reduces the relapse incidence in patients with acute myeloid leukemia. *Blood*. 2016;128(3):456-459.
32. Emery VC, Sabin CA, Cope AV, Gor D, Hassan-Walker AF, Griffiths PD. Application of viral-load kinetics to identify patients who develop cytomegalovirus disease after transplantation. *Lancet*. 2000;355(9220):2032-2036.
33. Ljungman P, Perez-Bercoff L, Jonsson J, et al. Risk factors for the development of cytomegalovirus disease after allogeneic stem cell transplantation. *Haematologica*. 2006;91(1):78.
34. Lilleri D, Gerna G, Furione M, et al. Use of a DNAemia cut-off for monitoring human cytomegalovirus infection reduces the number of preemptively treated children and young adults receiving hematopoietic stem-cell transplantation compared with qualitative pp65 antigenemia. *Blood*. 2007;110(7):2757.
35. Poncet D, Pauleau A-L, Szabadkai G, et al. Cytopathic effects of the cytomegalovirus-encoded apoptosis inhibitory protein vMIA. *J Cell Biol*. 2006;174(7):985-996.
36. Nakamura R, Battiwalla M, Solomon S, et al. Persisting post-transplantation cytomegalovirus antigenemia correlates with poor lymphocyte proliferation to cytomegalovirus antigen and predicts for increased late relapse and treatment failure. *Biol Blood Marrow Transplant*. 2004;10(1):49-57.
37. Parkman R, Cohen G, Carter SL, et al. Successful immune reconstitution decreases leukemic relapse and improves survival in recipients of unrelated cord blood transplantation. *Biol Blood Marrow Transplant*. 2006;12(9):919-927.
38. Bao X, Zhu Q, Xue S, et al. Cytomegalovirus induces strong antileukemic effect in acute myeloid leukemia patients following sibling HSCT without ATG-containing regimen. *Am J Transl Res*. 2016;8(2):653-661.
39. Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a retrospective, pharmacodynamic cohort analysis. *Lancet Haematol*. 2017;4(4):e183-e191.
40. Turki AT, Klisanin V, Bayraktar E, et al. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies. *Am J Transplant*. 2020;20(3):677-688.
41. Dokhelar MC, Wiels J, Lipinski M, et al. Natural killer cell activity in human bone marrow recipients: early reappearance of peripheral natural killer activity in graft-versus-host disease. *Transplantation*. 1981;31(1):61-65.
42. Vago L, Forno B, Sormani MP, et al. Temporal, quantitative, and functional characteristics of single-KIR–positive alloreactive natural killer cell recovery account for impaired graft-versus-leukemia activity after haploidentical hematopoietic stem cell transplantation. *Blood*. 2008;112(8):3488-3499.
43. Kanakry CG, Coffey DG, Towlerton AMH, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. *JCI Insight*. 2016;1(5):e86252.
44. Marty FM, Ljungman P, Chemaly RF, et al. Letermovir prophylaxis for cytomegalovirus in hematopoietic-cell transplantation. *N Engl J Med*. 2017;377(25):2433-2444.
45. Luznik L, Bolaños-Meade J, Zahurak M, et al. High-dose cyclophosphamide as single-agent, short-course prophylaxis of graft-versus-host disease. *Blood*. 2010;115(16):3224-3230.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

How to cite this article: Leserer S, Bayraktar E, Trilling M, et al. Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution. *Am J Hematol*. 2021;96:436–445. https://doi.org/10.1002/ajh.26094

Online Supporting Information to:

Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution

Saskia Leserer. M. Sc.[1, 2, 3], Dr. Evren Bayraktar, PhD[1, 2, 5], Prof. Mirko Trilling, PhD[4], Dr. Rashit Bogdanov, MD[1, 2], Dr. Esteban Arrieta-Bolaños, PhD[3, 6], Nikolaos Tsachakis-Mück[1], Dr. Pietro Crivello, PhD[3], Prof. Michael Koldehoff, MD[1], Fabienne Maaßen, M. Sc.[3, 4], Prof. R. Stefan Ross, MD[4], Prof. Katharina Fleischhauer, MD[3, 6], Prof. Dietrich W. Beelen, MD[1] and Dr. Amin T. Turki, MD, PhD[1, 2, 3]

1 Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

2 Computational Hematology Workgroup, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

3 Institute for Experimental Cellular Therapy, West-German Cancer Center, University Hospital Essen, Essen, Germany

4 Institute for Virology, University Hospital Essen, Essen, Germany

5 Chair of Applied Mathematics and Numerics III, TU Dortmund University, Dortmund, Germany

6 German Cancer Consortium (DKTK)

Supplementary Methods:

Assessments

All data concerning baseline patient-, donor-, HCT-characteristics and early HCT-outcome were prospectively documented in electronic forms. Clinical characteristics and laboratory parameters of patients after HCT were retrospectively analyzed. For inpatients, daily clinical assessment was obtained. Outpatients were assessed at each follow-up visit with extended intervals, depending on transplant-associated complications and individual clinical performance. OS was calculated from transplantation up to a follow-up 5-years after HCT or death of any cause. Patients with longer survival were censored. Cumulative relapse incidence (CRI) was calculated from day of HCT to diagnosis of relapse, NRM was determined from day of HCT to death.

Supportive Therapy

Supportive therapy and anti-infectious prophylaxis was identical for the entire cohort. With the beginning of the conditioning until discharge, in-patients were protected in reverse isolation single rooms with high-efficacy particle air filtration. Patients systematically received a combined intestinal decontamination medication as previously described [1] consisting of oral metronidazole at 400 mg three times daily and oral ciprofloxacin at 500 mg twice daily starting from day -14 until day +35 after HCT. Neutropenic patients' meals were prepared as decontaminated or germ-poor meals. Antiviral prophylaxis during neutropenia consisted of intravenous aciclovir at 250 mg three times daily. Antifungal prophylaxis consisted of oral posaconazole at 300 mg once daily from day+1 for HCT patients [2] with a minimal duration until day +100. Colony stimulating factors were not routinely applied. For the prevention of *Pneumocystis jirovecii*-pneumonia, patients received monthly pentamidin inhalations following admission and after discharge oral cotrimoxazole at 960 mg three times per week. HCT

patients exclusively received irradiated red blood cell and platelet transfusions and in-line leukocyte-filtered products.

The uniform pharmacological GVHD prophylaxis consisted of calcineurin inhibitors, in particular of 3 mg/kg body weight ciclosporin (CSP) starting from day -1 before HCT in combination with 15 mg/m^2 methotrexate (MTX) on day +1 and 10 mg/m^2 MTX on days +3, +6 and +11 after HCT [3,4]. Normal CSP target blood levels (range, 150-250 ng/ml) were controlled three times per week. Before patient discharge, intravenous CSP was substituted orally. From day +100 after HCT, CSP was continuously tapered for patients without clinical signs and symptoms of GVHD. Additional T cell depletion using polyvalent rabbit-anti-Jurkat-T lymphocyte globulin (ATG) was used for patients with HCT from MUD and MMUD and therefore expected high GVHD risk (n=546, 77%) on days -4, -3 and -2 with cumulative dosages of 30 mg/kg and 60 mg/kg, respectively. HCT patients were continuously monitored for CMV by PCR and a first-line pre-emptive treatment strategy using Ganciclovir 5 mg/kg twice daily for 14 days (or equivalent oral Valganciclovir) was applied in patients with CMV viremia >2000 copies/ml or if >1000 CMV genome copies/ml were detected in two subsequent blood samples.

Flow cytometry

For flow cytometry analysis, we collected whole blood from patients at different time points after HCT (months +1, +3, +6, +9 and +12). In this study, a total of 1 199 blood samples were analyzed at the BMT Flow Cytometry Laboratory, University Hospital Essen. Peripheral blood mononuclear cells (PBMC) were isolated using an automatic red blood cell lysing system (TQ-Prep, Beckman Coulter, Brea, CA), washed with fluorescence-activated cell sorting (FACS) buffer and stained for surface markers. No intracellular staining was performed. FACS analysis of the patient’s immune status was performed on an FC500 until late 2015 and afterwards with a NAVIOS flow cytometer (Beckman Coulter) using the manufacturer’s software. Protocol concordance between both cytometers was extensively tested using parallel runs of healthy controls (n=20) and

patient samples. A minimum of 15 000 lymphocytes were analyzed in each run to ensure adequate subset representation.

The following cell subsets within the $CD45^+$ lymphocyte gate were characterized by the FC500 flow cytometer: T Cells, $CD3^+$; T helper cells, $CD3^+/CD4^+$; activated T cells, $CD3^+/HLA\text{-}DR^+$; cytotoxic T cells, $CD3^+/CD8^+$; naïve helper T cells, $CD3^+/CD4^+/CD45RA^+$, memory helper T cells, $CD3^+/CD4^+/CD45RO^+$, B cells, $CD19^+$, NK cells, $CD16^+/CD56^+$, T cell receptor α/β, TCRα/β and T cell receptor γ/δ, TCRγ/δ. The characterization of cell subsets with the NAVIOS flow cytometer differed in its gating strategy for TCRα/β and TCRγ/δ, which depended on the $CD3^+$ gating. Through the implementation of this flow cytometer, the following subsets were introduced: naïve cytotoxic T cells, $CD3^+/CD8^+/CD45RA^+$; memory cytotoxic T cells, $CD3^+/CD8^+/CD45RO^+$; NKG2D-NK cells, $CD16^+CD56^+/CD314^+$; regulatory T cells, $CD3^+/CD4^+/CD25^+/CD127^+$low; effector T cells, $CD3^+/CD4^+/CD25^-/CD127^+$high.

References

1. Beelen DW, Elmaagacli A, Müller K-D, Hirche H, Schaefer UW. Influence of Intestinal Bacterial Decontamination Using Metronidazole and Ciprofloxacin or Ciprofloxacin Alone on the Development of Acute Graft-Versus-Host Disease After Marrow Transplantation in Patients With Hematologic Malignancies: Final Results and Long-Term Follow-Up of an Open-Label Prospective Randomized Trial. *Blood.* 1999;93(10):3267-3275.
2. Cornely OA, Maertens J, Winston DJ, et al. Posaconazole vs. Fluconazole or Itraconazole Prophylaxis in Patients with Neutropenia. *New England Journal of Medicine.* 2007;356(4):348-359.
3. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and Cyclosporine Compared with Cyclosporine Alone for Prophylaxis of Acute Graft versus Host Disease after Marrow Transplantation for Leukemia. *New England Journal of Medicine.* 1986;314(12):729-735.
4. BEELEN DW, QUABECK K, KAISER B, et al. SIX WEEKS OF CONTINUOUS INTRAVENOUS CYCLOSPORINE AND SHORT-COURSE METHOTREXATE AS PROPHYLAXIS FOR ACUTE GRAFT-VERSUS-HOST DISEASE AFTER ALLOGENEIC BONE MARROW TRANSPLANTATION. *Transplantation.* 1990;50(3):421-426.

Supplementary Tables

Supplementary Table S1. Patient baseline characteristics compared between patients with or without CMV reactivation (<500 copies/ml).

Characteristics	CMV reactivation (> 500copies/ml)		No CMV reactivation (< 500copies/ml)		*p*
	n	%	n	%	
Total enrolled and treated	351	50	354	50	
Median age at transplantation (range)	58	(17-76)	51	(18-76)	**<0.0001**
Male gender	189	54	216	61	0.057
Disease					0.367
Acute myeloid leukemia	152	43	151	43	
Myelodysplastic syndromes	56	16	36	10	
Acute lymphoblastic leukemia	29	8	38	11	
Chronic myeloid leukemia	11	3	17	5	
Chronic lymphocytic leukemia	9	3	8	2	
Chronic myelomonocytic leukemia	6	2	9	3	
Non-Hodgkin's lymphoma	41	12	38	11	
Hodgkin lymphoma	2	1	6	2	
Multiple myeloma	12	3	18	5	
Myelofibrosis	22	6	23	6	
Other hematologic disorders	11	3	10	3	
Graft source					0.356
PBSC	332	95	328	93	
BM	19	5	26	7	
HLA-matching/Donor Type					0.071
MRD	68	19	91	26	
MMRD	8	2	5	1	
MUD	198	56	201	57	
MMUD	77	22	57	16	
Recipient/Donor gender constellation					0.264
Female/Female	85	24	71	20	
Male/Male	144	41	169	48	
Female/Male	77	22	67	19	
Male/Female	45	13	47	13	
CMV Serology					**<0.0001**
R+/D-	116	33	38	11	
R+/D+	217	62	53	15	
R-/D+	16	5	72	20	
R-/D-	2	1	191	54	
Acute myeloid leukemia subgroup	**CMV reactivation**		**No CMV reactivation**		***p***
	n	**%**	**n**	**%**	
Total enrolled and treated	152	50	151	50	
ELN classification					0.195
Adverse	37	24	51	34	
Intermediate	90	59	78	52	
Favorable	25	16	22	15	
Conditioning					**0.009**
Myeloablative conditioning	93	61	114	75	
Reduced intensity conditioning	59	39	37	25	
Total body irradiation	48	32	98	65	**<0.0001**
In vivo T cell depletion/ATG	114	75	96	64	**0.035**

Abbreviations: *ATG,* anti-T lymphocyte globuline; *CMV,* Cytomegalovirus; *ELN,* European LeukemiaNet, *PBSC,* peripheral blood stem cell; *BM,* bone marrow; *HLA,* human leukocyte antigen; *MMRD,* mismatched-related; *MMUD,* mismatched-unrelated donors; *MRD,* matched-related donors; *MUD,* matched-unrelated donors

Supplementary Table S2. Cox regression analysis of CMV serostatus risk cohorts.

Risk factor	Cox regression analysis		
	HR	**95% CI**	***p***
Overall survival			
R+/D-	1.29	0.96 – 1.72	0.089
R+/D+	1.22	0.94 – 1.58	0.136
R-/D+	0.76	0.51 – 1.12	0.168
R-/D-	—	—	—
CMV R+ (R+/D+ & R+/D-)	**1.35**	**1.09 – 1.67**	**0.007**
CMV R- (R-/D- & R-/D+)	—	—	—
Non-relapse mortality			
R+/D-	**1.55**	**1.04 – 2.29**	**0.030**
R+/D+	1.38	0.97 – 1.98	0.077
R-/D+	0.86	0.51 – 1.47	0.586
R-/D-	—	—	—
CMV R+ (R+/D+ & R+/D-)	**1.51**	**1.12 – 2.02**	**0.006**
CMV R- (R-/D- & R-/D+)	—	—	—
Relapse			
R+/D-	0.86	0.57 – 1.28	0.455
R+/D+	0.98	0.70 – 1.38	0.906
R-/D+	0.94	0.59 – 1.50	0.783
R-/D-	—	—	—
CMV R+ (R+/D+ & R+/D-)	0.95	0.72 – 1.26	0.737
CMV R- (R-/D- & R-/D+)	—	—	—

Abbreviations: *CI,* Confidence interval; *CMV,* Cytomegalovirus; *HR,* hazard ratio; —, reference group.

Supplementary Table S3. Patient baseline characteristics of patient subgroups with CMV reactivation: Low CMV peak titers, intermediate- and high peak titers.

Characteristics	**CMV low peak titers**		**CMV intermediate peak titers**		**CMV high peak titers**		***p***
	n	**%**	**n**	**%**	**n**	**%**	
Total enrolled and treated	202	58	89	25	60	17	
Median age at transplantation (range)	57	(17-75)	58	(20-73)	58,5	(20-76)	0.557
Male gender	111	55	47	53	31	52	0.882
Disease							0.917
Acute myeloid leukemia	90	45	35	39	27	45	
Myelodysplastic syndromes	30	15	15	17	11	18	
Acute lymphoblastic leukemia	16	8	8	9	5	8	
Chronic myeloid leukemia	7	3	3	3	1	2	
Chronic lymphocytic leukemia	6	3	1	1	2	3	
Chronic myelomonocytic leukemia	5	2	1	1	0	0	
Non-Hodgkin's lymphoma	19	9	15	17	7	12	
Hodgkin lymphoma	1	0	1	1	0	0	
Multiple myeloma	6	3	3	3	3	5	
Myelofibrosis	13	6	5	6	4	7	
Other hematologic disorders	9	4	2	2	0	0	
Graft source							0.259
PBSC	188	93	85	96	59	98	
BM	14	7	4	4	1	2	
HLA-matching/Donor Type							**<0.0001**
MRD	51	25	14	16	3	5	
MMRD	4	2	2	2	2	3	
MUD	117	58	52	58	29	48	
MMUD	30	15	21	24	26	43	
Recipient/Donor gender constellation							0.631
Female/Female	52	26	17	19	16	27	
Male/Male	83	41	38	43	23	38	
Female/Male	39	19	25	28	13	22	
Male/Female	28	14	9	10	8	13	
CMV Serology							**0.015**
R+/D-	59	29	27	30	30	50	
R+/D+	132	65	56	63	29	48	
R-/D+	11	5	4	4	1	2	
R-/D-	0	0	2	2	0	0	
Acute myeloid leukemia subgroup	**CMV low peak titers**		**CMV intermediate peak titers**		**CMV high peak titers**		***p***
	n	**%**	**n**	**%**	**n**	**%**	
Total enrolled and treated	90	59	35	23	27	18	
ELN classification							0.084
Adverse	23	26	6	17	8	30	
Intermediate	53	59	26	74	11	41	
Favorable	14	16	3	9	8	30	
Conditioning							0.582
Myeloablative conditioning	52	58	23	66	18	67	
Reduced intensity conditioning	38	42	12	34	9	33	
Total body irradiation	29	32	10	29	9	33	0.904
In vivo T cell depletion using ATG	65	72	26	74	23	85	0.392

Abbreviations: PBSC, Peripheral blood stem cells, BM, Bone Marrow, MRD, Matched related donors, MMRD, Mismatched related donors, MUD, Matched unrelated donors, MMUD, Mismatched unrelated donors; R+, recipient positive serostatus, ELN, EuropeanLeukemiaNet, ATG, Anti-T-Lymphocyte Globulin.

Supplementary Table S4. Patient baseline characteristics compared between patient subgroups with CMV reactivation ≤d+30 and ≥d+31.

Characteristics	CMV ≤d+30		CMV ≥d+31		*p*
	n	%	n	%	
Total enrolled and treated	140	40	210	60	
Median age at transplantation (range)	56.5	(20-74)	58	(17-76)	0.068
Male gender	77	55	111	53	0.743
Disease					**<0.0001**
Acute myeloid leukemia	78	56	73	35	
Myelodysplastic syndromes	10	7	46	22	
Acute lymphoblastic leukemia	13	9	16	8	
Chronic myeloid leukemia	1	1	10	5	
Chronic lymphocytic leukemia	4	3	5	2	
Chronic myelomonocytic leukemia	0	0	6	3	
Non-Hodgkin's lymphoma	27	19	14	7	
Hodgkin lymphoma	0	0	2	1	
Multiple myeloma	3	2	9	4	
Myelofibrosis	0	0	22	10	
Other hematologic disorders	4	3	7	3	
Graft source					0.815
PBSC	133	95	198	94	
BM	7	5	12	6	
HLA-matching/Donor Type					0.111
MRD	19	14	49	23	
MMRD	4	3	4	2	
MUD	81	58	116	55	
MMUD	36	26	41	20	
Recipient/Donor gender constellation					0.180
Female/Female	30	21	55	26	
Male/Male	53	38	90	43	
Female/Male	33	24	44	21	
Male/Female	24	17	21	10	
CMV Serology					**0.015**
R+/D-	58	41	58	28	
R+/D+	79	56	138	66	
R-/D+	2	1	13	6	
R-/D-	1	1	1	0	
Acute myeloid leukemia subgroup	**CMV ≤d+30**		**CMV ≥d+31**		***p***
	n	**%**	**n**	**%**	
Total enrolled and treated	78	52	73	48	
ELN classification					0.068
Adverse	13	17	24	33	
Intermediate	51	65	39	53	
Favorable	14	18	10	14	
Conditioning					0.410
Myeloablative conditioning	45	58	47	64	
Reduced intensity conditioning	33	42	26	36	
Total body irradiation	26	33	21	29	0.600
In vivo T cell depletion using ATG	63	81	51	70	0.133

Abbreviations: PBSC, Peripheral blood stem cells, BM, Bone Marrow, MRD, Matched related donors, MMRD, Mismatched related donors, MUD, Matched unrelated donors, MMUD, Mismatched unrelated donors; R+, recipient positive serostatus, ELN, EuropeanLeukemiaNet, ATG, Anti-T-Lymphocyte Globulin.

Supplementary Table S5. Multivariate Cox regression of OS, NRM and relapse including interaction terms between CMV peak titers and other significant factors from initial multivariate analysis.

	Interaction Term		
	HR	**95% CI**	***p***
Overall survival			
High peak titers * aGVHD (2-4)	0.54	0.27 – 1.07	0.078
Intermediate peak titers * aGVHD (2-4)	1.07	0.51 – 2.28	0.852
Low peak titers * aGVHD (2-4)	0.77	0.46 – 1.30	0.326
<500 copies/ml * aGVHD (2-4)	—	—	—
High peak titers * cGVHD	0.81	0.40 – 1.62	0.546
Intermediate peak titers * cGVHD	**2.10**	**1.05 – 4.18**	**0.036**
Low peak titers * cGVHD	0.79	0.47 – 1.32	0.361
<500 copies/ml * cGVHD	—	—	—
High peak titers * Age>50	1.32	0.59 – 2.97	0.496
Intermediate peak titers * Age>50	**0.31**	**0.16 – 0.63**	**0.001**
Low peak titers * Age>50	0.69	0.40 – 1.19	0.182
<500 copies/ml * Age>50	—	—	—
Non-relapse mortality			
High peak titers * aGVHD (2-4)	0.45	0.19 – 1.06	0.067
Intermediate peak titers * aGVHD (2-4)	2.59	0.72 – 9.36	0.147
Low peak titers * aGVHD (2-4)	0.85	0.41 – 1.74	0.646
<500 copies/ml * aGVHD (2-4)	—	—	—
High peak titers * cGVHD	1.09	0.46 – 2.56	0.844
Intermediate peak titers * cGVHD	2.28	0.97 – 5.36	0.058
Low peak titers * cGVHD	0.88	0.43 – 1.81	0.728
<500 copies/ml * cGVHD	—	—	—
High peak titers * Age>50	1.30	0.42 – 4.01	0.650
Intermediate peak titers * Age>50	**0.22**	**0.09 – 0.57**	**0.002**
Low peak titers * Age>50	**0.37**	**0.17 – 0.81**	**0.014**
<500 copies/ml * Age>50	—	—	—
Relapse			
High peak titers * cGVHD	1.05	0.37 – 3.02	0.927
Intermediate peak titers * cGVHD	1.43	0.48 – 4.22	0.520
Low peak titers * cGVHD	0.92	0.49 – 1.73	0.799
<500 copies/ml * cGVHD	—	—	—

Abbreviations: *CI,* Confidence interval; *CMV,* Cytomegalovirus; *HR,* hazard ratio; aGVHD, acute Graft-versus Host disease, cGVHD, chronic Graft-versus Host disease
—, reference group.
* indicates interaction analysis using interaction terms.

Supplementary Table S6. Analysis of overall survival in cohorts stratified by different time points of initial CMV reactivation (≤d+10, ≤d+20, ≤d+30, ≤d+40, ≤d+50, ≤d+60 after HCT).

	n	**%**	**Median OS in months**	***p***
No CMV reactivation	354	50	*	
Reactivation (day+10)				
CMV reactivation ≤d+10	2	0	1	**<0.0001**
CMV reactivation >d+10	348	50	29	
Reactivation (day+20)				
CMV reactivation ≤d+20	21	3	12	**0.045**
CMV reactivation >d+20	329	47	36	
Reactivation (day+30)				
CMV reactivation ≤d+30	140	20	17	0.057
CMV reactivation >d+30	210	30	45	
Reactivation (day+40)				
CMV reactivation ≤d+40	259	37	25	0.112
CMV reactivation >d+40	91	13	50	
Reactivation (day+50)				
CMV reactivation ≤d+50	318	45	28	0.119
CMV reactivation >d+50	32	5	42	
Reactivation (day+60)				
CMV reactivation ≤d+60	341	48	29	0.119
CMV reactivation >d+60	9	1	*	

Abbreviations: *CMV*, Cytomegalovirus; *, Median OS not reached within 60 months.

Supplementary Figure Titles

Supplementary Figure 1. CONSORT flow diagram of patient selection. Applied selection criteria: 1) a minimum of ≥ 5 CMV PCR samples during the first 200 days after HCT, 2) use of CMV PCR assays with a detection limit below 500 copies/ml and 3) allogeneic HCT between 01/2012 and 12/2017.

Supplementary Figure 2. Passing-Bablok regression comparing both CMV assays. Non-parametric Passing-Bablok regression for comparison of validation assays of the two CMV qPCR kits (Qiagen and Abbott Molecular) used in this study. This internal validation assay was performed on 83 whole blood samples at the Institute for Virology of University Hospital Essen. Observed values, converted into a logarithmic scale, are shown by green circles. Both assays were considered comparable with an $r=0.939$ and 95% CI's of α and β of -0.011 – 0.485 and 0.913 – 1.048, respectively. The regression function (solid line) is almost overlapping with the identity function (x=y; dashed line).

Supplementary Figure 3. Visualization of the methodological workflow of this study. The methodological workflow used to analyze 705 patients after HCT is shown. Patients' CMV PCR data of 11 508 analyzed whole blood samples were integrated into two independent, kinetics-based risk models according to their 1) CMV peak titers: Low, intermediate or high, and 2) time point of first CMV viremia (≤d+30 or ≥d+31 after HCT). Within each model, clinical outcome and cellular immune reconstitution were correlated.

Supplementary Figure 4. CMV reactivation and serostatus constellation. (A) Kaplan-Meier OS analysis stratified by CMV reactivation (yes/no), from HCT until death by any cause. Data was censored at 60 months. **(B)** Correlation of CMV serostatus and CMV reactivation. Statistical significance was tested with the *two-sample t test* as $p<0.0001$ for all combinations, except for R+/D+ and R+/D- with $p=0.224$. **(C, D)** Kaplan-Meier OS analysis stratified by CMV serostatus subgroups.

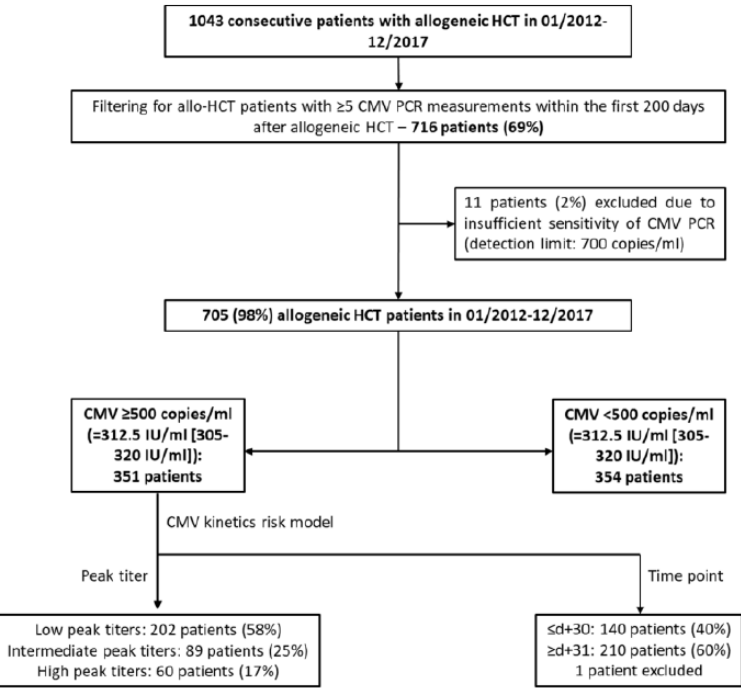
Supplementary Figure 5. CMV reactivation ≤d+30 after HCT is associated with increased mortality. (A) Histogram of initial CMV reactivation with a Gaussian normal distribution and a median on day +33. **(B)** Proportion of patients in relevant time point subgroups. **(C)** Kaplan-Meier OS analysis categorized as in (B), from HCT until death by any cause. Data was censored at 60 months after HCT. **(D)** Correlation of CMV serostatus with time point cohorts. Statistical significance was tested with the *two-sample t test* as $p<0.0001$ for all combinations, except for R+/D+ and R+/D- with $p=0.014$.

Supplementary Figure 6. Comparison of additional cellular immune reconstitution patterns of CMV peak titer cohorts. Peripheral blood lymphocyte subsets were measured by flow cytometry. Cell subsets within the CD45+ gate were characterized as follows: **(A)** Naïve helper T cells, $CD3^+/CD4^+/CD45RA^+$; **(B)** Memory helper T cells, $CD3^+/CD4^+/CD45RO^+$; **(C)** Activated T cells, $CD3^+/HLA\text{-}DR^+$; **(D)** B cells, $CD19^+$; **(E)** naïve cytotoxic T cells, $CD3^+/CD8^+/CD45RA^+$; **(F)** memory cytotoxic T cells, $CD3^+/CD8^+/CD45RO^+$. Median absolute cell numbers and the 95% CI were analyzed by the Mann-Whitney-U-test. A p-value < 0.05 was considered statistically significant and indicated in the figure with an arrow instead of an asterisk, $p < 0.01$ was indicated with two arrows, $p < 0.001$ was indicated with three arrows and $p < 0.0001$ was indicated with four arrows. An arrow shown in brackets refers to p-values < 0.05 – 0.10. Arrow direction illustrates higher or lower levels. Cohort and sample numbers: CMV < 500 copies/ml (purple line) around 1 month (n = 134), 3- (n = 110), 6- (n = 140), 9- (n = 121) and 12 months (n = 107); low peak titers (yellow line) around 1 month (n = 78), 3- (n = 60), 6- (n = 76), 9- (n = 64) and 12 months (n = 63); intermediate peak titers around 1 month (n = 35), 3- (n = 34), 6- (n = 35), 9- (n = 36) and 12 months (n = 26); high peak titers around 1 month (n = 21), 3- (n = 17), 6- (n = 12), 9- (n = 10) and 12 months (n = 18). **(E)** and **(F)** cohort and sample numbers: CMV no viremia (purple line) around 1 month (n = 24), 3- (n = 33), 6- (n = 44), 9- (n = 49) and 12 months (n = 44); low peak titers (yellow line) around 1 month (n = 21), 3- (n = 22), 6- (n = 33), 9- (n = 28) and 12 months (n = 29); intermediate peak titers around 1 month (n = 7), 3- (n = 5), 6- (n = 12), 9- (n = 13) and

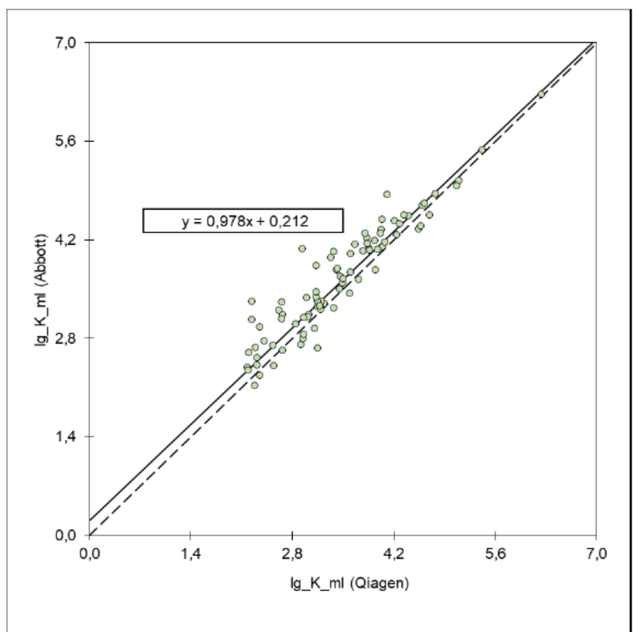
12 months (n = 6); high peak titers around 1 month (n = 4), 3- (n = 9), 6- (n = 5), 9- (n = 8) and 12 months (n = 11).

Supplementary Figures

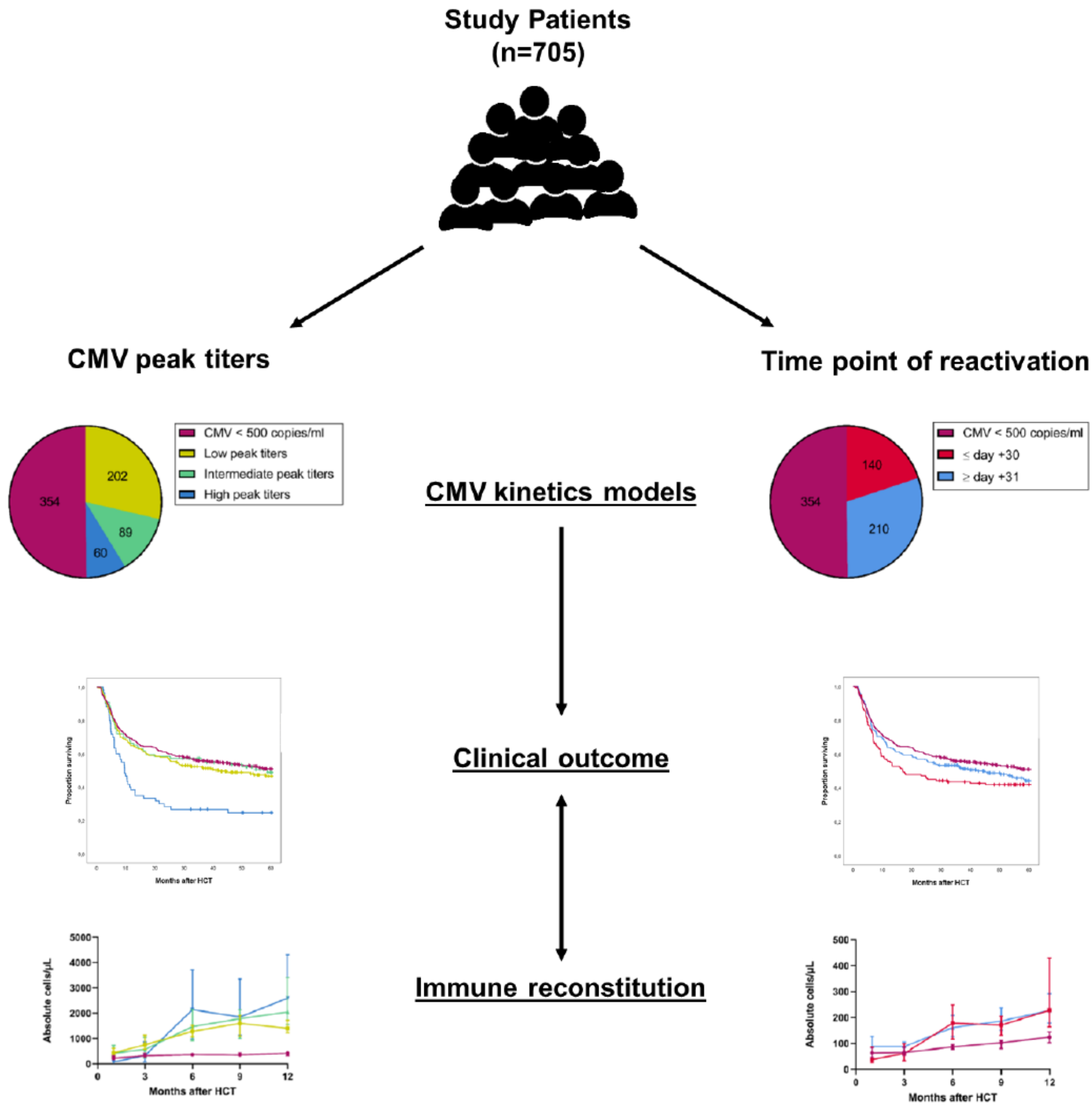
Supplementary Figure 1. CONSORT flow diagram of patient selection.



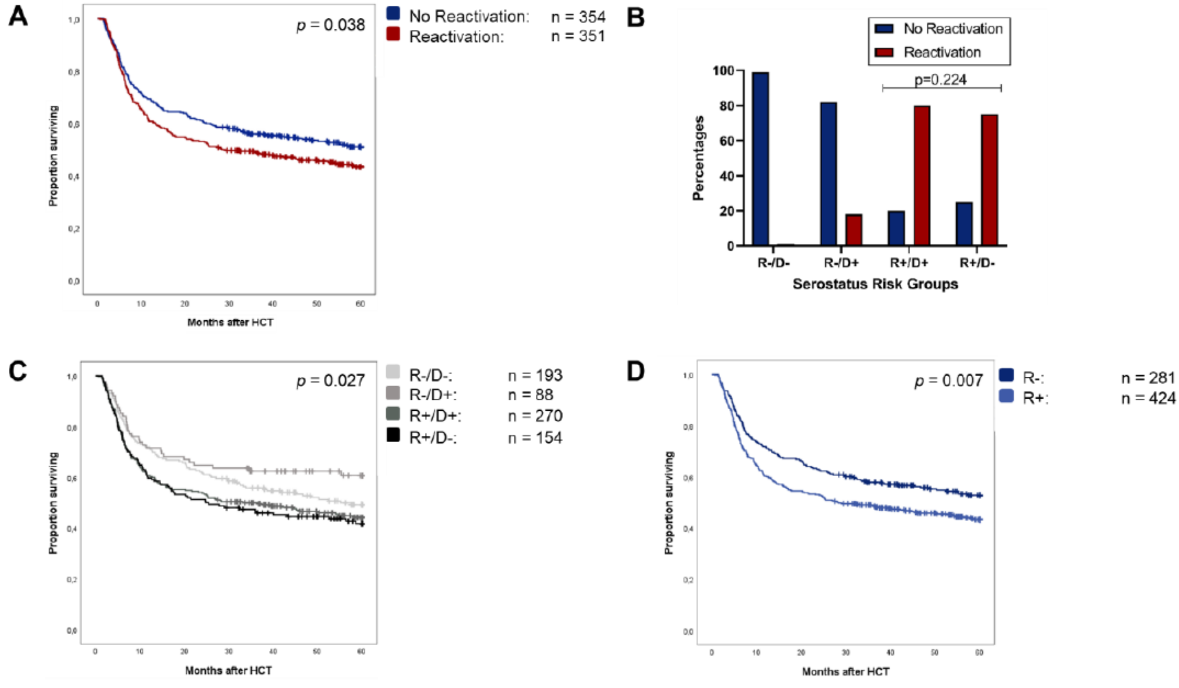
Supplementary Figure 2. Passing-Bablok Regression comparing both CMV assays.



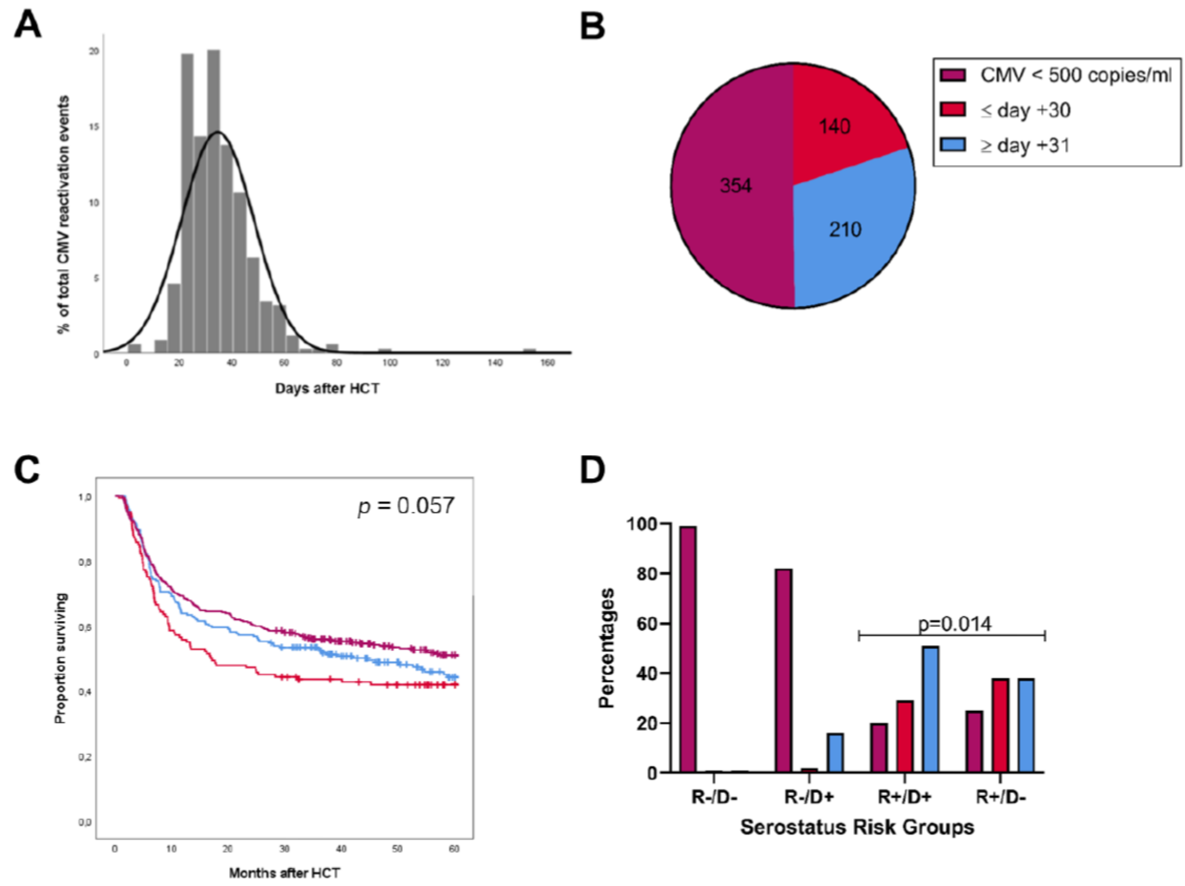
Supplementary Figure 3. Visualization of the methodological workflow of this study.



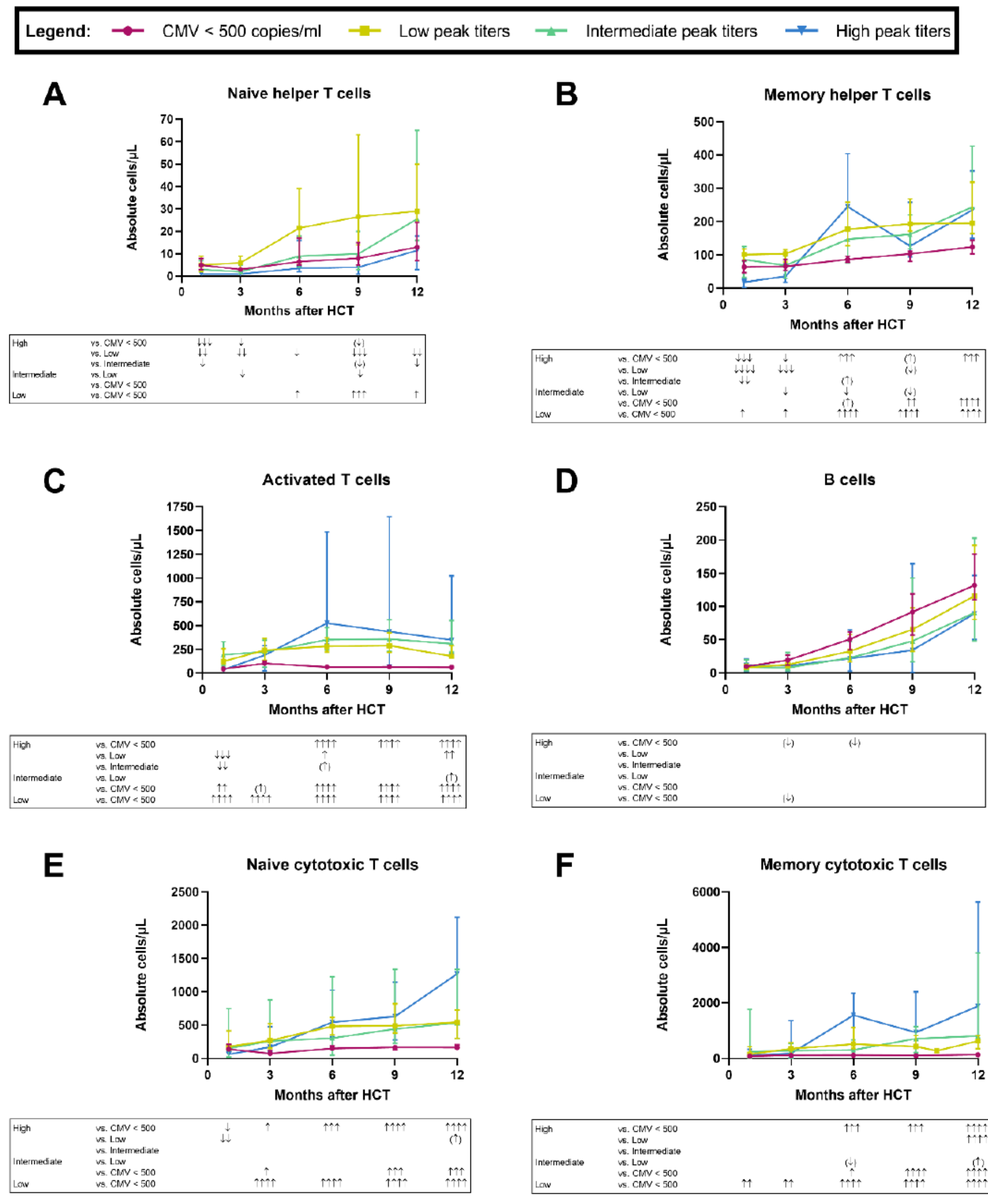
Supplementary Figure 4. CMV reactivation and serostatus constellation.



Supplementary Figure 5. CMV reactivation ≤d+30 after HCT is associated with increased mortality.



Supplementary Figure 6. Comparison of additional cellular immune reconstitution patterns of different peak titer cohorts.



6.2 Article II

Author contributions

Impact of CMV reactivation on relapse of acute myeloid leukemia after HCT is dependent on disease stage and ATG

Amin T. Turki, Nikolaos Tsachakis-Mück, **Saskia Leserer**, Pietro Crivello, Tobias Liebregts, Luisa Betke, Ferras Alashkar, Nils B. Leimkühler, Mirko Trilling, Katharina Fleischhauer and Dietrich W. Beelen

Original Article in **Blood Advances**[161]

Impact factor: 6.799 (2020)

Status: final version published online on December 30th, 2021, Blood advances (prepublished online on October 7th, 2021), licensed under CC BY-NC-ND 4.0, article included without any changes

DOI: 10.1182/bloodadvances.2021005509; PubMed-ID: 34619756

Complete citation of the original source:

Amin T. Turki, Nikolaos Tsachakis-Mück, Saskia Leserer, Pietro Crivello, Tobias Liebregts, Luisa Betke, Ferras Alashkar, Nils Bastian Leimkühler, Mirko Trilling, Katharina Fleischhauer, Dietrich W. Beelen; Impact of CMV reactivation on relapse of acute myeloid leukemia after HCT is dependent on disease stage and ATG. Blood Adv 2021; bloodadvances.2021005509. doi: https://doi.org/10.1182/bloodadvances.2021005509

Contributions:

Conception:	0 %	
Experimental work:	20 %	- data collection
Data analysis:	0 %	
Statistical analysis:	10 %	- data interpretation
Writing the manuscript:	20 %	- visualization of the results, writing the manuscript
Revising the manuscript:	10 %	- Revision of the reviewed manuscript

Prof. Dr. med. Dietrich W. Beelen

Saskia Leserer

REGULAR ARTICLE

blood advances

Impact of CMV reactivation on relapse of acute myeloid leukemia after HCT is dependent on disease stage and ATG

Amin T. Turki,[1,2] Nikolaos Tsachakis-Mück,[1] Saskia Leserer,[1,2] Pietro Crivello,[2] Tobias Liebregts,[1] Luisa Betke,[2] Ferras Alashkar,[1] Nils B. Leimkühler,[1] Mirko Trilling,[3] Katharina Fleischhauer,[2,4] and Dietrich W. Beelen[1]

[1]Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, [2]Institute of Experimental Cellular Therapy, West-German Cancer Center, [3]Institute for Virology, University Hospital Essen, Essen, Germany; and [4]German Cancer Consortium - Deutsches Konsortium für Translationale Krebsforschung (DKTK)

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

Key Points

- The impact of CMV reactivation on hematologic relapse after HCT is modulated by AML stage (CR1 or advanced) and in vivo T cell depletion.
- Following CMV reactivation, NRM was increased in CR1 patients without ATG, but not in patients with ATG or advanced disease stages.

Cytomegalovirus (CMV) reactivation is a frequent complication after allogeneic hematopoietic cell transplantation (HCT), whose impact on clinical outcome, in particular on leukemic relapse, is controversial. We retrospectively analyzed 687 HCT recipients with acute myeloid leukemia (AML) and ciclosporin-based immunosuppression to better understand the differential impact of CMV on transplant outcomes depending on AML disease stage and in vivo T cell depletion with antithymocyte globulin (ATG). Without ATG, CMV reactivation associated with significantly reduced relapse, yet its effect was more pronounced for advanced disease AML ($P = .0002$) than for patients in first complete remission (CR1, $P = .0169$). Depending on the disease stage, ATG exposure abrogated relapse protection following CMV reactivation in advanced stages ($P = .796$), while it inverted its effect into increased relapse for CR1 patients ($P = .0428$). CMV reactivation was associated with significantly increased nonrelapse mortality in CR1 patients without ATG ($P = .0187$) but not in those with advanced disease and ATG. Following CMV reactivation, only patients with advanced disease had significantly higher event-free survival rates as compared with patients without CMV. Overall, our data suggest that both ATG and disease stage modulate the impact of post-HCT CMV reactivation in opposite directions, revealing a level of complexity that warrants future studies regarding the interplay between antivirus and antitumor immunity.

Introduction

Cytomegalovirus (CMV) reactivation is a very common complication after allogeneic hematopoietic cell transplantation (HCT).[1-3] However, its impact on clinical outcome has been controversial: most studies associate CMV viremia and particularly the development of CMV end-organ disease with decreased overall survival (OS)[4,5] and with increased nonrelapse mortality (NRM) across different hematologic malignancies.[4-8] Conversely, other studies did not find such associations between CMV reactivation and NRM,[9,10] or observed comparable OS of patients with and without CMV reactivation.[7,11,12] Based on baseline characteristics, recent registry studies from Japan[13] and France[14] defined CMV risk scores for NRM and CMV reactivation that will need further prospective validation. Currently, the donor (D) and recipient (R) CMV serostatus are the standard risk indicators of CMV reactivation and OS after HCT.[15] The D−/R− serostatus was shown to associate with higher OS,[4,5] while the R+ serostatus associated

Submitted 11 June 2021; accepted 26 August 2021; prepublished online on *Blood Advances* First Edition 7 October 2021; final version published online 30 December 2021. DOI 10.1182/bloodadvances.2021005509.

Requests for data sharing may be submitted to Dietrich W. Beelen (dietrich.beelen@uk-essen.de).

The full-text version of this article contains a data supplement.

© 2021 by The American Society of Hematology. Licensed under Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0), permitting only noncommercial, nonderivative use with attribution. All other rights reserved.

with higher rates of both CMV reactivation and NRM.[4] As a consequence of these differential outcomes, many HCT centers use the CMV serostatus for donor selection.

A major controversy in ongoing discussions on CMV revolves around its potential protective impact on leukemic relapse. Reduced relapse rates in HCT recipients with CMV replication have been first reported in 1986,[16] and the effect of CMV seropositivity on relapse independent of chronic graft-versus-host disease (GVHD) was described in the early 2000s.[17] A study from our HCT department associated CMV pp65 antigenemia with reduced relapse, independent from D-R CMV serostatus.[9] Other studies confirmed this finding for HCT patients with acute leukemia,[7,18] chronic myeloid leukemia,[11] myeloproliferative disorders[12] and lymphoma[19] applying either pp65 detection or CMV-specific, quantitative polymerase chain reaction (qPCR) assays. Intriguingly, it was shown that this association between CMV and relapse was not observed in patients with antithymocyte globulin (ATG) exposure.[20] Accordingly, registry studies reported comparable relapse rates for patients with or without CMV reactivation.[4,21] Different CMV detection methods and thresholds defining reactivations complicated the comparison of results across different studies and countries. While qPCR has become the current standard for monitoring CMV in most countries, it is not approved for example, in Japan.[22] Differences in sensitivity between various assays had also been discussed.[23] Given these controversies, we retrospectively analyzed a large, longitudinal cohort of HCT recipients transplanted for acute myeloid leukemia (AML) at our center to better understand the differential impact of CMV on transplant outcomes depending on disease stage, detection technique, and in vivo T cell depletion with ATG.

Patients and methods

Patients

Between October 1997 and October 2017, 687 patients with AML underwent HCT with a uniform calcineurin inhibitor-based GVHD prophylaxis (predominantly ciclosporin plus methotrexate) in the Department of Bone Marrow Transplantation of the West-German Cancer Center at University Hospital Essen. Donors were HLA-matched related donors (MRD, 31%), 10/10 HLA-A-, -B, -C, -DRB1, -DQB1 matched unrelated donors (MUD, 62%), or 9/10 mismatched unrelated donors (MMUD, 7%; Table 1). HLA-DPB1 was not considered for donor-recipient matching. Patients receiving haploidentical HCT with post-transplant cyclophosphamide were not included. Assignment to ATG-prophylaxis was based on standardized clinical treatment protocols (detailed in supplemental Methods) for patients with higher GVHD risk. Patients were followed-up for 60 months after transplantation; surviving patients were censored at maximum follow-up. Early supportive and follow-up care was identical for all patients. The primary study endpoint was relapse, additional endpoints were NRM, acute and chronic GVHD, OS, and event-free survival (EFS). Details on patient treatment, HCT specific assessments, and endpoints are provided in the supplemental Methods section.

CMV monitoring

Starting with leukocyte reconstitution >500/µL, CMV titers were measured twice weekly at the Institute for Virology using qPCR[24] or CMV phosphoprotein pp65 antigenemia assay[25] until hospital discharge. Details of both assays are described in the supplemental Methods. Outpatient sampling was done weekly until week 16 after transplantation. Results were expressed as measured CMV copies/mL or as pp65 antigen expressing cells per 5×10^5 leukocytes. CMV reactivation was defined as a replication of >500 CMV copies per mL EDTA blood or as >5 pp65 antigen expressing cells per 5×10^5 white blood cells. In CMV *R*−/D− patients, CMV de novo replication was detected with the same methods. Only in 5 out of 203 R−/D− patients (2%) a primary CMV infection was detected over this long-term observation period.

Statistical analysis

For discrete variables, we applied the Fisher-Exact 2-tailed test. Continuous variables, described with median and extreme values (min-max), were studied with the Wilcoxon rank sum test. Cumulative incidences of relapse and NRM were calculated as time-dependent endpoints with mutually competing events. The homogeneity of the cumulative incidence functions was tested by the Gray method.[26] Corresponding subdistribution hazards and 95% confidence intervals (95% CI) were calculated using the Fine and Gray method.[27] OS and EFS were analyzed with the Kaplan-Meier method. The log-rank test compared the heterogeneity of survival distributions. *P*-values in the log-rank test were calculated for 2-sided 95% CIs, which was also adopted for Cox-regression analyses. For multiple testing, the significances were adjusted according to the method of Šidák,[28] and a *P*-value <.05 was accepted to indicate statistical significance. The multivariate Cox regression models for relapse included the following factors: AML disease stage at HCT, HLA disparities, gender constellation (female donor for male recipient vs others), bone marrow or PBSC, conditioning, CMV+ serostatus, CMV reactivation, and acute and chronic GVHD. Nonbaseline factors, such as CMV reactivation and acute and chronic GVHD, were integrated as variables into the multivariate models. All analyses were performed with Statistical Analysis Software (SAS, Release 9.4, Version 7.11 (7.100.1.2711); SAS Institute Inc., Cary, NC).

Ethics

This study was conducted in accordance with German legislation and the revised Helsinki Declaration. Study design and data acquisition was evaluated by the institutional review board of the University Duisburg-Essen (Protocol No. 18-8496-BO). All patients have given written consent to collection, electronic storage, and scientific analysis of anonymized HCT-specific patient data. We confirm that no patient can be identified by use of anonymized patient data.

Results

A total of 687 consecutive patients with AML underwent HCT with a uniform calcineurin inhibitor-based GVHD prophylaxis. A relevant fraction (N = 267, 39%) additionally received in vivo T cell depletion using ATG. Patient baseline characteristics including disease status at HCT; transplant, donor, and gender constellation; CMV serostatus; and conditioning regimen are detailed in Table 1.

CMV and relapse

The overall incidence of early CMV reactivation (before d+100) was up to 52% when measured by qPCR. CMV reactivation occurred significantly more frequently in patients with ATG exposure (48.7%) than in patients without ATG (27.8%, *P* < .0001).

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

Table 1. Patient baseline characteristics

N (%)	Overall cohort N (%)	No ATG N (%)	ATG	P
Total enrolled and treated, n (%)	687 (100)	420 (100)	267 (100)	
Median age at HCT (range)	50 (16-76)	48 (16-73)	55 (18-76)	<.0001
Male gender, n (%)	346 (51)	228 (54)	118 (44)	.0121
Acute myeloid leukemia	687 (100)	420 (100)	267 (100)	
First CR,* n (%)	293 (43)	164 (39)	129 (48)	.017
Advanced disease stages,† n (%)	394 (57)	256 (61)	138 (52)	
Cytomegalovirus serostatus constellation, n (%)				
D+/R−	52 (8)	29 (7)	23 (9)	<.0001
D+/R+	285 (41)	172 (41)	113 (42)	
D−/R+	147 (21)	76 (18)	71 (27)	
D−/R−	203 (30)	143 (34)	60 (22)	
Donor-recipient constellations				
MRD	214 (31)	214 (51)	0 (0)	<.0001
MUD	424 (62)	198 (47)	226 (85)	
MMUD	49 (7)	8 (2)	41 (15)	
D/R gender: f/m	77 (11)	58 (14)	19 (7)	.0063
Other	610 (89)	362 (86)	248 (93)	
Donor age, median (95% CI)	38 (20-64)	41 (22-64)	32 (20-52)	<.0001
Graft source				
PBSC	617 (90)	365 (87)	255 (96)	<.0001
BM	67 (10)	55 (13)	12 (4)	
Conditioning				
MAC	272 (40)	226 (54)	46 (17)	<.0001
RIC	412 (60)	191 (46)	221 (83)	<.0001

95% CI, 95% confidence interval; BM, bone marrow; D, donor; HCT, allogeneic hematopoietic stem cell transplantation; MAC, myeloablative conditioning; MRD, matched related donor transplant; MUD, matched unrelated donor transplant; PBSC, peripheral blood stem cells; R, recipient; RIC, reduced intensity conditioning.
*De novo AML in first complete remission (CR).
†All other disease stages that did not correspond to AML in first CR, such as AML in second remission.

No significant differences were detected within ATG dosage subgroups. In absence of ATG prophylaxis, CMV reactivation associated with significantly reduced relapse rates for all AML disease stages. The relapse incidence for patients with CMV reactivation was 0.18 (95% confidence interval [CI], 0.12-0.25) compared with 0.41 (95% CI, 0.35-0.46) for patients without CMV (Figure 1A, $P < .0001$). However, exposure to ATG abrogated this protective effect on relapse (Figure 1B, $P = .1935$). That same association and its ATG-dependent loss were confirmed for the subgroup of patients with advanced disease stages ($P = .0002$, Figure 1C-D). Also, in the no-ATG subgroup of AML patients in CR1 the relapse incidence was significantly lower with CMV reactivation (0.14, 95% CI 0.05-0.27) than without (0.35, 95% CI 0.26-0.43; Figure 1E, $P = .0169$). In addition to the previously described reduction of early relapse events in the presence of CMV reactivation after HCT, the present data also revealed a reduction in late relapse events (>24 months) for patients with CMV reactivation in the absence of ATG (Figure 1A,C,E). In contrast, the effect on late relapse events was again not detectable in the ATG-receiving cohorts (Figure 1B,D,F), which is indicative of the impact of ATG-susceptible cells, most likely T cells, in the containment of late relapse. Interestingly, the opposite was observed for CMV reactivation in AML patients in CR1 who had received ATG for in vivo T cell depletion (Figure 1F). Here, CMV reactivation associated with an inverse effect of significantly increased relapse ($P = .0428$). Due to the above-described differences between patients with or without ATG exposure, we separately analyzed the cohorts in a series of multivariate analyses including the above-mentioned covariables. For 420 patients without ATG, multivariate analysis confirmed CMV reactivation as an independent significant factor of relapse after HCT (Figure 2A; hazard ratio [HR] 0.42, 95% CI 0.26-0.68) along with chronic GVHD (HR 0.44, 95% CI 0.30-0.65) and positive donor serostatus (CMV+; HR 0.69, 95%CI 0.48-0.87). In the 267 patients that received ATG, multivariate analysis did not associate these cofactors with significant differences in relapse (Figure 2B). Both CMV+ donor serostatus (HR 0.82, 95% CI 0.54-1.24) and CMV reactivation did not reach significance after ATG exposure (HR 1.35, 95% CI 0.87-2.10). Of notice, the R−/D+ CMV serostatus alone also associated with reduced relapse in patients without ATG ($P = .0179$, Figure 2C) but had a numerically, nonsignificant, increased relapse with ATG (Figure 2D).

OS, NRM, EFS, and GVHD

OS did not significantly differ between the cohort with or without CMV reactivation ($P = .833$, supplemental Figure 1A), while the

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

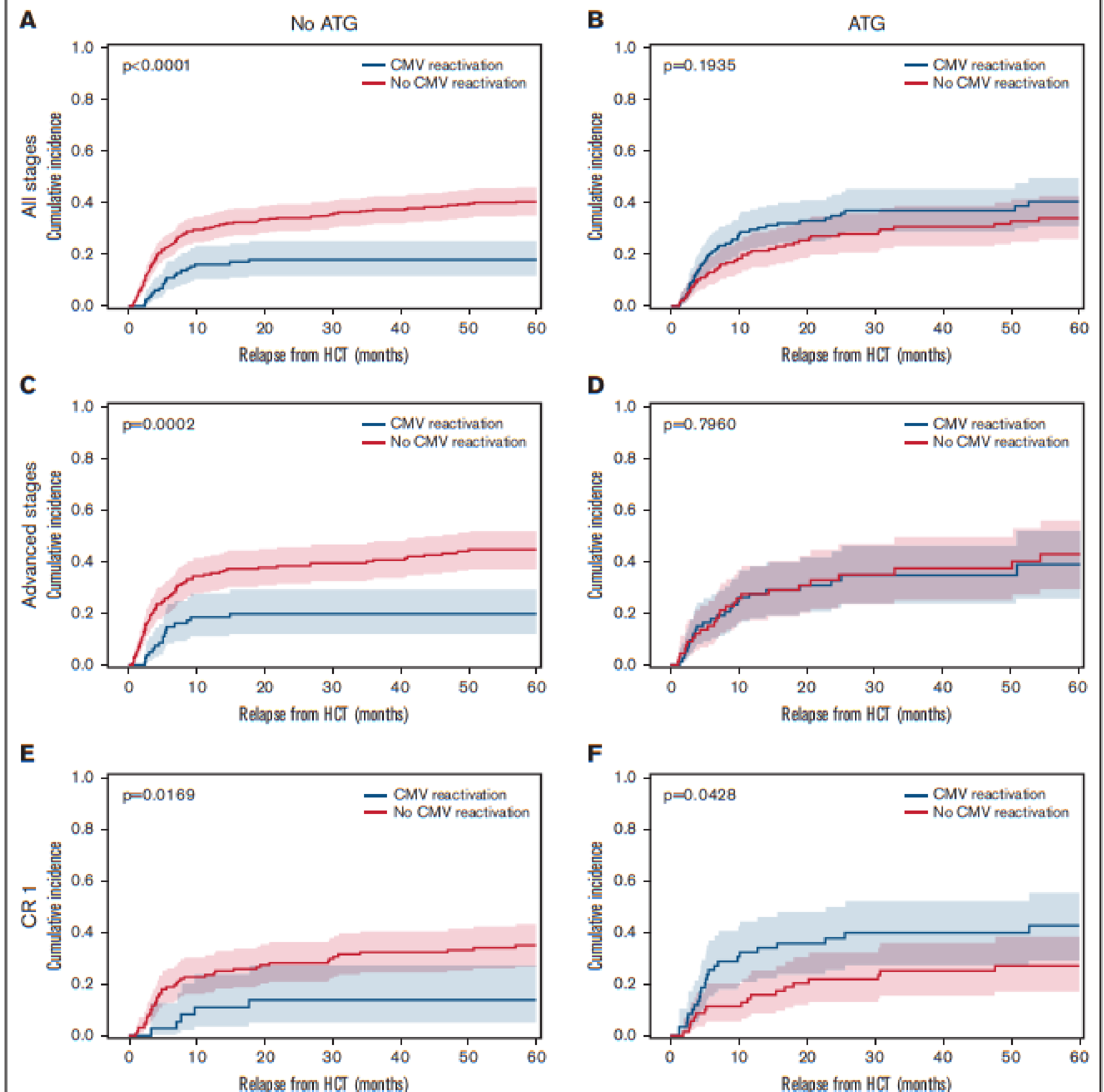


Figure 1. Cumulative relapse incidence depending on CMV reactivation, in vivo T cell depletion and disease stage. (A) Patient cohort without ATG (n = 420). (B) Patient cohort with ATG (n = 267). (C) Advanced disease stages subgroup without ATG (n = 256). (D) Advanced disease stages subgroup with ATG (n = 138). (E) AML in CR1 subgroup without ATG (n = 164). (F) AML in CR1 subgroup with ATG (n = 129). Cumulative incidence function of relapse (60 months censored) depending on CMV reactivation (blue) and absence of CMV reactivation (red). Median (line) with 95% confidence interval (CI) shaded. All *P* values refer to comparisons of strata with Gray's test.

NRM was significantly increased in patients with CMV reactivation (*P* = .0424, supplemental Figure 1B). Patients in CR1 had higher NRM following CMV reactivation, while patients with advanced AML stages had higher NRM than CR1 patients regardless of CMV reactivation (supplemental Figure 1C-D; Figure 3A-B). The increased NRM of patients in CR1 with CMV reactivation, however, only reached significance in the no-ATG subgroup (*P* = .0187, Figure 3C-D). In both disease constellations, ATG exposure associated with relatively lower NRM (Figure 3B,D) due to reduced GVHD (supplemental Table 1). The observed significant differences in relapse (Figure 1C,E) translated into significantly improved OS for AML patients with CMV reactivation and advanced disease (*P* = .0485). The 4 CMV serostatus risk categories (D+/R+, D+/R−, D−/R+, D−/R−) had a significant impact on OS only in

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

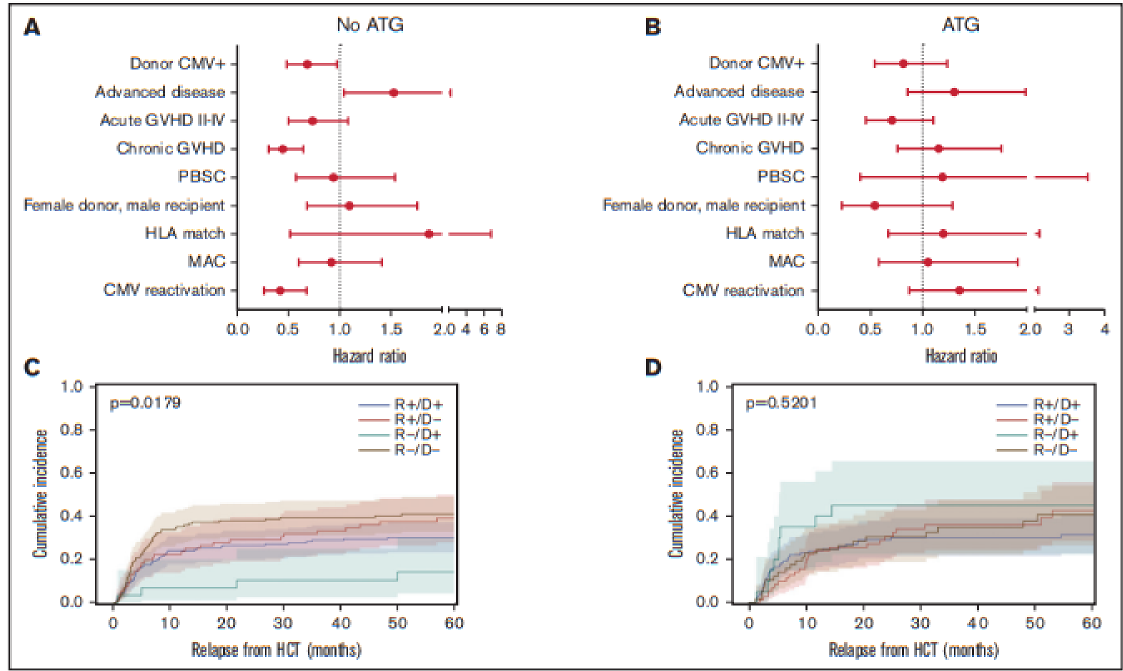


Figure 2. Multivariate analysis of relapse influencing variables and impact of pre-HCT serostatus constellation. (A) Patient cohort without ATG (n = 420). Multivariate analysis of relapse as time-dependent endpoint. Forest plots showing results from multivariate analysis including all significant covariates from univariate analysis with respect to relapse. (B) Patient cohort with ATG (n = 267). Multivariate analysis of relapse as time-dependent endpoint. Plots as in panel A. (C) Cumulative relapse incidence depending on CMV serostatus without ATG and (D) with ATG. Cumulative incidence function of relapse (60 months censored) compared by CMV serostatus (R+/D+ [blue], R+/D− [red], R−/D+ [green], and R−/D− [brown]. Median [line] with 95% CI shaded). C+D: *P* values according to Gray's test. Abbreviations: GVHD, graft-versus-host disease; HLA, human leukocyte antigen; MAC, myeloablative conditioning; PBSC, peripheral blood derived stem cells.

the absence of ATG (*P* = .0446, supplemental Figure 2A). Without ATG, positive donor serostatus (D+) also associated with significantly higher OS (*P* = .0059, supplemental Figure 2C). The EFS of the overall cohort showed no significant differences depending on CMV reactivation. However, when patients were stratified by disease stage and ATG exposure, CMV reactivation associated with significantly higher EFS in patients with advanced disease or without ATG, while the opposite was observed for patients in CR1 or with ATG exposure (supplemental Figure 3). As expected, the incidence of grades III-IV acute GVHD or extensive chronic GVHD was higher in patients without ATG exposure (supplemental Table 1). Both grades II-IV acute GVHD and CMV reactivation reduced the cumulative incidence of relapse (supplemental Figure 4). Interestingly, additive relapse reduction effects were observed for sequential events of acute GVHD and CMV reactivation. In the no-ATG subgroup the sequence of acute GVHD followed by CMV reactivation associated with reduced relapse, while this was not the case for the ATG subgroup with the same sequence. Although being a small subgroup, the sequence of CMV reactivation followed by acute GVHD resulted in the lowest relapse rate both with and without ATG (*P* = .0148).

qPCR and pp65 CMV detection methods

In order to analyze the impact of the CMV detection method on the reporting of CMV reactivation rates, we separately analyzed patients monitored by qPCR (n = 294) or pp65 antigen (n = 393) methods within each patient subset. In patients without ATG exposure, both methods led to comparable results. The cumulative incidence of early CMV reactivation detected by pp65 was 27.0% (95% CI 22.2-32.0, *P* = .39, supplemental Figure 5A) and 30.3% with qPCR (95% CI 21.7-39.5). In patients with ATG, however, the cumulative incidence of early CMV reactivation was significantly higher when measured by qPCR (52.6, 95% CI 45.3-59.4, *P* = .0176) than by pp65 (38.7, 95% CI 27.6-49.5), and the time to detection of CMV reactivation was shorter.

Discussion

This study adds several new facets to our understanding of the complexity of CMV reactivation after HCT. Our data independently confirms previous studies of reduced relapse risk for AML patients without ATG after CMV reactivation,[9,18] but it is also in full agreement with more recent reports,[4,20] which have shown that this effect is either abrogated or attenuated if ATG was administered. We show that the protective effect of CMV reactivation observed in patients with advanced disease not receiving ATG is inverted into a predisposing effect in CR1 patients with ATG and that this in turn leads to opposing effects of numerically higher or lower EFS, respectively. Together, our data show that the effect of CMV reactivation is inversely modulated by both the use of ATG and the

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

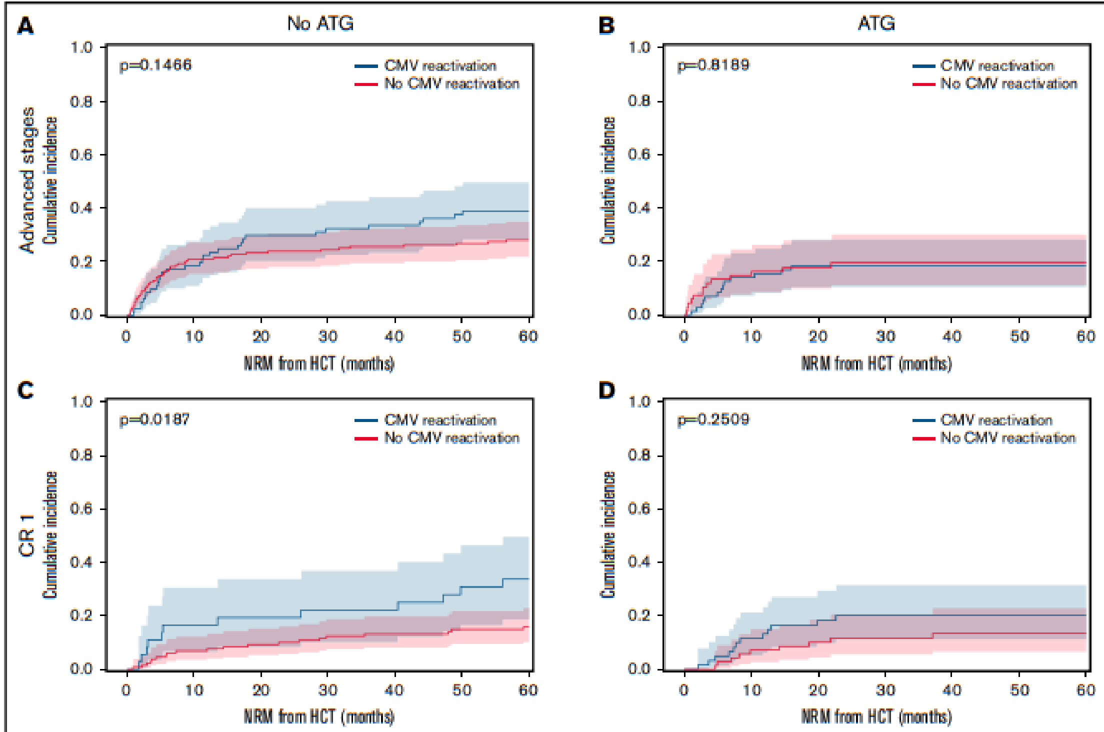


Figure 3. Cumulative incidence of NRM depending on CMV reactivation, in vivo T cell depletion and disease stage. Cumulative incidence function of nonrelapse mortality (NRM) with relapse as competing risk (60 months censored). Patients with CMV reactivation (blue) and without CMV reactivation (red). Median (line) with 95% CI shaded. (A) AML advanced disease stages without ATG (n = 256). (B) AML advanced disease stages with ATG (n = 138). (C) AML in CR1 without ATG (n = 164). (D) AML in CR1 with ATG (n = 129). All *P* values refer to comparisons of strata with Gray's test.

disease stage at transplant: while CMV reactivation has a positive effect due to lower relapse risks and no impact on NRM in patients with advanced disease not receiving ATG, it is deleterious in CR1 patients with ATG prophylaxis due to higher relapse rates. These findings have important implications for the reevaluation of a number of studies dealing with similar questions. To understand this seemingly paradox constellation, one also needs to integrate both the historical dimension of the discussion as well as novel insights into biological mechanisms. While the first study showing an impact of CMV reactivation on relapse exclusively used pp65 antigenemia monitoring,[9] as also did the subsequent confirmatory reports,[7,11,18] more recent studies used qPCR to confirm[10] or oppose[4,20] this finding. In our study, we compared the cumulative incidence of CMV reactivations depending on the detection method, and the number of CMV reactivations was higher in patients receiving ATG measured by qPCR. Beyond differences in the detection method, the definition of CMV reactivation cutoffs have been a matter of debate and heterogeneity between HCT studies.[10,29] Also, the proportion of patients who received ATG differed importantly between studies, ranging between 0%,[9] 17%[10] and 100%.[20] In order to overcome this bias, we separately analyzed CMV's association with relapse within the ATG subgroup (39%) and for all other patients. Furthermore, we distinguished AML disease stage subgroups, such as AML in CR1, which previous reports could not distinguish due to its small sample size[20] or due to registry data limitations.[4]

Despite previous reports discussing a biological effect of CMV viremia on leukemic relapse,[20,29,30] its exact mechanisms are still insufficiently understood. ATG exposure modulates how CMV replication affects the incidence of relapse after HCT,[20] and a recent study[31] highlighted the role of CMV kinetics and T cell subpopulations in this interaction. Indeed, the immune reconstitution of T helper cells and naïve T helper cells is impaired after ATG prophylaxis,[32] and also lower CD8+ T cell receptor (TCR) repertoire diversity has been reported compared with MUD patients without ATG exposure.[33,34] Poor T cell reconstitution may favor relapse.[30,35] CMV reactivation after HCT has complex implications on its host T cells, driving CD8+ activation,[36] narrowing the TCR repertoire[37] and might also influence the presentation of HLA mismatch antigens.[38] However, the overwhelming majority of published TCR repertoire data after CMV reactivation were obtained from HCT patients without ATG exposure.[33,36,39] Given the limited data involving ATG-exposed HCT patients with CMV,[33,38,40] one may speculate if the previously described CMV-induced skewing of the TCR repertoire[36] would be consistently detectable in AML patients with ATG or if additive effects would be observed. We observed relatively increased relapse rates for AML patients in CR1 with ATG, which

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

might possibly result from differential cross-reactive TCR profiles. In patients without ATG exposure, multivariate analysis associated CMV+ donor serostatus with reduced relapse. Lower TCR diversity has been described in HCT recipients from CMV+ donors,[33] and a previous large analysis of HCT donors revealed TCR repertoires specific to CMV+ donors,[41] which may possibly help to explain antirelapse effects as observed in our cohorts. Yet, the clinical impact of CMV+ donor serostatus is under discussion. A relevant European Society for Blood and Marrow Transplantation study focusing on CMV serostatus independent of the hematologic disease at HCT detected an increased relapse risk in MUD patients receiving a graft from a CMV+ donor but not in matched sibling donor HCT.[42] In 2 previous studies analyzing the impact of CMV reactivation on HCT patients with T cell depletion, such serostatus-association was not described[11] or analyzed.[20] The most recently described cytotoxic potential of CMV-induced CD57+/CD27− CD4+ T cells may reflect one mechanism of controlling CMV-infected myeloid cells.[43] With or without CMV reactivation, ATG exposed patients had more late relapse events without reaching a plateau. Novel ATG dose optimization strategies[32,44,45] may improve relapse incidence and modify CMV-dependent effects. While previous reports associated CMV reactivation both with reduced early (<12 months)[7] and later relapse,[9,18] our data highlight that a late-relapse (>18 months)-protective impact of CMV is only detected in patients without ATG. This study has limitations due to its retrospective character, the use of 2 different detection assays (qPCR, pp65), and the inclusion of data before the approval of Letermovir for the prophylaxis of CMV reactivation. Patients in the ATG cohort were older and had a higher proportion of MUD recipients and RIC conditioning than in the no-ATG cohort. The potentially resulting increased relapse events could have supported the detection of significant outcome associations. Still, RIC did not significantly impact the relapse rate in univariate and multivariate analysis including CMV reactivation and other cofactors. Despite serostatus proportions comparable to large registry studies,[4,5] the relatively small absolute number of patients with D+/R− serostatus might have influenced the OS rates for D+ patients. Both grades II-IV acute GVHD and CMV reactivation can occur sequentially and independently impact leukemic relapse. Sequential analysis of their interactions supported the hypothesis that acute GVHD may be more relevant as trigger of CMV reactivations[46] in the absence of ATG than in patients with ATG exposure. Due to the long patient inclusion period, only the first episode of CMV reactivation is documented. The increasing impact of haploidentical HCT is not covered by this study. Its strength is its large AML patient population with homogenous ciclosporin-based immunosuppression, which permitted differential subgroup analyses.

The clinical impact of CMV reactivation after HCT for the treatment of AML significantly depended on both disease stage and ATG, which differentially determined the impact of CMV reactivation on relapse rates and on other HCT outcomes. ATG prophylaxis and disease stage at HCT modulate the impact of post-HCT CMV reactivation in opposite directions, revealing a level of complexity that warrants future studies regarding the interplay between antivirus and antitumor immunity.

Acknowledgments

This study received support in part by Bundesministerium für Bildung und Forschung (BMBF) grant no. 031L0027D (D.W.B.), UDE-IFORES grant (A.T.T.) and Deutsche Forschungsgemeinschaft-UDE-UMEA grant FU 356/12-1 (A.T.T.). In part, support was received from DKMS grant number DKMS DKMS-SLS-MHG-2018-01 (P.C.). Open Access publication costs were covered by MERCUR-GYF-VI of the Mercator Foundation.

Authorship

Contribution: D.W.B. and A.T.T. designed the study; N.T.-M., T.L., and S.L. performed data collection; F.A. and L.B. participated in data acquisition; D.W.B., N.T.-M., and A.T.T. performed statistical analysis; A.T.T., D.W.B., P.C., K.F., and N.T.-M. interpreted data; M.T., N.B.L., and K.F. participated in data analysis; A.T.T. and D.W.B. wrote the manuscript; N.T.-M., P.C., K.F., and S.L. contributed to writing the manuscript; N.T.-M. contributed importantly to this manuscript with research performed in the framework of his MD thesis; and all authors had access to primary clinical trial data and read and approved the final manuscript.

Conflict-of-interest disclosure: A.T.T., consultancy for MSD, JAZZ Pharmaceuticals, CSL Behring. Travel subsidies from Neovii Biotech. All outside the submitted work. D.W.B. received travel subsidies from Medac, all outside the submitted work. The other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

ORCID profiles: A.T.T., 0000-0003-1347-3360; S.L., 0000-0002-4956-1760; P.C., 0000-0001-9668-5013; F.A., 0000-0001-7530-8892; N.B.L., 0000-0003-2530-4329; M.T., 0000-0003-3659-3541; K.F., 0000-0002-5827-8000; D.W.B., 0000-0001-5050-220X.

Correspondence: Dietrich W. Beelen, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen, Germany; e-mail: dietrich.beelen@uk-essen.de.

References

1. Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol Blood Marrow Transplant.* 2003;9(9):543-558.

2. George B, Kerridge IH, Gilroy N, et al. A risk score for early cytomegalovirus reactivation after allogeneic stem cell transplantation identifies low-, intermediate-, and high-risk groups: reactivation risk is increased by graft-versus-host disease only in the intermediate-risk group. *Transpl Infect Dis.* 2012;14(2):141-148.

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

3. Ljungman P. The role of cytomegalovirus serostatus on outcome of hematopoietic stem cell transplantation. *Curr Opin Hematol.* 2014;21(6):466-469.
4. Teira P, Battiwalla M, Ramanathan M, et al. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood.* 2016;127(20):2427-2438.
5. Schmidt-Hieber M, Labopin M, Beelen D, et al. CMV serostatus still has an important prognostic impact in de novo acute leukemia patients after allogeneic stem cell transplantation: a report from the Acute Leukemia Working Party of EBMT. *Blood.* 2013;122(19):3359-3364.
6. Broers AE, van Der Holt R, van Esser JW, et al. Increased transplant-related morbidity and mortality in CMV-seropositive patients despite highly effective prevention of CMV disease after allogeneic T-cell-depleted stem cell transplantation. *Blood.* 2000;95(7):2240-2245.
7. Green ML, Leisenring WM, Xie H, et al. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood.* 2013;122(7):1316-1324.
8. Camargo JF, Kimble E, Rosa R, et al. Impact of cytomegalovirus viral load on probability of spontaneous clearance and response to preemptive therapy in allogeneic stem cell transplantation recipients. *Biol Blood Marrow Transplant.* 2018;24(4):806-814.
9. Elmaagacli AH, Steckel NK, Koldehoff M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood.* 2011;118(5):1402-1412.
10. Manjappa S, Bhamidipati PK, Stokerl-Goldstein KE, et al. Protective effect of cytomegalovirus reactivation on relapse after allogeneic hematopoietic cell transplantation in acute myeloid leukemia patients is influenced by conditioning regimen. *Biol Blood Marrow Transplant.* 2014;20(1):46-52.
11. Ito S, Pophali P, Co W, et al. CMV reactivation is associated with a lower incidence of relapse after allo-SCT for CML. *Bone Marrow Transplant.* 2013;48(10):1313-1316.
12. Peric Z, Wilson J, Durakovic N, et al. Early human cytomegalovirus reactivation is associated with lower incidence of relapse of myeloproliferative disorders after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant.* 2018;53(11):1450-1456.
13. Kaito S, Nakajima Y, Hara K, et al. Heterogeneous impact of cytomegalovirus reactivation on nonrelapse mortality in hematopoietic stem cell transplantation. *Blood Adv.* 2020;4(6):1051-1061.
14. Beauvais D, Drumez E, Blaise D, et al. Scoring system for clinically significant CMV infection in seropositive recipients following allogenic hematopoietic cell transplant: an SFGM-TC study. *Bone Marrow Transplant.* 2021;56(6):1305-1315.
15. Ayuk F, Balduzzi A. Donor selection for adults and pediatrics. In: Carreras E, Dufour C, Mohty M, Kröger N, eds. The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies. Cham: Springer International Publishing; 2019:87-97.
16. Lönnqvist B, Ringdèn O, Ljungman P, Wahren B, Gahrton G. Reduced risk of recurrent leukaemia in bone marrow transplant recipients after cytomegalovirus infection. *Br J Haematol.* 1986;63(4):671-679.
17. Nachbaur D, Bonatti H, Oberaigner W, et al. Survival after bone marrow transplantation from cytomegalovirus seropositive sibling donors. *Lancet.* 2001;358(9288):1157-1159.
18. Takenaka K, Nishida T, Asano-Mori Y, et al. Cytomegalovirus reactivation after allogeneic hematopoietic stem cell transplantation is associated with a reduced risk of relapse in patients with acute myeloid leukemia who survived to day 100 after transplantation: the Japan Society for Hematopoietic Cell Transplantation Transplantation-related Complication Working Group. *Biol Blood Marrow Transplant.* 2015;21(11):2008-2016.
19. Koldehoff M, Ross SR, Dührsen U, Beelen DW, Elmaagacli AH. Early CMV-replication after allogeneic stem cell transplantation is associated with a reduced relapse risk in lymphoma. *Leuk Lymphoma.* 2017;58(4):822-833.
20. Busca A, Passera R, Pini M, et al. The use of ATG abrogates the antileukemic effect of cytomegalovirus reactivation in patients with acute myeloid leukemia receiving grafts from unrelated donors. *Am J Hematol.* 2015;90(6):E117-E121.
21. Remberger M, Ringdén O. Survival after bone-marrow transplantation. *Lancet.* 2002;359(9309):888.
22. Mori Y, Jinnouchi F, Takenaka K, et al. Efficacy of prophylactic letermovir for cytomegalovirus reactivation in hematopoietic cell transplantation: a multicenter real-world data. *Bone Marrow Transplant.* 2021;56(4):853-862.
23. Preiksaitis JK, Hayden RT, Tong Y, et al. Are we there yet? Impact of the first international standard for cytomegalovirus DNA on the harmonization of results reported on plasma samples. *Clin Infect Dis.* 2016;63(5):583-589.
24. Schnepf N, Scieux C, Resche-Riggon M, et al. Fully automated quantification of cytomegalovirus (CMV) in whole blood with the new sensitive Abbott RealTime CMV assay in the era of the CMV international standard. *J Clin Microbiol.* 2013;51(7):2096-2102.
25. Trenschel R, Ross S, Hüsing J, et al. Reduced risk of persisting cytomegalovirus pp65 antigenemia and cytomegalovirus interstitial pneumonia following allogeneic PBSCT. *Bone Marrow Transplant.* 2000;25(6):665-672.
26. Gray RJ. A class of K-sample tests for comparing the cumulative incidence of a competing risk. *Ann Stat.* 1988;16(3):1141-1154.
27. Fine JP, Gray RJ. A proportional hazards model for the subdistribution of a competing risk. *J Am Stat Assoc.* 1999;94(446):496-509.
28. Sidak Z. Rectangular confidence regions for the means of multivariate normal distributions. *J Am Stat Assoc.* 1967;62(318):626-633.
29. Elmaagacli AH, Koldehoff M. Cytomegalovirus replication reduces the relapse incidence in patients with acute myeloid leukemia. *Blood.* 2016;128(3):456-459.
30. Parkman R, Cohen G, Carter SL, et al. Successful immune reconstitution decreases leukemic relapse and improves survival in recipients of unrelated cord blood transplantation. *Biol Blood Marrow Transplant.* 2006;12(9):919-927.
31. Leserer S, Bayraktar E, Trilling M, et al. Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution. *Am J Hematol.* 2021;96(4):436-445.

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

32. Turki AT, Klisanin V, Bayraktar E, et al. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies. *Am J Transplant.* 2020;20(3):677-688.

33. Link-Rachner CS, Eugster A, Rücker-Braun E, et al. T-cell receptor-α repertoire of CD8+ T cells following allogeneic stem cell transplantation using next-generation sequencing. *Haematologica.* 2019;104(3):622-631.

34. Leick M, Gittelman RM, Yusko E, et al. T cell clonal dynamics determined by high-resolution TCR-β sequencing in recipients after allogeneic hematopoietic cell transplantation. *Biol Blood Marrow Transplant.* 2020;26(9):1567-1574.

35. Varanasi PR, Ogonek J, Luther S, et al. Cytomegalovirus-specific CD8+ T-cells are associated with a reduced incidence of early relapse after allogeneic stem cell transplantation. *PLoS One.* 2019;14(3):e0213739.

36. Suessmuth Y, Mukherjee R, Watkins B, et al. CMV reactivation drives post-transplant T cell reconstitution and results in defects in the underlying TCRβ repertoire. *Blood.* 2015;125(25):3835-3850.

37. Kanakry CG, Coffey DG, Towlerton AMH, et al. Origin and evolution of the T cell repertoire after posttransplantation cyclophosphamide. *JCI Insight.* 2016;1(5):e86252.

38. Meurer T, Crivello P, Metzing M, et al. Permissive HLA-DPB1 mismatches in HCT depend on immunopeptidome divergence and editing by HLA-DM. *Blood.* 2021;137(7):923-928.

39. Toya T, Taguchi A, Kitaura K, et al. T-cell receptor repertoire of cytomegalovirus-specific cytotoxic T-cells after allogeneic stem cell transplantation. *Sci Rep.* 2020;10(1):22218.

40. Link CS, Eugster A, Heidenreich F, et al. Abundant cytomegalovirus (CMV) reactive clonotypes in the CD8(+) T cell receptor alpha repertoire following allogeneic transplantation. *Clin Exp Immunol.* 2016;184(3):389-402.

41. Emerson RO, DeWitt WS, Vignali M, et al. Immunosequencing identifies signatures of cytomegalovirus exposure history and HLA-mediated effects on the T cell repertoire. *Nat Genet.* 2017;49(5):659-665.

42. Ljungman P, Brand R, Hoek J, et al; Infectious Diseases Working Party of the European Group for Blood and Marrow Transplantation. Donor cytomegalovirus status influences the outcome of allogeneic stem cell transplant: a study by the European group for blood and marrow transplantation. *Clin Infect Dis.* 2014;59(4):473-481.

43. Yeh AC, Varelias A, Reddy A, et al. CMV exposure drives long-term CD57+ CD4 memory T cell inflation following allogeneic stem cell transplant [published online ahead of print 3 June 2021]. *Blood.* 2021. 10.1182/blood.2020009492.

44. Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a multicentre, retrospective, pharmacodynamic cohort analysis. *Lancet Haematol.* 2017;4(4):e183-e191.

45. Kennedy VE, Chen H, Savani BN, et al. Optimizing antithymocyte globulin dosing for unrelated donor allogeneic hematopoietic cell transplantation based on recipient absolute lymphocyte count. *Biol Blood Marrow Transplant.* 2018;24(1):150-155.

46. Martins JP, Andoniou CE, Fleming P, et al. Strain-specific antibody therapy prevents cytomegalovirus reactivation after transplantation. *Science.* 2019;363(6424):288-293.

Downloaded from http://ashpublications.org/bloodadvances/article-pdf/6/1/28/1856158/advancesadv2021005509.pdf by guest on 18 December 2022

1 **Supplemental Information to:**

2 **Impact of CMV reactivation on relapse of acute myeloid leukemia after HCT is dependent**

3 **on disease stage and ATG**

4

5 Amin T. Turki[1, 3], Nikolaos Tsachakis-Mück[1], Saskia Leserer[1], Pietro Crivello[3], Tobias

6 Liebregts[1], Luisa Betke[3], Ferras Alashkar[1], Nils Leimkühler[1], Mirko Trilling[2], Katharina

7 Fleischhauer[3, 4] and Dietrich W. Beelen[1]

8

9 1 Department of Hematology and Stem Cell Transplantation, West-German Cancer Center,

10 University Hospital Essen, Essen, Germany

11 2 Institute for Virology, University Hospital Essen, Essen, Germany

12 3 Institute of Experimental Cellular Therapy, West-German Cancer Center, University Hospital

13 Essen, Essen, Germany

14 4 Deutsches Konsortium für Translationale Krebsforschung (DKTK)

15

16

17

18

19

20

21

22

1 **Supplemental Methods:**

2 *Patient treatment:*

3 Patients aged <50 years received a myeloablative conditioning using total body irradiation
4 (TBI) from a ^{60}Cobalt-source with a daily dose of 2.5 Gy on 4 consecutive days (cumulative
5 dose of 10 Gy with a reduced lung dose of 8 Gy) followed by intravenous cyclophosphamide
6 infusion (60 mg/kg bodyweight/day, cumulative dose 120 mg/kg bodyweight/day) or by
7 intravenous fludarabine (30 mg/m2 body surface/day, cumulative dose 150 mg/m2 body
8 surface/day). Reduced intensity conditioning consisted of fludarabine (30 mg/m2 body
9 surface/day, cumulative dose 150 mg/m2 body surface/day) combined with intravenous
10 busulfan (0.8mg/kg bodyweight/day, cumulative dose 6.4mg/ kg bodyweight) or treosulfan
11 (cumulative dose 14g/m² body surface).

12 Supportive therapy and anti-infectious prophylaxis was uniform and independent of the
13 conditioning intensity. With the beginning of the conditioning regimen until discharge, all in-
14 patients were treated in reverse isolation single rooms with high-efficacy particle air filtration.
15 In the absence of contraindications, all patients received a combined intestinal
16 decontamination medication as previously described[1] consisting of oral metronidazole at 400
17 mg three times daily and oral ciprofloxacin at 500 mg twice daily starting from day -14 until day
18 +35 after HCT. Antiviral prophylaxis during neutropenia consisted of intravenous aciclovir at
19 250 mg three times daily. Antifungal prophylaxis consisted of oral posaconazole at 200 mg
20 three times daily from day+1 for HCT patients[2] during the 2010s years or oral itraconazole at
21 200 mg twice daily from day +1 for HCTs during the 2000s years with a minimal duration until
22 day +100. Colony stimulating factors were not routinely applied. As pneumocystis-jirovecii
23 pneumonia prophylaxis patients received either monthly pentamidine inhalation or oral
24 cotrimoxazole at 960 mg three times per week from day +30. Neutropenic patients' meals were
25 prepared as decontaminated or germ-poor meals. Irradiated red blood cell and platelet
26 transfusions and in-line leukocyte-filtered products were exclusively during the entire HCT
27 course.

1 The uniform pharmacological GVHD prophylaxis of this study cohort consisted of 3 mg/kg body
2 weight ciclosporin (CSP) starting from day -1 before HCT in combination with 15 mg/m2
3 methotrexate (MTX) on day +1 and 10 mg/m2 MTX on days +3, +6 and +11 after HCT.[3, 4] For
4 inpatients, normal CSP target blood levels (range, 150-250 ng/ml) were controlled three times
5 weekly. Before patient discharge, intravenous CSP was substituted orally. From day +100 after
6 HCT, CSP was continuously tapered for patients without clinical signs and symptoms of GVHD.
7 From 2012, patients with increased risk of GVHD (e.g. HCT from MUD or MMUD) received
8 additional polyvalent rabbit-anti-Jurkat-T lymphocyte globulin (ATG Fresenius/Neovii) at a
9 dosage of 10mg/kg bodyweight on days -4, -3 and -2 (cumulative dosage: ATG 30mg/kg) or
10 at a dosage of 20mg/kg bodyweight on days -4, -3 and -2 (cumulative dosage: ATG 60mg/kg).

11 *Clinical endpoints and assessments*

12 Patient-, donor-, HCT- characteristics and HCT-outcome were documented in electronic forms
13 and retrospectively analysed. For inpatients, daily clinical assessment was obtained.
14 Outpatient follow-up was weekly early after discharge and was sequentially extended,
15 depending on clinical performance and transplant-associated complications. CMV reactivation
16 in peripheral blood samples was monitored twice weekly for inpatients starting with leukocyte
17 reconstitution >500/µl and weekly for outpatients beyond day +100. If several CMV reactivation
18 episodes occurred, only the interval to the first episode after HCT was analyzed. Patients with
19 CMV reactivation received a preemptive treatment with twice daily 5 mg/kg of patient body
20 weight ganciclovir for 14 days. In case of non-response to first-line treatment, foscarnet or
21 cidofovir was applied according to physician's choice and its toxicity profile.

22 Acute GVHD (aGVHD) was clinically assessed and classified according to published criteria
23 for aGVHD.[5-8] Its diagnosis was documented in electronic forms including staging of organ
24 involvement of skin, gut or liver and the date of aGVHD diagnosis. Chronic GVHD (cGVHD)
25 was diagnosed after day +100 based on characteristic clinical signs and symptoms according
26 to published criteria for cGVHD. For documentation of cGVHD before 2014, the Seattle criteria[9]

1 were applied whereas the NIH criteria [10] were used for more recent diagnoses. For purpose of
2 consistency, only the Seattle terminology was used throughout this manuscript.

3 Overall survival (OS) was calculated from day of transplantation to last follow-up or death of
4 any cause. Event-free survival (EFS) was calculated from day of transplantation to diagnosis
5 of relapse, persistence or death of any cause. Patients' EFS was censored at last follow-up.
6 For patients without relapse or persisting hematologic malignancy, non-relapse mortality
7 (NRM) was calculated from day of transplantation to death. Relapse was assessed as
8 hematologic marrow relapse (>5% blasts) or detection of extramedullar disease after HCT.
9 Cumulative relapse incidence was calculated from day of transplantation to diagnosis of
10 relapse or persistence of malignancy.

11 *CMV assays*

12 Between October 1997 and May 2011, pp65 antigenemia was measured on peripheral blood
13 leukocytes applied to slides by cytocentrifugation at the Institute for Virology as previously
14 described.[11] From May 2011 until February 2012, whole blood CMV DNA load was monitored
15 using the CMV-Roche PCR assay (Roche Diagnostics SA, Rotkreuz, Switzerland, detection
16 limit 700 copies/ml). Between February 2012 and August 2013, the Artus CMV Real-time PCR
17 Kit was used (Qiagen GmbH, Hilden, Germany; detection limit 150 copies/ml). From August
18 2013 to October 2017, CMV monitoring was performed with the CMV Real-time PCR Kit
19 (Abbott Molecular, Des Plaines, IL, USA; detection limit 40 copies/ml). Data obtained by these
20 CMV qPCR kits (Qiagen and Abbott Molecular) were comparable (r=0.939) in validation
21 assays by Passing-Bablok [12] regression.

22

23

24

25

1 **Supplemental References**

2 1. Beelen DW, Elmaagacli A, Müller K-D, Hirche H, Schaefer UW. Influence of Intestinal
3 Bacterial Decontamination Using Metronidazole and Ciprofloxacin or Ciprofloxacin Alone on
4 the Development of Acute Graft-Versus-Host Disease After Marrow Transplantation in Patients
5 With Hematologic Malignancies: Final Results and Long-Term Follow-Up of an Open-Label
6 Prospective Randomized Trial. *Blood*. 1999;93(10):3267.
7 2. Cornely OA, Maertens J, Winston DJ, et al. Posaconazole vs. fluconazole or
8 itraconazole prophylaxis in patients with neutropenia. *N Engl J Med*. Jan 25 2007;356(4):348-
9 59. doi:10.1056/NEJMoa061094
10 3. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with
11 cyclosporine alone for prophylaxis of acute graft versus host disease after marrow
12 transplantation for leukemia. *The New England journal of medicine*. Mar 20 1986;314(12):729-
13 35. doi:10.1056/nejm198603203141201
14 4. Beelen DW, Quabeck K, Kaiser B, et al. Six weeks of continuous intravenous
15 cyclosporine and short-course methotrexate as prophylaxis for acute graft-versus-host disease
16 after allogeneic bone marrow transplantation. *Transplantation*. Sep 1990;50(3):421-7.
17 5. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host
18 disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation*.
19 Oct 1974;18(4):295-304.
20 6. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD
21 Grading. *Bone Marrow Transplant*. Jun 1995;15(6):825-8.
22 7. Rowlings PA, Przepiorka D, Klein JP, et al. IBMTR Severity Index for grading acute
23 graft-versus-host disease: retrospective comparison with Glucksberg grade. *Br J Haematol*.
24 Jun 1997;97(4):855-64.
25 8. Harris AC, Young R, Devine S, et al. International, Multicenter Standardization of Acute
26 Graft-versus-Host Disease Clinical Data Collection: A Report from the Mount Sinai Acute
27 GVHD International Consortium. *Biology of blood and marrow transplantation : journal of the*

1 *American Society for Blood and Marrow Transplantation*. Jan 2016;22(1):4-10.
2 doi:10.1016/j.bbmt.2015.09.001
3 9. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in
4 man: A long-term clinicopathologic study of 20 seattle patients. *The American Journal of*
5 *Medicine*. 1980/08/01/ 1980;69(2):204-217. doi:https://doi.org/10.1016/0002-9343(80)90380-
6 0
7 10. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus
8 Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The
9 2014 Diagnosis and Staging Working Group Report. *Biology of Blood and Marrow*
10 *Transplantation*. 21(3):389-401.e1. doi:10.1016/j.bbmt.2014.12.001
11 11. Trenschel R, Ross S, Hüsing J, et al. Reduced risk of persisting cytomegalovirus pp65
12 antigenemia and cytomegalovirus interstitial pneumonia following allogeneic PBSCT. *Bone*
13 *marrow transplantation*. 2000/03/01 2000;25(6):665-672. doi:10.1038/sj.bmt.1702216
14 12. Passing H, Bablok. A new biometrical procedure for testing the equality of
15 measurements from two different analytical methods. Application of linear regression
16 procedures for method comparison studies in clinical chemistry, Part I. *Journal of clinical*
17 *chemistry and clinical biochemistry Zeitschrift fur klinische Chemie und klinische Biochemie*.
18 Nov 1983;21(11):709-20.

19
20
21
22
23
24

Supplemental Table 1: Transplant outcomes depending on additional in-vivo T cell depletion

	Overall Cohort	**No ATG**	**With ATG**	***p***
Total enrolled and treated, n (%)	687 (100)	420 (100)	267 (100)	
Acute GVHD characteristics				
- Grade 0-II	604 (88)	359 (85)	245 (91)	0.0160
- Grade III-IV	83 (12)	61 (15)	22 (9)	
Chronic GVHD characteristics				
Limited cGVHD	218 (32)	108 (26)	110 (41)	0.0027
Extensive cGVHD	169 (25)	120 (29)	49 (18)	<0.0001
No cGVHD	300 (44)	192 (46)	108 (40)	

GVHD, graft-versus host disease, cGVHD, chronic graft-versus-host disease, ATG, anti-thymocyte globulin

1 **Supplemental Figure Legends**

2 **Supplemental Figure 1: OS and NRM depending on CMV reactivation. A:** Kaplan Meier
3 analysis of OS of the overall AML cohort (n=687) comparison of strata with the logrank test
4 and **B:** Cumulative incidence function of NRM with relapse as competing risk (60 months
5 censored), comparison of strata with Gray's test. **C:** NRM of AML patients in CR1 at HCT
6 (n=293) **D:** NRM of AML patients with all other disease stages (n=394). Patients with CMV
7 reactivation (blue) and without CMV reactivation (red). Median (line) with 95% CI (shaded).

8 **Supplemental Figure 2: OS depending on CMV serostatus and in vivo T cell depletion**
9 **for additional GVHD prophylaxis.** Kaplan Meier analysis of OS (60 months censored).
10 Patients with CMV reactivation (blue) and without CMV reactivation (red). Median (line) with
11 95% CI (shaded). **A:** OS depending on CMV serostatus in patients without ATG **B:** OS
12 depending on CMV serostatus in patients with ATG **C:** OS depending on donor CMV
13 serostatus in patients without ATG **D:** OS depending on donor CMV serostatus in patients with
14 ATG. All p values according to the logrank test.

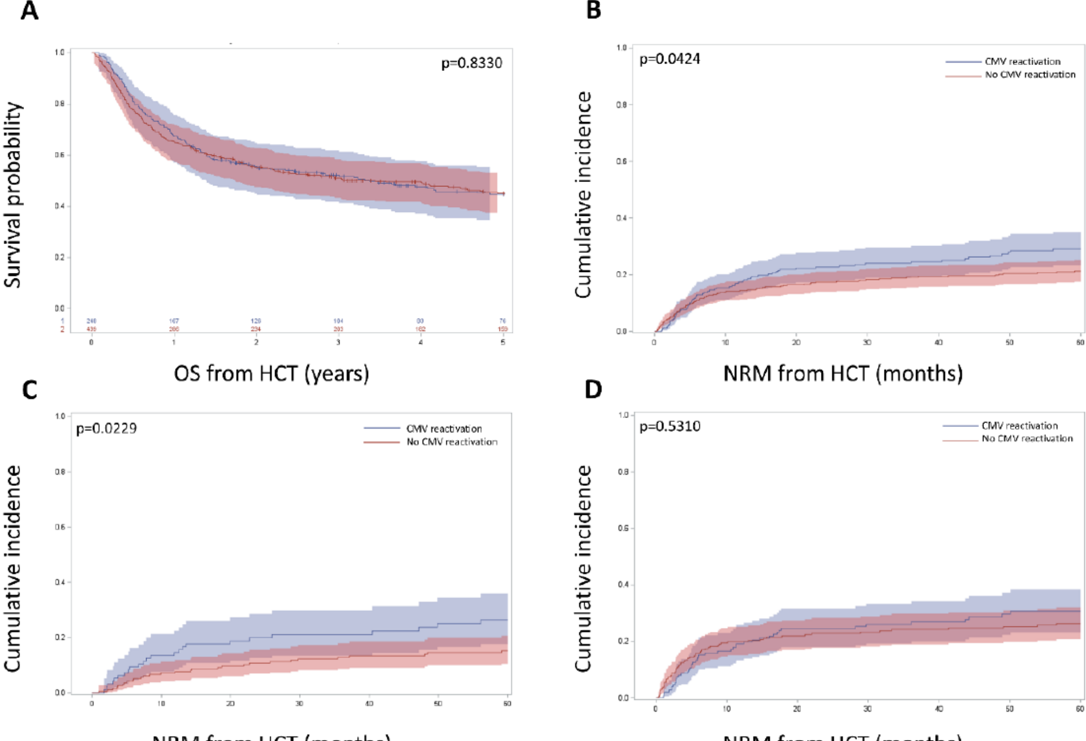
15 **Supplemental Figure 3: EFS after HCT depending on CMV reactivation. A:** Event-free
16 survival with relapse and mortality as competing risks (60 months censored). Patients with
17 CMV reactivation (blue) and without CMV reactivation (red). Median (line) with 95% CI
18 (shaded). **A:** Overall cohort (n=687) **B:** Advanced disease stages subgroup (n=394), C: AML
19 in CR1 (n=293), **D**: Patient without ATG (n=420), **E**: Patients with ATG (n=267). All p values
20 according to the logrank test.

21 **Supplemental Figure 4: Cumulative incidence of relapse stratified by grades II-IV aGVHD**
22 **and/or CMV reactivation in patients with and without ATG. A:** Patients without ATG
23 (n=420). Cumulative incidence function of relapse (60 months censored) stratified by patients
24 without grades II-IV acute GVHD (aGVHD) without CMV reactivation (blue, n=206), patients
25 with only CMV reactivation (dark orange, n=57), patients with only grades II-IV aGVHD (green,
26 n=97) and combinations of grades II-IV aGVHD and CMV reactivation. Grades II-IV aGVHD
27 followed by CMV reactivation (brown, n=52) and CMV reactivation followed by grades II-IV

aGVHD (violet, n=8) **B:** Patients with *in vivo* T cell depletion using ATG (n=267). Cumulative incidence function of relapse (60 months censored) stratified by patients without grades II-IV aGVHD without CMV reactivation (blue, n=94), patients with only CMV reactivation (dark orange, n=73), patients with only grades II-IV aGVHD (green, n=42) and combinations of grades II-IV aGVHD and CMV reactivation. Grades II-IV aGVHD followed by CMV reactivation (brown, n=45) and CMV reactivation followed by grades II-IV aGVHD (violet, n=13). Median (line) with 95% confidence interval (CI) shaded. P values refer to comparisons of all strata with Gray's test.

Supplemental Figure 5: Cumulative incidence of CMV reactivation depending on the detection method. A: Patients without ATG (n=420) **B:** Patients with *in vivo* T cell depletion using ATG (n=267). Cumulative incidence function of early CMV reactivation (<d+100) detected by PCR (blue) or pp65-antigenemia (red). Median (line) with 95% CI (shaded). Comparison of strata with Gray's test.

1 **Supplemental Figure 1: OS and NRM depending on CMV reactivation.**



2

3

4

5

6

7

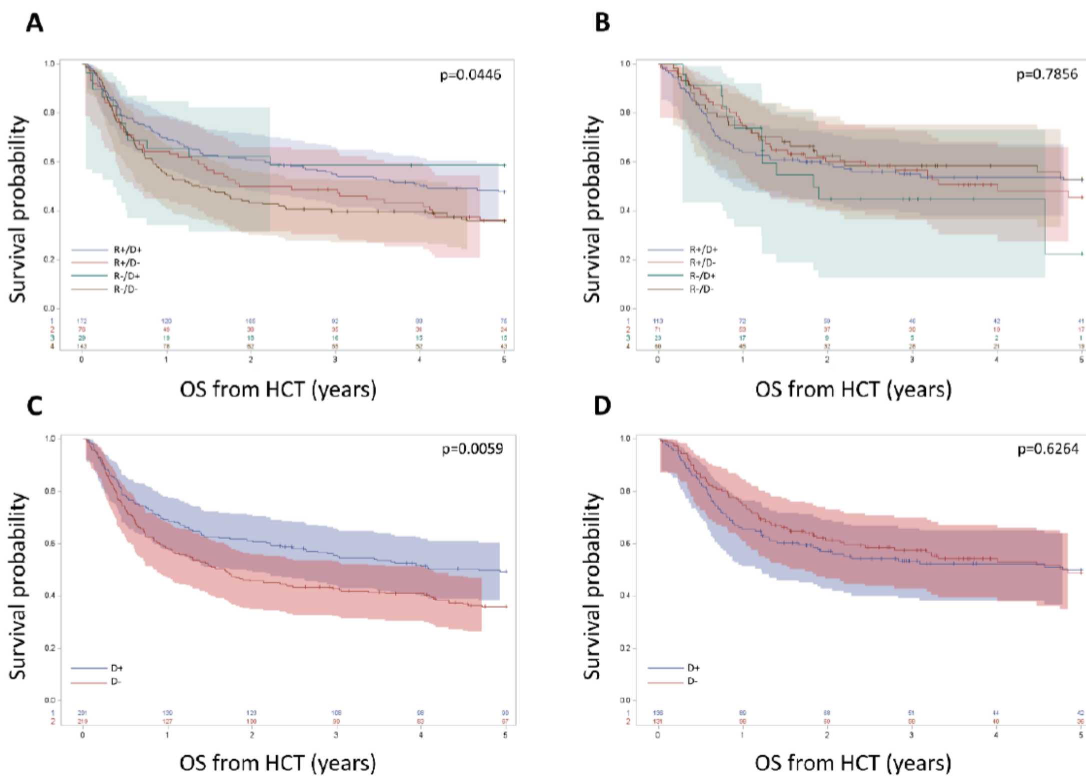
8

9

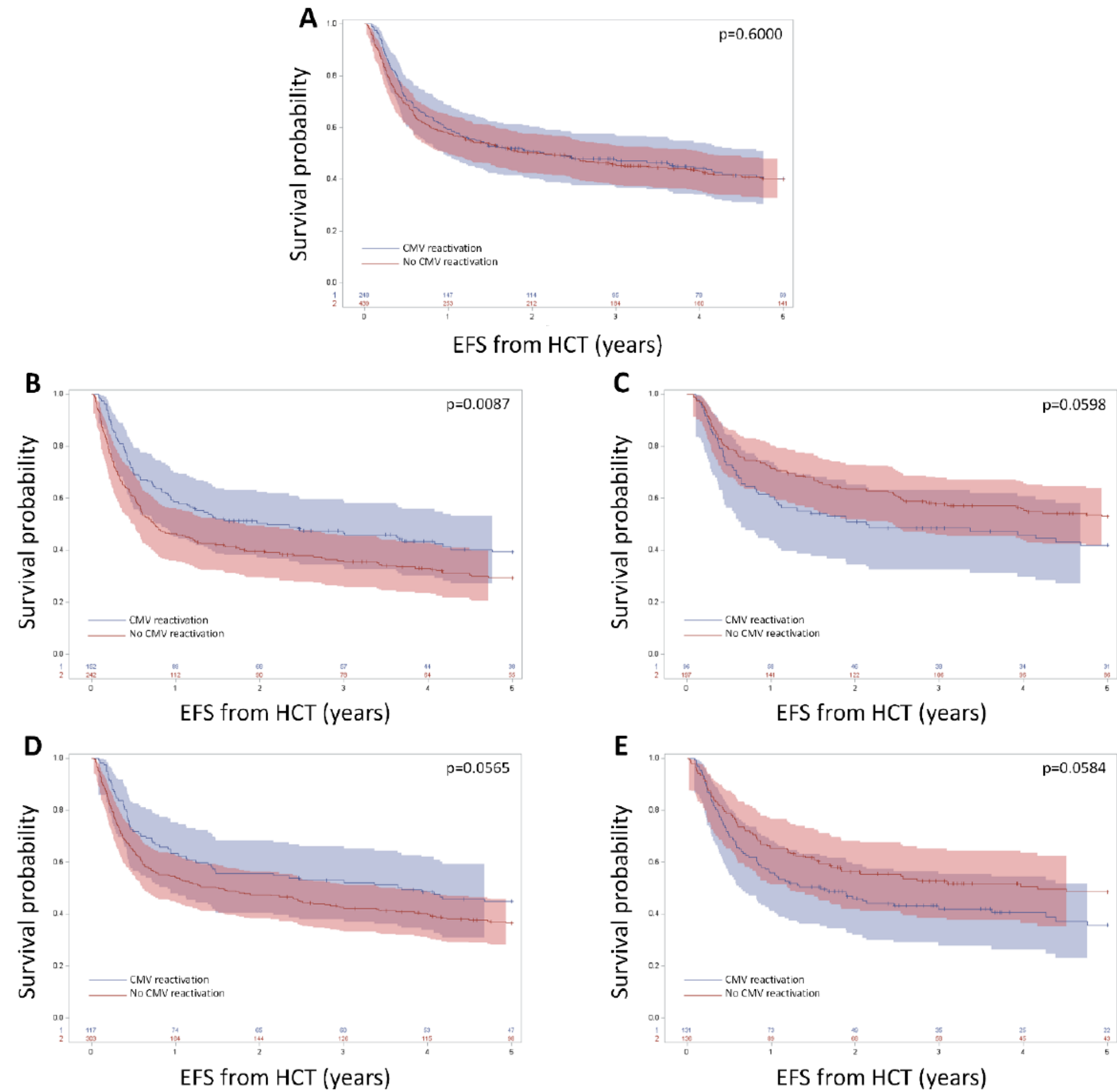
10

10

Supplemental Figure 2: OS depending on CMV serostatus and in vivo T cell depletion for additional GVHD prophylaxis.



1 **Supplemental Figure 3: EFS after HCT depending on CMV reactivation.**



2

3

4

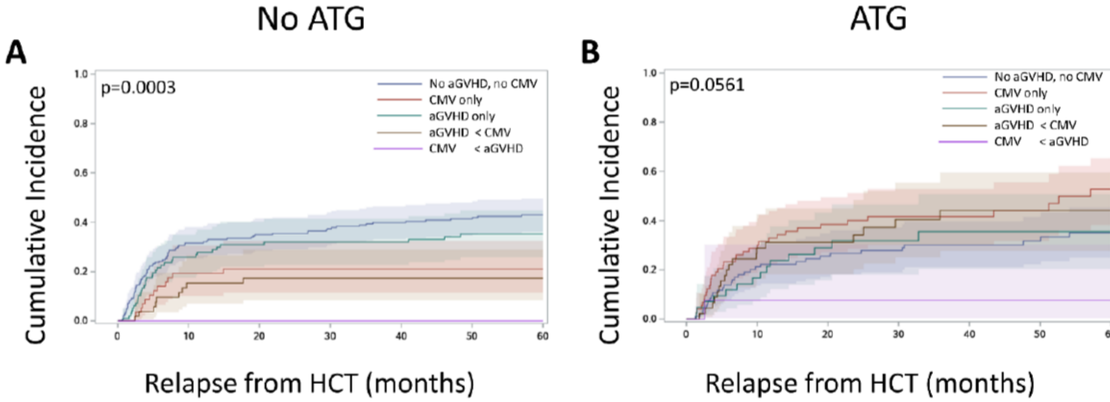
5

6

7

12

1 **Supplemental Figure 4: Cumulative incidence of relapse stratified by grades II-IV aGVHD**
2 **and/or CMV reactivation in patients with and without ATG.**



3

4

5

6

7

8

9

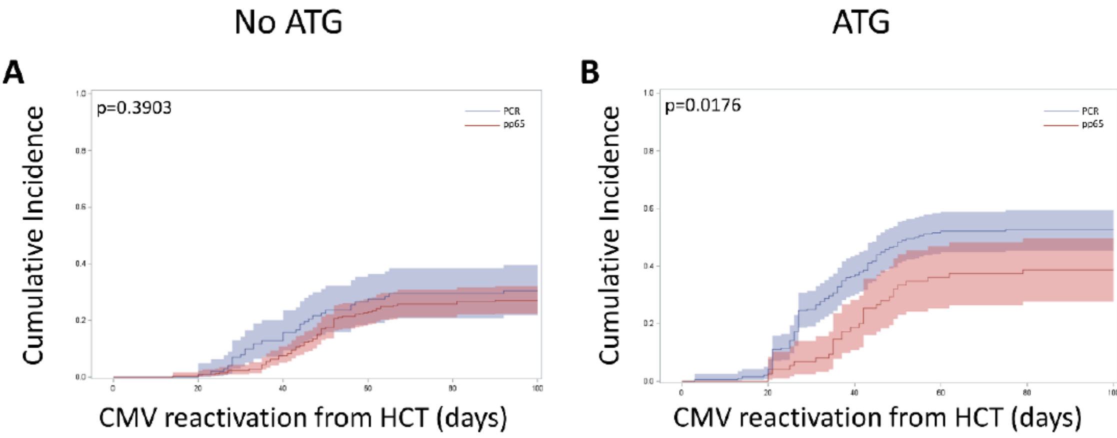
10

11

13

1 **Supplemental Figure 5: Cumulative incidence of CMV reactivation depending on the**
2 **detection method.**

3



4

5

6.3 Article III

Author contributions

Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies

Amin T. Turki, Vesna Klisanin, Evren Bayraktar, Lambros Kordelas, Rudolf Trenschel, Hellmut Ottinger, Nina K. Steckel, Nikolaos Tsachakis-Mück, **Saskia Leserer**, Markus Ditschkowski, Tobias Liebregts, Michael Koldehoff, Katharina Fleischhauer and Dietrich W. Beelen

Original Article in **American Journal of Transplantation**[162]

Impact factor: 7.338 (2019)

Status: published online on October 9th, 2019, American Journal of Transplantation, licensed under CC BY-NC 4.0, article included without any changes

DOI: 10.1111/ajt.15642; PubMed-ID: 31597002

Complete citation of the original source:

Turki, AT, Klisanin, V, Bayraktar, E, et al. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies. Am J Transplant. 2020; 20: 677– 688. https://doi.org/10.1111/ajt.15642

Contributions:

Conception:	0 %	
Experimental work:	25 %	- data collection
Data analysis:	0 %	
Statistical analysis:	0 %	
Writing the manuscript:	10 %	- visualization of the results
Revising the manuscript:	0 %	

____________________ ____________________

Prof. Dr. med. Dietrich W. Beelen Saskia Leserer

Received: 10 June 2019 | Revised: 6 September 2019 | Accepted: 25 September 2019
DOI: 10.1111/ajt.15642

AJT

ORIGINAL ARTICLE

Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies

Amin T. Turki[1,2] | Vesna Klisanin[1] | Evren Bayraktar[1,3] | Lambros Kordelas[1] | Rudolf Trenschel[1] | Hellmut Ottinger[1] | Nina K. Steckel[1] | Nikolaos Tsachakis-Mück[1] | Saskia Leserer[1] | Markus Ditschkowski[1] | Tobias Liebregts[1] | Michael Koldehoff[1] | Katharina Fleischhauer[2] | Dietrich W. Beelen[1]

[1]Department of Bone Marrow Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany
[2]Institute for Experimental Cellular Therapy, University Hospital Essen, Essen, Germany
[3]Chair of Applied Mathematics and Numerics, TU Dortmund University, Dortmund, Germany

Correspondence
Amin T. Turki and Dietrich W. Beelen
Emails: amin.turki@uk-essen.de; dietrich.beelen@uk-essen.de

Funding information
University of Duisburg-Essen UDE-IFORES; Bundesministerium für Bildung und Forschung, Grant/Award Number: 031L0027

Prophylaxis of graft-versus-host disease (GVHD) after allogeneic hematopoietic stem cell transplantation (HCT) remains challenging. Because prospective randomized trials of in-vivo T cell depletion using anti-T-lymphocyte globulin (ATLG) in addition to a calcineurin inhibitor and methotrexate (MTX) led to conflicting outcome results, we evaluated the impact of ATLG on clinical outcome, lymphocyte- and immune reconstitution survival models. In total, 1500 consecutive patients with hematologic malignancies received matched unrelated donor (MUD) HCT with cyclosporin and MTX (N = 723, 48%) or with additional ATLG (N = 777, 52%). In the ATLG cohort, grades III-IV acute (12% vs 23%) and extensive chronic GVHD (18% vs 34%) incidences were significantly reduced ($P < .0001$). Nonrelapse mortality (27% vs 45%) and relapse (30% vs 22%) differed also significantly. Event-free and overall survival estimates at 10 years were 44% and 51% with ATLG and 33% and 35% without ATLG ($P < .002$ and $<.0001$). A dose-dependent ATLG effect on lymphocyte- and neutrophil reconstitution was observed. At ATLG exposure, lymphocyte counts and survival associated through a logarithmically increasing function. In this survival model, the lymphocyte count optimum range at exposure was between 0.4 and 1.45/nL ($P = .001$). This study supports additional ATLG immune prophylaxis and is the first study to associate optimal lymphocyte counts with survival after MUD-HCT.

KEYWORDS
bone marrow/hematopoietic stem cell transplantation, clinical research/practice, flow cytometry, graft-versus-host disease (GVHD), graft-versus-leukemia (GVL)/graft versus tumor, hematology/oncology, immunosuppressant – polyclonal preparations: rabbit antithymocyte globulin, immunosuppression/immune modulation, mathematical model, translational research/science

Abbreviations: aGVHD, acute graft-versus-host disease; ALC, absolute lymphocyte count; AML, acute myeloid leukemia; aSCT, allogeneic stem cell transplantation; ATG, antithymocyte-globulin; ATLG, anti-T-lymphocyte-globulin; CI, confidence interval; CML, chronic myeloid leukemia; CMV, cytomegalovirus; CSP, cyclosporin; CTCAE, common terminology criteria for adverse events; EBV, Epstein-Barr virus; EFS, event-free survival; FACS, fluorescence-activated cell sorting; GVHD, graft-versus-host disease; HCT, hematopoietic cell transplantation; HLA, human leukocyte antigen; HR, hazard ratio; MDS, myelodysplastic syndromes; MMF, mycophenolate-mofetil; MUD, matched unrelated donors; NRM, nonrelapse mortality; OS, overall survival; PBSC, peripheral blood stem cells; RIC, reduced intensity conditioning.

[Correction added on February 6, 2019, after first online publication: The term globulin has been corrected in the title and throughout the article.]

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
© 2019 The Authors. American Journal of Transplantation published by Wiley Periodicals, Inc. on behalf of The American Society of Transplantation and the American Society of Transplant Surgeons

1 | INTRODUCTION

Effective prophylaxis of acute- and chronic graft-versus-host disease (GVHD) remains an unmet medical need after matched unrelated donor (MUD) allogeneic hematopoietic stem cell transplantation (HCT).[1] Both grades III-IV acute GVHD (aGVHD) and severe chronic GVHD (cGVHD) associate with substantial morbidity and nonrelapse mortality (NRM) after HCT. Standard prophylactic immunosuppressive regimens combining a calcineurin inhibitor, such as cyclosporin (CSP) with short-course MTX have shown limited efficacy to prevent grades III-IV aGVHD and severe cGVHD.[2,3] In several clinical studies, additional in-vivo T cell depletion has reduced the incidence of both aGVHD and cGVHD,[4-8] as a consequence it has become standard of care for MUD HCT in many transplant centers.[9] There are different polyclonal T cell depleting globulins: Rabbit anti-T–lymphocyte globulin (ATLG) and antithymocyte globulin (ATG)[9] and polyclonal horse ATG. In-vivo T cell depletion is equally used as a preparatory regimen prior to solid organ transplantation, in particular in patients with positive crossmatch or alloantibodies.[10] Two prospective randomized trials, which investigated in-vivo T cell depletion using ATLG as part of immune prophylaxis in myeloablative MUD HCT recipients, led to inconsistent outcome results concerning relapse incidence and its impact on overall survival (OS).[7,11] As a consequence, a number of questions have been discussed relating to study design, ATLG in different malignancies,[9,12] optimal ATLG dosage/kg body weight,[12] ATG-area under the curve (AUC)-dependent dosage,[13] lymphocyte-ATG dosage ratio[14] and immune reconstitution.[15,16]

In other HCT settings, such as myeloablative HCT from sibling donors, additional ATLG reduced the incidence of cGVHD without affecting OS.[17] In myeloablative and non-myeloablative MUD HCT trials ATG reduced aGVHD without increasing risk of relapse.[6,18] After reduced intensity conditioning (RIC), ATG reduced GVHD, increased relapse, and was associated with a comparable OS.[19] Large, retrospective registry analyses associated ATG with either reduced OS[20] or reported similar risk of relapse and OS in MUD patients.[21-23] Again, the recently presented innovative approaches analyzing the timing of ATG exposure[24] and its AUC serum levels[13] may help to solve the controversies concerning ATG and leukemic relapse.

In order to comprehend conflicting published results and overcome limitations of study design we hypothesized that homogenous data of a large single-center clinical cohort combined with studies of engraftment, immune reconstitution, and optimized ATLG dosage models should contribute to clarify the role of additional ATLG as immune prophylaxis. We evaluated the impact of ATLG in a large cohort of MUD HCT patients with hematologic malignancies and a long-term observation of up to 10 years. Due to center policy and data of previous randomized trials, exclusively ATLG was used.

2 | PATIENTS AND METHODS

2.1 | Patients

Between June 1991 and July 2016, a total of 1500 consecutive patients with hematologic malignancies underwent MUD-HCT with a uniform GVHD prophylaxis in combination with ATLG (N = 777, 52%) or without ATLG (N = 723, 48%) in the Department of Bone Marrow Transplantation of the West-German Cancer Center at University Hospital Essen. HLA-mismatch between patients and donors was allowed, but limited to a maximum of one single antigen or allele difference at the HLA loci A, B, C, DRB1, or DQB1 (9/10 match). HLA-DPB1 was not considered for donor-recipient matching. Assignment to ATLG-prophylaxis was based on standardized clinical treatment protocols of the center. Early supportive and follow-up care was identical for all patients. All data on baseline patient, donor, HCT characteristics and HCT outcome were documented prospectively in electronic forms. Clinical characteristics and laboratory parameters of patients after allogeneic stem cell transplantation were retrospectively analyzed. OS was calculated from transplantation to last follow-up visit or death of any cause. Patients were followed for up to 10 years after transplantation. Patients with longer survival were censored at maximum follow-up.

2.2 | Treatment

Patients of the no-ATLG cohort received a uniform pharmacological GVHD prophylaxis with 3 mg/kg body weight CSP starting from day −1 before HCT in combination with 15 mg/m^2 MTX on day +1 und 10 mg/m^2 MTX on days +3, +6, and +11 after HCT.[2,3] Patients of the ATLG cohort received additional polyvalent rabbit-ATLG (anti-Jurkat-T lymphocyte globulin; formerly ATG-Fresenius®, now Grafalon® Neovii, Neovii Biotech, Lexington, MA) at a dosage of 10 mg/kg body weight on days −4, −3, and −2 (cumulative dosage: ATLG 30 mg/kg) or at a dosage of 20 mg/kg body weight on days −4, −3, and −2 (cumulative dosage: ATLG 60 mg/kg). A total of 49 patients (6%) with ATLG dosages other than 30 or 60 mg/kg were excluded from multivariate ATLG analysis.

2.3 | Assessments

For inpatients, daily clinical assessment and standard laboratory parameters, such as peripheral blood cell parameters for hematologic regeneration, were obtained. Standard laboratory procedures were performed at the central laboratory of the University Hospital Essen. Hematologic regeneration was assessed daily for inpatients during the first 28 days after transplantation and weekly for outpatients during the following 2 months. Further outpatient follow-up intervals were sequentially extended, depending on clinical performance and transplant-associated complications. Transplant engraftment was defined as time from transplantation (day 0) to the first of 3 consecutive days with a measured leukocyte count of ≥1.000/μL, a neutrophil count of ≥500/μL, a lymphocyte count of ≥500/μL, and platelets of ≥20.000/μL, respectively. Acute GVHD (aGVHD) was clinically assessed and classified according to published criteria for aGVHD.[25-28] Staging data of aGVHD maximum organ involvement (skin, gut, or liver) and the date of first aGVHD diagnosis were prospectively collected in the institutional clinical database. Chronic GVHD (cGVHD) was diagnosed after day +100

based on characteristic clinical signs and symptoms according to published criteria for cGVHD. For prospective documentation of cGVHD before 2014, the Seattle criteria[29] were applied whereas the National Institutes of Health criteria[30] were used for more recent diagnoses. For purpose of consistency, only the Seattle terminology was used throughout this manuscript. OS was calculated from day of transplantation to death of any cause. Event-free survival (EFS) was calculated from day of transplantation to diagnosis of relapse, persistence, or death of any cause. Patients' EFS was censored at last follow-up. For patients without relapse or persisting hematologic malignancy, nonrelapse mortality (NRM) was calculated from day of transplantation to death. Cumulative relapse incidence (CRI) was calculated from day of transplantation to diagnosis of relapse or persistence of malignancy. NRM and CRI were considered as respective competing risks.

2.4 | Ethics

The study and data acquisition were conducted in accordance with German legislation, the revised Helsinki Declaration, evaluated and approved by the ethics committee of the University of Duisburg-Essen (Protocol No. 18-8299-BO). All patients have given written consent on collection, electronic storage, and scientific analysis of anonymized transplant-specific patient data. We confirm that no patient can be identified because of anonymized patient data.

2.5 | Statistical analysis

Statistical methods and software for clinical data analysis and predictive absolute lymphocyte count (ALC) models are detailed in the Appendix S1.

3 | RESULTS

3.1 | Patient characteristics

A total of 1500 consecutive patients with hematologic malignancies underwent MUD-HCT with a uniform immune prophylaxis of CSP and MTX alone (N = 723, 48%) or in combination with ATLG (N = 777, 52%). Baseline demographic characteristics including underlying hematologic disease, conditioning regimen, patient and donor HLA, and gender constellation are detailed in Table 1. Acute myeloid leukemia (AML) was the predominant disease in both cohorts (43% vs 38%). Established GVHD risk factors, such as HLA mismatch (9/10) or female donor to male recipient gender matching, were evenly distributed between cohorts. Differences between cohorts involved diagnosis of myelodysplastic syndromes (MDS, 12% vs 5%), chronic myeloid leukemia (CML, 9% vs 31%), myeloablative conditioning regimen (31% vs 73%), high-risk leukemia (60% vs 86%), peripheral blood stem cells as transplant source (95% vs 71%), median age at allogeneic HCT (54 vs 44 years), and the median year of allogeneic HCT (2012 vs 2004).

3.2 | Therapy and response

The addition of ATLG to standard immune prophylaxis with CSP and MTX resulted in significant clinical effects. After a median follow-up of 7.6 years from HCT, the OS estimate at 10 years was 51% for patients with ATLG and 35% for patients without ATLG ($P < .002$; Figure 1). The corresponding EFS was 44% with ATLG and 33% without ATLG ($P < .0001$). Non-relapse mortality (NRM) was significantly reduced in the ATLG cohorts. The 10-year cumulative incidence (CumIn) of NRM was 27% with ATLG and 45% without ATLG ($P < .0001$). Patients' GVHD characteristics are detailed in Table 2. Detailed analysis revealed in particular a reduction of aGVHD grades III-IV to 11% in ATLG patients compared to 22% in the no-ATLG cohort ($P < .0001$; Figure 2B). The use of ATLG resulted in a relative downgrading through all observed aGVHD grades, compared to the no-ATLG cohort (Table 2). A similar effect of relative downgrading was also observed with regards to cGVHD. The overall cGVHD incidence was 59% with ATLG and 62% without ATLG ($P = .04$; Table 2). For limited and extensive cGVHD the cohorts separated clearly: the CumIn of limited cGVHD was 41% with ATLG opposed to 26% without ATLG ($P < .0001$; Figure 2C). In contrast, extensive cGVHD was 18% with ATLG and 35% in the no-ATLG cohort ($P < .0001$; Figure 2D). Thus, the significant difference of NRM between patient cohorts with and without prophylactic ATLG appeared primarily attributable to significantly lower grades III-IV aGVHD and extensive cGVHD rates in patients with prophylactic ATLG.

As expected, the relapse rate was higher in the ATLG cohorts. The 10-year CumIn of hematologic relapse was 30% with and 22% without ATLG ($P < .008$). Still, OS remained significantly higher in the ATLG cohort (Figure 1), because relapse-related mortality did not increase after prophylactic ATLG (22%; 95% confidence interval [95% CI], 19%-25% and 20% without ATLG; 95% CI, 17%-23%). EFS was also higher for the ATLG patient cohorts with a probability at 10 years of 44% (95% CI, 36%-52%) after 60 mg/kg ATLG, 39% (95% CI, 34%-45%) after 30 mg/kg ATLG, and 33% (95% CI, 30%-37%) in patients without prophylactic ATLG ($P < .002$). Systematic review of replicative viral infections in a subset of 470 patients associated the administration of ATLG only with a significantly higher incidence of replicative cytomegalovirus infections as compared to no-ATLG (49%; 95% CI, 43%-55% vs 28%; 95% CI, 23%-33%; $P < .0001$).

3.3 | Multivariate analysis

Multivariate analysis (Table 3) confirmed observed differences with regard to OS. Both ATLG dosages associated with reduced mortality (hazard ratio [HR] 0.64; 95% CI 0.55-0.75 and HR 0.54; 95% CI, 0.42-0.70 for 30 and 60 mg/kg ATLG, respectively). As expected, the incidence of grades III-IV aGVHD (HR 2.17; 95% CI, 1.84-2.56) and of extensive cGVHD (HR 3.62; 95% CI, 3.04-4.32) associated with substantially increased mortality. High-risk disease stages also associated with increased mortality (HR 1.38, 95% CI 1.12-1.70, $P < .0005$;

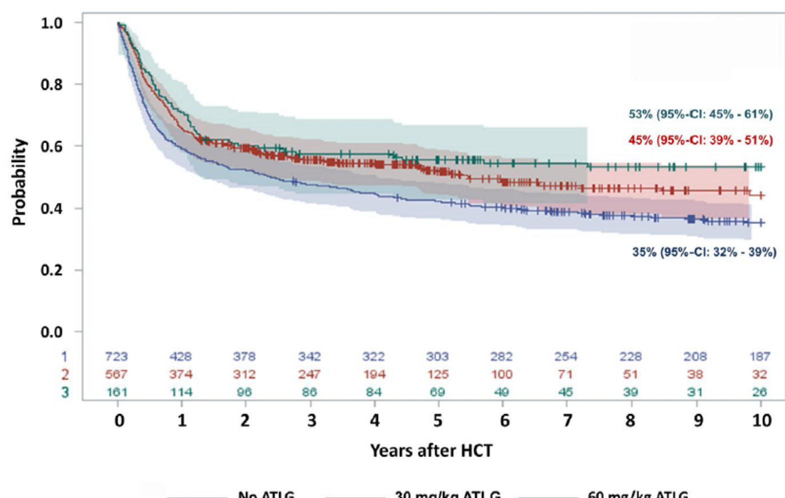
TABLE 1 Patient and transplant baseline characteristics

	ATLG n (%)	No ATLG n (%)	P
Total enrolled and treated	777 (52)	723 (48)	
Median age at transplantation (range)	54 (18-76)	44 (18-73)	<.0001
Age ≥60 y	238 (31)	97 (13)	<.0001
Male gender	408 (53)	433 (60)	.0042
Disease			
Acute myeloid leukemia	331 (43)	275 (38)	n.s.
Myelodysplastic syndromes	93 (12)	33 (5)	<.0001
Acute lymphoblastic leukemia	91 (12)	69 (10)	n.s
Chronic myeloid leukemia	69 (9)	226 (31)	<.0001
Chronic lymphocytic leukemia	12 (2)	17 (2)	n.s.
Chronic myelomonocytic leukemia	10 (1)	12 (2)	n.s.
Non-Hodgkin's lymphoma	56 (7)	50 (7)	n.s
Hodgkin lymphomas	12 (1)	2 (0)	.0133
Multiple myeloma	45 (6)	13 (2)	<.0001
Osteomyelofibrosis	43 (6)	25 (3)	n.s.
Other myeloproliferative disorders	9 (1)	1 (0)	.0218
Disease risk			
Standard[a]	313 (40)	103 (14)	<.0001
High[b]	464 (60)	620 (86)	<.0001
Conditioning			
Myeloablative conditioning	241 (31)	525 (73)	<.0001
Reduced intensity conditioning	536 (69)	198 (27)	<.0001
Total body irradiation	291 (37)	525 (73)	
Cumulative ATLG dosage			
ATLG 30 mg/kg	567 (72)	–	–
ATLG 60 mg/kg	161 (21)	–	–
Other ATLG dosage	49 (6)	–	–
Transplant and donor constellation			
PBSC	738 (95)	516 (71)	<.0001
BM	39 (5)	207 (29)	<.0001
Matched donor 10/10	525 (68)	513 (71)	n.s.
HLA mismatch donor	252 (32)	210 (29)	n.s.
Median year of transplantation (range)	2012 (1997-2016)	2004 (1991-2015)	<.0001
Recipient/donor gender constellation			
Female/female	177 (23)	155 (21)	n.s.
Male/male	337 (43)	352 (49)	.04
Female/male	192 (25)	135 (19)	.005
Male/female	71 (9)	81 (11)	n.s.

ATLG, anti-T-lymphocyte globulin; BM, bone marrow; HLA, human leukocyte antigen; MUD, matched unrelated donor; n.s., not significant; PBSC, peripheral blood stem cell.
[a]Standard risk: De-novo AML in first remission, ALL in first remission, MDS with single lineage dysplasia, and MDS with single lineage dysplasia and ring sideroblasts, CML in first chronic phase.
[b]High-risk stages were all other stages that did not correspond to standard risk stages, such as AML in second remission. Patients with myeloproliferative disorders, multiple myeloma, and lymphomas were only transplanted in high risk disease stages.

Table S1). In concordance with previously published studies, both ATLG dosages of 30 mg/kg (HR 0.52; 95% CI, 0.38-0.73) and 60 mg/kg (HR 0.29; 95% CI, 0.17-0.53) were associated with reduced incidence of grades III-IV aGVHD. In addition, aGVHD (HR 0.49; 95% CI, 0.32-0.74) and cGVHD were associated with reduced relapse incidence (Table 3) as were myeloablative conditioning regimens (HR

FIGURE 1 Comparison of overall survival depending on ATLG prophylaxis and dosage. Survival from day of allogeneic stem cell transplantation until death of any cause (95% confidence interval [CI] shaded). Data was censored at 120 mo after transplantation. Patients were categorized into no ATLG prophylaxis (n = 723, hatched) and different ATLG prophylaxis (n = 777, solid) dosage subgroups, ATLG 30 mg (n = 567), and ATLG 60 mg (n = 161). Patients with deviating ATLG dosages (n = 49) were excluded from this analysis. Graphs were plotted using the Kaplan-Meier survival analysis and cohorts were compared with the log-rank test. Vertical bars represent censored patients [Color figure can be viewed at wileyonlinelibrary.com]



0.64; 95% CI, 0.48-0.84; Table 3). In high-risk disease stages relapse incidence was not significantly influenced by prophylactic ATLG (HR 1.17; 95% CI, 0.87-1.57; Table S1). Multivariate analysis was also performed for the largest subgroup of patients with acute leukemia (n = 766). Within this subgroup, both ATLG dosages also associated with significantly reduced hazards with respect to grades II-IV and III-IV aGVHD, extensive cGVHD, and thereby significantly improved OS as compared to the no-ATLG cohort (Table 4). The additional comparison of patient subsets of different disease categories (leukemias, other myeloid malignancies, and other lymphoid malignancies) with prophylactic 30 or 60 mg/kg ATLG revealed no significant difference in the incidence of grades II-IV aGVHD (Table S4).

Significantly differing patient characteristics between ATLG and no-ATLG cohorts were analyzed with Cox regression analysis (Table S1). The median year of transplantation of all 1500 patients was 2009. Although both cohorts diverged with regards to the transplantation period, this variance did not associate with significant differences in OS analysis (P = .29; Table S1). Further, multivariate analysis revealed that the observed OS benefit associated with ATLG would have been even larger in absence of bias. Elements of bias in the no-ATLG cohort, which were associated with longer survival, were lower age, myeloablative conditioning regimen, and the diagnosis of CML. In a subgroup analysis excluding all CML patients from both cohorts, high-risk disease was well balanced between the ATLG and no-ATLG cohort (398 vs 394 patients). Within this patient subset, the use of ATLG was also associated with improved OS (HR 0.70; 95% CI, 0.56-0.88, P < .0005; Table S1).

3.4 | Correlative studies, lymphocyte and leukocyte dynamics models

In order to analyze the interaction of ATLG and absolute lymphocyte counts (ALC) before HCT, we first performed a categorical Cox regression analysis. Lymphocyte counts >0.1/nL at day −5 (before ATLG exposure) significantly correlated with higher OS (HR 0.68; 95% CI, 0.47-1.00, P = .05). We then constructed a noncategorical Cox regression model by adopting the natural logarithm of ALC on day −5 as continuous variable stratified for ATLG dosages. The result was a logarithmically increasing power function for lymphocytes >0.1/nL, meaning that patients with very low ALC (<0.1/nL) and patients with ALC > 1.45/nL both associated with increased hazard (Figure S1 and Table S2). The ALC optimum for patients with ATLG was between 0.4 and 1.45/nL at day −5. These effects were not reproducible in the no-ATLG cohort, where ALC did not correlate with OS at all (Table S2, Figure S2).

TABLE 2 Acute and chronic GVHD incidence by prophylactic ATLG administration

	ATLG (%)	No ATLG (%)	P
Acute GVHD grades			
0	21	15	.002
I	41	34	<.02
II	26	25	n.s
III	6	12	<.0001
IV	6	13	<.0001
II - IV	37	49	<.0001
III - IV	11	22	<.0001
Number of organ involvements with acute GVHD			
1	47	46	n.s
2	25	21	n.s
3	5	15	<.0001
Chronic GVHD grading			
Total incidence	59	62	.04
Limited cGVHD	41	26	<.0001
Extensive cGVHD	18	35	<.0001

cGVHD, chronic graft-versus-host disease; GVHD, graft-versus-host disease; n.s., not significant.

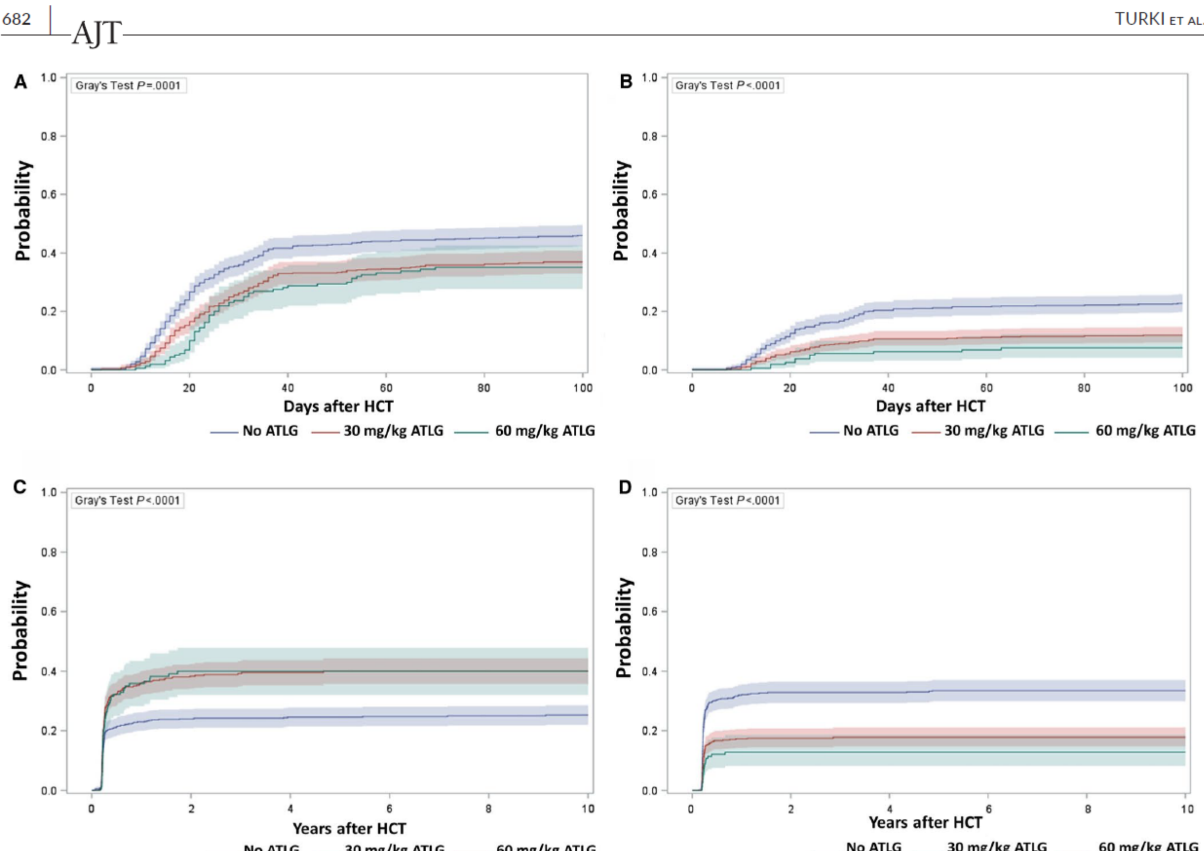


FIGURE 2 Time-dependent cumulative incidence of graft-versus-host disease. Time-dependent cumulative incidence (95% CI shaded) of acute GVHD grades II° - IV° (A) and III° - IV° (B). Cumulative incidence of limited (C) and extensive (D) chronic GVHD. Significance levels of cause-specific risk functions were tested according to Fine und Gray. A: No ATLG vs ATLG, $P = .0001$. B: No ATLG vs ATLG, $P < .0001$. C: No ATLG vs ATLG, $P < .0001$; 30 vs 60 mg/kg ATLG, n.s.; no ATLG vs 30 mg/kg ATLG, $P < .0001$, no ATLG vs 60 mg/kg ATLG, $P < .0009$. D: No ATLG vs ATLG, $P < .0001$; 30 vs 60 mg/kg ATLG, n.s.; no ATLG vs 30 mg/kg ATLG, $P < .0001$; no ATLG vs 60 mg/kg ATLG, $P < .0005$ [Color figure can be viewed at wileyonlinelibrary.com]

After HCT, leukocyte and lymphocyte recovery significantly differed between no-ATLG and ATLG cohorts. Furthermore, we detected significant differences in leukocyte recovery dynamics between the ATLG dosages of 30 and 60 mg/kg (Figure 3). Overall leukocyte recovery was significantly delayed in both ATLG cohorts as compared to the no-ATLG cohort ($P < .0001$). Remarkably, neutrophil recovery did not differ between the no-ATLG and 30 mg/kg ATLG cohorts but was significantly delayed with the use of 60 mg/kg ATLG (Figure 3B). Median time to platelet recovery (≥50 000/µL) was 22 days in the no-ATLG cohort (range, 10-99) compared to 23 days in the ATLG 30 mg/kg subgroup (range, 10-92) and 27 days (range, 10-96) in the ATLG 60 mg/kg subgroup. Clinical endpoints (OS, EFS, NRM) did not significantly differ between the cumulative ATLG dosages of either 30 mg/kg (N = 567) or 60 mg/kg (N = 161). Median time to lymphocyte recovery (>500/µL) was 21 days in the no-ATLG cohort (range, 10-92) compared to 24 days (range, 12-93) in the ATLG 30 mg/kg subgroup and 32 days (range, 14-98) in the ATLG 60 mg/kg subgroup. Interestingly, lymphocyte recovery dynamics did not only significantly differ between the no-ATLG and both ATLG cohorts ($P < .0001$) but also between each of the 30 and 60 mg/kg ATLG dosage subgroups ($P < .0001$; Figure 3C).

Cellular immune reconstitution after HCT revealed differences and similarities of ATLG and no-ATLG cohorts (Figure 4). T helper cell immune reconstitution was significantly faster in patients without ATLG ($P < .001$). This difference was more pronounced for naïve T helper cells than for memory T helper cells. B cell and NK cell recovery appeared faster in the ATLG cohort, but these differences were not significant. Cytotoxic T cell recovery was comparable between both cohorts (Figure 4C). Of notice, early T helper cell reconstitution showed a significant dose-dependent delay for patients with 30 and 60 mg/kg ATLG at month 3 after HCT (Table S3), which parallels the observed dose dependency with regard to neutrophil and total lymphocyte regeneration. Further, the ALC optimum model was associated with significantly higher T helper counts in patients within the optimum range before ATLG exposure as compared to patients with ATLG exposure outside the optimum ALC range ($P = .002$; Table S3). A normalized description (Appendix S1) of T cell recovery illustrated a decreasing T cell count variation with time after HCT (Figure S3). The large range of both average T cell counts and standard deviations at month 3 and 6 after HCT decreased to no difference at month 12. Maximum T cell counts were reached around 15 months after HCT.

TABLE 3 Multivariate Cox regression analysis

Predictor	HR	95% CI	P
Overall survival			
ATLG 30 mg/kg	0.642	0.549-0.752	<.0001
ATLG 60 mg/kg	0.543	0.423-0.697	<.0001
Male recipient with female donor	1.380	1.118-1.703	.0027
Patient age (10 y increments)	1.252	1.188-1.319	<.0001
Grades III-IV aGVHD	2.168	1.840-2.556	<.0001
Limited cGVHD[a]	0.216	0.180-0.260	<.0001
Extensive cGVHD[a]	3.624	3.037-4.324	<.0001
Grades II-IV aGVHD			
ATLG 30 mg/kg	0.716	0.584-0.877	.0013
ATLG 60 mg/kg	0.595	0.442-0.801	.0006
Male recipient with female donor	1.477	1.146 –1.875	.0019
PBSC	1.273	1.006-1.633	.0486
Grades III-IV aGVHD			
ATLG 30 mg/kg	0.518	0.380-0.734	<.0002
ATLG 60 mg/kg	0.289	0.165-0.531	<.0001
HLA mismatch	1.631	1.251-2.125	.0003
Donor age N	1.180	1.038-0.341	.0114
Relapse incidence			
Myeloablative conditioning	0.636	0.481-0.841	.0015
Grades III-IV aGVHD	0.492	0.328-0.740	.0007
Limited cGVHD[a]	0.281	0.220-0.358	<.0001
Extensive cGVHD[a]	0.202	0.148-0.277	<.0001

aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; CI, confidence interval; HR, hazard ratio; *P*, significance as *P* value.
[a]The Seattle classification was used for consistency purpose. See Methods section.

The normalized analysis revealed similar T cell recovery kinetics across major (eg, CD4+ and CD8+) T cell subsets (Figure S3). T cell recovery after HCT started at 60% of its maximum value in the ATLG cohort and at 45% of its maximum value in the no-ATLG cohort, which may in part be explained by the more frequent use of myeloablative conditioning.

4 | DISCUSSION

The addition of ATLG to a standard immune prophylactic regimen improved both OS and EFS. In the ATLG cohort both aGVHD and cGVHD severity were significantly downgraded resulting in reduced incidence of grades III-IV aGVHD and extensive cGVHD. This finding is consistent with previous randomized ATLG trials[7,11] and several retrospective registry analyses, which associated in vivo T cell depletion with reduced aGVHD and cGVHD.[20,21] In contrast to previous studies,[11,14] our data revealed an optimum ALC at ATLG exposure that significantly correlated with OS. Its correlate may be the observed ATLG-dose-dependency in overall leukocyte recovery (Figure 3) as well as in early immune reconstitution of helper T cells (Table S3).

Currently, the impact of ATLG on relapse incidence and relapse-associated mortality in the MUD setting is controversial. In line with previous reports,[11] the relapse rate was significantly higher in the ATLG cohort (30% vs 20%). Soiffer et al[11] reported a cumulative 2-year relapse incidence in the ATLG and no-ATLG cohorts of 32% vs 21%. Finke et al[7] observed no significant difference between both arms at 2 years posttransplant (28.9% vs 23.6%). Other studies[18-21] suggested that in-vivo T cell depletion might be more efficient in patients with RIC conditioning regimens. Although the relative

TABLE 4 Cox regression analysis in patients with acute leukemias (n = 766)

Predictor	HR	95% CI	P
Overall survival			
30 mg/kg ATLG (n = 304) vs no ATLG (n = 344)	0.621	0.497-0.776	<.0001
60 mg/kg ATLG (n = 105) vs no ATLG (n = 344)	0.634	0.464-0.867	.004
Grade II-IV aGVHD			
30 mg/kg ATLG (n = 304) vs no ATLG (n = 344)	0.735	0.551-0.982	.0374
60 mg/kg ATLG (n = 105) vs no ATLG (n = 344)	0.617	0.425-0.897	.0115
ATLG (n = 422) vs no ATLG (n = 344)	0.694	0.536-0.899	.0056
Grade III-IV aGVHD			
30 mg/kg ATLG (n = 304) vs no ATLG (n = 344)	0.570	0.351-0.926	.0231
60 mg/kg ATLG (n = 105) vs no ATLG (n = 344)	0.340	0.169-0.684	.0025
ATLG (n = 422) vs no ATLG (n = 344)	0.486	0.320-0.739	.0007
Chronic GVHD (all stages)			
30 mg/kg ATLG (n = 304) vs no ATLG (n = 344)	0.757	0.590-0.971	.0282
60 mg/kg ATLG (n = 105) vs no ATLG (n = 344)	0.687	0.510-0.925	.0133
ATLG (n = 422) vs no ATLG (n = 344)	0.723	0.579-0.903	.0043
Limited cGVHD			
30 mg/kg ATLG (n = 304) vs no ATLG (n = 344)	1.714	1.203-2.443	.0029
60 mg/kg ATLG (n = 105) vs no ATLG (n = 344)	1.775	1.186-2.658	.0053
ATLG (n = 422) vs no ATLG (n = 344)	1.693	1.224-2.341	.0015
Extensive cGVHD			
30 mg/kg ATLG (n = 304) vs no ATLG (n = 344)	0.374	0.250-0.559	<.0001
60 mg/kg ATLG (n = 105) vs no ATLG (n = 344)	0.310	0.179-0.538	<.0001
ATLG (n = 422) vs no ATLG (n = 344)	0.361	0.255-0.511	<.0001

aGVHD, acute graft-versus-host disease; cGVHD, chronic graft-versus-host disease; CI, confidence interval; HR, hazard ratio; *P*, significance as *P* value.

proportion of RIC patients was also larger in our ATLG cohort than in the no-ATLG cohort (69% vs 27%, $P < .0001$), multivariate analysis identified RIC as an independent risk factor associated with reduced OS. Patient age in the ATLG cohort was also significantly higher than in the two aforementioned prospective trials. Similar to reports from other transplant centers,[7,11] median patient age in our study steadily increased over the total observation period of 25 years, and resulted in differences of median age (54 vs 44 years) and RIC rates between ATLG and no-ATLG cohorts. Thus, the administration of ATLG at least partially compensated for the adverse bias of patient age >45 years and RIC on OS.

Exposure to T cell depleting agents has previously been associated with delayed hematological engraftment[31] or immune reconstitution.[32,33] Our data showed a dose-dependent difference in both lymphocyte and neutrophil regeneration between the 30 and 60 mg/kg ATLG cohorts (Figure 3). Interestingly, neutrophil engraftment did not differ between the 30 mg/kg ATLG subgroup and the no-ATLG cohort. In a recent analysis of immune reconstitution involving patients with or without ATLG, higher absolute numbers of CD3+, CD4+, naïve- and regulatory T cells as well as B cells associated with improved OS and reduced NRM.[15] We also found significantly lower T helper cell counts in the ATLG cohort after HCT (Figure 4), but no significant differences in cytotoxic T cell and B cell reconstitution. Our data showed an ATLG dose dependency in early T helper cell reconstitution (Table 3). The normalized analysis of immune reconstitution (Figure 3) revealed similar regeneration kinetics across different T cell subsets.

The observed differences to other published studies might also relate to direct or indirect drug interactions of standard immunosuppressive regimens and ATLG. Whereas Bacigalupo et al,[6] Finke et al,[7] Kröger et al,[17] and the present study exclusively used short-course MTX and CSP, patients in Soiffer et al[11] received tacrolimus in combination with short-course MTX or in Baron et al[19] tacrolimus was combined with mycophenolate mofetil. ATLG may be administered at different cumulative dosages (eg, 30 or 60 mg/kg body weight). The question of optimal ATLG dosage has been previously discussed by several authors and is still inconclusive.[12,34,35] Whereas in both prospective, randomized ATLG trials, the ATLG dose was 60 mg/kg,[7,11] other previous studies successfully applied 30 mg/kg resulting in a similar relapse risk compared to placebo.[17,34] The great majority of patients (72%) in our analysis received a cumulative dosage of 30 mg/kg, with sustained beneficial effect on OS along with a significant reduction of NRM, which was most probably related to a reduction of grades III-IV aGVHD and extensive cGVHD. Thus, the different ATLG doses may in part explain differences of clinical results compared to previous trials.

Beyond conventional dose categories, we investigated optimal ATLG dosing with respect to patient's ALC before administration of ATLG. Our data confirmed Soiffer et al,[11] who had shown increased hazard in a categorical analysis of lymphocyte counts <0.1/nL. Still, the association of lymphocyte counts and hazard rate was more complex. It followed a logarithmically increasing power function above lymphocyte counts of >0.1/nL, which showed an optimum range between lymphocytes of 0.4 and 1.45/nL (Table S1, Figure S1). Patients within this ALC range and ATLG exposure had a significantly improved OS as compared to patients outside this range (Table S2). As a consequence, both patients with very low ALC and those with higher ALC associated with reduced OS, when exposed to ATLG. In our hypothesis, the biological correlate of this model would relate to free excess ATLG due to a low binding capacity of residual recipient T cells at the time of ATLG administration, which in turn leads to more vigorous in-vivo donor T cell depletion after HCT (ALC < 0.4/nL). Conversely, ATLG might extensively be absorbed by recipient T cells, resulting in less effective donor T cell depletion after HCT (ALC > 1.45/nL). Patients within the optimum

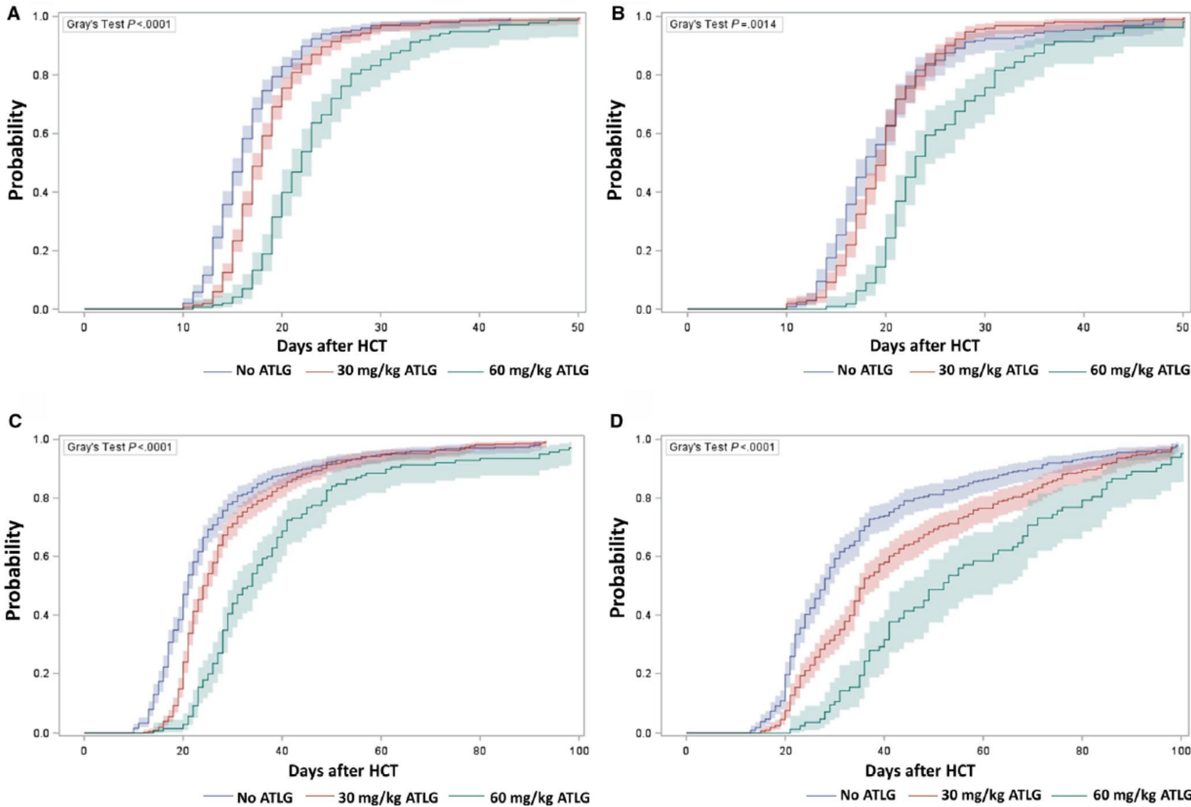


FIGURE 3 Time-dependent cumulative incidence of cellular regeneration. Engraftment dynamics are significantly delayed in high-dosed ATLG. Daily change of hematologic cell number was evaluated. Time-dependent cumulative incidence (95% confidence intervals shaded) of leukocyte engraftment ≥1.000/µL (A), neutrophil engraftment ≥500/µL (B), lymphocyte engraftment ≥500/µL (C), and lymphocyte regeneration ≥1.000/µL (D). Significance levels of cause-specific risk functions were tested according to Fine und Gray. A: All tests with *P* < .0001. B: No ATLG vs ATLG, *P* < .001; 30 vs 60 mg/kg ATLG, *P* < .004; no ATLG vs 30 mg/kg ATLG, n.s.; no ATLG vs 60 mg/kg ATLG, *P* < .0001. C: All tests, *P* < .0001. D: No ATLG vs ATLG, *P* < .0001; 30 vs 60 mg/kg ATLG, *P* < .006; no ATLG vs 30 mg/kg ATLG, *P* < .0001; no ATLG vs 60 mg/kg ATLG, *P* < .0001 [Color figure can be viewed at wileyonlinelibrary.com]

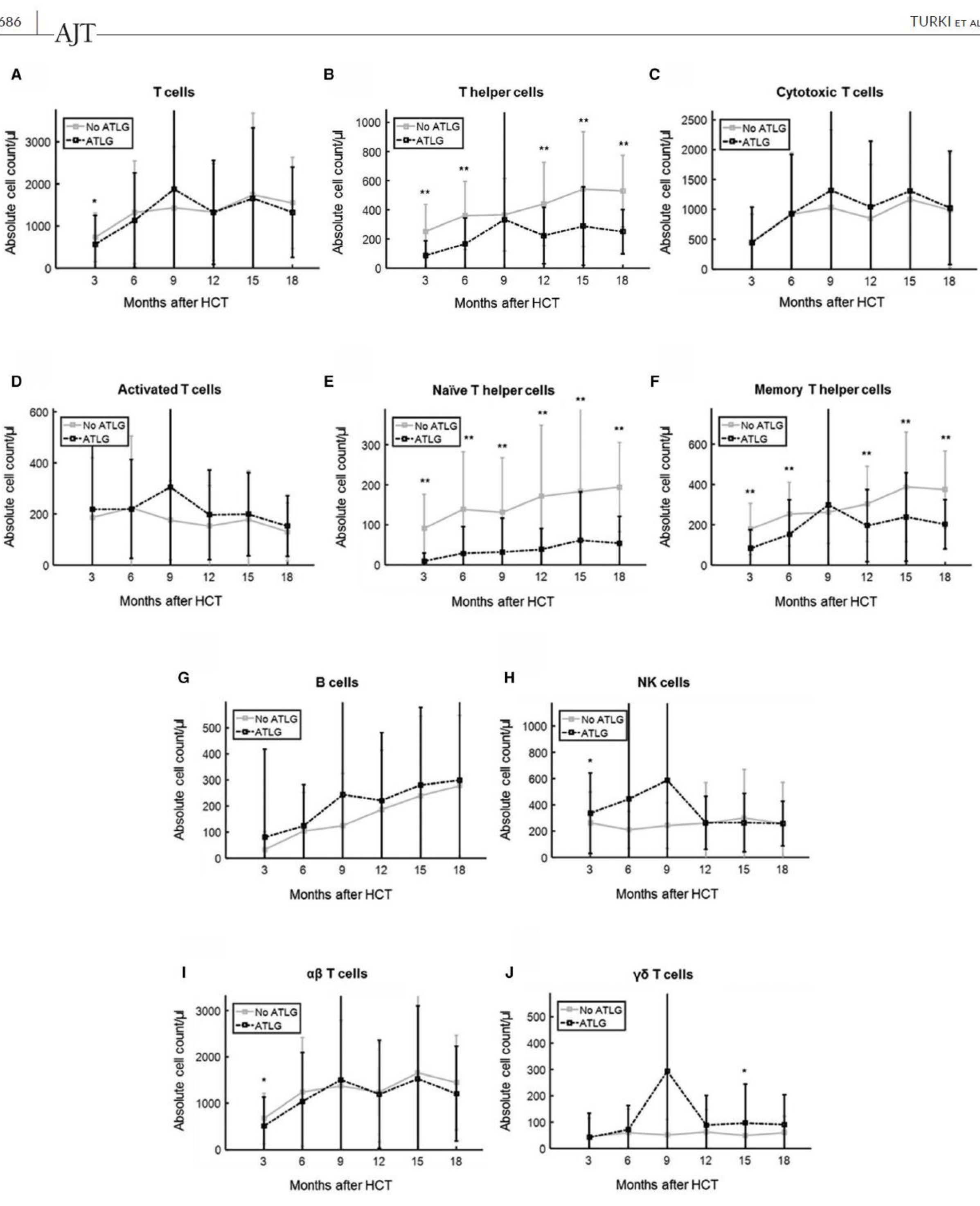


FIGURE 4 Comparison of cellular immune reconstitution between the ALTG and no-ATLG cohort. Lymphocyte subsets in the peripheral blood were measured by flow cytometry after HCT. Specific cell subsets within the CD45+ lymphocyte gate were characterized as follows: (A) T cells, CD3+; (B) T helper cells, CD3+/CD4+; (C) cytotoxic T cells, CD3+/CD8+; (D) activated T cells, CD3+/HLA-DR+; (E) naïve T helper cells, CD3+/CD4+/CD45RA+; (F) memory T helper cells, CD3+/CD4+/CD45RO+; (G) B cells, CD19+; (H) natural killer (NK) cells, CD16+/56+; (I) αβ T cells, CD3+/ αβ-T +; (J) γδ-T Cells, CD3+/γδ -T+. Absolute cell numbers after transplantation were analyzed by the two-sample *t* test. ATLG cohort around 3 mo (n = 221), 6- (n = 119), 9- (n = 105), 12- (n = 111), 15- (n = 93), and 18 mo (n = 80); no-ATLG cohort around 3 (n = 124), 6 (n = 64), 9 (n = 62), 12 (n = 48), 15 (n = 50), and 18 mo (n = 48). Mean values and the standard error of the mean are shown. A $P < .05$ was considered statistically significant and indicated in the figure with an asterisk, $P < .001$ was indicated with two asterisks

ALC range also had significantly improved early immune reconstitution compared to patients who received ATLG outside this range (Table S3). Together with our data on the ATLG dose dependency of leukocyte recovery dynamics, this is the first study to associate optimal ALC with OS and to support the concept of individualized ATLG dosing. Previous reports aiming for optimal ATG dosing using AUC-ATG dosage[13] or patient's lymphocyte count at the start of the preparative regimen[14] provided important evidence on individualized T cell depletion using ATG and supported these new dosage approaches. Maybe due to smaller sample size these data did not associate ALC and OS. Future prospective clinical trials are thus clearly warranted, which should investigate the concept of individualized ATLG or ATG dosage.

This study has a number of limitations due to its retrospective nature, a long recruiting period, the inclusion of different hematologic malignancies and conditioning regimens. We have included all possible elements of bias in multivariate and subgroup analyses and described the impact of each element of bias within both ATLG and no-ATLG cohorts. Despite the observed differences, multivariate analysis supported the study's conclusions. Furthermore, we have provided a large subgroup analysis of patients with acute leukemia, whose results are consistent with the findings in the entire study cohort.

In the face of two conflicting prospective clinical trials without upcoming further prospective studies on ATLG, large retrospective analyses may help to clarify its prophylactic role and thereby provide important hints for its use in clinical practice. Our single-center data, which is delineated from one of the largest ATLG patient cohorts, supports the addition of ATLG to the short-course MTX and CSP regimen. The use of ATLG effectively reduced grades III-IV aGVHD and extensive cGVHD translating into improved long-term EFS and OS after MUD-HCT. In addition, this study supports the concept of individualized dosing of ATLG. Within the ALC optimum of 0.4 and 1.45/nL, our data suggest an ATLG dosage of 30 mg/kg. Our data on the ATLG dose-dependency of leukocyte and T helper cell recovery dynamics and on the association of optimum ALC with improved OS provide a new perspective on in-vivo T cell depleting GVHD prophylaxis that should be pursued in future studies.

ACKNOWLEDGMENTS

Clinical research was supported by the University of Duisburg-Essen. This study was in part supported by Bundesministerium für Bildung und Forschung (BMBF) grant No. 031L0027 (DWB) and UDE-IFORES grant (ATT). The doctoral thesis of VK is part of this work. We would like to thank in particular Andrea Weggen, Ulrike Krumpf, and all coworkers of the BMT documentation team for assistance. We further thank all technicians of the BMT laboratory for assistance.

DISCLOSURE

The authors of this manuscript have potential conflicts of interest to disclose as described by the *American Journal of Transplantation*. ATT: Consultancy for MSD, JAZZ, CSL. Travel subsidies from Neovii Biotech outside the submitted work. VK received travel subsidies from Gilead and JAZZ, NKS received travel subsidies from MSD and DWB received travel subsidies from Medac, all outside the submitted work. The other authors declare no competing financial interests within the submitted work.

AUTHOR CONTRIBUTIONS

ATT and DWB designed the study. VK performed data collection; RT, HO, NKS, MD, LK, TL, and MK participated in clinical data acquisition. EB and DWB performed statistical analysis. EB and ATT developed the ALC model, and ATT, DWB, and EB interpreted data. VK, SL, and NTM participated in data analysis. ATT and DWB wrote the manuscript. VK, EB, LK, MK, and KF contributed to write the manuscript. KF corrected the manuscript. All the authors had access to primary clinical trial data, read and approved the final manuscript.

ORCID

Amin T. Turki https://orcid.org/0000-0003-1347-3360

Katharina Fleischhauer https://orcid.org/0000-0002-5827-8000

Dietrich W. Beelen https://orcid.org/0000-0001-5050-220X

DATA AVAILABILITY STATEMENT

Data are available on reasonable request due to privacy/ethical restrictions.

REFERENCES

1. Gooley TA, Chien JW, Pergam SA, et al. Reduced mortality after allogeneic hematopoietic cell transplantation. *N Engl J Med*. 2010;363(22):2091-2101.
2. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. *N Engl J Med*. 1986;314(12):729-735.
3. Beelen DW, Quabeck K, Kaiser B, et al. Six weeks of continuous intravenous cyclosporine and short-course methotrexate as prophylaxis for acute graft-versus-host disease after allogeneic bone marrow transplantation. *Transplantation*. 1990;50(3):421-427.
4. Genestier L, Fournel S, Flacher M, Assossou O, Revillard JP, Bonnefoy-Berard N. Induction of Fas (Apo-1, CD95)-mediated apoptosis of activated lymphocytes by polyclonal antithymocyte globulins. *Blood*. 1998;91(7):2360-2368.
5. Bacigalupo A, Lamparelli T, Bruzzi P, et al. Antithymocyte globulin for graft-versus-host disease prophylaxis in transplants from unrelated donors: 2 randomized studies from Gruppo Italiano Trapianti Midollo Osseo (GITMO). *Blood*. 2001;98(10):2942-2947.
6. Bacigalupo A, Lamparelli T, Barisione G, et al. Thymoglobulin prevents chronic graft-versus-host disease, chronic lung dysfunction, and late transplant-related mortality: long-term follow-up of a randomized trial in patients undergoing unrelated donor transplantation. *Biol Blood Marrow Transplant*. 2006;12(5):560-565.
7. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti-T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a

randomised, open-label, multicentre phase 3 trial. *Lancet Oncol.* 2009;10(9):855-864.
8. Socie G, Schmoor C, Bethge WA, et al. Chronic graft-versus-host disease: long-term results from a randomized trial on graft-versus-host disease prophylaxis with or without anti-T-cell globulin ATG-Fresenius. *Blood*. 2011;117(23):6375-6382.
9. Baron F, Mohty M, Blaise D, et al. Anti-thymocyte globulin as graft-%3cem%3eversus%3c/em%3e-host disease prevention in the setting of allogeneic peripheral blood stem cell transplantation: a review from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Haematologica*. 2017;102(2):224.
10. Ravindra KV, Sanoff S, Vikraman D, et al. Lymphocyte depletion and risk of acute rejection in renal transplant recipients at increased risk for delayed graft function. *Am J Transplant*. 2019;19(3):781-789.
11. Soiffer RJ, Kim HT, McGuirk J, et al. Prospective, randomized, double-blind, phase III clinical trial of anti–T-lymphocyte globulin to assess impact on chronic graft-versus-host disease-free survival in patients undergoing HLA-matched unrelated myeloablative hematopoietic cell transplantation. *J Clin Oncol*. 2017;35(36):4003-4011.
12. Mohty M, Malard F. Antithymocyte globulin for graft-versus-host disease prophylaxis after allogeneic hematopoietic stem-cell transplantation. *J Clin Oncol*. 2017;35(36):3993-3995.
13. Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a multicentre, retrospective, pharmacodynamic cohort analysis. *Lancet Haematol*. 2017;4(4):e183-e191.
14. Kennedy VE, Chen H, Savani BN, et al. Optimizing antithymocyte globulin dosing for unrelated donor allogeneic hematopoietic cell transplantation based on recipient absolute lymphocyte count. *Biol Blood Marrow Transplant*. 2018;24(1):150-155.
15. Gooptu M, Kim HaesookT, Chen Y-B, et al. Effect of antihuman T lymphocyte globulin on immune recovery after myeloablative allogeneic stem cell transplantation with matched unrelated donors: analysis of immune reconstitution in a double-blind randomized controlled trial. *Biol Blood Marrow Transplant*. 2018;24(11):2216-2223.
16. Hannon M, Beguin Y, Ehx G, et al. Immune recovery after allogeneic hematopoietic stem cell transplantation following Flu-TBI VERSUS TLI-ATG conditioning. *Clin Cancer Res*. 2015;21(14):3131-3139.
17. Kröger N, Solano C, Wolschke C, et al. Antilymphocyte globulin for prevention of chronic graft-versus-host disease. *N Engl J Med*. 2016;374(1):43-53.
18. Walker I, Panzarella T, Couban S, et al. Pretreatment with anti-thymocyte globulin versus no anti-thymocyte globulin in patients with haematological malignancies undergoing haemopoietic cell transplantation from unrelated donors: a randomised, controlled, open-label, phase 3, multicentre trial. *Lancet Oncol*. 2016;17(2):164-173.
19. Baron F, Zachée P, Maertens J, et al. Non-myeloablative allogeneic hematopoietic cell transplantation following fludarabine plus 2 Gy TBI or ATG plus 8 Gy TLI: a phase II randomized study from the Belgian Hematological Society. *J Hematol Oncol*. 2015;8:4.
20. Soiffer RJ, LeRademacher J, Ho V, et al. Impact of immune modulation with anti-T-cell antibodies on the outcome of reduced-intensity allogeneic hematopoietic stem cell transplantation for hematologic malignancies. *Blood*. 2011;117(25):6963-6970.
21. Baron F, Labopin M, Blaise D, et al. Impact of in vivo T-cell depletion on outcome of AML patients in first CR given peripheral blood stem cells and reduced-intensity conditioning allo-SCT from a HLA-identical sibling donor: a report from the Acute Leukemia Working Party of the European Group for Blood and Marrow Transplantation. *Bone Marrow Transplant*. 2014;49(3):389-396.
22. Rubio MT, D'Aveni-Piney M, Labopin M, et al. Impact of in vivo T cell depletion in HLA-identical allogeneic stem cell transplantation for acute myeloid leukemia in first complete remission conditioned with a fludarabine iv-busulfan myeloablative regimen: a report from the EBMT Acute Leukemia Working Party. *J Hematol Oncol*. 2017;10(1):31.
23. Wakamatsu M, Terakura S, Ohashi K, et al. Impacts of thymoglobulin in patients with acute leukemia in remission undergoing allogeneic HSCT from different donors. *Blood Adv*. 2019;3(2):105-115.
24. Dabas R, Jamani K, Kangarloo SB, et al. Antirelapse effect of pretransplant exposure to rabbit antithymocyte globulin. *Blood Adv*. 2019;3(9):1394.
25. Glucksberg H, Storb R, Fefer A, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. *Transplantation*. 1974;18(4):295-304.
26. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15(6):825-828.
27. Rowlings PA, Przepiorka D, Klein JP, et al. IBMTR Severity Index for grading acute graft-versus-host disease: retrospective comparison with Glucksberg grade. *Br J Haematol*. 1997;97(4):855-864.
28. Harris AC, Young R, Devine S, et al. International, multicenter standardization of acute graft-versus-host disease clinical data collection: a report from the Mount Sinai Acute GVHD International Consortium. *Biolog Blood Marrow Transplant*. 2016;22(1):4-10.
29. Shulman HM, Sullivan KM, Weiden PL, et al. Chronic graft-versus-host syndrome in man: a long-term clinicopathologic study of 20 Seattle patients. *Am J Med*. 1980;69(2):204-217.
30. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group Report. *Biol Blood Marrow Transplant*. 2015;21(3):389-401.e381.
31. Admiraal R, Lindemans CA, van Kesteren C, et al. Excellent T-cell reconstitution and survival depend on low ATG exposure after pediatric cord blood transplantation. *Blood*. 2016;128(23):2734-2741.
32. Lowdell MW, Craston R, Ray N, Koh M, Galatowicz G, Prentice HG. The effect of T cell depletion with Campath-1M on immune reconstitution after chemotherapy and allogeneic bone marrow transplant as treatment for leukaemia. *Bone Marrow Transplant*. 1998;21(7):679–686.
33. Bosch M, Dhadda M, Hoegh-Petersen M, et al. Immune reconstitution after anti-thymocyte globulin-conditioned hematopoietic cell transplantation. *Cytotherapy*. 2012;14(10):1258–1275.
34. Ayuk F, Diyachenko G, Zabelina T, et al. Comparison of two doses of antithymocyte globulin in patients undergoing matched unrelated donor allogeneic stem cell transplantation. *Biol Blood Marrow Transplant*. 2008;14(8):913-919.
35. Locatelli F, Bernardo ME, Bertaina A, et al. Efficacy of two different doses of rabbit anti-T-lymphocyte globulin to prevent graft-versus-host disease in children with haematological malignancies transplanted from an unrelated donor: a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2017;18(8):1126-1136.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Turki AT, Klisanin V, Bayraktar E, et al. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies. *Am J Transplant*. 2020;20:677–688. https://doi.org/10.1111/ajt.15642

Online Supporting Information to:

Optimizing anti-T-lymphocyte globuline dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies

Amin T. Turki,[1, 2] Vesna Klisanin,[1] Evren Bayraktar,[1, 3] Lambros Kordelas,[1] Rudolf Trenschel,[1] Hellmut Ottinger,[1] Nina K. Steckel,[1] Nikolaos Tsachakis-Mück,[1] Saskia Leserer, [1] Markus Ditschkowski,[1] Tobias Liebregts,[1] Michael Koldehoff,[1] Katharina Fleischhauer[2] and Dietrich W. Beelen[1]

[1] Department of Bone Marrow Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

[2] Institute of Experimental Cellular Therapy, University Hospital Essen, Essen, Germany

[3] Chair of Applied Mathematics and Numerics III, TU Dortmund University, Germany

Supplementary Methods:

Correlative studies

We collected blood serum and from the anti-Jurkat-T-lymphocyte globuline (ATLG)- and no-ATLG therapy cohort at different time-points after transplantation. All analyses were performed at the BMT laboratory of University Hospital Essen. Peripheral blood mononuclear cells (PBMC) were isolated using an automatic red blood cell lysing system (TQ-Prep, Beckman Coulter, Brea, CA), washed with Fluorescence-activated cell sorting (FACS) buffer and stained with surface markers. No intracellular staining was performed. FACS analysis of the patient's immune status was performed on a FC500 and NAVIOS flow cytometer (Beckman Coulter) using the manufacturer's software. A minimum of 15,000 lymphocytes were analyzed to ensure adequate subset analysis. Specific cell subsets within the CD45+ lymphocyte gate were characterized as following: T cells, CD3+; T helper cells, CD3+/CD4+; activated T cells, CD3+/HLA-DR+;; cytotoxic T cells, CD3+/CD8+; naïve T helper cells, CD3+/CD4+/CD45RA+,memory T helper Cells, CD3+/CD4+/CD45RO+; B cells, CD19+; Natural killer (NK) cells, CD16+/CD56+; αβ T cells, CD3+/ αβ-T +; γδ-T Cells, CD3+/ γδ -T +.

Statistical Analysis

While examining the significance of the contingency between the two cohorts of patients (ATLG vs no-ATLG) we adopted the established Fisher-Exact two-tailed test for discrete variables. When the variables of interest were continuous, described with median and extreme values (min-max), we used the Wilcoxon rank sum test. These two tests were used to study the patient baseline characteristic and GVHD characteristics. The OS was analyzed with the Kaplan-Meier method so that event probabilities of time-to-event intervals without competing events were obtained. While analyzing the survival of different cohorts, the log-rank test was chosen to compare heterogeneity of the survival distributions. The *p*-values in the log-rank test were calculated for two-sided 95% confidence intervals; this was also the adopted convention for Cox-regression analysis. When we had multiple testing, the significances were adjusted according to the method of Šidák (Šidák, 1967), and $p\text{-value} < 0.01$ was accepted to indicate statistical significance. The results of survival analyses for 3 strata (two different ATLG-dose cohorts and a no-ATLG cohort) were obtained as in Figure 1 with this approach. A cox proportional hazards model was adopted to calculate the hazard rate. Multivariate and univariate Cox-Regression analyses were performed with Statistical Analysis Software (SAS, Release 9.4, Version 7.100.1.2711, 2015, SAS/STAT User's Guide 14.1; Cary, NC, USA). Cox-Regression analysis of clinical endpoints involved OS, the incidence and severity of acute and chronic GVHD, CRI, NRM, EFS, and hematological regeneration. The onset of acute or chronic GVHD was examined as a time-dependent variable. Only variables with a significance level <0.05 in univariate analysis were considered for multivariate models. After adjustment for all other significant variables in the model, a significance level of $p\text{-value} < 0.01$ was accepted. While building models based on multivariate Cox-Regression analysis, the following variables have been considered as potential influencing factors: *(i)* patient and donor age, *(ii)* patient and donor gender constellation (female donor for male recipient versus other constellations), *(iii)* underlying disease and stage of disease at the time of transplantation, stem

cell sources (BM versus PBSC), *(iv)* conditioning regimen, *(v)* GVHD prophylaxis with or without ATLG, *(vi)* ATLG dosage and HLA disparities. Clinical endpoints for analysis included OS, the incidence and severity of acute and chronic GVHD, CRI, NRM, EFS, and hematological regeneration. Interactions of competing events, the *hematological regeneration* (competing event: primary graft rejection, NRM and recurrence), the *NRM* (competing event: recurrence) and the *CRI* (competing event: NRM), were studied using cumulative incidence rates. Homogeneity of the cumulative incidence functions is tested by the Gray method (Gray, 1988); the corresponding *subdistribution hazards* and the *confidence intervals* (95% CI) were calculated using the Fine and Gray method (Fine and Gray, 1999).

For development of a Lymphocyte-based dosing ATLG model, we used Statistical Analysis Software (SAS, Release 9.4, Version 7.100.1.2711, 2015, SAS/STAT User's Guide 14.1; Cary, NC, USA). Lymphocyte data was measured on day -5 before HCT. For a total of 564 patients, lymphocyte data could be obtained and was correlated with the total administered ATLG dosage. Cox proportional hazards model was adopted to calculate the hazard rate. Multivariate and univariate Cox-Regression analysis was performed for competing risks and subgroup analysis using SAS software. The statistical significance of T Lymphocyte changes in flow cytometry after transplantation was evaluated by comparing both ATLG and no-ATLG cohorts with a *two-sample T-test* in Matlab (Mathworks, Natick, MA).

Immunosuppressive and supportive therapy prophylaxis description

The uniform, immunosuppressive pharmacological GVHD prophylaxis consisted of 3 mg/kg body weight intravenous ciclosporin (CSP) starting from day -1 before HCT in combination with 15 mg/m2 methotrexate (MTX) on day +1 und 10 mg/m2 MTX on days +3, +6 and +11 after HCT.(1, 2) Normal CSP target blood levels (range, 150-250 ng/ml) were controlled three

times weekly. Patients of the ATLG cohort received additional polyvalent rabbit-ATLG at a dosage of 10 mg/kg bodyweight on days -4, -3 and -2 (cumulative dosage: ATLG 30 mg/kg) or at a dosage of 20 mg/kg bodyweight on days -4, -3 and -2 (cumulative dosage: ATLG 60 mg/kg). Before patient discharge, intravenous CSP was substituted orally. From day +100 after HCT, CSP was continuously tapered for patients without clinical signs and symptoms of GVHD.

Supportive therapy and anti-infectious prophylaxis was identical for patients of both ATLG and no-ATLG cohorts and independent of the conditioning intensity. With the beginning of the conditioning regimen until discharge, all in patients were treated in reverse isolation single rooms with high-efficacy particle air filtration. In the absence of contraindications, all patients received a combined intestinal decontamination medication as previously described [(3)] consisting of oral metronidazole at 400 mg three times daily and oral ciprofloxacin at 750 mg twice daily starting from day -14 until day +35 after HCT. Antiviral prophylaxis during neutropenia consisted of intravenous aciclovir at 250 mg three times daily. Antifungal prophylaxis consisted of oral posaconazole at 200 mg three times daily from day+1 for HCT patients [(4)] during the 2010s years or oral itraconazole at 200 mg twice daily from day +1 for HCTs during the 2000s years with a minimal duration until day +100. Colony stimulating factors were not routinely applied. As pneumocystis-jirovecii pneumonia prophylaxis patients received either monthly pentamidin inhalation or oral cotrimoxazol at 960 mg three times per week from day +30. Neutropenic patients' meals were prepared as decontaminated or germ-poor meals. Irradiated red blood cell and platelet transfusions and in-line leukocyte-filtered products were exclusively during the entire HCT course.

Supplementary Table S1. Cox-Regression analysis of possible confounders.

Predictor	HR	99% CI	*p*
With respect to overall survival from transplantation			
Age (10 years of increase)	1.192	1.115 – 1.275	< 0.0005
Transplantation year (5 years of increase)	1.032	0.956 – 1.114	0.286
Transplantation period			
2000 - 2009	1.004	0.753 – 1.340	0.971
2010 - 2016	1.077	0.807 – 1.437	0.509
Myeloablative conditioning	0.795	0.665 – 0.950	0.001
PBSC	1.023	0.856 – 1.301	0.806
High risk disease stages$	1.384	1.123 – 1.704	< 0.0005
With respect to relapse from transplantation			
Reduced intensity conditioning	1.218	1.063 – 0.196	< 0.0005
High risk disease stages$	1.172	0.867 – 1.586	0.176
Subgroup analyses			
With respect to overall survival from transplantation			
AML/MDS	1.219	1.021 – 1.456	0.004
AML/MDS stratified according to high risk disease stages$	1.351	1.223 – 1.624	< 0.0005
CML	0.560	0.435 – 0.721	< 0.0005
CML stratified according to high risk disease stages$	0.481	0.371 – 0.624	< 0.0005
High risk disease stages$ (CML excluded) stratified according to ATLG use	0.702	0.558 – 0.881	< 0.0005
With respect to relapse, patients surviving ≤ 5 years from transplantation			
Advanced disease stages	1.401	1.033 – 1.899	< 0.0005

Abbreviations: *HR*: Hazard ratio; *CI*: Confidence interval; *p*: Significance as p-value

$ Standard risk: De-novo AML in 1st remission, ALL in 1st remission, MDS with single lineage dysplasia, and MDS with single lineage dysplasia and ring sideroblasts, CML in 1st chronic phase. High-risk stages were all other stages that did not correspond to standard risk stages, such as AML in 2nd remission. Patients with myeloproliferative disorders, multiple myeloma and lymphomas were only transplanted in high risk disease stages.

Supplementary Table S2. Cox-Regression analysis for HR of competing risks in a Lymphocyte-based dosing ATLG model

Predictor	**HR**	**99% CI**	**p**
With respect to overall survival from transplantation and stratified by ATLG dosage (n=564)			
Logarithm of lymphocyte counts *	1.28	1.12 – 1.47	<0.0005
lymphocyte counts in [0.4, 1.45]/*nl* (n = 230)	0.68	0.53 – 0.86	0.002
With respect to overall survival from transplantation in ATLG cohort (n = 399)			
lymphocyte counts ≥ 0.1/*nl* (n = 351)	0.68	0.47 – 1.00	0.05
lymphocyte counts in [0.4, 1.45]/*nl* (n = 187)	0.62	0.47 – 0.82	0.001
With respect to overall survival from transplantation in no-ATLG cohort (n = 164)			
lymphocyte counts ≥ 0.1/*nl* (n = 116)	0.90	0.60 – 1.36	0.62
lymphocyte counts in [0.4, 1.45]/*nl* (n = 43)	0.90	0.51 – 1.36	0.27

HR: Hazard ratio; *CI*: Confidence interval; *p*: Significance as p-value

* Lymphocyte data was measured on day -5 before HCT. Total patients with available lymphocyte data on d-5: n=564. Patients were stratified into 3 groups, ATLG 30 mg/kg, ATLG 60mg/kg and no-ATLG. In an unstratified analysis of all patients, no significant results were obtained. Natural logarithm is adopted, corresponding to ~2.72 times of the lymphocyte counts increase the Hazard with a factor of 1.28

Supplementary Table S3. Comparison of early immune reconstitution (3 months after transplantation) using multicolor flow cytometry with respect to ATLG dosage and ALC counts before ATLG.

	Median of absolute cell count (cells/µl)	p*
T cells (CD3+) (n=346), analysis of dose-dependent effects		
No ATLG (versus ATLG 30 mg)	572	0.00003
ATLG 30 mg (versus ATLG 60 mg)	326	0.07
ATLG 60 mg (versus no ATLG)	278	0.00002
T helper cells (CD3+/CD4+) with respect to ALC analysis, only ATLG patients (n=152)		
ALC ≤0.1/nl (versus ALC >0.1/nl)	1	0.02
ALC >0.1/nl	328	
Within optimal ALC (0.4/nl-1.4/nl; n=80) versus outside-optimum ALC	398	0.005
Outside-optimum ALC (<0.4/nl or >1.4/nl; n=72)	284	
T helper cells (CD3+/CD4+)(n=346), analysis of dose-dependent effects		
No ATLG (versus ATLG 30 mg)	217	<0.00001
ATLG 30 mg (versus ATLG 60 mg)	62	0.003
ATLG 60 mg (versus no ATLG)	38	<0.00001
T helper cells (CD3+/CD4+) with respect to ALC analysis, only ATLG patients (n=152)		
ALC ≤0.1/nl (versus ALC >0.1/nl)	0	0.02
ALC >0.1/nl	54	
Within optimal ALC (0.4/nl-1.4/nl; n=80) versus outside-optimum ALC	68	0.002
Outside-optimum ALC (<0.4/nl or >1.4/nl; n=72)	47	

ATLG: Anti T lymphocyte globulin; ALC: Absolute lymphocyte count on day -5 before HCT; *p*: Significance as p-value

*Median absolute cell counts were compared using Wilcoxon-rank sum test. Given the hypothesis that ATLG exposure decreases T cell counts, we compared the ATLG dose cohorts with a right-sided test. ALC cohorts were compared with a two-sided Wilcoxon-rank sum test.. p-values <0.05 were considered as significant values. Lymphocyte data was measured on day -5 before HCT. Total patients with available lymphocyte and flow cytometry data on d-5: n=214, of whom n=152 were exposed to ATLG.

Supplementary Table S4. Cox-Regression analysis of disease cohorts.

Predictor	HR	95% CI	p
With acute grades II-IV GVHD as end event, stratified by disease status and ATLG exposure*			
Other myeloid malignancies (n=521) versus all acute leukemias (n=766)	1.025	0.764 – 1.375	0.8696
Other lymphoid malignancies (n=207) versus all acute leukemias (n=766)	0.899	0.625 – 1.293	0.5641
With acute grades II-IV GVHD as end event, stratified by ATLG exposure			
Other myeloid malignancies (n=521) versus all acute leukemias (n=766)	1.093	0.826 – 1.445	0.5331
Other lymphoid malignancies (n=207) versus all acute leukemias (n=766)	0.944	0.661 – 1.346	0.7491

HR: Hazard ratio; *CI*: Confidence interval; *p*: Significance as p-value

* Disease status refers to high risk and standard risk disease. Patients were categorized into 3 disease categories: Acute leukemias (AML and ALL), other myeloid malignancies (CML, MPN, MDS, CMML) and other lymphoid malignancies (CLL, NHL, HD and MM)

Supplementary Figure Legends:

Supplementary Figure S1: Illustration of the results of Cox-Regression in supplementary Table 1 for a given hazard rate H_0.

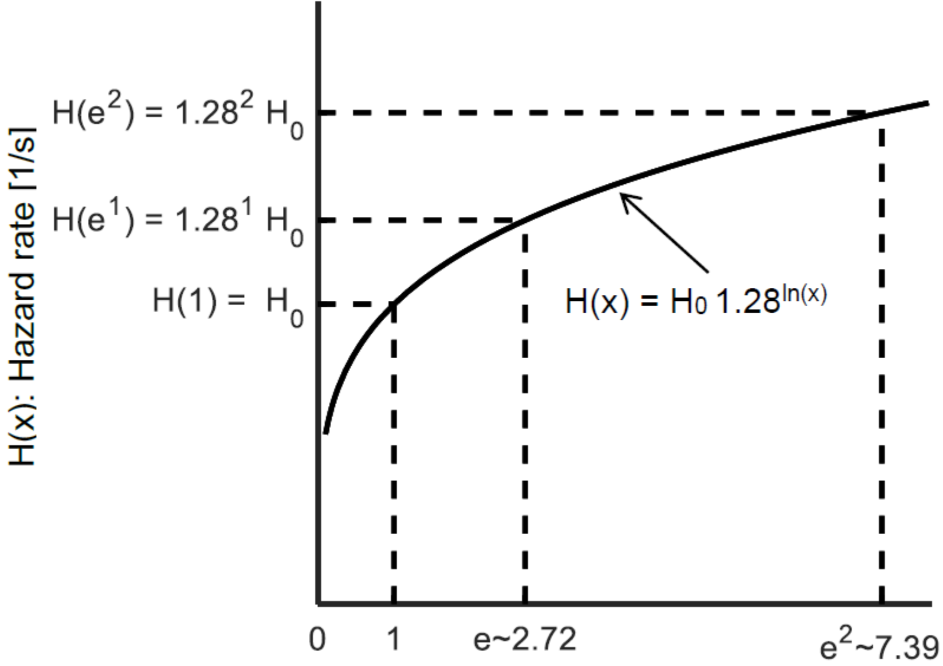
Lymphocyte data was measured on day -5 before HCT. Patients with available lymphocyte data on d-5, n=564. Patients were stratified into 3 groups, ATLG 30mg/kg, ATLG 60mg/kg and no-ATLG

Supplementary Figure S2: Lymphocyte count spectrum at ATLG exposure.

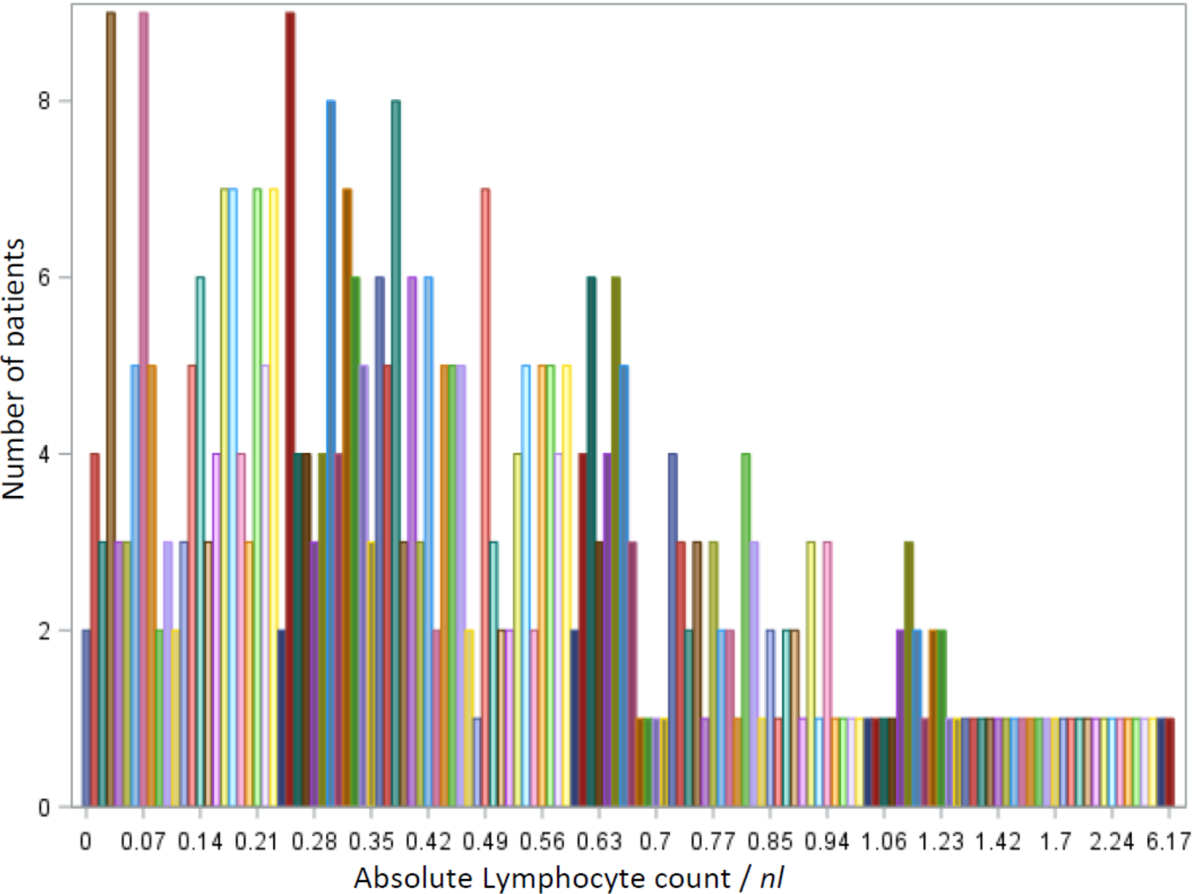
Bar plot of the ATLG sub-cohort with available measured lymphocyte data on day -5 before HCT (n=564).

Supplementary Figure S3: Normalized T Lymphocyte regeneration. Lymphocyte subsets in the peripheral blood were analyzed as in Supplementary Figure 3. The normalized lymphocyte evolution after HCT was calculated from absolute cell numbers after HCT, with 1 representing the maximum count of the patient during the study period. Mean values and the standard error of the mean are shown. A p-value < 0.05 was considered statistically significant and indicated in the figure with an asterix.

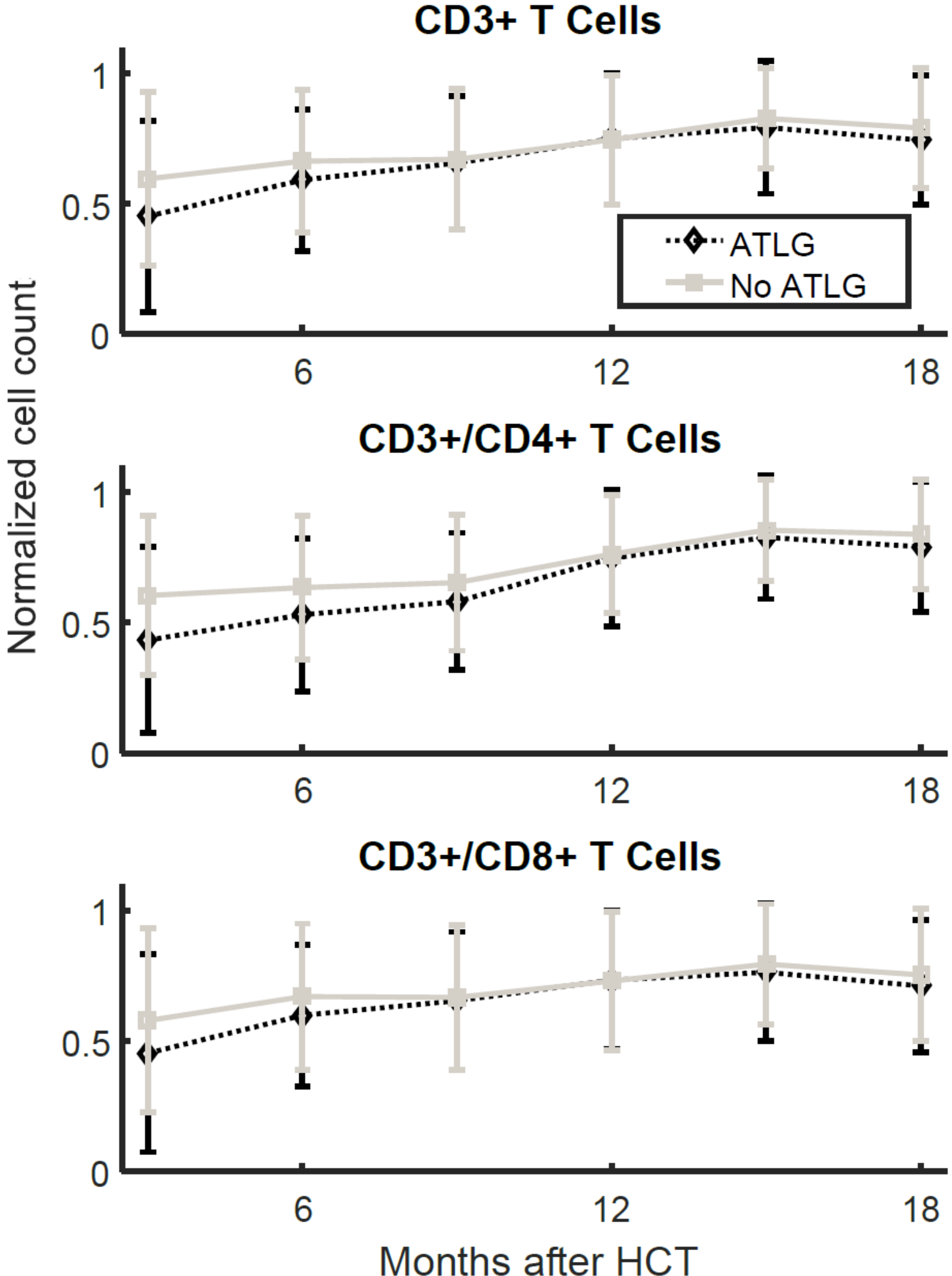
Supplementary Figure S1:



Supplementary Figure S2:



Supplementary Figure S3:



Supplementary References

1. Storb R, Deeg HJ, Whitehead J, Appelbaum F, Beatty P, Bensinger W et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. The New England journal of medicine 1986;314(12):729-735.
2. Beelen DW, Quabeck K, Kaiser B, Wiefelsputz J, Scheulen ME, Graeven U et al. Six weeks of continuous intravenous cyclosporine and short-course methotrexate as prophylaxis for acute graft-versus-host disease after allogeneic bone marrow transplantation. Transplantation 1990;50(3):421-427.
3. Beelen DW, Elmaagacli A, Müller K-D, Hirche H, Schaefer UW. Influence of Intestinal Bacterial Decontamination Using Metronidazole and Ciprofloxacin or Ciprofloxacin Alone on the Development of Acute Graft-Versus-Host Disease After Marrow Transplantation in Patients With Hematologic Malignancies: Final Results and Long-Term Follow-Up of an Open-Label Prospective Randomized Trial. Blood 1999;93(10):3267.
4. Cornely OA, Maertens J, Winston DJ, Perfect J, Ullmann AJ, Walsh TJ et al. Posaconazole vs. fluconazole or itraconazole prophylaxis in patients with neutropenia. N Engl J Med 2007;356(4):348-359.

6.4 Article IV

Author contributions

Cellular immune reconstitution analysis reveals distinct phenotypes and clinically relevant heterogeneity among patient cohorts receiving either anti-T-lymphocyte globulin or post-transplant cyclophosphamide in HCT

Saskia Leserer, Theresa Graf, Rashit Bogdanov, Martina Franke, Ulrike Buttkereit, Nils Leimkühler, Katharina Fleischhauer, H. Christian Reinhardt, Dietrich W. Beelen, Amin T. Turki

Original Article in NA

Impact factor: NA

Status: Manuscript in preparation

DOI: NA; PubMed-ID: NA

Complete citation of the original source: NA

Note: A peer-reviewed version of this manuscript was published as “Time series clustering of T cell subsets dissects heterogeneity in immune reconstitution and clinical outcomes among MUD-HCT patients receiving ATG or PTCy” in Frontiers of Immunology on March 20th, 2023[163].

Contributions:

Conception:	30 % - study design, cohort definition
Experimental work:	70 % - data collection, data validation
Data analysis:	60 % - data processing, data filtering, data matching, development of time series clustering
Statistical analysis:	60 % - clinical analysis, analysis of immune reconstitution data, data interpretation
Writing the manuscript:	35 % - visualization of the results, literature research, writing the manuscript, preparation of the supplemental material
Revising the manuscript:	NA

Prof. Dr. med. Dietrich W. Beelen

Saskia Leserer

Cellular immune reconstitution analysis reveals distinct phenotypes and clinically relevant heterogeneity among patient cohorts receiving either anti-T-lymphocyte globulin or post-transplant cyclophosphamide in HCT

Saskia Leserer[1, 2, 3], Theresa Graf[1, 2], Rashit Bogdanov[1, 2], Martina Franke[1], Ulrike Buttkereit[1], Nils Leimkühler[1], Katharina Fleischhauer[3, 4], H. Christian Reinhardt[1], Dietrich W. Beelen[1] and Amin T. Turki[1, 2, 3]

[1] Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

2 Computational Hematology Lab, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

3 Institute for Experimental Cellular Therapy, West-German Cancer Center, University Hospital Essen, Essen, Germany

4 German Cancer Consortium (DKTK)

Keywords: GVHD prophylaxis, immune reconstitution patterns, GVHD protection, time series clustering

Text word count, 4870; abstract word count, 232. 5 figures; 2 tables, 6 supplementary figures, 5 supplementary tables and 50 references.

Corresponding author:

Amin T. Turki, MD PhD, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, University of Duisburg-Essen, Hufelandstrasse 55, 45122 Essen; email: amin.turki@uk-essen.de; Phone: +49-201-723-83686; Fax +49-201-723-5961

Key Points:

1. GVHD prevention with ATG and PTCy results in the predominance of distinct T cell fractions after HCT, still with comparable clinical outcomes
2. Analysis of immune reconstitution by dynamic time warping identified clinically relevant subgroups in ATG cohort

Authors' ORCID:

Saskia Leserer, M. Sc. https://orcid.org/0000-0002-4956-1760

Dr. Nils B. Leimkühler, MD https://orcid.org/0000-0003-2530-4329

Prof. Katharina Fleischhauer, MD https://orcid.org/0000-0002-5827-8000

Prof. Dietrich W. Beelen, MD https://orcid.org/0000-0001-5050-220X

Dr. Amin T. Turki, MD PhD https://orcid.org/0000-0003-1347-3360

Abstract

Graft-versus-host disease (GVHD) prophylaxis with anti-T-lymphocyte globulin (ATG) and post-transplant cyclophosphamide (PTCy) effectively reduces the incidence of GVHD after stem cell transplantation. While clinical differences between both agents in different donor settings have been previously described, their respective impact on cellular immune reconstitution is only sparsely known. We retrospectively analyzed clinical outcomes and immune reconstitution in 384 HCT recipients with matched-unrelated- (MUD) or haploidentical donors either receiving ATG (n=304, all MUD) or PTCy (n=80, n=35 MUD, n=45 haplo). Using conventional analysis and time-series clustering, these patients were studied both on a populational- and individual level. GVHD prophylaxis with PTCy was slightly more effective than ATG in preventing grades II-IV acute GVHD (55.3% MUD-PTCy, 57.6% haplo-PTCy and 68.3% MUD-ATG; p=0.032). In all other clinical outcomes, no significant differences were observed. Cellular reconstitution suggested that distinct T cell populations mediated GVHD protection. In PTCy patients, GVHD prophylaxis resulted in elevated regulatory T cell levels, while protection in patients with ATG was likely conveyed by higher levels of γδ T or NKT cells. Analysis of individual patients' cellular immune reconstitution, using a new approach with time-series clustering using dynamic time warping further dissected heterogeneity of reconstitution and identified patients with impaired transplant outcomes. Overall, our data suggest a differential impact of ATG and PTCy on cellular immune reconstitution and further provide guidance on choosing the most appropriate agent for individual patients in the MUD setting.

Abbreviations

aGVHD	acute graft-versus-host disease
ATG	anti-T-lymphocyte-globulin
BMT	bone marrow transplantation
CD	cluster of differentiation
cGVHD	chronic graft-versus-host disease
CI	confidence interval
CIBMTR	Center for International Blood and Marrow Transplant Research
CRI	cumulative incidence of relapse
DTW	dynamic time warping
e.g.	exempli gratia
FACS	fluorescence-activated cell sorting
GVHD	graft-versus-host disease
HCT	hematopoietic stem cell transplantation
HLA	human leukocyte antigen
HR	hazard ratio
kg	kilogram
mg	milligram
MMF	mycophenolate mofetil
(M)MUD	(mis)matched-unrelated donor
NRM	non-relapse mortality
OS	overall survival
PTCy	post-transplant cyclophosphamide
SHR	subdistribution hazard ratio
TCR	T cell receptor
Treg	regulatory T cell

1 Introduction

Despite the introduction of high-resolution human leukocyte antigen typing for donor selection, graft-versus-host disease (GVHD) remains the most frequent complication and a major cause of mortality after allogeneic hematopoietic cell transplantation (HCT)[1,2]. The continuous increase of HCT with alternative donor sources, such as matched- or mismatched unrelated donors (MUD, MMUD) or haploidentical donors[3] required improved GVHD prophylaxis strategies beyond baseline calcineurin inhibitors. Proliferating alloreactive T cells are the leading mediators of acute GVHD (aGVHD)[4], contribute to the pathogenesis of chronic GVHD (cGVHD)[5] and are indeed promising targets for preventing excessive alloreactivity. During the last decade, the addition of *in vivo* T cell depletion by anti-T-lymphocyte globulin (ATG) or alemtuzumab have become the standard-of-care in MUD-HCT in most European centers[6]. More recently, post-transplant cyclophosphamide (PTCy) has proven to be a safe and feasible choice for GVHD prophylaxis in patients with haploidentical-[7-9] or MUD donors[10,11]. Hence, previous studies compared the efficacy of ATG and PTCy as GVHD prophylaxis in different HCT settings, showing comparable GVHD incidences in haploidentical patients[12] and lower incidences of aGVHD II-IV in unrelated donor-HCT with PTCy[13]. While the clinical impact of both agents has been well scrutinized, comparative immune reconstitution studies are very scarce and highlight differential results based on the analyses of cohorts with distinct conditioning regimens[14,15]. ATG is well known to delay the reconstitution of $CD3^+$ and $CD4^+$ T cells, in particular of T helper cells, up to 12 months post-HCT[16,17], while PTCy preserves regulatory T cells allowing its rapid recovery[18] and has a blunting effect on NK cell alloreactivity[19]. The existence of alternative T cell depletion protocols has increased the heterogeneity of treatment protocols across the world, which comes along with potentially reduced comparability between centers and regimens. It has also led to a competition for the best T cell depletion system, which is not yet determined[20].

The potentially differential effects of ATG or PTCy on HCT patients' immune reconstitution may support HCT physicians in their choice in different alternative donor settings. Previously, e.g. a sufficient reconstitution of $CD4^+$ T cells was associated to lower patient mortality[21]. Similarly, early helper T cell reconstitution and clinical patient outcome could be improved by optimized dosing of ATG[17,22]. Based on the hypothesis that differences in immune reconstitution allow to optimize GVHD prophylaxis for individual patients, we studied the cellular immune reconstitution in patients with MUD transplantation using either ATG or PTCy as GVHD prophylaxis as well as patients transplanted from haploidentical donors with PTCy. Immune reconstitution patterns were comparatively analyzed and correlated to clinical outcomes with the primary endpoint of aGVHD grades II-IV in order to identify different cellular mechanisms for GVHD protection. Beyond cohort

comparisons, we developed a method to investigate individual longitudinal immune reconstitution data with the purpose to dissect the heterogeneity in immune reconstitution, leading to a better differentiation of clinical outcomes in patients with the same GVHD prophylaxis.

2 Patients and methods

2.1. Study population

This retrospective study included 551 patients with allogeneic HCT between January 2017 and May 2020 at the Department of Hematology and Stem Cell Transplantation of the West-German Cancer Center, University Hospital Essen. Patients were screened for the following inclusion criteria: administration of *in vivo* T cell depletion with 1) anti-T-lymphocyte globulin (ATG) or 2) post-transplant cyclophosphamide (PTCy) as GVHD prophylaxis for HCT from haploidentical related- or 10/10 matched unrelated donors (MUD, CONSORT diagram, Supplemental Figure 1). A total of 384 patients were eligible for downstream analysis.

GVHD prophylaxis consisted of baseline calcineurin inhibitor-based immunosuppression combined with *in vivo* T cell depletion using either ATG or PTCy. Anti-T-lymphocyte globulin (ATG Neovii, Rapperswill, CH; ATG) (n=304) was applied at a dose of 10mg/kg or 20mg/kg bodyweight on three consecutive days between day -4 and day -2 before HCT based on standardized protocols, followed by ciclosporin and methotrexate starting at day -1. PTCy (n=80) was administered on day +3 and +4 (50 mg/kg body weight per day) post-HCT followed by tacrolimus and mycophenolate-mofetil (MMF) starting on day +5. Out of these 80 patients receiving PTCy as GVHD prophylaxis 45 patients (56%) were transplanted with HCTs derived from haploidentical donors and 35 patients (44%) derived from MUD donors.

Early supportive and follow-up care followed the same internal and was considered identical for all patients. Patients were followed-up until the last documented clinical assessment or death by any cause. Surviving patients were censored at maximum follow-up of 12 months. All patients have given written informed consent to collection, electronic storage, and scientific analysis of anonymized HCT-specific patient data in accordance with German legislation and the revised Helsinki Declaration. We confirm that no patient can be identified through use of anonymized patient data. Study protocol approval was obtained by the institutional review board of the University Duisburg-Essen (Protocols N° 17-7675BO and N° 18-8299-BO).

2.2 *Assessments*

Baseline data concerning patient-, donor-, HCT characteristics and HCT-outcome were documented prospectively in electronic forms. Laboratory parameters and clinical characteristics of patients after HCT were retrospectively analyzed. Clinical assessment was obtained daily for inpatients and at each visit for outpatients. Acute GVHD (aGVHD) is defined as GVHD organ involvement of skin, gut and/or liver until 100 days post-HCT. aGVHD was clinically assessed and classified according to consensus grading for aGVHD[23]. Diagnosis of chronic GVHD (cGVHD)

occurred after day +100 based on characteristics symptoms and clinical signs according to the published NIH criteria for cGVHD[24]. Overall survival was defined as the period from transplantation to a 12-months follow-up or death by any cause. Cumulative relapse incidence (CRI) was calculated as the time from the day of transplantation to the day of documented relapse to original disease or persistence of malignancy. For patients without diagnosed relapse or persisting malignancy, non-relapse mortality (NRM) was determined as the time from day of transplantation to death.

2.3 Monitoring of immune reconstitution, comparative analysis and time-series clustering

Immune reconstitution after HCT was studied in peripheral blood samples from patients at months +1, +3, +6, +9, and +12 after HCT. A total of 1262 samples were analyzed by flow cytometry at the BMT Laboratory, University Hospital Essen. Details on sample preparation, gating strategy and antibodies are detailed in the Supplemental Methods. For each individual point in time, median counts of immune subsets were compared between MUD-ATG, MUD-PTCy and Haplo-PTCy cohorts using Mann-Whitney U test (GraphPad Prism 9.0.0, GraphPad Software, LLC, San Diego California).

Detailed information about the analysis of individual patient' longitudinal immune reconstitution is provided in the Supplementary Method section. In short, we defined two distinct multi-dimensional immune cell clustering models integrating two different subsets of T cells: 1) "GVHD-associated" T cells: $CD3^+/CD4^+/CD25^+/CD127^+$low regulatory T cells, $CD3^+$/HLA-DR^+ activated T cells, $TCR\alpha/\beta^+$ and $TCR\gamma/\delta^+$ T cells and 2) "broad spectrum" T cells: $CD3^+/CD4^+$ helper T cells, $CD3^+/CD4^+/CD45RA^+$ naïve helper T cells, $CD3^+/CD8^+$ cytotoxic T cells and $CD3^+/CD8^+/CD45RO^+$ memory cytotoxic T cells. To apply these models, all patients (n=384) were filtered for 1) at least three flow cytometry measurements within +12 months post-HCT and 2) measurement of the first flow cytometry ≤ d+45 post-HCT. Data filtering resulted in a patient subgroup of 180* patients eligible for clustering analysis (n=147* MUD-ATG, n=15 MUD-PTCy, and n=18 Haplo-PTCy; *"broad spectrum" T cell model included 4 additional patients). After filtering, individual patient time-series data underwent linear interpolation to fill up missing data points. Individual longitudinal immune reconstitution was then studied within each patient cohort by partitional clustering of time-series data. Here, partitional clustering was performed using dynamic time warping (DTW) as distance measure[25] with 36 different function-specific configurations tested. The performance of clustering configurations was evaluated by the silhouette coefficient[26] and model robustness was tested via a 10-fold resampling approach (see Supplementary method section). Data interpolation, DTW and time-series clustering were performed using R[27] packages *R stats*[27] and *dtwclust*[28] (R version 3.6.3, R core Team, https://www.r-project.org/).

2.4 Statistical analysis

Patient baseline characteristics were analyzed with Chi-square test and one-way ANOVA where appropriate (GraphPad Prism 9.0.0). The primary endpoint of this study was the incidence of grades II-IV aGVHD. Secondary endpoints were the overall incidence of 100-day aGVHD, 1-year relapse and NRM, 1-year cGVHD as well as 1-year overall survival. The onset of all-grade aGVHD and aGVHD II-IV in the studied subgroups was calculated with the Kaplan-Meier method, obtaining event probabilities of time-to-event intervals. Furthermore, the cumulative incidence of all-grade aGVHD and aGVHD II-IV was analyzed in a competing risk analysis considering death before d+100 as competing event. Complementary competing risk analysis was performed for cGVHD, which considered death within 12 months after HCT as competing event. The secondary endpoint of 1-year OS was analyzed via Kaplan-Meier analysis[29]; hazards were calculated by a Cox proportional hazard model[30]. The secondary endpoints of NRM and relapse were considered as competing events to each other and analyzed by competing risk analysis. P-values <0.05 were accepted as indication for statistical significance. Clinical outcome analyses were done using the R[27] packages *survival*[31], *survminer*[32] and *cmprsk*[33] (R version 3.6.3, R core Team, https://www.r-project.org/).

3 Results

3.1. Patient characteristics

The alternative donor HCT cohorts included in this study (MUD-ATG, n=304), MUD-PTCy (n=35) and haplo-PTCy, n=45) were balanced for age, gender, disease, graft source, conditioning and CMV recipient/donor serostatus (Suppl. Table 2). The gender mismatch proportion was highest for MUD-PTCY patients (18%); high-risk gender mismatch (F donor/M recipient) was significantly higher in the haplo-PTCY cohort compared to all others ($p<0.0001$). Median study follow-up was 12 months.

3.2. GVHD prophylaxis with PTCy associated with significantly reduced acute GVHD

The cumulative incidence of grades II-IV aGVHD differed significantly between the study cohorts (MUD-ATG 68.3%; haplo-PTCY 57.6% and MUD-PTCY 55.3%, p=0.032, Figure 1A, Table 1), with the lowest frequency of grade II-IV aGVHD in haplo-PTCy patients (Figure 1C). Similarly, the incidence of all-grade aGVHD was numerically lowest in the MUD-PTCy cohort (p=0.081, Figure 1B). Median time to both aGVHD II-IV and all-grade aGVHD was significantly longer in patients receiving PTCy as compared to the MUD-ATG cohort (p=0.032, Suppl. Figure 2A, MUD-ATG: 22 days, MUD-PTCy 24 days and haplo-PTCy 57 days; and for all-grade aGVHD 20 and 21 days for haplo- and MUD-PTCy vs 17 days in MUD-ATG, p=0.049, Suppl. Figure 2B). Fine and Gray competing risk regression corroborated these results, revealing significantly lower aGVHD subdistribution hazards for both PTCy cohorts compared to MUD-ATG (haplo-PTCy: SHR 0.77, 95%CI 0.60-1.00, p=0.05; MUD, PTCy: SHR 0.68, 95%CI 0.48-0.97, p=0.034, Suppl. Table 3). For grades II-IV aGVHD, this effect was differentially pronounced between donor settings (haplo-PTCy: SHR 0.54, 95%CI 0.33-0.86, p=0.010; MUD-PTCy: p=0.220, Suppl. Table 3). Inclusion of different pre-HCT parameters (e.g. conditioning regimen or CMV serology) into the analysis of aGVHD II-IV revealed no further significant covariates (detailed in Table 2). Both, all-grade cGVHD and moderate-severe cGVHD at 12 months after HCT were comparable between cohorts (p=0.436 and p=0.511, respectively, Figure 1D, E), although the incidence of severe cGVHD was lower in the MUD-ATG cohort (Figure 1F). MUD-PTCy patients developed cGVHD at higher frequencies compared to the other subgroups, while the fraction of documented cGVHD in the haplo-PTCy and MUD-ATG groups did not show any differences (Figure 1F). OS, NRM and relapse at 12 months did not significantly differ between cohorts (Figure 1G-I, Suppl. Table 3).

3.3. Strategies for T cell depletion associated with distinct T cell subsets involved in aGVHD prophylaxis

The comparative analysis of patients' cellular immune reconstitution revealed significant differences in T cell subsets, which paralleled the observed clinical differences in aGVHD incidence. Since T cells are considered to be the major mediators in aGVHD, these differences provide alternative mechanisms of immune modulation induced by ATG or PTCy as strategy for T cell depletion. Throughout the entire observation period of 12 months after HCT, ATG patients had significantly lower absolute counts within the helper T cell compartments (Figure 2A-E) compared to patients receiving PTCy. This pattern was also observed for TCR α/β T cells through month 6 (Figure 2G). Interestingly, the absolute regulatory T cell (Treg) counts were also significantly higher through 6 months after HCT in patients receiving PTCy as T cell depletion (Figure 2D) as compared to the MUD-ATG cohort. Further differentiation of the PTCy cohorts revealed constantly higher Tregs levels in haplo-PTCy, while the medians in MUD-PTCy patients plateaued after the first 6 months. Contrary to this generally observed T cell pattern, the median TCR γ/δ T cell counts (Figure 2H) in the MUD-ATG cohort were significantly higher compared to patients receiving PTCy. In particular, the MUD-PTCy subgroup had very low TCR γ/δ T cell levels. Of notice, T cell subsets of both PTCy cohorts although comparable up to month 6 later plateaued or even declined in the MUD-PTCy cohort, which was consistently observed for helper T cells, Tcon, TCRα/β, TCRγ/δ, naïve helper T cells and Tregs (Figure 2). Interestingly, early NKT cell counts were significantly higher in the MUD-ATG cohort as compared to the PTCy subgroups (months 1 and 3, Suppl. Figure 3F). The reconstitution of further subsets as for example cytotoxic- and activated T cells subsets (Suppl. Figure 3B-E), as well as NK- and B cells (Suppl. Figure 3G-H) were comparable for ATG and PTCy cohorts.

The decreasing $CD3^+$ T cell levels in MUD-PTCy patients from month 6 led to comparable $CD3^+$ numbers to MUD-ATG patients at month 12. This finding was consistent throughout the majority of T cell subsets, with little exceptions, equalizing the above-described early differences in immune reconstitution of MUD patients between both T cell depleting regimens in the long-term. The distinct immune reconstitution patterns also correlated with significant clinical differences in grades II-IV aGVHD between cohorts and raised the question, whether different cellular mechanisms conveyed aGVHD prophylaxis in the different T cell depleting regimens. In the PTCy cohorts, the lower incidence of grade II-IV aGVHD (Figure 1A), correlated with higher early Treg counts at months 1 and 3 after HCT (Figure 2D). Contrarily, the strong reconstitution of TCR γ/δ cells (Figure 2H) or NKT cells (Suppl. Figure 3F) likely mediates a GVHD-protective effect in patients receiving ATG. Despite such strikingly different immune reconstitution profiles, the most

relevant clinical outcomes OS, relapse and NRM at 12 months after HCT did not differ between cohorts, indicating comparable clinical efficacy of the different T cell depleting protocols.

3.4. Time-series immune clustering reveals heterogeneity of phenotypes and outcomes within the ATG and PTCy cohorts

Beyond the comparison of pooled immune reconstitution data in ATG- and PTCy cohorts, we next sought to analyze cellular recovery with an approach which is able to dissect heterogeneity and consequently distinguish patient outcomes within these cohorts. Here, we developed multi-dimensional parameter models integrating longitudinally measured reconstitution data of several T cell subsets for each patient. Building on immunologic evidence from past studies we designed a first model using T-cell subsets with known mechanistic impact in GVHD[34,35] ("GVHD-associated" T cell model). The second model included relevant T cell populations of both the $CD4^+$ and $CD8^+$ T cell compartment[36,37] ("broad spectrum" T cell model). In order to manage their complexity both models were limited to four T cell subsets, i.e. regulatory-, activated-, αβ-, and γδ T cells for the "GVHD-associated"- as well as helper-, naïve helper, cytotoxic-, and memory cytotoxic T cells for the "broad spectrum"- models. These multi-dimensional models were analyzed for patterns within each patient subgroup (MUD-ATG, MUD-PTCy and haplo-PTCy) by time-series clustering in order to dissect their heterogeneity. Identified clusters were correlated to clinical outcomes and revealed differences in survival and GVHD. The methodological workflow is detailed in the supplementary methods and illustrated in Figure 3A. Clinical outcomes of patients included in this multi-dimensional analysis (n=180) were comparable to those of the overall cohort (Figure 3B-D), making a selection bias unlikely.

Applying the "GVHD-associated" T cell model to the MUD-ATG cohort we identified two distinct clusters (Figure 3E, F), defined by both a good and robust silhouette coefficient in the optimal cluster configuration (7_1: *Sil*=0.524) and a balanced patient distribution (cluster 1: n=53; cluster 2: n=94; Suppl. Figure 4A-C). Activated- and αβ T cells, which revealed the most striking differences over time and higher absolute counts in cluster 1, contributed primarily to the clustering. This was also observed in the corresponding cluster centroids (Figure 3F), additionally indicating distinct reconstitution shapes. Patients with higher absolute counts of activated and αβ T cells had significantly lower NRM (p=0.032, Figure 3H) and relapse (p=0.01, Figure 3I) resulting in excellent 1-year OS (98% vs. 79%, p=0.0023, Figure 3G) for MUD-ATG patients in cluster 1. Comparative outcome analysis including both PTCy subgroups (MUD-PTCY n=15 and haplo-PTCy n=18) revealed significantly decreased OS for MUD-ATG patients in cluster 2 and for MUD-PTCy patients compared to haplo-PTCy and MUD-ATG cluster 1 patients (p=0.0053) due to increased NRM rates (p=0.077) (Figure 4A, B). Relapse incidence was similar between both

PTCy-subgroups but lower compared to MUD-ATG cluster 2 (p=0.057, Figure 4C). While in this analysis the cumulative incidence of aGVHD II-IV was also lower in haplo- and MUD-PTCy patients (Figure 4D), no cGVHD difference between ATG and PTCy subgroups was observed (Figure 4E). Within the MUD-ATG cohort, the clustering revealed a numerically lower cGVHD rate for patients in cluster 1 (p=0.061, Figure 4F). The established clustering approach was also applicable for haplo- and MUD-PTCy patients utilizing the "GVHD-associated" T cell model (Figure 4G-J), yielding lower silhouette coefficients in robust configurations compared to the clustering in the MUD-ATG cohort (Suppl. Figure 4D-I). Similar to the results in the MUD-ATG cohort, the clustering of PTCy patients was also strongly impacted by the evolution of activated- and αβ T cells (Figure 4G-J). However, the statistical comparison of cluster-defined cohorts did not yield to meaningful results for clinical outcome, due to the low patient numbers after pre-processing. This analysis method performs best within larger collectives, as in the ATG cohorts.

A second clustering model integrating the reconstitution of "broad spectrum" T cells also resulted in the identification of two separate clusters in the MUD-ATG dataset. Here, the clusters were mainly influenced by higher levels of cytotoxic and memory cytotoxic T cells in cluster 2 (Figure 5A, B). The selected optimal cluster configuration showed a comparable silhouette coefficient (2_1: *Sil*=0.536) to the above-described "GVHD-associated" T cell clustering model, and both good robustness and patient distribution (cluster 1: n=105 and cluster 2: n=46; Suppl. Figure 5A-C). Of note, clinical analysis of these MUD-ATG clusters in the "broad-spectrum" T cell model revealed analog results to the "GVHD-associated" T cell model (Figure 5C-E), with only a marginal significance for NRM. Strikingly, albeit both models integrate biologically different T cell subsets their degree of similarity was 92.5% as revealed by cluster model comparison (Figure 5F). Patient re-allocation between the two models was also minimal (n=11, 7.5%, Figure 5G). Patient baseline characteristics were also similar between those clusters e.g. for patient age or underlying disease (Supp Table 5, 6). The clustering of PTCy patients in the "broad spectrum" T cell model, showed overall lower and more unstable silhouette coefficients (Suppl. Figure 5D-I). In the conventional statistical analysis of immune reconstitution, helper T cells were less affected by PTCy and may have impacted their clustering. Yet, comparable to the clustering in the MUD-ATG subgroup this model's clustering of PTCy patients was also mainly influenced by the cytotoxic T cell compartment (Supp. Figure 6A-D).

4 Discussion

Optimizing GVHD prophylaxis in allogeneic HCT with T cell depleting regimens, such as ATG or PTCy, is one key to effectively manage GVHD in patients with alternative donor HCT. The clinical effects of both agents after HCT have been studied by several groups, with the aim to identify the optimal strategy for each donor setting. Here, we focused on cellular immune reconstitution in each cohort and found that GVHD prophylaxis with PTCy, both in haploidentical- and MUD-donors, exhibited a slightly superior efficacy over ATG to prevent grade II-IV aGVHD. This effect was paralleled by striking differences in immune reconstitution patterns between the groups, indicating different underlying aGVHD-protective mechanisms. In PTCy patients, significantly higher regulatory T cell counts associated to its GVHD preventive effect, while GVHD protection in ATG patients associated with higher levels of γδ T- and NKT cells. Next, we employed a different approach to analyze heterogeneous longitudinal immune reconstitution data. Using time-series clustering of multi-dimensional flow cytometry data we were able to identify two underlying clusters of ATG patients with distinct reconstitution patterns, one of which was associated with poor HCT outcomes. These findings corroborate previous results, which have implied different clinical implications for established T cell depletion protocols[11,13]. They also identify the temporal dynamics of T cell reconstitution after HCT as potential mediator of the observed differential effects. Most importantly, this approach can be leveraged to dissect heterogeneity in cellular immune reconstitution patterns and support the identification of the optimal GVHD prophylaxis protocol for individual patients. Our data indicate that early cellular immune reconstitution data may be used as early biomarkers of clinical outcome events after HCT.

Very recently, PTCy was shown to be more effective than ATG in showing an improved OS and reduced relapse incidence in the haploidentical-[12], whereas it associated with a more pronounced protection from aGVHD II-IV in the unrelated donor setting[13]. The latter finding is supported by our data. However, more importantly our data point to relevant heterogeneity within the different T cell depletion regimens. The cumulative incidence of aGVHD II-IV in our ATG cohort was higher than in the two randomized clinical trials using the same ATG product[38,39]. However, incidences in retrospective real world data studies are highly variable[13,14,40-43] also reporting similar values[13]. For PTCy patients, the incidence of aGVHD II-IV was also higher compared to previous studies[11,13,14], but confirms comparable incidence of haplo- and MUD settings[11]. Differences of aGVHD II-IV incidences in PTCy cohorts may relate to distinct immunosuppressive strategies, which was in our patients predominantly based on TAC compared to CsA in the latter study[13]. Our data confirmed a previous report[13] finding no statistical significances in other transplant outcomes although this comparison is limited by different follow-up periods. Data from the CIBMTR registry showed

significantly lower grades II-IV aGVHD in MUD vs. haploidentical HCT in the reduced-intensity setting but not after myeloablative conditioning[11]. Similarly, we also found comparable frequencies for aGVHD II-IV in MUD and haploidentical transplants. Furthermore, corresponding to a perfectly controlled GVHD[20], the impairment of alloreactivity also translates into a decreased graft-versus-leukemia effect. In recent studies, relapse incidence was either comparable in MUD grafts using uniform prophylaxis[11] or reduced in haploidentical transplants compared to matched transplant with PTCy studied in a mixed prophylaxis regimen[10]. Our study used a uniform prophylaxis strategy for MUD and haploidentical transplants, showing similar relapse incidences compared to patients with ATG, therefore validating the CIBMTR data[11].

Cellular immune reconstitution of patients receiving PTCy and ATG in MUD and haploidentical donor HCT have not yet been extensively compared. The few existing studies primarily compared cohorts with either ATG or PTCy to control groups not receiving any specific T cell depletion. In these studies, ATG was associated with a slow recovery of CD3^{+} T cells, due to a delayed[16,17] dose-dependent recovery of CD4^{+} T cells[17], which likely reduced aGVHD. In contrast, PTCy was described with a sparing effect[18] on and a preferential recovery of regulatory T cells after HCT[44]. PTCy was also found to eliminate proliferative and putatively alloreactive mature NK cells[19]. In a recent study including mixed donor settings after reduced-intensity conditioning higher percentages of CD4^{+}-, regulatory- and αβ T cells were observed in the PTCy group while it was the case for NK cells and monocytes in the ATG cohort[15]. In our study, we were able to validate increased levels of helper- and regulatory T cells independently in the MUD- and haplo-PTCy setting compared to MUD-ATG. In another recent study analyzing immune reconstitution after both prophylactic protocols in myeloablative HCT with mixed donor settings, ATG associated with a faster reconstitution of CD8^{+} T cells, NKT and γδ T cells while CD4^{+} T cells were increased after PTCy[14]. Some of our data are well in line with those observations, except for the faster reconstitution of CD8^{+} T cells after ATG, which was mostly comparable to our PTCy groups. However, due to the separate analysis of MUD-PTCy and haplo-PTCy we identified significant differences in the reconstitution of regulatory T cells, which were not seen in a study with mixed cohorts[14]. Our analyses revealed higher early cell counts and a faster reconstitution of the CD4^{+} compartment in PTCy patients, especially of naïve helper and regulatory T cells, throughout the first six months after transplantation. In PTCy patients, early higher total numbers of CD3^{+} T cells and cytotoxic T cells were also seen, but differences to ATG patients were not as pronounced. Higher levels of T cells without increased aGVHD in PTCy patients after HCT might be explained by the fact, that PTCy does not eliminate alloreactive T cells, but instead leads to a functional impairment of these cells which can be sufficient to prevent newly formed donor T cells from

causing GVHD[44]. MUD-PTCy and haplo-PTCy patients had higher αβ T cell levels than MUD-ATG patients early after transplant. αβ T cells have been described to exhibit a stronger alloreactive potential compared to γδ T cells[34], yet we observed at the same time less aGVHD in our PTCy cohorts. The parallel increase in regulatory T cell levels, which have previously been implied to mediate aGVHD-protective effects[35] may explain this otherwise paradox finding. Our finding of increased cell counts of γδ T cells and NKT cells in the first months post-HCT in ATG patients is in line with a previous study showing high levels of γδ T cells in patients with ATG prophylaxis and lower incidence of aGVHD[45]. This supports the relevance of γδ T cells in aGVHD protection in patients treated with ATG, despite its mechanism remains a matter of debate[46]. The presence of low γδ T cell levels in PTCy patients might be counterbalanced by elevated regulatory T cell levels. The increased absolute NKT cell counts in ATG patients appear complementary, as they are able to produce anti-inflammatory cytokines and attenuate GVHD through a Th2 polarization[47-49]. Contrary to a recent pooled analysis[14] of different donor sources, the number of cytotoxic- or activated T cells as well as NK and B cells was comparable between ATG and PTCy patients.

The limitations of such pooled analyses may be overcome by our new analysis method using time series clustering of multi-dimensional cellular data. It allows to individually analyze T cell reconstitution within larger cohorts and exposes its heterogeneity within, which is to our knowledge the first of its kind. This approach reflects the pattern of reconstitution along with its' actual counts, an information that is no more comprehensible after transformation by dimensionality reduction[50]. The ability to distinguish smaller sets within patient cohorts and relate individual reconstitution patterns to clinical outcomes is another asset. Although the results from our multi-dimensional clustering approach, appeared to depend strongly on the cell counts, the reconstitution shape also contributed to the respective differentiations, which is a particularity of DTW, conceived as method for the shape-based alignment of sequences. Both of our T cell models extracted specific T cell subsets, which dominated the clustering process, such as αβ T cells in the "GVHD-associated" model. Differences between the pooled analysis and our clustering approach are mostly visible in the distinct absolute cell counts of αβ T cells. This dissimilarity results from the use of median values in the pooled approach, which compensates for potential outliers whereas the time-series clustering integrates actual values on an individual basis. Although our models exhibited data from the period of aGVHD development we could not observe any differences concerning aGVHD II-IV in both multi-dimensional models within the ATG cohort. However, both cluster models in the ATG group associated to similar patient outcomes in OS, NRM and relapse reflecting the strength of this approach. Both models started from distinct T cell subsets but were able to identify clusters with a high similarity exhibiting patients with poor outcomes.

Despite the relevant size of this retrospective immune reconstitution dataset, the PTCy cohorts were limited in size for the quantitative models, which encourages its validation on datasets from different centers in future studies. Still, flow cytometry data require harmonization if merged between centers, as this approach has proven its potential to differentiate heterogenous reconstitution patterns and to extract patients with distinct clinical outcomes from HCT cohorts. Furthermore as for any computational models, data pre-processing was an essential step. Since we only included data of patients with at least three consecutive flow cytometry measurements, this could have introduced a bias for patients with a longer survival. Further limitations are due to missing functional assays parallel to the detailed quantitative assessment of immune cells, which does not reflect the functionality of cells. Future studies might integrate both the new quantitative approach together with functional and genetic T cell assays to investigate the distinct effects of different T cell depletion protocols on the functional capacity of effector cells.

4.1 Conclusion

Employing the analysis of cellular reconstitution patterns, we show that GVHD protection appears to be driven by different T cell subsets in patients receiving either PTCy or ATG for GVHD prophylaxis, namely either regulatory T cells after PTCy or γδ T and/or NKT cells after ATG, respectively. Leveraging T cell reconstitution and temporal resolution, we were able to dissect the heterogenous cellular immune reconstitution landscape and thereby identify individuals with poor outcomes after transplantation based on their immune reconstitution profiles, which revealed its potential as biomarkers.

Acknowledgement:

This study was in part supported by the Bundesministerium für Bildung und Forschung (BMBF) through grant 031L0027 (DWB) and by the Deutsche Forschungsgemeinschaft UDE-UMEA grant FU 356/12-1 (ATT) and grant #FL843/1-1 (KF), and the Deutsche José Carreras Leukämiestiftung, grant 20R/2019 (KF). We would like to thank all technicians of the BMT Flow Cytometry Laboratory for assistance. B.Sc. Aleksandra Pillibeit supported the documentation of study patients.

Authorship Contribution:

ATT, SL, RB designed the study. SL and RB, and performed data collection; MF, UB, and NL participated in data acquisition. SL and TG performed statistical analysis. ATT, SL, TG, KF and DWB interpreted the data. RB and NL participated in data analysis. ATT, DWB, RB and NL provided clinical expertise. ATT and SL wrote the manuscript. DWB, UB, NL and KF contributed to write the manuscript. All authors had access to primary clinical trial data, read and approved the final manuscript.

Conflict-of-interest disclosure

The authors of this manuscript have potential conflicts of interest to disclose. ATT: Consultancy for MSD, JAZZ, CSL. Travel subsidies from Neovii Biotech outside the submitted work. DWB received travel subsidies from Medac, all outside the submitted work. The other authors declare no competing financial interests within the submitted work.

Data Availability Statement

On reasonable request, primary data is available from the corresponding author in accordance with ethical restrictions.

Supporting Information Statement

Additional supporting information may be found online in the Supporting Information section at the end of the article.

References

1. Styczyński J, Tridello G, Koster L, et al. Death after hematopoietic stem cell transplantation: changes over calendar year time, infections and associated factors. *Bone Marrow Transplantation*. 2020;55(1):126-136.
2. Phelan R, Arora, M., Chen, M. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR US summary slides; 2020.
3. Passweg JR, Baldomero H, Chabannon C, et al. Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. *Bone Marrow Transplantation*. 2021.
4. Ferrara JLM, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet (London, England)*. 2009;373(9674):1550-1561.
5. Zeiser R, Blazar BR. Pathophysiology of Chronic Graft-versus-Host Disease and Therapeutic Targets. *New England Journal of Medicine*. 2017;377(26):2565-2579.
6. Bacigalupo A. ATG in allogeneic stem cell transplantation: standard of care in 2017? Point. *Blood Advances*. 2017;1(9):569-572.
7. Luznik L, O'Donnell PV, Symons HJ, et al. HLA-Haploidentical Bone Marrow Transplantation for Hematologic Malignancies Using Nonmyeloablative Conditioning and High-Dose, Posttransplantation Cyclophosphamide. *Biology of Blood and Marrow Transplantation*. 2008;14(6):641-650.
8. McCurdy SR, Kanakry JA, Showel MM, et al. Risk-stratified outcomes of nonmyeloablative HLA-haploidentical BMT with high-dose posttransplantation cyclophosphamide. *Blood*. 2015;125(19):3024-3031.
9. Sugita J, Kagaya Y, Miyamoto T, et al. Myeloablative and reduced-intensity conditioning in HLA-haploidentical peripheral blood stem cell transplantation using post-transplant cyclophosphamide. *Bone Marrow Transplantation*. 2019;54(3):432-441.
10. Sanz J, Galimard J-E, Labopin M, et al. Post-transplant cyclophosphamide after matched sibling, unrelated and haploidentical donor transplants in patients with acute myeloid leukemia: a comparative study of the ALWP EBMT. *Journal of Hematology & Oncology*. 2020;13(1):46.
11. Gooptu M, Romee R, St Martin A, et al. HLA Haploidentical versus Matched Unrelated Donor Transplants with Post-Transplant Cyclophosphamide based prophylaxis. *Blood*. 2021.
12. Nagler A, Kanate AS, Labopin M, et al. Post-transplant cyclophosphamide versus anti-thymocyte globulin for graft-versus-host disease prevention in haploidentical transplantation for adult acute lymphoblastic leukemia. *Haematologica*. 2021;106(6):1591-1598.
13. Bailén R, Kwon M, Pascual-Cascón MJ, et al. Post-transplant cyclophosphamide for GVHD prophylaxis compared to ATG-based prophylaxis in unrelated donor transplantation. *Ann Hematol*. 2021;100(2):541-553.
14. Massoud R, Gagelmann N, Fritzsche-Friedland U, et al. Comparison of immune reconstitution between anti-T-lymphocyte globulin and post-transplant cyclophosphamide as acute graft-versus-host disease prophylaxis in allogeneic myeloablative peripheral blood stem cell transplantation. *Haematologica*. 2021.
15. Retière C, Willem C, Guillaume T, et al. Impact on early outcomes and immune reconstitution of high-dose post-transplant cyclophosphamide vs anti-thymocyte globulin after reduced intensity conditioning peripheral blood stem cell allogeneic transplantation. *Oncotarget*. 2018;9(14).
16. Gooptu M, Kim HT, Chen YB, et al. Effect of Antihuman T Lymphocyte Globulin on Immune Recovery after Myeloablative Allogeneic Stem Cell Transplantation with Matched Unrelated Donors: Analysis of Immune Reconstitution in a Double-Blind Randomized Controlled Trial. *Biol Blood Marrow Transplant*. 2018;24(11):2216-2223.

17. Turki AT, Klisanin V, Bayraktar E, et al. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies. *Am J Transplant*. 2020;20(3):677-688.
18. Kanakry CG, Ganguly S, Zahurak M, et al. Aldehyde Dehydrogenase Expression Drives Human Regulatory T Cell Resistance to Posttransplantation Cyclophosphamide. *Science Translational Medicine*. 2013;5(211):211ra157-211ra157.
19. Russo A, Oliveira G, Berglund S, et al. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood*. 2018;131(2):247-262.
20. Bacigalupo A. HLA still matters in allogeneic transplants. *Blood*. 2021;138(3):209-211.
21. Bartelink IH, Belitser SV, Knibbe CAJ, et al. Immune Reconstitution Kinetics as an Early Predictor for Mortality using Various Hematopoietic Stem Cell Sources in Children. *Biology of Blood and Marrow Transplantation*. 2013;19(2):305-313.
22. Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a retrospective, pharmacodynamic cohort analysis. *The Lancet Haematology*. 2017;4(4):e183-e191.
23. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15(6):825-828.
24. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host Disease: I. The 2014 Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant*. 2015;21(3):389-401.e381.
25. Sardá-Espinosa A. Time-Series Clustering in R Using the dtwclust Package. *The R Journal*. 2019;11:22.
26. Rousseeuw PJ. Silhouettes: A graphical aid to the interpretation and validation of cluster analysis. *Journal of Computational and Applied Mathematics*. 1987;20:53-65.
27. R Core Team (2021). R: A language and environment for statistical computing.: R Foundation for Statistical Computing, Vienna, Austria; 2021.
28. Sardá-Espinosa A (2019). dtwclust: Time Series Clustering Along with Optimizations for the Dynamic Time Warping Distance. R package version 5.5.6.
29. Kaplan EL, Meier P. Nonparametric Estimation from Incomplete Observations. *Journal of the American Statistical Association*. 1958;53(282):457-481.
30. Cox DR. Regression Models and Life-Tables. *Journal of the Royal Statistical Society Series B (Methodological)*. 1972;34(2):187-220.
31. Therneau T (2021). A Package for Survival Analysis in R: R package version 3.2-11.
32. Kassambara A, Kosinski M, Biecek P (2021). survminer: Drawing Survival Curves using 'ggplot2': R package version 0.4.9.
33. Bob Gray (2020). cmprsk: Subdistribution Analysis of Competing Risks: R package version 2.2-10.
34. Handgretinger R, Schilbach K. The potential role of γδ T cells after allogeneic HCT for leukemia. *Blood*. 2018;131(10):1063-1072.
35. Trenado A, Sudres M, Tang Q, et al. Ex Vivo-Expanded CD4⁺CD25⁺ Immunoregulatory T Cells Prevent Graft-versus-Host-Disease by Inhibiting Activation/Differentiation of Pathogenic T Cells. *The Journal of Immunology*. 2006;176(2):1266-1273.
36. Tomblyn M, Chiller T, Einsele H, et al. Guidelines for Preventing Infectious Complications among Hematopoietic Cell Transplantation Recipients: A Global Perspective. *Biology of Blood and Marrow Transplantation*. 2009;15(10):1143-1238.
37. Ogonek J, Kralj Juric M, Ghimire S, et al. Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol*. 2016;7:507.
38. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti-T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a randomised, open-label, multicentre phase 3 trial. *The Lancet Oncology*. 2009;10(9):855-864.

39. Soiffer RJ, Kim HT, McGuirk J, et al. Prospective, Randomized, Double-Blind, Phase III Clinical Trial of Anti–T-Lymphocyte Globulin to Assess Impact on Chronic Graft-Versus-Host Disease–Free Survival in Patients Undergoing HLA-Matched Unrelated Myeloablative Hematopoietic Cell Transplantation. *Journal of Clinical Oncology*. 2017;35(36):4003-4011.

40. Mohty M, Labopin M, Balère ML, et al. Antithymocyte globulins and chronic graft-vs-host disease after myeloablative allogeneic stem cell transplantation from HLA-matched unrelated donors: a report from the Sociéte Française de Greffe de Moelle et de Thérapie Cellulaire. *Leukemia*. 2010;24(11):1867-1874.

41. Brissot E, Labopin M, Moiseev I, et al. Post-transplant cyclophosphamide versus antithymocyte globulin in patients with acute myeloid leukemia in first complete remission undergoing allogeneic stem cell transplantation from 10/10 HLA-matched unrelated donors. *Journal of Hematology & Oncology*. 2020;13(1):87.

42. Moiseev IS, Pirogova OV, Alyanski AL, et al. Graft-versus-Host Disease Prophylaxis in Unrelated Peripheral Blood Stem Cell Transplantation with Post-Transplantation Cyclophosphamide, Tacrolimus, and Mycophenolate Mofetil. *Biology of Blood and Marrow Transplantation*. 2016;22(6):1037-1042.

43. Kennedy VE, Chen H, Savani BN, et al. Optimizing Antithymocyte Globulin Dosing for Unrelated Donor Allogeneic Hematopoietic Cell Transplantation Based on Recipient Absolute Lymphocyte Count. *Biology of Blood and Marrow Transplantation*. 2018;24(1):150-155.

44. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. *J Clin Invest*. 2019;129(6):2357-2373.

45. Minculescu L, Marquart HV, Ryder LP, et al. Improved Overall Survival, Relapse-Free-Survival, and Less Graft-vs.-Host-Disease in Patients With High Immune Reconstitution of TCR Gamma Delta Cells 2 Months After Allogeneic Stem Cell Transplantation. *Front Immunol*. 2019;10:1997.

46. Ye W, Kong X, Zhang W, Weng Z, Wu X. The Roles of γδ T Cells in Hematopoietic Stem Cell Transplantation. *Cell Transplantation*. 2020;29:0963689720966980.

47. Kohrt HE, Pillai AB, Lowsky R, Strober S. NKT cells, Treg, and their interactions in bone marrow transplantation. *European Journal of Immunology*. 2010;40(7):1862-1869.

48. Kronenberg M, Engel I. On the road: progress in finding the unique pathway of invariant NKT cell differentiation. *Current Opinion in Immunology*. 2007;19(2):186-193.

49. Mavers M, Maas-Bauer K, Negrin RS. Invariant Natural Killer T Cells As Suppressors of Graft-versus-Host Disease in Allogeneic Hematopoietic Stem Cell Transplantation. *Frontiers in immunology*. 2017;8:900-900.

50. Tinnevelt GH, Kokla M, Hilvering B, et al. Novel data analysis method for multicolour flow cytometry links variability of multiple markers on single cells to a clinical phenotype. *Scientific Reports*. 2017;7(1):5471.

Tables

Table 1. Calculated cumulative incidences at 1-year post-HCT for aGVHD (II-IV), cGVHD (moderate-severe), relapse and NRM in the respective patient subgroups.

	Cumulative Incidences						
	MUD-ATG		MUD-PTCy		Haplo-PTCy		*p*
	%	95% CI [%]	%	95% CI [%]	%	95% CI [%]	
Acute GVHD	88.2	84.6 – 91.9	80.0	67.8 – 94.4	88.9	80.2 – 98.6	0.081
Acute GVHD II-IV	68.3	61.4 – 76.0	55.3	39.4 – 77.6	57.6	38.9 – 85.2	**0.032**
Chronic GVHD	29.7	24.7 – 35.7	41.4	27.7 – 62.0	41.8	27.5 – 63.3	0.436
Chronic GVHD (moderate-severe)	12.1	8.6 – 17.0	16.2	7.2 – 36.2	20.3	10.3 – 40.0	0.511
Relapse	16.9	13.0 – 21.9	14.4	6.4 – 32.4	20.9	11.6 – 37.7	0.661
NRM	18.2	14.3 – 23.3	20.5	10.6 – 39.7	23.2	13.5 – 40.2	0.728

Abbreviations: *CI,* Confidence interval.
Death within 100 days and 1-year post-HCT were regarded as competing event towards acute and chronic GVHD, respectively. Relapse and NRM were considered competing events to each other.

Table 2. Univariate Competing risk regression analysis of pre-transplant factors for the outcome of aGVHD grades II-IV.

Covariate	Competing risk regression		
	SHR	**95% CI**	***p***
Subgroup			
MUD-ATG	—	—	—
Haplo-PTCy	0.54	0.33 – 0.86	**0.010**
MUD-PTCy	0.73	0.44 – 1.21	0.220
Disease			
AML	—	—	—
others	0.97	0.73 – 1.30	0.840
Recipient Age			
<50 years	—	—	—
≥50 years	1.13	0.80 – 1.59	0.500
Recipient Sex			
Male	—	—	—
Female	0.99	0.74 – 1.33	0.940
Donor Age			
<30 years	—	—	—
≥30 years	0.98	0.73 – 1.30	0.870
Donor Sex			
Male	—	—	—
Female	0.91	0.66 – 1.25	0.570
Conditioning			
MAC	—	—	—
RIC	1.03	0.76 – 1.40	0.850
Total body irradiation			
Yes	—	—	—
No	0.93	0.69 – 1.25	0.610
ECOG			
0-1	—	—	—
2-3	0.72	0.38 – 1.34	0.300
CMV Serology			
R-/D-	—	—	—
R-/D+	0.56	0.30 – 1.04	0.066
R+/D-	0.93	0.57 – 1.50	0.760
R+/D+	1.00	0.72 – 1.38	1.000

Abbreviations: *CI,* Confidence interval; *SHR,* subdistribution hazard ratio; —, reference group. Death was regarded as competing event towards aGVHD grades II-IV.

Figure Legends

Figure 1. Cumulative Incidence of severe acute GVHD is significantly increased in MUD-ATG subgroup. Studied patients comprise the following subgroups: MUD-ATG (green), MUD-PTCy (red) and haplo-PTCy (blue). Cumulative incidence of **(A)** aGVHD (II-IV) and **(B)** all grade aGVHD within 100 days post-HCT. **(C)** Occurrence of aGVHD grades within 100 days post-HCT in the studied subgroups illustrated in percentages. Cumulative incidence of **(D)** moderate-severe cGVHD and **(E)** all grade cGVHD. **(F)** Occurrence of cGVHD grades within 1-year post-HCT in the studied subgroups illustrated in percentages. **(G)** One-year overall survival (OS) compared between study subgroups. **(H,I)** Cumulative Incidences of NRM and relapse. Equality of cumulative incidences functions (CIF's) across the studied subgroups was compared by Gray's test for competing risks. *P*-values < 0.05 were considered as statistically significant.

Figure 2. Increased cell counts within helper T cell subsets for patients with PTCy as GVHD prophylaxis. Immune reconstitution of T cell subsets within one year after HCT. T-lymphocyte subsets in the peripheral blood were characterized by multicolor flow cytometry. T helper cell subsets were gated on $CD45^+$ cells and were identified as followed: **(A)** Helper T cells, $CD3^+/CD4^+$; **(B)** Naïve helper T cells, $CD3^+/CD4^+/CD45RA^+$; **(C)** Memory helper T cells, $CD3^+/CD4^+/CD45RO^+$. Furthermore the **(D)** regulatory T cells, $CD3^+/CD4^+/CD25^+/CD127^+$low and **(E)** conventional T cells, $CD3^+/CD4^+/CD25^-/CD127^+$high were gated among the $CD3^+/CD4^+$ cells. In **(F)** the ratio the Treg/Tcon ratio is shown. **(G, H)** illustrate the T cell receptors α/β, TCRα/β and T cell receptor γ/δ, TCRγ/δ, respectively. These were gated within the $CD3^+$ gate. Median absolute cell numbers were analyzed by the Mann-Whitney-U-test testing each group against the others at every time point. P-values < 0.05 were considered as statistically significant and are indicated with asterisks ($p < 0.1$, (*); $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; and $p < 0.0001$, ****). Median values and sample numbers of the respective cohorts as well as the *p*-values are summarized in the attached excel file.

Figure 3. Clustering of individual patient data provides a more differentiated picture in clinical outcomes. (A) Depiction of time-series clustering workflow integrating the steps of data pre-processing, clustering, and clinical analysis. **(B-D)** Clinical outcome analysis for HCT patients (n=151 MUD-ATG, n=18 haplo-PTCy, and n=15 MUD-PTCy) that were included into time-series clustering approach in their respective study subgroups: **(B)** One-year OS; cumulative incidences of **(C)** NRM and **(D)** relapse within one-year post-HCT. **(E,F)** Individual patient immune cell data clustering in the MUD-ATG cohort using data of "GVHD-associated" T cells: $CD3^+/CD4^+/CD25^+/CD127^+$low regulatory T cells, $CD3^+/HLA\text{-}DR^+$ activated T cells, TCRα/β$^+$ and

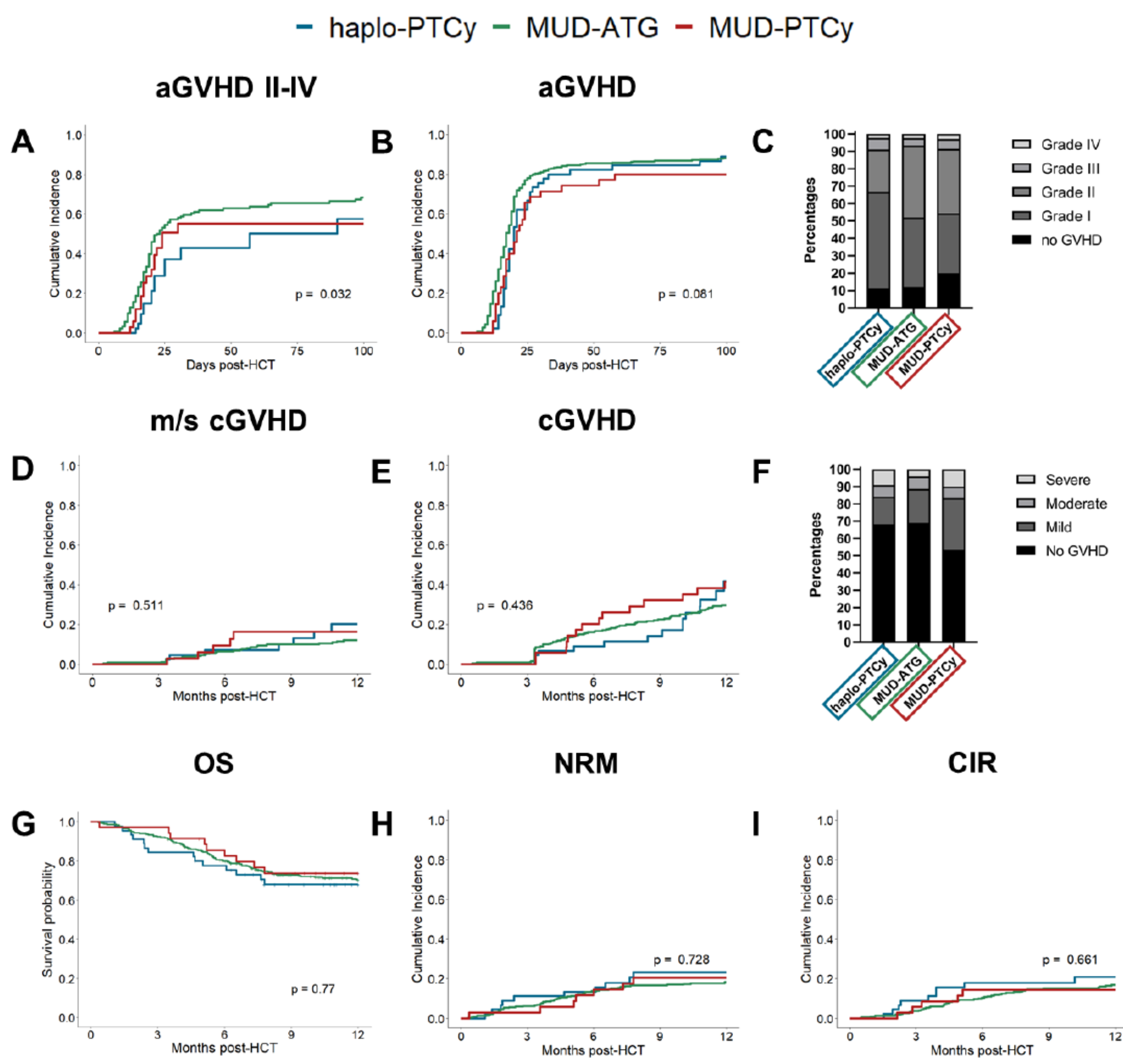
TCR γ/δ^+ T cells, The graph in **(E)** depicts each patients' individual reconstitution pattern in the respective subset; **(F)** includes the medoid samples of each subset calculated via partition around medoids (PAM). **(G-I)** Clinical outcome analysis for the MUD-ATG cohort using the cluster affiliation produced via the above shown time-series clustering. **(G)** One-year OS; cumulative incidences of **(H)** NRM and **(I)** relapse within one-year post-HCT.

Figure 4. "GVHD-associated" T cell model emphasizes long-term survivors. (A-E) Clinical outcome analysis integrating all patient subgroups after time-series clustering of MUD-ATG patients in the "GVHD-associated" T cell model. **(A)** One-year OS; cumulative incidences of **(B)** NRM and **(C)** relapse within one-year post-HCT; **(D)** cumulative incidence of aGVHD grades II-IV within 100 days post-HCT; **(E)** cumulative incidence of cGVHD. **(F)** Cumulative incidence of cGVHD in MUD-ATG patients only. **(G-J)** Individual patient immune cell data clustering in the **(G,H)** MUD-PTCy cohort and **(I,J)** haplo-PTCy cohort using data of "GVHD-associated" T cells: $CD3^+/CD4^+/CD25^+/CD127^+$low regulatory T cells, $CD3^+/HLA\text{-}DR^+$ activated T cells, $TCR\alpha/\beta^+$ and TCR γ/δ^+ T cells, illustrated in distinct boxes. The graphs in **(G,I)** depict each patients' individual reconstitution pattern in the respective subset; **(H,J)** include the medoid samples of each subset calculated via partition around medoids (PAM).

Figure 5. Time-series clustering of „broad spectrum" T cells reveals comparable patient survival to the "GVHD-associated" T cell model. (A,B) Individual patient immune cell data clustering in the MUD-ATG cohort using data of "broad spectrum" T cells: $CD3^+/CD4^+$ helper T cells, $CD3^+/CD4^+/CD45RA^+$ naïve helper T cells, $CD3^+/CD8^+$ cytotoxic T cells and $CD3^+/CD8^+/CD45RO^+$ memory cytotoxic T cells, The graph in **(A)** depicts each patients' individual reconstitution pattern in the respective subset; **(B)** includes the medoid samples of each subset calculated via DTW barycenter averaging (DBA). **(C-E)** Clinical outcome analysis for the MUD-ATG cohort using the cluster affiliation produced via the above shown time-series clustering. **(C)** One-year OS; cumulative incidences of **(D)** NRM and **(E)** relapse within one-year post-HCT. **(F)** Overlap between tautomeric clusters of the "GVHD-associated"- and the "broad spectrum" T cell model. **(G)** Transition of patients between the clusters of both models.

Figures

Figure 1. Cumulative Incidence of severe acute GVHD is significantly increased in MUD-ATG subgroup.



26

Figure 2. Increased cell counts within helper T cell subsets for patients with PTCy as GVHD prophylaxis.

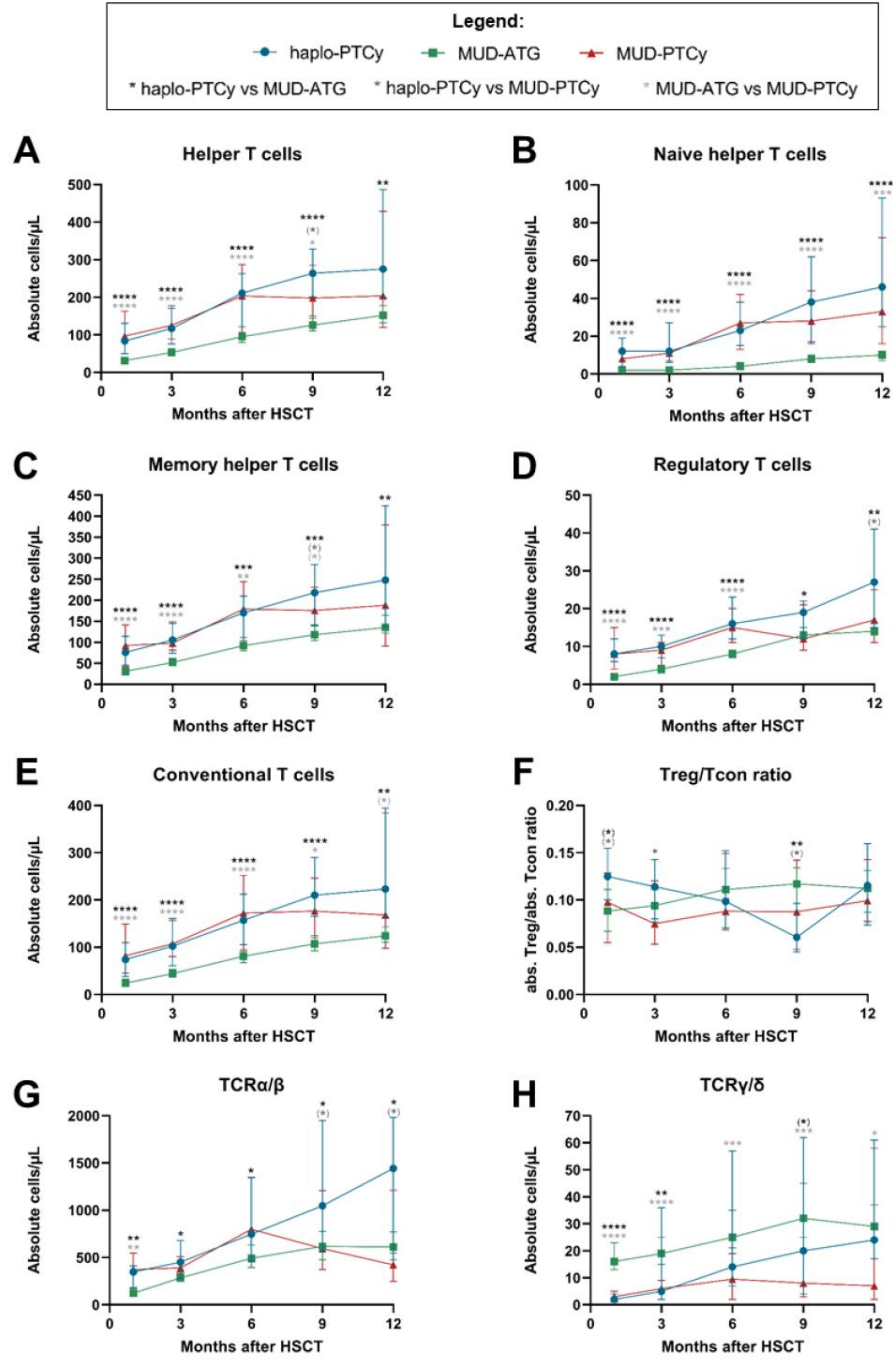
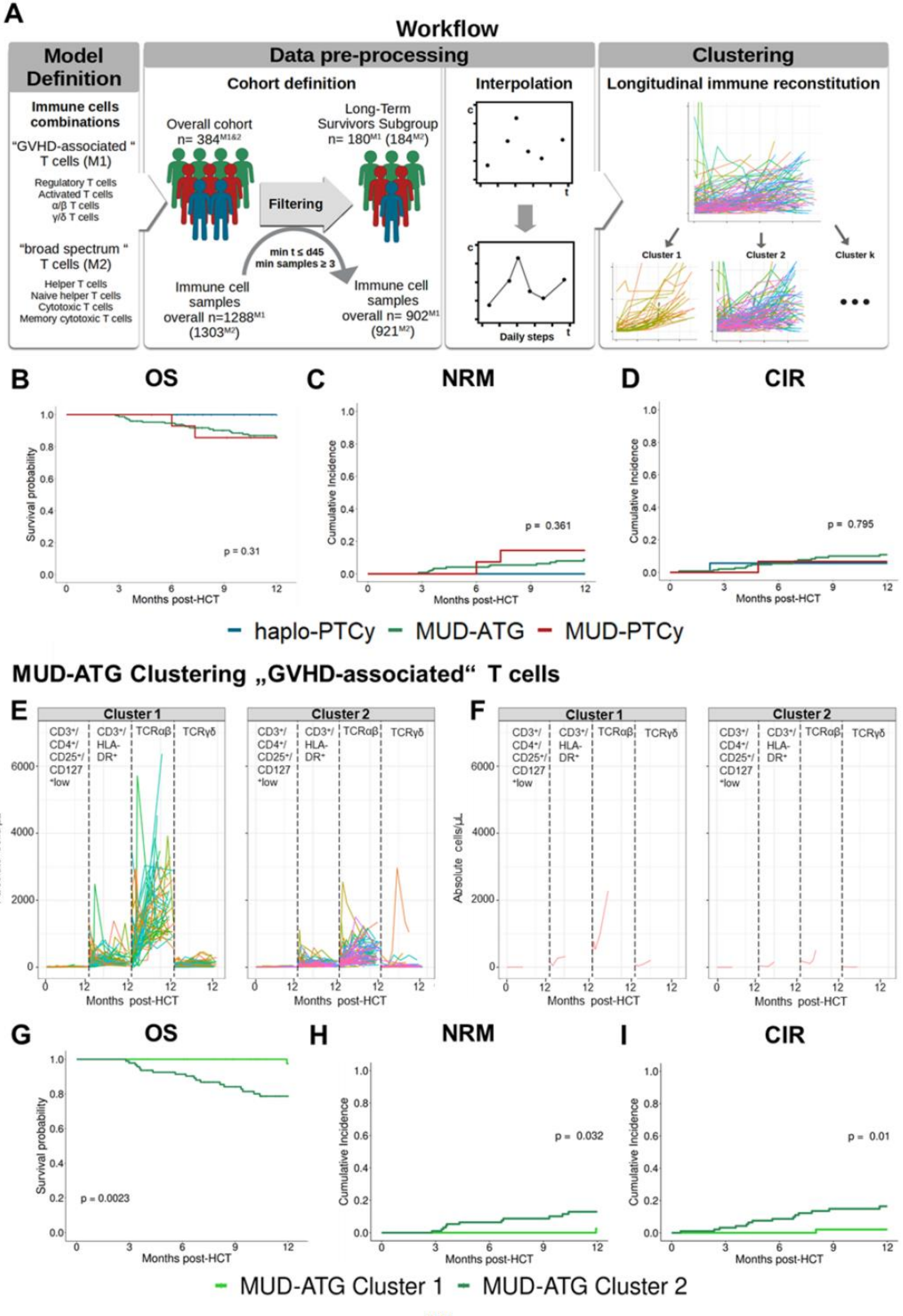


Figure 3. Clustering of individual patient data provides a more differentiated picture in clinical outcomes.



28

Figure 4. "GVHD-associated" T cell model emphasizes long-term survivors.

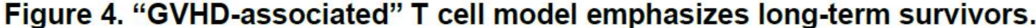
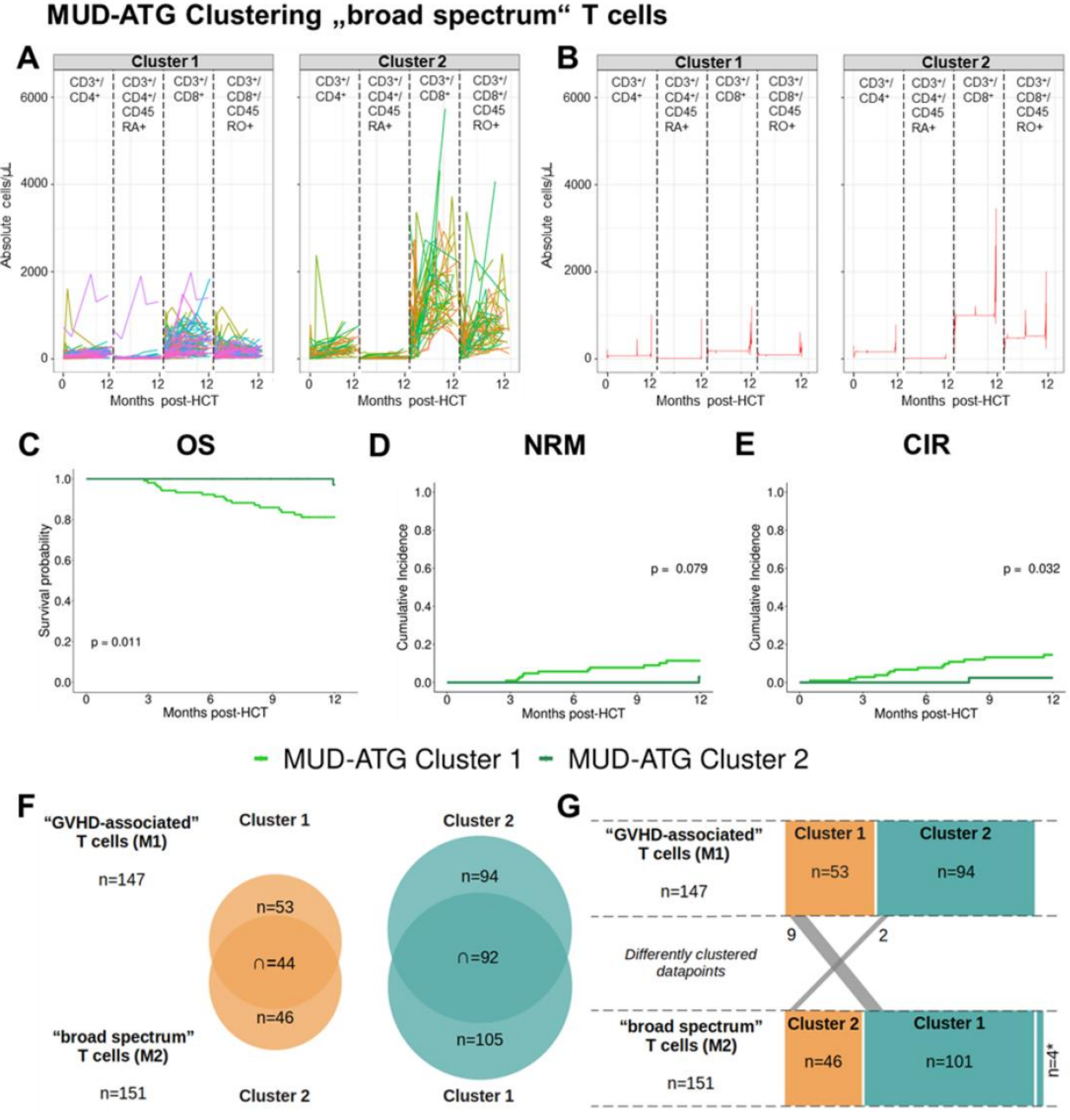


Figure 5. Time-series clustering of „broad spectrum“ T cells reveals comparable patient survival to the “GVHD-associated” T cell model.



Online Supporting Information to:

Cellular immune reconstitution analysis reveals distinct phenotypes and clinically relevant heterogeneity among patient cohorts receiving either anti-T-lymphocyte globulin or post-transplant cyclophosphamide in HCT

Saskia Leserer[1, 2, 3], Theresa Graf[1, 2], Rashit Bogdanov[1, 2], Martina Franke[1], Ulrike Buttkereit[1], Nils Leimkühler[1], Katharina Fleischhauer[3, 4], Dietrich W. Beelen[1] and Amin T. Turki[1, 2, 3]

[1] Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

2 Computational Hematology Workgroup, Department of Hematology and Stem Cell Transplantation, West-German Cancer Center, University Hospital Essen, Essen, Germany

3 Institute for Experimental Cellular Therapy, West-German Cancer Center, University Hospital Essen, Essen, Germany

4 German Cancer Consortium (DKTK)

Supplementary Methods:

Analysis of Immune Reconstitution

For flow cytometry analysis patient samples were prepared by isolating peripheral blood mononuclear cells (PBMC) using an automated red blood cell lysing system (TQ-Prep, Beckman Coulter, Brea, CA), washing with fluorescence-activated cell sorting (FACS) buffer and subsequently staining with surface markers (Supplementary Table 2). There was no examination of intracellular markers. To capture the patient's full immune status, two complimentary panels were measured. All 1262 samples run on the same NAVIOS flow cytometer (Beckman Coulter, Brea, CA) with the same antibodies and FACS compensation parameters using the manufacturer's software. Adequate subset analysis was ensured by analysis of a minimum of 15000 lymphocytes.

The first panel characterized immune cell subsets as follows: T Cells, $CD3^+$; T helper cells, $CD3^+/CD4^+$; cytotoxic T cells, $CD3^+/CD8^+$; regulatory T cells, $CD3^+/CD4^+/CD25^+/CD127^+$low; conventional T cells, $CD3^+/CD4^+/CD25^-/CD127^+$high; naïve helper T cells, $CD3^+/CD4^+/CD45RA^+$; memory helper T cells, $CD3^+/CD4^+/CD45RO^+$; naïve cytotoxic T cells, $CD3^+/CD8^+/CD45RA^+$; memory cytotoxic T cells, $CD3^+/CD8^+/CD45RO^+$; B cells, $CD19^+$. All mentioned subsets were gated on the $CD45^+$ lymphocyte gate despite the regulatory- and conventional T cells which were among the $CD3^+/CD4^+$ subset. In the second panel the following immune cell subsets were gated on the $CD45^+$ lymphocyte gate: Activated T cells, $CD3^+$/HLA-DR^+; $NKG2D^+$-NK cells, $CD1656^+/CD314^+$. T cell receptor α/β, TCRα/β and T cell receptor γ/δ, TCRγ/δ were gated on the $CD3^+$ gate.

Time-series clustering

Time-series (TS) clustering was used to classify and visualize longitudinal patient-wise immune reconstitution data into structures with maximal similarity. TS clustering utilizes the application of a specific distance measure, called dynamic time warping (DTW), to transform longitudinal series into readable data for clustering algorithms[1,2]. DTW can compare time series that are shifted in time and identify those which are similar in shape[1,2]. Concerning the partitional clustering an essential factor that needs to be included is the use of time series prototypes, as the resulting prototypes function as cluster centroids resembling the average time series of a cluster[2]. The number of clusters, *k*, also defines the number of randomly initialized centroids[2]. The distance between all series within the data as well as all centroids is determined, and each time series is assigned to its closest centroid within a cluster[2]. The DTW algorithm creates a local cost matrix to find an optimal warping path (=minimal distance) between two time series[2]. These steps are repeated as long as the defined iteration limit is

reached or the builded clusters are constant[2]. In the context of DTW the only TS prototypes that can be used are the DTW barycenter averaging and the partition around medoids[2]. The performance of the clustering algorithms can be evaluated by internal cluster validity indices such as the silhouette coefficient (*Sil*)[2,3]. The silhouette coefficient ranges between -1 to +1 where positive values generally indicate a separation of clusters with +1 showing the optimum[3].

Time-series clustering was applied to two different multi-dimensional T cell models comprising 1) "GVHD-associated" T cells: $CD3^+/CD4^+/CD25^+/CD127^+$low regulatory T cells, $CD3^+$/HLA-DR^+ activated T cells, $TCR\alpha/\beta^+$ and TCR γ/δ^+ T cells and 2) "broad spectrum" T cells: $CD3^+/CD4^+$ helper T cells, $CD3^+/CD4^+/CD45RA^+$ naïve helper T cells, $CD3^+/CD8^+$ cytotoxic T cells and $CD3^+/CD8^+/CD45RO^+$ memory cytotoxic T cells. Longitudinal immune reconstitution data were pre-processed by filtering of the dataset as well as an interpolation of individual patient data points. Inclusion criteria were 1) a minimum of three available flow cytometry measurements within +12 months after HCT and 2) first flow cytometry measurement ≤ d+45 post-HCT. Next, data gaps between discrete data points were filled by linear interpolation resulting in daily data. After data pre-processing n=147 patients remained in the MUD-ATG cohort ("GVHD-associated" T cell model, n=151 "broad spectrum" T cell model) as well as n=18 and n=15 in the haplo-PTCy and MUD-PTCy cohorts, respectively. DTW was applied on the interpolated data and the resulting distance matrix was used for data clustering. Here, we tested two different clustering algorithms, a partitional and a hierarchical one. Initial experiments using the hierarchical clustering showed acceptable performance results but suffered from dysbalanced patient distribution within the clusters and required inappropriate computational costs leading to its exclusion. Model development was therefore limited to the partitional clustering. Using the partitional clustering algorithm we did a hyperparameter tuning integrating following features: pre-defined cluster numbers (k=2, k=3, k=4), method for distance measurement (DTW vs. DTWbasic) and prototype functions. In total, 36 several feature combinations were analyzed, and the best-performing setting was evaluated by comparing the silhouette coefficient of all possible combinations. To identify the most robust model, we performed a 10-fold data resampling of random 2/3 datasets to evaluate the robustness of each configuration by the variability of silhouette coefficients within the resampling. The final cluster configurations were selected based on 1) high silhouette coefficient, 2) the robustness of the configuration and 3) sufficient patient distribution within the clusters. Method development of the time-series clustering was done utilizing the dataset of the MUD-ATG cohort and was later applied to both PTCy subgroups. However, these were difficult to evaluate due to small patient numbers.

References

1. Aghabozorgi S, Seyed Shirkhorshidi A, Ying Wah T. Time-series clustering – A decade review. *Information Systems*. 2015;53:16-38.
2. Sardá-Espinosa A. Time-Series Clustering in R Using the dtwclust Package. *The R Journal*. 2019;11:22.
3. Rousseeuw PJ. Silhouettes: A graphical aid to the interpretation and validation of cluster analysis. *Journal of Computational and Applied Mathematics*. 1987;20:53-65.

Supplementary Tables

Supplemental Table 1: Surface markers for immune reconstitution monitoring via FACS analysis

Antibody	Clone	Isotype	Label	Manufacturer	Reference Number
CD45	J33	IgG1	KrO	Coulter	B36294
CD3	UCHT1	IgG1	PB	Coulter	A93687
CD4	13B8.2	IgG1	APC750	Coulter	A94682
CD8	B9.11	IgG1	APC	Coulter	IM2469
CD45RA	ALB11	IgG1	FITC	Coulter	AO7786
CD45RO	UCHL1	IgG2a	ECD	Coulter	B49192
CD25	B1.49.9	IgG2a	PE	Coulter	A07774
CD19	J3-119	IgG1	PC5.5	Coulter	B49211
CD127	R34.34	IgG1	PC7	Coulter	A64618
CD314 (NKG2D)	ON72	IgG1	PE	Coulter	A08934
HLA-DR	Immu-357	IgG1	PC5.5	Coulter	B20024
CD16	3G8	IgG1	PC7	Coulter	6607118
CD56	N901(NKH-1)	IgG1	PC7	Coulter	A21692
CD14	RMO52	IgG2a	APC750	Coulter	A86052
TCR α/β		IgG1	APC	Miltenyi	130-113-527
TCR γ/δ	11F2	IgG1	FITC	Miltenyi	130-113-503

Supplemental Table 2: Patient baseline characteristics for patient subgroups with either ATG or PTCy as GVHD prophylaxis.

Characteristics	MUD-ATG		MUD-PTCy		Haplo-PTCy		p
	n	%	n	%	n	%	
Total enrolled and treated	304	80	35	9	45	12	
Median age at transplantation (range)	59	(19-77)	56	(20-70)	56	(18-78)	0.249
Male gender	170	56	23	66	28	62	0.430
Disease							0.754
Acute myeloid leukemia	147	48	16	46	23	51	
Myelodysplastic syndromes	40	13	3	9	4	9	
Myeloproliferative neoplasia	27	9	4	11	2	4	
Acute lymphoblastic leukemia	26	9	4	11	7	16	
Chronic myeloid leukemia	16	5	1	3	1	2	
Chronic myelomonocytic leukemia	8	3	0	0	0	0	
Non-Hodgkin's lymphoma	29	10	4	11	4	9	
Multiple myeloma	1	0	1	3	1	2	
Aplastic Anemia	3	1	1	3	1	2	
Other hematologic disorders	7	2	1	3	2	4	
Graft source							0.307
PBSC	302	99	34	97	45	100	
BM	2	1	1	3	0	0	
HLA-matching/Donor Type							**<0.0001**
MUD	304	100	35	100	0	0	
Haploidentical	0	0	0	0	45	100	
Conditioning							0.167
Myeloablative conditioning	204	67	18	51	28	62	
Reduced intensity conditioning	100	33	17	49	17	38	
TBI containing	120	39	11	31	19	42	0.587
GVHD Prophylaxis							**<0.0001**
CsA	0	0	0	0	1	2	
CsA + MTX	254	84	0	0	0	0	
CsA + MMF	11	4	0	0	0	0	
TAC + MMF	25	8	35	100	44	98	
Other	14	5	0	0	0	0	
Recipient/Donor gender constellation							**<0.0001**
Female/Female	71	23	9	26	10	22	
Male/Male	152	50	20	57	15	33	
Female/Male	63	21	3	9	7	16	
Male/Female	18	6	3	9	13	29	
CMV Serology							0.132
R+/D-	34	11	9	26	8	18	
R+/D+	141	46	12	34	23	51	
R-/D+	28	9	5	14	4	9	
R-/D-	101	33	9	26	10	22	

Abbreviations: *ATG*, anti-T-lymphocyte globulin; *BM*, bone marrow; *CMV*, cytomegalovirus; *CsA*, cyclosporin A; *D*, donor; *haplo*, haploidentical; *MMF*; mycophenolate mofetil; *MTX*, methotrexate; *MUD*, matched unrelated donor; *n*, number of cases; *p*; p-value; *PBSC*, peripheral blood stem cells; *PTCy*, post-transplant cyclophosphamide; *R*, recipient; *TBI*, total body irradiation.

Supplemental Table 3: Univariate Competing risk regression analysis for respective outcomes at 1-year post-HCT.

Outcome	Competing risk regression		
	SHR	95% CI	*p*
Acute GVHD			
MUD-ATG	—	—	—
Haplo-PTCy	0.77	0.60 – 1.00	**0.050**
MUD-PTCy	0.68	0.48 – 0.97	**0.034**
Acute GVHD II-IV			
MUD-ATG	—	—	—
Haplo-PTCy	0.54	0.33 – 0.86	**0.010**
MUD-PTCy	0.73	0.44 – 1.21	0.220
Chronic GVHD			
MUD-ATG	—	—	—
Haplo-PTCy	1.04	0.58 – 1.84	0.900
MUD-PTCy	1.48	0.84 – 2.60	0.180
Chronic GVHD (moderate-severe)			
MUD-ATG	—	—	—
Haplo-PTCy	1.17	0.46 – 2.99	0.740
MUD-PTCy	1.24	0.44 – 3.51	0.690
Overall Survival*			
MUD-ATG	—	—	—
Haplo-PTCy	1.17	0.67 – 2.06	0.582
MUD-PTCy	0.87	0.44 – 1.72	0.684
Relapse			
MUD-ATG	—	—	—
Haplo-PTCy	1.36	0.66 – 2.80	0.410
MUD-PTCy	0.90	0.35 – 2.29	0.820
NRM			
MUD-ATG	—	—	—
Haplo-PTCy	1.31	0.67 – 2.56	0.430
MUD-PTCy	1.11	0.52 – 2.41	0.780

Abbreviations: *CI,* Confidence interval; *SHR,* subdistribution hazard ratio; —, reference group. Overall survival was analyzed with Cox proportional hazard regression yielding Hazard ratios (HR). Death and relapse were regarded as competing events towards acute and chronic GVHD. Relapse and NRM were competing events to each other.

Supplemental Table 4: Patient baseline characteristics from patients in cluster 1 of the “GVHD-associated” T cell model and cluster 2 from the “broad spectrum” T cell model.

Characteristics	**„GVHD-associated“ T Cells, Cluster 1**		**„broad spectrum“ T Cells, Cluster 2**		***p***
	n	**%**	**n**	**%**	
Total enrolled and treated	53	100	46	100	
Median age at transplantation (range)	61	(20-73)	61,5	(20-73)	0.838
Male gender	18	34	17	37	0.756
Disease					>0.999
Acute myeloid leukemia	33	62	30	65	
Myelodysplastic syndromes	8	15	6	13	
Myeloproliferative neoplasia	3	6	2	4	
Acute lymphoblastic leukemia	3	6	3	7	
Chronic myeloid leukemia	0	0	0	0	
Chronic myelomonocytic leukemia	1	2	1	2	
Non-Hodgkin’s lymphoma	2	4	2	4	
Multiple myeloma	0	0	0	0	
Aplastic Anemia	1	2	1	2	
Other hematologic disorders	2	4	1	2	
Graft source					>0.999
PBSC	53	100	46	100	
BM	0	0	0	0	
Conditioning					0.843
Myeloablative conditioning	31	58	26	57	
Reduced intensity conditioning	22	42	20	43	
TBI containing	16	30	15	33	0.796
GVHD Prophylaxis					0.823
CsA	0	0	0	0	
CsA + MTX	48	91	42	91	
CsA + MMF	1	2	1	2	
TAC + MMF	3	6	3	7	
Other	1	2	0	0	
Recipient/Donor gender constellation					0.983
Female/Female	17	32	15	33	
Male/Male	16	30	15	33	
Female/Male	18	34	14	30	
Male/Female	2	4	2	4	
CMV Serology					0.970
R+/D-	6	11	5	11	
R+/D+	42	79	37	80	
R-/D+	3	6	3	7	
R-/D-	2	4	1	2	

Abbreviations: *ATG*, anti-T-lymphocyte globulin; *BM*, bone marrow; *CMV*, cytomegalovirus; *CsA*, cyclosporin A; *D*, donor; *haplo*, haploidentical; *MMF*; mycophenolate mofetil; *MTX*, methotrexate; *MUD*, matched unrelated donor; *n*, number of cases; *p*; p-value; *PBSC*, peripheral blood stem cells; *PTCy*, post-transplant cyclophosphamide; *R*, recipient; *TBI*, total body irradiation.

Supplemental Table 5: Patient baseline characteristics from patients in cluster 2 of the "GVHD-associated" T cell model and cluster 1 from the "broad spectrum" T cell model.

Characteristics	**„GVHD-associated" T Cells, Cluster 2**		**„broad spectrum" T Cells, Cluster 1**		***p***
	n	**%**	**n**	**%**	
Total enrolled and treated	94	100	105	100	
Median age at transplantation (range)	56	(19-75)	56	(19-75)	0.864
Male gender	56	60	60	57	0.728
Disease					>0.999
Acute myeloid leukemia	45	48	50	48	
Myelodysplastic syndromes	6	6	8	8	
Myeloproliferative neoplasia	11	12	12	11	
Acute lymphoblastic leukemia	12	13	12	11	
Chronic myeloid leukemia	8	9	9	9	
Chronic myelomonocytic leukemia	2	2	3	3	
Non-Hodgkin's lymphoma	7	7	7	7	
Multiple myeloma	0	0	0	0	
Aplastic Anemia	1	1	1	1	
Other hematologic disorders	2	2	3	3	
Graft source					>0.999
PHSC	94	100	105	100	
BM	0	0	0	0	
Conditioning					0.888
Myeloablative conditioning	69	73	78	74	
Reduced intensity conditioning	25	27	27	26	
TBI containing	42	45	46	44	0.902
GVHD Prophylaxis					0.963
CsA	0	0	0	0	
CsA + MTX	83	88	91	87	
CsA + MMF	1	1	2	2	
TAC + MMF	6	6	7	7	
Other	4	4	5	5	
Recipient/Donor gender constellation					0.959
Female/Female	22	23	24	23	
Male/Male	51	54	55	52	
Female/Male	16	17	21	20	
Male/Female	5	5	5	5	
CMV Serology					0.934
R+/D-	5	5	6	6	
R+/D+	30	32	36	34	
R-/D+	10	11	13	12	
R-/D-	49	52	50	48	

Abbreviations: *ATG*, anti-T-lymphocyte globulin; *BM*, bone marrow; *CMV*, cytomegalovirus; *CsA*, cyclosporin A; *D*, donor; *haplo*, haploidentical; *MMF*; mycophenolate mofetil; *MTX*, methotrexate; *MUD*, matched unrelated donor; *n*, number of cases; *p*; p-value; *PBSC*, peripheral blood stem cells; *PTCy*, post-transplant cyclophosphamide; *R*, recipient; *TBI*, total body irradiation.

Figure Legends

Supplemental Figure 1. CONSORT flow diagram for selection of study cohort. All 551 patients with HCT between January 2017 and May 2020 were screened. For study inclusion, the following selection criteria were applied: allogeneic HCT from haploidentical- or matched unrelated donors (MUD) with either ATG or PTCY as GVHD prophylaxis.

Supplemental Figure 2. Clinical outcome comparison between patients with ATG or PTCY as GVHD prophylaxis did not reveal significant differences. (A, B) Time to acute GVHD II-IV and acute GVHD (all grades) within 100 days post-HCT calculated with the Kaplan-Meier method. **(C, D)** Time to moderate-severe chronic GVHD and chronic GVHD (all grades) within 12 months post-HCT, as obtained by Kaplan-Meier method. *P*-values < 0.05 were considered as statistically significant.

Supplemental Figure 3. Reconstitution of cytotoxic T cell subsets, NK and B cells. Immune reconstitution 12 months after HCT. T cell subsets were gated within the $CD45^+$ gate as follows: **(A)** T cells, $CD3^+$; **(B)** Cytotoxic T cells, $CD3^+/CD8^+$; **(C)** Naïve cytotoxic T cells, $CD3^+/CD8^+/CD45RA^+$; **(D)** Memory cytotoxic T cells, $CD3^+/CD8^+/CD45RO^+$; **(E)** Activated T cells, $CD3^+/HLA\text{-}DR^+$; **(F)** NKT cells, $CD3^+/CD1656^+/CD314^+$; **(G)** NK cells, $CD1656^+/CD314^+$; **(H)** B cells, $CD19^+$. Median absolute cell numbers and the 95% CI were analyzed by the Mann-Whitney-U-test. P-values < 0.05 were considered as statistically significant and are indicated with asterisks ($p < 0.1$, $^{(*)}$; $p < 0.05$, *; $p < 0.01$, **; $p < 0.001$, ***; and $p < 0.0001$, ****). Median values and sample numbers of the respective cohorts as well as the *p*-values are summarized in the attached excel file.

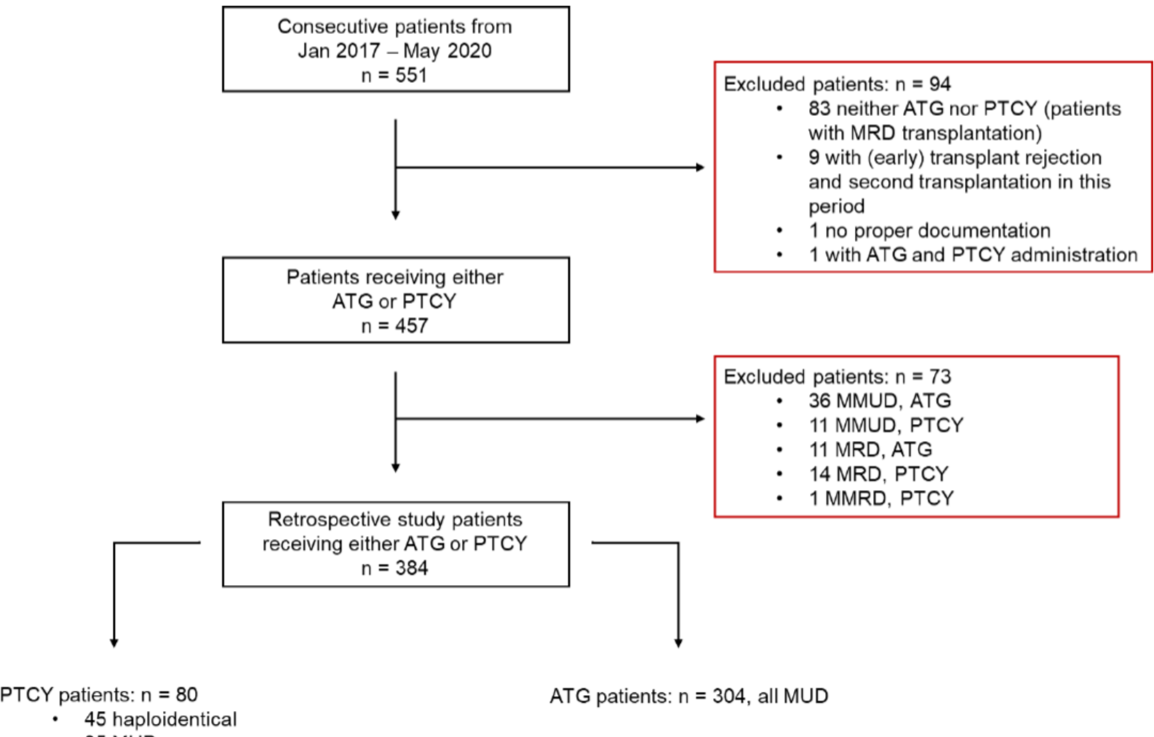
Supplemental Figure 4. Overview of clustering results in the "GVHD-associated" T cell model. (A-B) Selection of parameter configurations **(A)** in the entire MUD-ATG dataset with their respective silhouette coefficients and **(B)** results from 10x resampling for robustness testing. **(C)** Distribution of MUD-ATG patients within the selected configuration. **(D-F)** Selection of parameter configurations in the **(D)** entire cohort and **(E)** in 10x resampling for MUD-PTCy patients and **(F)** patient distribution within the selected parameter configuration. **(G-I)** Selection of parameter configurations in the **(G)** entire cohort and **(H)** in 10x resampling for haplo-PTCy patients and **(I)** patient distribution within the selected parameter configuration. Parameter configurations with concurrent good silhouette coefficients in the entire dataset and the most stable results in resampling are illustrated in orange.

Supplemental Figure 5. Overview of clustering results in the "broad spectrum" T cell model. (A-B) Selection of parameter configurations **(A)** in the entire MUD-ATG dataset with their respective silhouette coefficients and **(B)** results from 10x resampling for robustness

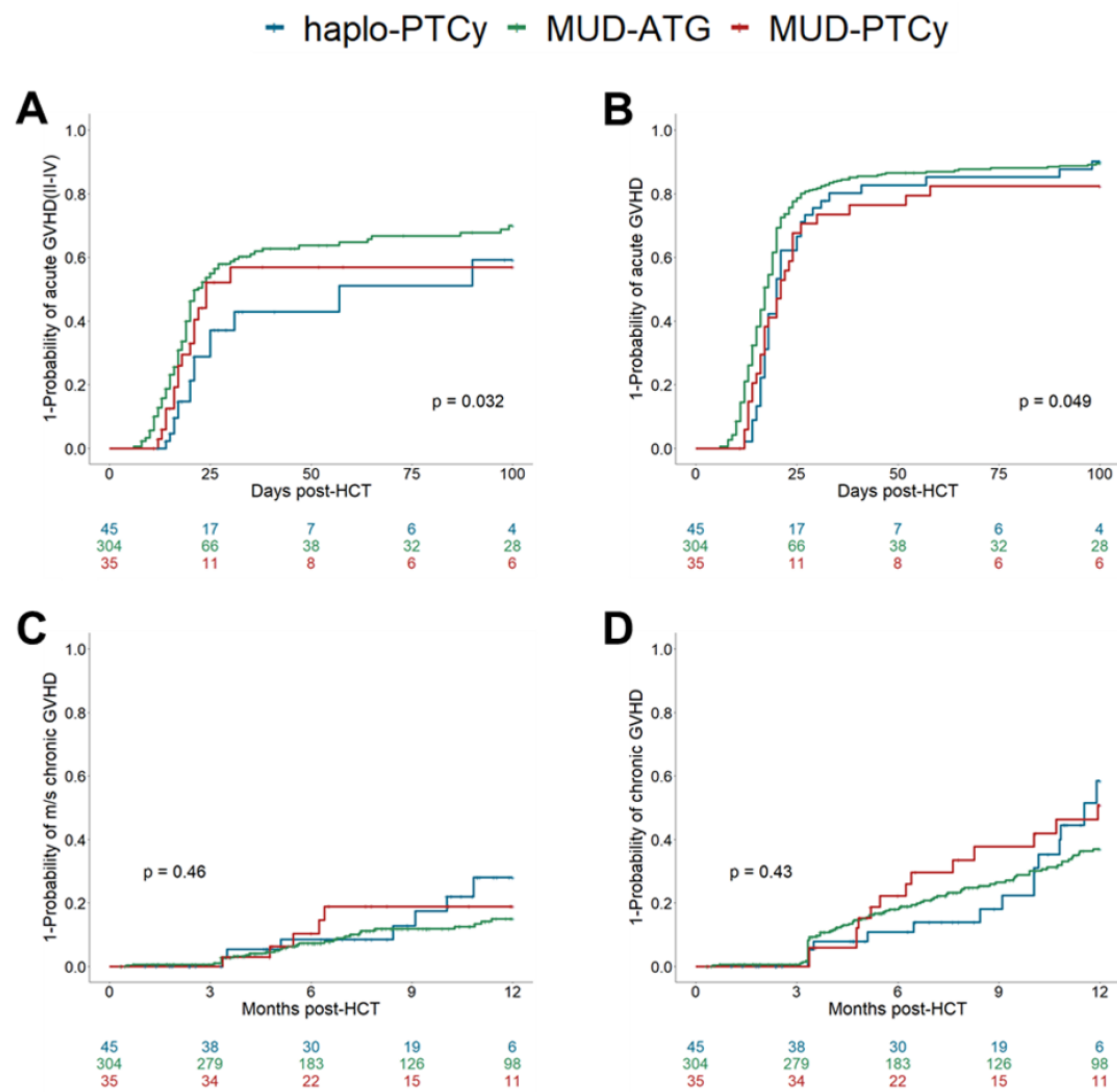
testing. **(C)** Distribution of MUD-ATG patients within the selected configuration. **(D-F)** Selection of parameter configurations in the **(D)** entire cohort and **(E)** in 10x resampling for MUD-PTCy patients and **(F)** patient distribution within the selected parameter configuration. **(G-I)** Selection of parameter configurations in the **(G)** entire cohort and **(H)** in 10x resampling for haplo-PTCy patients and **(I)** patient distribution within the selected parameter configuration. Parameter configurations with concurrent good silhouette coefficients in the entire dataset and the most stable results in resampling are illustrated in orange.

Supplemental Figure 6. Clustering results of PTCy patients in the "broad spectrum" T cell model. (A-D) Individual patient immune cell data clustering in the **(A,B)** MUD-PTCy cohort and **(C,D)** haplo-PTCy cohort using data of "broad spectrum" T cells: $CD3^+/CD4^+$ helper T cells, $CD3^+/CD4^+/CD45RA^+$ naïve helper T cells, $CD3^+/CD8^+$ cytotoxic T cells and $CD3^+/CD8^+/CD45RO^+$ memory cytotoxic T cells, illustrated in distinct boxes. The graphs in **(A,C)** depict each patients' individual reconstitution pattern in the respective subset; **(B,D)** include the medoid samples of each subset calculated via DTW barycenter averaging (DBA) and partition around medoids (PAM), respectively.

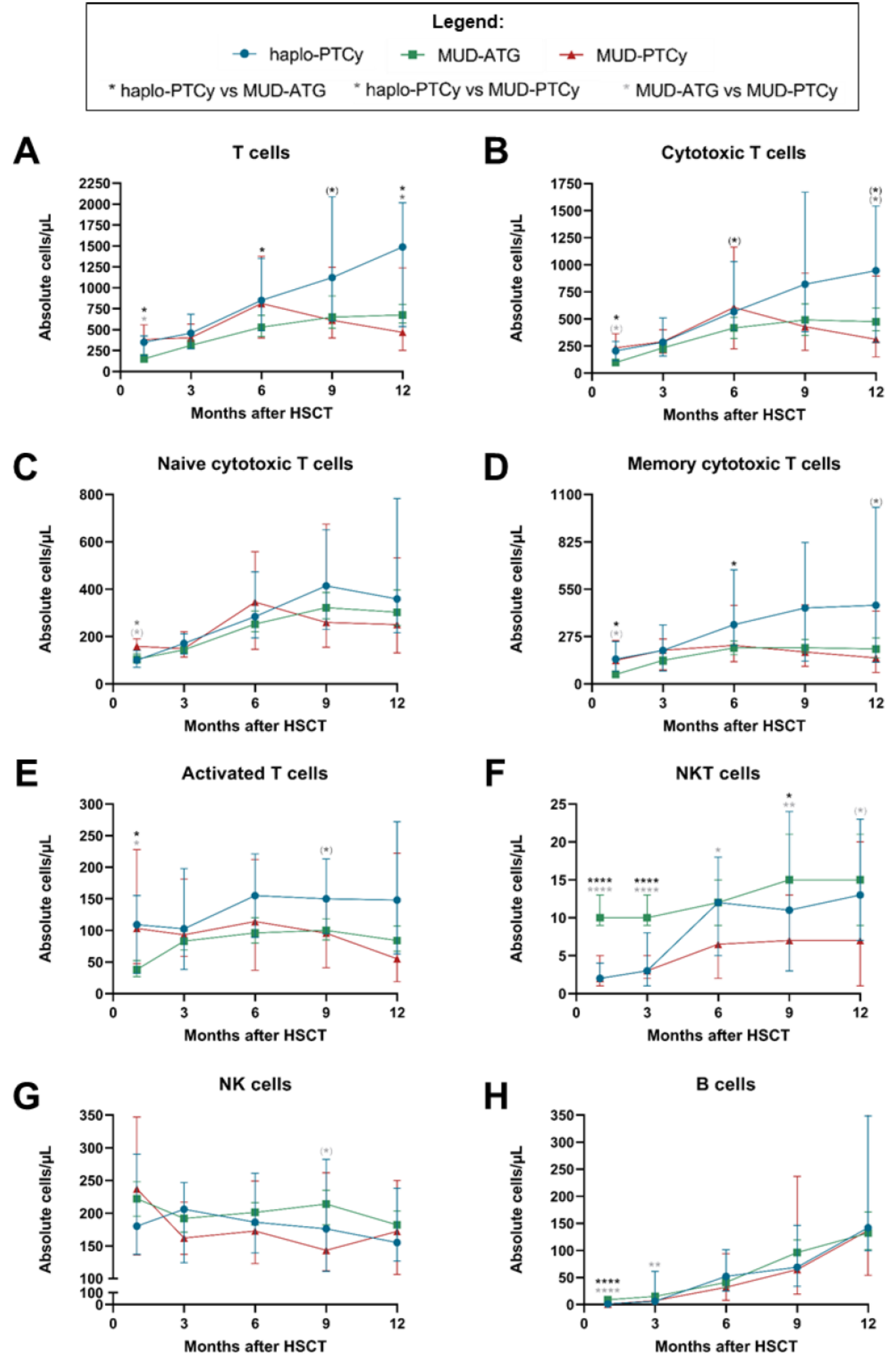
Supplemental Figure 1. CONSORT flow diagram for selection of study cohort.



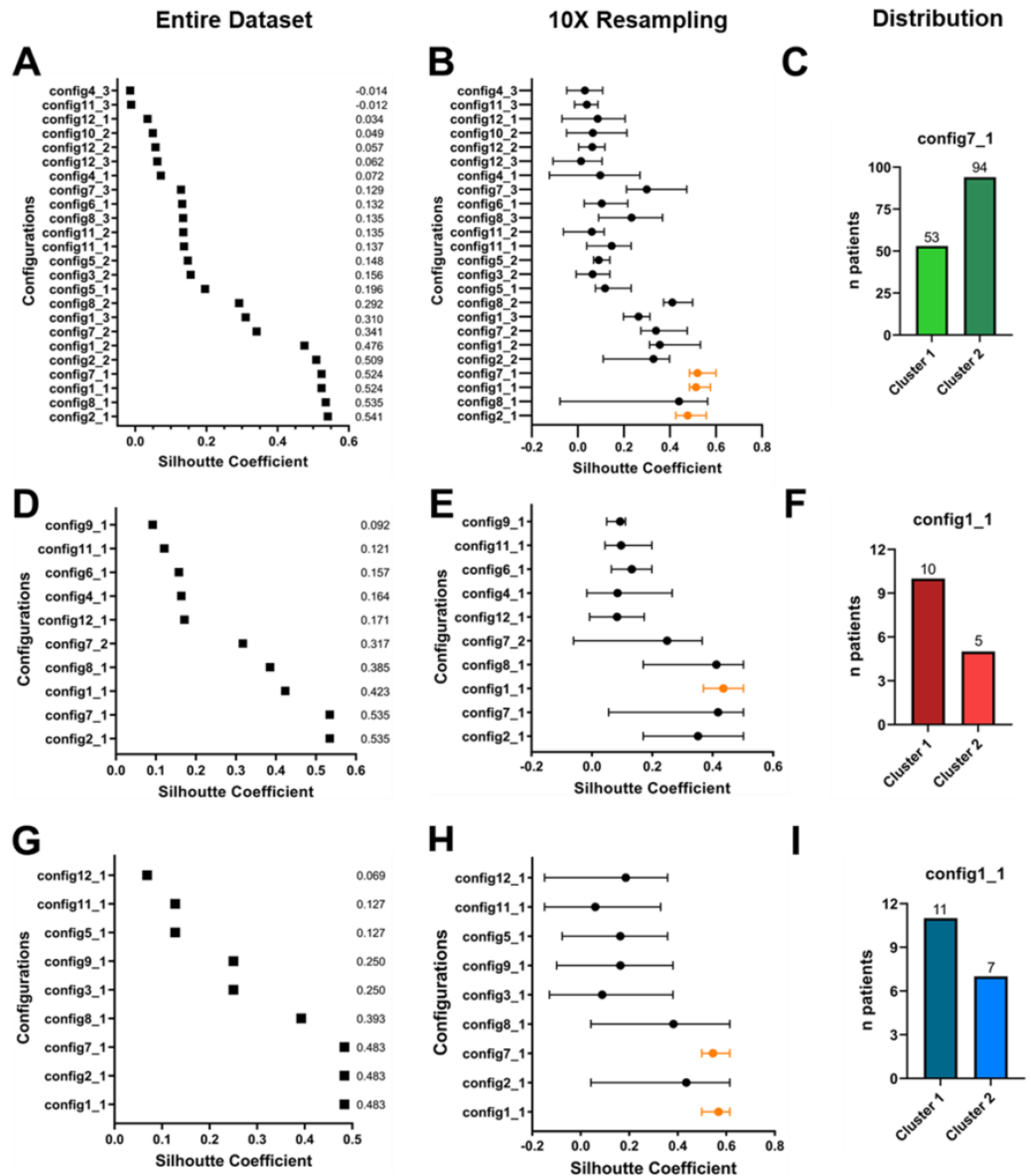
Supplemental Figure 2. Clinical outcome comparison between patients with ATG or PTCY as GVHD prophylaxis did not reveal significant differences.



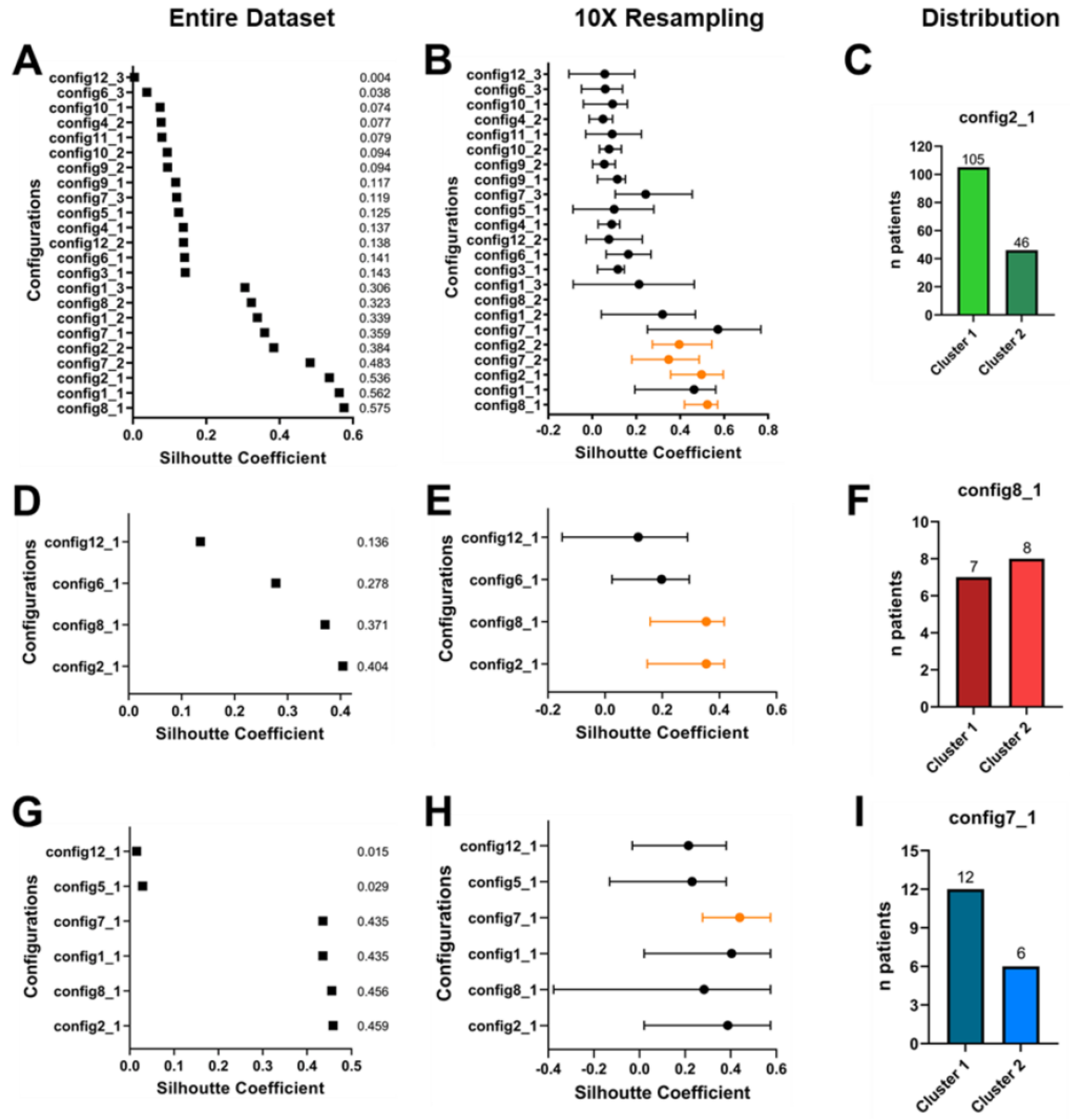
Supplemental Figure 3. Reconstitution of cytotoxic T cell subsets, NK and B cells.



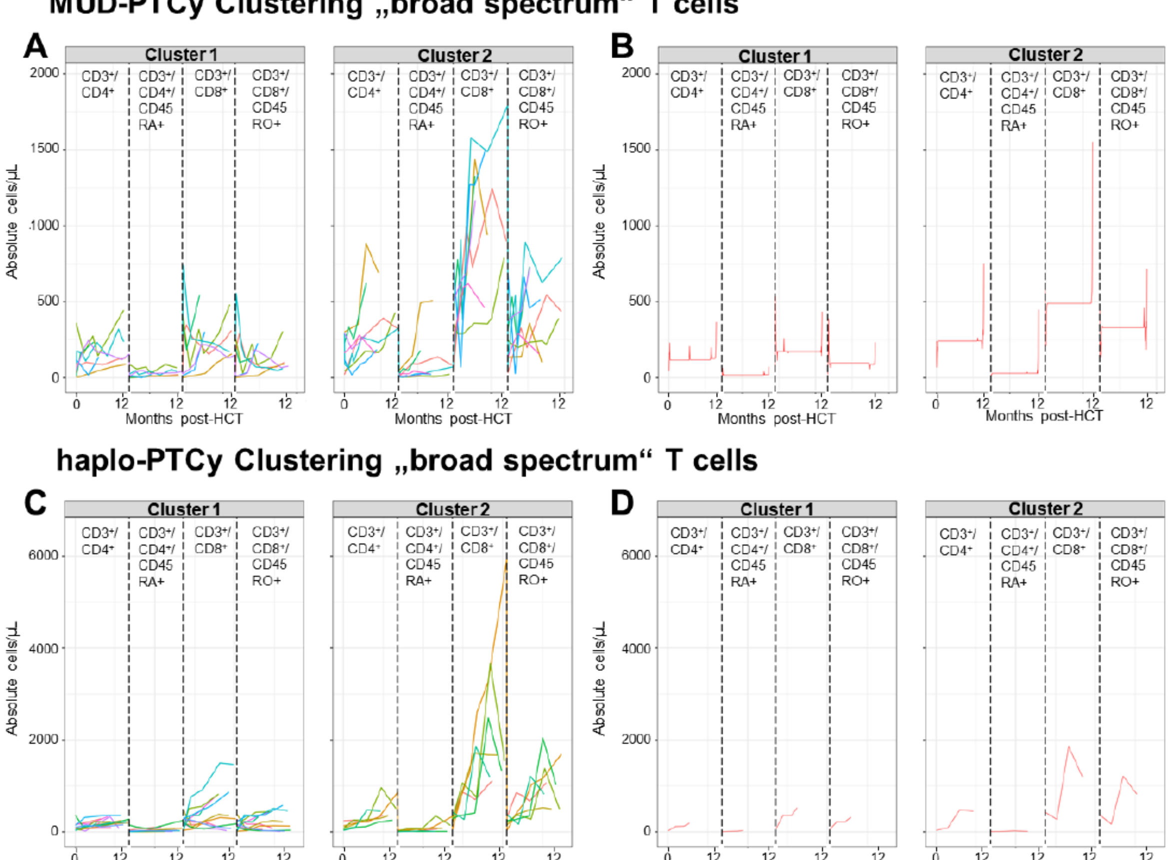
Supplemental Figure 4. Overview of clustering results in the "GVHD-associated" T cell model.



Supplemental Figure 5. Overview of clustering results in the "broad spectrum" T cell model.



Supplemental Figure 6. Clustering results of PTCy patients in the "broad spectrum" T cell model.



7 Discussion

In this thesis, different data-driven approaches, ranging from conventional medical statistics to unsupervised machine learning, were employed to study the immune reconstitution of allogeneic HCT-recipients after transplantation and its relation to different transplant-related factors, in particular CMV reactivation and GVHD prophylaxis to better understand the complex processes in the context of alloreactivity. These analyses were specifically focused on the recovery of T cells as these are the key modulators of the alloreactive effects in GVHD and GVL[58,164]. First, I attempted to dissect the distinct influences of CMV reactivation kinetics on immune recovery, alloreactivity, and hence clinical outcomes of patients with different hematologic malignancies **(Article 1)**. CMV reactivation was associated to increased NRM as well as reduced relapse, depending on the CMV peak viral load within the first 200 days after HCT. The observed dependency of clinically relevant events on peak viral loads was further reflected in distinct T cell reconstitution patterns, attributable to CMV's immune modulatory capacities. In a second study I was able to confirm a beneficial effect of CMV reactivation on AML relapse reduction in patients without ATG **(Article 2)**, whereas this effect was completely abrogated or even inverted in ATG-exposed recipients depending on their disease stage. The ATG-induced abrogation/inversion of relapse protection points to an effective depletion of CMV-boosted alloreactive T cells, which is also reflected by a reduction of severe GVHD events in these patients. This finding might also explain the relapse reduction in patients with intermediate CMV peak titers observed in **Article 1**. In a retrospective ATG study **(Article 3)** I identified a decreased alloreactivity after ATG exposure as shown by a significant reduction of grades III-IV aGVHD and extensive cGVHD rates and detected a dose-dependent ATG-effect on neutrophil and lymphocyte reconstitution, particularly observed in the $CD4^+$ helper T cell subsets. Further, I established an optimal blood lymphocyte count range at the time of ATG exposure correlating to superior survival rates, which supports a potential clinical benefit of individual ATG dosage adjustments. A comparative retrospective analysis of ATG- and PTCy-based GVHD prophylaxis **(Article 4)** revealed significantly decreased incidences of grades II-IV aGVHD in patients with PTCy administration compared to ATG patients. This was paralleled by distinct cellular immune reconstitution fractions that might be responsible for aGVHD protective effects in each setting, namely regulatory T cells and γδ T- as well as NKT cells in PTCy- and

ATG-exposed patients, respectively. I also developed to my knowledge a new time series clustering approach for individualized longitudinal immune reconstitution data analysis, which differentiated heterogenous recovery data and revealed distinct cohorts with respect to their clinical outcomes.

Since amplification of the GVL effect while minimizing GVHD[45] is unfortunately still an unmet goal to date, this thesis aimed at developing data-driven models to characterize the impact of different transplant-related factors on immune reconstitution, especially T cell recovery. By these models I sought to contribute to the clarification of T cell immunity after HCT and to provide new potential sources for harnessing alloreactivity and tilt the balance towards the curative effects of allogeneic HCT. The models presented in this thesis integrated the transplant-related factors of 1) CMV reactivation as discussed to promote GVL activity[165] or at least to support relapse reduction[112,121,124] and 2) medical aGVHD prophylaxis[58]. Both CMV reactivation and aGVHD are shown to be bidirectionally related, promoting increased risks for each other[166,167]. Here, aGVHD and the use of immunosuppressive or T cell depleting regimens associate with delayed immune reconstitution, which favors the occurrence of various infectious complications[64,65] and in particular CMV reactivation. Interestingly, patients during or after CMV replication also have an increased risk to develop aGVHD, likely as a consequence of increased inflammation and cytokine release, particularly in the intestinal tract[168].

In a first step, I addressed the effect of CMV reactivation on immune reconstitution, particularly on T cell reconstitution, to shed light onto the heterogeneous impact of cellular recovery on alloreactivity and thus onto clinical outcomes. Using two CMV kinetics models I was able to clarify previous data concerning increased NRM-[54,119,169] as well as reduced relapse incidences[121,123-125,170] in patient with CMV reactivation suffering from different underlying hematological malignancies by a dependency on CMV peak viral loads. Patients with high peak titers (>100 000 copies/mL) suffered from increased NRM, which translated into reduced OS. Conversely, patients having reactivations with intermediate peak titers (20 000-100 000 copies/mL) had lower risks of relapse **(Article 1, Figure 1F)**, thus providing a possible explanation for inconsistencies in previous analyses[121,123-125,170], which were mostly restricted to binary comparisons using the differentiation between the presence and absence of CMV reactivation[171]. While the first CMV kinetics paper used narrow and low thresholds for

their studies[172], the presented study focused on the wide range of the viral burden within the patient cohort which made it possible to identify clinically relevant cut-off levels for CMV peak titers. Focusing on patients' immune recovery, subgroups divided by these CMV peak titers could be associated to several distinct T cell patterns **(Article 1, Figure 2 & Supp. Figure 6)**. I was able to identify an association of early impaired T cell levels to high CMV peak titer reactivations and therefore a high vulnerability to NRM. The shown data further confirmed the already described immune modulatory effect of CMV on patient immune recovery[55,173,174] and supports a CMV viral load dose-dependency of this effect, in which T cell reconstitution might be achieved through clonal expansion[106,175] in high peak titers and via thymopoesis in others[70]. These presumably differential mechanisms of T cell reconstitution might result in distinct TCR diversity and functionality of T cell subsets[106], which needs to be proven in future prospective studies. Comparable immune modulatory capabilities, as observed in T cells, were only noticed in the NK cell subset for patients with high peak titers **(Article 1, Figure 2D)**, which is in line with previous findings about the stimulation of NK cell recovery by CMV[104,105] and further supports the importance of this subset as pathogen defense after allogeneic HCT[176]. However, no effect of the CMV viral load was notable for B cell recovery **(Article 1, Supp. Figure 6D)**, as it seems to be generally delayed in patients with CMV reactivation potentially due to preceding or subsequent aGVHD events[47]. Generally, the presented data suggest a crucial role of the augmented T cell subset reconstitution after CMV reactivation in reducing leukemic relapse, which is also in line with previous studies[127,177] and the well-established importance of T cells in alloreactive processes[58,164]. Reduced relapse rates in patients with intermediate peak titers **(Article 1, Table 1)** were therefore mediated either by a sufficient GVL activity, as indicated by early elevated T cell levels, or might include other mechanisms, for example: 1) a direct anti-leukemic activity of CMV[178,179], 2) the cross-recognition of CMV-infected and leukemic cells by γδ T cells[180] or 3) heterologous immunity via cross-reactive CMV-induced memory T cells[181]. In summary these findings emphasize the importance of the viral burden, which was also recently validated by Duke *et al.*[113] and differentiates patients with manageable risks from those, which are vulnerable to increased risks of NRM. Moreover, this study highlights insights into the relationship between replicative CMV infections and relapse protection via the CMV peak titer model after allogeneic HCT, by indicating that a CMV-augmented T cell reconstitution might also contribute to the observed GVL effect.

Second, I analyzed the impact of the *in vivo* T cell depleting agent ATG[54,127] and the influence of the AML disease stage[112] on the relationship of CMV reactivation and leukemic relapse in a cohort of AML patients following allogeneic HCT. This analysis revealed a complex interaction of CMV reactivation and AML relapse, in which the protective effect of CMV replication against relapse was highly dependent on both disease stage and the use of ATG. CMV reactivation associated with a reduced relapse incidence in patients without ATG irrespective of disease stage **(Article 2, Figure 1A, C, E)**, while this protective effect was completely abrogated by ATG administration. Moreover, ATG even increased the relapse risk of patients treated in CR1 **(Article 2, Figure 1F)**. These observations are in good agreement with a previous hypothesis about the ability of ATG to mitigate the beneficial effect of CMV reactivation[127]. Both, the complete abrogation as well as the inversion of relapse protection indicate a loss of GVL activity, which is further supported by the observed reduced aGVHD incidence upon ATG exposure. These findings suggest a clinically relevant contribution of a CMV-specific T cell response in reducing AML relapse after allogeneic HCT, which has never been noted after conventional chemotherapy. Since the mechanisms of leukemic relapse protection as a consequence of CMV reactivation are still under debate, it is difficult to make any decisive conclusions about the impact of ATG on this effect. Of note, the bidirectional relationship of CMV replication and aGVHD is also covered by our data, as illustrated by the remarkable relapse protection in patients without ATG in the sequential analysis of early CMV reactivation and aGVHD **(Article 2, Supp. Figure 4A).** This observation might also support the previous hypothesis that in non-ATG patients the main driver for CMV reactivation is aGVHD and the inherent functional impairment of humoral immunity, which is necessary to prevent viral reactivation[93]. This is mirrored by the relapse protection in the same subgroup, potentially through the induction of GVL by alloreactive T cells.

Both articles highlight the distinct influence of CMV reactivation and its viral load on NRM and leukemic relapse after allogeneic HCT. This impact can be further modulated by other transplant-related factors such as GVHD prophylaxis with ATG and the disease stage at transplantation. On a cellular level both studies support a substantial contribution of CMV replication to an augmented reconstitution of alloreactive T cells, which mediates relapse protection. Although these data underpin a central role of T cell reconstitution dynamics in these interactions, they do not provide evidence for the

actual mechanism(s) underlying relapse protection. Beside T cell alloreactivity the developed CMV peak titer model is also consistent with a potential anti-leukemic contribution by a direct virus-versus-leukemia effect[178,179] as indicated by comparable blood T cell levels in the low- and intermediate peak titer subgroups, of which only the latter associated with reduced relapse. Further, different levels of inflammation caused by CMV replication between the low and intermediate peak titer subgroups could modulate the intensity of the response and activity of T cells. These considerations need to be further scrutinized in future studies, providing a more detailed characterization of immune reconstitution after CMV reactivation by including data of specific T cell subsets, e.g. regulatory-, αβ-, and γδ T cells, as well as TCR repertoire analysis. It remains unclear at present, whether the reduced relapse risk observed with intermediate CMV peak titers, is influenced by either ATG or disease stage leaving further space for future studies.

These studies could have potential clinical implications for ongoing strategies of donor selection and CMV prophylaxis. Some findings suggest benefits of D+ for R- in transplants without *in vivo* T cell depletion obtained by ATG **(Article 1, Supp. Figure 4C & Article 2, Supp. Figure 2A, C)**. Furthermore, these data emphasize that not every single episode of CMV reactivation is a threat to patients and that CMV reactivation can have indeed a positive influence on the curative potential of allogeneic HCT, depending on additional factors. This observation might be relevant to the current practices of CMV prophylaxis as it questions the administration of letermovir to all CMV-positive recipients[115] and supports the use of additional transplant-related factors and immunologic parameters in clinical decision-making as well as a timely monitoring of CMV replication early after transplantation to guide prophylaxis and treatment of CMV reactivation after allogeneic HCT.

The regulation of alloreactivity through intensified GVHD prophylaxis and its influence on patients' immune reconstitution and transplant outcomes was subject of another retrospective study in this thesis. These data validated the administration of ATG in addition to a standard GVHD prophylactic regimen as highly effective to reduce grades III-IV aGVHD as well as extensive cGVHD in both analyzed cumulative dosages of 30 or 60 mg/kg ATG **(Article 3, Table 3-4)**. Unlike in previous studies[182,183], GVHD reduction translated into significantly decreased NRM rates after ATG exposure, confirming a general trend towards decreased NRM already observed previously[57].

The reduced alloreactive potential was further illustrated by the expected increased incidence of relapse in ATG patients, which, however, did not lead to increased relapse-related mortality. The observations of reduced NRM and increased relapse were in line with previous data from this thesis **(Article 2)**. Furthermore, I was able to demonstrate a differential effect of ATG on T cell reconstitution. While cytotoxic T cell recovery was comparable between ATG and non-ATG patients, the reconstitution of blood helper T cells showed a dose-dependent delay **(Article 3, Supp. Table 3)**. Interestingly, the comparison of αβ T cell levels, which are known to bear the most alloreactive potential[184], between ATG and non-ATG patients only revealed significant differences at month +3 post-HCT. Despite comparable αβ T cell levels recipients with ATG had a significant reduction of severe GVHD **(Article 3, Table 2)**, which was unexpected given the previously described important role of this subset[184]. In addition, the proposed model for an optimum absolute blood lymphocyte count (ALC) range of 0.4-1.45/nL supports previous attempts for individualized *in vivo* T cell depletion strategies using ATG[185,186]. Patients within the optimum ALC range at ATG exposure significantly correlated to increased OS and improved early $CD4^+$ T cell immune reconstitution compared to patients outside this optimum **(Article 3, Supp. Table 2-3)**.

I pursued the analysis of the influence of GVHD prophylaxis in relation to its effect on alloreactivity with a comparative study of ATG and PTCy. This study revealed a high efficacy of PTCy and ATG in the reduction of grades II-IV aGVHD **(Article 4, Table 1 & Figure 1A)** without differences in the incidence of leukemic relapse **(Article 4, Figure 1I)**. The comparable relapse incidence of the patient subgroups with PTCy or ATG is interesting but not conclusive, as I did not include control groups without ATG or PTCy, limiting the evaluation of possible effects on GVL activity. However, the 1-year relapse incidence of the three subgroups appeared to be comparable to previous results obtained following myeloablative conditioning regimens[182,183,187]. The above-mentioned observations were not accompanied by a significant reduction of cGVHD or NRM. These results are well in line with previous comparisons of both prophylactic strategies, which evaluated their clinical outcomes in the MUD setting[188]. However, recent data on reduced aGVHD and NRM after RIC regimens in MUD compared to haploidentical transplant recipients using PTCy[187] are not comparable with these results as I analyzed a patient cohort, which received conditioning regimens of varying intensity. Comparative analyses of immune reconstitution using both agents

are sparse. By analyzing the cellular recovery of the different study cohorts **(Article 4, Figure 2 & Supp. Figure 3)**, I found evidence that likely different cellular fractions contribute to GVHD protection dependent on the respective agent. While in patients with PTCy GVHD protection could be accomplished through regulatory T cells as previously described in mouse models by the Baltimore group[189], protection in ATG patients might be mediated by elevated blood levels of γδ T cells, although their role remains controversial at present[190-194] and needs to be further evaluated. As a complementary mechanism, NKT cells **(Article 4, Supp. Figure 3F)** can produce anti-inflammatory cytokines such as IL-4, which promote a Th2 polarization and consequently hinder GVHD[195-197]. Overall, the comparative analysis showed a less pronounced effect of PTCy on the helper T cell compartment **(Article 4, Figure 2A-E),** while both protocols led to a similar cytotoxic T cell reconstitution **(Article 4, Supp. Figure 3B-D)**. Additionally, within the scope of this paper I successfully developed a novel approach for analyzing multidimensional individual longitudinal reconstitution data from different cellular subsets, using dynamic time warping and time series clustering **(Article 4, Figure 3-5 & Supp. Figure 4-6)**. This novel approach was able to differentiate heterogenous reconstitution patterns and to characterize patients with worse transplant outcomes, especially in the preponderant MUD-ATG cohort. A significant advantage of this approach was the analysis of individual patients rather than a patient pool, thus reflecting actual cell counts and reconstitution patterns. This technique might further contribute to resolve the question for the best prophylaxis system, as it has the ability to identify patient subgroups with superior responses to the respective applied prophylactic regimen. Due to a limited patient number this was not completely attainable in the PTCy cohort. Furthermore, this new approach is applicable to other research questions, for example the impact of the CMV viral load on immune reconstitution and relapse reduction, potentially leading to a better understanding of the interplay between CMV reactivation, immune competence after transplantation and clinical outcomes as a consequence of GVL and GVHD.

In summary, these two consecutive studies **(Articles 3&4)** both focused on the effects of different GVHD prophylactic strategies, showing an effective reduction of GVHD after the administration of ATG and PTCy, which is accompanied by a profound modulation of the T cell recovery. This can be attributed to the distinct mechanisms of action of ATG and PTCy. While the depletion of alloreactive T cells by ATG in blood

and peripheral lymphoid tissues is a well-known effect[198], the use of PTCy may not result in the elimination of alloreactive T cells as it has been previously shown in several mouse models[199,200]. Besides prompting replication stress in dividing cells[201], it's mechanism of action includes the induction of functional impairment and constrained early expansion of alloreactive T cells. This is paired by robust suppressive mechanisms, for example a preferential, rapid recovery of regulatory T cells[199,200],as a consequence of high expression levels of aldehyde dehydrogenase leading to cyclophosphamide resistance in these cells[202]. Like PTCy, ATG is described to induce the recovery of regulatory T cells as well as NKT cells[198], which presumably contribute further to GVHD protection[203,204]. These different mechanisms might explain the more pronounced reduction observed in several helper T cell subsets of ATG patients. The induction of regulatory T cells after both agents as well as of NKT cells after ATG cannot be directly delineated from this thesis' data, which for various reasons failed to reproduce these effects. Those reasons include the missing evaluation of the regulatory T- and NKT cell recovery in the study comparing the cellular reconstitution of patients with and without ATG **(Article 3)** as well as the lack of control groups without ATG and PTCy in the comparative study of both prophylactic regimens **(Article 4)**. From the results of the comparative study, one might suspect that GVHD prophylaxis with additional PTCy might be the better choice in the MUD setting, due to a faster T cell reconstitution and mostly comparable clinical outcomes to the ATG setting. However, this evidence might be biased as the ATG subgroup consisted of patients getting different cumulative dosages, which were shown to affect helper T cell reconstitution in a dose-dependent manner **(Article 3)**. In contrast to the presented data, some recent studies were not able to find differences in acute or chronic GVHD incidences between ATG and PTCy in the MUD[205] and even in haploidentical transplant settings[206]. Others, however, revealed a significantly lower all-grades and extensive cGVHD incidence for ATG patients in the MRD setting[207] while in the MMUD setting a significantly decreased incidence of aGVHD III-IV for PTCy patients was observed[208]. In the summary of the respective clinical evidence to date, both prophylactic regimens are effective to reduce detrimental acute and chronic GVHD. However, their immunosuppressive activity inevitably leads to mitigation of GVL activity and consequently to an abrogation of relapse protection[127,182]. Consequently, other strategies which simultaneously minimize GVHD and augment GVL reactions are of utmost importance to consistently decrease high relapse rates[34,57]. More recently,

different approaches targeting this unmet clinical need involve the adoptive infusion of immune cells, allograft engineering techniques, the use of new pharmacological agents as well as the use of specific cytokine combinations being evaluated in clinical- and preclinical models[164,209-211]. These strategies include the use of donor lymphocyte infusions, which is the standard allogeneic cellular therapy for disease relapse management post-HCT[209,212], the utilization of αβ- or naïve- T cell depleted grafts[164], or the generation of leukemia-specific T cells[164,210]. In this regard, the use of αβ- or naïve- T cell depleted grafts might be of particular interest to optimize the GVL effect without aggravating GVHD. Another promising strategy, based on the depletion of $CD4^+$ T cells showed distinct effects in form of decreased GVHD and increased GVL[213]. These examples highlight the need for specific subset selection, which allow GVL optimization that appears unattainable with either ATG or PTCy.

In general, the CMV peak titer- **(Article 1)** and ALC model **(Article 3)**, as well as the newly established clustering approach to compare individualized reconstitution patterns **(Article 4)** highlight the strength of sophisticated data-based- or even machine learning models to resolve the complexity of medical processes like the cellular reconstitution after allogeneic HCT as exemplified by this thesis. The joint integration of a variety of factors, e.g. viral peak titers, T cell cut-offs and GVHD protocols, into comprehensive models is required to better characterize the complex interplay between patient immunity, donor graft source, and external influences (e.g. infections) to ultimately predict HCT outcomes. In the future, this has the potential to become a clinically important tool of personalized medical decision-making. Based on the underlying complexity this might only be achieved through advanced machine learning approaches, for example artificial neural networks[153] rather than by classical statistical methods. The implementation of these approaches is challenging as it requires large amounts of data to be trained and validated[153]. Since the introduction of TCR- or mass cytometry analysis[214,215], which have the ability to produce such huge datasets, this appears as a realistic approach. While such models could improve clinical decision-making through predictions of various individual patient outcomes, they might lack the understanding of potential causal relationships between different variables and their significance leading to these outcomes[154]. In contrast, the data-driven models included in this thesis, although only based on comparably small amounts of data, offer such an understanding.

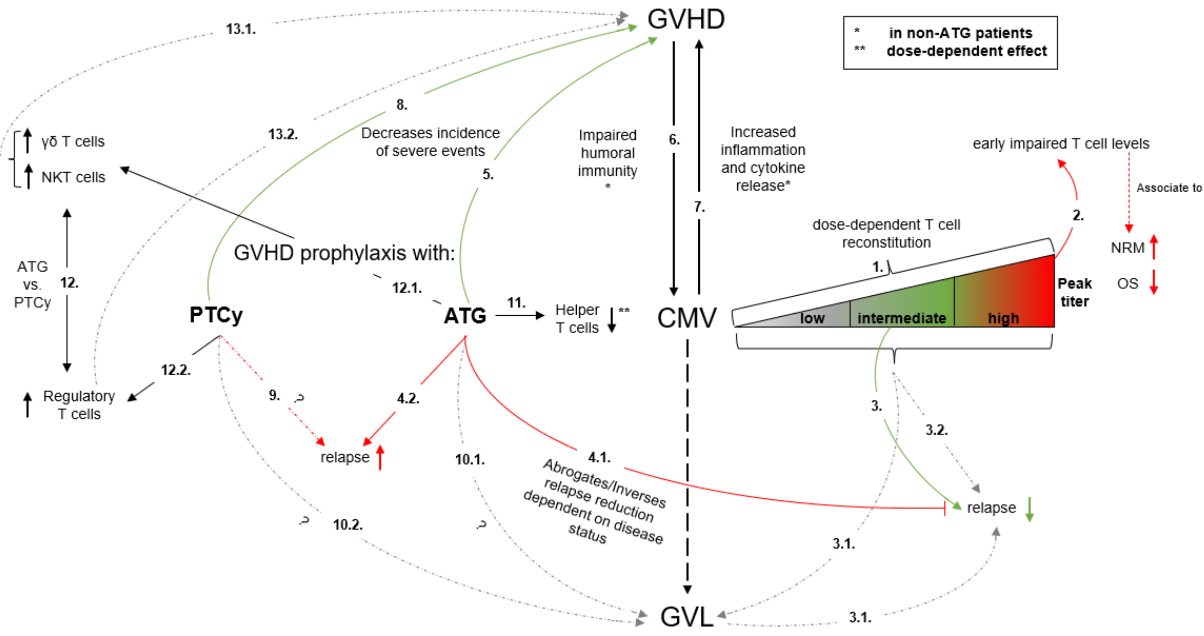


Figure 6: Summary of thesis results. 1. CMV modulates T cell reconstitution post-HCT depending on its peak titers. **2.** High CMV peak titers exhibit early impaired T cell levels associating with increased NRM and reduced OS. **3.** Intermediate peak titers are associated with reduced relapse through a yet unknown mechanism. However, the demonstrated data provided evidence for two possible mechanisms: **3.1.** Elevated peak titers of CMV reactivation trigger the reconstitution of alloreactive T cells which then contribute to reduced relapse via mediation of GVL. This positive effect is only observed in intermediate peak titers as high peak titers induce a high vulnerability to NRM. **3.2.** Patients with intermediate peak titers could benefit from CMV reactivation by a direct virus-versus-leukemia effect. **4.1.** ATG abrogates/inverses the relapse reduction depending on the underlying disease stage at transplantation, potentially driven by the impairment of alloreactive T cells otherwise mediating GVL. **4.2.** ATG is associated with increased relapse incidences but **5.** at the same time decreases the incidence of severe GVHD events. **6.** In non-ATG patients, GVHD might be a trigger for CMV by impaired humoral immunity[93] whereas **7.** in turn an increased inflammation and cytokine release after CMV reactivation may stimulate alloreactivity and hence GVHD[168]. **8.** Compared to ATG, PTCy appears to be more effective in preventing severe GVHD events, while **9.** showing relapse incidences comparable to ATG patients. **10.1.** ATG is well known to deplete alloreactive T cells while **10.2.** PTCy was shown to lead to a functional impairment of alloreactive T cells[199,200] which could also impact the GVL potential. **11.** Concerning cellular immune reconstitution, ATG induces a dose-depending depleting effect which is most pronounced in helper T cells. **12.** GVHD prophylaxis with ATG and PTCy results in differences of the blood cellular recovery. While in **12.1.** ATG patients, γδ T- and NKT cell levels are increased compared to those of PTCy patients, **12.2.** PTCy patients exhibit increased regulatory T cell levels. **13.1.** Both, γδ T- and NKT cells after ATG as well as **13.2.** regulatory T cells after PTCy are likely involved in the reduction of severe GVHD events.

Altogether, the herein presented results enlighten the understanding of how distinct transplant-related factors, as for example CMV reactivation or the choice of GVHD

prophylaxis, influence the reconstitution of T cells and hence the peculiarity of alloreactivity **(Figure 6)**. In particular, these results contribute to the knowledge that the reconstitution of T cells is generally augmented through CMV reactivation but that this effect is very differently pronounced depending on the blood CMV virus titer and leads to a differential impact on patient outcomes. The results highlighted in this thesis are in line with the hypothesis that CMV-augmented T cell alloreactivity reduces the leukemic relapse risk, which is abrogated by their *in vivo* depletion through prophylactic application of ATG. However, this might not be the only contributor to the beneficial impact of CMV as these data also provide evidence for a direct anti-leukemic effect of CMV. In this context, the hypothesized mechanistic combination of T cell alloreactivity and direct virus-versus-leukemia effects needs to be further evaluated by combined TCR analysis and functional CMV assays. The presented data further support the hypothesis of a bidirectional relationship between CMV replication and aGVHD, which is probably modulated by the use of *in vivo* T cell depletion with ATG. Reconstitution of the helper T cell compartment was shown to be highly impaired after ATG , which presumably led to the reduction of severe GVHD events. In contrast, the reconstitution of cytotoxic T cells was similar between patients with or without ATG prophylaxis. Subsequent work, which was focused on comparative cellular reconstitution analysis between ATG and PTCy uncovered differences in discrete cellular fractions, which despite of their different immune modulatory mechanisms of action, could both be involved in the protection from GVHD. In particular, elevated regulatory T cell levels after PTCy might be responsible for a more substantial reduction of severe GVHD as compared to ATG, while maintaining the anti-leukemic alloreactivity. In summary, I was able to confirm previous studies with regard to increasing incidences of malignant relapse after allogeneic HCT with more vigorous prophylactic interventions against GVHD. This emphasizes the clinical need for innovative prophylactic strategies, which are highly effective against GVHD and which at the same time preserve or even enhance the anti-leukemic activity. The results of this thesis can provide additional information to the understanding of the interplay between transplant-related factors and the reconstituting T cell subsets and the balance between GVHD and GVL. The findings of the CMV-augmented GVL activity as well as the differential impact of ATG and PTCy on cellular recovery may contribute to improvements of individualized clinical decision-making.

8 References

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

2. Niederwieser D, Baldomero H, Atsuta Y, et al. One and Half Million Hematopoietic Stem Cell Transplants (HSCT). Dissemination, Trends and Potential to Improve Activity By Telemedicine from the Worldwide Network for Blood and Marrow Transplantation (WBMT). *Blood*. 2019;134(Supplement_1):2035-2035.

3. Passweg JR, Baldomero H, Chabannon C, et al. Hematopoietic cell transplantation and cellular therapy survey of the EBMT: monitoring of activities and trends over 30 years. *Bone Marrow Transplantation*. 2021.

4. Gratwohl A, Pasquini MC, Aljurf M, et al. One million haemopoietic stem-cell transplants: a retrospective observational study. *The Lancet Haematology*. 2015;2(3):e91-e100.

5. Storb R. HSCT: Historical Perspective. In: Carreras E, Dufour C, Mohty M, Kröger N, eds. The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies. Cham: Springer International Publishing; 2019:3-9.

6. Chabannon C, Kuball J, Bondanza A, et al. Hematopoietic stem cell transplantation in its 60s: A platform for cellular therapies. *Science Translational Medicine*. 2018;10(436):eaap9630.

7. Beelen DW, Mytilineos, Joannis. Jahresbericht des Deutschen Registers für Stammzelltransplantation (DRST); 2019.

8. Savani BN, Labopin M, Blaise D, et al. Peripheral blood stem cell graft compared to bone marrow after reduced intensity conditioning regimens for acute leukemia: a report from the ALWP of the EBMT. *Haematologica*. 2016;101(2):256-262.

9. Champlin RE, Schmitz N, Horowitz MM, et al. Blood stem cells compared with bone marrow as a source of hematopoietic cells for allogeneic transplantation. *Blood*. 2000;95(12):3702-3709.

10. Penack O, Peczynski C, Mohty M, et al. How much has allogeneic stem cell transplant–related mortality improved since the 1980s? A retrospective analysis from the EBMT. *Blood Advances*. 2020;4(24):6283-6290.

11. Pavletic ZS, Bishop MR, Tarantolo SR, et al. Hematopoietic recovery after allogeneic blood stem-cell transplantation compared with bone marrow transplantation in patients with hematologic malignancies. *Journal of Clinical Oncology*. 1997;15(4):1608-1616.

12. Armitage JO. Bone Marrow Transplantation. *New England Journal of Medicine*. 1994;330(12):827-838.

13. Dini G, Arcese W, Barbanti M, et al. Peripheral blood stem cell collection from G-CSF-stimulated unrelated donors for second transplant. *Bone Marrow Transplant*. 1998;22 Suppl 5:S41-45.

14. Kekre N, Antin JH. Hematopoietic stem cell transplantation donor sources in the 21st century: choosing the ideal donor when a perfect match does not exist. *Blood*. 2014;124(3):334-343.

15. Yakoub-Agha I. Transplantations from HLA-identical siblings versus 10/10 HLA-matched unrelated donors. *Seminars in Hematology*. 2016;53(2):74-76.

16. Tiercy JM. How to select the best available related or unrelated donor of hematopoietic stem cells? *Haematologica*. 2016;101(6):680-687.

17. Association WMD. WMDA Global Trend Report 2020 - Summary Slides; 2020.

18. Duarte RF, Labopin M, Bader P, et al. Indications for haematopoietic stem cell transplantation for haematological diseases, solid tumours and immune disorders: current practice in Europe, 2019. *Bone Marrow Transplantation*. 2019;54(10):1525-1552.

19. Bornhäuser M, Bethge, Wolfgang, Einsele, Hermann, Kobbe, Guido, Kröger, Nicolaus, Mielke, Stephan, Müller, Carlheinz, Mytilineos, Joannis, Ottinger, Hellmut, Schönemann, Constanze. DAG-KBT Leitlinien: Spenderauswahl zur allogenen Stammzelltransplantation; 2016.

20. Finke J, Schmoor C, Bethge WA, et al. Prognostic Factors Affecting Outcome after Allogeneic Transplantation for Hematological Malignancies from Unrelated Donors: Results from a Randomized Trial. *Biology of Blood and Marrow Transplantation*. 2012;18(11):1716-1726.

21. Seo S, Usui Y, Matsuo K, et al. Impact of the combination of donor age and HLA disparity on the outcomes of unrelated bone marrow transplantation. *Bone Marrow Transplantation*. 2021.

22. Gratwohl A, Hermans J, Niederwieser D, van Biezen A, van Houwelingen HC, Apperley J. Female donors influence transplant-related mortality and relapse incidence in male recipients of sibling blood and marrow transplants. *Hematol J*. 2001;2(6):363-370.

23. Ljungman P, Brand R, Hoek J, et al. Donor Cytomegalovirus Status Influences the Outcome of Allogeneic Stem Cell Transplant: A Study by the European Group for Blood and Marrow Transplantation. *Clinical Infectious Diseases*. 2014;59(4):473-481.

24. Seebach JD, Stussi G, Passweg JR, et al. ABO Blood Group Barrier in Allogeneic Bone Marrow Transplantation Revisited. *Biology of Blood and Marrow Transplantation*. 2005;11(12):1006-1013.

25. Fleischhauer K, Shaw BE, Gooley T, et al. Effect of T-cell-epitope matching at HLA-DPB1 in recipients of unrelated-donor haemopoietic-cell transplantation: a retrospective study. *Lancet Oncol*. 2012;13(4):366-374.

26. Yakoub-Agha I, Mesnil F, Kuentz M, et al. Allogeneic marrow stem-cell transplantation from human leukocyte antigen-identical siblings versus human leukocyte antigen-allelic-matched unrelated donors (10/10) in patients with standard-risk hematologic malignancy: a prospective study from the French Society of Bone Marrow Transplantation and Cell Therapy. *J Clin Oncol*. 2006;24(36):5695-5702.

27. Schetelig J, Bornhäuser M, Schmid C, et al. Matched Unrelated or Matched Sibling Donors Result in Comparable Survival After Allogeneic Stem-Cell Transplantation in Elderly Patients With Acute Myeloid Leukemia: A Report From the Cooperative German Transplant Study Group. *Journal of Clinical Oncology*. 2008;26(32):5183-5191.

28. Gragert L, Eapen M, Williams E, et al. HLA Match Likelihoods for Hematopoietic Stem-Cell Grafts in the U.S. Registry. *New England Journal of Medicine*. 2014;371(4):339-348.

29. Ballen KK, Koreth J, Chen Y-B, Dey BR, Spitzer TR. Selection of optimal alternative graft source: mismatched unrelated donor, umbilical cord blood, or haploidentical transplant. *Blood*. 2012;119(9):1972-1980.

30. Spierings E, Fleischhauer K. Histocompatibility. In: Carreras E, Dufour C, Mohty M, Kröger N, eds. The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies. Cham: Springer International Publishing; 2019:61-68.

31. Luznik L, O'Donnell PV, Symons HJ, et al. HLA-Haploidentical Bone Marrow Transplantation for Hematologic Malignancies Using Nonmyeloablative Conditioning and High-Dose, Posttransplantation Cyclophosphamide. *Biology of Blood and Marrow Transplantation*. 2008;14(6):641-650.

32. McCurdy SR, Luznik L. How we perform haploidentical stem cell transplantation with posttransplant cyclophosphamide. *Blood*. 2019;134(21):1802-1810.

33. Ballen K. Umbilical Cord Blood Transplantation: Challenges and Future Directions. *STEM CELLS Translational Medicine*. 2017;6(5):1312-1315.

34. Shouval R, Fein JA, Labopin M, et al. Outcomes of allogeneic haematopoietic stem cell transplantation from HLA-matched and alternative donors: a European Society for Blood and Marrow Transplantation registry retrospective analysis. *The Lancet Haematology*. 2019;6(11):e573-e584.

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

36. Trowsdale J, Knight JC. Major histocompatibility complex genomics and human disease. *Annu Rev Genomics Hum Genet*. 2013;14:301-323.

37. Robinson J, Barker DJ, Georgiou X, Cooper MA, Flicek P, Marsh SGE. IPD-IMGT/HLA Database. *Nucleic Acids Res*. 2020;48(D1):D948-d955.

38. Bertaina A, Andreani M. Major Histocompatibility Complex and Hematopoietic Stem Cell Transplantation: Beyond the Classical HLA Polymorphism. *International Journal of Molecular Sciences*. 2018;19(2):621.

39. Petersdorf EW. Histocompatibility. Thomas' Hematopoietic Cell Transplantation; 2015:112-127.

40. Zakrzewski JL, Goldberg GL, Smith OM, van den Brink MR. Enhancing T cell reconstitution after hematopoietic stem cell transplantation: a brief update of the latest trends. *Blood Cells Mol Dis*. 2008;40(1):44-47.

41. Ruggeri L, Capanni M, Urbani E, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science*. 2002;295(5562):2097-2100.

42. Spierings E. Minor histocompatibility antigens: past, present, and future. *Tissue Antigens*. 2014;84(4):374-360.

43. Masouridi-Levrat S, Simonetta F, Chalandon Y. Immunological Basis of Bone Marrow Failure after Allogeneic Hematopoietic Stem Cell Transplantation. *Frontiers in immunology*. 2016;7:362-362.

44. Felix NJ, Allen PM. Specificity of T-cell alloreactivity. *Nat Rev Immunol*. 2007;7(12):942-953.

45. Blazar BR, Hill GR, Murphy WJ. Dissecting the biology of allogeneic HSCT to enhance the GvT effect whilst minimizing GvHD. *Nat Rev Clin Oncol*. 2020;17(8):475-492.

46. Phelan R, Arora, M., Chen, M. Current use and outcome of hematopoietic stem cell transplantation: CIBMTR US summary slides; 2020.

47. Tomblyn M, Chiller T, Einsele H, et al. Guidelines for Preventing Infectious Complications among Hematopoietic Cell Transplantation Recipients: A Global Perspective. *Biology of Blood and Marrow Transplantation*. 2009;15(10):1143-1238.

48. Zeiser R, Dreger, Peter, Finke, Jürgen, Greinix, Hildegard, Holler, Ernst, Luft, Thomas, Mielke, Stephan, Scheid, Christof, Wolff, Daniel. DAG-KBT Leitlinien: GVHD-Prophylaxe und Therapie; 2016.

49. Lee SJ, Vogelsang G, Flowers MED. Chronic graft-versus-host disease. *Biology of Blood and Marrow Transplantation*. 2003;9(4):215-233.

50. van den Brink MR, Porter DL, Giralt S, et al. Relapse after allogeneic hematopoietic cell therapy. *Biol Blood Marrow Transplant*. 2010;16(1 Suppl):S138-145.

51. Ogonek J, Kralj Juric M, Ghimire S, et al. Immune Reconstitution after Allogeneic Hematopoietic Stem Cell Transplantation. *Front Immunol*. 2016;7:507.

52. Gratwohl A, Brand R, Frassoni F, et al. Cause of death after allogeneic haematopoietic stem cell transplantation (HSCT) in early leukaemias: an EBMT analysis of lethal infectious complications and changes over calendar time. *Bone Marrow Transplantation*. 2005;36(9):757-769.

53. Annaloro C, Serpenti F, Saporiti G, et al. Viral Infections in HSCT: Detection, Monitoring, Clinical Management, and Immunologic Implications. *Frontiers in Immunology*. 2021;11(3482).

54. Teira P, Battiwalla M, Ramanathan M, et al. Early cytomegalovirus reactivation remains associated with increased transplant-related mortality in the current era: a CIBMTR analysis. *Blood*. 2016;127(20):2427-2438.

55. Stern L, Withers B, Avdic S, et al. Human Cytomegalovirus Latency and Reactivation in Allogeneic Hematopoietic Stem Cell Transplant Recipients. *Frontiers in Microbiology*. 2019;10(1186).

56. Sahin U, Toprak SK, Atilla PA, Atilla E, Demirer T. An overview of infectious complications after allogeneic hematopoietic stem cell transplantation. *Journal of Infection and Chemotherapy*. 2016;22(8):505-514.

57. Styczyński J, Tridello G, Koster L, et al. Death after hematopoietic stem cell transplantation: changes over calendar year time, infections and associated factors. *Bone Marrow Transplantation*. 2020;55(1):126-136.

58. Ferrara JLM, Levine JE, Reddy P, Holler E. Graft-versus-host disease. *Lancet (London, England)*. 2009;373(9674):1550-1561.

59. Jagasia MH, Greinix HT, Arora M, et al. National Institutes of Health Consensus Development Project on Criteria for Clinical Trials in Chronic Graft-versus-Host

Disease: I. The 2014 Diagnosis and Staging Working Group report. *Biol Blood Marrow Transplant*. 2015;21(3):389-401.e381.

60. Leen AM, Tripic T, Rooney CM. Challenges of T cell therapies for virus-associated diseases after hematopoietic stem cell transplantation. *Expert Opin Biol Ther*. 2010;10(3):337-351.

61. Champlin R. Relapse of Hematologic Malignancy After Allogeneic Hematopoietic Transplantation. Thomas' Hematopoietic Cell Transplantation; 2015:836-844.

62. Vago L, Perna SK, Zanussi M, et al. Loss of Mismatched HLA in Leukemia after Stem-Cell Transplantation. *New England Journal of Medicine*. 2009;361(5):478-488.

63. Dazzi F, Fozza C. Disease relapse after haematopoietic stem cell transplantation: Risk factors and treatment. *Best Practice & Research Clinical Haematology*. 2007;20(2):311-327.

64. Bosch M, Khan FM, Storek J. Immune reconstitution after hematopoietic cell transplantation. *Current Opinion in Hematology*. 2012;19(4):324-335.

65. Dudakov JA, Perales M-A, van den Brink MRM. Immune Reconstitution Following Hematopoietic Cell Transplantation. Thomas' Hematopoietic Cell Transplantation; 2015:160-169.

66. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. *Nature Reviews Cancer*. 2004;4(1):11-22.

67. Storek J. Immunological reconstitution after hematopoietic cell transplantation - its relation to the contents of the graft. *Expert Opin Biol Ther*. 2008;8(5):583-597.

68. Naik P, Verma A, Pedicano J, et al. Tempo of neutrophil recovery and the definition of myeloid engraftment after allogeneic hematopoietic stem cell transplantation (HSCT) in patients not receiving growth factors post-transplant. *Biology of Blood and Marrow Transplantation*. 2004;10:84.

69. Ramaprasad C, Pouch S, Pitrak DL. Neutrophil function after bone marrow and hematopoietic stem cell transplant. *Leukemia & Lymphoma*. 2010;51(5):756-767.

70. Wils E-J, van der Holt B, Broers AEC, et al. Insufficient recovery of thymopoiesis predicts for opportunistic infections in allogeneic hematopoietic stem cell transplant recipients. *Haematologica*. 2011;96(12):1846-1854.

71. Onoe T, Kalscheuer H, Chittenden M, Zhao G, Yang Y-G, Sykes M. Homeostatic Expansion and Phenotypic Conversion of Human T Cells Depend on Peripheral Interactions with APCs. *The Journal of Immunology*. 2010;184(12):6756-6765.

72. Hokland M, Jacobsen N, Ellegaard J, Hokland P. Natural killer function following allogeneic bone marrow transplantation. Very early reemergence but strong dependence of cytomegalovirus infection. *Transplantation*. 1988;45(6):1080-1084.

73. Cichocki F, Cooley S, Davis Z, et al. CD56dimCD57+NKG2C+ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia*. 2016;30(2):456-463.

74. Small TN, Keever CA, Weiner-Fedus S, Heller G, O'Reilly RJ, Flomenberg N. B-Cell Differentiation Following Autologous, Conventional, or T-Cell Depleted Bone

Marrow Transplantation: A Recapitulation of Normal B-Cell Ontogeny. *Blood*. 1990;76(8):1647-1656.

75. Seggewiss R, Einsele H. Immune reconstitution after allogeneic transplantation and expanding options for immunomodulation: an update. *Blood*. 2010;115(19):3861-3868.

76. Fry TJ, Mackall CL. Immune reconstitution following hematopoietic progenitor cell transplantation: challenges for the future. *Bone Marrow Transplantation*. 2005;35(1):S53-S57.

77. Mackall CL, Fleisher TA, Brown MR, et al. Distinctions between CD8+ and CD4+ T-cell regenerative pathways result in prolonged T-cell subset imbalance after intensive chemotherapy. *Blood*. 1997;89(10):3700-3707.

78. Dumont-Girard F, Roux E, van Lier RA, et al. Reconstitution of the T-Cell Compartment After Bone Marrow Transplantation: Restoration of the Repertoire by Thymic Emigrants. *Blood*. 1998;92(11):4464-4471.

79. Hakim FT, Memon SA, Cepeda R, et al. Age-dependent incidence, time course, and consequences of thymic renewal in adults. *The Journal of Clinical Investigation*. 2005;115(4):930-939.

80. Gandhi MK, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis*. 2004;4(12):725-738.

81. Gibson W. Structure and Formation of the Cytomegalovirus Virion. In: Shenk TE, Stinski MF, eds. Human Cytomegalovirus. Berlin, Heidelberg: Springer Berlin Heidelberg; 2008:187-204.

82. Landolfo S, Gariglio M, Gribaudo G, Lembo D. The human cytomegalovirus. *Pharmacol Ther*. 2003;98(3):269-297.

83. Verma S, Benedict CA. Sources and signals regulating type I interferon production: lessons learned from cytomegalovirus. *Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research*. 2011;31(2):211-218.

84. Biron CA, Byron KS, Sullivan JL. Severe Herpesvirus Infections in an Adolescent without Natural Killer Cells. *New England Journal of Medicine*. 1989;320(26):1731-1735.

85. Crough T, Khanna R. Immunobiology of human cytomegalovirus: from bench to bedside. *Clin Microbiol Rev*. 2009;22(1):76-98, Table of Contents.

86. Picarda G, Benedict CA. Cytomegalovirus: Shape-Shifting the Immune System. *The Journal of Immunology*. 2018;200(12):3881-3889.

87. Freeman J, R. B. The 'Indirect' Effects of Cytomegalovirus Infection. *American Journal of Transplantation*. 2009;9(11):2453-2458.

88. Griffiths P, Baraniak I, Reeves M. The pathogenesis of human cytomegalovirus. *The Journal of Pathology*. 2015;235(2):288-297.

89. Cho SY, Lee DG, Kim HJ. Cytomegalovirus Infections after Hematopoietic Stem Cell Transplantation: Current Status and Future Immunotherapy. *Int J Mol Sci*. 2019;20(11).

90. Déchanet J, Merville P, Bergé F, et al. Major expansion of gammadelta T lymphocytes following cytomegalovirus infection in kidney allograft recipients. *J Infect Dis*. 1999;179(1):1-8.

91. Blyth E, Withers B, Clancy L, Gottlieb D. CMV-specific immune reconstitution following allogeneic stem cell transplantation. *Virulence*. 2016;7(8):967-980.

92. Jonjić S, Pavić I, Polić B, Crnković I, Lucin P, Koszinowski UH. Antibodies are not essential for the resolution of primary cytomegalovirus infection but limit dissemination of recurrent virus. *J Exp Med*. 1994;179(5):1713-1717.

93. Martins JP, Andoniou CE, Fleming P, et al. Strain-specific antibody therapy prevents cytomegalovirus reactivation after transplantation. *Science*. 2019;363(6424):288-293.

94. Rasmussen L, Matkin C, Spaete R, Pachl C, Merigan TC. Antibody response to human cytomegalovirus glycoproteins gB and gH after natural infection in humans. *J Infect Dis*. 1991;164(5):835-842.

95. Zuhair M, Smit GSA, Wallis G, et al. Estimation of the worldwide seroprevalence of cytomegalovirus: A systematic review and meta-analysis. *Reviews in Medical Virology*. 2019;29(3):e2034.

96. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med*. 2005;202(5):673-685.

97. Einsele H, Roosnek E, Rufer N, et al. Infusion of cytomegalovirus (CMV)-specific T cells for the treatment of CMV infection not responding to antiviral chemotherapy. *Blood*. 2002;99(11):3916-3922.

98. Elkington R, Shoukry NH, Walker S, et al. Cross-reactive recognition of human and primate cytomegalovirus sequences by human CD4 cytotoxic T lymphocytes specific for glycoprotein B and H. *Eur J Immunol*. 2004;34(11):3216-3226.

99. Boeckh M, Ljungman P. How we treat cytomegalovirus in hematopoietic cell transplant recipients. *Blood*. 2009;113(23):5711-5719.

100. Einsele H, Ljungman P, Boeckh M. How I treat CMV reactivation after allogeneic hematopoietic stem cell transplantation. *Blood*. 2020;135(19):1619-1629.

101. Nichols WG, Corey L, Gooley T, Davis C, Boeckh M. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. *J Infect Dis*. 2002;185(3):273-282.

102. Camargo JF, Komanduri KV. Emerging concepts in cytomegalovirus infection following hematopoietic stem cell transplantation. *Hematology/Oncology and Stem Cell Therapy*. 2017;10(4):233-238.

103. Boeckh M, Leisenring W, Riddell SR, et al. Late cytomegalovirus disease and mortality in recipients of allogeneic hematopoietic stem cell transplants: importance of viral load and T-cell immunity. *Blood*. 2003;101(2):407-414.

104. Foley B, Cooley S, Verneris MR, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood*. 2012;119(11):2665-2674.

105. Foley B, Cooley S, Verneris MR, et al. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol*. 2012;189(10):5082-5088.

106. Suessmuth Y, Mukherjee R, Watkins B, et al. CMV reactivation drives post-transplant T cell reconstitution and results in defects in the underlying TCRβ repertoire. *Blood*. 2015:blood-2015-2003-631853.

107. Einsele H, Hebart H, Kauffmann-Schneider C, et al. Risk factors for treatment failures in patients receiving PCR-based preemptive therapy for CMV infection. *Bone Marrow Transplant*. 2000;25(7):757-763.

108. Melendez-Munoz R, Marchalik R, Jerussi T, et al. Cytomegalovirus Infection Incidence and Risk Factors Across Diverse Hematopoietic Cell Transplantation Platforms Using a Standardized Monitoring and Treatment Approach: A Comprehensive Evaluation from a Single Institution. *Biology of blood and marrow transplantation : journal of the American Society for Blood and Marrow Transplantation*. 2019;25(3):577-586.

109. Goldsmith SR, Abid MB, Auletta JJ, et al. Post-Transplant Cyclophosphamide (PTCy) is Associated with Increased Cytomegalovirus Infection: A CIBMTR Analysis. *Blood*. 2021.

110. Walker CM, van Burik JA, De For TE, Weisdorf DJ. Cytomegalovirus infection after allogeneic transplantation: comparison of cord blood with peripheral blood and marrow graft sources. *Biol Blood Marrow Transplant*. 2007;13(9):1106-1115.

111. George B, Pati N, Gilroy N, et al. Pre-transplant cytomegalovirus (CMV) serostatus remains the most important determinant of CMV reactivation after allogeneic hematopoietic stem cell transplantation in the era of surveillance and preemptive therapy. *Transpl Infect Dis*. 2010;12(4):322-329.

112. Takenaka K, Nishida T, Asano-Mori Y, et al. Cytomegalovirus Reactivation after Allogeneic Hematopoietic Stem Cell Transplantation is Associated with a Reduced Risk of Relapse in Patients with Acute Myeloid Leukemia Who Survived to Day 100 after Transplantation: The Japan Society for Hematopoietic Cell Transplantation Transplantation-related Complication Working Group. *Biol Blood Marrow Transplant*. 2015;21(11):2008-2016.

113. Duke ER, Williamson BD, Borate B, et al. CMV viral load kinetics as surrogate endpoints after allogeneic transplantation. *The Journal of Clinical Investigation*. 2021;131(1).

114. Erard V, Guthrie KA, Seo S, et al. Reduced Mortality of Cytomegalovirus Pneumonia After Hematopoietic Cell Transplantation Due to Antiviral Therapy and Changes in Transplantation Practices. *Clinical Infectious Diseases*. 2015;61(1):31-39.

115. Marty FM, Ljungman P, Chemaly RF, et al. Letermovir Prophylaxis for Cytomegalovirus in Hematopoietic-Cell Transplantation. *N Engl J Med*. 2017;377(25):2433-2444.

116. Mori Y, Jinnouchi F, Takenaka K, et al. Efficacy of prophylactic letermovir for cytomegalovirus reactivation in hematopoietic cell transplantation: a multicenter real-world data. *Bone Marrow Transplantation*. 2020.

117. Ljungman P, Schmitt M, Marty FM, et al. A Mortality Analysis of Letermovir Prophylaxis for Cytomegalovirus (CMV) in CMV-seropositive Recipients of Allogeneic

Hematopoietic Cell Transplantation. *Clinical Infectious Diseases*. 2019;70(8):1525-1533.

118. Zamora D, Duke ER, Xie H, et al. Cytomegalovirus-specific T-cell reconstitution following letermovir prophylaxis after hematopoietic cell transplantation. *Blood*. 2021;138(1):34-43.

119. Schmidt-Hieber M, Labopin M, Beelen D, et al. CMV serostatus still has an important prognostic impact in de novo acute leukemia patients after allogeneic stem cell transplantation: a report from the Acute Leukemia Working Party of EBMT. *Blood*. 2013;122(19):3359-3364.

120. Solano C, Vázquez L, Giménez E, et al. Cytomegalovirus DNAemia and risk of mortality in allogeneic hematopoietic stem cell transplantation: Analysis from the Spanish Hematopoietic Transplantation and Cell Therapy Group. *Am J Transplant*. 2020.

121. Elmaagacli AH, Steckel NK, Koldehoff M, et al. Early human cytomegalovirus replication after transplantation is associated with a decreased relapse risk: evidence for a putative virus-versus-leukemia effect in acute myeloid leukemia patients. *Blood*. 2011;118(5):1402-1412.

122. Green ML, Leisenring WM, Xie H, et al. CMV reactivation after allogeneic HCT and relapse risk: evidence for early protection in acute myeloid leukemia. *Blood*. 2013;122(7):1316-1324.

123. Ito S, Pophali P, Co W, et al. CMV reactivation is associated with a lower incidence of relapse after allo-SCT for CML. *Bone Marrow Transplant*. 2013;48(10):1313-1316.

124. Löunnqvist B, Ringdegn O, Ljungman P, Wahren B, Gahrton G. Reduced risk of recurrent leukaemia in bone marrow transplant recipients after cytomegalovirus infection. *British Journal of Haematology*. 1986;63(4):671-679.

125. Peric Z, Wilson J, Durakovic N, et al. Early human cytomegalovirus reactivation is associated with lower incidence of relapse of myeloproliferative disorders after allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplantation*. 2018;53(11):1450-1456.

126. Remberger M, Ringden O. Survival after bone-marrow transplantation. *Lancet*. 2002;359(9309):888.

127. Busca A, Passera R, Pini M, et al. The use of ATG abrogates the antileukemic effect of cytomegalovirus reactivation in patients with acute myeloid leukemia receiving grafts from unrelated donors. *Am J Hematol*. 2015;90(6):E117-121.

128. Barnes DW, Loutit JF. Treatment of murine leukaemia with x-rays and homologous bone marrow. II. *Br J Haematol*. 1957;3(3):241-252.

129. Billingham RE. The biology of graft-versus-host reactions. *Harvey Lect*. 1966;62:21-78.

130. Ghimire S, Weber D, Mavin E, Wang XN, Dickinson AM, Holler E. Pathophysiology of GvHD and Other HSCT-Related Major Complications. *Frontiers in immunology*. 2017;8:79-79.

131. Choi SW, Levine JE, Ferrara JL. Pathogenesis and management of graft-versus-host disease. *Immunol Allergy Clin North Am*. 2010;30(1):75-101.

132. Hill GR, Ferrara JLM. The primacy of the gastrointestinal tract as a target organ of acute graft-versus-host disease: rationale for the use of cytokine shields in allogeneic bone marrow transplantation. *Blood*. 2000;95(9):2754-2759.

133. Sprent J, Schaefer M. Properties of purified T cell subsets. I. In vitro responses to class I vs. class II H-2 alloantigens. *Journal of Experimental Medicine*. 1985;162(6):2068-2088.

134. Trenado A, Sudres M, Tang Q, et al. Ex Vivo-Expanded CD4⁺CD25⁺ Immunoregulatory T Cells Prevent Graft-versus-Host-Disease by Inhibiting Activation/Differentiation of Pathogenic T Cells. *The Journal of Immunology*. 2006;176(2):1266-1273.

135. Welniak LA, Blazar BR, Murphy WJ. Immunobiology of Allogeneic Hematopoietic Stem Cell Transplantation. *Annual Review of Immunology*. 2007;25(1):139-170.

136. Wysocki CA, Panoskaltsis-Mortari A, Blazar BR, Serody JS. Leukocyte migration and graft-versus-host disease. *Blood*. 2005;105(11):4191-4199.

137. Przepiorka D, Weisdorf D, Martin P, et al. 1994 Consensus Conference on Acute GVHD Grading. *Bone Marrow Transplant*. 1995;15(6):825-828.

138. Harris AC, Young R, Devine S, et al. International, Multicenter Standardization of Acute Graft-versus-Host Disease Clinical Data Collection: A Report from the Mount Sinai Acute GVHD International Consortium. *Biol Blood Marrow Transplant*. 2016;22(1):4-10.

139. Weisdorf DJ, Snover DC, Haake R, et al. Acute upper gastrointestinal graft-versus-host disease: clinical significance and response to immunosuppressive therapy. *Blood*. 1990;76(3):624-629.

140. Schoemans HM, Lee SJ, Ferrara JL, et al. EBMT-NIH-CIBMTR Task Force position statement on standardized terminology & guidance for graft-versus-host disease assessment. *Bone Marrow Transplant*. 2018;53(11):1401-1415.

141. Chernick MR. The What, Why, and How of Biostatistics in Medical Research. The Essentials of Biostatistics for Physicians, Nurses, and Clinicians; 2011:1-14.

142. Altman D. Practical statistics for medical research; 1990.

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

144. Cox DR. Regression Models and Life-Tables. *Journal of the Royal Statistical Society Series B (Methodological)*. 1972;34(2):187-220.

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

146. Iacobelli S, de Wreede LC. Statistical Methods in HSCT and Cellular Therapies. In: Carreras E, Dufour C, Mohty M, Kröger N, eds. The EBMT Handbook: Hematopoietic Stem Cell Transplantation and Cellular Therapies. Cham: Springer International Publishing; 2019:41-46.

147. Chernick MR. Survival Analysis. The Essentials of Biostatistics for Physicians, Nurses, and Clinicians; 2011:158-174.

148. Stare J, Maucort-Boulch D. Odds Ratio, Hazard Ratio and Relative Risk. *Metodoloski zvezki*. 2016;13:59-67.

149. Scrucca L, Santucci A, Aversa F. Regression modeling of competing risk using R: an in depth guide for clinicians. *Bone Marrow Transplantation*. 2010;45(9):1388-1395.

150. Daly L.E. BGJ. Associations: Chance, Confounded or Causal? Interpretation and Uses of Medical Statistics; 2000:57-85.

151. Kononenko I. Machine learning for medical diagnosis: history, state of the art and perspective. *Artificial Intelligence in Medicine*. 2001;23(1):89-109.

152. Alashwal H, El Halaby M, Crouse JJ, Abdalla A, Moustafa AA. The Application of Unsupervised Clustering Methods to Alzheimer's Disease. *Frontiers in Computational Neuroscience*. 2019;13(31).

153. Rajkomar A, Dean J, Kohane I. Machine Learning in Medicine. *New England Journal of Medicine*. 2019;380(14):1347-1358.

154. Sidey-Gibbons JAM, Sidey-Gibbons CJ. Machine learning in medicine: a practical introduction. *BMC Medical Research Methodology*. 2019;19(1):64.

155. Panje CM, Glatzer M, von Rappard J, et al. Applied Swarm-based medicine: collecting decision trees for patterns of algorithms analysis. *BMC Medical Research Methodology*. 2017;17(1):123.

156. Shouval R, Labopin M, Bondi O, et al. Prediction of Allogeneic Hematopoietic Stem-Cell Transplantation Mortality 100 Days After Transplantation Using a Machine Learning Algorithm: A European Group for Blood and Marrow Transplantation Acute Leukemia Working Party Retrospective Data Mining Study. *Journal of Clinical Oncology*. 2015;33(28):3144-3151.

157. Arai Y, Kondo T, Fuse K, et al. Using a machine learning algorithm to predict acute graft-versus-host disease following allogeneic transplantation. *Blood Advances*. 2019;3(22):3626-3634.

158. He K, Wu Z, Fujiwara H, et al. Computational analysis of continuous body temperature provides early discrimination of graft-versus-host disease in mice. *Blood Advances*. 2019;3(23):3977-3981.

159. Amir E-aD, Davis KL, Tadmor MD, et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. *Nature Biotechnology*. 2013;31(6):545-552.

160. Leserer S, Bayraktar E, Trilling M, et al. Cytomegalovirus kinetics after hematopoietic cell transplantation reveal peak titers with differential impact on mortality, relapse and immune reconstitution. *American Journal of Hematology*. 2021;96(4):436-445.

161. Turki AT, Tsachakis-Mück N, Leserer S, et al. Impact of CMV reactivation on relapse of acute myeloid leukemia after HCT is dependent on disease stage and ATG. *Blood Advances*. 2021.

162. Turki AT, Klisanin V, Bayraktar E, et al. Optimizing anti-T-lymphocyte globulin dosing to improve long-term outcome after unrelated hematopoietic cell transplantation for hematologic malignancies. *Am J Transplant*. 2020;20(3):677-688.

163. Leserer S, Graf T, Franke M, et al. Time series clustering of T cell subsets dissects heterogeneity in immune reconstitution and clinical outcomes among MUD-HCT patients receiving ATG or PTCy. *Frontiers in Immunology*. 2023;14.

164. Biernacki MA, Sheth VS, Bleakley M. T cell optimization for graft-versus-leukemia responses. *JCI Insight*. 2020;5(9).

165. Yoon J-H, Lee S, Kim H-J, et al. Impact of cytomegalovirus reactivation on relapse and survival in patients with acute leukemia who received allogeneic hematopoietic stem cell transplantation in first remission. *Oncotarget*. 2016;7(13).

166. Cantoni N, Hirsch HH, Khanna N, et al. Evidence for a bidirectional relationship between cytomegalovirus replication and acute graft-versus-host disease. *Biol Blood Marrow Transplant*. 2010;16(9):1309-1314.

167. Bueno F, Solano C, Vázquez L, et al. Assessment of the association between cytomegalovirus DNAemia and subsequent acute graft-versus-host disease in allogeneic peripheral blood stem cell transplantation: A multicenter study from the Spanish hematopoietic transplantation and cell therapy group. *Transplant Infectious Disease*. 2021;n/a(n/a):e13627.

168. Miller HK, Braun TM, Stillwell T, et al. Infectious Risk after Allogeneic Hematopoietic Cell Transplantation Complicated by Acute Graft-versus-Host Disease. *Biol Blood Marrow Transplant*. 2017;23(3):522-528.

169. Broers AE, van Der Holt R, van Esser JW, et al. Increased transplant-related morbidity and mortality in CMV-seropositive patients despite highly effective prevention of CMV disease after allogeneic T-cell-depleted stem cell transplantation. *Blood*. 2000;95(7):2240-2245.

170. Elmaagacli AH, Koldehoff M. Cytomegalovirus replication reduces the relapse incidence in patients with acute myeloid leukemia. *Blood*. 2016;128(3):456-459.

171. Manjappa S, Bhamidipati PK, Stokerl-Goldstein KE, et al. Protective effect of cytomegalovirus reactivation on relapse after allogeneic hematopoietic cell transplantation in acute myeloid leukemia patients is influenced by conditioning regimen. *Biol Blood Marrow Transplant*. 2014;20(1):46-52.

172. Green ML, Leisenring W, Xie H, et al. CMV Viral Load and Mortality after Hematopoietic Cell Transplantation: A Cohort Study in the Era of Preemptive Therapy. *The Lancet Haematology*. 2016;3(3):e119-e127.

173. Lakshmikanth T, Olin A, Chen Y, et al. Mass Cytometry and Topological Data Analysis Reveal Immune Parameters Associated with Complications after Allogeneic Stem Cell Transplantation. *Cell Reports*. 2017;20(9):2238-2250.

174. Itzykson R, Robin M, Moins-Teisserenc H, et al. Cytomegalovirus shapes long-term immune reconstitution after allogeneic stem cell transplantation. *Haematologica*. 2015;100(1):114-123.

175. Link CS, Eugster A, Heidenreich F, et al. Abundant cytomegalovirus (CMV) reactive clonotypes in the CD8+ T cell receptor alpha repertoire following allogeneic transplantation. *Clinical & Experimental Immunology*. 2016;184(3):389-402.

176. Lodoen MB, Lanier LL. Natural killer cells as an initial defense against pathogens. *Current opinion in immunology*. 2006;18(4):391-398.

177. Varanasi PR, Ogonek J, Luther S, et al. Cytomegalovirus-specific CD8+ T-cells are associated with a reduced incidence of early relapse after allogeneic stem cell transplantation. *PLOS ONE*. 2019;14(3):e0213739.

178. Bao X, Zhu Q, Xue S, et al. Cytomegalovirus induces strong antileukemic effect in acute myeloid leukemia patients following sibling HSCT without ATG-containing regimen. *Am J Transl Res*. 2016;8(2):653-661.

179. Koldehoff M, Lindemann M, Opalka B, Bauer S, Ross RS, Elmaagacli AH. Cytomegalovirus induces apoptosis in acute leukemia cells as a virus-versus-leukemia function. *Leuk Lymphoma*. 2015;56(11):3189-3197.

180. Scheper W, van Dorp S, Kersting S, et al. γδT cells elicited by CMV reactivation after allo-SCT cross-recognize CMV and leukemia. *Leukemia*. 2013;27(6):1328-1338.

181. van den Heuvel H, Heidt S, Roelen DL, Claas FH. T-cell alloreactivity and transplantation outcome: a budding role for heterologous immunity? *Curr Opin Organ Transplant*. 2015;20(4):454-460.

182. Soiffer RJ, Kim HT, McGuirk J, et al. Prospective, Randomized, Double-Blind, Phase III Clinical Trial of Anti–T-Lymphocyte Globulin to Assess Impact on Chronic Graft-Versus-Host Disease–Free Survival in Patients Undergoing HLA-Matched Unrelated Myeloablative Hematopoietic Cell Transplantation. *Journal of Clinical Oncology*. 2017;35(36):4003-4011.

183. Finke J, Bethge WA, Schmoor C, et al. Standard graft-versus-host disease prophylaxis with or without anti-T-cell globulin in haematopoietic cell transplantation from matched unrelated donors: a randomised, open-label, multicentre phase 3 trial. *The Lancet Oncology*. 2009;10(9):855-864.

184. Handgretinger R, Schilbach K. The potential role of γδ T cells after allogeneic HCT for leukemia. *Blood*. 2018;131(10):1063-1072.

185. Admiraal R, Nierkens S, de Witte MA, et al. Association between anti-thymocyte globulin exposure and survival outcomes in adult unrelated haemopoietic cell transplantation: a retrospective, pharmacodynamic cohort analysis. *The Lancet Haematology*. 2017;4(4):e183-e191.

186. Kennedy VE, Chen H, Savani BN, et al. Optimizing Antithymocyte Globulin Dosing for Unrelated Donor Allogeneic Hematopoietic Cell Transplantation Based on Recipient Absolute Lymphocyte Count. *Biology of Blood and Marrow Transplantation*. 2018;24(1):150-155.

187. Gooptu M, Romee R, St Martin A, et al. HLA Haploidentical versus Matched Unrelated Donor Transplants with Post-Transplant Cyclophosphamide based prophylaxis. *Blood*. 2021.

188. Bailén R, Kwon M, Pascual-Cascón MJ, et al. Post-transplant cyclophosphamide for GVHD prophylaxis compared to ATG-based prophylaxis in unrelated donor transplantation. *Ann Hematol*. 2021;100(2):541-553.

189. Ganguly S, Ross DB, Panoskaltsis-Mortari A, et al. Donor CD4+ Foxp3+ regulatory T cells are necessary for posttransplantation cyclophosphamide-mediated protection against GVHD in mice. *Blood*. 2014;124(13):2131-2141.

190. Minculescu L, Marquart HV, Ryder LP, et al. Improved Overall Survival, Relapse-Free-Survival, and Less Graft-vs.-Host-Disease in Patients With High

Immune Reconstitution of TCR Gamma Delta Cells 2 Months After Allogeneic Stem Cell Transplantation. *Front Immunol*. 2019;10:1997.

191. Ye W, Kong X, Zhang W, Weng Z, Wu X. The Roles of γδ T Cells in Hematopoietic Stem Cell Transplantation. *Cell Transplantation*. 2020;29:0963689720966980.

192. Wu N, Liu R, Liang S, et al. γδ T Cells May Aggravate Acute Graft-Versus-Host Disease Through CXCR4 Signaling After Allogeneic Hematopoietic Transplantation. *Frontiers in Immunology*. 2021;12(2831).

193. Kordelas L, Buttkereit U, Lindemann M, et al. αβ-T-cell depleted donor lymphocyte infusion for leukemia relapse after allogeneic stem cell transplantation. *Bone Marrow Transplantation*. 2017;52(12):1668-1670.

194. de Witte MA, Janssen A, Nijssen K, et al. αβ T-cell graft depletion for allogeneic HSCT in adults with hematological malignancies. *Blood Advances*. 2021;5(1):240-249.

195. Kohrt HE, Pillai AB, Lowsky R, Strober S. NKT cells, Treg, and their interactions in bone marrow transplantation. *European Journal of Immunology*. 2010;40(7):1862-1869.

196. Kronenberg M, Engel I. On the road: progress in finding the unique pathway of invariant NKT cell differentiation. *Current Opinion in Immunology*. 2007;19(2):186-193.

197. Mavers M, Maas-Bauer K, Negrin RS. Invariant Natural Killer T Cells As Suppressors of Graft-versus-Host Disease in Allogeneic Hematopoietic Stem Cell Transplantation. *Frontiers in immunology*. 2017;8:900-900.

198. Mohty M. Mechanisms of action of antithymocyte globulin: T-cell depletion and beyond. *Leukemia*. 2007;21(7):1387-1394.

199. Wachsmuth LP, Patterson MT, Eckhaus MA, Venzon DJ, Gress RE, Kanakry CG. Post-transplantation cyclophosphamide prevents graft-versus-host disease by inducing alloreactive T cell dysfunction and suppression. *J Clin Invest*. 2019;129(6):2357-2373.

200. Radojcic V, Luznik L. Mechanism of action of posttransplantation cyclophosphamide: more than meets the eye. *The Journal of Clinical Investigation*. 2019;129(6):2189-2191.

201. Al-Homsi AS, Roy TS, Cole K, Feng Y, Duffner U. Post-Transplant High-Dose Cyclophosphamide for the Prevention of Graft-versus-Host Disease. *Biology of Blood and Marrow Transplantation*. 2015;21(4):604-611.

202. Kanakry CG, Ganguly S, Zahurak M, et al. Aldehyde Dehydrogenase Expression Drives Human Regulatory T Cell Resistance to Posttransplantation Cyclophosphamide. *Science Translational Medicine*. 2013;5(211):211ra157-211ra157.

203. Feng X, Kajigaya S, Solomou EE, et al. Rabbit ATG but not horse ATG promotes expansion of functional CD4+CD25highFOXP3+ regulatory T cells in vitro. *Blood*. 2008;111(7):3675-3683.

204. Lan F, Zeng D, Higuchi M, Higgins JP, Strober S. Host conditioning with total lymphoid irradiation and antithymocyte globulin prevents graft-versus-host disease: the role of CD1-reactive natural killer T cells. *Biology of Blood and Marrow Transplantation*. 2003;9(6):355-363.

205. Brissot E, Labopin M, Moiseev I, et al. Post-transplant cyclophosphamide versus antithymocyte globulin in patients with acute myeloid leukemia in first complete remission undergoing allogeneic stem cell transplantation from 10/10 HLA-matched unrelated donors. *Journal of Hematology & Oncology*. 2020;13(1):87.

206. Nagler A, Kanate AS, Labopin M, et al. Post-transplant cyclophosphamide versus anti-thymocyte globulin for graft-versus-host disease prevention in haploidentical transplantation for adult acute lymphoblastic leukemia. *Haematologica*. 2021;106(6):1591-1598.

207. Battipaglia G, Labopin M, Hamladji R-M, et al. Post-transplantation cyclophosphamide versus antithymocyte globulin in patients with acute myeloid leukemia undergoing allogeneic stem cell transplantation from HLA-identical sibling donors: A retrospective analysis from the Acute Leukemia Working Party of the European Society for Blood and Marrow Transplantation. *Cancer*. 2021;127(2):209-218.

208. Battipaglia G, Labopin M, Kröger N, et al. Posttransplant cyclophosphamide vs antithymocyte globulin in HLA-mismatched unrelated donor transplantation. *Blood*. 2019;134(11):892-899.

209. Chang Y-J, Zhao X-Y, Huang X-J. Strategies for Enhancing and Preserving Anti-leukemia Effects Without Aggravating Graft-Versus-Host Disease. *Frontiers in Immunology*. 2018;9(3041).

210. Lulla PD, Naik S, Vasileiou S, et al. Clinical effects of administering leukemia-specific donor T cells to patients with AML/MDS after allogeneic transplant. *Blood*. 2021;137(19):2585-2597.

211. Schmid C. A step closer to GVL without GVHD. *Blood*. 2021;137(19):2565-2566.

212. Xia G, Truitt RL, Johnson BD. Graft-versus-Leukemia and Graft-versus-Host Reactions after Donor Lymphocyte Infusion Are Initiated by Host-Type Antigen-Presenting Cells and Regulated by Regulatory T Cells in Early and Long-Term Chimeras. *Biology of Blood and Marrow Transplantation*. 2006;12(4):397-407.

213. Ni X, Song Q, Cassady K, et al. PD-L1 interacts with CD80 to regulate graft-versus-leukemia activity of donor CD8+ T cells. *The Journal of Clinical Investigation*. 2017;127(5):1960-1977.

214. Woodsworth DJ, Castellarin M, Holt RA. Sequence analysis of T-cell repertoires in health and disease. *Genome Medicine*. 2013;5(10):98.

215. Spitzer Matthew H, Nolan Garry P. Mass Cytometry: Single Cells, Many Features. *Cell*. 2016;165(4):780-791.

9 Appendix

9.1 Abbreviations

A

ABO	human blood type and blood group system
aGVHD	acute graft-versus-host disease
ALC	absolute lymphocyte count
AML	acute myeloid leukemia
APC	antigen presenting cells
ATG	anti-T-lymphocyte globulin

B

BM	bone marrow

C

CB	cord blood
CD	cluster of differentiation
cGVHD	chronic graft-versus-host disease
CI	confidence interval
CIBMTR	Center for International Blood and Marrow Transplant Research
CMV	cytomegalovirus
CR	complete remission
CTL	cytotoxic T lymphocytes

D

D	donor
DLI	donor lymphocyte infusion
DNA	Deoxyribonucleic acid

E

EFS	event-free survival

G

gB(H)	glycoprotein B(H)
G-CSF	granulocyte-colony stimulating factor
GI	gastrointestinal

GVHD	graft-versus-host disease
GVL	graft-versus leukemia

H

hCMV	human Cytomegalovirus
HIV	human immunodeficiency viruses
HLA	human leukocyte antigen
HPE	homeostatic peripheral expansion
HR	hazard ratio
HSC	hematopoietic stem cell
HSCT	hematopoietic stem cell transplantation

I

i.e.	id est
IE-1(2)	immediate-early protein 1(2)
IL	interleukin
INF	interferon

K

kg	kilogram
KIR	killer cell immunoglobulin-like receptors

L

LPS	lipopolysaccharide
L	ligand

M

mHAgs	minor histocompatibility antigens
mg	milligram
MHC	major histocompatibility complex
MIP-1α	macrophage inflammatory protein-1alpha
ML	machine learning
mm	millimeter
MMUD	mismatched-unrelated donor
MRD	matched-related donor

MUD	matched-unrelated donor
N	
NK	natural killer
NKT	natural killer T
NRM	non-relapse mortality
O	
OS	overall survival
P	
PB	peripheral blood
PBSC	peripheral blood stem cell
pp50(65)	phosphoprotein 50(65)
PTCy	post-transplant cyclophosphamide
R	
R	recipient
RIC	reduced-intensity conditioning
T	
TCR	T cell receptor
Th	T helper
TNF	tumor necrosis factor
U	
UD	unrelated donor
V	
vs.	versus

9.2 List of Figures

Figure 1: Developments in HCT from 1990-2018. 11

Figure 2: Frequent impairments of clinical outcome after HCT. 14

Figure 3: Reconstitution of innate and adaptive immunity after HCT................ 16

Figure 4: Phases of CMV infection and worldwide distribution. 18

Figure 5: Pathophysiology of acute GVHD (aGVHD)... 21

Figure 6: Summary of thesis results... 164

9.3 Danksagung

Zu Beginn möchte ich mich bei meinem Doktorvater Prof. Dr. med. Dietrich W. Beelen bedanken für Ihre stetige Unterstützung und Lehre sowie der Bereitschaft zur Übernahme der Betreuung trotz Ihres damals nahenden und nun mehr aktiven Ruhestandes.

Mein weiterer Dank gilt Dr. med. Dr. phil. Amin T. Turki für die Übernahme der direkten wissenschaftlichen Betreuung, die Erteilung vielzähliger Ratschläge und die Möglichkeit zur frequenten Weiterbildung durch den Besuch von Konferenzen, Workshops und Vorträgen. Vielen Dank für dein Vertrauen und insbesondere für deine Zeit, die du dir trotz vielseitiger Aufgaben genommen hast, um mich zu fördern und zu unterstützen.

Des Weiteren möchte ich dem zweiten Gutachter dieser Arbeit Prof. Dr. rer. nat. Ulf Dittmer meinen Dank aussprechen zur Bereitschaft der Übernahme dieser zeitaufwendigen Aufgabe.

Weiterhin gilt mein Dank den Mitarbeiterinnen des KMT Labors unter der Leitung von Frau Dr. Buttkereit sowie der Arbeitsgruppe von Frau Prof. Fleischhauer für die herzliche Aufnahme, Einarbeitung und den stetigen (wissenschaftlichen) Austausch welche unerlässlich für meine Entwicklung waren.

Letztlich möchte ich allen aktiven sowie ehemaligen Kollegen/innen meiner Arbeitsgruppe danken ohne die der interdisziplinäre Austausch aus den Bereichen Medizin, Biologie, Mathematik und Informatik nicht möglich gewesen wäre. Danke an Dr. Evren Bayraktar für das „an die Hand nehmen“ zu Beginn meiner Promotion. Vielen Dank an Aleksandra Pillibeit für deine tatkräftige Unterstützung in der Dokumentation sowie deine stets hilfsbereite Art, dein offenes Ohr und deine Zusprache. Ein großes Dankeschön an Theresa Graf für die harmonische Zusammenarbeit, dein Verständnis in jeglichen Situationen sowie deine experimentelle Unterstützung.

Ein ausdrücklicher Dank gilt meinen Eltern, Sandra und Stefan Leserer, meinem Bruder Niklas, und meinen Freunden ohne deren Unterstützung, Rückhalt, motivierenden Worte und Zusprache Ich heute nicht da wäre, wo ich bin. Insbesondere möchte hier meinem Partner Patrick Ständler vom Ganzen Herzen danken. Danke, dass du jeden Tag für mich da bist, danke für deine Geduld, dein Verständnis und deine Unterstützung. Du bist mein Ruhepol, mein Gegenstück, mein Lieblingsmensch!

9.4 Conference Contributions

9.4.1 Talks

March 2021, 47th Annual Meeting of the European Society for Blood and Marrow Transplantation (EBMT) 2021, Virtual

- **Presentation title:** “Comparative Analysis of Immune Reconstitution in HSCT patients with PT-CY and ATG reveals distinct T cell immune reconstitution patterns”
- **Authors: Saskia Leserer**, Theresa Graf, Rashit Bogdanov, Aleksandra Pillibeit, Nils Leimkühler, Martina Franke, Ulrike Buttkereit, Katharina Fleischhauer, H. Christian Reinhardt, Dietrich W. Beelen and Amin T. Turki

June 2020, Graduate School of Biomedical Science (BIOME); Core: Transplantation Medicine, Virtual

- **Presentation title:** “Early Cytomegalovirus viremia after allogeneic hematopoietic cell transplantation”
- **Authors: Saskia Leserer**

January 2020, Graduate School of Biomedical Science (BIOME); Core: Cellular and Molecular Immunology, Essen, Germany

- **Presentation title:** “Early Cytomegalovirus viremia kinetics after allogeneic hematopoietic cell transplantation correlate with survival and relapse”
- **Authors: Saskia Leserer**

May 2019, Annual Conference of the “Deutsche Arbeitsgemeintschaft für Knochenmark- und Blutstammzelltransplantation e.V. (DAG-KBT)“ 2019

- **Presentation title:** “Cytomegalovirus reactivation kinetics as predictors of survival and relapse after allogeneic cell transplantation for hematologic malignancies”
- **Authors: Saskia Leserer**, Evren Bayraktar, Nikolaos Tsachakis-Mück, Mirko Trilling, Katharina Fleischhauer, Dietrich W. Beelen and Amin T. Turki

December 2018, Graduate School of Biomedical Science (BIOME); Core: Cellular and Molecular Immunology, Essen, Germany

- **Presentation title:** Kinetics of human Cytomegalovirus reactivation after hematopoietic stem cell transplantation
- **Authors: Saskia Leserer**

9.4.2 Posters

October 2020, Annual Meeting of the Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie (DGHO), Virtual

- **Poster title:** “Combining recipient serostatus and early CD4-T cell immune reconstitution to improve CMV serostatus-based risk assessment after allogeneic hematopoietic cell transplantation”
- **Authors: Saskia Leserer**, Esteban Arrieta-Bolaños, Katharina Fleischhauer, Theresa Graf, Dietrich W. Beelen and Amin T. Turki

June 2020, 25th Annual Congress of the European Hematology Association (EHA), Virtual

- **Poster title:** “Improved CMV risk assessment after HCT by combining recipient serostatus and early CD4-T cell reconstitution”
- **Authors: Saskia Leserer**, Esteban Arrieta-Bolaños, Katharina Fleischhauer, Dietrich W. Beelen and Amin T. Turki

November 2019, Retreat of the Graduate School of Biomedical Science (BIOME), Bonn, Germany

- **Poster title:** Cytomegalovirus reactivation kinetics correlate with survival and relapse after allogeneic hematopoietic cell transplantation for hematologic malignancies
- **Authors: Saskia Leserer**, Evren Bayraktar, Nikolaos Tsachakis-Mück, Michael Koldehoff, Mirko Trilling, Katharina Fleischhauer, Dietrich W. Beelen and Amin T. Turki

October 2019, Annual Meeting of the Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie (DGHO), Berlin, Germany

- **Poster Title:** Cytomegalovirus reactivation kinetics as predictors of survival and relapse after allogeneic cell transplantation for hematologic malignancies
- **Authors: Saskia Leserer**, Evren Bayraktar, Nikolaos Tsachakis-Mück, Michael Koldehoff, Mirko Trilling, Katharina Fleischhauer, Dietrich W. Beelen and Amin T. Turki

March 2019, 45th Annual Meeting of the European Society for Blood and Marrow Transplantation (EBMT) 2019, Frankfurt, Germany

- **Poster title:** Cytomegalovirus reactivation kinetics and peak titers as novel predictors of survival and relapse after allogeneic cell transplantation for hematologic malignancies
- **Authors: Saskia Leserer**, Evren Bayraktar, Nikolaos Tsachakis-Mück, Michael Koldehoff, Lara Kasperidus, Esteban Arrieta-Bolanos, Mirko Trilling, Katharina Fleischhauer, Dietrich W. Beelen and **Amin T. Turki**

- Presented by: Amin T. Turki

December 2018, 17th Research Day of the University Hospital Essen, Essen, Germany

- **Poster title:** Do the dynamics of human Cytomegalovirus reactivation influence the relapse incidence of patients after hematopoietic stem cell transplantation?
- **Authors: Saskia Leserer**, Evren Bayraktar, Amin T. Turki, Dietrich W. Beelen

November 2018, Retreat of the Graduate School of Biomedical Science (BIOME), Cologne, Germany

- **Poster title:** Do the dynamics of human Cytomegalovirus reactivation influence the relapse incidence of patients after hematopoietic stem cell transplantation?
- **Authors: Saskia Leserer**, Evren Bayraktar, Amin T. Turki, Birgit Goitowski, Dietrich W. Beelen

9.5 Curriculum vitae

9.6 Stellungnahmen

Bestätigung des Eigenanteils an Publikationen

Hiermit bestätige ich, **Prof. Dr. med. Dietrich W. Beelen**, die Darstellung zu den Anteilen von Frau Saskia Leserer an Konzeption, Durchführung und Abfassung jeder Publikation (Chapter 6 – Articles) gemäß der Promotionsordnung der Fakultät für Biologie zur Erlangung des Doktorgrades Dr. rer. nat.

Essen, den _______________ ______________________

Prof. Dr. Dietrich W. Beelen

Erklärung der Urheberrechte der Publikationen

Hiermit erkläre ich, **Saskia Leserer**, dass ich mit der Veröffentlichung der Publikationen (Chapter 6 – Articles) im Rahmen dieser Dissertation keine Urheberrechte verletze.

Essen, den _______________ ______________________

Saskia Leserer

9.7 Eidesstattliche Erklärung

Hiermit erkläre ich, gem. § 7 Abs. (2) d) + f) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient, bei der Abfassung der Dissertation nur die angegebenen Hilfsmittel benutzt und alle wörtlich oder inhaltlich übernommenen Stellen als solche gekennzeichnet habe.

Essen, den _______________ ______________________

Saskia Leserer

Hiermit erkläre ich, gem. § 7 Abs. (2) e) + g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

Essen, den _______________ ______________________

Saskia Leserer

Hiermit erkläre ich, gem. § 6 Abs. (2) g) der Promotionsordnung der Fakultät für Biologie zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema **„Investigation of the impact of CMV reactivation and GVHD prophylaxis on alloreactivity after hematopoietic cell transplantation by data-driven analysis“** zuzuordnen ist, in Forschung und Lehre vertrete und dem Antrag von **Saskia Leserer** befürworte und die Betreuung auch im Falle eines Weggangs, wenn nicht wichtige Gründe dem Entgegenstehen, weiterführen werde.

Essen, den _______________ ______________________

Prof. Dr. med. Dietrich W. Beelen