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Depletion of CD45RA+ naïve T Cells for Haploidentical Stem Cell Transplantations: Graft Generation Under Good Manufacturing Practice and Establishment of a Flow Cytometric Quality Control for Clinical-Scale Grafts

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To my parents

Table of Contents

1. Abstract

In haploidentical stem cell transplantation (SCT), achieving a balance between graft versus host disease (GvHD), graft versus leukemia effect (GvL) and bridging the vulnerable phase of aplasia against viral infections is still a challenge. Graft preparation strategies attempt to achieve this balance by removing and retaining harmful and helpful cells. At this point it is known that T cell subpopulations hold different properties concerning GvHD promotion and immunocompetence towards pathogens. CD45RA+ naïve T cells show the greatest, while CD45RO+ memory T cells show less alloreactive potential but provide immunocompetence. CD45RA depletion is a promising new approach to graft processing that potentially combines GvHD prevention, GvL promotion and transfer of immunological competence by removing potentially harmful CD45RA+ naïve T cells and retaining CD45RO+ memory cells. This work focused on manufacturing CD45RA-depleted grafts within a one- or two-step approach, as well as a feasibility assessment of the process and the establishment of a 10-color fluorescence activated cell sorting (FACS) measurement panel for clinical-scale graft generation. CD45RA depletions were conducted from granulocyte-colony stimulated factor (G-CSF) mobilized peripheral blood stem cells (PBSC) applying two different strategies, direct depletion of CD45RA+ cells (one-step approach), or depletion following preceding CD34 selection. A 10-color FACS measurement panel was established ensuring quality control and enabling preliminary data acquisition on CD45RA co-expression for cell loss estimations. Residual virus-specific T cells after depletion were measured using MHC multimers. It was observed that the depletion antibody occupied the cell binding sites, resulting in insufficient binding of the fluorescent dye for subsequent FACS measurement. Therefore, three FACS antibodies were tested and compared, and CD45RA-PE (clone:2H4) was found to be the best choice for reliable cell detection. To further characterize residual T cells, two homing markers, CD62L and CCR7, were compared, with particular attention paid to the expression of the surface markers

after cooling. Both markers were complementary to each other, resulting in the decision to include an additional FACS measuring tube whenever samples are cooled or further T cell characterization is needed. With a median log depletion of -3.9 (one-step) and -3.8 (two-step) data showed equally efficient removal of CD45RA+CD3+ T cells for both approaches. Close to complete B cell removal was obtained without additional reagent use. However, also close to complete NK cell loss occurred due to high CD45RA co-expression. Stem cells recovered at a median of 52% (range: 49.7 - 67.2%) after one-step CD45RA depletion. CD45RO+ memory T cells recovery was statistically not differing between both approaches. Virus-specific T cells were detectable after depletion, suggesting that virus-specific immunocompetence is transferable. In conclusion, CD45RA depletions are equally feasible for both approaches when performed from fresh, non-cryopreserved starting products, show reliable reduction of CD45RA and B cells, but also result in co-depletion of NK cells. Stem cell recovery and NK cell losses must be considered carefully especially regarding overcoming HLA barriers, pathogen protection during aplasia, early engraftment an GvL. Therefore, a combination of CD45RA-depleted products with already established other processing methods to ensure sufficient stem and NK cells is desirable to allow high clinical flexibility.

2. Zusammenfassung

Innerhalb der haploidenten Stammzelltransplantationen (SZT) stellt das Erreichen eines Gleichgewichts zwischen Graft versus Host Reaktion (Graft versus Host Disease, GvHD), Graft versus Leukämie (GvL) Effekt, sowie die Überbrückung der vulnerablen Phase während der Aplasie gegenüber Virusinfektionen eine Herausforderung dar. Die Vorbereitung von Stammzellpräparaten zielt darauf ab, durch Entfernen und Erhalten unerwünschter und erwünschter Zellen, dieses Gleichgewicht zu erreichen. Derzeit ist bekannt, dass T-Zell-Subpopulationen unterschiedliches GvHD-Potenzial und Immunkompetenz besitzen. Vor allem naive CD45RA+ T-Zellen besitzen ein hohes alloreaktives Potenzial, während CD45RO+ T-Zellen vor allem Immunkompetenz vermitteln. Die CD45RA-Depletion ist ein vielversprechender neuer Ansatz für die Transplantatverarbeitung, der durch die Entfernung von CD45RA+ Zellen und die Erhaltung von CD45RO+ Gedächtniszellen, eine effektive Kombination der gewünschten Effekte bietet.

Diese Arbeit konzentrierte sich auf die Herstellung von CD45RA-depletierten Transplantaten in einem ein- oder zweistufigen Verfahren, auf die Bewertung der Durchführbarkeit des Prozesses und die Etablierung einer 10-Farben-FACS-Antikörperauswahl zur Herstellung von Transplantaten im klinischen Maßstab. CD45RA-Depletionen wurden aus G-CSF-mobilisierten peripheren Blutstammzellen (PBSC) durchgeführt, wobei zwei verschiedene Strategien angewandt wurden: direkte Depletion von CD45RA+-Zellen (einstufiger Ansatz) oder Depletion nach vorheriger CD34-Selektion (zwei-stufiger Ansatz). Die Etablierung einer 10-Farb FACS Antikörperauswahl erfolgte zur Gewährleistung einer Qualitätskontrolle und vorläufigen Datenerfassung des zu erwartenden Zellverlust im Rahmen der Depletion aufgrund der CD45RA Ko-Expression. Verbleibende virusspezifische T-Zellen wurden nach der Depletion mit MHC-Multimeren gemessen. Diese Arbeit konnte zeigen, dass durch die Bindung des Depletionsantikörpers auf den Zelloberflächen, die fluoreszierenden Antikörper

der FACS Messung nicht alle gleichermaßen binden konnten. Daher wurden drei FACS-Antikörper getestet und verglichen, wobei CD45RA-PE (Klon:2H4) den zuverlässigen Zellnachweis bot. Zur weiteren T-Zell-Charakterisierung wurden zwei Homing-Marker, CD62L und CCR7, unter Berücksichtigung der Oberflächenmarkerveränderungen bei Kühlung verglichen. Mit dem Ergebnis, dass sich die beiden Marker komplementär zueinander zeigten und somit in Kühlungssituationen oder bei Notwendigkeit einer weiteren T-Zell-Charakterisierung durch ein zusätzliches FACS-Messröhrchen beide integriert wurden. Mit einer medianen Log Depletion von -3,9 (einstufig) und -3,8 (zweistufig) zeigten beide Ansätze eine gleichwertig effiziente Entfernung von CD45RA+CD3+ T-Zellen. B-Zellen wurden nahezu vollständig ohne zusätzliche Reagenzien entfernt. Wegen der hohen CD45RA-Koexpression kam es jedoch auch zu einem nahezu vollständigen NK-Zellverlust. Stammzellen konnten im Median zu 52 % (49,7 - 67,2 %, einstufig) wiedergefunden werden. CD45RO+ Gedächtnis-T-Zellen konnten gleichermaßen effizient in beiden Ansätzen wiedergefunden werden. Virusspezifische T-Zellen waren nach der Depletion nachweisbar, was darauf hindeutet, dass virusspezifische Immunkompetenz übertragbar ist. Zusammenfassend bedeutet dies, dass CD45RA-Depletionen in beiden Ansätzen gleichermaßen durchführbar sind, wenn sie aus frischen, nicht-kryokonservierten Ausgangsprodukten erfolgen. Sie zeigen eine zuverlässige Reduktion von CD45RA+- und B-Zellen, führen aber auch zu einer Ko-Depletion von NK-Zellen. Stamm- und NK-Zellverlust müssen, vor allem in Hinblick auf das Überwinden von HLA-Barrieren, dem Pathogenschutz, frühes Engraftment, sowie Erhalt eines GvL-Effekts besondere Berücksichtigung finden. Daher ist eine Kombination von CD45RA-depletierten Produkten mit bereits etablierten anderen Verarbeitungsmethoden zur Sicherstellung ausreichender Stamm- und NK-Zellen Mengen wünschenswert, um eine hohe klinische Flexibilität zu ermöglichen.

3. Introduction

3.1 Childhood Leukemia

With 30% of all childhood malignancies in pediatric oncology, leukemia is the most frequent malignancy, followed by malignancies of the central nervous system (CNS, 24%) and lymphomas (15%).[1]

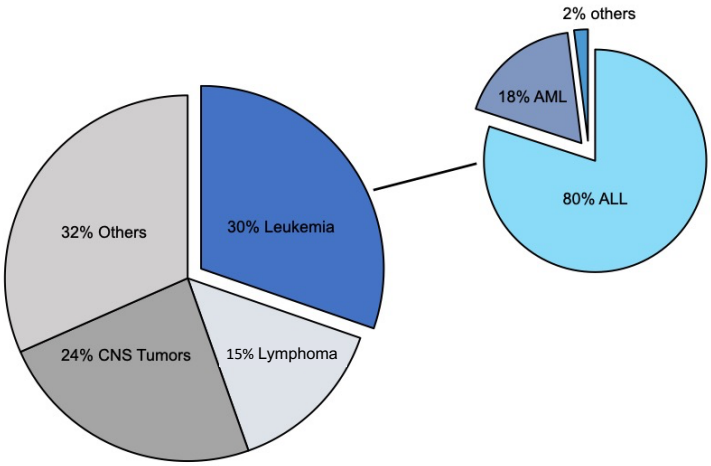

Figure 1 Childhood Malignancies. *Within all childhood malignancies, childhood leukemia has the largest share: 80% of the affected children suffer from an acute lymphoid leukemia and 18% from an acute myeloid leukemia. 24% of childhood malignancies are tumors of the central nervous system (CNS), 15% are lymphoma and 32% are other malignancies with origin in liver, spleen, bone etc. Figure edited based on data obtained from Deutsches Kinderkrebsregister*[1]

Leukemia is a malignant disease of the hematopoietic system. The fundamental mechanism of leukemia is based on malignant degeneration of leucocytes and their autonomous proliferation within the bone marrow. Due to the proliferation, physiological hematopoiesis is replaced by malignant cells, called (leukemic) blasts, which impairs the development of hematopoietic cells. Leukemia usually presents with anemia, thrombocytopenia, granulocytopenia and impairment of the T and B cell system, resulting in to fatigue, pallor, increased bleeding tendency and increased vulnerability to infections. Leukemic may also infiltrate extramedullary organs (e.g., spleen, liver) resulting in organ specific symptoms.[2,3]

Depending on the clinical course and affected cell lines involved, leukemias can be classified in chronic or acute and myeloid or lymphatic. 80% of childhood leukemias are acute lymphatic leukemias (ALL), followed by 18% acute myeloid leukemias (AML).[2,3] Both, ALL and AML develop from progenitor cells of the respective cell lines. Depending on which progenitor cell is affected, ALL and AML can be further subdivided based on cytomorphological and cytogenetic alterations. In order to find the right therapy regimen and to estimate the eventual outcome of the disease, cytogenetic alterations of the respective leukemia type are most accurate measures.[3] Therapy is risk-adjusted according to their subtypes and further risk factors, imbedded in therapy optimization studies and can be divided in two major principals - remission therapy and supportive care. Supportive care includes all therapeutic measures to prevent complications and side effects during the course of therapy such as infections, anemia, tumor lysis syndrome. Remission therapy aims to achieve complete remission (CR). Complete remission is defined as the achievement of less than 5% leukemic blasts in the bone marrow, normalization of the blood count and disappearance of disease specific symptoms. To achieve CR high intensity (radio-) chemotherapy is considered standard procedure. Stem cell transplantation is a therapy option for patients with high-risk leukemia types, for therapy non-responders or for disease relapse, and other malignancies or non-malignant disease compromising the hematopoietic system, when patients are in remission.[2–4] Over the years, SCT has improved significantly as a treatment option, particularly with the improved use of medications and measures to prevent complications and risks. Nevertheless, SCT is fraught with risks, potentially life-threatening and associated with long-term consequences. Therefore, the indication for SCT must be thoroughly weighed and the risk of the underlying disease outweighs the risk of performing SCT.

Indications for SCT range from malignancies such as acute lymphatic leukemia (ALL), acute myeloid leukemia (AML), myelodysplastic Syndrome (MDS), chronic

myeloid leukemia (CML) to malignant lymphoma, solid tumors such as neuroblastoma, Wilms tumor, Ewing sarcoma and brain tumors, to non-malignant diseases such as severe aplastic anemias, autoimmune diseases, immunodeficiencies, Fanconi-anemias.[5]

3.2 Stem Cell Transplantation

SCT is a transmission of blood stem cells (SC) from a healthy donor to a recipient. The superordinate goal of the SCT is proliferation of the transmitted cells as well as regeneration of the hematopoiesis within the recipient. In other words, the goal is the replacement of non-functional, malignant or missing cells by healthy immunocompetent cells.[5,6] The SCT process is assembled by several steps: myeloablative conditioning, donor selection, selection of an adequate SC source and SC processing, if necessary. After the indications for SCT are met, donor selection as well as SC source, depending on the underlying disease and donor availability are conducted. Meanwhile patients undergo a conditioning regime. Once remission is attained, SC processing occurs and subsequent SCT takes place.[7]

Myeloablative conditioning using high intensity (radio-) chemotherapy is conducted prior to SCT. Different cytostatic drugs such as cyclophosphamide, etoposide, fludarabine, melphalan, busulfan, thiothepa or treosulfan are used. Additional anti-lymphocyte substances like anti-thymocyte-globulin (ATG) or Alemtuzumab (Campath) are used preventively in order to avoid graft rejection through remaining recipient lymphocytes.[6,8]

Myeloablative conditioning aims to eliminate all malignant cells circulating within the patient and creates bone marrow space for the transmitted healthy donor SC in order to enable engraftment. Engraftment describes the accrual of transmitted cells within the empty bone marrow spaces and the initiation of a new hematopoietic system. This phenomenon usually occurs between 10 to 28 days post transplantation. However, myeloablative conditioning cannot differentiate

between malignant and heathy cells, therefore healthy cells are also eliminated leading to aplasia after SCT and can present a severe threat of life due to lack of infectious protection for the patient.[8]

SCT can be distinguished in autologous and allogenic SCT. In autologous SCT the patient is both - donor and recipient. This means healthy stem cells from the patient are collected before undergoing radio-chemotherapy, are then preserved and reinfused to the patient after chemotherapy allowing an intensified radio-chemotherapy treatment and is e.g., a common approach for neuroblastoma therapy. In contrast, allogenic SCT (allo-SCT) pursues the goal of replacing the patients SC, and therefore the whole hematopoietic system by a healthy donors' stem cells and its related hematopoietic system.[6]

Stem cells can be obtained from bone marrow extraction, collection of peripheral blood stem cells (PBSC) and from umbilical cord. While the collection of umbilical cord stem cells plays a subordinated role for malignancy therapy in Germany, stem cell extraction from bone marrow or collection from peripheral blood through apheresis is more common. The extraction of bone marrow stem cells from the donors' iliac crest is an inpatient procedure and conducted under general anesthesia and sterile conditions. Within this process up to 1.5 liters of bone marrow are taken from a healthy donor and then transfused into the patient. SC collections using peripheral blood stem cells apheresis is less invasive need doesn't general anesthesia and can be conducted as an outpatient procedure. In this case the donor receives subcutaneous injected growth factor (G-CSF, granulocyte stimulating factor) for four to five days ahead of the collection, which stimulates and mobilizes hematopoietic stem cells from the bone marrow to the peripheral blood. Stem cells then circulate within the blood stream and can be collected through apheresis. Blood is drawn similarly to a blood donation, then supplied to a cell separator. Cells are then separated according to their weight using density centrifugation, allowing a precise collection of mononuclear cells (lymphocytes and monocytes). After separation blood components (e.g., red

blood) and plasma cells are returned to the donor, while stem cells and immunological cells (T, B, and natural killer cells) remain within a collection bag. Cells within this collection bag are referred to as peripheral blood stem cells (PBSC) and may need to undergo immunological processing in case of mismatched SCT.[5]

3.2.1 Allogenic Stem Cell Transplantation

Finding a well-matched donor is an important prerequisite for any SCT. Therefore, matching by major histocompatibility complex (MHC) is the fundament for any donor selection. The MHC is a genetic region located on the short arm of chromosome 6 that encodes numerous cell surface tissue markers and are inherited as a genetic unit. This genetic unit is also referred to as the haplo-type. In humans, the MHC is referred to as humane lymphocyte antigen (HLA). Within the HLA system, there are two major antigen groups - class I HLA antigens (HLA-A, HLA-B and HLA-C) and class II HLA antigens (HLA-DR, HLA-DP and HLA-DQ). Both antigen classes are characterized by strong polymorphism.[5,9] Precise HLA characterization of the donor and recipient is performed using DNA sequencing, which allows accurate representation of class I and II HLA antigens and their alleles. HLA A, B, C, DR and DQ are important for donor selection. Depending on the HLA match, three levels of donors can be distinguished. Matched sibling donors (MSD) with an HLA match of 10 out of 10, matched unrelated donors (MUD) with an HLA match of 9 to 10 out of 10, and mismatched unrelated (MMUD) or family donors (MMFD) with an HLA match of 8 or less (see table 1).[10]

HLA Matching Types	Extend of HLA Matching
Matched sibling donor (MSD)	10/10 identical HLA alleles
Matched unrelated donor (MUD)	9-10/10
Mismatched unrelated / family donor (MMUD/ MMFD)	≤ 8/10

***Table 1 HLA Matching Classification**. Table edited based on Peters et al* [9]

While HLA-identical siblings (MSD) are considered gold standard, research has shown that the precise allele matching influences the outcome of a SCT. Data has shown that the level of matching is closely connected to occurrence of complications (e.g. graft rejection) and is also crucial for therapy decisions such as choice of conditioning or Graft versus host disease (GvHD) prophylaxis regims.[9,10] For patients lacking a MSD or MUD, but needing a SCT in a timely manner, haploidentical SCT (haplo-SCT) is a considerable therapy option.[11,12]

3.2.2 Haploidentical Stem Cell Transplantation

Haplo-SCT is a special form of SCT and is characterized by matching only half of the HLA characteristics between donor and recipient (5/10). In this case, HLA disparity is greater than in matched transplants. While MSDs are available for 20-25% of patients requiring SCT and MUDs can be found in 60%, 15-20% of patients lack a suitable donor or enough time for donor acquisition, leaving haplo-SCT as the only therapeutic option. In a haplo-SCT scenario, parents or siblings of the patient can be considered as donors. They are considered good options because they are highly motivated to donate, can be acquired quickly and easily, and are available if a second donation or donor lymphocyte infusion (DLI) is needed.[13] Due to greater HLA disparity, graft-versus-host disease (GvHD) and graft rejection occur more frequently with haplo-SCT.[12,14] Measures to prevent these immunologic responses include efficient removal of alloreactive T cells and the delivery of large SC amounts, called megadose, to overcome HLA

barriers.[15,16] The absence of T cells in grafts can be achieved by combined T and B cell depletion (CD3/19 depletion) or CD34+ selection. Additional depletion of B cells is a preventive measure to reduce Epstein-Bar-Virus (EBV)-associated lymphoproliferative diseases.[12] CD34 selections provide highly purified CD34+ stem cells. PBSCs can also be split to combine both CD3/19 and CD34 selections. To minimize the immunologic responses emphasized in haplo-SCT, T cell depletion (TCD), myeloablative conditioning, additional immunosuppressive therapy and SC megadose are important, but lead to a delay in immune reconstitution, especially in T cell reconstitution.[11,14,16] Myeloablative conditioning increases organ toxicity and aplasia. In turn, aplasia poses an elevated risk of infection, while overall treatment-related mortality (TRM) increases.[12,17] Therefor, researchers and physicians are constantly trying to reduce the intensity of conditioning to lower organ toxicity and shorten the time of aplasia to decrease overall TRM.[17]

3.2.3 Stem Cell Processing

Before stem cell grafts can be transplanted, they undergo SC processing. SC processing is an important component in SCT from PBSC and is especially important for haplo-SCT due to greater HLA disparity. The processing is performed by immunomagnetic separation. All cells of interest are therefore labeled with an antibody paired with a magnetic bead. All labeled cells then pass through a column placed in a magnetic field that retains all target cells. The antibodies used are highly specific and based on high-resolution cluster of differentiation (CD-) markers expressed on the cell surface.[18]

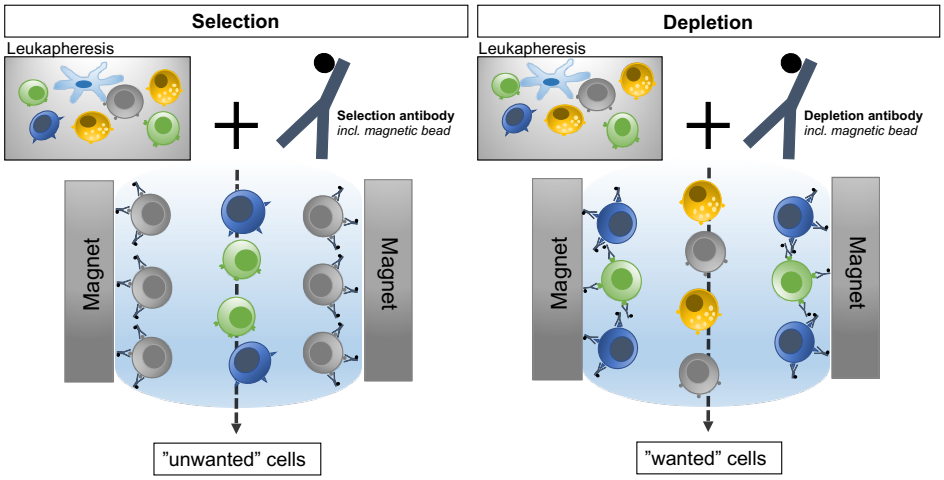

***Figure 2 Stem Cell Processing**. Stem cell are processed by immunomagnetic separation. All cells of interest are labeled with an antibody paired with a magnetic bead. All labeled cells then pass through a column that is placed in a magnetic field and retains all labeled cells. depletion all unwanted cells are labeled and only wanted cells pass through the magnetic field.*

Because this work focuses on haplo-SCT and both CD34 selection and CD3/19 depletion are most commonly used in haplo-SCT settings, these two processing methods are described below. CD34 selection is a method to obtain highly purified CD34+ SCs. SCs are labeled with a CD34 antibody paired with a magnetic bead. While all unlabeled cells pass and are collected in the so-called non-target cell collection bag (NTCB), labeled CD34+ stem cells are retained by the magnet, allowing a specific selection of SCs. Thus, CD34 selection is a positive selection process. After all cells have passed the magnetic field, the column is removed from the magnetic field and rinsed to collect all labelled cells and transfer them to the cell collection bag (CCB).

CD3/CD19 depletion, on the other hand, is referred to as negative selection. While the principal of using antibodies paired with magnetic beads is the same, the target cells differ. In this case CD3+ T cells and CD19+ B cells are labeled and retained as they pass through the column. All unlabeled cells pass through and are now the cells of interest and collected in the CCB.[19]

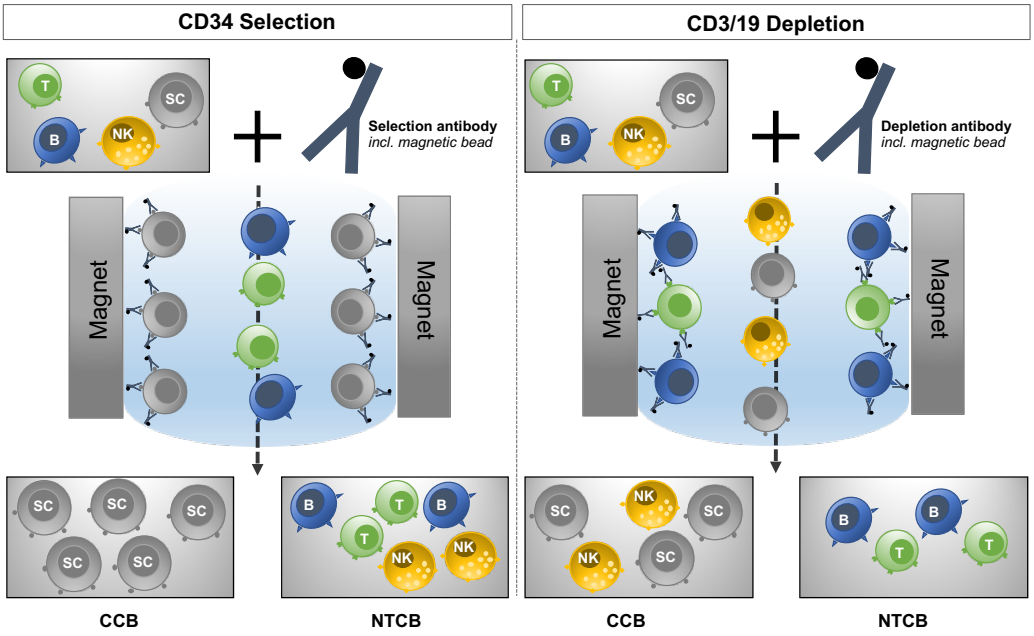

Figure 3 CD34 Selection and CD3/19 Depletion. *The cells from a leukapheresis are incubated with a specific selection or depletion antibody. For CD34 selection, an antibody against CD34+ is used and for CD3/19 depletion an antibody against CD3+ and CD19+ cells is used. The antibody is paired with a magnetic bead. As the cells pass through the magnetic field, all antibody-carrying cells are retained and unlabeled cells pass through. The cell mixture from the leukapheresis is then separated into two bags: a cell collection bag (CCB), containing wanted cells such as SC in CD34 selections, and a non-*target cell bag (NTCB), containing all remaining unwanted cells. The composition of CCB and NTCB varies depending on the used antibody. Abbreviations: SC=Stem cells, B= B cells, T= T cells, NK= Natural killer cells.

While both principals use the same technical method, the main difference is the composition of collected cells within the CCB. CCBs after CD34 selection contain over 90% CD34+ SCs, while cell composition after CD3/19 depletion consists of variety of cells including CD34+ SCs, natural killer (NK) and antigen presenting cells (dendritic cells and monocytes).

Previous research has shown that additional NK and antigen presenting cells (APC) promote faster engraftment, immune reconstitution and the beneficial Graft vs. leukemia effect. SCT from CD34 selections provide a graft enriched with CD34+ SCs, without possible beneficial immunological cells. However, due to greater HLA disparity in haplo-SCT CD34 selection is often necessary to overcome HLA barriers.[12,17] To obtain sufficient numbers of SCs while preserving

beneficial NK and AP cells, both methods can be combined. For technical execution of magnetic cell isolation, the use of CliniMACS technology from Miltenyi Biotec (Bergisch Gladbach) is the standard in Europe.

3.2.4 Immune System and T Cell Populations

Before addressing immunological phenomena and the possible complications of SCT, it is necessary to understand fundamental principles of the immune system and the cell populations involved.

The immune system can be divided into innate and an adoptive immune system. In particular, the recovery of the adoptive immune system is important for the well-being of patients after transplantation, as it is highly specialized in the elimination of potentially life-threatening viruses and degenerated tumor cells. For this reason, this chapter takes a close look at T cell populations and examines NK cells as part of the innate immune system. T cells play a central role within the SCT process. Finding the right balance between beneficial and harmful immunological effects remains a major challenge for successful SCT treatments. T cells make up 60-70% of lymphocytes. Lymphocytes can develop an “immunologic memory” that enables a rapid and efficient immune response to pathogens and malignant cells.[18,20] To become immunocompetent, naïve T cells undergo several developmental stages, beginning within the bone marrow, from where they migrate to the thymus until they emigrate to secondary lymphoid organs where they await contact with an antigen. After antigen contact, they transform into immunocompetent T cells. Antigen-presenting cells (APCs) such as dendritic cells (DC), macrophages and B cells process circulating antigens. The antigens are then presented to naïve T cells via MHC receptors, forming the so-called MHC-antigen-complex. T cells recognize the MHC-Antigen-complex and bind it with their T cell receptor (TCR). This leads to initialization of T cell activation and proliferation. The T cells proliferate with the help of autocrine (self-induced) interleukin-2 (IL-2) ensuring efficient numbers of T cells to adequately fight infectious agents.[18,20,21]

T cells (CD3+CD56-) can be divided into naïve or immunocompetent T cells and CD8+ T cytotoxic (T_C) cells or CD4+ T helper (T_H) cells. T regulatory cells (T_{reg}) are derived from T_H cells and are defined as CD3+CD4+CD25+^{bright}. T_H cells are responsible for supporting the innate immune system and therefore increase the efficiency of antigen eradication. T_H cell activation occurs through the presentation of antigen fragments by APCs, especially dendritic cells. T_H cells then differentiate into inflammatory T_{H1} cells or T_{H2} cells. Both support the innate immune system through their release of cytokines (IL10, INF-γ, TGF-β) that activate macrophages and B cells. T_{reg} cells are responsible for immunologic regulation between T cell subsets and can prevent graft rejection and the occurrence of GvHD after allo-SCT.[22–25] Tc cells identify virus-infected or malignant cells and can eradicate them. Activation of Tc cells occurs through APCs with HLA-class-I molecules. Tc cells carry cell-dissolving proteins that lead to apoptosis of infected or degenerated cells. After SCT, Tc cells are of great importance because they are able to eradicate life-threatening viruses (e.g., Cytomegalovirus, Epstein-Barr-Virus) or fungi (e.g., Aspergillus) during aplasia. In addition to pathogen specificity, Tc cells enable helpful GvL effect.[3,21,26] Naïve T cells (T_N) are antigen-inexperienced T cells with CD45RA+ CD45RO- surface markers, whereas T memory (T_M) cells have already acquired immunocompetence (CD45RA-CD45RO+). Previous studies have shown that T_N cells can cause severe GvHD, whereas T_M cells cause only mild to no GvHD. The higher GvHD potential has been explained by a more diverse TCR repertoire in inexperienced T cells. During T cell development, TCRs form high specificity such that T_M cells have lower receptor diversity and thus have lower alloreactivity and consequently lower GvHD potential.[27,28] After activation, naïve T cells switch from their CD45RA+ isoform to their CD45RO+ isoform. The T cells expand and differentiate primarily into effector T cells (T_{EM}) and central memory T cells (T_{CM}) and then carry the lymph node homing markers CD62L and CCR7.[29] T_{CM} cells (CD45RO+CD62L+/CCR7+) are known to have a long lifespan. Due to their positivity for homing markers, T_{CM} are mainly found in lymph nodes and spleen.

T_{EM} cells (CD45RO+CD62L-/CCR7-) have a shorter lifespan and are found in peripheral lymphoid tissue.[26] Studies have shown that both subtypes of T Memory cells can promote GvL, with T_{EM} cells show no GvHD potential, and T_{CM} cells having only mild GvHD potential.[27,30]

B cells are also part of the adaptive immune system. B cells are CD19+ and are characterized primarily by their ability to produce antigen-/pathogen- specific antibodies (immunoglobulins). Immunoglobulins can also be manufactured and are used, for example, for vaccinations. Substitution of immunoglobulins after SCT is a good therapeutic option to mimic B cell function. The development of B cell development takes place solely in the bone marrow. After maturation, they circulate in the blood stream or are found in lymphoid tissue. They carry a specific B cell receptor (BCR) consisting of membranous IgM and IgD. To activate B cells, the BCR binds antigens, internalizes and processes them and present their fragments on the cell surface using HLA-class-II molecules. T_H cells then recognize the presented antigen and activate the B cells through cytokines. The B cells then differentiate into B Memory and plasma cells. Plasma cells produce antibodies against specific antigens to neutralize or opsonize them. B memory cells are able to respond quickly and in the same manner upon second antigen contact.[18,20,21]

NK cells (CD3-CD56+), as part of the innate immune system, respond to virus-infected, degenerated cells and mycobacteria without prior antigen contact and are thus part of the “first line of defense” in antigen eradication. NK cells are able to kill their target cells using membrane-destroying perforin, leading to cell death.[21] Activation of NK cells is initiated by interferons distributed by themselves or by T cells. NK cells play an important role within SCT by protecting against infections and facilitating GvL effect that ultimately prevents disease relapse.[31] NK cells have also been described to have beneficial effects on engraftment after SCT and can be used as part of post-SCT immunotherapy.[17,32,33]

3.2.5 Complications and Risk Factors

As discussed in the previous sections, allo-SCTs, especially haplo-SCTs, pose a challenge in terms of their immunological conditions due to the larger HLA mismatch. Therefore, measures are taken to minimize immunological complications, such as myeloablative conditioning, immunosuppression, or additional pharmaceutical T cell depletions. However, these measures pose a risk of their own. Complications associated with SCTs are diverse, and it is often difficult to distinguish between adverse complications and desirable effects (e.g., engraftment), because they may share the same symptoms. The most important complications associated with SCT are organ toxicity and infections and are briefly discussed in the following section.

Organ toxicity due to conditioning is one of the major complications. The combination of high-intensity chemotherapy, total body irradiation (TBI) and immunosuppressive therapy can damage the liver, kidney, heart and lungs. Patients with a long history of disease and overall high toxicity are particularly affected. While some complications associated with conditioning regimes such as hair loss, inflammations of mucous membranes, and the skin irritation are considered temporary, others serious complications can have long-term consequences. Due to organ toxicity, patients may develop endothelial-leakage-syndrome, vein-occlusive-disease or microangiopathy, affecting virtually all organ system. To minimize complications due to organ toxicity, lower intensity conditioning regimes are pursued in the setting of SCT.[34] The immune status of a patient is constantly changing after SCT and is characterized by aplasia and impairment of cellular and humoral immunity, which may cause viral, bacterial or fungal infections as opportunistic infections or viral reactivations. In addition, the conditioning regimes previously performed cause damage to protective barriers and the need for implantation of permanent intravenous catheters increase the risk for bacterial infections. Viral infections can cause severe life-threatening complications after SCT. For this reason, standard preventive measures, such as

preemptive antibiotics, high hygiene standard, isolation and constant immunologic monitoring are of great importance for a good therapeutic outcome.[7,35–37] While most pathogens are harmless in the healthy patient state, they can pose serious threat to transplanted patients. Due to the impairment of cellular immunity reactivation and/or *de novo* infections with adenovirus and herpes viruses, cytomegalovirus (CMV) or Epstein-Barr-Virus (EBV) are possible and especially life-threatening in the first year after transplantation.[38] In addition to bacterial and viral infections, fungal infections with Candida and Aspergillus may also occur during the first year after SCT.[7] Since organ toxicity and infections continue to pose a threat of life to patients, research is focused on how to reduce chemotherapy and immunosuppression intensity without compromising the effective removal of malignant cells or provoke unwanted immunological reactions, while preserving desirable immunological responses such as the graft versus leukemia effect. Because the possible immunologic responses in SCT are rather complex, they are discussed separately in the following chapter.

3.2.6 Transplant-Related Donor-Recipient-Reactions

Besides organ toxicity and infections, transplant-related reactions are one of the relevant complications of SCT. Transplant-related reactions can be divided into three major reaction groups

- immunological Host versus Graft reaction/ Transplant reaction (HvG),
- immunological Graft versus Host reaction (GvHD),
- immunological Graft versus Leukemia effect (GvL).

Host versus Graft Reaction /Transplant Rejection

Host versus graft reaction (HvG) is referred to as recipient-induced graft rejection. Transplant rejection occurs in up to 2% of HLA-matched transplants and in 20-30% of HLA-unmatched transplants. Residual recipient T cells recognize donor HLA molecules and promote graft rejection. The risk of graft rejection is particularly high in haplo-SCT, where residual recipient T cells promote rejection

and potentially counteracting donor T cells are absent.[18,34] Other risk factors for graft rejection include low SC counts in the graft and inadequate (high) GvHD prophylaxis. For early detection of graft rejections post transplantation chimerism analysis is performed and DLI or adjustment of immunosuppressive therapy may be initiated.[39]

Graft versus Host Reaction

Graft versus Host Reaction (GvHD) is one of the major complications of allo-SCT. Although only 40-60% of GvHD reactions that occur require treatment, general occurrence is fairly common, with approximately 60% in HLA identical family members and up to 80% in HLA-identical unrelated donors.[34] Therefore, GvHD prophylaxis and its treatment remain one of the greatest challenges in SCT. GvHD is an immunologic transplant response triggered by residual mature donor T cells in the graft. Residual T cells recognize the recipient's HLA antigens on tissue and organs as foreign and then promote severe inflammatory disease. GvHD generally affects all tissues, but to a great extent skin, intestine and liver leading to rashes, diarrhea and liver dysfunction, thus affecting patients' quality of life.[18] GvHD is predominantly induced by the interaction of T cell with major HLA-antigens (unrelated or mismatched SCT), but can also be induced by minor HLA-antigens (HLA identical siblings).[40] The development of GvHD can be divided into three pathophysiological phases: 1. tissue damage and APC activation, 2. T cell activation and 3. subsequent cell damage by activated donor T cells. In particular, CD3+CD8+ cytotoxic T cells mediate cytotoxic damage to host cells and promote apoptosis.[24] The development of GvHD begins with tissue damage due to intense conditioning. Tissue damage leads to the release of great numbers of inflammatory cytokines resulting in upregulation of HLA expression and adhesion molecules on APCs. APCs then mediate the activation of donor T cell, leading to sever GvHD. At the same time tissue membranes become more permeable, allowing (physiological) bacteria (e.g., intestinal flora) to mediate further cytokine release. Activated donor T cells then promote an immune

response that activates even more T cells through the release of IL-2.[24] GvHD can occur in an acute or chronic form. In the past, acute and chronic forms were distinguished by time of onset, but today a distinction based on manifestation rather than time seems more accurate.[41] Accurate differentiation between the two forms is often difficult. Acute GvHD (aGvHD) is usually limited to manifestation on the skin, gastrointestinal tract and liver. Chronic GvHD (cGvHD), on the other hand, presents multifaceted, similar to a systemic autoimmune disease. Chronic GvHD can manifest in all organs and cause dysfunction and atrophy. Both forms lead to impaired quality of life and are potentially life-threatening making their prevention and effective treatment important.[5,34] Acute GvHD is classified according to Glucksberg criteria, which define the extent and number of organs affected individually and then combine these to obtain an overall severity grading. The severity graduation then allows an evaluation of treatment options and their necessity (Table 2).[34,40]

Grade	Skin	Intestine	Liver	Limitations in everyday life
I (light)	1-2	0	0	None
II (moderate)	1-3	1	1	Light
III (severe)	2-3	2-3	2-3	Moderate
IV (life threatening)	2-4	2-4	2-4	Significant

Table 2 Clinical Grading of aGvHD*.Grading of an aGvHD includes evaluation of skin, intestine, liver and limitations in everyday life. The severity is then ranked from grade I (light) to IV (life threatening). Table edited based on Ball et al.*[24]

Chronic GvHD can be assessed using the diagnostic recommendations of the National Institute of Health (NIH) cGvHD Consensus Group. The scoring system is based on a thorough examination of the organs (lungs, GI tract and liver) including eyes, mouth, joints, scalp and body hair etc. All sites are assessed individually and possible overlap between acute and chronic forms is considered.

The established scoring system is used to guide treatment planning decisions.[41] Risk factors that favor the development of GvHD include HLA disparity, source of stem cells, donor age, prior conditioning and other factors.[42,43] To prevent severe GvHD, immunosuppressive therapies and graft processing methods are used that aim to remove immunologically active cells that promote GvHD.

Graft versus Leukemia Effect

While transplant rejection and GvHD are feared complications of SCT, not all immunological phenomena are undesirable, such as the graft versus leukemia effect (GvL). In the GvL effect, alloreactive donor T and NK cells recognize minor HLA antigens of leukemia cells remaining after conditioning and eradicate them. This effect has been shown to be beneficial in preventing leukemia relapse and also allows the use of nonmyeloablative conditioning regimes, minimizing complications such as organ toxicity or pancytopenia.[18,44,45] While depletion of T cells is an effective method of preventing the occurrence of life-threatening GvHD, it also prevents the eradication of residual malignant cells, which in turn increases the risk of disease relapse.[45] Therefore, allowing GvL effect to occur is part of a successful leukemia treatment.[46,47] Due to the greatest HLA disparity, GvL (and GvHD) effect is most commonly seen with haplo-SCT.[48,45] Although strategies such as donor lymphocyte infusions (DLI) after T cell depletion (TCD) can realize an adequate GvL effect without harmful GvHD, finding the right equilibrium between the two continues to be subject of current research.[46]

3.3 Immune Reconstitution and Immunosuppressive Therapy

After SCT, patients are in aplasia, meaning they are left without having a functioning immune system due to prior conditioning. The time span between aplasia and complete immune reconstitution is critical after SCT. Without a well-functioning, fully reconstituted immune system, patients are at high risk of developing potentially life-threatening bacterial, viral and fungal infections.[35] In particular, reactivation and *de novo* infections of Epstein-Barr-Virus (EBV),

cytomegalovirus (CMV) and adenovirus (AdV) are common and threatening due to the lack of virus specific T cells.[7,49] Therefore, early immune reconstitution after SCT is of great importance to the therapeutic outcome and patient mortality. Engraftment occurs 12 to 21 days after SCT and means that more than 1000 leucocytes/µl, 500 neutrophilic granulocytes/µl and 20000 thrombocytes/µl are detectable in the patient's peripheral blood count. This marks the beginning of the immune reconstitution. Subsequently all other cells of the innate and adoptive immune system return, such as NK cells and T cells. Whereas NK cells return relatively quickly and reach normal cell counts after 1 to 4 months, complete reconstitution of T cells takes up to year.[50–52] Reconstitution of NK and T cells is particularly important as they protect against infections and enable GvL effect, thus prevent disease relapse.[31] Immune reconstitution is influenced by numerous factors, including the degree of HLA matching between donor and recipient, the immunosuppressive therapy used, the degree of remission before SCT, the source of SCs, the graft engineering method and composition, the age of donor and the occurrence of infections.[31,51,53–55] Although not all influencing factors are changeable, immunosuppressive therapy and graft processing methods are potentially adjustable. Immunosuppression is essential, especially in haplo-SCT, where HLA disparity between donor and recipient is greater than in matched SCT.

To minimize GvHD after SCT, immunosuppressive therapy with various pharmaceuticals (Cyclosporin A, methylprednisolone etc.) is performed. In addition, infectious prophylaxis and close monitoring of emerging bacterial, viral or fungal infections are performed to provide early therapeutic coverage.[7]

However, extensive immunosuppression leads to prolongation of lymphoblastic deficiency, resulting in higher infection rates and TRM. For this reason, immunosuppression must be minimized to the extent that is just sufficient to prevent severe GvHD but does not prolong lymphoblastic deficiency, increase the risk of disease relapse, or impair GvL and thus provokes graft rejection.

For this reason, researchers are eager to find methods of graft processing methods, such as CD45RA depletion, that promote little or no GvHD, while transferring immunocompetent cells and preserving GvL.

3.4 CD45RA Depletion

CD45RA depletion is a graft processing method in which naïve CD45RA+ T cells are removed from peripheral blood stem cell products (PBSC). Depletion is performed using magnetic bead paired antibodies and CliniMACS technology (see 3.2.3 and 5.4.4.). The CD45RA depletion process is an alternative to already established methods and aims to improve unresolved problems such as TRM due to persistent lymphoblast deficiency, infections, organ toxicity as well as the occurrence of GvHD in haplo-SCT. Knowing the benefit of maintaining T cells to ensure GvL, prevent disease relapse and ensure immunologic protection from pathogens, researchers continue to search for ways to transfer an adequate amount of T cells without promoting GvHD.[45–47] Compared to established methods such as CD3/19 depletion or CD34 selection, CD45RA depletion provides more T cells simultaneously to CD34+ SC. CD45RA depletion can transfer useful CD45RA-CD45RO+ T memory cells (T_M) in much higher cell numbers. Previous studies have shown that T_M promote only mild to no GvHD but maintain GvL.[28,56,57] In addition, it is discussed that CD45RO+ memory cells improve early immune reconstitution, shorten viremia due to preservation of donor virus-specific T cells, and do not require serotherapy compared with depletion of pan T cells.[58] In the clinical setting, CD45RA depletion requires a similar amount of work compared with CD3/19 depletion and CD34 selection and can be performed in a one- or two-step procedure. The one-step approach is performed as direct depletion without prior manipulation of the G-CSF-stimulated graft. Performing CD45RA depletion using the two-step approach describes CD45RA depletion after preceding CD34 selection from the respective CD34-negative fraction. The combination of CD34 selection and CD45RA depletion

may be useful to obtain sufficient CD34+ stem cells and overcome HLA barriers in haplo-SCT settings.[15,57,59]

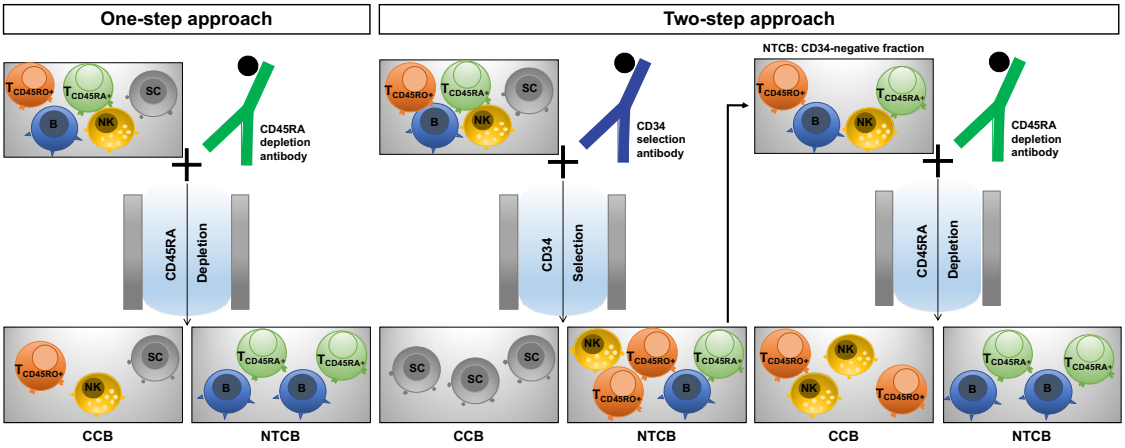

Figure 4 CD45RA Depletion: One- and Two-Step Approach. *CD45RA depletion can be performed using two different approaches: In the one-step approach, CD45RA depletions are performed as direct depletions from G-CSF stimulated PBSCs. CD45RA+ T and B cells are depleted, while CD45RA- cells are collected in the cell collection bag (CCB). SC and NK cells can also be labeled and depleted depending on their CD45RA co-expression, but are mainly collected in the CCB. In the two-step approach, CD45RA depletions are performed from the negative fraction (non-target cell bag, NTCB) after preceding CD34 selection. This results in two CCBs, one with highly purified CD34+ stem cells, and one CD45RA- CCB. SC=Stem cells, B= B cells, T= T cells, NK= Natural killer cells, $T_{CD45RO+}$=T memory cells, $T_{CD45RA+}$= naïve T cells*

4. Objectives

The therapy of childhood malignancies with haplo-SCT is a great therapeutic achievement in pediatric oncology and is currently well advanced. However, preventing the occurrence of GvHD, handling vulnerability to opportunistic viral infections during lymphoblastic deficiency, and simultaneously maintaining GvL remain major challenges. Recent research has demonstrated that that T cell subpopulations hold different properties in mediating GvHD, conferring immunocompetence, and preserving of GvL. CD45RA depletion as a graft preparation method provides a promising combination of prevention of life-threatening GvHD while transferring immunocompetence and preserving in GvL by eliminating naïve CD45RA+ T cells. [28,56,57] The aim of this research is to evaluate the feasibility of CD45RA depletions from G-CSF stimulated SC grafts, including cryopreserved grafts. The evaluation included prior establishment of a 10-color FACS measurement panel to meet laboratory quality control requirements.

The depletion process as well as the establishment of a 10-color FACS measurement panel was based on good manufacturing practice (GMP) principals. Using the established 10-color FACS measurement panel, preliminary data acquisition was performed to assess CD45RA co-expression on all SCT-relevant cell populations, allowing estimation of potential cell losses during the CD45RA depletion process. A particular focus of the study was the efficiency comparison between a one- and two-step approach. The aim of this work was to critically evaluate CD45RA depletion considering depletion quality of CD45RA+ cells, recovery of desired cells, determination of graft composition after depletion, and quantification of transferable beneficial cell populations including virus-specific T cells (AdV, EBV, CMV). This work aimed to elaborate the potential benefits and challenges of the depletion process and to provide recommendations for the implementation of the CD45RA depletion process in clinical practice.

5. Materials and Methods

5.1 Samples

For the establishment of a 10-color measurement panel, analysis of CD45RA co-expressions and for CD45RA depletion quality control n=20 surplus samples (1mL) after stem cell apheresis were analyzed (Ref. 502/16). Samples were all anonymized through the cooperating DRK-Blutspendedienst Baden-Württemberg – Hessen (Standort Frankfurt am Main). Conduction of preceding apheresis was covered by a separate ethics vote (Ref. 329/10).

For further characterization of CD45RA co-expressions on stem cells, n=7 cryopreserved highly purified stem cell products after CD34 selection from deceased patients were used. Used samples were stored for several years after deceasing of the respective patient and were then authorized for disposal as well as anonymized before becoming part of this research project.

All samples used for the conduction of CD45RA depletions were anonymized before becoming part of this research project and no individual-related data was identifiable. For CD45RA depletion n=3 fresh unmanipulated PBSC samples and n=4 fresh samples discarded after CD34 selection (NTCB) were used. In order to compare the feasibility between depletions of fresh and cryopreserved starting products, n=2 cryopreserved PBSC were CD45RA-depleted. For homing marker selection, measurements were conducted from anonymized surplus blood samples (n=3).

5.2 Material

5.2.1 Equipment and Software

Equipment	Manufacturer
Barkey Plasmatherm	Barkey, Leopoldshoehe, Germany
Centrifuge Multifuge 3SR+	Heraeus, Hanau, Germany
Centrifuge Multifuge 4KR	Heraeus, Hanau, Germany
Centrifuge Rotina 46	Hettich, Tattling, Germany
Clean bench HeraSafe KS18	Thermo Electron, Langenselbold, Germany
Cryogenic tank Biosafe ® MD	Cryotherm, Kirchen/Siege, Germany
Flow Cytometer FC500	Beckman Coulter, Krefeld, Germany
Flow Cytometer NAVIOS 3L10C	Beckman Coulter, Krefeld, Germany
Hematocytometer Coulter ® Ac.T diff ™	Beckman Coulter, Krefeld, Germany
Pipette Eppendorf Research (10- 1000µL)	Eppendorf, Hamburg, Germany
Refrigerator	Liebherr, Ochsenhausen, DE
Shaker SI100	Pharmacia Diagnostics, Uppsala, Sweden
Sterile Tubing Connector TSCD- II	Terumo, Phenix, USA
Vortex device Vortex-Genie 2G-560E	Scientific Industries, New York, USA
Welding machine CompoSeal ®	Fresenius Kabi, Bad Homburg, Germany

Table 3 Equipment for Flow cytometric measurements and sample preparations.

Software	Manufacturer
GraphPad Prism 5.0	GraphPad Software, La Jolla, USA
Kaluza Analysis 2.1	Beckman Coulter, Krefeld, Germany
Navios 1.2	Beckman Coulter
CXP System 2.2	Beckman Coulter

Table 4 Software for Flow cytometric measurements and statistical analysis.

5.2.2 Consumables

Product	Manufacturer
Combi Stopper	Braun, Melsungen, Germany
Eppendorf Tube 1,5mL	Eppendorf, Hamburg, Germany
FACS ® Tube 5mL	BD Biosciences, San Jose, USA
Heidelberg Connections (Luer Lock)	Braun, Melsungen, Germany
Infu-Star infusion-manifold 3 way	MiroMed, Frankfurt, Germany
Pall-Filter	Haemonetics, Massachusetts, USA
Pipette filter tip (10-1000µL)	Starlab, Hamburg Germany
Pipette filter tip sterile (10-1000µL)	Starlab, Hamburg, Germany
Three-way valve correct translation	Braun, Melsungen, Germany
Transfer Set Coupler	Miltenyi Biotec, Großwallstadt, Germany
Tubes (5mL)	Sarstedt, Germany

Table 5 Consumablesfor flow cytometry and CliniMACS depletion preparation.

5.2.3 Chemicals, Buffers and Kits

Product	**Manufacturer**
Coulter Clenz®	Beckman Coulter, Krefeld, Germany
Dimethylsulfoxid (DMSO)	Sigma-Aldrich, Steinheim, Germany
DNA-Dye 7-AAD	Beckman Coulterm Krefeld, Germany
DNase I (1mg/ml)	Roche, Mannheim, Germany
FACS clean	Becton Dickinson, San Jose, USA
FACS flow	Becton Dickinson, San Jose, USA
Flow- Count™ Fluorospheres	Beckman Coulter, Krefeld, Germany
Flow-Check™ Fluorospheres	Beckman Coulter, Krefeld, Germany
Human serum albumin, HSA 20%	Octapharm, Dessau, Germany
IO3 Test lysing solution	Beckman Coulter, Krefeld, Germany
IsoFlow™ Sheat Fluid	Beckman Coulter, Krefeld, Germany
Phosphate buffered saline solution (PBS)	PAA Laboratories, Linz, Austria
Stem-Kit Reagents	Beckman Coulter, Krefeld, Germany
X-Vivo 10 without Phenolred	Cambrex, Verviers, Belgium

Table 6 Chemicals, Buffers and Kits for sample preparation.

5.2.4 Materials for Immunological Separation

Product	Manufacturer
CliniMACS CD45RA reagent	Miltenyi Biotec, Bergisch Gladbach, Germany
CliniMACS DTS tubing set	Miltenyi Biotec, Bergisch Gladbach, Germany
CliniMACS PBS/EDTA buffer	Miltenyi Biotec, Bergisch Gladbach, Germany

Table 7 CliniMACS Depletion Supplies.

5.2.5 Antibodies and MHC Dextramers ®

Antibody	Clone	Fluorescent	Manufacturer
Anti-CD3	UCHT-1	APC	*Beckman Coulter*
Anti-CD4	13B8.2	APC-A750	Beckman Coulter
Anti-CD4	13B.2	PC5	Beckman Coulter
Anti-CD8	B9.11	APC-A700	Beckman Coulter
Anti-CD8	SFCI21Thy2D3	PC7	Beckman Coulter
Anti-CD14	RMO52	PB	Beckman Coulter
Anti-CD14	RMO52	ECD	Beckman Coulter
Anti-CD19	J3-119	APC-A700	Beckman Coulter
Anti-CD19	J2-119	ECD	Beckman Coulter
Anti-CD25	2A3	PC7	Beckman Coulter
Anti-CD34	581	PC7	Beckman Coulter
Anti-CD45	J.33	KO	Beckman Coulter

Anti-CD45RA	ALB11	FITC	Beckman Coulter
Anti-CD45RA	2H4	PB	Beckman Coulter
Anti-CD45RA	2H4	PE	Beckman Coulter
Anti-CD45RO	UCHL1	ECD	Beckman Coulter
Anti-CD45RO	UCHL1	PE	Beckman Coulter
Anti-CD56	N901	PC7	Beckman Coulter
Anti-CD62L	DREG56	FITC	Beckman Coulter
Anti-127	R34.34	PE	Beckman Coulter

Table 8 Flow Cytometric Antibodies.

Specific virus	**HLA allele**	**Peptide sequence**	**Manufacturer**
CMV	HLA - A* 0201 HLA - A* 0301	pp65 pp65	Immudex, Copenhagen, Denmark
EBV	HLA - A* 0201 HLA - A* 0201 HLA - B* 0801 HLA - B* 3501	BMLF1 LMP2A EBNA3A EBNA1	Immudex, Copenhagen, Denmark
AdV	HLA - A* 0101	Hexon	Immudex, Copenhagen, Denmark

Table 9 MHC Dextramers ®.

5.3 Methods

5.3.1 Flow Cytometry

Flow cytometry, also called fluorescence-activated cell sorting (FACS), is a measurement method for quantifying and specifying cells in a suspension. Cells are identified and counted based on their size, intracellular characteristics and surface antigen expression. Flow cytometry is an established measurement method that can characterize thousands of cells in just a few seconds. To identify them, the cells must be labeled with fluorochrome- paired antibodies directed against specific cell surface markers. In a laminar sample stream, the labeled cells then pass individually through a monochromatic laser beam. After the monochromatic light impinges on the passing cell, the light is scatters in a cell-characteristic manner and subsequently detected. Forward scattering (FSC) detects the forward deflected light and allows characterization of the cells by size. Side scattering (SSC) detects light deflected at a 90° angle to the side and allows characterization of the complexity and granularity of the cells. At the same time, intensity of the fluorescent dye is measured, allowing characterization of the cells by antigen expression. Characterization and quantification of surface antigens is possible because the monochromatic light from the laser strikes a cell, which is then partially absorbed by the fluorescent dye and then emits light at a higher wavelength that is eventually detected. The emitted light is characteristic for each fluorescent antibody, known as its emission spectrum, and enables the correct characterization of cells. Fluorescent antibodies are highly specific, but overlap of emitted light is possible. Complex optic systems with filters, mirrors and lenses are used to separate and recognize cells in a highly specific manner. Photomultipliers convert the light into an electrical signal. The emitted and filtered light correlates with the intensity of the fluorescence and thus with the expression of the cell antigen.[19]

Fluorescent	Emission maximum (nm)
7-AAD (7-Aminoactinomycin D)	660 nm
APC (Allophycocyanin)	660 nm
APC -A700 (APC – Alexa Fluor 700)	719 nm
APC -A750 (APC – Alexa Fluor 750)	780 nm
ECD (PE-Texas Red)	620 nm
FITC (Fluorescein-Isothiocyanat)	520 nm
KO (Krome Orange ™)	528 nm
PB (Pacific Blue)	455 nm
PC 5 (Phycoerythrin-Cyanin 5.5)	694 nm
PC 7 (PE-Cyanin 7)	767 nm
PE (Phycoerythrin)	578 nm

Table 10 Used Fluorescents and Corresponding Emission Maxima.

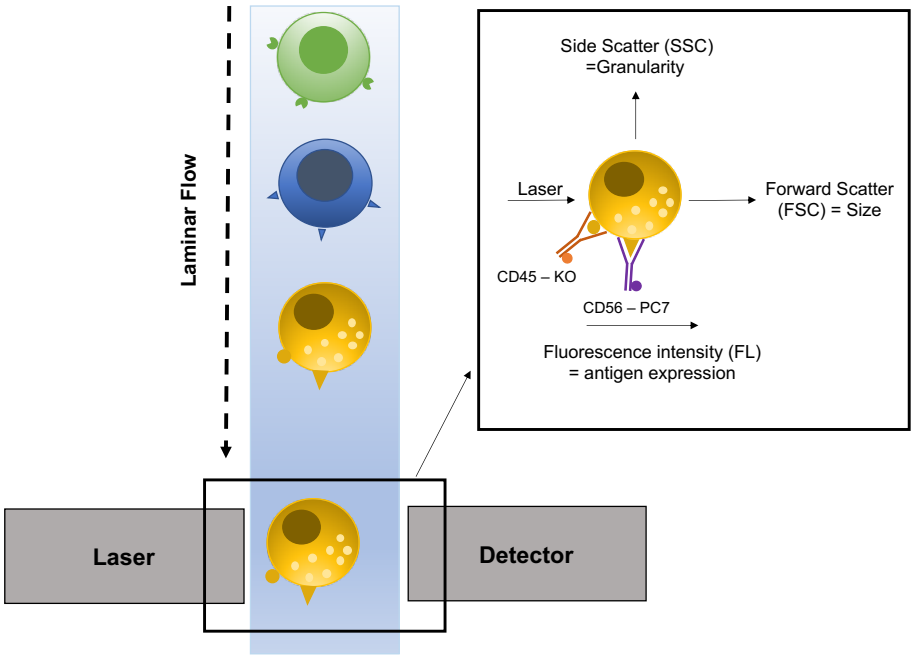

Figure 5 Flow Cytometric Measurement. *Flow cytometry enables the characterization of cells based on their morphological properties (size, granularity) and their surface antigens. Cells individually pass through a laser beam, then scatter and emit light in highly cell-specific amounts, enabling cell characterization. While forward scattering (FSC) detects the size of each cell, side scattering (SSC) determines granularity. Emission of specific wavelengths provide details about surface antigen expression.*

For internal quality control, measurements were performed using Navios flow cytometer (Beckman Coulter) and FC-500 (Beckman Coulter) and standard filter configurations. Navios is a multi-color, multi-laser flow cytometer with 10 color and 3 lasers (blue 488nm, red 635 nm, purple 405 nm). FC-500 analyzes samples with 5 colors and a dual laser technology (blue 488 nm, red 635 nm). The use of more than one laser allows a broader spectrum of fluorescence emission and thus the simultaneous detection of multiple CD markers. Liquid stability and optical alignment in the flow cytometer were tested using Flow-Check Pro™ Fluorospheres (Beckman Coulter). Immuno-Trol cells (Beckman Coulter) and CD-Chex CD34 (Streck) were used to ensure stability of measurements. Antibodies were titrated and assayed separately (0.5 µl, 1.0 µl, 2.5 µl, 5.0 µl, 7.5 µl, 10.0 µl, 20.0 µl).

Phenotyping of cells using 5- or 10-color flow cytometry

Cell immunophenotyping refers to the identification of cell populations based on their antigen patterns using monoclonal fluorescent antibodies and flow cytometry. Identification is conducted using specific surface proteins, called cluster of differentiation (CD). Depending on their expression pattern, leukocytes can be subdivided and characterized. The assignment of the cells according to their respective antigen expression pattern is shown in Table 11.

Subpopulation	Antigen Expression
Leucocytes	CD45+
T cells	CD3+
T Helper cells	CD3+ CD4+
T cytotoxic cells	CD3+ CD8+
NK like T cells	CD3+ CD56+
T regulatory cells	CD3+ CD4+ CD25+^{bright}
Naïve T cells	CD45RA+ CD62L+CCR7+
Effector RA+ T cells	CD45RA+ CD62L-CCR7-
Central Memory T cells	CD45RO+ CD62L + CCR7+
Effector Memory T cells	CD45RO+ CD62L-CCR7-
NK cells	CD56+ CD3-
B cells	CD19+
Stem cells	CD34+
Monocytes	CD14+

Table 11 Surface Marker Phenotyping for Relevant Cell Populations.

Relevant subpopulations were measured using 5- and 10-color measuring panels allowing for parallel detection of 5 or 10 antibodies.

The vitality dye 7-AAD (7-aminoactinomycin) was used in the analysis to distinguish between vital and dead cells. Single and double platform methods were used for the analysis. Single platform measurement allows simultaneous determination of absolute cell number and cell identification during flow cytometric measurement. Latex beads (Flow-Count ™ Fluorospheres) were used for absolute cell count determination according to internal laboratory standard. A defined bead concentration was used to calculate the cell number per sample volume. The dual platform does not provide absolute cell counts simultaneously with the flow cytometric measurement, but uses an additional hemocytometer (Beckman Coulter, Ac.T diff counter) to determine the absolute leukocyte count/µl. Cell counts for subpopulations were then calculated as a percentage of previously measured leukocytes.

Sample preparation

A no-wash procedure was used to analyze the various lymphocyte subpopulations (T, B, and NK cells) and their various other subpopulations. Antibodies from 1.0 to 10 µL were used, after being titrated according to the best signal to noise ratio. Antibodies were then added to 100 µl of the previously 1:50 attenuated (PBS and 5% HSA) samples.

After thorough blending using a vortex, samples were incubated for 15 minutes in darkness and at room temperature (RT). After antibody incubation, 2 mL of Beckman Coulter IO Test 3 (NH_4Cl) was used to perform adequate erythrocyte lysis in PBSC samples, followed by 10 minutes of incubation as before. No lysis was required for stem cell samples. Instead, PBS + 5 % HSA was added to obtain adequate sample volumes. Finally, Flow-Count™ latex beads were added, followed immediately by flow cytometric measurement.

CD45RA relevant specifications

The CD45RA depletion antibody is paired with a magnetic particle so that the magnetic field of the CliniMACS® device (see 5.3.5) retains the labeled cells during the depletion process. Therefore, depletion antibodies are comparatively large molecules that attach to CD45RA+ cells, which can lead to steric hindrance or, in other words, occupation of antigen binding sites when stained with a fluorescent dye for detection purposes after depletion. This means that due to the molecule size of the depletion antibody, the antibodies used for quality control after depletion have fewer antigen regions to bind to. For this reason, there is a risk that CD45RA+ cells will be molecularly masked and more difficult to detect after labeling with the depletion antibody. Knowing this phenomenon and aiming to avoid molecular masking, three different CD45RA antibodies were tested to determine the best choice for creating the 10-color FACS panel. Three CD45RA antibodies were tested: Pacific Blue, FITC and PE.

5.3.2 CD62L/CCR7 Surface Marker Expression After Cooled Storage

Stem cell products can be stored at 2 to 8 °C for up to 72 h prior to administration to recipients. Memory T cells can be further differentiated based on their ability to carry coming markers (CD62/CCR7). To ensure reliable detection and differentiation of Memory T cells even after cooling, a series of cooling assays were performed. Both CD3+CD4+ and CD3+CD8+ cells were considered. Sample preparation was performed as previously described. However, NH_4CL lysis was required because peripheral blood was used. Measurements were conducted at four time points (0 h, 24 h, 48 h and 72 h). In the meantime, samples were stored at 2 to 8 °C.

5.3.3 Detection of Virus-specific T Cells Within Depleted Grafts

Fluorescent MHC multimer antibodies were used to detect virus-specific T cells. Fluorescent MHC multimers are antibodies configured by multiple identical MHC complexes. Each MHC complex consists of an MHC molecule and a presented

antigen-specific peptide. However, a single TCR has a low affinity for the MHC complex, which complicates antigen-specific T cell recognition. By using MHC multimer technology, multiple TCR from a single T cell are bound to the MHC-complex, increasing antibody-antigen-avidity and thus resulting in more stable binding between T cells and MHC-multimer. MHC multimers are available for the detection of CD4+ T cells via MHC class II molecules and for the detection of CD8+ T cells via MHC class I molecules. In addition, various antigens are available, e.g. for malignancies, autoimmune deficiencies, and, being importantly for this work, viral infections such as CMV, AdV and EBV.[60–62]

Depending on how many MHC molecules are bound to the basic structure of the antibody, MHC multimers can be tetra-, penta- or dextramers. By coupling a fluorescent dye to the basic structure, flow cytometric detection is possible.

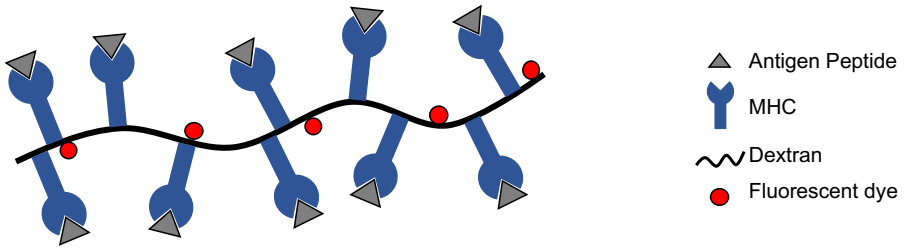

Figure 6 Dextramer Technology. *MHC multimer antibodies are composed by a dextran framework, several MHC-antigen-peptide-complexes and fluorescent dye for FACS detection. The optimized number of MHC molecules facilitates a reliable and stable bound between MHC multimer and a specific T cell. Illustration edited based on Immudex product sheet.*[63]

In this work, different MHC multimers were used depending on HLA typing of the donor. The dextramers therefore carry a donor specific HLA allele and the specific virus peptide sequence.

Detected Virus	HLA Allele	Peptide Sequence
CMV	HLA - A* 0201	pp65
	HLA - A* 0301	pp65
EBV	HLA - A* 0201	BMLF1
	HLA - A* 0201	LMP2A
	HLA - B* 0801	EBNA3A
	HLA - B* 3501	EBNA1
AdV	HLA - A* 0101	Hexon

Table 12 Virus-Specific T Cell DetectionUsing HLA-Allele Matching MHC Multimers.

Sample preparation for frequency detection was performed as follows. T cells ($1x10^6$) were transferred to a FACS® tube and washed with 2 mL PBS + 0.5% HSA (5 minutes, 300 g, 8/7, RT).

All dextramers used were conjugated with PE as a fluorescent dye, therefore each HLA/peptide sequence was incubated separately for 10 minutes. After incubating with the dextramer, 1 µl CD4 APC A750, 2.5 µl CD8 APC A700, 5 µl CD3 APC, 5 µl CD45KO and 10 µl 7AAD were added and incubated for an additional 15 minutes at room temperature and in the dark.

After incubation, all samples were washed one more time with 2 mL PBS + 0.5% HSA (5 minutes, 300 g, 8/7, RT). Finally, after removing the excess, all cells were transferred to 350 µL phosphate buffered saline (PBS) + 0.5 % human serum albumin (HSA) mixed thoroughly, and measured using Navios flow cytometer. A negative control measurement was conducted to for each measurement to ensure correct identification of the positive and negative fraction. The frequency of antigen-specific T cells was measured before and after CD45RA depletion.

5.3.4 Defrosting Process of Cryopreserved Stem Cell Products

As part of the feasibility evaluation of CD45RA depletion from cryopreserved starting products, the preserved products had to be thawed prior to CD45RA depletion processing. Cryopreserved products were removed from storage and thawed under visual control using the Barkey Plasma Therm until they were only partially iced (approximately 4 minutes). Meanwhile, the medium was preheated to 37°C. Immediately after thawing, the products were washed twice with PBS + 0.5 % HSA (10 minutes, 400 g, 9/1, 20°C,). After draining the supernant, NaCl and Heparin were added. In some cases, DNAse was additional added to prevent clotting. If clotting was too advanced despite anticoagulation measures, the product was filtered with a PALL filter. The DNAse was incubated at room temperature for 30 minutes, mixed continuously and washed twice as before. FACS measurements were then performed to evaluate the thawing process.

5.3.5 CliniMACS Technology

Stem cell processing was performed using CE- certified CliniMACS® technology (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The CliniMACS® plus instrument is a semi-automatic cell processing device that can be placed in a clean room to meet GMP regulations. Its tubing set enables completely sterile handling. Cell processing follows the principle of positive or negative selection. The underlying concept of graft purification with the CliniMACS device follows immunomagnetic depletion of unwanted naïve CD45RA cells. The main component of the systems is the magnetic column, which is placed in the magnetic field of the instrument. The labeled cell mixture is applied to the column and peristaltically pumped through the tubing set to ensure continuous flow rates. Magnetically labeled cells are retained in the magnetic field while unlabeled cells pass through the column. Depending on the calculations of the integrated computer system, the cells are applied several times and then collected in their dedicated cell collection bag (CCB) or non-target cell collection bag (NTCB).

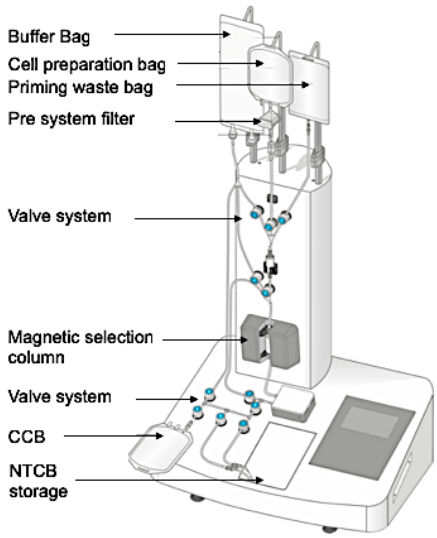

Figure 7 CliniMACS Plus Instrument. *The CliniMACS device enables cell separation of magnetically labeled and unlabeled cells. Cell suspensions are peristaltically pumped through the device and through the magnetic column, which is located in the magnetic field. As cells pass through the magnetic field, magnetically labeled cells are retained while unlabeled cells are allowed to pass. After processing by the instrument, desired and undesired cells are collected within the non-target cell bag (NTCB) or cell collection bag (CCB). Figure edited and translated from Miltenyi Biotec technical data sheet.*[64]

CD45RA depletion using CliniMACS technology

In CD45RA depletion, cells are labeled with the CD45RA depletion antibody of the CliniMACS Depletion Kit. The CD45RA depletion antibody recognizes the 220 kDa isoform of the CD45 antigen, which is expressed in large amounts by all hematopoietic cells and leukocytes ("common leukocyte antigen", LCA). The sample sizes used for CD45RA depletion were particularly small because excess samples were used, resulting in a reduction of the depletion antibody to a total of 1.5 to 3 ml per depletion. The manufacturer's recommendations for 7.5 mL antibody volume required a total cell concentration of 50 x 10^9 WBC and 20 x 10^9 CD45RA+ cells. The cell concentration limits were met but adjusted according to the reduced sample size. Cell concentrations were measured with the established FACS panel and cross-referenced with the Total Nucleated Cell Count (ACT-diff., Beckman Coulter). After accurate adjustment of all cell

concentrations, cells were incubated with the depletion antibody in an adjusted incubation volume (50 mL) for 30 minutes at room temperature and stored on a rotator. After incubation, cells were washed twice with phosphate buffered saline (PBS) containing 5% HSA (15 min, 300 g, no brake, RT) and transferred to the CliniMACS cell processor. All depletion runs were performed using depletion 3.1 software and a DTS tubing set (Miltenyi Biotech, Bergisch Gladbach, Germany). After completion of the depletion process, CD45RA+ depleted cell populations were analyzed for cell composition, purity and viability. In addition to quality control, negative controls were performed using spiking method i.e., CD45RA+ cells from the NTCB were added to the quality control samples, which allowed accurate gating in FACS analysis. CD45RA depletion was performed using two GMP-compliant purification strategies. In the one-step approach, direct CD45RA depletion was performed from CCS-F- stimulated excess stem cell samples. In the two-step approach, CD45RA depletion was performed from the negative fraction (NTCB) after a preceding CD34 selection The procedure the same, only the starting products differed.

5.3.6 Statistical Methods

Statistical analysis and graph generation were performed using GraphPad Prism 6 software (GraphPad Software, San Diego, USA).

The Mann-Whitney-Test was used to calculate the statistical significance between each group.

The Mann-Whitney-Test was the significance test of choice because the N-numbers used were small, there was no normal distribution in the data, and therefore no T-test could be applied. Differences were considered significant if $p<0.05$.

Flow cytometric measurements were analyzed using Kaluza® and Navios software (Beckman Coulter, Krefeld, Germany).

6. Results

6.1 Establishment of a 10 Color FACS Panel for Quality Control

The establishment of a 10 color FACS measurement panel formed the backbone for all experiments in this work. It was used for preliminary experiments such as CD45RA co-expression measurements. It was also used for quality control (QC) measurements to ensure graft safety and to evaluate the depletion process. The establishment of a 10 color FACS measurement panel was performed considering general principals of fluorescents and FACS measurement. First, the CD markers of particular interest for this project were identified: CD45RA, CD34, CD19, CD3, CD56 and the homing markers CD62L and CCR7. To achieve the highest possible data quality, fluorochromes were matched to the antigen expression of the cell population of interest, i.e., CD antigens with low expression were stained with bright fluorochrome-conjugated antibodies and vice versa to minimize the interaction between highly and less expressed antigens. To avoid spillover, the key markers were placed so that they were energized by different lasers, allowing spatial separation. In addition, the highly expressed leukocyte marker CD45 was stained with Krome Orange and placed on a silent channel.

Since CD34 is the stem cell marker and therefore has relatively low antigen expression, a bright fluorochrome such as APC A750 (APC-Alexa Fluor 750) was required to ensure reliable detection. Since APC A750 is a tandem fluorochrome that is relatively unstable when exposed to light, pipetting needed to be conducted in the dark. Antibody volumes were set according to titration to achieve optimal antibody reagent concentration for best signal-to-noise ratio and minimization of background pollution. Other antibodies that were needed for accurate cell characterization but did not require CD45RA-specific selection were used according to the already established internal quality control. In addition to careful selection of fluorochrome-conjugated antibodies, any measurement overflows were corrected using Navios software. To validate the new protocol, measurements were performed with two flow cytometers (Navios and FC500)

and with different CD dyes to verify the accuracy of the measurements. When comparing the collected data, no significant deviations were found between the two flow cytometric measurements, thus demonstrating the reliability of the established 10-color measurement panel.

6.1.1 Staining of CD45RA+ Cells for Processing Evaluation

Appropriate staining and development of an accurate FACS measurement panel for CD45RA+ cells were critical. A bright and stable fluorescent dye was required for accurate quantification and identification of CD45RA+ cells. The CD45RA depletion antibody used is an antibody targeting the 220kDa isoform of the common leukocyte antigen (LCA). The occupation of binding sites with CD45RA depletion antibody complicated flow cytometric quality control. Therefore, three different CD45RA-antibodies with different fluorochromes and clones were tested to determine the best choice for the 10-color FACS panel. The following antibodies were analyzed: CD45RA-FITC (clone: ALB11), CD45RA-Pacific Blue (clone: 2H4) and CD45RA-PE (clone: 2H4). To make decisions on the best antibody choice, the staining index was used as an evaluation tool. It was used to evaluate the brightness of the stain. It is a measure of how well stained cells can be distinguished from unstained cells and consequently, how reliably stained cells can be detected. Because the depletion antibody presumptively inhibits FACS antibodies, the use of dyes with a high staining index was critical to detect CD45RA+ cells after staining with the depletion antibody and the FACS detection antibody. The CD45 depletion antibody was considered a disruptive factor for the stain index as it decreased the staining index and thus the resolution, ultimately resulting in less accurate detection of the respective cell population.

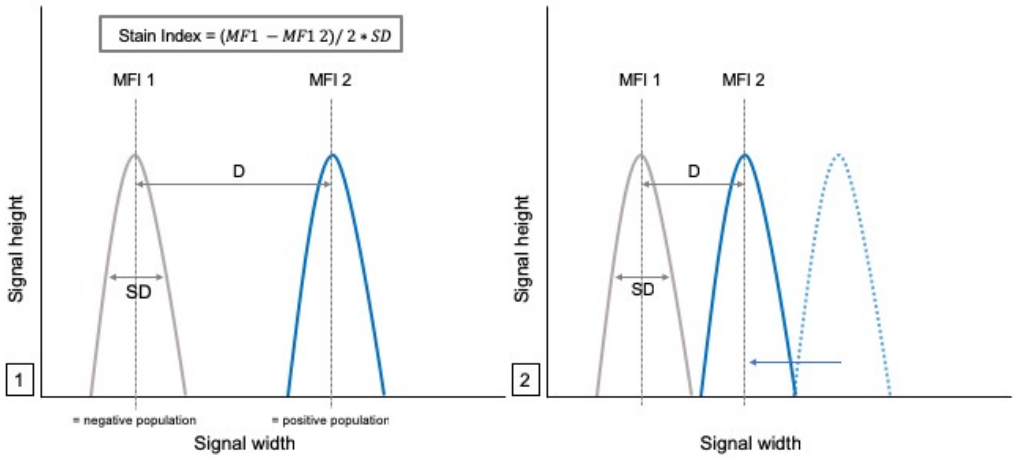

Figure 8 Stain Index. *The stain index describes the effective brightness of a fluorescent dye depending on the difference (D) between antigen negative (grey, Mean fluorescent Index (MFI 1)) and positive (blue, MFI 2) populations. 1. Shows that the ratio of separation between constant variables. 2. Shows the possible effect on the stain index when disruptive factors e.g., CD45RA depletion antibody or other influential effects (e.g., autofluorescence, non-specific staining) bias the stain index and therefore the degree of high-resolution population separation. The stain index is calculated via the difference of both mean fluorescent index divided by two times the standard deviation of both populations.*

Changes in the staining index were observed in flow cytometric measurements of CD45RA+ cells before and after labeling with the CD45RA-depletion antibodies. The respective changes are shown for three different flow cytometric dyes (see Figure 9, below).

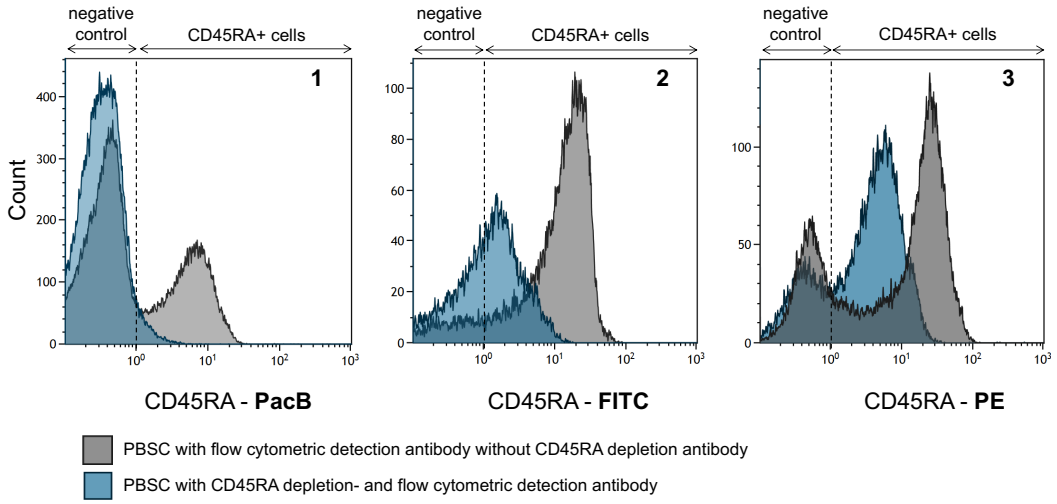

Figure 9 Stain Index Changes Due to Steric Hindrance. *Shown are three overlay plots of PBSCs stained with (blue) or without depletion antibody (grey). The log histograms shown illustrate the intensity distribution of CD45RA+ cells stained with the respective antibodies (x-axis) plotted against measurement events (y-axis). A negative control was used to distinguish the populations. Three FACS antibodies were tested and compared. CD45RA-Pacific Blue (Graph 1) showed inadequate separation between CD45RA+ and CD45RA- cells after additional contact with the depletion antibody, while CD45RA-FITC (plot 2) showed better separation and CD45RA-PE (plot 3) shows the most adequate separation. Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

Figure 9 shows CD45RA+ cells stained with a flow cytometric detection antibody. A negative control was used to distinguish the populations. All signals shown in blue represent CD45RA+ cells carrying both the FACS antibody and the CD45RA depletion antibody. The signal level correlated with the intensity of fluorescent light detected by the flow cytometer and thus with the binding ability of the corresponding fluorescent dye. CD45RA+ cells stained with the fluorescent antibody Pacific blue were indistinguishable from CD45RA- cells once exposed to the depletion antibody (Graph 1). In comparison, the use of FITC to stain CD45RA+ cells allowed a clearer distinction between CD45RA+ cells and CD45RA- cells. However, the results of CD45RA+ cells stained with the depletion antibody and FITC were close to the negative control (CD45RA- cells). Due to the lower fluorochrome brightness of FITC, staining with CD45RA-FITC was not optimal for detecting cells with low CD45RA expression. In contrast, CD45RA+

cells labeled with both the depletion antibody and the FACS antibody PE showed the best differentiation between CD45RA+ cells carrying the depletion antibody, those not carrying it, and CD45RA- cells (negative control). This led to the decision to use using PE as the fluorescent of choice for CD45RA+ cell to ensure that all CD45RA+ cells are detected even after staining with the depletion antibody and to prevent molecular masking. The use of PE as the fluorescent agent of choice ensured adequate quality control after each depletion process, as depleted products can be measured without missing potentially remaining CD45RA+ cells. In addition, PE is known to be particularly stable and bright, which made it a suitable choice for important CD markers. In view of a clearer separation of the CD45RA-negative and -positive fraction, CD45RA-PE was selected as a suitable staining antibody. It is interesting to note that CD45RA-PE and CD45RA-PacB have the same antigen clones, but the detection of cells labeled with the depletion antibody was not possible with the CD45AR-PacB antibody. This may be due to the lower staining index and fluorochrome brightness of the CD45RA-PacB antibody.

Blue laser					Red laser			Purple laser		
FL1	**FL2**	**FL3**	**FL4**	**FL5**	**FL6**	**FL7**	**FL8**	**FL9**	**FL10**	
FITC	**PE**	**ECD**	**FL4**	**PC7**	**APC**	**APC A700**	**APC A750**	**PB**	**KO**	**+ Beads**
CD62L	CD45RA	CD45RO	7AAD	CD56	CD3	CD19	CD34	CD14	CD45	
5.0 µl	**2.5 µL**	**5.0 µL**	**10.0 µl**	**2.5 µL**	**5.0 µl**	**1.0 µl**	**2.5 µl**	**1.0 µL**	**5.0 µl**	

Table 13 10-Color FACS Panel for Quality Control *of the CD45RA depletion process. The 10-color FACS panel includes all relevant cell populations for reliable quality control measurements before and after thevCD45RA depletion process. The table shows which surface antigen-specific fluorescent dye was detected in the respective channel (FL1-10) and laser (blue, red or purple). The antibody volumes shown below are based on titration experiments to obtain the best signal-to-noise ratio. The addition of beads allowed a dual platform measurement. Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.1.2 CD62L/CCR7 Surface Marker Expression after Cooled Storage

To ensure correct T cell identification and counting even after possible cooling times of up to 72 hours before SCT, the stability of the homing markers CD62 and CCR7 was tested during cooling (see 5.3.2).

To evaluate the stability of each CD marker, changes in mean fluorescence intensity (MFI) within 72 hours were analyzed. Samples were refrigerated and measured after 0 h, 24 h, 48 h and 72 h. The analysis performed showed no significant differences between the two antibodies. However, visual evaluation showed that CCR7 provided more distinct differentiation compared to CD62L when samples were cooled.

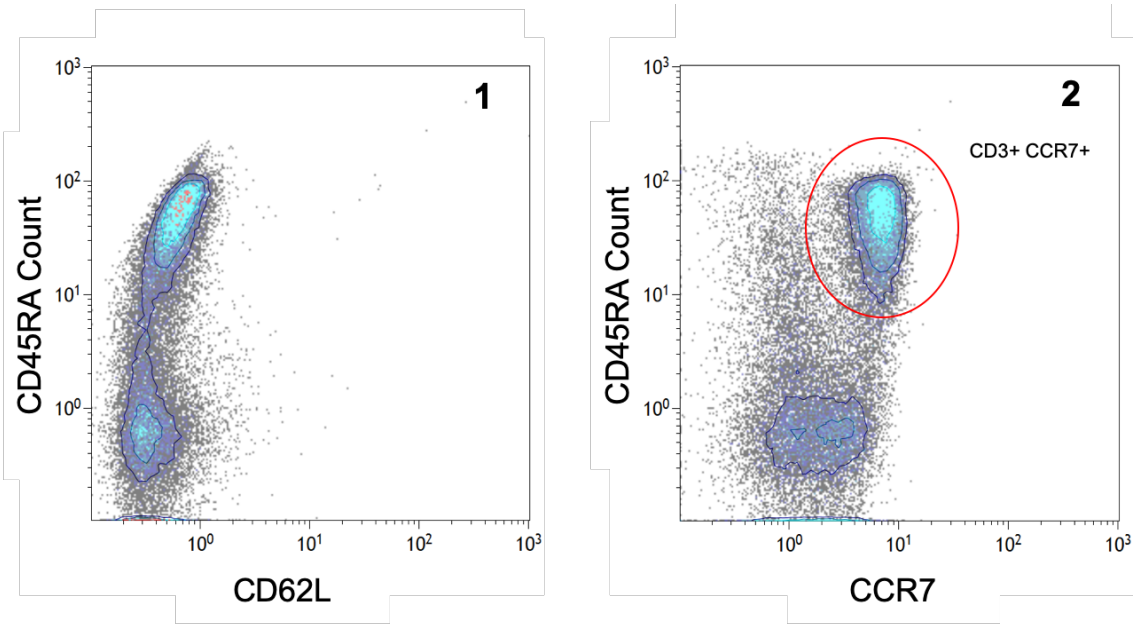

Figure 10 Exemplary FACS Plot Comparing Measurement Resolutions *of CD62L and CCR7 homing markers on cell surfaces after cooling for 48h. CD62L and CCR7 markers allow further CD3 cell characterization and identification of CD62L/CCR7+ T memory cells. Visual evaluation showed that CCR7 provided more distinct differentiation compared to CD62L when samples were cooled. Samples were measured at four points in time (0 h, 24 h, 48 h, 72 h).*

Figure 10 shows two FACS plots of an exemplary PBSC measurement using CCR7 and CD62L for additional CD3+ characterization. While CD62L did not

allow accurate differentiation between positive and negative population, cooled samples stained with CCR7 did. Based on this finding, CCR7 was used as the marker of choice when samples were cooled. In cases where samples are not previously cooled but processed immediately after extraction, CD62L may provide better resolution. This led to the conclusion that inclusion of both antibodies in the CD45RA measurement panels would provide the most accurate identification possible. CCR7 was therefore included as an additive in a second measuring tube for FACS analysis, containing CD antibodies needed for further T cell subset measurements (see table 14) and allowing reliable CD45RA detection after cooling.

Blue laser					**Red laser**			**Purple laser**		
FL1	**FL2**	**FL3**	**FL4**	**FL5**	**FL6**	**FL7**	**FL8**	**FL9**	**FL10**	
FITC	**PE**	**ECD**	**FL4**	**PC7**	**APC**	**APC A700**	**APC A750**	**PB**	**KO**	**+ Beads**
CD62L	CD45RA	CD45RO	7AAD	CD56	CD3	CD8	CD4	CCR7	CD45	
5.0 µl	**2.5 µL**	**5.0 µL**	**10.0 µl**	**2.5 µL**	**5.0µl**	**1.0 µl**	**2.5 µl**	**1.0 µL**	**5.0 µl**	

Table 14 Additional 10-color FACS Panel for T cell Characterization and Reliable T_M Detection After Cooling. *An additional 10-color FACS panel was established to adequately detect T memory cells in situations where samples are cooled or further T cell characterization (CD4+,CD8+) is required. The table shows which surface antigen-specific fluorescent dye was detected in each channel (FL1-10) and laser (blue, red or purple). The antibody volumes shown below are based on titration experiments to obtain best signal-to-noise ratio. The addition of beads allows a dual platform measurement. Results are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.1.3 Quality Control and Gating Strategy within Measurements

A gating strategy was established for quality control before and after CD45RA depletion to ensure accurate graft composition enumeration and minimize intra-laboratory variations. Residual erythrocytes included after lysis were excluded using a gate placed on all viable CD45+ cells in a CD45RA/side-scatter (SS) plot. The viability dye 7AAD was used to distinguish dead and viable cells. Monocytes

were detected using CD14. Stem cell detection and enumeration were performed according to the International Society of Hematotherapy and Graft Engineering (ISHAGE) guidelines. To ensure accurate gating after depletion, a quality control spike measurement was added as well as additional NTCB measurement to ensure that no residual CD45RA+ cells were overlooked due to inadequate gating. Figure 11 shows the integration of a quality control spike measurement for.

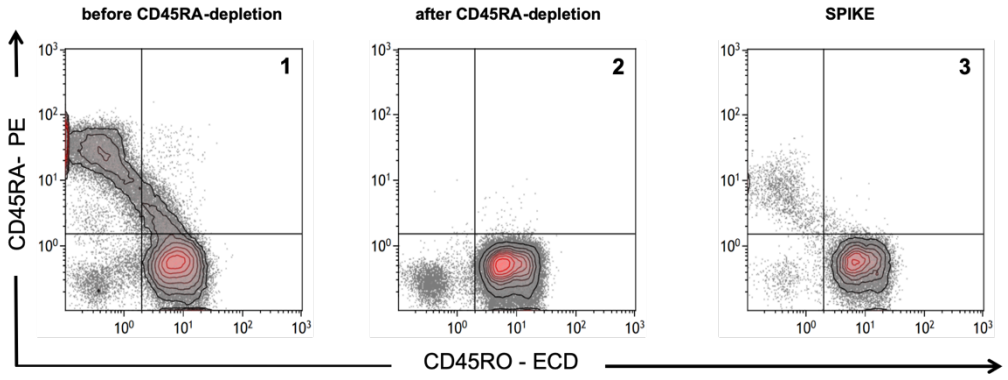

Figure 11 Spike Measurement after CD45RA Depletion *within the quality control for the depletion procedures. The three plots show CD45RA and CD45RO expressions gated on WBCs. Plot 1 shows the measurements before depletion, while plot 2 shows measurements after depletion. To ensure valid measurements of remaining CD45RA+ T cells after depletion, a spike control (plot 3) was established. For this purpose, a 1:6 mixture of cells from CCB and NTCB was measured after CD45RA depletion Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.2 Co-Expression of CD45RA on Relevant Cell Populations

To provide an accurate estimate of the probable cell loss due to CD45RA depletion, co-expression of CD45RA was measured on all relevant cell populations using the established 10-color FACS panel (see table 13). To obtain sufficient data, n=20 PBSCs, n=3 fresh CCBs after CD34 selection and n=7 cryopreserved CCBs after CD34 selection were analyzed.

6.2.1 Analysis of Peripheral Blood Stem Cell Products

Analysis of CD45RA co-expression on relevant cell populations in n=20 PBSC samples showed that the surface marker CD45RA is co-expressed at varying

percentages. With a median of 99.9% (range 99.4 to 99.9%) close to all CD19+ B cells co-expressed the CD45RA marker. NK cells also showed a comparatively high CD45RA co-expression with median 95.2% (range 56.5 to 98.8%).

T cells showed a co-expression of median 61.6% (range 37.8 to 99.3%). Similar to T cells, natural killer-like T cells co-expressed CD45RA at a median of 59.8% (range 31.6 to 92.8%). A median of 16.9% (range 9.3 to 30.4%) CD34+ stem cells co-expressed CD45RA. Thus, in some PBSCs, almost one third of the contained stem cells carried the additional surface marker.

Cell population	**CD45RA Co-Expression**
CD34+ Stem cells	16.9% (range 9.3 to 30.4%)
CD19+ B cells	99.9% (range 99.4 to 99.9%)
CD56+ NK cells	95.2% (range 56.5 to 98.8%).
CD3+ T cells	61.6% (range 37.8 to 99.3%).
NK-like T cells	59.8% (range 31.6 to 92.8%)

***Table* 15 CD45RA Co-Expression Measurements**. *CD45RA co-expression was analyzed in n= 20 PBSC samples on Stem cells, B cells, NK cells, T and NK-like T cells. Results are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

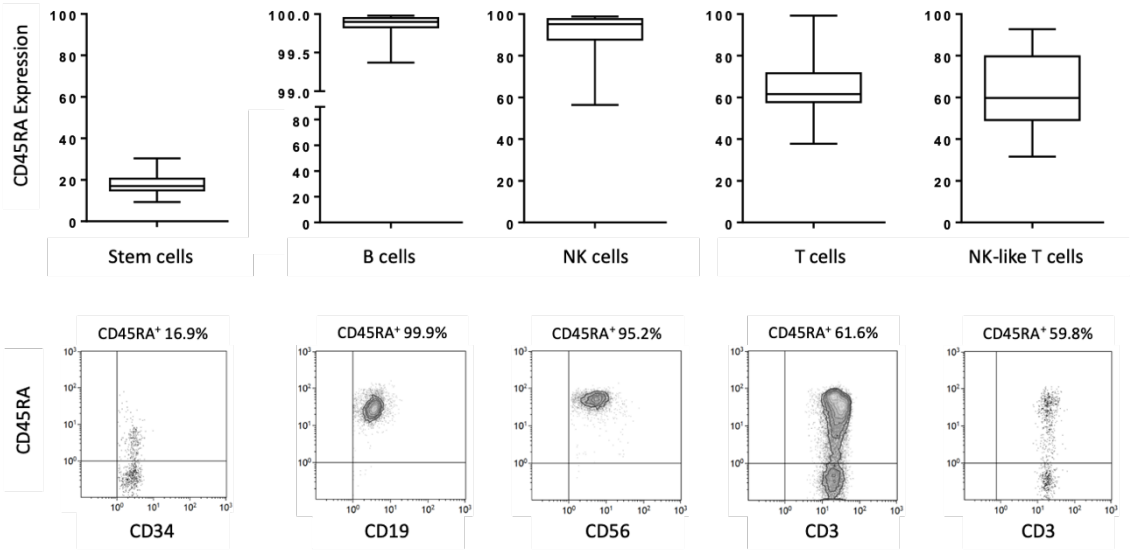

Figure 12 CD45RA Co-Expression Measurements *. CD-45RA co-expression measurements on stem cells and leucocyte sub sets illustrated as Box-Whisker plots (upper row) and FACS plots (lower row). CD45RA co-expression was analyzed in n= 20 PBSC samples on stem cells, B cells, NK cells, T and NK-like T cells. Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.2.2 Analysis of Highly Purified CD34+ Stem Cell Products

PBCS used for CD45RA co-expression measurements showed a high percentage of CD45RA co-expression on CD34+ stem cells. However, the percentage of included stem cells in PBSC samples was sometimes quite low, so that erroneous measurements could not be excluded with certainty. For this reason, highly purified CD34+ stem cells were additionally measured after CD34 selection (CCB). The data thus obtained allowed additional validation of the data already obtained. To further optimize the obtained data on CD45RA co-expression on CD34+ stem cells, both fresh (n=3) and cryopreserved highly purified stem cell products (n=7) were measured and analyzed. The median CD45RA co-expression on stem cells was 22.4% (range: 21.9 to 22.4%) for fresh and 15.9% (range: 7.56 to 17.53%) for cryopreserved CCBs. Comparison of CD45RA co-expression on stem cells within PBSCs and CCBs revealed no statistically significant difference (p=0.94; Mann-Whitney-Test).

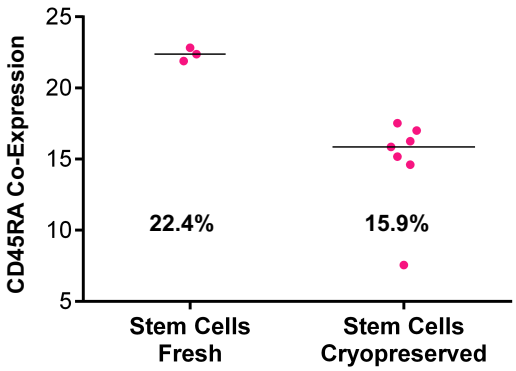

Figure 13 CD45RA Co-Expression on CD34+ Stem Cells. *CD45RA co-expression on stem cells was measured on highly purified CD34+ stem cells after CD34 selection (n=3) and compared with data collected from cryopreserved CD34+ stem cells (n=7).*

6.3 CD45RA Depletion from Cryopreserved Grafts

In clinical practice, surplus stem cell products not needed for immediate patient therapy are cryopreserved and are available later as a therapeutic option, e.g., in case of disease relapse or insufficient engraftment. Cryopreserved grafts have different depletion characteristics compared to fresh products, and processing is challenging. The ability to flexibly administer SC products at a later stage of therapy is desirable for clinical practice. Processed SC products are then available without repeated donor SC collection, allowing therapeutic freedom in choosing the best processing method for a patient's situation.

N=20 excess products from deceased patients served as samples for performing CD45RA depletion after cryopreservation. A thawing procedure was required to perform CD45RA depletion from cryopreserved grafts. Although adequate measures were taken to prevent coagulation and agglutination of the grafts, agglutination could not be prevented. CD45RA depletion was not possible due to exceptional agglutination during depletion preparation. Due to clumping of the depletion column of the CliniMACS device, cell loss was disproportionately high

and did not allow further use of the collected data. Within the scope of this work, it was not possible to perform CD45RA depletion from cryopreserved SC grafts.

6.4 Evaluation of CD45RA Depletion Processing

For evaluation of a depletion process and quality control of the depleted product two parameters were of high importance.

1. Recovery data – “Does the depleted product contain all important cell populations? Can they be rediscovered?”
2. Log depletion data – “Were all unwanted cells removed?”

Both, recovery data and log depletion data were examined, focusing on the differences between a one – and two-step approach.

6.4.1 Recovery Data

The median with total white blood cell (WBC) recovery after CD45RA depletion was of 30.1% (range: 16.4 to 45.8%) in the one-step approach (n=3), and slightly lower than the median total WBC recovery obtained in the two-step approach (n=4) which was of 42.05% (range: 28.5 to 61.5%).

Rinsing the depletion column after CD45RA depletion to detect the lost cells revealed that 44% (n=1) of all cells contained in the depletion column were CD45RO+ lymphocytes (see Figure 14 below).

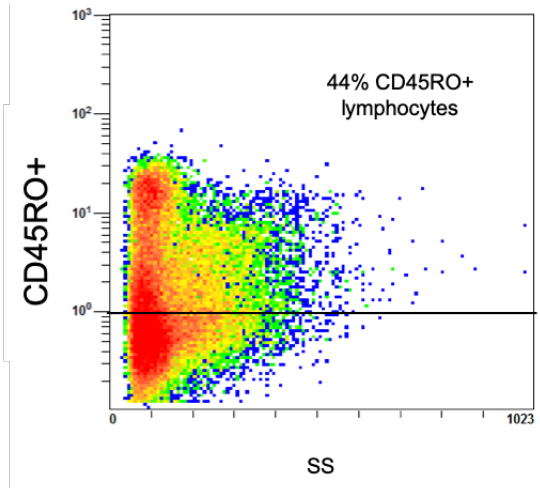

Figure 14 Measurement after Depletion Column Rinsing. *Measurement of cells contained within one CD45RA depletion column after depletion. The FACS plot shows that 44% of all cells contained within the depletion column are CD45RO+ cells (gated on WBCs).*

Within the one-step approach (n=3) stem cells recovered at a median of 52.0% (range: 49.7 to 57.2%). Because the two-step approach used NTCB after CD34 selection, the recovery of CD34+ stem cells was measurable only with the one-step approach. T Memory (T_M) cells recovered at a median of 24.2% (range: 5.8 to 45.6%, one-step, n=3) and 42.1% (range: 6.5 to 52.4%, two-step, n=4). No statistically significant difference was found between the two methods in terms of T_M recovery (p= 0.6, Mann-Whitney-Test). Also, no difference was observed in CD4+ and CD8+ memory subsets between the two purification approaches (p=0.06, Mann-Whiney-Test).

NK cell recovery was only at median 1.5% (range: 1.4 to 12.6%) for the one-step approach (n=3) and at median of 1.8% (range: 0.1 to 3.4%) for the two-step approach (n=4). These results were consistent with the high CD45RA co-expression on NK cells (median of 95.2%) detected in the preliminary measurements of CD45RA co-expression. The results are summarized in table 16 and displayed in figure15.

	Recovery Data in %	
Cell Population	**One-Step (n=3)**	**Two-Step (n=4)**
CD34+ stem cells	52% (49.7 to 67.2%)	
CD3+CD4+ T cells	20.0% (4.9 to 32.0%)	22.4% (13.4 to 33.2%)
CD3+CD8+ T cells	4.3% (1.4 to 8.5%)	8.8% (3.2 to 13.8%)
CD3+CD45RO+ T_M cells	24.2% (5.8 to 45.6%)	42.1% (6.5 to 52.4%).
CD3+CD4+CD45RO+ T_M cells	38.8% (10.6 to 53.0%)	37.5% (23.7 to 48.3%)
CD3+CD8+CD45RO+ T_M cells	13.1% (4.8 to 22.6%)	20.2% (11.0 to 25.4%)
CD3-CD56+ NK cells	1.5 % (1.1 to 12.6%)	1.8% (0.1 to 3.4%)

Table 16 Recovery Data after One (n=3) – and Two-Step CD45RA Depletion. *CD45RA depletions were performed in a one-step and two-step process. The table shows recovery data for SC, T-,T_M – and NK cells. SC recovery data are shown only for the one-step approach because SC are not part of the starting product in two-step depletions. Results and table are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

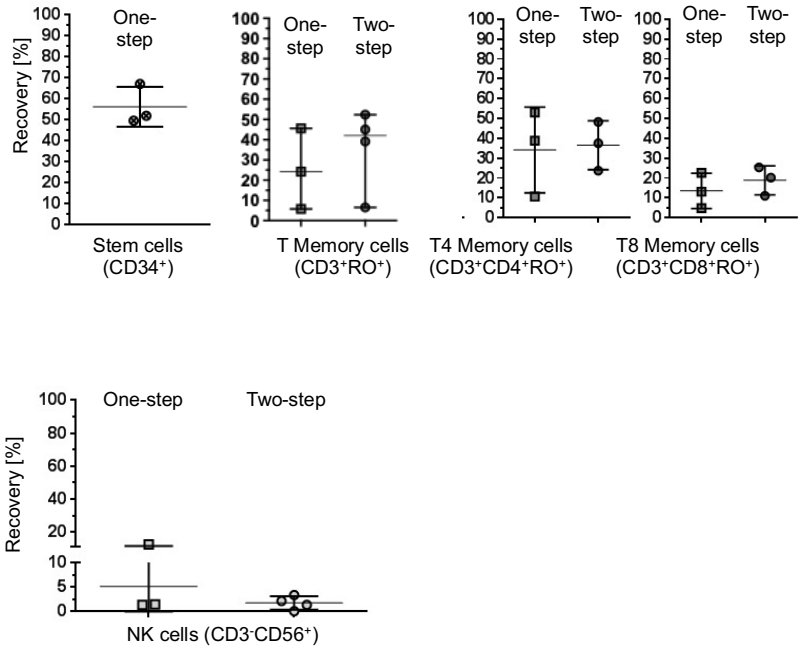

Figure 15 Recovery Datafor One- (n=3) and Two-step (n=4) CD45RA Depletion. CD45RA depletions were performed in a one-step and two-step process. *The figure shows recovery data for SC,T_M – and NK cells. For the two-step approach only T_M recovery data are shown because the starting product did not contain SC. Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.4.2 Log Depletion

For quality control and product safety, validation of adequate removal of unwanted cell populations, in this case naïve T cells as well as B cells, was of great importance. A reliable parameter to assess the quality of the depletion process is the so-called log depletion. Log depletion is calculated as follows: Log(CD45RA+ cells (abs) before/ CD45RA+ cells after depletion). Smaller log depletion values imply better efficiency of the depletion process. A log depletion value of 0 indicates that no depletion has taken place.

The log depletion data for CD45RA+CD3+ T cells was not differing to a statistically significant extent (p=0.87, Mann-Whitney-Test) between both approaches with a median log depletion of -3.9 (range: -4.1 to -3.8, one-step) and -3.8 (range: -5.1 to -3.2; two-step).

B cell depletion showed no statistically significant difference between both approaches (p=0.31, Mann-Whitney-Test). The median log was at log -3.6 (range: -4.0 to -2.9) within the two-step approach, compared to median of log -3.2 (range: -3.3 to -2.9) within the one-step approach. The results are summarized in table 17 and displayed in figure 16.

	Log Depletion Data	
Cell Population	**One-Step (n=3)**	**Two-Step (n=4)**
CD45RA+CD3+ T cells	-3.9 Log (-4.1 to -3.8)	-3.8 Log (-5.1 to -3.2)
CD19+ B cells	-3.2 Log (-3.3 to -2.9)	-3.6 Log (-4.0 to -2.9)

Table 17 Log Depletion Data *after one(n=3) – and two-step(n=4) CD45RA depletion. Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

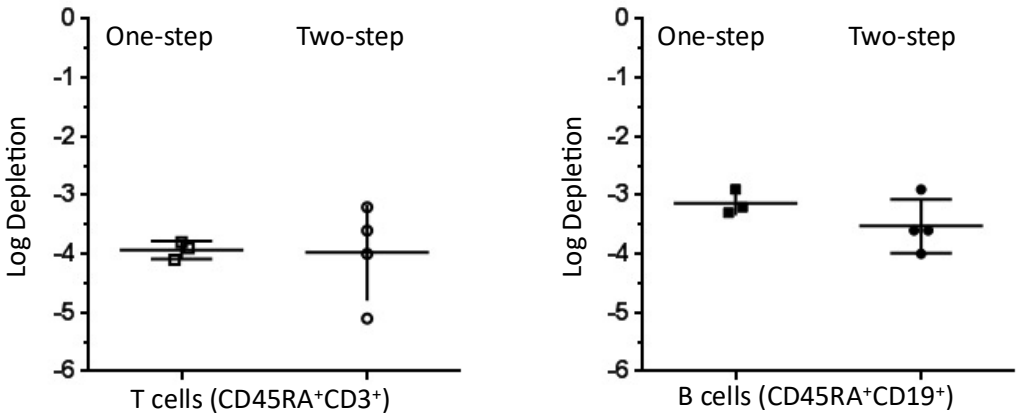

Figure 16 Log Depletion Analysis of log depletion data comparing the one- (n=3) and two-step approach (n=4). *The efficiency of depletion of CD45RA+ T and B cells is quantified using log depletion data and is calculated as follows: Log depletion= Log (CD45RA+ cells (abs) before /CD45RA+ cells (abs) after). A log depletion of 0 means that no depletion occurred. Smaller values indicate better depletion quality. Results and figure are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.5 Cell Composition of CD45RA-Depleted Products

The analysis of the overall composition of CD45RA-depleted stem cell products was another important quality control step. Knowledge of cell composition enables researchers and clinical users (e.g., physicians) to make predictions about possible outcomes after successful treatment. Figure 17 shows an example cell composition before and after performing CD45RA depletion. To demonstrate a cell composition that includes all cell populations before and after CD45RA depletion, an unmanipulated PBSC stimulated with G-CSF was used (one-step procedure).

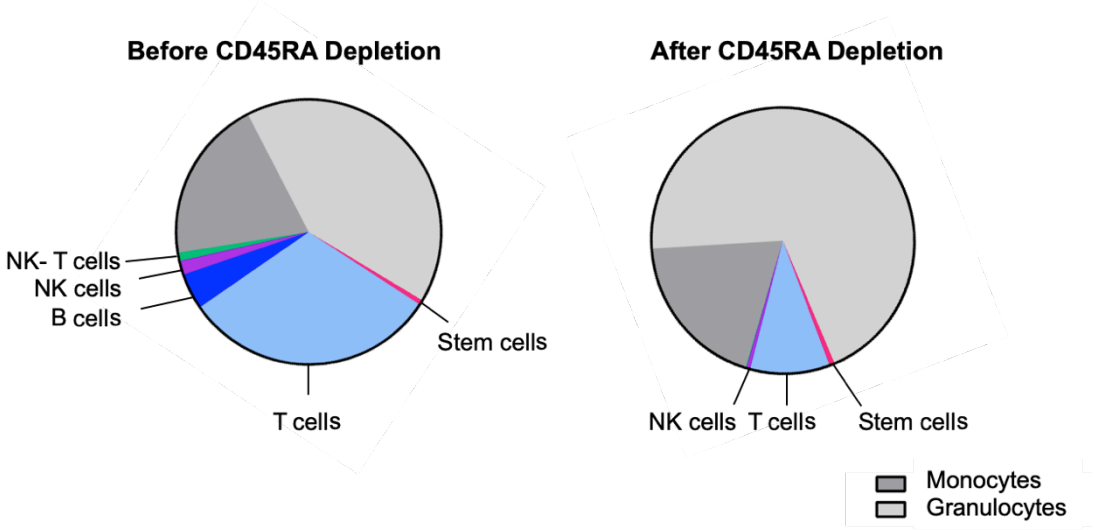

Figure 17 Cell Composition Overview before and after CD45RA Depletions. *The pie chart shows an example cell composition before and after depletion from an unmanipulated PBSC. After CD45RA depletion, almost no NK T and B cells are detectable in the graft. T cells were reduced but not completely eliminated, indicating that only CD45RA+ T cells are removed by the depletion process. NK and stem cells were still present in very low percentages.*

Before depletion, naïve CD45RA+ T cells (T_N) comprised a median of 21.6% (range: 20.6 to 26.0%) of the T cell population, whereas immunocompetent memory CD45RO+ T cells (TM) comprised a median of 10.0% (range: 6.2 to 16.7%). After CD45RA depletion in a one-step approach, grafts contained a median of 0.009% (range: 0.006 to 0.013%) T_N cells and 0.011% (range: 0.007

to 0.04%) after performing the two-step approach. T_M cells, on the other hand, were presented at a median of 9.9% (range: 5.9 to 32.8%; one-step) and 14.9% (range: 3.7 to 41.9%; two-step), suggesting that the majority of the remaining T cells were CD45RO+ TM cells.

Most B cells were removed independently from the approach, leaving a median of 0.02% (range 0.01 to 0.02%, one-step; 0.001 to 0.05%, two-step) of B cells within the overall cell composition. NK cells were represented at a median of 0.3% (range: 0.1 to 0.5%) within the one-step approach and at a median of 0.11% (range: 0.03 to 0.27%) within the two-step approach.

The change in the CD4/CD8 ratio was particularly striking. CD8+ T_c cells were removed in a greater proportion than CD4+ T_H cells, increasing the ratio in both approaches. An increase in the CD4/CD8 ratio from 1.8 (range: 1.8 to 2.0, one-step) to 6.7 (range: 6.1 to 9.1, one-step) and from 2.0 (range: 1.9 to 2.1, two-step) to 5.1 (range: 4.8 to 8.8, two-step) was observed. These results are summarized in Table 18 below.

		One-Step		Two-Step	
Cell population	**Gate**	**Before (%)**	**After (%)**	**Before (%)**	**After (%)**
T cells (CD3+)	% WBC	31.5 (26.9 - 42.7)	9.9 (5.9 - 32.84)	31.5 (19.9 - 42.9)	15.0 (3.7 - 41.9)
T helper (CD3+CD4+)	% T cells	64.3 (63.2 - 63.4)	87.9 (86.9 – 89.1)	66.6 (65.1 - 68.4)	84.9 (84.5 - 90.4)
T cytotoxic (CD3+CD8+)	% T cells	35.7 (32.7 – 35.7)	13.2 (9.8 – 14.2)	33.0 (31.8 – 34.1)	16.6 (10.2 – 17.5)
CD4/CD8 ratio		1.8 (1.8 – 2.0)	6.7 (6.1 – 9.1)	2.0 (1.9 – 2.1)	5.1 (4.8 – 8.8)
Memory T (CD3+CD45RO+)	% WBC	10.0 (6.2 – 16.7)	9.9 (5.9 – 32.8)	10.5 (4.8 – 17.7)	14.9 (3.7- 41.9)
Naïve T (CD3+CD45RA+)	% WBC	21.6 (20.6 – 26.0)	0.009 (0.006 – 0.013)	21.5 (14.1 – 25.2)	0.011 (0.007 – 0.04)
T regulatory (CD3+CD4+CD25+^{bright})	% T4 cells	4.8 (0.7 – 6.3)	7.2 (6.4 – 8.1)	5.4 (4.6 – 7.6)	8.8 (8.5 – 9.0)
B cells (CD19+)	% WBC	5.9 (4.3 – 9.1)	0.02 (0.01 - 0.02)	8.0 (4.6 – 9.3)	0.02 (0.002 – 0.05)
NK cells (CD3-CD56+)	% WBC	2.9 (1.7 – 4.9)	0.3 (0.1 – 0.5)	3.1 (0.9 – 3.6)	0.11 (0.11 -0.27)
NK-like T cells (CD3+CD25+)	% WBC	1.3 (1.0 – 1.7)	0.4 (0.1 – 0.4)	1.1 (0.6 – 1.3)	0.17 (0.04 – 0.5)
Monocytes (CD14+)	% WBC	27.0 (20.2 – 30.7)	19.6 (14.5 – 32.0)	32.6 (23.9 – 37.8)	31.1 (12.1 – 44.9)
Granulocytes (Side Scatter^{high}, Forward Scatter^{low})	% WBC	30.2 (17.4 – 41.7)	70.5 (35.2 – 79.6)	31.8 (6.3 – 55.6)	53.5 (14.3 – 83.5)

Table 18 Cell Composition before and after CD45RA-Depletion *as direct depletion (one-step) and following a preceding CD34 selection (one-step). Results and table are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

6.5.1 Virus-Specific T cells

Virus-specific T cells were detectable before and after CD45RA depletion. Frequency measurements of EBV, ADV and CMV specific T cytotoxic (T_c)cells resulted in low frequencies, however were clearly detectable among CD3+CD8+ T_c cells. The detection of AdV-specific T_c cells was possible at very low frequencies with median 0.05% (range: 0.01 to 0.29%). CMV-specific T_c cells were slightly more frequently detectable with median 0.41% (range: 0.12 to 0.81%). EBV-specific T_c cells were most frequently with a median of 0.66% with a noticeably large range from 0.02 up to 10.1%. No statistically significant differences were found between the two methods with respect to the frequency of virus-specific T cell detection (EBV: p=0.54, Mann-Whitney-Test, CMV: p=0.2, Mann-Whitney-Test; ADV: a calculation of significance was not possible due to the low n-number). Virus specific T cells recovered at median 25.1% (range: 0.23 to 84.5%) after CD45RA depletion.

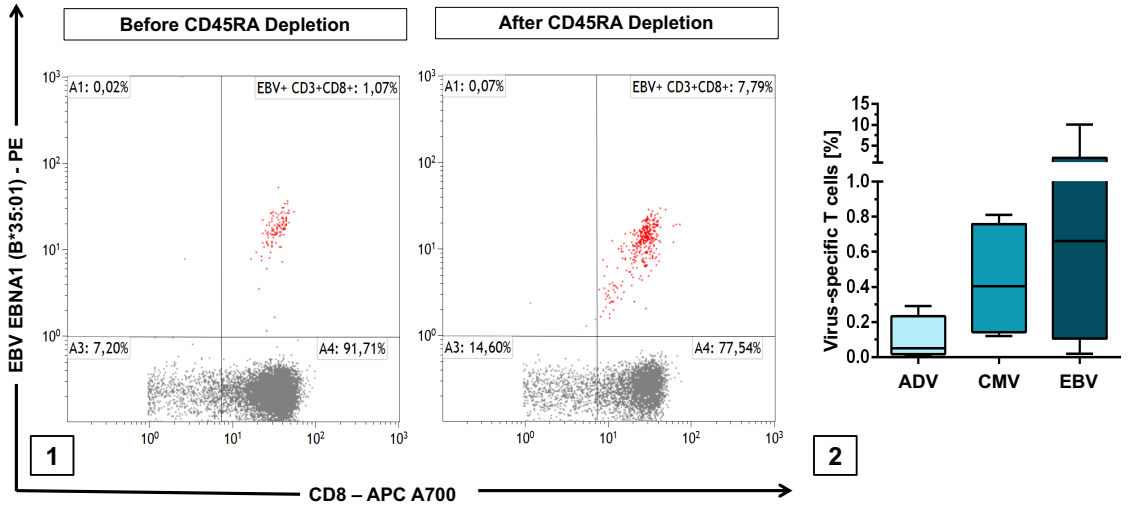

Figure 18 Detection of Virus-Specific T cells *in CD45RA depletion. Virus specific T cells were identified by staining with MHC-I and MHC-II multimers corresponding to the donor HLA type. 1.Shows an example measurement of EBV-specific T_c cells before and after CD45RA depletion (EBNA1 (B*35:01) dextramer). 2. Shows the frequency distribution of detected virus-specific T cells after CD45RA depletion. Measurements were performed in the one-step (n=3) and two-step (n=4) approaches (EBV: n=15, CMV: n=5, ADV: n=5). Results are part of a publication by Bremm et al. to which I contributed as a co-author.*[65]

7. Discussion

7.1 Establishment of a 10-color Measurement Panel

To ensure reliable and safe graft manufacturing for clinically used CD45RA-depleted stem cell grafts, the establishment of a 10-color flow cytometric measurement panel was essential for this work. The focus here was on the accurate detection and quantification of CD45RA+ cells before and after the depletion process.

Reliable detection of CD45RA+ cells was particularly challenging because the CD45RA antibody occupied binding sites that complicated cytometric quality control by interfering with the binding ability of fluorescent dyes for FACS measurements. The CD45RA depletion antibody used for depletion with CliniMACS technology is an antibody that targets the 220kDa isoform of the common leukocyte antigen (LCA) and is comparatively large. The phenomenon of binding site occupation has already been described by Bremm *et. al.* in the generation of TCR$\alpha\beta$/CD19 depleted grafts.[66] While the size of the depletion antibody is unalterable, the choice of fluorescent dye is of great importance to minimize the interfering effects of the depletion antibody and to ensure accurate cell identification after depletion. Therefore, three different CD45RA dyes were tested using different clones and their detection reliability was compared using the stain index. This showed that CD45RA-PE (clone 2H4) provided the clearest differentiation between cells labeled with the CD45RA depletion antibody and unlabeled cells within the quality control. Interestingly, CD45RA-PE and CD45RA-PacB have the same antigen clones, but detection of cells labeled with the depletion antibody was not possible with the CD45RA-PacB antibody. This is best explained by the lower staining index and fluorochrome brightness of the CD45RA-PacB antibody.

CD45RA-PE was chosen as the dye of choice (n=20) and then confirmed by comparison measurements between two cytometers (FC500 and Navios,

Beckman Coulter). The number of samples was sufficient, and the additional flow cytometric comparison showed no discrepancy between the flow cytometric instruments. Thus, CD45RA-PE fluorescent dye allowed reliable detection of CD45RA+ cells even after they have come into contact with the CD45RA depletion antibody and experienced potential receptor blockage. Another aspect considered in the generation of the QC measurement panel was to reserve the possibility of further differentiation between naïve CD45RA+ and CD45RO+ memory T cells. Further differentiation of the remaining T memory cells into central and effector memory T cells (T_{CM}, T_{EM}) may be of interest for future investigations. Therefore, CD62L and CCR7, both known markers for the corresponding cell differentiation, were considered and compared to be incorporated in the measurement panel. The applicability of CD62L for further cell differentiation has been investigated in previous studies. In these, it was described that CD62L is prone to antigen shedding, particularly in graft processing procedures in which G-CSF mobilized grafts or cryopreserved grafts were used.[67,68] However, Verfuerth *et. al.*[69] described that no CD62L shedding occurred within a 24-hour period when samples were stored at room temperature or 4 °C. The aim of this study was to establish a measurement panel that would allow characterization of cells, including memory T cells, according to their degree of differentiation, independent of storage temperature. Therefore, CD62L and CC7R were tested and compared while refrigerated for 72 hours. Comparison of the mean fluorescence intensity of both antigen markers within a 72-hour period revealed no significant differences between the two antibodies, but visual gating analysis showed a slight superiority of CCR7 over CD62L after samples were refrigerated for three days. Considering this, it would have been possible to use only CCR7 to distinguish between TCM and TEM in the future. However, CD62L showed a tendency toward clearer flow cytometric resolution for fresh grafts compared to CCR7, which could not be proven statistically but may be of help in clinical practice. Considering the respective properties of the antigen markers and the fact that not all grafts are cooled prior to processing,

both markers were included to ensure high resolution for TCM and TEM differentiation without the risk of incorrect measurements due to cooling. For this purpose, CCR7 was included in an additional measurement tube that can be referred to whenever grafts need to be cooled. The inclusion of both markers therefore will therefore allow greater flexibility in the future with regard to graft storage conditions.

7.2 Cryopreservation and CD45RA- Depleted Grafts

This study investigated the feasibility of CD45RA depletion from previously cryopreserved SC grafts. Cryopreservation is a useful tool in therapeutic strategies that allows preservation of remaining SC grafts as they might be needed later. Given the advantages of CD45RA depletions, the possibility of combining CD45RA depletion and cryopreservation seems desirable. However, cryopreserved products have different processing properties compared to fresh products, which complicates the depletion process. In the context of this work, performing a CD45RA depletion was not possible due to rapid clotting. As an alternative to performing CD45RA depletion from a cryopreserved graft, it might be useful to perform CD45RA depletion from fresh G-CSF stimulated grafts or peripheral donor lymphocytes and subsequently cryopreserve them. This approach would be similar to a post-transplant DLI approach[70] but would provide more flexibility and donor independence within the course of a treatment and could be an addition to current post-transplant practice. Often, two apheresis are taken from the donor to perform a combination of CD34 selection and CD3/19 depletion. The remaining product could be CD45RA-depleted and cryopreserved for later use. Instead of using G-CSF stimulated grafts, cryopreservation of unstimulated grafts from the donor could be considered as a reserve for later use. However, data has shown that G-CSF stimulated products are superior in terms of viability and recovery after defrosting.[32,71]

7.3 Feasibility Evaluation and Clinical Application

In this work, CD45RA depletion from G-CSF stimulated PBSC was performed using two different GMP-compliant processing strategies: direct depletion of CD45RA+ cells (one-step approach) and depletion of the negative fraction (NTCB) after a preceding CD34 selection (two-step approach).

Both processing strategies were investigated and evaluated using standard quality-specifying properties, such as log depletion and cell recovery data, as well as overall cell composition after depletion.

7.3.1 Removing Unwanted Cells: Log Depletion Data

Log depletion data describe the efficiency with which target cells, in this case CD45RA+ cells, are removed from the graft. For both processing strategies, cell reduction was possible at a similar quality with log -3.9 (one-step approach) and log -3.8 (two-step approach) and is similar to data previously published by other research groups for CD3/19 and TCR$\alpha\beta$/CD19 depletion strategies.[66,71] Compared with data from research groups that investigated CD45RA depletion processing and obtained more efficient cell reduction data (log –4.75 [72], log -4.2 [73] and log -4.4 [74]), the data obtained in this work showed less efficient cell reduction. This could be because all processed samples had very small volumes, as they were obtained as excess of G-CSF stimulated graft collections. Log depletion data may dependent on the initial target cell concentration, as other research groups have described in context of TCD.[75] This explanation seems reasonable, considering that the superior log data were generated from starting products that were not reduced in volume.[72–74]

In addition to the efficient reduction of CD45RA+ naïve T cells, B cells were also almost completely removed. This result is in line with the data obtained in the preliminary CD45RA co-expression measurements, which showed that B cells co-express CD45RA as a surface molecule at a median of 99.9%. This can be

considered an advantage of CD45RA depletions as they do not require additional anti-CD19 reagents.

7.3.2 Retaining Wanted Cells: Recovery Data

In contrast to efficient target cell reduction, recovery data is important to describe the efficiency with which beneficial cell populations can be retained in a SC graft. Consideration of recovery data is particularly important for SC, CD45RO+ T Memory cells and NK cells.

CD45RO+ T Memory recovery and residual T cells

As opposed to the efficient reduction of alloreactive CD45RA+ cells, CD45RO+ memory T cells are retained as part of the CD45RA depletion process. CD45RO+ memory T cells are reported to be immunologically competent, already differentiated upon antigen contact and capable to efficiently targeting pathogens.[26] Studies in mice, as well as *in vitro* mixed lymphocyte reactions (MLR) with human cell products, demonstrated differing reactions between naïve and memory T cells to contact with host antigen. In these studies, naïve CD45RA+ T cells showed higher alloreactive potential than CD45RO+ (CD45RA-) T cells.[28,76] In addition to the lower alloreactive potential, T Memory cells show specific immunocompetence towards viral pathogens (CMV, EBV, AdV). MLR assays have shown that CD45RO+ T cells are more responsive to pathogen antigens compared with CD45RA+ cells.[77] Triplett *et al.*[58] further described that the provision of T memory cells in stem cell grafts seems to reduce the duration as well as overall incidence of viremia of CMV, EBV or AdV.

Data collected in this work showed that when CD45RA depletion was used, T memory were retrieved at a median recovery of 24.2% (one-step approach) and 42.1% (two-step approach).

In this study, the frequencies with which virus-specific T cells were detectable in the pool of T memory cells appears to be comparatively low. However, data on the generation of virus-specific T cells as supportive therapy for severe viral

infections have shown that, despite low frequencies, an adequate virus-specific T cell response can be generated if a T memory cell pool is provided followed by an *in vivo* proliferation.[78,79]

The remaining CD45RO+ Memory T cells showed a noticeable shift in the CD4/CD8 ratio. The ratio showed a disproportionate loss of CD8+ cells compared to CD4+ cells, leading to the assumption that CD8+ cells co-express additional CD45RA surface markers and are therefore removed by the depletion process. This finding is consistent with data from other research groups postulating that CD8+ cells co-express more CD45RA surface markers.[80,81] Several studies have investigated the importance of the CD8+ T_c cell amounts after SCT, and concluded that CD8+ cell counts are related to overall survival. These findings indicate that low CD8+ counts are associated with higher relapse rates and infections due to the lack of cytotoxic effect of CD8+ T cells..[31,82] However, a large-scale multicenter analysis by Salzmann-Manirique *et al*.[83] showed, among other data, that T_c cells recover more rapidly after SCT and therefore, the CD4/CD8 ratio tends to decrease. In addition to the faster recovery of CD8+ after CD45RA depletion, it seems arguable that residual T_M cells take over T_c functions related to GvL promotion and protection against pathogens in lymphoblastic deficiency. Concurrent with the increase in CD4/CD8 ratio within the remaining CD45RO+ T cell population, evaluation of T cell subset composition showed that T_{reg} cells were retained in a comparatively high amount. This may be attributed to the fact that proportional CD8+ loss results in a higher remaining amount of CD4+ cells, which explains the higher amount of T_{reg} cells as they form a CD4+ cell subset. Several studies have shown that T_{reg} cells have immunosuppressive properties and prevent GvHD, whereas they do not inhibit GvL.[84–86]

Nguyen *et al.*[87] further argued that T_{reg} cells can enhance immune reconstitution by preventing GvHD-induced tissue damage, including thymic tissue. Protection of thymic and extrathymic lymphoid tissues enables T-cell generation and the development of an earlier TCR repertoire.[87] CD45RA depletion enables SCT

grafts that contain a greater amount of transferrable T cells, than pan-TCDs and suggests that CD45RA-depleted grafts facilitate GvL. As long as GvL is maintained, GvHD appears to be less frequent. Triplett *et al.*[77] described a better response to therapy in patients who had GvHD after SCT with CD45RA-depleted grafts. Bleakley *et al*.[27] further reported that different types of GvHD were observed in clinical trials of 35 patients who underwent two-step CD45RA depletion (with preceding CD34 selection). Although the incidence of aGvHD was similar in CD45RA-depleted grafts compared with pan-TCD, the incidence of cGvHD was significantly lower in CD45RA-depleted grafts. Furthermore, all cases of aGvHD responded to corticosteroid.[27] While the concept of GvL preservation with simultaneous GvHD prevention and transfer of immunocompetence is desirable, it is questionable whether CD45RA-depleted grafts can meet all criteria. Distler *et al.*[76] have described that CD45RA-depleted grafts carry a risk of alloreactivity due to differential immunologic capabilities within memory T subsets, as they contain central memory T cells, which in turn lack an efficient GvL effect. During maturation, T cells specify their TCR repertoire to have lower receptor diversity and thus lower alloreactivity, while becoming highly immunocompetent.[27,28] Therefore, it might not be entirely accurate to conclude that transfer of residual T memory cells will certainly enabled GvL.

Stem cell recovery

To overcome HLA barriers in haplo-SCT settings, a sufficient amount of SC is indispensable.[13] Therefore, data on SC recovery is important to ensure transplant success. In this work, SC recovery was measured with a median of 52.0% (range: 49.7 to 67.2%) using the one-step approach. Compared with the approach used by Shook et al.[88], in which one-step CD45RA depletion was performed in combination with CD3 depletion, resulting in a median CD34+ recovery of 59.2%, the CD34+ recovery data obtained in this work are within a reasonable range. However, in the data on CD3/19 depletion and CD34 selection collected by Huenecke et al.[71], which resulted in a median of 85% and 73%, respectively, SC

recovery was shown to result in a significantly higher number of recovered stem cells in the grafts than in this work. Therefore, it is necessary to find an explanation for why SC recovery data show less promising results in the case of CD45RA depletion. It is important to consider that the data on SC recovery collected in this work might not be sufficiently conclusive due to the relatively small number of cases (n=3). In addition, the one- and two-step methods were not directly compared. The collected data from the other research groups suggest that CD34 selections ensure high SC recoveries. The conclusion that CD34 selections results in significantly higher SC amounts may not be entirely accurate according to Keever-Taylor *et al*.[89] who published data showing quite substantial variations in CD34+ recovery data (29.0 to 125.6%). Craig *et al.*[90] and Fritsch *et al.*[91] previously described the co-expression of CD45RA on SC. These findings are consistent with the CD45RA co-expression measurements collected in this work, showing that the median co-expression of CD45RA on SC is 16.9%. In the published data of Bleakley *et al.*[57] median CD45RA co-expression on SC was reported to be 25.6%. Therefore, preliminary estimates of SC loss are consistent to some extent with the actual loss of SC but, this does not explain the full extent of cell loss seen in this work. In addition to SC loss due of CD45RA co-expression, further cell loss could be explained by the fact that the CD45RA depletion antibody is particularly large-scaled and therefore more likely to lead to the formation of cell conglomerates. It would be conceivable that the depletion antibody then forms something imaginable as a meshwork in which cells can easily become trapped and therefore do not recover from the depletion process but remain within the depletion column. This theory is in line with the overall cell loss found within this work. However, it could be argued that this is also true for other depletion methods (CD3/19, TCR$\alpha\beta$/CD19), which calls this theory into question. Because data obtained by other research groups show less overall cell loss and better SC recovery, it is also possible that the apparently inferior data obtained in this work are due to the fact that only small amounts of G-CSF stimulated products were used.

NK Cell Recovery

The provision of NK cells in SCT grafts constitutes for a large part of SCT research. As part of the innate immune system, NK cells have the immediate potential to clear viral infections, are discussed to influence engraftment duration and play an important role in providing GvL.[17,18] Sisinni *et al.*[72] , who investigated the outcomes of SCT after CD45RA depletion, suggested that deficiency of NK cells leads to higher incidence of HHV-6 encephalitis in transplanted patients and published that 8 out of 25 patients receiving CD45RA depleted products became infected with HHV-6.

Assuming that the absence of NK cells and their important properties can seriously harm patient receiving haplo-SCT, the data on NK cell recovery must be given special consideration. However, in this work, almost all NK cells were lost after processing, regardless of the approach chosen (one-step or two-step).

This finding was not surprising considering that preliminary measurements of CD45RA co-expression on NK cells showed that nearly almost all NK cells (median: 95.2%) were also CD45RA+ and had already been expected to be lost. A previous study conducted by Shook *et al.*[88] showed different data and published that the log depletion of NK cell in the context of CD45RA depletion was only at log values of -1.1, leaving a large number of NK cells in the graft. This enormous discrepancy between the results cannot be explained and is also in contrast to data collected by other research groups such as Bleakley *et al.*[57] Their study describes that CD56+ NK cells were depleted with a median log depletion of -3.34, which is more consistent with the NK cell loss described in this work.[57]

The importance of NK cell preservation, especially regarding protection against pathogens and the impact on early engraftment, is controversial in most processing strategies, including the CD45RA depletion strategies. Triplett *et al.*[58,77] investigated a combination of CD45RA depletion with an additional NK infusion in a DLI fashion, to provide more NK cells and ensure their beneficial properties. However, data analysis suggested that the reduction of viremia

duration was due to the additional T memory cells rather than the addition of NK cells.[58,77] In addition to those findings, Bleakley *et al.*[27] among others, demonstrated rapid engraftment (median: 13 days) after CD45RA depletions using the two-step approach without additional NK cell infusions. Regardless of the potential amount of NK cells transferred in the CD45RA-depleted SC graft or DLI, it should be considered that NK cells return relatively quickly after SCT, reaching normal cell counts after 1-4 months, whereas complete T cell reconstitution takes up to several years.[50–52]

7.4 Clinical Application of CD45RA Depletions

Because the evaluation of CD45RA depletion provides many individual results, it is quite complex to consider them in their entirety and focus on how to translate the findings into applicable strategies for clinical use. A brief summary of the results just discussed seems useful to recall relevant parameters for performing a successful SCT. CD45RA depletions provide adequate reduction of unwanted C45RA+ naïve T cells and close to complete removal of B cell, while preserving the beneficial CD45RO+ T memory cells. However, when only CD45RA processing is used as a one-step approach, CD34+ SC are co-depleted from the graft, which is unacceptable, especially in haplo-SCT. In addition to SC loss, the loss of NK cells must also be considered, especially regarding the protection against pathogens during aplasia after SCT, earlier engraftments and preservation of the GvL effect.

The aim of this work was to investigate whether CD45RA depletions from G-CSF stimulated grafts are a suitable alternative to already established methods (CD3/19 depletion, CD34 selection and others), are feasible and applicable in clinical practice. The results obtained call into question the sole use of CD45RA depletions in clinical practice. However, due to their ability to transfer immunocompetent memory T cells, CD45RA depletions are of great added value to SCT, so it is inevitable to discuss ways to include them in one way or another. The collected data suggests that CD45RA depletions are feasible and show an

efficient removal of CD45RA+ cells, but compensation of possibly insufficient SC amounts must be considered. Sufficient SC amounts could be achieved by combination of CD45RA depletion and CD34 selection. Due to small case numbers (n=3) and possible substantial range between SC recovery data within CD34 selections, no clear recommendation can be made for either approach.[89]

Several research groups have already combined CD45RA depletion with other processing methods. In particular, the combination of CD45RA depletion with preceding CD34 selection from G-CSF stimulated grafts has already been applied clinically.[27,57,92] This approach allows the provision of immunologically beneficial CD45RO+ T memory cells and a sufficient amount of SC.

Other approaches must be considered for NK cells compensation. As described earlier, NK cells are known to function as protective cells for the initial response in destroying pathogen antigens and residual tumor cells. In addition, they appear to have a positive effects on engraftment after SCT.[17,32,83] For this reason, it is desirable to be able to transfer NK cells to the recipient in SC grafts. Unfortunately, most NK cells are depleted in CD45RA depletion. Although previous studies have also postulated, in part, that an additional supply of NK cells is not necessary to ensure engraftment, GvL or immunologic competence during aplasia, lack of NK cells has also shown to hold a risk towards HHV-6 infection vulnerability.[72] There is a substantial evidence that NK cells are effective in combating viral infections (EBV, CMV, AdV) when administered as a donor lymphocyte infusion (DLI) after transplantation.[17,58,77,78,88] Therefore, in any constellation, it is important to consider the potential benefits of additional NK cell supply.

Triplett *et al*.[58,77] performed separate NK cell isolation from unstimulated leukapheresis as an addition to CD45RA-depleted grafts to provide NK cells. Other research groups combined TCR$\alpha\beta$/CD19 and CD19 depletions followed by CD45RA-depleted donor lymphocyte infusions (DLI).[70,93] Grafts depleted of alpha and beta T cells have been reported to ensure fast engraftment, have low GvHD

potential and have a beneficial effect on immune recovery.[94–96] However, the return of a properly functioning immune system, protecting the patient from viral infections is delayed.[97] CD45RA-depleted DLI could thus fill this protective gap.[70]

Similarly, CD45RA-depleted DLIs could be combined with CD3/19 depletions to provide NK, T cells and additional memory T cells. Currently, there are no study published study investigating this approach. CD3/19 depletions are often combined with CD34 selections to maintain the balance between SC and residual T cells. Concretely, CD34 selections are usually performed to compensate for possible excessive amounts of T cell or to provide additional SC to overcome HLA barriers.[71] Thus, if CD45RA depletions are now combined with CD3/19 depletions, this might require additional CD34 selection making this approach ultimately very impractical, time-consuming, and expensive.

The potential of T memory to cause GvHD is currently not fully understood. The published data suggest that depletion of CD45RA+ cells does not completely eliminate the risk of GvHD occurrence.[27,76,77,88] For this reason, the dosage of cells supplied by CD45RA-depleted DLI should be critically considered. Maschan *et al.*[70] described that low-dose memory T cell infusions after are safe for haplo-SCT after TCR$\alpha\beta$/CD19 depletions at doses of 25-100 x10^3/kg. Maung *et al.*[98] described that not acute GvHD occurred at a maximum of 1x10^7 CD3+ cells/kg from CD45RA-depleted DLIs. However, the sample sizes in this study were very small, so the exact number of transmissible cells cannot yet be determined.

The combination of CD34 and CD56 selection in one step and additional subsequent CD45RA depletion represents another possible concept for graft processing. Combined CD34 and CD56 selection would provide a CCB containing purified SC and NK cells. Subsequent CD45RA depletion from the respective NTCB would supply CD45RO+ cells. In terms of time and resource requirements, this combination is comparable to a combined CD34 selection and CD3/19 depletion graft processing approach and provides all desired cell populations.

Overall, the data obtained in this study show that CD45RA depletion from G-CSF-mobilized grafts is feasible at a good quality but should not be recommended as the sole measure. For example, a combination of CD45RA depletions with a preceding CD34 selection represents a reasonable choice. Because most NK cells are co-depleted, it is advisable to deliver NK cells by other means. Delivery of NK cells by DLI represents a useful tool for posttransplant cell boosting and can be combined with TCR/CD19 or CD3/19 depletions.

8. Perspectives

This work has dealt with the establishment of a 10-color FACS- measurement panel. In particular, the antibody selection for the detection of CD45RA+ cells posed a challenge. The final decision was made in favor of the FACS antibody CD45RA-PE. Interestingly, two of the antibodies tested (CD45RA-PE and CD45RA-PacB) had the same antigen clone, but detection of cells labeled with the depletion antibody was not possible with the CD45RA-PacB antibody. In the context of this work, the low staining index and lower fluorochrome brightness of the CD45RA-PacB antibody was used as the best explanatory approach to this issue. However, an influence of the specificity of the antigen epitope would also be possible and should be further investigated in future studies.

This study demonstrated that CD45RA depletions are feasible and allow effective elimination of CD45RA+ naïve T cells while preserving CD45RO+ immunocompetent T cells. Furthermore, this study showed that CD45RA depletions can be performed with comparable quality to established methods such as CD3/19 depletions and CD34 selections. However, in this work, CD45RA depletions were found to be associated with higher cell loss, presumably due to very small sample sizes, and therefore CD45RA processing needs to be performed with larger sample sizes to assess cell loss on a clinical scale. In addition to the overall cell loss, this study confirmed the previously described loss of NK and stem cells due to co-expression of CD45RA. While SC loss as described in this work is also shown in other publications, the use of combination of CD45RA depletions with prior CD34 selection seems to be the strategy of choice for most research groups [27,57,92], a satisfactory solution to compensate NK cell loss, apart from DLI is currently not available.[70] As described in detail, NK cells have great potential to be of benefit in SCT, and it is likely that NK cell maintenance may enhance the benefits of CD45RA depletion. For this reason, it is important to investigate possible combination strategies for CD45RA depletions with NK cell- preserving strategies, such as NK selections or CD3/19

depletions. Another perspective to be explored is the application of CD45RA depletions from cryopreserved grafts as a therapeutic back-up in case of impending disease relapse or viral infection. Since depletions of cryopreserved G-CSF-stimulated starting products are currently not possible, it would be desirable to find ways to retrospectively deplete cryopreserved grafts. It is envisionable that CD45RA depletions from graft surpluses will be performed as part of established processing strategies, such as a combination of CD34 selection and CD3/19 depletion, and subsequently cryopreserved for later use as a donor -independent DLI alternative with lower GvHD potential.

In addition to investigating possible combinations strategies for processing, further investigation of virus-specific protective competence of retained CD45RO+ T_M cells must to be part of the research agenda. This and several other studies, have convincingly shown that transferred T cells after CD45RA depletions carry specific virus competence for potentially life-threatening infections (CMV, EBV, AdV) even at partially low frequencies. However, in addition to viral infections, opportunistic fungal infections must also be considered, as they can lead to severe deterioration of the patient's overall condition. Although antifungal drugs are available, it would be desirable to transfer T cells with specific antifungal competence. To achieve further progress in in reducing lymphoblast deficiency and TRM, it is desirable to further minimize conditioning and immunosuppression therapies. CD45RA depletions are a promising approach to achieve this goal, especially in combination with additional NK cell therapy. Further studies should focus on quantifying GvL promotion by CD45RO+ cells and on how this might help minimizing conditioning regimens. This could be done, for example, by cytotoxicity assays of CD45RO+ cells against degenerated cells. In conclusion, CD45RA depletion is a promising new option for stem cell preparation that needs to be further developed and clinically explored.

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11. Schriftliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Medizin der Johann Wolfgang Goethe-Universität Frankfurt am Main zur Promotionsprüfung eingereichte Dissertation mit dem Titel:

Depletion of CD45RA+ naïve T Cells for Haploidentical Stem Cell Transplantations: Graft Generation under Good Manufacturing Practice and Establishment of a Flow Cytometric Quality Control for Clinical-Scale Grafts

in dem Zentrum der Kinder – und Jugendmedizin, Klink für Kinder- und Jugendmedizin, Schwerpunkt Stammzelltransplantation und Immunologie, Universitätsklinikum der Goethe Universität Frankfurt am Main, unter Betreuung und Anleitung von Prof. Dr. Peter Bader, ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Darüber hinaus versichere ich, nicht die Hilfe einer kommerziellen Promotionsvermittlung in Anspruch genommen zu haben.

Ich habe bisher an keiner in- oder ausländischen Universität ein Gesuch um Zulassung zur Promotion eingereicht. Die vorliegende Arbeit wurde bisher nicht als Dissertation eingereicht.

Vorliegende Ergebnisse der Arbeit wurden in folgendem Publikationsorgan veröffentlicht:

Melanie Bremm, Theresa Krastel, Claudia Cappel, Olga Zimmermann, Lisa-Marie Pfeffermann, Verena Katzki, Halvard Bonig, Richard Schäfer, Eva Rettinger, Michael Merker, Sebastian Bremm, Kirsten Schaefer, Thhomas Klingebiel, Jan Soerensen, Peter Bader, Sabine Huenecke, Depletion of CD45RA+ T cells: Advantages and disadvantages of different purification methods, Journal of Immunological Methods, Volume 492, 112960, 2021

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I. List of Figures

II. List of Tables

III. List of Abbreviations

7-AAD	7-Aminoactinomycin D
AdV	Adenovirus
ALL	Acute lymphoblastic leukemia
Allo-(SCT)	Allogenic (Stem cell transplantation)
AML	Acute myeloid leukemia
APC	antigen-presenting cell
APC	Allophycocyanin
APC-A	Allophycocyanin- Alexa Fluor
APC-Cy	Allophycocyanin-Cyanin
ATG	Anti- thymocyte globulin
BCR	B cell receptor
CCB	Cell collection bag
CD	Cluster of differentiation
CIK	Cytokine-induced killer cells
CML	Chronic myeloid leukemia
CR	Complete remission
CSA	Cyclosporin A

DC	Dendritic cell
DLI	Donor lymphocyte infusion
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein-Barr-Virus
ECD	Phycoerythrin-Texas Red
FACS	Fluorescent activated cell sorter
FCS	fetal calf serum
FCS/SS	Forward Scatter/Side Scatter
FITC	Fluorescein-Isothiocyanat
G-CSF	Granulocyte-Colony stimulating factor
GMP	Good manufacturing practice
GvHD, aGvHD, cGvHD	(acute, chronic) graft versus host disease
GvLE	Graft versus leukemia effect
haplo-(SCT)	Haploidentical (SCT)
HSA	Human serum albumin
HHV	Humane herpes virus
HLA	Histocompatibility antigen

HvG	Host versus graft
IFN	interferon
IL	Interleukin
ISHAGE	International Society of Hematotherapy and Graft Engineering
LCA	Common Leukocyte Antigen
MDS	Myelodysplastic Syndrome
MFD	Matched family donor
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MMFD	Mismatched family donor
MMUD	Mismatches unrelated donor
MSD	Matched sibling donor
NK (cell)	Natrual killer cell
NTCB	Non-target cell bag
PBSC	Peripheral blood stem cells
PC	Phycoerythrin-Cyanin
PE	Phycoerythrin
PerCP	Peridinin-Chlorophyll

PTLD	post-transplant lymphoproliferative disorder,
QC	Quality control
SCT	Stem cell transplantation
T_C	T Cytotoxic cell
TCD	T cell depletion
T_{CM}	T Central memory cell
TCR	T cell receptor
T_{EM}	T Effector memory cell
T_H	T Hepler cell
T_M	T Memory cell
T_N	Naïve T cell
T_{reg}	T regulatory cell
WBC	White blood cells

V. Publication

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Depletion of CD45RA$^+$ T cells: Advantages and disadvantages of different purification methods

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ABSTRACT

Background: Recently, new advances were made regarding the depletion of CD45RA$^+$ naïve T cells from haploidentical grafts as they are suspected to be the most alloreactive.
Methods: Within this project we investigated CD45RA-depletion from G-CSF mobilized PBSC by two different purification strategies according to GMP, specifically direct depletion of CD45RA$^+$ cells (one-step approach), or CD34-positive selection followed by CD45RA-depletion (two-step approach).
Results: With log −3.9 and − 3.8 the depletion quality of CD45RA$^+$ T cells was equally for both approaches together with a close to complete CD19$^+$ B cell depletion. However, due to a high expression of CD45RA the majority of NK cells were lost within both CD45RA depletion strategies. Stem cell recovery after one-step CD45RA-depletion was at median 52.0% (range: 49.7–67.2%), which was comparable to previously published recovery data received from direct CD34 positive selection. Memory T cell recovery including CD4$^+$ and CD8$^+$ memory T cell subsets was statistically not differing between both purification approaches. The recovery of CD4$^+$ and CD8$^+$ T cells was as well similar, but overall a higher amount of cytotoxic than T-helper cells were lost as indicated by an increase of the CD4/CD8 ratio.
Conclusions: CD45RA-depletion from G-CSF mobilized PBSC is feasible as one- and two-step approach and results in sufficient reduction of CD45RA$^+$ T cells as well as B cells, but also to a co-depletion of NK cells. However, by gaining two independent cell products, the two-step approach enables the highest clinical flexibility in regard to individual graft composition with precise dosage of stem cells and T cells.

1. Introduction

CD45 is a receptor-linked tyrosine phosphatase which is expressed on all leukocytes regulating receptor signaling via direct cell contact by its extracellular domain or by activating various Src family kinases. Alternative splicing of three adjacent exons within the CD45 gene results in up to eight different isoforms of the CD45 receptor. The CD45 isoforms CD45RA and CD45RO are used to differentiate between naïve and memory T cells. Naïve T cells express CD45RA carrying the largest extracellular domain, whereas memory T cells express the shortest

Abbreviations: CCB, cell collection bag; CD, cluster of differentiation; CMV, cytomegalovirus; DLI, donor lymphocyte infusion; DTS, Depletion tubing set; EBV, Epstein-Barr-Virus; E:T, effector to target; FACS, fluorescence-activated cell sorting; G-CSF, granulocyte-colony stimulating factor; GMP, good manufacturing practice; GvHD, graft-versus-host disease; GvL/T, graft-versus-leukemia/tumor effect; IFN, interferon; IL, interleukin; NK, natural killer; NTCB, non-target cell bag; ns, not significant; MFI, mean fluorescence intensity; MHC, major histocompatibility complex; PBSC, peripheral blood stem cells; QC, quality control; SCT, hematopoietic stem cell transplantation; TCD, T cell depletion; TCR, T cell receptor; NK-T, NK-like T cells.

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isoform which facilitates T cell activation (Altin and Sloan, 1997; Rhee and Veillette, 2012). In mouse models it has been shown, that naïve T cells cause severe graft-versus-host-disease (GvHD) whereas memory T cells only cause mild or no GvHD (Anderson et al., 2003; Chen et al., 2007; Dutt et al., 2007). Memory T cells are descendants of naïve T cells that maturated following antigen contact in the presence of co-stimulatory signals. They can be divided into the subgroups of central – and effector memory T cells which both express CD45RO, but can be distinguished via the homing receptors CD62L and CCR7 which are also expressed by naïve T cells. CD62L and CCR7 are expressed on central memory T cells which reside mainly in the lymph nodes, blood and spleen; whereas CD62L and CCR7 negative effector memory cells are found in non-lymphoid tissues (Lefrancois and Marzo, 2006).

Precise T cell depletion (TCD) of allografts is an established approach to reduce the incidence and severity of GvHD especially for haploidentical hematopoietic stem cell transplantation (SCT) (Aversa et al., 2001; Klingebiel et al., 2004; Klingebiel et al., 2005). First ex vivo techniques resulted in indirect TCD by the selection of $CD34^+$ stem cells (Aversa et al., 2001; Koehl et al., 2008). However, CD34-selection was associated with delayed immune reconstitution, reduced graft-versus-leukemia/tumor effect (GvL/T) and an increased frequency of opportunistic infections (Gordon et al., 2002; Lang et al., 2005; Bethge et al., 2006; van Burik et al., 2007). Therefore, combined depletion of all $CD3^+$ T cells and $CD19^+$ B cells was established, resulting in grafts including various immunocompetent cells in addition to stem cells (Bader et al., 2011). This strategy might also be combined with CD34-selection and seemed beneficial regarding engraftment and GvL/T-effect (Huenecke et al., 2016). Aiming for further enhancement of GvL/T-effect, in a refinement, only $TCR\alpha\beta^+$ T cells and $CD19^+$ B cells were removed from the graft, as $TCR\gamma\delta^+$ T cells were reported to exert strong cytotoxicity against tumor cells (Bremm et al., 2017; Locatelli et al., 2017). Nevertheless, the problem of opportunistic infections remains challenging (Laberko et al., 2017). Within the cohort of T cells, naïve T cells seem to have the highest alloreactive potential, presumably attributable to the great diversity of the naïve T-cell receptor repertoire (Nikolich-Zugich et al., 2004; Distler et al., 2011). By selective depletion of potentially alloreactive $CD45RA^+$ cells, memory T cells remain in grafts and could mediate pathogen specific immunity and therefore shorten viremia due to preservation of virus specific donor T cells (Bleakley et al., 2014; Teschner et al., 2014; Touzot et al., 2015). However, CD45RA expression is not restricted to naïve T cells. Also $CD19^+$ B cells co-express CD45RA, which makes an additional CD19 depletion for avoiding EBV-associated post-transplant lymphoproliferative disease (PTLD) dispensable. Furthermore, NK cells and $CD34^+$ stem cells express CD45RA to some extent (Krzywinska et al., 2016). A two-step procedure involving preceding CD34-selection before depletion of $CD45RA^+$ cells might be an applicable strategy to retain CD45RA-co-expressing stem cells (Bleakley et al., 2015; Touzot et al., 2015; Triplett et al., 2018). With log −3.43 Bleakley et al. described a nearly complete NK cell depletion whereas Shook et al. and Teschner et al. reported significant proportions of NK cells in CD45RA depleted mobilized grafts (Bleakley et al., 2014; Teschner et al., 2014; Shook et al., 2015). Within this project a laboratory scale comparison of the one-step and two-step approach was performed to provide individual composition of differently manufactured CD45RA-depleted grafts.

2. Material and methods

2.1. Sample material

To establish and refine a 10-color flowcytometric panel for quality control, CD45RA expression was analyzed on various lymphocyte subpopulations in $n = 20$ surplus samples from PBSC collections (ethics committee approval number 502/16). Surplus samples ($n = 3$) were also used for testing one-step CD45RA-depletion approach. For the two-step approach the negative fraction after CD34-selection was used for further CD45RA-depletion ($n = 4$, ethics committee approval number 329/10). Samples for one- and two-step approach were gained from six different donors. Of one donor, samples for one-step and two-step CD45RA-depletion were available.

2.2. CD45RA-depletion via CliniMACS

CD45RA-depletion was performed on a CliniMACS device (Miltenyi Biotec, Bergisch Gladbach, Germany). Two different approaches were tested including a direct CD45RA-depletion of the PBSC referred to as one-step approach and a second alternative including CD34-selection followed by CD45RA-depletion of the negative fraction referred to as two-step approach (Fig. 1).

One-step approach: After thrombocyte reduction, CD45RA microbeads (Miltenyi Biotec) were introduced according to the application capacity of 20×10^9 $CD45RA^+$ cells and 50×10^9 total nucleated cells and incubated for 30 min. Afterwards cells were washed twice, followed by immunomagnetic depletion on the automated CliniMACS device using a Depletion tubing set (DTS) and depletion program 3.1.

Two-step approach: Two-step approach was performed as descried in Bleakley et al. (2014). As a first step, positive enrichment of $CD34^+$ stem cells was performed with the automated CliniMACS device (Miltenyi Biotec) using the appropriate clinical-grade CD34 microbeads obeying good manufacturing practice (GMP) as described previously (Koehl et al., 2008). As a second step, $CD45RA^+$ cells were depleted from the negative fraction (non-target cell bag; NTCB) resulting from CD34-selection. After thrombocyte reduction, CD45RA microbeads (Miltenyi Biotec) were introduced according to the application capacity of 20×10^9 $CD45RA^+$ cells and 50×10^9 total nucleated cells and incubated for 30 min. Afterwards cells were washed twice, followed by depletion on the automated CliniMACS device, using a DTS and depletion program 3.1.

2.3. Flowcytometric analysis

For CD45RA expression analysis of various lymphocyte subpopulations, a new 10-color flowcytometric single platform panel applying Flow Count™ Fluorospheres (Beckman Coulter, Brea, USA) was created on a Navios™ flowcytometer (Beckman Coulter, Krefeld, Germany) with three lasers (violet 405 nm, blue 488 nm and red 638 nm) and standard filter configuration except FL4 695BP for 7AAD emission. Stained CytoComp™ cells (Beckman Coulter, O'Callaghan's Mills, Ireland) were applied to compensate the fluorescence overlap. The flowcytometer fluidic stability and the optical alignment were tested using Flow-Check Pro™ Fluorospheres (Beckman Coulter, Brea, California). All antibodies were applied within two panels and are listed in Suppl. Table 1. In addition to CD45RA-PE (clone: 2H4, Beckman Coulter), CD45RA-FITC (clone: ALB11, Beckman Coulter) and CD45RA-Pacific Blue (clone: 2H4) were tested.

2.4. Detection of virus-specific T cells

Cytomegalovirus (CMV)-, Epstein-Barr-Virus (EBV)- and Adenovirus (AdV)-specific T cells were identified by staining with various MHC-I and MHC-II multimers according to the manufacturer's instructions. CMV specific T cells: pp65 (A*02:01, A*03:01; Immudex, Copenhagen, Denmark). EBV-specific T cells: LMP2A (A*02:01), EBNA1 (B*35:01), EBNA3A (B*07:02 and B*08:01), BMLF1 (A*02:01; Immudex) and EBNA1 (DRB1*04:01; ProImmune). AdV-specific T cells: 5Hexon (A*0101; Immudex). Furthermore, the cells were additionally stained with the following antibodies: CD3 (APC, clone: UCHT1, Beckman Coulter), CD4 (APC A750, clone: 13B8.2, Beckman Coulter), CD8 (APC A700, clone: B9.11, Beckman Coulter) and CD45 (KO, clone: B9.11, Beckman Coulter). Measurements were performed in $n = 3$ PBSCs (one-step) and $n = 4$ products following CD34-selection (two-step) and following depletion, respectively. For each patient one up to five specific

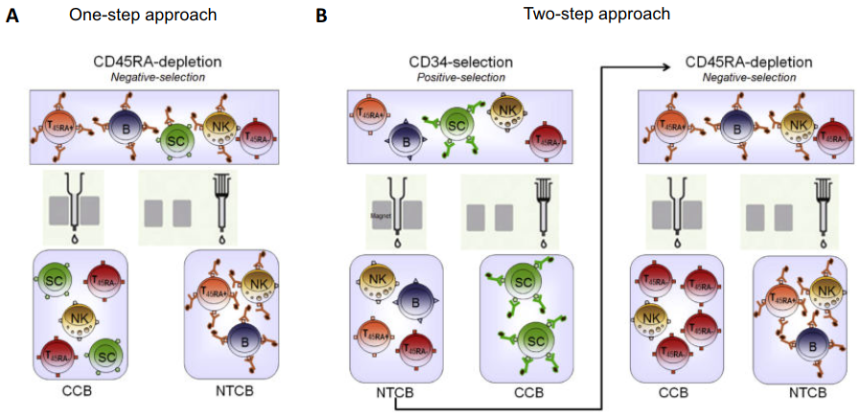


Fig. 1. CD45RA-depletion scheme via CliniMACS.
Depletion of CD45RA$^+$ naïve T cells via CliniMACS device was investigated by two different strategies. (A) One-step approach: direct depletion of CD45RA$^+$ cells from G-CSF stimulated PBSC. CD45RA$^+$CD3$^+$ naïve T cells and CD45RA$^+$CD19$^+$ B cells can be labelled and depleted. Depending on their CD45RA co-expression level, NK cells and stem cells are also labelled and adversely depleted from the graft. (B) Two-step approach: within a two-step procedure, stem cells are primary selected via CD34 positive selection. In a second step, CD45RA$^+$ cells, CD45RA$^+$CD3$^+$ naïve T cells, CD45RA$^+$CD19$^+$ B cells but also CD45RA expressing NK cells, were depleted from the negative fraction (non-target cell bag,; NTCB) from the CD34-selection.

multimers could be introduced depending on the patient's MHC-type (data not shown).

2.5. Statistics

Statistical analysis was performed using GraphPad Prism 6 for Windows (GraphPad Software, San Diego, USA) applying Mann-Whitney test. Flowcytometric measurements were evaluated using Kaluza® or Navios Software (Beckman Coulter).

3. Results

3.1. Flowcytometric quality control of CD45RA$^+$ cells

Precise quantification of CD45RA expression is essential for quality control of CD45RA-depleted cell products. Occupation of binding sites

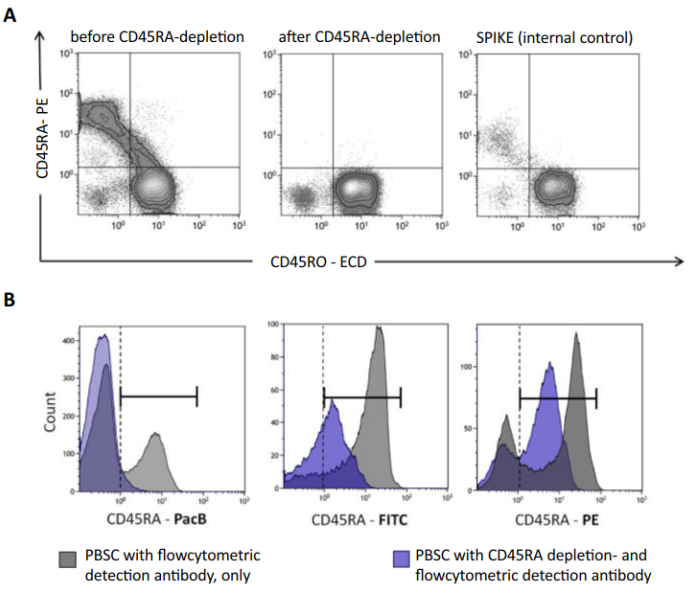


Fig. 2. Staining of CD45RA$^+$ cells via flowcytometry. (A) FACS plots showing CD45RA vs. CD45RO expression gated on WBCs before (left) and after CD45RA-depletion (middle). To achieve correct detection of residual CD45RA$^+$ T cells a 1:6 mixture of cells from the target and non-target cell bag (previously 1:50 diluted with PBS) following CD45RA-depletion is displayed at the right (SPIKE). A definitely lower CD45RA MFI was detected on the cells within SPIKE (right) that are labelled with depletion antibody compared to PBSC (left) (B) Overlay plots of PBSC stained with CD45RA depletion antibody (dark grey) and without staining (light grey) in individual analyses testing three different CD45RA FACS antibodies. CD45RA-Pacific Blue (left) was not suitable as CD45RA$^+$ and CD45RA$^-$ cells cannot be separated whereas CD45RA-FITC (middle) and CD45RA-PE (right) showed adequate staining results. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

with CD45RA-depletion antibody hampered flowcytometric quality control (QC). A QC SPIKE was applied to adjust the gates for residual CD45RA$^+$ T cells which show reduced MFI because the receptor might be partially blocked by the depletion antibody (Fig. 2A). Therefore, CD45RA-FITC (ALB11), CD45RA-Pacific Blue and CD45RA-PE (both 2H4) were tested in different concentrations to optimize flowcytometric analysis. With the CD45RA-PacB antibody no distinct discrimination between CD45RA negative cells and cells labelled with CD45RA depletion antibody was possible. Consequently, undesired residual CD45RA$^+$ cells might not be detected via CD45RA-PacB (Fig. 2B, left plot) and therefore falsify QC. Adequate staining results were obtained using CD45RA-FITC and CD45RA-PE. However, because of a lower fluorochrome brightness of FITC, staining with CD45RA-FITC might not be optimal for the detection of cells with low CD45RA expression. With regard to more distinct separation of the CD45RA negative and positive fraction, CD45RA-PE was selected as suitable staining antibody. CD45RA-PE and CD45RA-PacB target the equal clone, however with CD45RA-PacB antibody the detection of depletion antibody labelled CD45RA$^+$ cells was not possible. This might be due to a lower staining index and fluorochrome brightness of CD45RA-PacB antibody (Fig. 2B).

3.2. CD45RA expression on stem cells and leukocyte subsets

We examined CD45RA expression on stem cells, B cells, NK cells, T cells, NK-like T cells, monocytes and granulocytes in G-CSF mobilized grafts. A median of 16.9% (range: 9.3–30.4%) of CD34$^+$ stem cells additionally express CD45RA (Fig. 3A). With a median of 99.9% (range: 99.4–99.9%) CD45RA expression was measurable on nearly all B cells (Fig. 3B). A comparably high expression of in median 95.2% (range: 56.5–98.8%) was detected on NK cells, whereas T cells and NK-like T cells showed median CD45RA expression of 61.6% (range: 37.8–99.3%) and 59.8% (range: 31.6–92.8%, Fig. 3C-E), respectively. Monocytes and granulocytes showed a CD45RA surface expression of in median 14.9% (range: 2.5–38.4%) and 1.5% (range: 0.5–5.6%).

3.3. Comparison of CD45RA-depletion via one- and two-step approach

Laboratory scale CD45RA-depletion on a CliniMACS device was investigated following a one-step approach of direct CD45RA-labeling and depletion of CD45RA$^+$ T cells from the negative fraction after preceding CD34-selection (two-step approach). The results are summarized in Table 1 and displayed in Fig. 4. Removal of CD45RA$^+$CD3$^+$ T cells was similar between one- and two-step approach, reaching a median depletion of log −3.9 (range: −4.1 to −3.8) and − 3.8 (range: −5.1 to −3.2), respectively. Regarding B-cell depletion determined via CD20 expression, both approaches were as well not differing to a statistically significant extent as indicated by log −3.2 (range:-3.3 to −2.9) and − 3.6 (range: −4.0 to −2.9) (Fig. 4A and Table 1). Since the final cell product after the two-step approach does not contain stem cells, the recovery of CD34$^+$ stem cells is shown for one-step approach, only. Median stem cell recovery after one-step CD45RA-depletion was at median 52.0% (range: 49.7–67.2%) (Fig. 4B and Table 1). Thereby, the amount of CD45RA$^+$CD34$^+$ progenitor cells dropped from 21.8% before to 1.1%. Importantly, the majority of NK cells was lost within both CD45RA-depletion approaches, resulting in a median recovery of 1.5% (range: 1.4–12.6%) and 1.8% (range: 0.1–3.4%) following one-step and two-step CD45RA-depletion (Fig. 4B), respectively. These results are in line with the high CD45RA expression of 95.2% detected on NK cells as

Table 1
Depletion quality and Recoveries following one- and two-step CD45RA-depletion.

	One-step	Two-step
Removal of CD45RA$^+$CD3$^+$ T cells	−3.9 Log (−4.1 to −3.8)	−3.8 Log (−5.1 to −3.2)
Removal of CD19$^+$ B cells	−3.2 Log (−3.3 to −2.9)	−3.6 Log (−4.0 to −2.9)
Recovery of CD34$^+$ stem cells	52.0% (49.7–67.2%)	
Recovery of CD3$^+$CD4$^+$ T cells	20.0% (4.9–32.0%)	22.4% (13.4–33.2%)
Recovery of CD3$^+$CD8$^+$ T cells	4.3% (1.4–8.5%)	8.8% (3.2–13.8%)
Recovery of CD3$^+$ CD45RO$^+$ Memory cells	24.2% (5.8–45.6%)	42.1% (6.5–52.4%)
Recovery of CD3$^+$CD4$^+$CD45RO$^+$ Memory cells	38.8% (10.6–53.0%)	37.5% (23.7–48.3%)
Recovery of CD3$^+$CD8$^+$CD45RO$^+$ Memory cells	13.1% (4.8–22.6%)	20.2% (11.0–25.4%)
Recovery of CD3$^-$CD56$^+$ NK cells	1.5% (1.4–12.6%)	1.8% (0.1–3.4%)

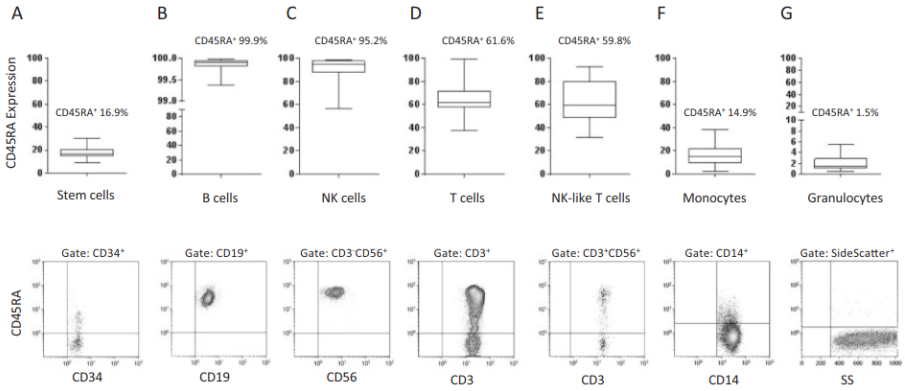


Fig. 3. FACS analysis of CD45RA co-expression on stem cells and leukocyte subsets.
Expression of CD45RA on stem cells, lymphocytes, monocytes and granulocytes was analyzed in n = 20 PBSC samples. Lymphocytes were defined via CD45 expression and granularity (side scatter). Monocytes were defined as CD45$^+$CD14$^+$ cells and granulocytes were gated as CD45$^+$CD14$^-$ cells with high granularity (side scatter). Results are shown as Box-Whisker plots (upper row) combined with a representative FACS plot (lower row). (A) A median of 16.9% stem cells showed an additional expression CD45RA. (B) With 99.9% nearly all CD19$^+$ B cells were CD45RA$^+$. (C) A median of 95.2% of NK cells revealed a CD45RA expression. (D) 61.6% of the T cells and (E) 59.8% of NK-like T cells, (F) 14.9% of monocytes and (G) 1.5% of granulocytes showed CD45RA co-expression.

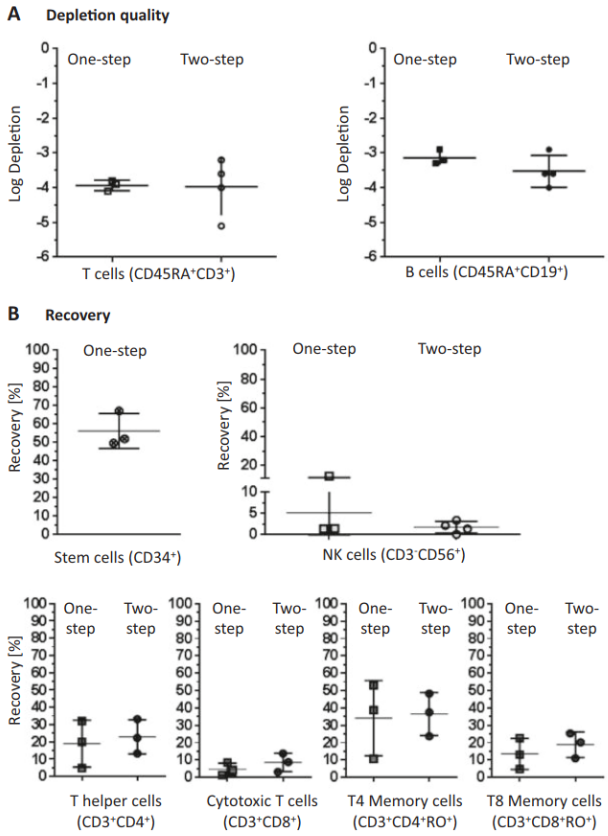


Fig. 4. Recovery and depletion quality.
CD45RA-depletion was performed via one-step ($n = 3$) and two-step ($n = 4$) approach. (A) Depletion quality for $CD45RA^+$ T cells and $CD19^+$ B cells is indicated via log depletion. Log depletion is calculated as follows: Log ($CD45RA^+$ cells (abs) after/ $CD45RA^+$ cells (abs) before depletion). A log depletion of 0 implies no depletion. The smaller the log depletion value the better the depletion efficacy. Depletion of $CD45RA^+CD3^+$ T cells was similar between one- and two-step approach, reaching a median depletion of log −3.9 (range: −4.1 to −3.8) and − 3.8 (range: −5.1 to −3.2), respectively. $CD45RA^+CD19^+$ B cell depletion was as well not differing to a statistically significant extent as indicated by log −3.2 (range:-3.3 to −2.9) and − 3.6 (range: −4.0 to −2.9). (B) Recovery of $CD34^+$ stem cells, $CD3^-CD56^+$ NK cells and T cell subsets. Since the final cell product after the two-step approach does not contain stem cells, the recovery of $CD34^+$ stem cells is shown for one-step approach only, which was at median 52.0% (range:49.7–67.2%). No difference in the recovery of NK cells was detected comparing one- and two-step approach. Thereby, the majority of NK cells was lost within both CD45RA-depletion approaches, resulting in a median recovery of 1.5% (range: 1.4–12.6%) and 1.8% (range: 0.1–3.4%) following one-step and two-step CD45RA-depletion. Memory T cell recovery following CD45RA-depletion was statistically not differing at median 24.2% (range: 5.8–45.6%) with the one-step approach and 42.1% (range: 6.5–52.4%) with the two-step approach. Also no difference between both purification approaches was observed for $CD4^+$ and $CD8^+$ memory T cell subsets. The recovery of $CD4^+$ T helper and $CD8^+$ cytotoxic T cells was similar with one- and two-step approach, but higher for $CD4^+$ T helper cells (median 20.0% and 22.4%) compared to $CD8^+$ cytotoxic T cells (median 4.3% and 8.8%).

shown in Fig. 3. Memory T cell recovery following CD45RA-depletion was statistically not differing at median 24.2% (range: 5.8–45.6%) with the one-step approach and 42.1% (range: 6.5–52.4%) with the two-step approach. Also no difference between both purification approaches was observed for $CD4^+$ and $CD8^+$ memory T cell subsets. Differentiation of $CD45RO^+$ cells into effector- and central memory T cells was not possible due to reduced expression of CD62L and CCR7 on the cells. The recovery of $CD4^+$ T helper and $CD8^+$ cytotoxic T cells was similar with one- and two-step approach, but higher for $CD4^+$ T helper cells (median 20.0% and 22.4%) compared to $CD8^+$ cytotoxic T cells (median 4.3% and 8.8%).

In one case, we had the possibility to receive samples of the same product for 1-step and 2-step approach. The analysis of these depletions showed lower $CD3^+CD45RO^+$ memory T-cell recovery (1-step: 25.5%, 2-step: 42.4%), but a slightly higher $CD3^+CD45RA^+$ naïve T-cell Log depletion (1-step, −4.1, 2-step: −3.6) for 1-step vs. 2-step approach, however not to a statistically significant extent (data not shown).

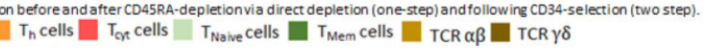
3.4. Cell composition following CD45RA-depletion of G-CSF mobilized PBSC

Cell composition before and after CD45RA-depletion is presented in Table 2. After depletion cell products contained a median of 0.009% (range: 0.006–0.013%) and 0.011% (range: 0.007–0.04%) $CD45RA^+$ naïve T cells, whereas the frequency of $CD45RO^+$ memory cells was at 9.9% (range: 5.9–32.8%) and 14.9% (range: 3.7–41.9%) for one-step and two-step approach, respectively. Almost all of these remaining T cells expressed TCRαβ. B cells and NK cells showed low frequencies of in median 0.02% (range: 0.01–0.02%) and 0.3% (range: 0.1–0.5%) following one-step and 0.02% (range: 0.002–0.05%) and 0.11% (range: 0.03–0.27%) for two-step approach, respectively. Interestingly, as already shown in Fig. 4B, within CD45RA-depletion a higher amount of cytotoxic T cells than T-helper cells was lost, which resulted in an increase of the CD4/CD8 ratio for one- and two-step approach. Among the T helper cells in median 7.2 and 8.8% were of regulatory phenotype following one- and two step CD45RA depletion, respectively.

Specific T cells against AdV, CMV and EBV were verifiable in CD45RA-depleted products. Median frequencies of virus-specific T cells referring to $CD8^+$ cytotoxic T cells were 0.05% (range: 0.01–0.29%), 0.41% (range: 0.12–0.81%) and 0.66% (range: 0.02–10.1%) for AdV, CMV and EBV-specific T cells, respectively (data not shown). No differences in the presence of virus-specific T cells were detected between one- and two-step CD45RA-depletion. Median recovery of virus-specific T cells was 25.1% (range: 0.23–84.5%).

Table 2
Cell composition before and after CD45RA-depletion via direct (one-step) and following CD34-selection (tw0-step).

Cell population	Gate	One-step before [%]	One-step after [%]	Two-step before [%]	Two-step after [%]
T cells (CD3$^+$)	% WBC	31.5 (26.9 - 42.7)	9.9 (5.9 - 32.84)	31.5 (19.9 - 42.9)	15.0 (3.7 - 41.9)
T helper (CD3$^+$CD4$^+$)	% T cells	64.3 (63.4 - 64.3)	87.9 (86.9 - 89.1)	66.6 (65.1 - 68.4)	84.9 (84.5 - 90.4)
T cytotox (CD3$^+$CD8$^+$)	% T cells	35.7 (32.7 - 35.7)	13.2 (9.8 - 14.2)	33.0 (31.8 - 34.1)	16.6 (10.2 - 17.5)
Ratio CD4/CD8		1.8 (1.8 - 2.0)	6.7 (6.1 - 9.1)	2.0 (1.9 - 2.1)	5.1 (4.8 - 8.8)
Memory T (CD3$^+$CD45RO$^+$)	% WBC	10.0 (6.2 - 16.7)	9.9 (5.9 - 32.8)	10.5 (4.8 - 17.7)	14.9 (3.7 - 41.9)
Naïve T (CD3$^+$CD45RA$^+$)	% WBC	21.6 (20.6 - 26.0)	0.009 (0.006 - 0.013)	21.5 (14.1 - 25.2)	0.011 (0.007 - 0.04)
TCRαβ (CD3$^+$TCRαβ$^+$)	% T cells	97.2 (95.0 - 97.7)	98.7 (97.5 - 99.2)	97.4 (96.4 - 98.3)	99.4 (96.8 - 99.5)
TCRγδ (CD3$^+$TCRγδ$^+$)	% T cells	4.8 (0.7 - 6.3)	0.9 (0.1 - 2.2)	2.2 (1.0 - 3.1)	0.3 (0.02 - 0.4)
Tregs (CD3$^+$CD4$^+$CD25$^+$ CD127$^{mid/dim}$)	% T4 cells	4.8 (0.7 - 6.3)	7.2 (6.4 - 8.1)	5.4 (4.6 - 7.6)	8.8 (8.5 - 9.0)
B cells (CD19$^+$)	% WBC	5.9 (4.4 - 9.1)	0.02 (0.01 - 0.02)	8.0 (4.6 - 9.3)	0.02 (0.002 - 0.05)
NK cells (CD3$^-$CD56$^+$)	% WBC	2.9 (1.7 - 4.9)	0.3 (0.1 - 0.5)	3.1 (0.9 - 3.6)	0.11 (0.03 - 0.27)
NK-like T cells (CD3$^+$CD56$^+$)	% WBC	1.3 (1.0 - 1.7)	0.4 (0.1 - 0.4)	1.1 (0.6 - 1.3)	0.17 (0.04 -0.5)
Monocytes (CD14$^+$)	% WBC	27.0 (20.2 - 30.7)	19.6 (14.5 - 32.0)	32.6 (23.9 - 37.8)	31.1 (12.1 - 44.9)
Granulocytes (Side Scatter^{high}, Forward Scatter^{low})	% WBC	30.2 (17.4 - 41.7)	70.5 (35.2 - 79.6)	31.8 (6.3 - 55.6)	53.5 (14.3 - 83.5)



Abbreviations: NK - natural killer, T - T cell, TCR - T cell receptor, Treg - regulatory T cell, WBC - white blood cells

4. Discussion

Within this study we investigated the depletion of CD45RA$^+$ cells from G-CSF stimulated PBSCs applying two different GMP-conform purification strategies with regard to graft composition. Direct depletion of CD45RA$^+$ cells (one-step approach) and CD45RA-depletion of the negative fraction of preceding CD34-selection (two-step approach) have already been deployed for the generation of grafts within a clinical setting. Shook et al. transplanted eight patients with a combination of CD3 and CD45RA-depleted stem cell grafts (Shook et al., 2015). CD45RA-depletion was performed via one-step depletion resulting in a median CD34 recovery of 59.2% (range: 24–68.9%), which is similar to our results of 52.0% (range: 49.7–67.2%) stem cell recovery for this approach. Since the final cell product after the two-step approach does not contain stem cells, the recovery of CD34$^+$ stem cells could not be directly compared between the one- and the two-step approach. However, median recovery rates, including wide ranges, were comparable to previously published data received from direct CD34 positive selection, i.e. 73% (range: 26.9–99.0%) for $n = 66$ CD34-selections (Huenecke et al., 2016) and 65% (range 29.9–125.6%) for $n = 84$ CD34 enrichments (Keever-Taylor et al., 2012). Nevertheless, it needs to be considered, that depending on the CD45RA expression on CD34$^+$ stem cells, to some extend also CD34$^+$CD45RA$^+$ stem cells will be affected by CD45RA-depletion as well. From our collected data indicating that at median 16.9% (range: 9.3–30.4%) of stem cells express CD45RA we can confirm previously published data indicating 25.6% co-expression as described by Bleakley et al. (2014). Craig et al. described that the most primitive CD34$^+$ cells are CD45RO$^+$ and express low or undetectable levels of CD45RA. Upon differentiation into myeloid and lymphoid lineages a down-regulation of CD45RO and up-regulation of CD45RA might take place (Craig et al., 1994). An interesting study by Radtke et al. described that CD34$^+$CD45RA$^-$CD90$^+$ stem cell phenotype supports rapid recovery of multilineage hematopoiesis in nonhuman primate transplant model, which might give a hint that following one-step CD45RA-depletion stem cells essential for the engraftment remain even though the CD34$^+$CD45RA$^+$ compartment is lost. Nevertheless, in one-step CD45RA-depletion losing of up to 30% of CD34$^+$CD45RA$^+$ cells is possible in addition to the loss attributable to cell washing and

separation. Planning patient studies requires adapting desired CD34$^+$ cell doses for transplantation as they might differ from existing protocols for transplantation with CD3/19- or TCRαβ-depleted grafts. To avoid co-depletion of stem cells, several approaches have been published where CD45RA-depletion is conducted from the negative fraction after CD34-selection (Bleakley et al., 2014; Bleakley et al., 2015; Touzot et al., 2015; Triplett et al., 2015; Sisinni et al., 2018; Triplett et al., 2018).

With a CD45RA target cell reduction of log −3.9 and log −3.8 our results for T-cell depletion efficiency were similar for both depletion strategies and in line with our results obtained for CD3- and TCRαβ-depletion (Huenecke et al., 2016; Bremm et al., 2017), but however are slightly lower compared to published data for CD45RA-depletion achieving log −4.75 (Sisinni et al., 2018), log −4.2 (Touzot et al., 2015) and log −4.4 (Teschner et al., 2014).

Remaining CD45RO$^+$ memory T cells within grafts after CD45RA-depletion aim to reduce the incidence of infectious complications. Triplett et al. described, that providing a pool of memory cells for SCT seems to reduce the incidence and duration of viremia attributable to CMV, EBV or AdV (Triplett et al., 2018). In our analysis, memory T cell recovery was 24.2% and 42.1% for one- and two-step approach, respectively. Differentiation of memory T cells into effector and central-memory subgroups was not possible due to reduced expression of CD62L and CCR7 on the cell surface. Comparable results were already described for CD62L on G-CSF mobilized PBSC (De Boer et al., 1998; Lundqvist et al., 2013).

Mueller et al. similarly described that CD45RA depletion leads to an increase of CD4/CD8 T cell ratio. This might be a consequence of CD45RA expression on a greater fraction of CD8$^+$ versus CD4$^+$ T cells (Cossarizza et al., 1996; Muller et al., 2018). Furthermore, we observed a nearly complete depletion of TCRγδ$^+$ T cells. These cells are described to recognize malignant cells and to mediate innate antitumor immunity. Furthermore, they are suspected to have graft facilitating properties but seem not to mediate GvHD (Boismenu and Havran, 1997; Lamb Jr. and Lopez, 2005).

Profound B cell elimination without the addition of anti-CD19 reagent is an advantage of CD45RA-depletion. NK cells, which are potent effector cells in the defense of viral infections and residual tumor cells, co-expressed CD45RA with a median of 95.2% (Farag and Caligiuri, 2006; Vivier et al., 2008). In line with our results, Krzywinska et al. published data about CD45 isoforms on NK cells describing that most NK cells in healthy humans are CD45RA$^+$CD45RO$^-$ (Krzywinska et al., 2016). Shook et al. reported NK cell log-depletion of only −1.01 (Shook et al., 2015) and Teschner et al. described significant proportions of NK cells following CD45RA depletion, but we nearly lost all NK cells independent of the CD45RA-depletion strategy what is also in line with previously published data (Muller et al., 2018). Indeed, NK cells play an important role within the immune system, exerting GvL/T effect and mediating pathogen specific immunity after SCT. Bleakley et al. showed an early reconstitution of NK cells following SCT with CD45RA-depleted grafts, which might protect the patients from undesired events following SCT (Bleakley et al., 2015). In further studies, the distribution of these regenerated NK cells into CD56^{++}CD16$^-$ (CD56^{bright}), CD56^{++}CD16$^+$ (CD56$^{intermediate=int}$) and CD56$^+$CD16^{++} (CD56^{dim}) NK cells might be of interest, as Huenecke et al. showed differences in these subgroups might relate to GvHD development and infections (Huenecke et al., 2017). Sisinni et al. observed an unexpectedly high rate of HHV-6 encephalitis in 8/25 patients following SCT with unaccompanied CD45RA-depleted stem cells, suspecting the lack of NK cells to play a role in the development of infections (Sisinni et al., 2018). To provide NK cells in addition to a CD45RA-depleted graft, Triplett et al. investigated separate NK cell isolation from steady-state leukocytapheresis (Triplett et al., 2015; Triplett et al., 2018). Other groups successfully combined TCRαβ/CD19-depleted stem cell grafts comprising a variety of immunocompetent including NK cells with a subsequent CD45RA-depleted donor lymphocyte infusion for therapeutic antiviral boost (Brodszki et al., 2016; Maschan et al., 2018).

5. Conclusions

In this manuscript we investigated two purification strategies for the depletion of CD45RA$^+$ T cells: direct depletion of CD45RA$^+$ cells (one-step approach) and CD45RA-depletion of the negative fraction of a preceding CD34-selection (two-step approach). Thereby, we focused on the feasibility and on a descriptive comparison of the advantages and disadvantages of both purification strategies. In miss-matched hematopoietic SCT, both, efficient T cell depletion and sufficient stem cell collection is of equally crucial importance. Our data indicated that CD45RA-depletion from G-CSF mobilized PBSC is feasible as one- and two-step approach. Both purification approaches achieved sufficient reduction of CD45RA$^+$ naïve T cells and CD19$^+$ B cells. In addition, even though recovery of CD34$^+$ stem cells could not be directly compared between the one- and two-step approaches, recovery after one-step CD45RA-depletion was comparable to previously published recovery data received from direct CD34 positive selection. However, both approaches resulted in an undesirable co-depletion of NK cells, potentially compromising the NK cell mediated GvL/T effect.

An advantage of the one-step approach is the reduced duration and lower manufacturing costs compared to the two-step approach, where an additional CD34 selection is performed on the day prior to CD45RA$^+$ depletion. On the other hand, especially in the field of haploidentical SCT, we find this separated collection of stem cells and T cells, leading to two independent cell products, as the key advantage of the two-step approach. This enables the highest clinical flexibility in regard to individual graft composition with precise dosage of stem cells and T cells. In addition, leftover material can be cryopreserved and used as DLI or for subsequent stem cell boosts, which can be administered independent of patients' post-transplant immunosuppression status.

Ethics approval and consent to participate

Informed consent was given with approval from the Ethical Review Board of the Medical Faculty of the University Hospital Frankfurt to use remaining material (502/16 and 329/10).

Consent for publication

Not applicable.

Availability of data and materials

All data are available in the manuscript or upon request to the authors.

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Authors' contributions

Conceptualization: MB, SH, CC, JS; Formal analysis: TKr, OZ, LMP; Funding acquisition: MB, SH, KS, PB, TKl; Investigation: MB, SH, TKr, CC, HB, JS; Methodology: TKr, OZ, LMP, VK; Project administration: MB, SH, PB; Software: TKr, MB, SH, SB; Supervision: PB, TKl; Visualization: SH, SB; Writing - original draft: MB, SH; Writing - review & editing: CC, MB, SH, TKr, HB, LMP, ER, MM, SB, KS, RS, OZ, VK.

Declaration of Competing Interest

None of the authors have any competing interests in the manuscript.

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Appendix A. Supplementary data

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