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Skin tissue resident memory T cells in patients after allogeneic hematopoietic stem cell transplantation

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Abstract

Tissue resident memory T cells (TRM) do not recirculate in the blood but reside in organs for an extended period of time, where they perform various functions on the frontline of potential infections to ensure immune defense. TRMs continue to be insufficiently characterized and studying their properties and phenotypes remains a significant challenge, particularly in humans.

The aim of this study was to investigate the longevity and potential residency cell markers associated with TRMs in human skin, which represents one of the most important pathogen contact surfaces. Skin and blood samples were obtained from 38 patients who underwent an allogeneic hematopoietic stem cell transplantation (allo-HSCT). The samples were digested enzymatically and mechanically, and T cells were isolated and phenotyped by flow cytometry. Subsequently, the T cell DNA was extracted and STR-PCR analysis was performed to determine whether host-derived resident T cells could be identified following allo-HSCT.

For a total of 6 patients among whom stem cell transplantation occurred as long as 818 days prior to the analysis host-derived TRMs were identified demonstrating the enormous longevity of TRMs in humans. Based on the analysis of numerous surface molecules expressed by the cells obtained from the blood and skin, a comprehensive view of the T cell population dynamics could be observed in patients after stem cell transplantation. Furthermore, alleged residency markers such as CD69 and CD103 could be critically examined. The results indicated that particularly $CD4^+$ T helper cells, which have been neglected in many previous studies on TRMs, contribute to a large proportion of TRMs.

Further research on this population in humans, for which the study model presented in this thesis can be further modified, offers a promising and relevant approach to obtaining a comprehensive understanding of the physiology and pathology of the immune system.

Kurzzusammenfassung

Gewebsresidente Gedächtnis-T-Zellen (TRM) zirkulieren nicht im Blut, sondern verbleiben über einen längeren Zeitraum in Organen, wo sie an erster Position stehen, um bei potenziellen Infektionen die Immunabwehr sicherzustellen. TRM wurden bisher nur unzureichend charakterisiert und die Untersuchung ihrer Eigenschaften und ihres Phänotyps stellt insbesondere beim Menschen bis heute eine große Herausforderung dar.

Ziel dieser Arbeit war es, die Dauer der Persistenz und spezifische Oberflächenmoleküle von TRMs in der menschlichen Haut als einer der wichtigsten Kontaktflächen mit Krankheitserregern zu untersuchen. Haut- und Blutproben wurden von 38 Patienten entnommen, die sich zuvor einer allogenen hämatopoetischen Stammzelltransplantation (allo-SZT) unterzogen hatten. Diese Proben wurde enzymatisch und mechanisch verdaut und die T-Zellen mittels Durchflusszytometrie auf ihren Phänotyp untersucht und isoliert. Anschließend wurde ihre DNS extrahiert und eine STR-PCR-Analyse durchgeführt, um festzustellen, ob nach einer allo-SZT vom Stammzellempfänger stammende residente T-Zellen identifiziert werden konnten.

Bei insgesamt 6 Patienten, bei denen die Stammzelltransplantation bis zu 818 Tage vor der Probenentnahme stattgefunden hatte, wurden vom Stammzellempfänger stammende TRMs identifiziert. Dies demonstriert die enorme Langlebigkeit der TRMs beim Menschen. Auf Basis der Analyse zahlreicher Oberflächenmoleküle, die von den aus dem Blut und der Haut gewonnenen Zellen exprimiert werden, konnte ein umfassender Überblick über die Dynamik der T-Zellpopulation bei Patienten nach allo-SZT gewonnen werden. Außerdem konnten vermeintliche Residenzmarker wie CD69 und CD103 kritisch untersucht werden. Die Ergebnisse deuten darauf hin, dass vor allem $CD4^+$ T-Helferzellen, die in vielen früheren Studien über TRMs wenig beachtet wurden, einen großen Anteil an TRMs haben.

Die weitere Erforschung dieser Population am Menschen, für die das in der vorliegenden Arbeit vorgestellte Studienmodell weiter modifiziert werden kann, stellt einen vielversprechenden und relevanten Ansatz dar, um ein umfassendes Verständnis der Physiologie und Pathologie des Immunsystems zu erlangen.

1 Introduction and scientific background

1.1 Tissue-resident memory T cells

1.1.1 T cells are varied and provide comprehensive protection of the body

T cells are essential components of the adaptive immune system, with manifold functions (Bonilla & Oettgen, 2010). They are responsible for defense against viral infections and are involved in the defense against microorganisms, such as bacteria and parasites (Kumar, Connors, & Farber, 2018). Additionally, T cells play a role in the elimination of malignantly transformed cells (S. Jiang & Yan, 2016). To perform these myriad functions, T cells are in constant contact with the majority of cells of the body. Via major histocompatibility complex (MHC) molecules, they can recognize pathological changes in the cells (Rossjohn et al., 2015). T cells represent an extremely versatile and heterogeneous cell type, which ensures that the body can mount an optimized and balanced immune response.

$CD8^+$ cytotoxic T cells react with class I MHC molecules, which are found on nearly all cells in the body. When T cells detect MHC molecules presented by virus-infected cells or tumor cells, T cells eliminate the MHC-presenting cells through cell lysis or the activation of programmed cell death pathways (Russell & Ley, 2002). $CD4^+$ T helper cells assume important mediator functions, activating other immune cells like B cells through the binding of CD40 ligand (CD40L) with CD40, which is expressed on the surfaces of antigen-presenting cells (APCs) or through the secretion of various cytokines following antigen recognition (Elgueta et al., 2009; Jinfang Zhu, Yamane, & Paul, 2010). Secreted cytokines can either support the cellular or humoral immune response or induce inflammatory processes. However, as is the case for cytokines secreted by regulatory T cells (T_{reg}), they can also induce an immunosuppressive effect to prevent either an inadequate or extreme immune response (Jinfang Zhu et al., 2010).

As crucial ability for enabling the long-term protection of the body against repeated exposure to infectious agents, T cells are capable of differentiation into memory T cells, which will be discussed in the following section.

1.1.2 Immunological memory is essential for an effective immune system and consists of different T cell populations

The formation of immunological memory is a fundamental aspect of effective immune system function (Farber, Netea, Radbruch, Rajewsky, & Zinkernagel, 2016). Antigen-specific T cells are generated that exist in the body for many years following antigen contact (Jameson, 2002). Each naive T cell expresses a cell-specific T cell receptor (TCR), which is produced through somatic recombination and can bind to a specific antigen (Glusman et al., 2001). TCRs are composed of two protein chains. The largest T cell population, the αβ T cells (αβTCs), express an α and a β chain, whereas a smaller T cell population, the γδ T cells (γδTCs), express TCRs consisting of a γ and a δ chain (Glusman et al., 2001; Vantourout & Hayday, 2013). γδTCs are less well-studied and can be regarded as a distinct population, associated with different properties and functions than the (αβTCs), which are described in the following section (Zarin, Chen, In, Anderson, & Zúñiga-Pflücker, 2015).

After differentiation and selection in the thymus, T cells initially circulate as naive T cells in the blood and secondary lymphatic organs (SLOs), such as lymph nodes, where APCs display ingested antigens (Weninger, Crowley, Manjunath, & von Andrian, 2001). Each naive T cell passes through numerous lymph nodes over time, which allows each T cell to be able to protect large parts of the body against its cognate antigen, as lymph nodes collect antigens from larger parts of the body.

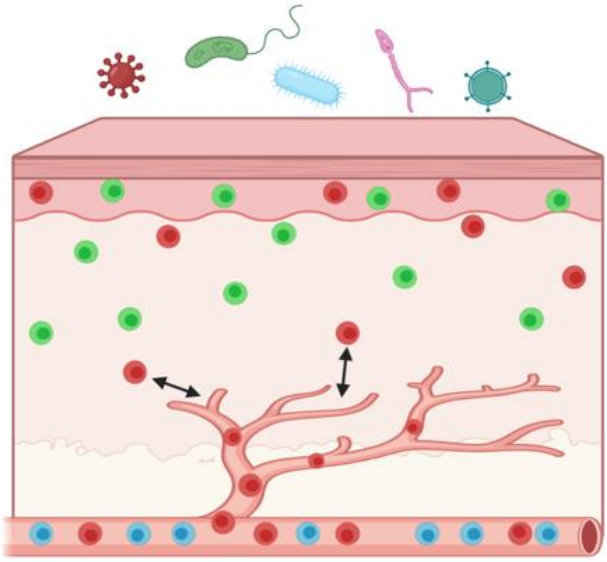


Figure 1.1 Migratory patterns of distinct T cell populations

Central memory T cells (blue) do not migrate to NLT, effector memory T cells (red) continuously circulate between blood and NLT. In contrast to these populations, TRM (green) do not circulate in the blood or lymph but are resident in NLT over an extended period of time, enabling them to respond immediately to local pathogens.

When naive T cells encounter and respond to a specific antigen in the lymph node, they become activated, expanding and differentiating into a variety of subpopulations (Busch, Kerksiek, & Pamer, 2000; Huster et al., 2004; Meng, Yasumoto, & Tsai, 2006; Reinhardt, Khoruts, Merica, Zell, & Jenkins, 2001). Initially, T cells predominantly differentiate into effector T cells (TEFFs), which fight acute infection. Subsequently after the infection, a large proportion of these TEFFs perish, and the small fraction that remains eventually transform into memory T cells (Farber, Yudanin, & Restifo, 2014; Murali-Krishna et al., 1998; Samji & Khanna, 2017). Memory T cells can exist for a long time, even in the absence of antigens, which guarantees an accelerated and effective immune response during reinfection (Lau, Jamieson, Somasundaram, & Ahmed, 1994).

In many respects, memory T cells represent a heterogeneous cell population. Sallusto et al. were the first group to differentiate memory T cell subsets. Based on the expression of the lymph node-homing chemokine receptors CCR7 and the selectin CD62L (Gunn et al., 1999), they divided memory T cells into two subpopulations (Sallusto, Lenig, Förster, Lipp, & Lanzavecchia, 1999). Central memory T cells (TCMs) express CCR7 and CD62L, which enables them to

enter and monitor SLOs from the blood, similar to naive T cells. When reactivated, TCMs can rapidly respond by stimulating B cells and dendritic cells and by proliferating to generate numerous TEFFs. In contrast, the $CCR7^{-}CD62L^{-}$ effector memory T cells (TEMs) were described to circulate continuously between blood and non-lymphatic tissue (NLT) but do not enter SLOs. When antigens are recognized, TEM perform effector functions to fight pathogens by producing inflammatory cytokines like Interferon gamma (IFNγ) or secreting cytotoxic proteins like Perforin (Sallusto et al., 1999; von Andrian & Mackay, 2000). Subsequent studies, in which memory T cells were registered in NLT, interpreted all of those cells as steadily circulating TEMs (Reinhardt et al., 2001). Sallustos´ studies were used to justify that findings reported by studies performed using T cells isolated from the blood could be regarded as representative for all memory T cells (including those found in NLT), which made experimental work extremely accessible because large numbers of T cells can easily be isolated from blood samples for research purposes (Schenkel & Masopust, 2014).

Recently, however, another memory T cell population has been described in the literature, which is currently the subject of intensive research (Rosato, Beura, & Masopust, 2017). These tissue-resident memory T cells (TRMs) do not circulate in the blood or lymph but are resident in NLT over an extended period. Current knowledge regarding this population, its characteristics, and its indispensable role in the immunological protection of the body are discussed below.

1.1.3 TRMs in this thesis

At first, potential mechanisms of tissue retention will be introduced, which provide a prolonged persistence of such T cells in peripheral, non-lymphatic tissue. Second, existing approaches to study this rare cell population will be explained and the approach of the presented study will be implemented in that background. Third and finally, existing theories about the potential role of such TRMs in tissue homeostasis and inflammation will be presented.

1.1.4 TRMs can develop from different precursors and remain in tissue with the help of surface molecules

The exact cytogeny of TRMs has not yet been clarified. Several studies have suggested that TCMs may serve as TRM precursors (Gaide et al., 2015; Gehad et al., 2018; Osborn et al., 2019) and a similar role has been suggested for TEMs (Van Braeckel-Budimir, Varga, Badovinac, & Harty, 2018). Other authors have suggested that TRMs may derive directly from TEFFs, which are generated after antigen recognition by naive T cells and enter the tissue during acute infection (Iijima & Iwasaki, 2015). Watanabe et al. (2015) have further identified a new subset of memory T cells, which they proposed to be direct precursors of TRMs (Watanabe et al., 2015). These cells, which they termed migratory memory T cells (TMMs), are similar in many aspects to TCMs and TEMs but have a $CCR7^{+}CD62L^{-}$ phenotype. Overall, there seem to be various specific T cell lineages preconditioned to develop preferentially into TRM (Kok, Masopust, & Schumacher, 2021).

TRM precursor cells enter NLT during the course of an infection. This well-researched process is regulated by numerous cytokines that induce lymphocyte rolling on activated endothelial cells, allowing T cells to enter the NLT (Iijima & Iwasaki, 2015).

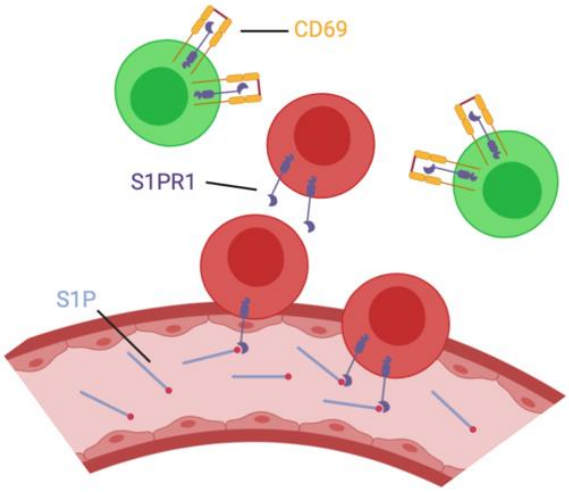


Figure 1.2 Role of CD69 in preventing tissue egress of TRM

By interfering with the S1P receptor-1 (S1P1), CD69 limits the influence of sphingosine-1-phosphate (S1P) on circulatory retention of T cells and thereby enables TRM to reside in tissues.

As a basic condition for residency, once inside the tissue, the cells must prevent tissue egress. Desensitization of the influence of sphingosine-1-phosphate (S1P), a signaling substance that T cells can sense and follow, resulting in circulatory retention, is considered to be an important step in this process (Matloubian et al., 2004) (Fig. 1.2). The increased expression of the lectin CD69 is an effective mechanism for limiting the influence of S1P. CD69 was originally identified as a classic early activation marker, observed on the cell surface two to three hours after TCR stimulation (Cibrián & Sánchez-Madrid, 2017; Sancho, Gómez, & Sánchez-Madrid, 2005). CD69 functions to prevent tissue egress in the presence of lymphocyte-activating stimuli such as interferon α/β (Freeman, Hammarlund, Raue, & Slifka, 2012). This effect was initially observed in SLOs by Hall et al. in 1965 and is thought to temporarily ensure tissue retention to allow for the optimization of the immune response (Hall & Morris, 1965). CD69 acts by interfering with S1P receptor-1 (S1P1) (Shiow et al., 2006), which is necessary for tissue egress; thus, CD69 facilitates T cell residence (Cyster & Schwab, 2012; Matloubian et al., 2004). Additionally, TCR stimulation down-regulates genes, such as Kruppel-like factor 2 (*KLF2*), that promote T cell egress via the S1PR1 and CCR7 pathway (Carlson et al., 2006).

Additionally, to become a resident TRM, the T cells are able to anchor themselves within their environments, which can be achieved through the expression of CD103 (also known as integrin αe), a protein that forms a complex with integrin β7 (Fig. 1.3). This complex binds with E-cadherin, which is present in the epithelium, enabling the tissue retention of lymphocytes (Cepek et al., 1994; Pauls et al., 2001). Several TRMs express additional receptors for proteins found in the extracellular matrix, such as CD49a, and chemokine receptors, such as CXCR6, which might contribute to anchoring TRMs in the surrounding tissue. However, these methods do not occur uniformly for all T cells in all tissues and the detailed differentiation process remains unclear (Cheuk et al., 2017; Hombrink et al., 2016; Kumar et al., 2017).

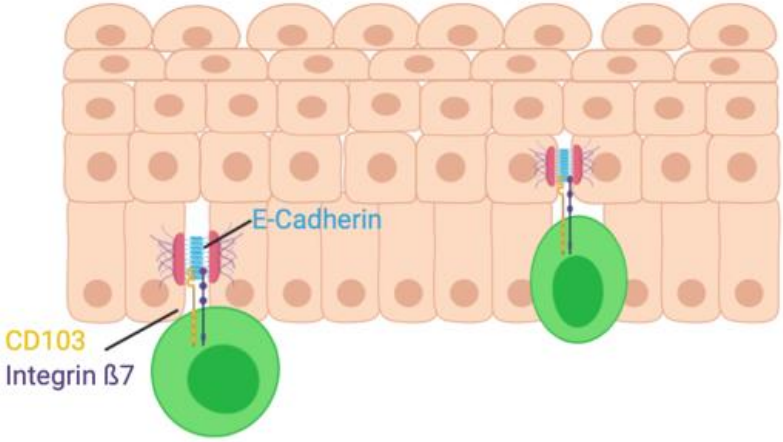


Figure 1.3 Role of CD103 in enabling tissue retention of TRM

By expressing CD103, which forms a complex with integrin β7, TRM are able to bind with E-cadherin, thereby preventing their egress from NLT.

Overall, the further differentiation of TRMs is significantly dependent on the location, which allows for TRM to adapt optimally to the various conditions present in the respective organs. For example, the cytokines transforming growth factor (TGF)-β and interleukin (IL)-15, whose presence varies significantly depending on localization, play major roles in the differentiation of several TRMs, but not in all organs (Casey et al., 2012; Mackay et al., 2013; Schenkel et al., 2016). Unlike TEMs, however, prolonged cognate antigen stimulation is only of minor importance for the development of TRMs (Casey et al., 2012; Mackay et al., 2012). In summary, TRMs use different methods to become long-term resident T cells in different tissues, depending both on the individual cell line and the tissue involved.

1.1.5 TRMs have numerous functions and provide substantial opportunities for the future of medicine

The functions of TRMs in the immune system and local tissue homeostasis are extremely diverse. In a local infection, the body is dependent on an immediate immune response. Without the protection of TRMs, the stimulation of TCMs by antigens in the draining lymph node can take a long time, allowing increased damage to occur before a sufficient TEFF response can occur. TRMs, which “occupy the frontline sites of infection ”(Schenkel & Masopust, 2014) such as

skin and mucosal sites, can ensure an immediate immune response to previously encountered pathogens.

TRMs can respond to infection through several pathways. First, they can exert a direct cytolytic effect to eliminate infected cells (D. Masopust, 2001). Second, they can significantly proliferate when reactivated to generate an enlarged secondary TRM population (Beura, Mitchell, et al., 2018). Third, TRMs are responsible for an essential task, designated the “sensing and alarm function” (Schenkel, Fraser, Vezys, & Masopust, 2013), which results in cytokines, such as tumor necrosis factor-alpha (TNF-α), IFN-γ, or IL-2, being produced and released into the surrounding tissues, increasing resistance to pathogens by recruiting further immune cells (Ariotti et al., 2014; Glennie et al., 2015; Schenkel, Fraser, Beura, et al., 2014; Schenkel et al., 2013).

In studies performed in mice, TRMs have been shown to provide protection against local reinfection or the reactivation of viruses in numerous tissues (Gebhardt et al., 2009; X. Jiang et al., 2012; Khanna, Bonneau, Kinchington, & Hendricks, 2003; David Masopust et al., 2010; Teijaro et al., 2011; L. M. Wakim, Woodward-Davis, & Bevan, 2010). For example, Gebhardt et al. used an elegant mouse model to demonstrate that virus-specific $CD8^+$ TRMs provided enhanced protective immunity against rechallenge with herpes simplex virus-1 (Gebhardt et al., 2009). This antiviral effect has also been demonstrated in human studies (Jia Zhu et al., 2007, 2013). Additionally, protection mediated by TRMs against reinfection with parasites (Fernandez-Ruiz et al., 2016; Glennie et al., 2015), bacteria (Hansen et al., 2018; N. M. Smith et al., 2018; Snyder et al., 2019) and fungi (Conti et al., 2014) have been described.

Furthermore, in recent years, numerous studies have indicated the positive effects of TRMs against cancer. Evidence has suggested that tumor-localized TRMs can effectively limit tumor growth and prolong overall survival in patients with various cancers (Boddupalli, Bar, et al., 2016; Djenidi et al., 2015; Ganesan et al., 2017; Malik et al., 2017; Nizard et al., 2017; Webb, Milne, Watson, DeLeeuw, & Nelson, 2014). TRMs can additionally play a central role in the development of new, effective vaccines. Since the initial discovery of TRMs, many promising approaches have been explored, in which the generation of TRMs has provided local protection against infections and diseases, including cancer. (Fernandez-Ruiz et al., 2016; Hansen et al., 2018; Nizard et al.,

2017; Shin & Iwasaki, 2012; Stary et al., 2015). Further research into TRMs, therefore, offers many promising opportunities for future medicine.

1.1.6 TRMs are involved in the development of diseases and may provide therapeutic targets

Since their discovery, TRMs have been associated with the development and maintenance of various diseases. This association was initially demonstrated, with particular clarity, for fixed drug eruptions (FDE). In FDE, erythematous lesions on the skin or mucosa are following repeated exposure to a particular drug at the lesion location. Within these sites, T cells with TRM-like phenotypes were identified as resident T cells and were significantly involved in lesion development (Shiohara, 2009; Teraki, Kokaji, & Shiohara, 2006). Subsequently, additional skin diseases were associated with the presence of TRMs, including TRM enrichment at skin sites affected by the depigmentation disorder vitiligo (Cheuk et al., 2017; Malik et al., 2017). By blocking CD122, a subunit of the IL-15 receptor, which is expressed by TRMs, TRMs could be depleted, and the course of the disease could be stopped (Richmond et al., 2018). TRMs also appear to play a role in the development and maintenance of psoriasis, which has been demonstrated in several studies (Cheuk et al., 2017, 2014; Khalil, Bardawil, Kurban, & Abbas, 2020; Matos et al., 2017). In addition to skin diseases, tissue-specific diseases, such as asthma (Hondowicz et al., 2016), Crohn's disease (Kleinschek et al., 2009) and multiple sclerosis (Sasaki et al., 2014) appear to be influenced by TRMs. Additionally, both in solid allogeneic organ transplantation and stem cell transplantation, a connection between TRMs and the development of graft-versus-host-disease (GvHD) has been suggested (Divito et al., 2020; David Masopust & Soerens, 2019; C. O. Park & Kupper, 2015; Snyder et al., 2019; Strobl et al., 2020; Tkachev et al., 2021). Further research into TRMs could, therefore, contribute to our understanding of various severe diseases and the identification of new therapeutic options.

1.1.7 TRMs are heterogeneous and difficult to phenotype, posing significant challenges for researchers

For a long time, T cells were exclusively examined through the evaluation of blood samples, and these results were used to draw conclusions that were considered to be generalizable to all T cells in the body (David Masopust & Soerens, 2019). Blood samples are relatively non-invasive, can be readily obtained, and contain many T cells, which are easy to isolate and examine. Since the discovery of TRMs in nearly all NLT and the realization that they represent a distinct T cell population with unique properties, the assumption that T cells in the blood are representative of all T cells is no longer considered valid. However, the study of TRMs poses significant challenges to scientists for various reasons.

TRMs, both within the same organ (such as the skin) and across different organs, display large heterogeneity in phenotypes, functions, and maintenance (van der Gracht, Behr, & Arens, 2021). Therefore, the unambiguous identification of TRMs remains challenging. To distinguish TRMs from other T cell populations, scientists have attempted to identify markers that are expressed specifically by TRMs. The proteins CD69 and CD103, whose role in TRM development and maintenance was described above, are the most frequently discussed potential TRM marker proteins. The suitability of these surface molecules to act as markers for TRMs, in general, and for skin, in particular, is discussed below.

During initial attempts to phenotype TRMs in mice, high expression levels of CD69 were repeatedly observed in various tissues (Gebhardt et al., 2009; Teijaro et al., 2011; L. M. Wakim et al., 2010). Subsequent mouse experiments have resulted in the production of substantial evidence that CD69 is significantly involved in TRM differentiation (Mackay et al., 2013), which can be explained by the effects described above (Mackay et al., 2015). Based on these and subsequent research results, a majority of TRMs are thought to express CD69, resulting in CD69 being used in many studies as a TRM marker (David Masopust & Soerens, 2019). However, CD69 cannot generally be regarded as an optimal TRM because TRMs have been described in many organs that lack CD69 expression (Steinert et al., 2015). Furthermore, recently stimulated non-resident T cells may express CD69 as a marker of recent activation (Sancho et al., 2005;

Shiow et al., 2006) and the expression of CD69 can be transient, which can complicate conclusions regarding the duration of residency.

Because TRMs also display high levels of CD103 expression, CD103 has additionally been discussed as a potential residence marker (Gebhardt, Palendira, Tscharke, & Bedoui, 2018; Gebhardt et al., 2009; L. M. Wakim et al., 2010). As described above, CD103 may be involved in the formation and maintenance of TRMs. However, CD103 does not appear to be required in all tissues, and many TRMs do not reliably present CD103 as a surface marker (Casey et al., 2012; C. J. Smith, Caldeira-Dantas, Turula, & Snyder, 2015; Steinert et al., 2015). In general, the expression of CD103 in TRMs appears to be variable across different organs and their compartments.

Human skin contains approximately 20 billion T cells, some of which are circulating T cells, but most of which appear to be TRMs (Clark et al., 2006; Watanabe et al., 2015). Based on an examination of the surface molecules expressed by TRMs in the skin, CD69 is often considered to be a reliable residence marker, and authors assume that it is expressed by nearly all skin TRMs (Ho & Kupper, 2019; Watanabe et al., 2015). In several studies, the expression of CD103 has shown increased heterogeneity in the skin. A large population of TRMs identified in the epidermis expresses a $CD8^+CD103^+$ phenotype. However, $CD8^+CD103^-$ TRMs have also been identified (Casey et al., 2012; Ho & Kupper, 2019; Mackay et al., 2013). $CD4^+$ TRMs have primarily been localized to the dermis and only a small fraction of these express CD103 (Ho & Kupper, 2019; C. O. Park et al., 2018).

Most current knowledge regarding TRMs has been acquired through the study of complex mouse models, primarily due to the lack of reliable TRM markers. In many studies, for example, the parabiosis technique has been applied (Beura, Wijeyesinghe, et al., 2018; Collins et al., 2016; Iijima & Iwasaki, 2014a; X. Jiang et al., 2012; Schenkel et al., 2013), in which two congenic mice are conjoined, which causes the vasculature of the two individuals to form anastomoses. After one to two weeks, the immune cells from both mice in the shared circulation equilibrate. By observing which tissue compartments do not present a balance between immune cells from both individuals, conclusions are drawn regarding residence. Alternatively, TRM research frequently involves tissue transplantation, which allows T cell populations to be monitored over time (Gebhardt et al., 2009; Glennie et al., 2015; David Masopust et al., 2010).

Another frequent experimental model involves the *in vivo* labeling of T cells from defined compartments, after which their further movements can be registered. Monoclonal antibodies (mAB) (Anderson et al., 2014, 2012; Tkachev et al., 2021) and fluorescent cell-staining dyes, such as carboxyfluorescein succinimidyl ester (CFSE) (Ely, Cookenham, Roberts, & Woodland, 2006; L. M. Wakim et al., 2010) are common applications for this purpose. Other studies have attempted to monitor cellular movements using transgenic cells that express “Kaede,” a photoconvertible fluorescent protein (S. L. Park et al., 2018; Tomura & Kabashima, 2013).

For technical and ethical reasons, experiments comparable to these mouse models are difficult to perform in humans. However, knowledge generated using mouse models must be critically evaluated to determine whether and how it can be translated into an understanding of the human immune system (Mestas & Hughes, 2004). Therefore, novel experimental models in the human setting are urgently necessary to expand this knowledge in the human context. A promising new approach based on allogeneic hematopoietic stem cell transplantation in human patients is presented in this thesis.

1.2 Allogeneic hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is the final curative therapy option for many patients with diseases of the myeloid or lymphatic systems (Copelan, 2006). The basic principle of HSCT is the transfer of pluripotent stem cells from a donor to a recipient and can be characterized as either autologous HSCT (auto-HSCT) and allogeneic HSCT (allo-HSCT). In auto-HSCT, patients receive their own stem cells, which are collected prior to chemotherapy or radiotherapy, such that patients serve as both the donor and the recipient. Patients who undergo allo-HSCT receive stem cells from another human leukocyte antigen (HLA)-compatible individual (Ljungman et al., 2006).

The indications for allo-HSCT are manifold and include non-malignant diseases, such as rare forms of anemia (Beelen & Bornhäuser, 2016). However, the most common indications are cancers of the myeloid or lymphatic systems, such as acute myeloid leukemia (AML) (Duarte et al., 2019). If auto-HSCT is not an option for a patient for one of several possible reasons, or the

underlying disease recurs after an auto-HSCT procedure, allo-HSCT is the preferred treatment option (Sureda et al., 2015). Allo-HSCT relies on the graft-versus-leukemia effect (GvL), in addition to the development of new donor hematopoiesis. GvL describes the process through which immune cells formed by the new hematopoietic system can attack remaining malignant cells to reduce the risk of recurrence (Horowitz et al., 1990; Weiden et al., 1979; Weiden, Sullivan, Flournoy, Storb, & Thomas, 1981). However, this process is also associated with the increased risk of GvHD occurrence (see below).

1.2.1 Donor stem cells can be obtained from a variety of sources and are selected based on tissue compatibility

Donor selection is primarily based on agreement between the HLA characteristics of the donor and recipient, which serves as a measure of tissue compatibility (Copelan, 2006). HLA complexes are more commonly known as major histocompability complexes (MHCs), which are the protein complexes used to signal the presence of foreign antigens and peptides on the cell surface. HLA complexes are encoded by *MHC*, a gene region on chromosome 6 (Murphy & Weaver, 2018). Depending on their functions and lengths, HLA molecules can be divided into three classes (HLA-I, HLA-II, and HLA-III) (Mehra, 2010). When selecting donors, particular attention is paid to those from classes HLA-I (HLA-A, -B, -C) and HLA-II (HLA-DRB1, -DQB1) (Duarte et al., 2019).

The alleles that encode HLA antigens are inherited according to Mendelian inheritance (Murphy & Weaver, 2018). Therefore, a patient's nuclear family, particularly siblings, are typically screened to identify HLA-matched related donors. Alternatively, HLA-matched unrelated donors can be identified through the use of large international donor databases. In case of failure to identify an HLA-identical (HLA-ident) donor, the transplantation of stem cells from a haploidentical related donor or a mismatched unrelated donor (MMUD) can be performed (Bornhäuser, 2016; Duarte et al., 2019).

The degree to which HLA alleles match can significantly influence the success of allo-HSCT with regard to graft failure and the development of severe GvHD (Beatty et al., 1985; Bussel, Berkowitz, McFarland, Lynch, & Chitkara, 1988; Goulmy et al., 1996; Powles et al., 1983). However, some disparities are more permissive than others, and these can increasingly be compensated through improved clinical management techniques or graft pretreatment.

(Aversa et al., 1998; G. B. Ferrara, 2001; Fleischhauer et al., 2006; S. J. Lee et al., 2007; Pidala et al., 2013).

Hematopoietic stem cells can be obtained using several techniques (Carreras, Dufour, Mohamad, & Kröger, 2019; Copelan, 2006). The originally developed method continues to be applied today at many transplant centers, which involves the removal of bone marrow using one or more punctures of the posterior iliac crest. However, for the donor, the removal of hematopoietic stem cells from the peripheral blood represents a much more convenient strategy. For the use of this technique, stem cells are first mobilized by the administration of hormones, such as granulocyte-colony stimulating factor (G-CSF), to increase the quantities of circulating stem cells in the donor blood, from whence they can be isolated (Hopman & DiPersio, 2014). Umbilical cord blood can also be used as a source for HSCs (Carreras et al., 2019; Copelan, 2006).

1.2.2 Numerous different regimens can be used to kill the patient's hematopoietic stem cells

If a suitable stem cell donor has been identified, allo-HSCT can be performed. The patient first receives what is known as conditioning therapy, which consists of whole-body irradiation, chemotherapy, or both, in addition to the application of biologicals and antibodies, as necessary. Conditioning has the following three goals: 1) the elimination of as many malignant cells as possible (in cases of cancer); 2) the induction of immunosuppression to ensure engraftment and prevent primary graft failure and GvHD development; and 3) the creation of niches in the bone marrow in which the stem cells can settle, although this remains controversial (Carreras et al., 2019; Wulf, 2016).

Several different conditioning regimens can be selected, which differ in intensity. Blurred distinctions can be made between myeloablative (MAC), non-myeloablative (NMA), and reduced-intensity conditioning (RIC) regimens (Giralt et al., 2009). Originally, only high-dose MAC regimens were used (Thomas, Bruckner, Banaji, & Weiden, 1977), which result in such severe damage to the patient’s bone marrow that autologous recovery without stem cell support is no longer possible (Bacigalupo et al., 2009).

However, MAC regimens are highly toxic and cannot be used in elderly patients, who bear the highest risk for hematological diseases. Other less aggressive conditioning regimens were identified for these patients, resulting in

the development of the NMA and RIC regimens (Giralt et al., 2009; Slavin et al., 1998). NMA is widely defined as “a regimen that will cause minimal cytopenia and does not require stem cell support“ (Bacigalupo et al., 2009). Conditioning regimens that do not fit unambiguously into the definitions of MAC and NMA are defined as RIC and typically cause profound lymphopenia, which may be reversible but requires clinical stem cell support (Bacigalupo et al., 2009; Wulf, 2016). Distinguishing among these categories can be difficult, and individual conditioning regimes frequently cannot be clearly defined.

Chemotherapy involves the use of numerous substances in various combinations and doses, and the protocols that are used for treatment depend on the transplant centers performing the treatment, which are generally involved in prospective studies (Wulf, 2016). A conditioning regimen that is frequently used at the Klinikum Rechts der Isar at the Technical University of Munich is the combination of fludarabine and treosulfan. Fludarabine is a strongly immunosuppressive purine analog that expresses synergistic effects with alkylators by inhibiting DNA replication and repair (Carreras et al., 2019; J.-H. Lee et al., 2013). Due to reduced toxicity and high tolerability, fludarabine is frequently used as a replacement for the previously popular alkylator cyclophosphamide. (Khouri et al., 1998; Rambaldi et al., 2015). Treosulfan is a cytotoxic drug that causes DNA-alkylation, chromosomal aberrations, and interstrand cross-linking and is frequently used instead of the previously used busulfan due to reduced toxicity (Casper, 2004; Remberger et al., 2017). Furthermore, total body irradiation (TBI), which should be limited to 12 Gy and fractionated due to high toxicity, is often recommended, particularly in young patients (Shank et al., 1990; Wulf, 2016).

To date, limited reliable data regarding which patients should receive which conditioning regimes are available. Contrary to initial assumptions, studies have shown that the intensity of conditioning regimes has no significant influence on overall survival of patients suffering from myelodysplastic syndrome (MDS) (Kröger et al., 2017; Martino, 2006). Currently, the choice of conditioning regimen is individually adjusted, based on patient age, risk profile, disease progress, and comorbidities, which can all influence transplantation success (Carreras et al., 2019; Malcovati et al., 2013; Wulf, 2016).

1.2.3 Circulating immune cells are depleted by conditioning and recover under the clinical control of blood and bone marrow, while tissue is not observed

After central venous administration, transplanted stem cells form new blood formation islands in bone marrow niches and extramedullary organs, such as the liver and spleen. Within 14–30 days, engraftment occurs, which is defined as a blood neutrophil concentration >500/µl for three consecutive days (Kim & Armand, 2013). In addition, the engraftment is verified by regular chimerism analyses. Chimerism describes the appearance of cells with different genetic origins in an organism, which, in this case, refers to the presence of allogeneic cells in the blood and bone marrow. Chimerism analyses can be performed using various laboratory methods, with high sensitivity, and chimerism is an important parameter to verify following allo-HSCT (Carreras et al., 2019). First, chimerism analysis provides information regarding graft success by indicating whether existing blood cells are being formed, as desired, by donor stem cells (Grigoleit, 2016). Second, the presence of mixed chimerism is a prognostically unfavorable factor in terms of recurrence development (Bader et al., 2004; Bornhauser et al., 2009) and can be used for the early detection of relapses. The aim is to achieve complete donor chimerism, in which the entire blood formation process is initiated from donor stem cells (Grigoleit, 2016).

The restoration of T cell populations in the blood after allo-HSCT occurs via two different pathways and in two phases (C. L. Mackall, Granger, Sheard, Cepeda, & Gress, 1993; Crystal L. Mackall & Gress, 1997; Ogonek et al., 2016; Rufer, Helg, Chapuis, & Roosnek, 2001). First, an increase in the $CD8^+$ T cell population can be observed, due predominantly to the homeostatic peripheral expansion of memory cells present among the transplanted cells (Fry & Mackall, 2005; Rufer et al., 2001). This process is thymus-independent and is triggered primarily by cytokines, such as IL-7, causing an inversion of the CD4/CD8-ratio shortly after transplantation, since reconstitution of T helper cells is more dependent on the second pathway (Ogonek et al., 2016; Petersen et al., 2003; PORMAN et al., 1982; UEDA et al., 1984). The resulting T cell population is only immunocompetent to a limited extent because the TCR repertoire will be limited to T cells contained in the transplant, without the ability to protect against a broad antigen spectrum (Fry & Mackall, 2005). Several months to years after transplantation, naive T cells regenerate via a thymus-dependent

pathway (C. L. Mackall et al., 1993; Crystal L. Mackall et al., 1995, 1997). Normalization can take two to three years and depends on factors, such as age, conditioning therapy, and stem cell source (Arnaout et al., 2014; Mehta & Rezvani, 2016). Currently available data regarding TRM susceptibility to conditioning and the dynamics of T cell regeneration in the skin following allo-HSCT are insufficient (Stewart et al., 2018), which is astonishing given the significance of the skin for immune defense and as the primary site for the occurrence of GvHD, and should be rectified by increased study.

1.2.4 Graft-versus-tumor effect and Graft-versus-host disease are important aspects to be considered in allo-HSCT

Due to the presence of allogenic T cells, allo-HSCT can result in complex reactions, which can significantly influence the success or failure of allo-HSCT (Negrin, 2015). The graft-versus-tumor effect (GvT, similar to GvL) is a major advantage of allo-HSCT over other therapeutic methods that can be applied to hematological cancer. However, Graft-versus-host disease (GvHD), which is one of the most significant complications associated with allo-HSCT, is frequently lethal and is closely associated with both GvT and GvL (Ghimire et al., 2017; Jamil & Mineishi, 2015).

The development of these reactions is often described using a three-phase model (Falkenburg & Jedema, 2017; J. L. Ferrara, Levine, Reddy, & Holler, 2009; Zeiser & Blazar, 2017; Zeiser, Socié, & Blazar, 2016): The first phase begins with tissue damage in the body, caused by conditioning regimens and the underlying disease and resulting in the massive release of proinflammatory cytokines and microbial components, such as lipopolysaccharides (LPSs), due to barrier defects, activating APCs throughout the body. These, in turn, activate allogeneic T cells during the second phase, which differentiate, proliferate, and release further proinflammatory signals. During the third phase, allogeneic T cells are directed against the cells of the recipient, causing massive damage via effector pathways.

Similar to physiological immune reactions, the detection of recipient cells is mediated by the presented peptides of HLA molecules. In HLA-matched allo-HSCT, both the recipient and donor cells express the same HLA molecules. However, HLA molecules present self-peptides, also referred to as minor histocompatibility antigens, which originate from intracellular proteins (Neefjes,

Jongsma, Paul, & Bakke, 2011) and are recognized by allogeneic T cells as nonself-peptides (Griffioen, van Bergen, & Falkenburg, 2016).

In GvT, allogeneic T cells attack cancer cells; however, in GvHD, allogenic T cells attack healthy cells, which can be divided into acute GvHD (aGvHD) or chronic GvHD (cGvHD). aGvHD typically develops within the first weeks to months (according to outdated definitions, within the first 100 days) after transplantation, whereas classic cGvHD occurs months (according to outdated definitions, after 100 days) after transplantation(Filipovich et al., 2005; Ghimire et al., 2017). However, the timing of onset may vary; therefore, the current diagnosis is primarily based on clinical and histological criteria, more than on the time after allo-HSCT (Filipovich et al., 2005; Jagasia et al., 2015). aGvHD primarily manifests in the skin, liver, and gastrointestinal tract (GI tract), where it causes epithelial necrosis and can be classified according to severity (grade I-IV) (Glucksberg et al., 1974; Rowlings et al., 1997). cGvHD has a somewhat more complex pathophysiology that involves B cells (Jamil & Mineishi, 2015) and can manifest in nearly all organs, causing fibrosis and atrophy (Zeiser, Dreger, Finke, Greinix, & Wolff, 2016). Repeated speculation has been raised in the literature regarding the potential roles played by TRMs in the manifestation of GvHD after transplantation of both solid organs and bone marrow. (David Masopust & Soerens, 2019; C. O. Park & Kupper, 2015; Sacirbegovic et al., 2016; Snyder et al., 2019). Recent studies suggested host-derived TRMs to possibly promote GvHD after allo-HSCT in several tissues including skin (Divito et al., 2020; Kong et al., 2021; Strobl et al., 2020; Tkachev et al., 2021). However, the underlying mechanisms associated with such involvement are unknown and require further research.

Determining the appropriate balance between GvHD prophylaxis and treatment capable of preventing severe GvHD while ensuring the maximum GvT effect is complex and challenging (Falkenburg & Jedema, 2017). In GvHD, prophylaxis typically involves combinations of a calcineurin inhibitor to prevent the activation of the transcription factor nuclear factor of activated T-cells (NFAT), such as cyclosporin A (CyA) or tacrolimus, and a cytostatic substance, such as methotrexate (MTX; folic acid antagonist) or mycophenolate mofetil (MMF; guanosine synthesis inhibitor) (Brazelton, 1996; Zeiser & Blazar, 2017; Zeiser, Dreger, et al., 2016). In addition, antithymocyte globulin (ATG), which causes T cell depletion via various mechanisms, can be used (Finke et al.,

2009; Mohty, 2007). Post-transplant treatment with cyclophosphamide to deplete T cells has also been shown to be effective for GvHD prevention (Ganguly et al., 2014; Luznik et al., 2010).

If GvHD develops, despite prophylaxis, glucocorticoids, such as prednisolone or dexamethasone, are the primary treatment option (Zeiser & Blazar, 2017; Zeiser, Dreger, et al., 2016). In case of GvHD resistance to glucocorticoids, the above-mentioned prophylactic drugs, in addition to other therapeutic approaches, such as antibodies (for example, Rituximab, an antibody against B cell marker CD20), can be considered as therapeutic alternatives (Wolff et al., 2013). Despite multiple prophylactic and therapeutic options, GvHD remains a common fatal complication after transplantation and further exploration of the role of TRMs in its pathogenesis may improve the medical management of patients.

1.2.5 Infections are the major complications of allo-HSCT

Invasive procedures, such as allo-HSCT, are not always successful, and depending on the graft source, conditioning regimen, and HLA matching, up to 10% of patients can develop graft failure, which is frequently lethal (Fleischhauer et al., 2006). However, even patients who undergo successful transplantations experience a time period characterized by cellular immune deficiencies. After an aplastic phase of severe neutropenia, until engraftment is established, which can last up to 30 days after conditioning, patients continue to present significantly reduced levels of natural killer cells and T cells, which can last for up to 100 days after conditioning (Ogonek et al., 2016; Seggewiss & Einsele, 2010). Unsurprisingly, patients frequently suffer from infections during this time.

Viral diseases are major causes of morbidity and mortality following HSCT (Arnaout et al., 2014). The most commonly encountered viruses are members of the human herpesvirus (HHV) group, such as the cytomegalovirus (HHV-5; CMV) and the Epstein-Barr virus (HHV-4; EBV) (Ogonek et al., 2016). After initial infection, these viruses can develop lifelong latency in various cell types, including hematopoietic lineage cells (Dupont & Reeves, 2016; Reddehase & Lemmermann, 2019). Immunodeficiency after allo-HSCT leads to CMV infections in a large number of patients, either due to reactivation or due to new infections from infected grafts (Arnaout et al., 2014; Schuster et al.,

2017), which can develop into life-threatening CMV disease, associated with multiorgan manifestations, such as pneumonia and gastroenteritis (Reddehase & Lemmermann, 2019). Therefore, close monitoring must be performed to detect infections as early as possible, and antiviral prophylaxis is typically administered, using drugs such as ganciclovir, valganciclovir, and foscarnet (Carreras et al., 2019).

The possible role played by TRMs in the reactivation of latent viruses after allo-HSCT has not yet been sufficiently investigated and remains to be addressed. Because TRMs are known to form in response to virus exposure and can persist and control latent viruses (Gebhardt et al., 2009; C. J. Smith et al., 2015), this process represents an promising opportunity to reduce morbidity associated with viral diseases after allo-HSCT.

1.3 Aims

The overall aim of the present study was to identify and characterize TRMs using recipient-derived TRMs obtained from the skin of patients following allo-HSCT. By examining the T cells from patient skin and blood samples in parallel, the following questions were addressed: (1) Can TRMs survive conditioning therapy and persist in tissues like human skin after transplantation? (2) What is the phenotype of TRMs in human skin and how reliable are the frequently used residency markers for the identification of TRMs? (3) Do TRMs have an impact on the clinical outcome of patients after allo-HSCT?

TRM research is challenging, and most of our knowledge regarding this important T cell population has been obtained from the study of mouse models, making the findings only limitedly transferable to humans (David Masopust & Soerens, 2019; Mestas & Hughes, 2004). Studies have frequently reported heterogeneous results to these questions, indicating that TRMs vary within different tissues. Therefore, studying TRMs in human skin is important because it represents the first contact area for most pathogens and appears to contain vast quantities of TRMs (Watanabe et al., 2015).

Furthermore, the model presented here can be used to study the development of T cell populations in the blood and skin tissue of patients after allo-HSCT. Because allo-HSCT patients develop a severe immune deficiency, developing a comprehensive understanding of the immune system dynamics following allo-HSCT is essential to the prevention of life-threatening infections. This comprehensive knowledge must include TRMs, which, in the case of lymphopenia in the blood, can offer significant opportunities for maintaining an immune defense and potentially influence the occurrence of GvHD. In summary, gaining a deeper understanding of the phenotypes and functions of TRMs is critically important to the development of new, effective, preventive, and therapeutic options for modern medicine.

2 Materials

2.1 Reagents

CountBright™ Absolute Counting Beads	Thermo Fisher Scientific (Waltham, USA)
2-Mercaptoethanol (50 mM)	Thermo Fisher Scientific (Waltham, USA)
CD3 (human) monoclonal antibody (TR66)	Enzo Life Sciences GmbH (Lörrach, DE)
Ethanol ≥70% (v/v), TechniSolv®	VWR International (Radnor, USA)
Fetal Bovine Serum	Biochrom (Berlin, DE)
Ficoll® Paque Plus	Sigma-Aldrich (Steinheim, DE)
Fludarabine phosphate	Sigma-Aldrich (Steinheim, DE)
Gibco™ Amphotericin B	Thermo Fisher Scientific (Waltham, USA)
GlutaMAX™ Supplement	Thermo Fisher Scientific (Waltham, USA)
Heparin Natrium 25 000 ratiopharm	Shop-Apotheke B.V. (Venlo, NL)
Human Serum	Sigma-Aldrich (Steinheim, DE)
MEM Non-Essential Amino Acids Solution (100X)	Thermo Fisher Scientific (Waltham, USA)
Penicillin-Streptomycin	Sigma-Aldrich (Steinheim, DE)
Remel™ PHA Purified	Thermo Fisher Scientific (Waltham, USA)
Sheath Fluid	BD Biosciences (Heidelberg, DE)
Sodium Pyruvate (100 mM)	Thermo Fisher Scientific (Waltham, USA)
Treosulfan	Sigma-Aldrich (Steinheim, DE)
Trypsin-EDTA (0.05%)	Thermo Fisher Scientific (Waltham, USA)

2.2 Solutions and buffers

Buffers

Wash buffer

1% FCS

In RPMI 1640 supplemented with Hepes

MACS buffer

1% FCS

2mM EDTA (pH8)

In PBS

Complete medium

10% FCS

1% MEM Non-Essential Amino Acids Solution (100X)

2mM L-Alanyl-L-Glutamine

1mM Sodium Pyruvate

100 IU/ ml Penicillin

100 µg/ ml Streptomycin

0.05mM b-Mercaptoethanol

In RPMI 1640 without Hepes

Skin storage medium

10% FCS

1% MEM Non-Essential Amino Acids Solution (100X)

2mM L-Alanyl-L-Glutamine

1mM Sodium Pyruvate

1% 100 IU/ ml Penicillin

100 µg/ ml Streptomycin

In RPMI 1640 supplemented with Hepes

Skin digestion medium

10% FCS

1% MEM Non-Essential Amino Acids Solution (100X)

2mM L-Alanyl-L-Glutamine
1mM Sodium Pyruvate
100 IU/ ml Penicillin
100 µg/ ml Streptomycin
1% Amphotericin B
In RPMI 1640 without Hepes

Freezing medium
40% FCS
10% DMSO
In RPMI supplemented with Hepes

Cell Culture Media

Fibroblast culture medium
20% FCS
100 IU/ ml Penicillin
100 µg/ ml Streptomycin
in MEM-Medium

Cloning medium
All ingredients filtered:
5% Human serum
1% MEM Non-Essential Amino Acids Solution (100X)
2mM L-Alanyl-L-Glutamine
1mM Sodium Pyruvate
100 IU/ ml Penicillin
100 µg/ ml Streptomycin
0,05mM b-Mercaptoethanol
2 IU/ml IL-2
In RPMI 1640 without Hepes

2.3 Antibodies

	Dilution	Clone	
Alexa Fluor 647 anti-human CD103 (Integrin αE)	1:150	Ber-ACT8	Biolegend (London, UK)
Alexa Fluor 647 anti-human CD127 (IL-7Rα)	1:200	A019D5	Biolegend (London, UK)
Alexa Fluor 700 anti-human CD69	1:640	FN50	Biolegend (London, UK)
BV421 anti-human TCR γ/δ	1:40	B1	Biolegend (London, UK)
BV510 anti-human CD8a	1:200	RPA-T8	Biolegend (London, UK)
BV650 anti-human CD25	1:50	BC96	Biolegend (London, UK)
BV786 anti-human CD103	1:1000	Ber-ACT8	BD Biosciences (Heidelberg, DE)
FITC anti-human CD45	1:50	2D1	Biolegend (London, UK)
FITC anti-human CD45RA	1:200	HI100	Biolegend (London, UK)
PE anti-human CD3	1:200	SK7	Biolegend (London, UK)
PE-Cy7 anti-human CD4	1:400	SK3	BD Biosciences (Heidelberg, DE)
PE/Dazzle 594 anti-human CD127 (IL-7Rα)	1:400	A019D5	Biolegend (London, UK)
PE/Dazzle 594 anti-human CD45RA	1:200	HI100	Biolegend (London, UK)
PerCP/Cyanine5.5 anti-human CD45RO	1:100	UCHL1	Biolegend (London, UK)

2.4 Commercial reagent kits

PE Annexin V Apoptosis Detection Kit I	Sigma-Aldrich (Steinheim, DE)
QIAamp DNA Micro Kit	Qiagen (Hilden, DE)
Whole Skin Dissociation Kit, human	Miltenyi Biotec (Bergisch Gladbach, DE)
Zombie NIR™ Fixable Viability Kit	Biolegend (London, UK)

2.5 Laboratory consumables

Biopsy punch 4mm	SMS medipool (Gauting, DE)
Biosphere® Fil. Tips	Sarstedt AG & Co. KG (Nümbrecht, DE)
Bottle top vacuum filtration systems, PES	VWR International (Radnor, USA)
CELLSTAR® centrifuge tubes	Greiner Bio-One GmbH (Frickenhausen, DE)
Clear polystyrene microplates, 384-well	VWR International (Radnor, USA)

Disposable Serological Pipets	Greiner Bio-One GmbH (Frickenhausen, DE)
Falcon® Multiwell Flat-Bottom Plates with Lids, Sterile, Corning®	Thomas Scientific (Swedesboro, NJ, USA)
Falcon™ Cell Strainers Corning® 35-100µm	Omnilab-Laborzentrum GmbH & Co.KG (Bremen, DE)
gentleMACS C Tubes	Miltenyi Biotec (Bergisch Gladbach, DE)
MACS® LS Columns	Miltenyi Biotec (Bergisch Gladbach, DE)
Microcentrifuge tubes, protein LoBind / DNA LoBind, Eppendorf®	VWR International (Radnor, USA)
Multiwell cell culture plates, Falcon®	VWR International (Radnor, USA)
NH4 Heparin Röhrchen	Zentrallager MRI (München, DE)
Original-Perfusor®-Syringes 50 ml	B. Braun (Melsungen, DE)
Pipette Tips	Greiner Bio-One GmbH (Frickenhausen, DE)
Reagiergefäße mit anhängendem Deckel	Zentrallager MRI (München, DE)
SepMate™-50 tubes	STEMCELL Technologies GmbH (Cologne, DE)

2.6 Laboratory instruments

BD FACSAria™ Fusion	BD Biosciences (Heidelberg, DE)
BD FACSAria™ III	BD Biosciences (Heidelberg, DE)
BD LSRFortessa™	BD Biosciences (Heidelberg, DE)
CytoFLEX S	Beckman Coulter (Brea, USA)
gentleMACS Dissociator	Miltenyi Biotec (Bergisch Gladbach, DE), joint use AG Knolle (Munich, DE)
MACS® MultiStand	Miltenyi Biotec (Bergisch Gladbach, DE)
QuadroMACS™ Separator	Miltenyi Biotec (Bergisch Gladbach, DE)
SH800S Cell Sorter	Sony Biotechnology (San Jose, USA), joint use AG Knolle (Munich, DE)

2.7 Software

FlowJo Tree Star	FlowJo (Ashland, USA)
GraphPad Prism	GraphPad Software Inc. (California, USA)
IBM® SPSS® Statistics Base GradPack	SPSS Inc.(Chicago, USA)

26 for Mac Microsoft Office	Microsoft (Redmont, USA)

3 Methods

3.1 General study concept

Skin biopsies and blood samples were taken from patients who had received allogeneic hematopoietic stem cell transplantation (allo-HSCT) in the past. The skin biopsies were transferred into a single-cell suspension (SCS) in an established laboratory procedure by enzymatic and mechanical digestion of the extracellular matrix. Peripheral blood mononuclear cells (PBMC) were isolated from the blood. The two SCSs were then stained with fluorescent antibodies onto defined surface markers and measured by flow cytometry. $CD3^{+}CD45^{+}$ T cells from skin were separated from other cells via cell sorting. The DNA was isolated, and the amount of recipient-derived DNA in the T cells isolated from the skin was determined. For the study, patients were only included when it had been ensured that only donor-derived T cells circulated in the blood rather than the recipient's own T cells. Therefore, it could be concluded with certainty that recipient-derived T cells present in the skin had resided there at least since the time of conditioning, that is, were TRMs.

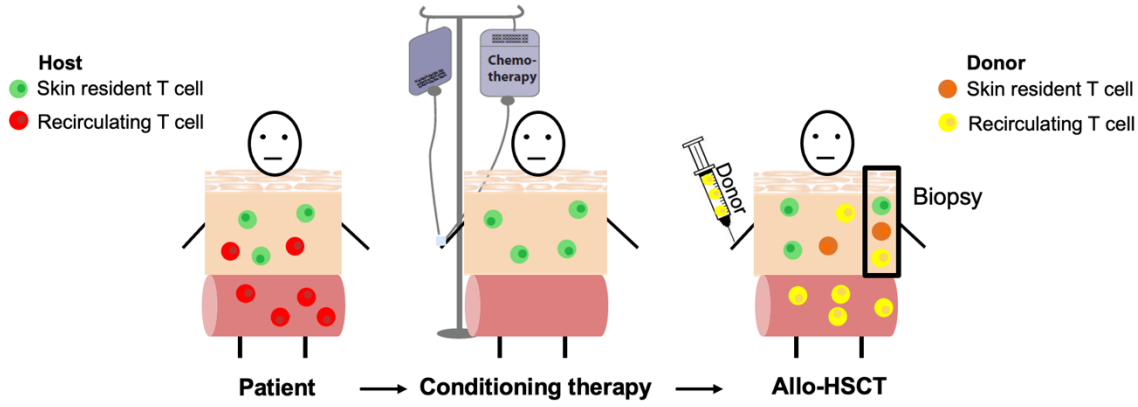


Figure 3.1 Rationale of the general study design

Conditioning therapy is performed to deplete the patient's hematopoietic system. Donor-derived hematopoietic stem cells are then administered to establish an allogeneic immune system. Regular checks are made to ensure that bone marrow and blood cells are exclusively donor-derived. Therefore, it can be concluded that any host-derived T cell registered in the skin of the patient after allo-HSCT resided there at least since the time of conditioning therapy (kindly provided by C. Zielinski, modified).

3.2 Patient cohort

3.2.1 Acquisition of patients

Blood and skin samples were taken from patients who received an allogeneic stem cell transplantation. Patients for this project were recruited in the bone marrow transplantation outpatient clinic (KMT Ambulanz) and hematological stations of Klinikum Rechts der Isar (MRI) of Technische Universität München (TUM). Samples were collected from October 2018 to May 2019. Suitable patients were identified based on the medical record and the digital patient system. Requirements for participation were a stable general condition, no concurrent skin GvHD and a thrombocyte count of over 10,000/µl blood to ensure adequate wound healing. All eligible patients were asked to participate in the study after consultation with the treating physician. A detailed explanation of the planned intervention and the scientific background was provided. 60–70% of the patients agreed to participate, while 30–40% declined to participate because of individual reasons, including fear of pain or inflammation. The intervention was performed in a suitable room within the facility.

3.2.2 General characteristics of the patient cohort

Age [years]	
Median	57
Mean	53.82
Range	22–79
Sex	
Male	n = 27 (71,1%)
Female	n = 11 (28,9%)
Diagnosis [a]	
MDS/AML	n = 18 (47.4%)
MMY	n = 6 (15.8%)
MPS	n = 4 (10.5%)
NHL	n = 4 (10.5%)
ALL	n = 3 (7.9%)
SAA	n = 2 (5.3%)
OMF	n = 1 (2.6%)
Time after allo-HSCT [days]	
median	325,5
mean	611,7895
range	20–5587

Table 3.1 Characteristics of the patient cohort

[a] MDS/AML = Myelodysplastic syndrome / Acute myeloid leukemia, MMY = Multiple myeloma, MPS = Myeloproliferative syndromes, NHL = Non-Hodgkin-Lymphoma, ALL = Acute lymphatic leukemia, SAA = Severe aplastic anemia, OMF = Osteomyelofibrosis

Overall, 38 patients were analyzed for T cell chimerism in the skin. Table 3.1 gives an overview of the patient group examined in this study. The cohort included patients of each age group between 22 and 79 years with patients aged 50-59 years representing the largest proportion.

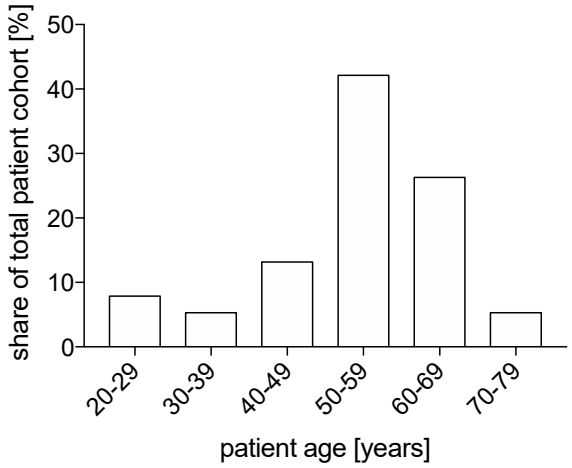


Figure 3.2 Patient age

Shown is the proportion of the total patient cohort represented by the different age groups of patients in years. n=38

There was a broad spectrum of initial diagnoses based on which patients of the cohort were indicated for an allo-HSCT. These included myelodysplastic syndrome (MDS) or AML, myeloproliferative syndromes (MPS), non-hodgkin-lymphoma (NHL), acute lymphatic leukemia (ALL), severe aplastic anemia (SAA) and osteomyelofibrosis (OMF).

For determining the time after allo-HSCT as the time interval between transplantation and sample collection, the day of stem cell transplantation was considered as day 0. The aim was to obtain a comprehensive insight into the dynamics of the T cell populations in the skin of patients after allo-HSCT. Therefore, the patient cohort included patients shortly after transplantation as well as patients transplanted several years ago. The range was 20-5578 days after allo-HSCT.

3.2.3 Stem cell source

66.7% of patients received the stem cell donation from an unrelated foreign donor, the majority of whom were HLA-ident to the stem cell recipients. Only four of the stem cell donations from unrelated donors had single HLA mismatches in HLA-A or HLA-C with the stem cell recipients. Among the related stem cell donors, 50% were HLA-ident, and another 50% were HLA-haploident to the stem cell recipients (table 3.2). Sex-matching was evenly distributed with 52.7% sex-matched stem cells and 47.3% sex-mismatched stem cells.

		Absolute	**Percentage**
Valid	HLA-ident unrelated donor	20	55.6
	HLA-ident related donor	6	16.7
	HLA-haploident related donor	6	16,.7
	HLA-A mm [a] unrelated donor	2	5.6
	HLA-C mm [a] unrelated donor	2	5.6
	Total	36	100.0
Missing		2	
Total		38	

Table 3.2 HLA-matching of the stem cell source

[a] single HLA mismatch in HLA-A / HLA-C

3.2.4 History of medical treatment

The patients received different conditioning regimens for HSCT based on their varying general physical status, underlying disease, and time interval between stem cell transplants with changing guidelines. These differed in terms of the drug combinations used for chemotherapy and the TBI used in multiple patients. By far the most common conditioning regimen used was the combination of fludarabine (usually in doses of 30 mg/m^2) and treosulfan (usually in doses of 14 000 mg/m^2). It was used in 21 patients (56.8%) with different underlying diseases and age groups. In addition or as an alternative to chemotherapy, 8 patients (21.6%) received TBI in doses between two and 12 Gray (Gy) as part of conditioning therapy (table 3.3).

Chemotherapy		**TBI [Gy]**	
Fludarabine, Treosulfan	n = 21 (56.8%)		
FLAMSA-RIC [a]	n = 3 (8.1%)		
CLAMSA-RIC [b]	n = 2 (5.4%)	+2	n=1 (2.7%)
		+4	n=1 (2.7%)
Cyclophosphamid	n = 2 (5.4%)	+12	n=1 (2.7%)
Fludarabine, Cyclophosphamid	n = 2 (5.4%)	+2	n=1 (2.7%)
		+12	n=1 (2.7%)
Fludarabine, Cyclophosphamid, Melphalan	n = 2 (5.4%)		
Etoposid	n = 1 (2.7%)	+12	n=1 (2.7%)
Fludarabine	n = 1 (2.7%)	+12	n=1 (2.7%)
Fludarabine, BCNU, Melphalan	n = 1 (2.7%)		
Fludarabine, Cyclophosphamid, Cytarabin	n = 1 (2.7%)	+2	n=1 (2.7%)
Fludarabine, Melphalan	n = 1 (2.7%)		

Table 3.3 Conditioning therapy received for allo-HSCT

[a] Conditioning according to FLAMSA-RIC protocol (based on Schmid et al. 2015): Combination of Fludarabine, Amsacrine, Cytarabine with RIC through Treosulfan/busulfan and cyclophosphamide. [b] Conditioning according to CLAMSA-RIC protocol: Combination of Clofarabine, Amsacrine, Cytarabine with RIC through Fludarabine/busulfan and cyclophosphamide.

To reduce the occurrence of rejection and GvHD, all patients received various immunosuppressants in different combinations, adapted to the individual patient status. Administered to 75.7% of the patients, ATG and MMF were the substances most frequently used. ATG was usually administered in doses of 10/20/30 mg per kg body weight from d-3 to d-1, while the standard dosage of MMF was 2*1g/d from d0. In 23 patients (62.2%), CsA was used usually from d-

1 at a dose of 5 mg/kg. Other substances administered to patients in varying combinations included tacrolimus (0.03mg/kg), post-Tx cyclophosphamide (50mg/kg), and MTX (10-15mg/m^2). Later in the course, 27 patients (73.0%) received steroids in the form of prednisolone for GvHD prophylaxis or therapy (table 3.4).

Immunosuppressant	Frequency
ATG	n=28 (75.7%)
MMF	n=28 (75.7%)
Prednisolone	n=27 (73.0%)
Cyclosporin A	n=23 (62.2%)
Tacrolimus	n=14 (37.8%)
Cyclophosphamide	n=6 (16.2%)
Methotrexate	n=3 (8.1%)

Table 3.4 Immunosuppression received in the context of allo-HSCT

3.2.5 Control group

Abdominal control skin and PBMCs were obtained from healthy patients undergoing plastic surgery or from healthy blood donors (n=18 (13 female, 5 male)). Due to patient anonymization by the performing plastic surgeons, precise data regarding the characteristics of the control groups could not be collected. However, it was ensured that healthy control subjects were not exposed to any relevant medical treatment, and all material was obtained with informed consent. Samples were processed according to the same procedure as those obtained from patients after allo-HSCT.

3.3 Procedures in the context of skin in blood samples

3.3.1 Sample collection

After thorough disinfection, blood samples of up to 9 ml were collected from an intravenous arm access or a central venous catheter in heparin tubes. Because of easy accessibility, low mechanical stress, and for cosmetic reasons, the abdomen was chosen as the collection site for skin biopsies. Samples were taken from inconspicuous, noninflamed skin areas. The procedure was performed after careful disinfection of the skin area, considering aseptic conditions. The skin was locally anesthetized by subcutaneous injection of Mecain. After the

onset of anesthesia, one or two skin punch biopsies of 4 mm diameter were taken. Bleeding was stopped and the wound was treated with a skin suture, which was removed after five to seven days.

3.3.2 Transport and storage

Transport and storage of the biopsies to the laboratory was performed in an Eppendorf tube filled with skin storage medium. To minimize contamination of the tissue with blood, the skin biopsies were washed twice with skin storage medium immediately after collection. The weight was determined using a precision balance. Subsequently, the skin biopsies were then stored in skin storage medium on ice until further processing.

3.3.3 Digestion of skin

Skin biopsies were transformed into single-cell suspensions through enzymatic and mechanical digestion using the Whole Skin Dissociation Kit (human) according to the manufacturer's instruction. By avoiding the addition of enzyme P to the gentleMACS C tube, relevant surface molecules such as CD3 were spared. Subsequently, skin biopsies were incubated in a water bath at 37°C for 15–16 hours to maximize the cell yield. After incubation, the enzyme action was stopped by adding 0.5 ml FCS containing skin medium. To obtain a SCS, samples were mechanically digested by running the program h_skin_01 on gentleMACS dissociator. Rapid centrifugation was performed to ensure that the entire volume was collected at the bottom of the tube. The cell suspension then was filtered through a 70-µm strainer into another tube. To collect all cells, the gentleMACS C tube was rinsed twice with 2 ml skin medium, which was additionally filtered into the new tube.

3.3.4 PBMC isolation

PBMCs were isolated on the day of blood collection. The method of density gradient centrifugation using the density gradient medium Ficoll at room temperature was used. The blood was mixed with PBS in a ratio of 1:1. A Falcon tube was pre-filled with the density gradient medium Ficoll. The blood PBS mixture was slowly filled onto this medium, taking care not to mix with the Ficoll and form two phases. The tube was centrifuged for 30 minutes at the lowest acceleration and without brake at 805 rcf. During this process, the individual blood

components separated (see figure), with the PBMCs appearing as a narrow ring above the Ficoll layer in the tube. This ring was carefully removed with the pipette and filled into a new tube. The PBMCs were purified by suspending them in PBS and then centrifuging at 453 rcf with maximum acceleration and brake. After centrifugation, the supernatant was discarded. ells were purified two more times by resuspending them in wash buffer and centrifuging at 290 rcf for 10 minutes with maximum acceleration and brake, discarding the supernatant each time. As last step, the PBMCs were suspended in washbuffer and stored at 4°C until further processing.

Alternatively, the first step of PBMC isolation, especially for small blood volumes, was performed using SepMate tubes according to the manufacturer's specifications.

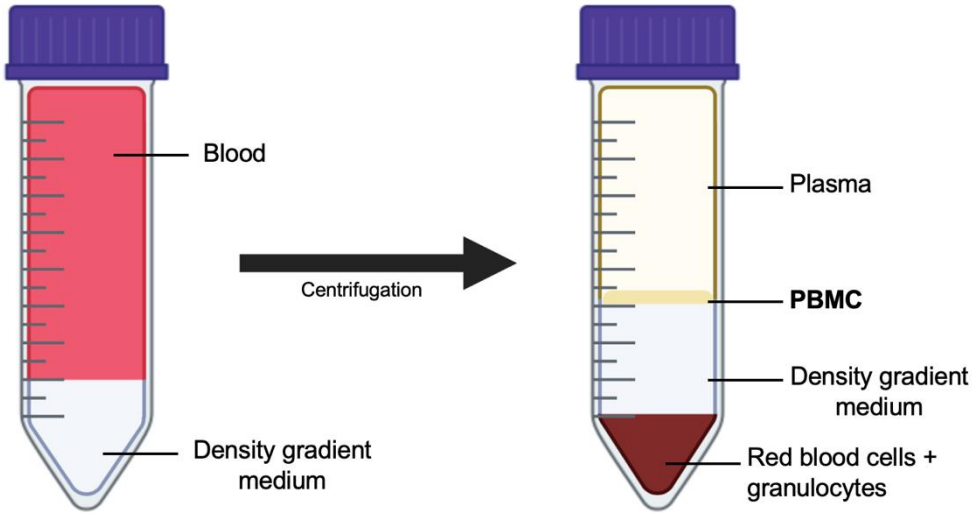


Figure 3.3 Isolation of PBMCs from whole blood samples

Schematic overview of the experimental process of PBMC isolation using the density gradient centrifugation method.

3.4 Flow cytometry

3.4.1 Staining

Cells were stained with antibodies, proteins, and DNA intercalators labeled with fluorescent dyes.

3.4.1.1 Live or dead staining with Zombie NIR

To remove proteins prior to staining with DNA intercalator Zombie NIR, the cells were washed twice by suspending them in PBS and centrifuging at 453 rcf and

4°C for five minutes, discarding the supernatant each time. The cells were suspended for staining in 200 µl PBS, and Zombie NIR was added according to the indicated dilution. This was followed by incubation in the dark for 20 min on ice and purifying cells with wash buffer.

3.4.1.2 Antibody staining

After purifying with MACS buffer, cells were resuspended in 100-200 µl medium. By preparing a master mix specifically for small volumes and simultaneous staining of multiple samples, antibodies were added at the indicated dilution. Cells were incubated on ice in the dark for 30 min and then purified again with MACS buffer.

3.4.2 Cell sorting and cell analysis

3.4.2.1 Instrument preparation

For analyzing and sorting of cells, depending on availability, the following flowcytometry devices were used: Cytoflex (Beckman Coulter), BD LSRFortessa™, BD FACSAria™ Fusion, BD FACSAria™ III and SH800S Cell Sorter. Device preparation and maintenance was carried out according to the manufacturer's specifications.

Nozzles of sizes 70 µm and 85 µm were used. Using single stained PBMC as controls, appropriate compensations were prepared for each experiment and checked at close intervals before sorting. For this purpose, a sample was prepared for each fluorochrome used by staining PBMC with that fluorochrome only.

3.4.2.2 Cell preparation

After staining, the cells were suspended in MACS buffer. The volumes were adjusted to the cell number. To avoid clogging by agglomerates, the cell suspension was filtered through a 30-50 µm strainer immediately prior to sorting.

Eppendorf- or FACS tubes were used as collection tubes. To ensure the highest possible viability of the cells, the tubes were coated with proteins before sorting. For this purpose, they were filled with FCS and rotated periodically to allow coating at each site of the tube. After at least one hour of coating, the FCS was removed and 400 µl of complete medium was added.

3.4.2.3 Cell sorting

The cells were taken up at the lowest possible flow rate to protect the cells. Information about the cells was recorded for later analysis. Gates were adjusted to the population to be sorted after a small number of cells had been recorded. After sorting, a re-analysis of the sorted cells was performed to ensure the quality of the sort.

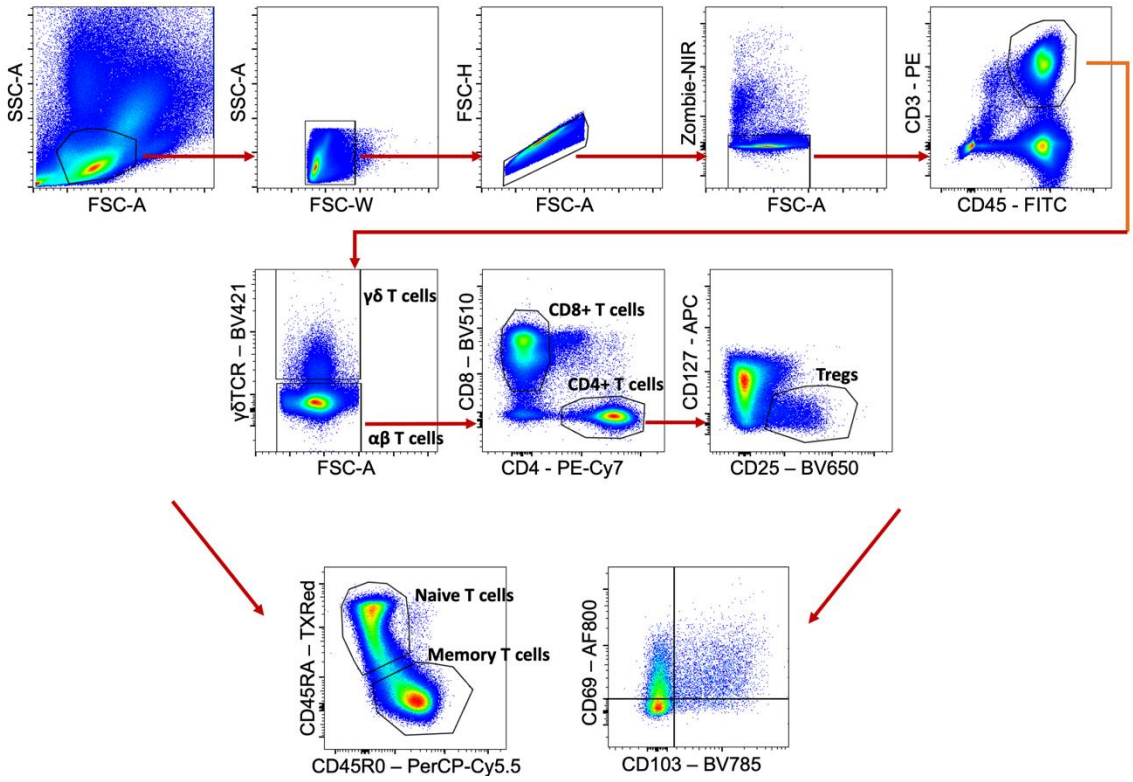


Figure 3.4 Gating strategy for the identification of immune cell subsets

PBMC and cells from skin punch biopsies were stained with antibodies directed against lineage specific surface markers. Shown is the exemplary gating strategy for immune subset identification and quantification from cells of a skin sample.

3.4.2.4 Analysis

After recording the cells, the information was evaluated, and the compensations were checked again using the FlowJo software. The recorded markers and the cell types defined by them are summarized in Table 3.4. In the analysis of the data, the following gating strategy was generally followed (figure 3.4):

Firstly, the forward scatter area (FSC-A) was plotted against the side scatter area (SSC-A), and a gate was set where lymphocytes were expected in terms of size and granularity (complexity). For excluding cell doublets, two gates were set by plotting height (FSC-H) and width (FSC-W) against SSC-A

and FSC-A. After selecting only viable cells via a live or dead viability dye such as Zombie NIR or PI, cells were discriminated in gamma (ɣ) delta (ϱ) T cells and alpha (α) beta (β) T cells. In the latter, a distinction was made between $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells. In the $CD4^+$ population, regulatory T cells (T_{reg}) were gated based on the expression of CD25 and the absence of CD127. Furthermore, the above-mentioned cell types were examined for the expression of CD45RA, CD45RO, CD69 and CD103.

Marker	Property / Cell type
CD3+ CD45+	T cell
CD3+ CD45+ γδTCR+	Gamma (γ) delta (δ) T cell
CD3+ CD45+ γδTCR-	Alpha (α) beta (β) T cell
CD3+ CD45+ γδTCR- CD4+	T helper cell
CD3+ CD45+ γδTCR- CD8+	Cytotoxic T cell
CD3+ CD45+ γδTCR- CD4+ CD25+ CD127-	Regulatory T cell (T_{reg})
CD3+ CD45+ CD45RO+	Memory T cell
CD3+ CD45+ CD45RA+	Naive T cell
CD3+ CD45+ γδTCR- CD4+ CD69+	$CD69^+$ T helper cell
CD3+ CD45+ γδTCR- CD4+ CD103+	$CD103^+$ T helper cell
CD3+ CD45+ γδTCR- CD8+ CD69+	$CD69^+$ cytotoxic T cell
CD3+ CD45+ γδTCR- CD8+ CD103+	$CD103^+$ cytotoxic T cell

Table 3.5 Surface molecules for cell identification

For phenotypic analysis via FACS, cells were stained with fluorescent antibodies targeting specific molecules on the cell surface, characteristic for distinct cell types.

3.5 Cell counting

3.5.1 Microscopic counting

A Neubauer counting chamber was used for cell counting. For the cell suspension, 20 µl was applied to the counting chamber, and cells were counted on at least two of four quadrants under a light microscope. The total cell count was calculated based on the determined number and sample volume.

3.5.2 Flow cytometric calculation of the cell count using counting beads

By adding a defined amount of CountBright™ Absolute Counting Beads for flow cytometry and subsequent flow cytometric evaluation, the cell number could be calculated according to the manufacturer's specifications. As a basis for the cal-

culation of the skin cells per mm^2, the skin biopsies were considered as a circle with a radius of 2 mm and an area of $A = \pi r^2$. Therefore, the following formula was used for calculation: $\mathrm{cellnumber/mm2} = \frac{\text{Absolute cellnumber}}{\pi * (2\mathrm{mm})^2}$.

3.6 Cloning

Since only small amounts of patient material were available and patients suffer from leukopenia shortly after stem cell transplantation, often only a few cells could be isolated from the skin. However, to perform a reliable short tandem repeat (STR)-PCR-analysis, the DNA of at least 1000 cells was required. If this cell count was clearly undercut, clones of the T cells were grown. The basic idea is that T cells could be placed individually in wells and then stimulated to proliferate. After culturing, high numbers of cells originating from one cell and containing the same DNA were present in each well. A mixture of a defined number of clones of each seeded T cell was then sorted. This would allow the subsequent STR-PCR analysis of the mix to reconstruct the original ratio of donor to recipient.

3.6.1 Feeder cells

Feeder cells were used in cultures to support the T cells in proliferation. By delivering growth factors to the medium and activating cells through direct contact, they increased cloning efficiency. Proliferation of the feeder cells was arrested by irradiation. Human PBMCs were used as feeder cells for the T cell clones.

3.6.1.1 CFSE labeling

To clearly distinguish the feeder cells from T cell clones during subsequent sorting, feeder cells were labeled with CFSE. CFSE (10mM) was diluted in PBS (1:10000) and stored in the dark at room temperature until use on the same day. After isolation of PBMC (see above), PBMC were washed twice with PBS at 1500 rpm and room temperature to remove proteins from the medium. After the second wash, the cells were resuspended in 1 ml of PBS and added to 1 ml of the CFSE dilution, resulting a dilution of 1:20000. An incubation time of eight minutes in darkness at room temperature followed. To ensure ideal mixing, the suspension was swiveled during incubation using a rotator. At the end of the incubation period, the reaction was stopped by slowly adding 20 ml complete

medium while constantly swiveling. The cells were purified twice with complete medium at 4°C and 1500 rpm and then resuspended in wash buffer.

3.6.1.2 Irradiation

After CSFE-staining, the cells were irradiated to stop proliferation. Suspended in wash buffer, the cells were irradiated on ice for 30 minutes at 45 Gy. Immediately afterwards, the cells were purified with wash buffer and put on ice until further processing.

3.6.2 Cloning procedure

3.6.2.1 Seeding of clones

T cells were sown in 384-well plates. A cloning mix was produced, of which 50 µl per well were distributed with a multichannel pipette. This mix contained:

- 200 T cells: only one T cell should be sown per well to ensure that the result of the subsequent STR-PCR analysis remained representative for the original T cell population. To make this as likely as possible, only 200 T cells were sown on 384 wells.
- $20*10^6$ feeder cells: irradiated and CFSE-labeled PBMC, suspended in
- 20 ml cloning medium containing Il-2 to stimulate T cell proliferation.
- 20 µl phytohemagglutinin (PHA) to stimulate T cell proliferation.

As control, several wells were filled with cloning mix without T cells. The plates were carefully and loosely wrapped with foil to avoid drying out and, simultaneously, not interrupt the oxygen supply. An incubation at 37°C and 5% CO_2 followed.

3.6.2.2 Growth control

Growth controls were carried out at regular intervals. Proliferation of the T cell clones was observed microscopically and macroscopically. In the microscope, successful cloning revealed agglomerate rates of T cells, which could be clearly distinguished from feeder cells by size and number. Macroscopically, a grayish staining at the bottom of the well could be detected with advanced proliferation.

3.6.2.3 Transfer of clones

12–14 days after cloning, macroscopically visible clones were transferred to 96-well plates for the first time in order to provide sufficient space and nutrients for further T cell proliferation. The wells were pre-filled with 50 µl of warm cloning medium. Carefully, the clones were removed from the bottom of the 384-well plate by multiple pipetting and transferred to the 96-well plate. The plate was incubated at 37°C and 5% CO_2.

In the following time, further proliferating clones were transferred from the 384-well plate to the 96-well plate. Growth controls were performed on the 96-well plate. The medium was changed with increasing consumption of the nutrients, which could be recognized by the yellowing of the medium. For this, 50 µl of medium were removed superficially from the well and fresh, preheated medium was carefully added.

3.6.2.4 Sorting of clones

After growth of a sufficient number of individual T cell clones, the 96-well plate filled with clones was centrifuged, and the supernatant was carefully removed. Then the clones were resuspended with 100 µl of MACS buffer each, and the cell suspension was transferred into one FACS tube each without mixing the clones together. To check cell viability, propidium iodide (PI) was added directly before the sorting process. Subsequently, 1000 viable clones from each sown T cell were sorted by flow cytometry. T cell clones could be clearly distinguished from feeder cells based on size and granularity. In addition, only cells without signal in the FITC channel were detected, thus excluding feeder cells previously labeled with CFSE. The DNA was then isolated from the mix of T cell clones and tested for chimerism.

3.7 Cultivation of fibroblasts

To obtain pure recipient DNA for subsequent STR-PCR analysis, fibroblasts were cultured from the digested skin biopsies. After filtering the digested cell suspension, tissue residues visible on the 70-µm filter were transferred to the wells of a 24-well plate. The filter was then inverted and rinsed with 0.5 ml of fibroblast culture medium heated to 37°C into the well to detach additional tissue residues from the filter. To allow adherent fibroblast growth, care was taken

to ensure that the tissue adhered to the bottom of the well. This was followed by incubation at 37°C and 5% CO_2. After three days, another 0.5 ml of warm fibroblast culture mediums were added. Depending on further fibroblast growth, old medium was removed at intervals of 3–5 days and replaced with 1 ml warm fibroblast culture medium. Regular growth controls were performed under the microscope. Trypsinization was performed to dissolve the adherent fibroblasts.

3.8 Magnetic activated cell sorting

Magnetic activated cell sorting (MACS) is an elegant technique that allows rapid sorting of specific cells from a cell mixture. First, characteristic molecules on the surface of the cells to be sorted are labeled with antibodies coupled with magnetic particles. A cell suspension is then passed through a column placed in a strong magnetic field which retains cells expressing the labeled surface molecules in the columns. When the magnetic field is removed, the cells can be eluted.

3.8.1 Cell labeling

PBMC were suspended in MACS buffer and centrifuged at 1500 rpm for five minutes before labeling. The supernatant was removed, and the cell pellet was resuspended in 500 µl MACS buffer. To an appropriate dilution, 5 µl of biotin-aCD3 antibody was added. This was followed by an incubation period of 30 min on ice. The cells were resuspended with MACS buffer and centrifuged. After removal of the supernatant, the cells were resuspended in 290 µl MACS buffer and 10 µl streptavidin micro-beads were added. Cells were incubated on ice for an additional 20 min and then washed with MACS buffer. The cell pellet was resuspended with 3 ml of MACS buffer.

3.8.2 Cell separation

A pre-cooled MACS column was filled with 3 ml MACS buffer. Care was taken throughout the process to ensure that the column did not dry out. After passing through the MACS buffer, the cell suspension was filtered through a 30-µm strainer and added to the column. The tube previously containing the cell suspension was rinsed twice, each time with 3 ml of MACS buffer, which was then added to the column after filtration. After all the liquid had passed through the

column, the column was removed from the multistand and placed on another tube. 3 ml of MACS buffer was added to the column. By forcing the MACS buffer through the column into the tube at low pressure using the enclosed plunger, the cells were eluted. The procedure was repeated one more time.

3.9 Determination of chimerism by STR-PCR analysis

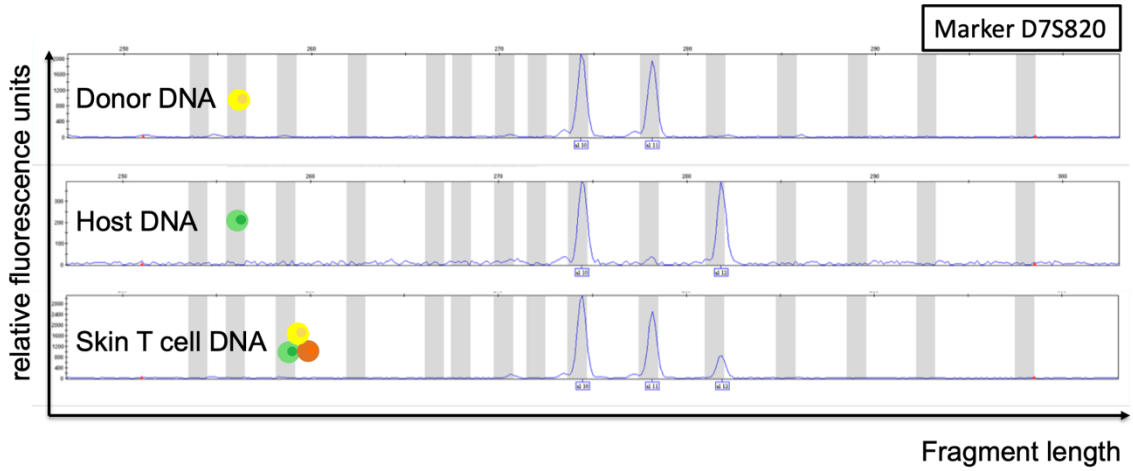


Figure 3.5 STR-PCR analysis

Electropherogram (GeneMapper® software) from marker D7S820 in STR-PCR analysis demonstrating host DNA in T cells isolated from the skin of one patient. The x-axis depicts the fragment length, and the y-axis depicts the intensity of the fluorescent signal in relative fluorescent units (RFU).

To determine chimerism, the DNA of T cells isolated from the skin was compared with the DNA of the donor and recipient by STR-PCR analysis. The donor's DNA was obtained from PBMCs in the patient's blood, which were ensured to be derived exclusively from the donor's stem cells. The recipient's DNA was isolated from skin fibroblasts.

STR analysis is an established method for clinical and forensic purposes. It compares two or more genetic individuals based on microsatellites called short tandem repeats, short base repeats on specific gene loci. Within alleles, these occur in different repeats, allowing individuals to be reliably distinguished from one another.

DNA isolation was performed using the QIAamp® DNA Micro Kit according to the manufacturer's instructions for isolating DNA from small amounts of blood. Cells were first centrifuged at 400 rcf for 10 minutes and the supernatant was carefully pipetted off. The volume was then made up to 100 µl total volume with buffer AL. The procedure was continued according to the attached protocol. Finally, the DNA was dissolved in 20 µl of purified water.

As an alternative to cloning for analysis of DNA with low cell number (<900 cells), DNA amplification was applied to multiple samples. This was done using the REPLI-g single cell kit according to the manufacturer's specifications.

For STR-PCR analysis, which was performed in collaboration with Helmholtz Zentrum München, the AmpFLSTR™ Identifiler™ PCR Amplification Kit (Applied Biosystems™) was used. It uses 15 STR loci and amelogenin to compare individuals. Polymerase chain reaction (PCR) was performed on a T100™ thermal cycler (BioRad). The diluted PCR products were mixed with Hi-Di™ formamide and GeneScan™ 500 LIZ™ dye size standard (Applied Biosystems™) and analyzed on the ABI 3730 DNA sequencer (Applied Biosystems™). Analysis was performed using GeneMapper software (Applied Biosystems™) version 5.

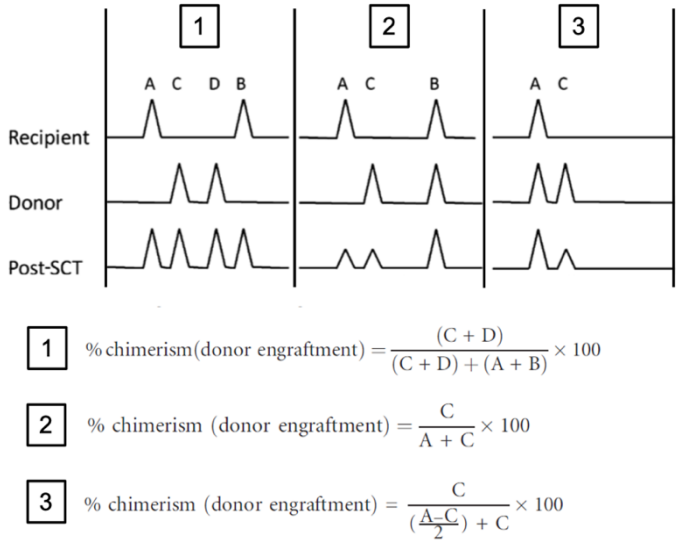


Figure 3.6 Calculation of T cell chimerism

Graphical representation of three possible allelic constellations. Percentage of chimerism was calculated depending on the allelic constellation for each marker. A, B: recipient alleles; C, D: donor alleles; Post-SCT: post stem cell transplantation. Modified from J. L. Clark et al. (2015).

The percentage of total skin T cell chimerism (STCC) was determined by calculating the percentage of chimerism for each analyzed marker and then calculating the mean value (according to J. L. Clark et al. (2015)).

3.10 Study approval

The study concept was ethically approved by the Institutional Review Board of the Technical University of Munich (195/15s, 146/17s, and 491/16s). All experiments working with human blood and tissue samples were performed in accordance with the ethical principles for medical research involving human subjects of the Declaration of Helsinki.

3.11 Statistics

Statistical analyses were performed with the statistical software programs IBM® SPSS® Statistics and Graphpad Prism.

If normally distributed, quantitative data sets were compared using the unpaired Student's t-test. To check for normal distribution, data was analyzed graphically by interpreting Boxplots and additional use of the Shapiro-Wilk test for normality. In the case of skewed deviation, the nonparametric Mann-whitney U test was performed. For measuring the degree of association between two variables, Spearman's correlation coefficient was used. By performance of Pearson's chi-squared test and two-tailed Fisher's exact test, categorical clinical patient variables were studied.

Significance was defined as a p value < 0.05. Statistical significance is expressed as n.s. for $p > 0.1$, (*) for $p < 0.1$, * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

4 Results

STR-PCR analyses were performed in collaboration with Helmholtz Zentrum München. All other results presented in this dissertation were generated exclusively by the work performed by Tonio Brinkschmidt in the context of his doctoral thesis.

4.1 T cell chimerism in skin

4.1.1 STR-PCR analyses reveal host-derived T cells respectively skin TRM for six patients

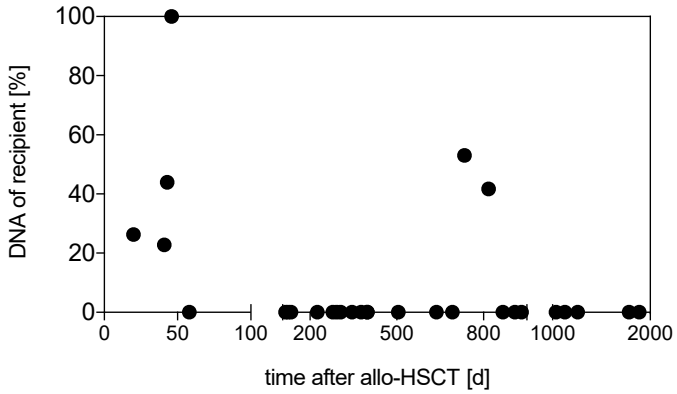


Figure 4.1 Proportion of recipient-derived DNA in skin T cells

Each symbol represents the percentage of host-derived DNA in T cells isolated from the skin of a single patient, presented as a function of the time following allo-HSCT (in days); n = 30

T cells were isolated from the skin, and DNA was isolated and compared against both stem cell donor and recipient DNA using short tandem repeat polymerase chain reaction (STR-PCR) analysis. STR-PCR analysis was performed once per patient per time point for 38 patients. Six patient data points were excluded for substantially low cell numbers and technical issues. Furthermore, two patients were excluded due to the detection of mixed chimerism in the blood during the STR-PCR analysis, preventing the exclusion of circulating recipient-derived T cells in the skin.

In six (20.0%) patients, skin T cell chimerism (STCC) could be detected. In these patients, complete donor chimerism was observed among T cells in the blood, both by routine cytogenetic analyses performed in the clinic and by the

STR-PCR analysis that was performed for this study. Four of these patients were analyzed within the first 50 days after allo-HSCT, with the earliest timepoint occurring 20 days after allo-HSCT. The other two patients were sampled more than two years after transplantation (734 and 818 days after transplantation). The proportion of recipient-derived DNA in the investigated skin T cells ranged from 22.82% to 100.00%, with a median of 42.82%. Thus, a distinct portion of the T cells identified in the skin of these patients were shown to be recipient-derived TRM. In 24 (80.0%) patients, no host-derived T cells could be detected in the skin biopsies. In the group of patients for whom STCC was detected, the period between transplantation and biopsy was significantly shorter than in patients without STCC ($p < 0.05$).

4.1.2 No association between skin T cell chimerism and the collected clinical patient data can be detected

Age [years]	
median	57
mean	51,83
range	22-64
Sex	
male	n = 3 (50.0%)
female	n = 3 (50.0%)
Diagnosis [a]	
MDS/AML	n = 2 (33.3%)
MMY	n = 2 (33.3%)
MPS	n = 1 (16.7%)
SAA	n = 1 (16.7%)
Conditioning	
Fludarabine, Treosulfan	n = 4 (66.7%)
Cyclophosphamide	n = 1 (16.7%)
FLAMSA-RIC	n = 1 (16.7%)
Immunosuppression	
ATG	n = 5 (83.3%)
Prednisolone	n = 5 (83.3%)
MMF	n = 4 (66.7%)
Cyclosporin A	n = 4 (66.7%)
Tacrolimus	n = 3 (50.0%)
Methotrexate	n = 1 (16.7%)
Sex matching	
Sex matched	n = 3 (50.0%)
Sex mismatched	n = 3 (50.0%)
HLA matching of stem cell donor	
HLA-ident unrelated donor	n = 4 (66.7%)
HLA-ident related donor	n = 2 (33.3%)

Table 4.1 Characteristics of patients with skin T cell chimerism

[a] MDS/AML= Myelodysplastic syndrome / Acute myeloid leukemia, MMY= Multiple myeloma, MPS= Myeloproliferative syndromes, SAA= Severe aplastic anemia, FLAMSA-RIC = fludarabine, amsacrine, and cytarabine-reduced-intensity conditioning, ATG = anti-thymocyte globulin, MMF = mycophenolate mofetil, HLA = human leukocyte antigen

Patients with STCC did not differ significantly from the total patient population in terms of general patient characteristics (age, sex, and diagnosis) or medical treatment (conditioning and immunosuppression). Furthermore, all patients received $CD34^+$ T cells from HLA-ident donors and did not show significant differences relative to the total population in terms of the donor stem cell sources (sex matching and HLA matching). Interestingly, none of the patients with STCC received TBI; however, the difference between patients with and without STCC treated with TBI was not significant. The same was additionally true for post-Tx-cyclophosphamide for immunosuppression, with which no patient with STCC was treated.

When examining the clinical outcomes of patients, no significant difference was identified in the occurrence of aGvHD or cGvHD in the skin or inner organs between the two patient groups (Fig. 4.2). Furthermore, the reactivation of viruses, such as CMV, was not significantly influenced by the occurrence of STCC.

	skin T cell chimerism		
skin aGvHD	+	-	total
yes	3	14	17
no	3	10	13
total	6	24	30

χ2=0.713 p=1.000

	skin T cell chimerism		
organ aGvHD	+	-	total
yes	2	7	9
no	4	17	21
total	6	24	30

χ2=0.842 p=1.000

	skin T cell chimerism		
skin cGvHD	+	-	total
yes	1	5	6
no	5	13	18
total	6	24	30

χ2=0.192 p=0.358

	skin T cell chimerism		
organ cGvHD	+	-	total
yes	0	5	5
no	6	19	25
total	6	24	30

χ2=0.221 p=0.553

Figure 4.2 GvHD in skin and inner organs

Correlation analysis of clinical metadata with the presence or absence of skin T cell chimerism. Chi-squared test and Fisher's exact test (two-sided) comparing the presence of skin T cell chimerism in patients with and without history of acute / chronic GvHD in skin and inner organs.

4.2 Immunomonitoring in the skin of patients after allo-HSCT

4.2.1 T cell numbers are reduced after allo-HSCT and recover over time

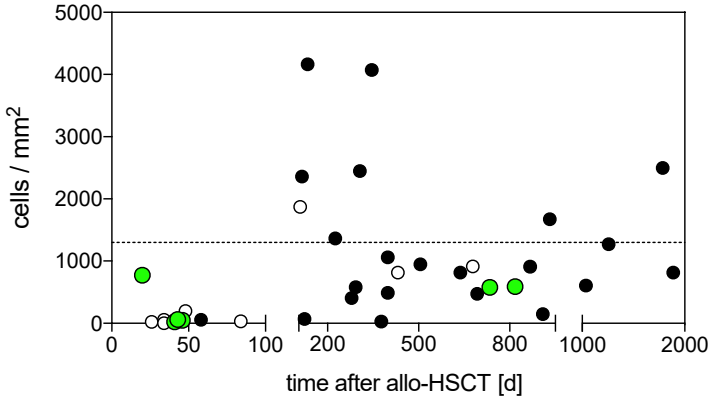


Figure 4.3 Absolute T cell numbers per mm² of skin

Each symbol represents the T cell count/mm² skin of a single patient (n=38), presented as a function of time after allo-HSCT. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no analyses of skin T cell chimerism was possible. Dashed line indicates the mean absolute T cell number obtained from healthy donors.

The absolute cell counts per mm² were calculated as described in chapter 3.5.2. The values ranged from close to 0 to maximum values of over 4,000 cells/mm². A strong reduction in cell numbers compared to healthy donors was noticeable, particularly shortly after transplantation.

However, significantly correlating with the time after allo-HSCT, mean cell numbers of T cells/mm² rose (r_{SP} = 0.473, p < 0.01) and approached the average value of approximately 1,300 cells/mm², which was measured in healthy donors. Noticeably, no significant difference in cell numbers was identified between patients with and without STCC.

4.2.2 αβ T cells represent the major T cell population as the numbers of γδ T cells remain on a low level after allo-HSCT

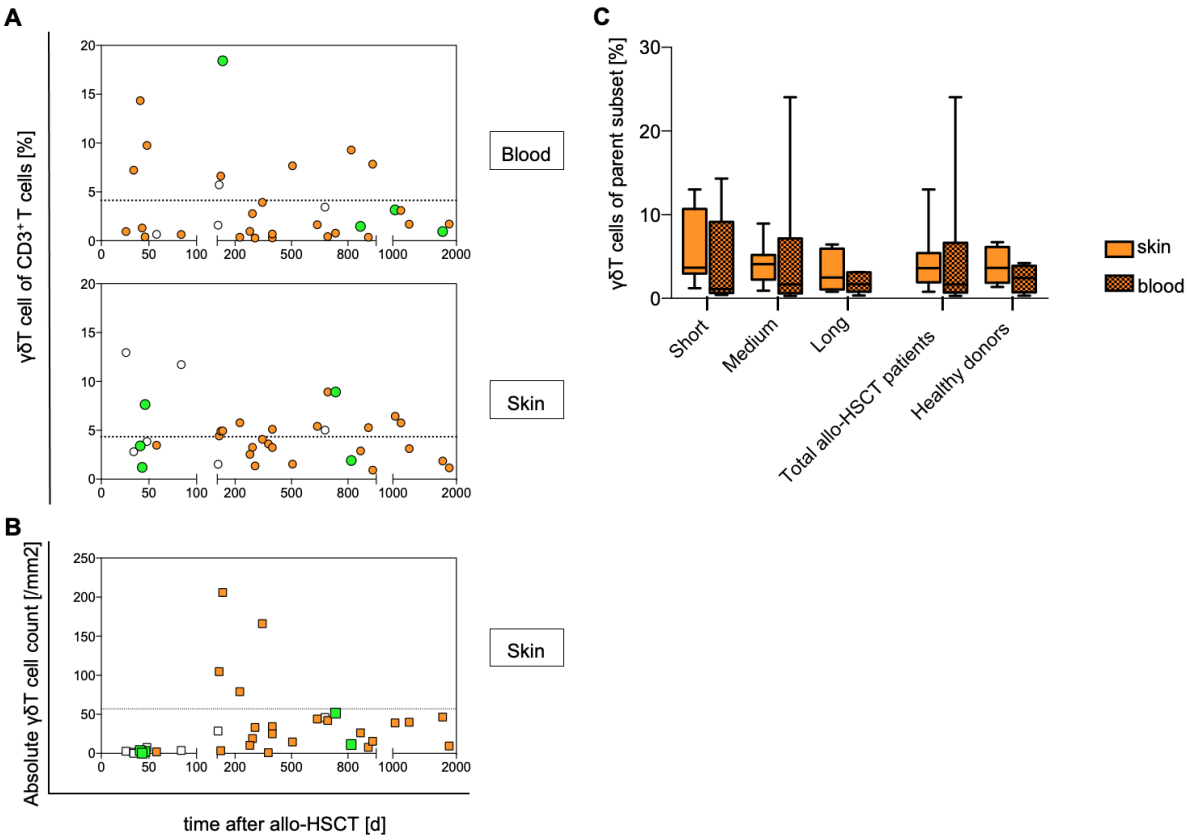


Figure 4.4 Expression of γδ TCR of T cells in skin and blood

(A) Frequency of γδ T cells among total T cells obtained from the skin (top) and blood (bottom). (B) Absolute numbers of γδ T cells in the skin. Each symbol represents the data collected from a single patient, presented as a function of time after allo-HSCT. Data were generated using flow cytometry analyses. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no clear analyses for skin T cell chimerism were possible. Dashed lines indicate the mean values obtained from healthy donors. (C) Frequency of γδ T cells among total T cells obtained from patients sampled in time periods short (<100d), medium (100-1000d) and long (>1000d) after allo-HSCT, from patients after allo-HSCT in total and from healthy donors (n=18).

To gain clearer insights into the dynamics of T cell populations in the blood and skin shortly after stem cell transplantation, the phenotypes of the isolated T cells were examined. First, T cells were tested for the expression of γδ TCR. T cells with γδ TCR were identified at low proportions in the skin and blood of the majority of the patients, similar to those observed for healthy donors. No significant difference in the frequency of γδ T cells was identified between patient groups

with and without STCC. The general trend of increasing absolute cell numbers in skin over time after transplantation was also reflected in the γδ T cell population. However, no striking features in the temporal development of γδ T cells after transplantation between blood to the skin could be observed. Therefore, further analyses focused on αβ T cells as the major T cell subset.

4.2.3 Inversion of the CD4/CD8-ratio shortly after transplantation is reflected in the skin to a lesser extent than in blood

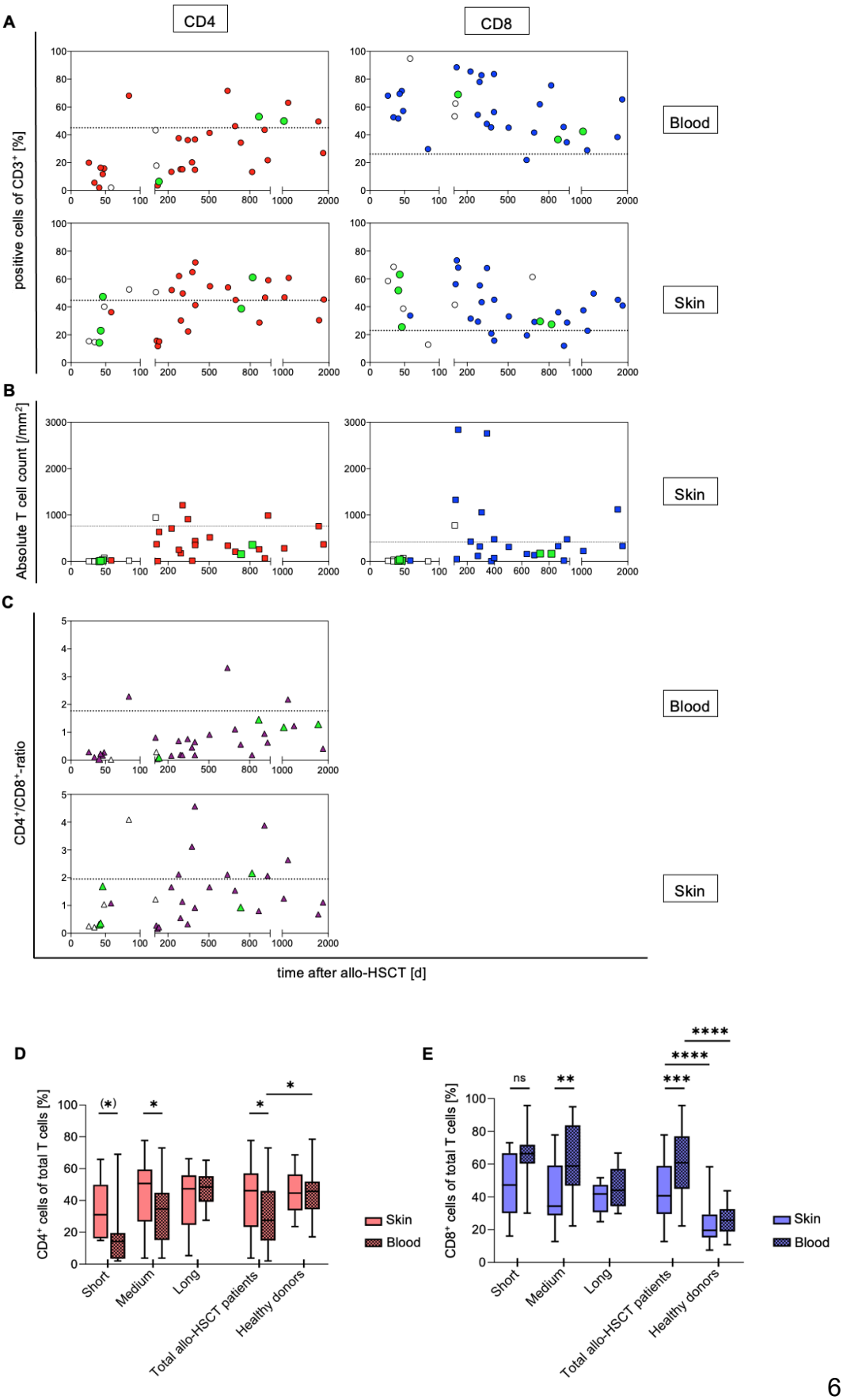


Figure 4.5 Frequency of T helper cells vs. cytotoxic T cells in skin and blood
(A) Frequency of $CD4^+$ T cells (left) and $CD8^+$ T cells (right) among total T cells obtained from the blood (top) and skin (bottom). (B) Absolute numbers of skin expressing CD4 (left) and CD8 (right). (C) Ratio of CD4/CD8 T cells obtained from the blood (top) and skin (bottom). Each symbol represents the data collected from a single patient (n=38), presented as function of time after allo-HSCT. Data were generated by flow cytometry analyses. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no analyses for skin T cell chimerism was possible. Dashed lines indicate the mean values obtained from healthy donors. (D,E) Frequency of $CD4^+$ (D) T cells and $CD8^+$ (E) T cells among total T cells obtained from patients sampled in time periods short (<100d), medium (100-1000d) and long (>1000d) after allo-HSCT, from patients after allo-HSCT in total and from healthy donors (n=18).

As essential phenotypic T cell markers for T helper cells and cytotoxic T cells, the expression of CD4 and CD8 was analyzed subsequently. The proportion of $CD8^+$ T cells in the blood was substantially increased shortly after transplantation, whereas the percentage of $CD4^+$ T cells was considerably diminished and only increased within the next few years. A similar dynamic could be observed among the T cell populations isolated from the skin of both patients with and without STCC, even though the absolute cell numbers were considerably diminished for both T cell populations in the skin. However, in the skin, the percentage of $CD4^+$ T cells increased significantly after HSCT ($r_{sp} = 0.408$, $p < 0.05$), whereas the percentage of $CD8^+$ T cells decreased ($r_{sp} = -0.363$, $p < 0.05$). Initially, the CD4/CD8 ratio in the skin was inverted similar to the blood and increased significantly with time after allo-HSCT ($r_{sp} = 0.408$, $p < 0.05$).

However, the inversion of the CD4/CD8-ratio was less pronounced in the skin than in the blood. In patients after allo-HSCT, the expression of CD4 in skin T cells was significantly higher compared to blood T cells, while CD8 was expressed to a significantly lower extent in skin compared to blood. This was particularly true for patients who were sampled in timespans short and medium after allo-HSCT. While the percentages of blood derived $CD4^+$ T cells of patients after allo-HSCT were significantly decreased compared to values obtained from healthy donors, skin CD4 T cell fractions showed stable values resembling the healthy patients' values. These results indicate that $CD4^+$ T cells were either more highly recruited to the skin after transplantation or resided in the skin tissue to a greater extent than $CD8^+$ T cells.

4.2.4 Regulatory T cells can be found at fluctuating levels in skin and blood after allo-HSCT

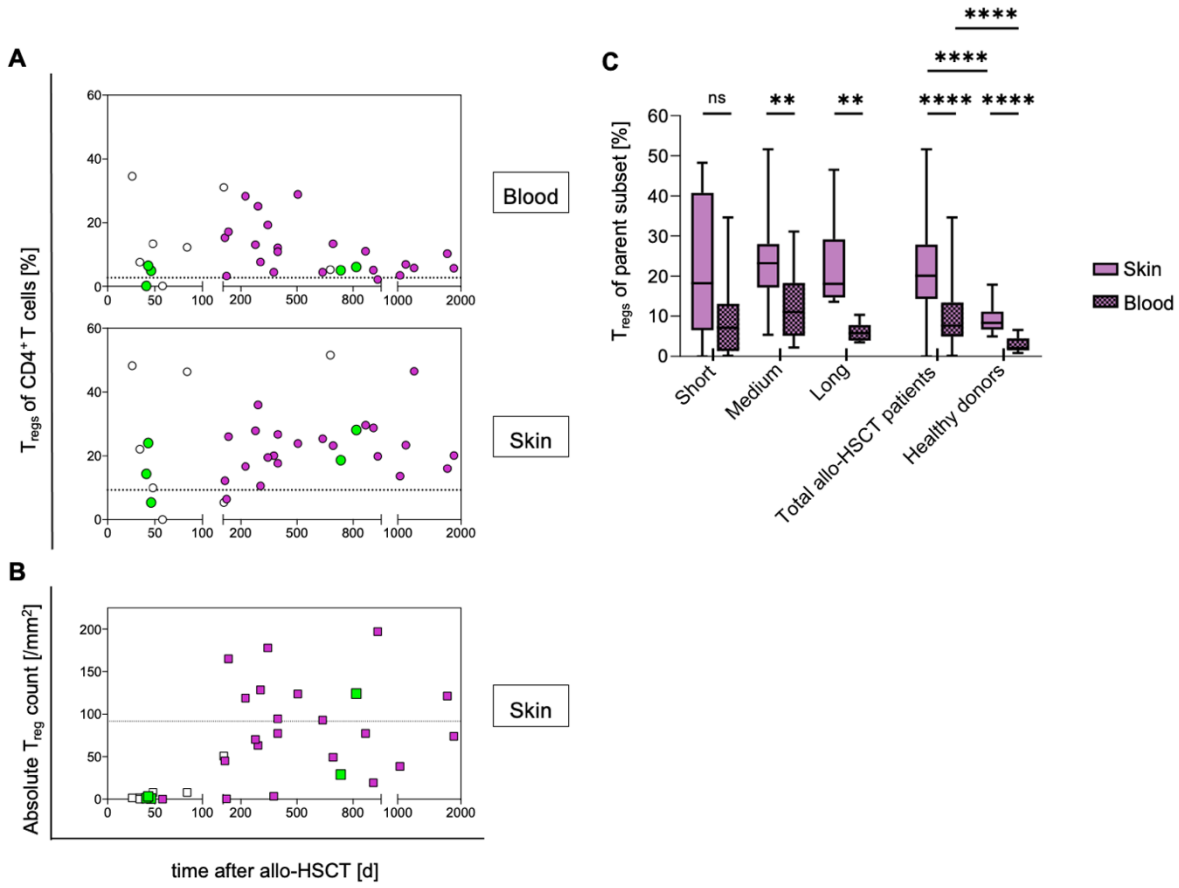


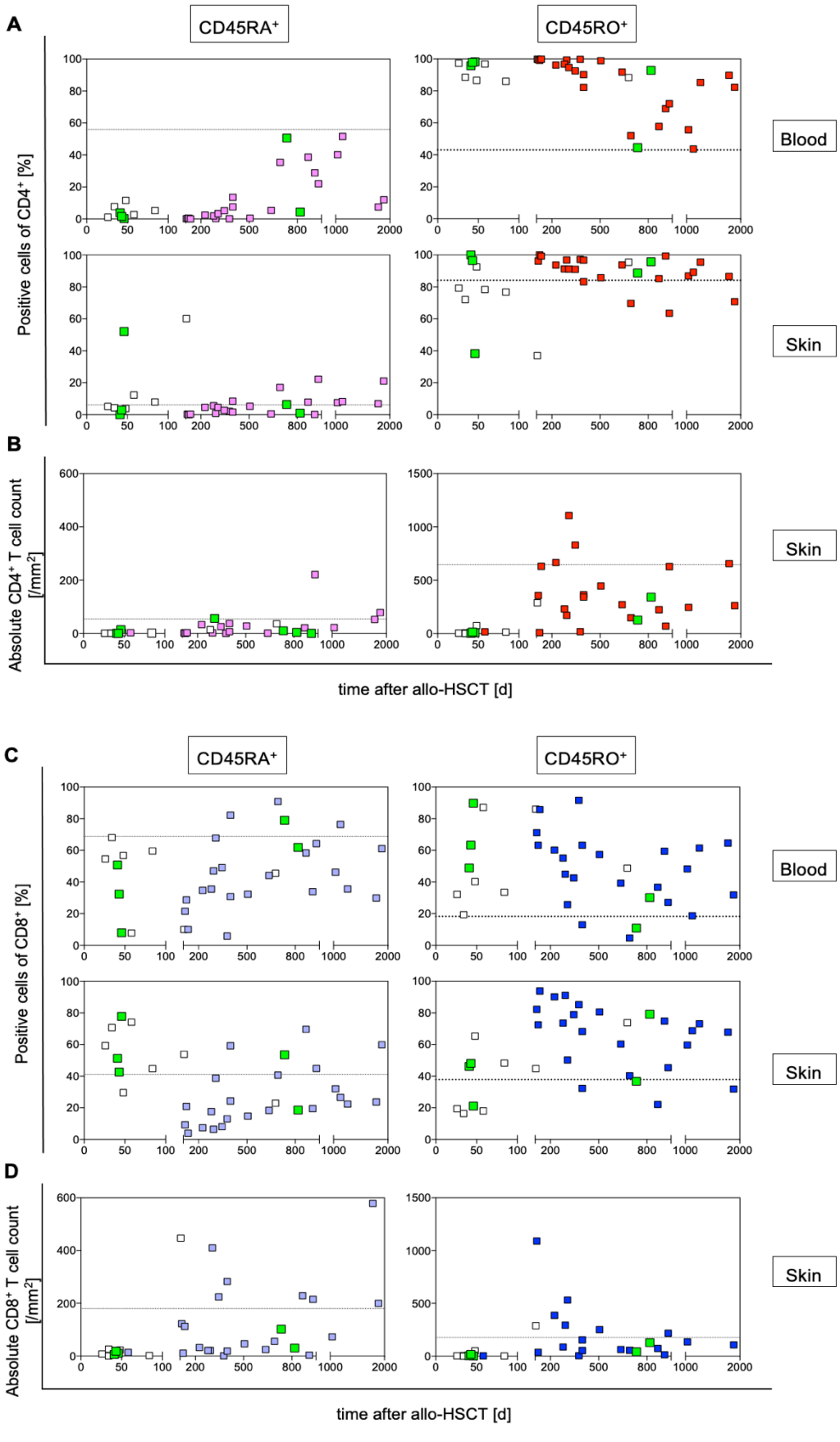
Figure 4.6 Frequency of T_{reg} in skin and blood

(A) Frequency of T_{reg} cells among $CD4^+$ T cells obtained from the skin (top) and blood (bottom). (B) Absolute numbers of T_{reg} cells in skin. Each symbol represents the data collected from a single patient (n=38), expressed as a function of time after allo-HSCT. The data were generated by flow cytometry analyses. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no clear analyses for skin T cell chimerism was possible. Dashed lines indicate the mean values obtained from healthy donors. (C) Frequency of T_{reg} cells among $CD4^+$ T cells cumulated from patients who were sampled short (<100d), medium (-1000d) and long (>1000d) after allo-HSCT, from patients after allo-HSCT in total and from healthy donors (n=18).

Regulatory T cells (T_{reg}), which represent an important control element of the functional immune responses, were characterized as $CD3^+$ $CD45^+$ γδTCR^- $CD4^+$ $CD25^+$ $CD127^-$, and their proportions were examined in the samples. Similar to healthy donors, T_{reg} were found at higher proportions in the skin than in the blood of patients who received transplantation, and the absolute T cell

numbers in the skin appeared to be reconstituted after initial lymphopenia. No clear trend was observed over time after transplantation or correlated with the occurrence of STCC. Interestingly, patients after allo-HSCT showed significantly increased frequencies of T_{reg} both in skin and blood compared to healthy donors. The further characterization of T_{reg}, using surface markers, such as CD69 and CD103, was not possible in a high fraction of the patients due to the low cell numbers. However, the increased proportion of T_{reg} both in blood and skin of patients after allo-HSCT indicated that this cell type represents an integral part of the $CD4^+$ T cell population, with significant relevance for a functional immune response, particularly in tissues, such as the skin.

4.2.5 T helper cells in the skin do primarily show a memory phenotype after allo-HSCT, in contrast to cytotoxic T cells



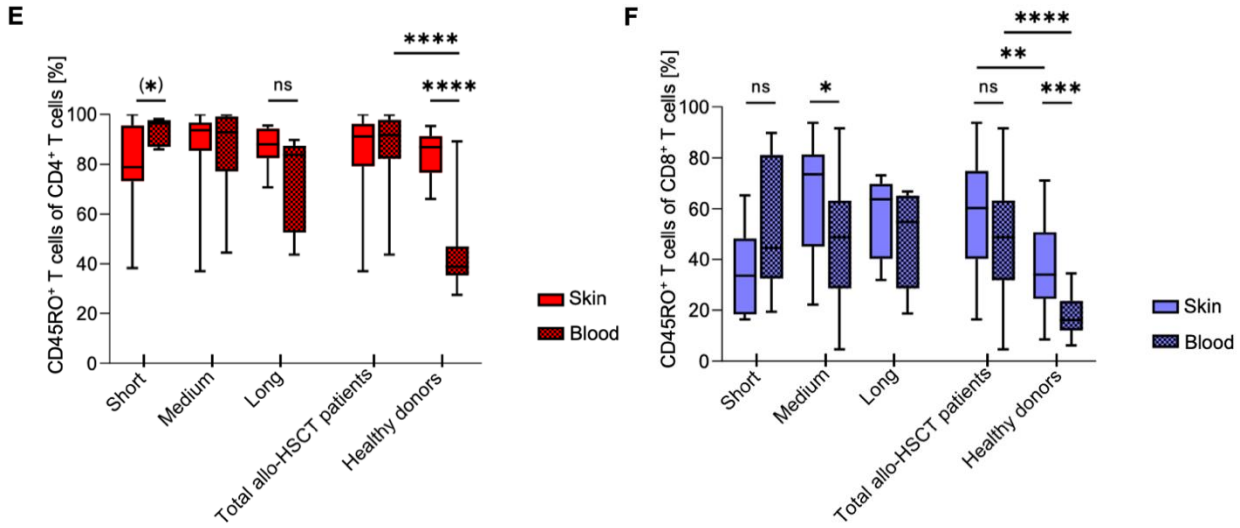


Figure 4.7 CD45RA and CD45RO expression of T helper cells vs. cytotoxic T cells in skin and blood

(A,C) Frequency of CD45RA$^+$ T cells (left) and CD45RO$^+$ T cells (right) among CD4$^+$ (A) and CD8$^+$ (C) T cells obtained from blood (top) and skin (bottom). (B,D) Absolute numbers of skin CD4$^+$ (B) and CD8$^+$ (D) T cells expressing CD45RA (left) and CD45RO (right). Each symbol represents data collected from a single patient (n=38), expressed as a function of time after allo-HSCT. The data were generated by flow cytometry analyses. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no analyses for skin T cell chimerism was possible. Dashed lines indicate the mean values obtained from healthy donors. (E,F) Frequency of CD45RO expressing cells among CD4$^+$ (E) and CD8$^+$ (F) T cells cumulated from patients who were sampled short (<100d), medium (-1000d) and long (>1000d) after allo-HSCT, from patients after allo-HSCT in total and from healthy donors (n=18).

To obtain additional information about the functional history of the cells, CD4$^+$ and CD8$^+$ T cells from blood and skin were analyzed for the expression of markers for naive (CD45RA) and memory (CD45RO) T cells. The expression of these markers in the skin corresponded approximately to the course observed in the blood. Due to the low cell counts shortly after transplantation, trends were difficult to determine based on absolute cell numbers. The difference in distribution between naive and memory T cells was noticeable when comparing CD4$^+$ and CD8$^+$ T cells. Interestingly, CD4$^+$ T cells in the blood primarily expressed CD45RO, while the CD45RA$^+$ cell fraction only recovered over the following years after transplantation. This was in stark contrast to values of CD4$^+$ T cells

obtained from healthy blood donors that showed significantly lower percentages of CD45RO. The proportions of $CD45RA^+$ and $CD45RO^+$ T cells among $CD4^+$ T cells in the blood were significantly correlated with time after allo-HSCT [r_{sp}(naive) = 0.639, r_{sp}(memory) = −0.612, $p < 0.01$]. A similar trend was evident among $CD4^+$ T cells in the skin, which were generally $CD45RO^+$, with few exceptions. The $CD45RA^+$ T cell populations approached the state of healthy donors only over the course of the following years.

The situation was different for the cytotoxic T cell population. Particularly during the first 100 days after transplantation, many cells expressed CD45RA in both the skin and blood. The $CD8^+$ T cells for the majority of patients were to less than 50% $CD45RO^+$ in the skin; in the blood, the distribution was more heterogeneous. After more than 100 days after transplantation, patients showed an increased percentage of $CD45RO^+$ T cells in blood and skin among $CD8^+$ T cells, which decreased slowly over time but was substantially more heterogeneous than among the $CD4^+$ T cells. Overall, a substantially higher percentage of $CD4^+$ T cells displayed a classical memory phenotype compared with that in $CD8^+$ T cells, which might indicate that T helper cells are more commonly conditioning therapy surviving resident memory cells.

4.2.6 Shortly after transplantation, skin T helper cells show the highest expression of CD69

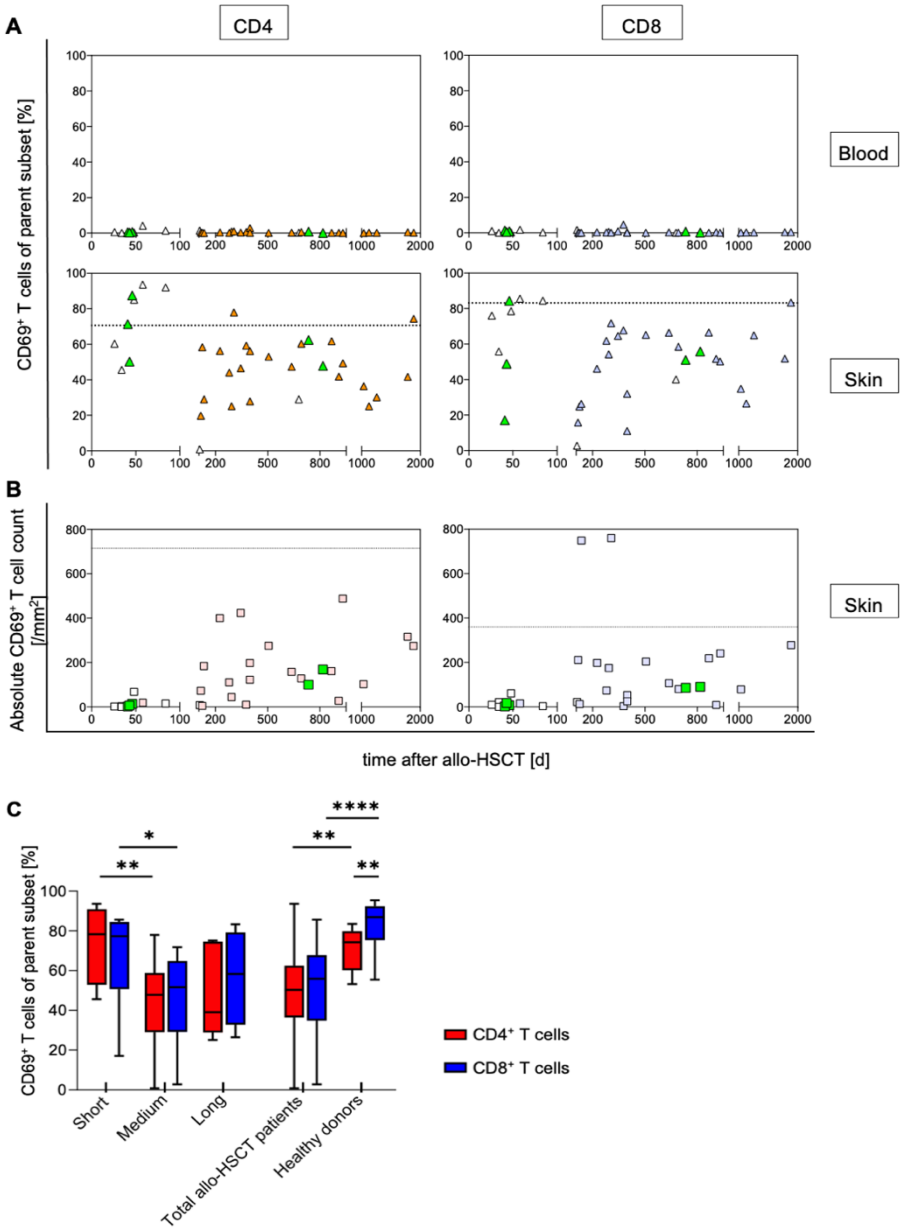


Figure 4.8 CD69 expression in T helper cells vs. cytotoxic T cells in skin

(A) Frequency of CD69 expressing cells among $CD4^+$ T cells (left) and $CD8^+$ T cells (right) obtained from blood (top) and skin (bottom). (B) Absolute numbers of skin $CD4^+$ T cells (left) and $CD8^+$ T cells (right) expressing CD69. Each symbol represents the data collected from a single patient (n=38), expressed as a function of time after allo-HSCT. The data were generated by flow cytometry analyses. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no analyses for skin T cell chimerism was possible. Dashed lines indicate the mean values obtained from healthy donors. (C) Frequency of CD69 expressing cells among $CD4^+$ and $CD8^+$ T cells cumulated from patients who were sampled short (<100d), medium (-1000d) and long (>1000d) after allo-HSCT, from patients after allo-HSCT in total and from healthy donors (n=18).

The expression of CD69 was evaluated to determine the residence of the different cell populations and to verify the validity of CD69 as a residency marker. In contrast to blood cells, a high percentage of $CD69^+$ T cells was found in cells isolated from the skin. Interestingly, skin cells of patients after allo-HSCT overall expressed CD69 to a lower extent compared to cells obtained from healthy donors. However, this was not true for patients who were sampled in the first 100 days after allo-HSCT. Both $CD4^+$ and $CD8^+$ T cells expressed CD69 to significantly higher levels compared to patients who were sampled in timepoints later than 100 days.

Another interesting observation arose when looking at the total patients´ expression of CD69 compared to healthy donors. While in healthy donors expression of CD69 among $CD8^+$ T cells was significantly higher compared to $CD4^+$ T cells, skin T cells of patients after allo-HSCT showed similar expression levels of CD69. These results suggested an increased existence of $CD69^+$ - and possibly resident – T helper cells in the skin of patients after allo-HSCT compared to cytotoxic T cells.

4.2.7 CD103 expression is significantly higher in T helper cells compared to cytotoxic T cells in the skin of patients after allo-HSCT

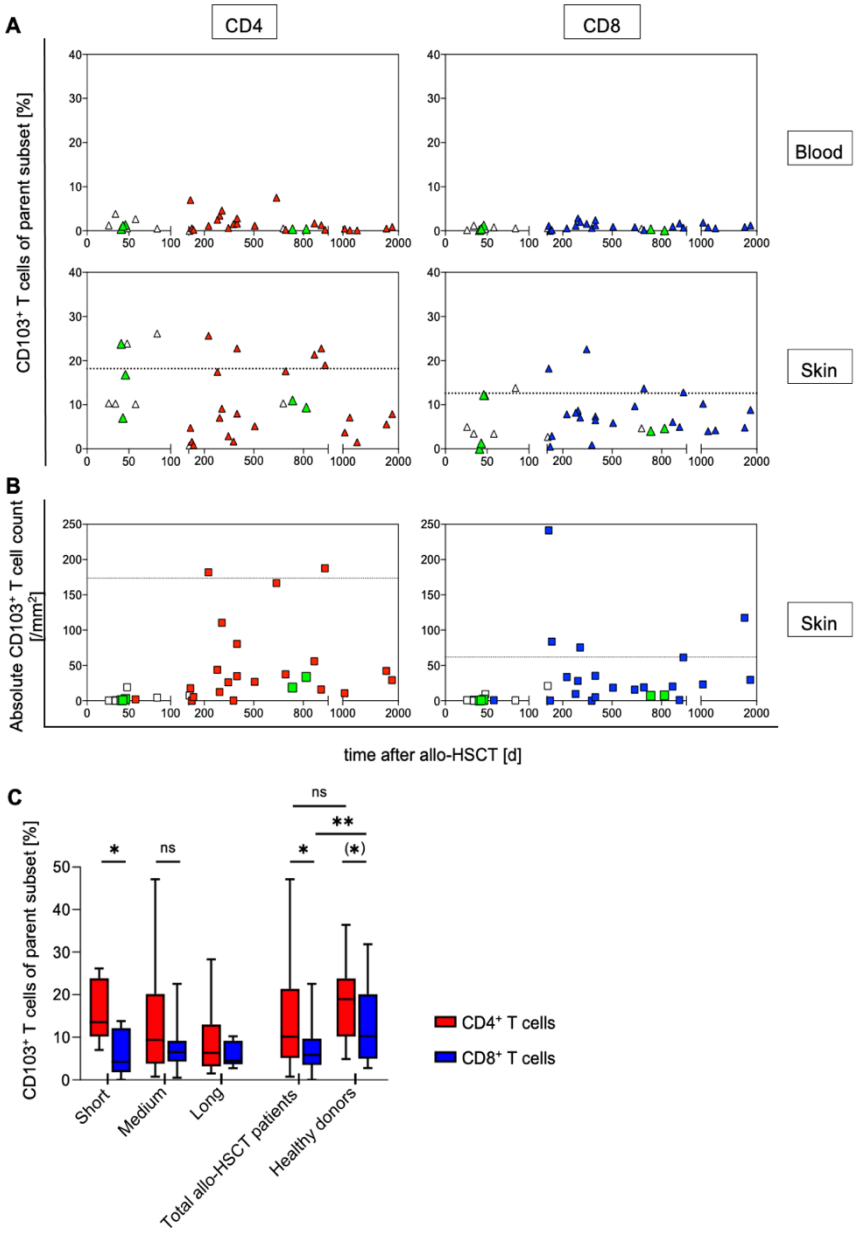


Figure 4.9 CD103 expression in T helper cells vs. cytotoxic T cells in skin

(A) Frequency of CD103 expressing cells among CD4⁺ T cells (left) and CD8⁺ T cells (right) obtained from blood (top) and skin (bottom). (B) Absolute numbers of skin CD4⁺ T cells (left) and CD8⁺ T cells (right) expressing CD103. Each symbol represents the data collected from a single patient (n=38), expressed as a function of time after allo-HSCT. The data were generated by flow cytometry analyses. Green symbols indicate patients with proven T cell chimerism in the skin. Colorless symbols indicate patients for whom no analyses for skin T cell chimerism was possible. Dashed lines indicate the mean values obtained from healthy donors. (C) Frequency of CD103 expressing cells among CD4⁺ and CD8⁺ T cells cumulated from patients who were sampled short (<100d), medium (-1000d) and long (>1000d) after allo-HSCT, from patients after allo-HSCT in total and from healthy donors (n=18).

Simultaneously, the expression of CD103, a putative residency marker, was investigated in different T cell populations. Overall and in particular shortly after transplantation, T helper cells showed significantly higher expression of CD103 than cytotoxic T cells. The comparison between patients after allo-HSCT and healthy donors revealed a significantly decreased frequency of CD103 expressing cells among the $CD8^+$ T cell population, which could not be observed among the $CD4^+$ T cells. Notably, differences in CD103 expression were particularly pronounced in patients with STCC. In both patients with STCC (42% and 53%) more than two years after transplantation, approximately 10% of $CD4^+$ T cells expressed CD103 compared with only 4% of $CD8^+$ T cells. These results suggest that T helper cells, more than cytotoxic T cells, play a part in TRM populations and their generation in human skin. However, the results also indicate the existence of TRMs that do not constantly express CD103.

5 Discussion

5.1 Methods discussion

5.1.1 Human experimental model

The data collected in this study were obtained from patients who had undergone allo-HSCT. Abdominal skin biopsies (Ø 4mm) and blood samples were taken from these patients, from which T cells were isolated and examined to determine their phenotypic and genetic origins. This elegant study model had several advantages over previous approaches. First, it was a human study model, which must be emphasized because human study models provide the most reliable information about the human immune system, and most TRM research has been derived from mouse models (Masopust & Soerens, 2019).

However, working with human skin samples increases the complexity of sample procurement. Research is restricted to small biopsies of the tissue, which contain only a limited number of T cells, which may explain why so few studies of TRMs in humans have been performed thus far (Mueller & Mackay, 2016; Schenkel & Masopust, 2014). One complicating factor in the study model used here was that some patients were sampled very shortly after allo-HSCT. During this phase, cell numbers were significantly reduced by chemotherapy. In addition, only one to two biopsies could be obtained from each patient to minimize the risk of infection and prevent dangerous wound healing situations in patients with severely weakened immune and coagulation systems. Additionally, patients were frequently unwilling to undergo further physical interventions within the scope of the study until after a certain time interval following the transplantation. Furthermore, although complete donor chimerism in the bone marrow was a prerequisite for participation in the study, this was only clinically determined by a bone marrow biopsy after the sufficient stabilization of the patient's health condition. Therefore, information regarding the state of chimerism was often only available 25-50 days after stem cell transplantation. Several patients were excluded due to a lack of complete donor chimerism in bone marrow and blood. In summary, obtaining results from patients, especially in the immediate short term after transplantation, represented a major challenge for this study.

However, a close connection with the university hospital made it possible to contact a high number of potential study participants. By providing a major

outpatient department and an efficient clinic infrastructure, the Klinikum rechts der Isar substantially supported the working conditions in place. In addition, the procedures followed in this study were approved by the ethics committee for research based on a human clinical model.

The lack of reliable TRM markers and knowledge has led a majority of researchers to examine a variety of alternative experimental designs, such as mouse models using parabiosis (Beura, Wijeyesinghe, et al., 2018; Collins et al., 2016; Iijima & Iwasaki, 2014a; X. Jiang et al., 2012; Schenkel et al., 2013). Within this framework, the blood circulation of two mice is connected for a few weeks before analyzing distinct tissues. However, such a major surgical intervention is inevitably associated with inflammation and modified immune reactions.

Similar issues are encountered when using common experimental models based on tissue transplantation (Gebhardt et al., 2009; Glennie et al., 2015; Masopust et al., 2010). By performing operations or frequently injecting substances, inflammatory processes are likely to be induced, which can influence subsequent immune behavior and lead to biased observations.

Another frequent approach utilized to study TRMs has been the *in vivo* labeling of T cells from defined compartments with mABs (Anderson et al., 2014, 2012) or staining using fluorescent dyes, such as CFSE (Ely et al., 2006; L. M. Wakim et al., 2010), in order to register their movements or to distinguish circulating cells from TRM. Although these strategies are promising, they are also associated with several disadvantages. Determining whether all applicable cells are stained and whether the staining process results in functional changes or damages can be difficult. The dye effects can also be diminished through proliferation and increased protein-turnover (Masopust & Soerens, 2019). In addition, although they do not circulate in the blood, TRMs can come into direct contact with the blood, for example, in the liver, and can erroneously be considered as circulating cells based on their staining (Fernandez-Ruiz et al., 2016; Gebhardt et al., 2018). Similar problems should be considered when attempting to deplete only circulating T cells using antibodies or when attempting to use other pharmacological methods to regulate T cell expression or behavior (X. Jiang et al., 2012; Schenkel et al., 2013; Watanabe et al., 2015).

Previous T cell studies have primarily been performed in mouse models; however, the knowledge gained studying mouse immune system models can

only be applied to the human immune system to a limited extent (Mestas & Hughes, 2004). For example, classical laboratory mice live in abnormally hygienic, specific pathogen-free environments, which massively influences their immune system development and distinguishes them from humans (Beura et al., 2016). These conditions can also influence the expression of immune cell markers, such as CD69 (Beura, Wijeyesinghe, et al., 2018). Therefore, the development of sophisticated and experimental human models, such as the one presented in this thesis, remains essential for obtaining more reliable knowledge regarding TRMs in humans.

Another advantage of this study is the examination of patients after allo-HSCT, which allows for clinical parameters to be measured. The development of GvHD, the reactivation of latent viruses, such as CMV, and other parameters can be recorded and compared against observation regarding the T cell populations in the skin of patients, which makes this approach highly relevant from a clinical point of view.

5.1.2 Tissue digestion

Skin biopsies were transformed into single-cell suspensions through enzymatic and mechanical digestion. Digestion of the entire sample has been criticized for potentially resulting in false results due to vascular contamination (Schenkel & Masopust, 2014). However, this criticism can primarily be applied to samples obtained from highly vascularized lung tissues, which has been associated with large quantities of T cells in the vasculature, even after perfusion with PBS (Anderson et al., 2014, 2012). This potential for contamination may be significantly reduced when studying skin biopsies, particularly when examining the epidermis, which has no capillarization. In this study, the nearly complete lack of naive-phenotype T cells, which are abundant in blood samples, in the single-cell suspensions derived from the skin of healthy patients supported the suitability of the described procedure for the study of TRMs. Furthermore, to remove any potential blood residue, the skin biopsies were washed thoroughly prior to digestion. However, the potential for residual blood in the skin tissue to influence the results of the STR analysis could not be completely excluded. However, blood cell contamination would only distort the results quantitatively, if at all. Because those patients who presented blood chimerism were excluded before performing additional analyses, the detection of recipient-derived DNA in isolat-

ed T cells supported the existence of TRMs in the human skin after allo-HSCT, even if blood contamination was present.

To obtain a maximized cell yield, an optimized method was used to transform the skin sample into a single-cell suspension. In this framework, digestion was performed using a long-term and gentle enzymatic treatment for 15–16 hours (in contrast with the majority of other studies, which typically use significantly higher enzyme concentrations combined with incubation times of no longer than one hour (X. Jiang et al., 2012; Preza, Yang, Elliott, Anton, & Ochoa, 2015; Schenkel et al., 2013; Steinert et al., 2015) combined with subsequent mechanical dissociation. This method allowed for the isolation of approximately 1.3×10^3 T cells/mm^2 from skin samples obtained from healthy individuals who were undergoing plastic surgery, which exceeded the number of cells obtained through common collagenase digestion (Clark et al., 2006). However, Clark et al. (2006) previously estimated that 1×10^4 T cells/mm^2 could be found in skin; therefore, only a portion of T cells are likely to be efficiently isolated through digestion-based methods, which has also been described by other authors (Preza et al., 2015; Steinert et al., 2015). However, the procedure applied during this study allowed the highest possible cell yield to be obtained while minimizing potential cell stress.

Despite using this method, the available samples and cell numbers remained limited, to the extent that the separate analyses of the epidermis and dermis were not possible due to low cell counts. Therefore, some anatomical and spatial information regarding the cells was lost during the digestion process. However, this limitation can be applied to the majority of the other existing experimental models used during TRM research. Approaches that have been applied to overcome this disadvantage in human experimental settings have included microscopy and pre-digestion with dispase, followed by manual separation with forceps (Cheuk et al., 2017; Klicznik et al., 2019; Strobl et al., 2020; Watanabe et al., 2015). However, these processes are inevitably associated with additional manipulation and stress for the tissue, which may result in cell death and reduced cell yields. Furthermore, the mixing of T cells derived from the epidermis and dermis during pre-digestion or manual separation cannot be reliably excluded. In addition, several surface proteins might be sensitive to digestion procedures, which must be carefully considered when choosing the digestion process (Autengruber, Gereke, Hansen, Hennig, & Bruder, 2012).

5.1.3 Addressing low cell counts

As described above, a major disadvantage associated with this study model was the low number of T cells obtained from the skin biopsies. If the number of cells obtained was below 1,000, the number had to be increased in order to obtain a sufficient amount of DNA for STR-PCR analysis. To address this potential limitation, cloning was introduced. In this elegant model, each isolated cell was cloned, and then an equal number of clones was analyzed for each originally seeded cell. The advantage of this method was that, in theory, both the original host and donor-recipient T cells would be reflected in the subsequent STR analysis. However, several issues were encountered when applying this method. In contrast with the 20%–50% cloning efficiency observed for blood cells, T cells isolated from the skin showed a lower cloning efficiency of approximately 10%, which may indicate that the skin cells were in poor condition after digestion and sorting or did not survive well outside of their original cell structure, as has been described by several authors (Brunner, Arnold, Wasem, Herren, & Frutschi, 2001; Linda M. Wakim et al., 2012). As a result, the clones of only a few cells could be analyzed, which limited their representativeness. In one extreme case, only clones from 27 cells could be analyzed and were 100% host-derived. Despite the limited successful cloning, in this case, these results indicated that the skin contained host-derived TRM. In patients from whom no clones for host-derived T cells could be identified, the complete absence of host-derived T cells could, therefore, not be determined with certainty. In addition, this method was technically demanding and required a high degree of resource consumption and man-power due to the long sorting time.

Instead, the whole-genome amplification method was utilized, which does not attempt to increase the number of cells but, rather, directly amplifies the DNA obtained from all isolated cells. After applying this method, subsequent STR analyses could be performed without difficulty, and clear results could be obtained, even when analyzing low cell numbers. This method was preferred due to the results being more reliable than those obtained using other methods and the procedures being easier to implement. However, due to the use of different techniques for obtaining sufficient amounts of DNA, the relative comparability of the quantitative results of STCC might be limited.

5.1.4 Determination of chimerism

All isolated cells were analyzed by STR-PCR analysis to identify donor and host-derived T cells. To perform this analysis, 15 STR loci and *amelogenin* were co-amplified and compared with donor-derived DNA isolated from peripheral blood mononuclear cells and host-derived DNA isolated from skin fibroblasts. The simultaneous analysis of 16 different gene loci makes this a reliable, well-proven method for determining the genetic origins of cells (Alonso, 2012). However, as previously described, a minimum quantity of DNA is necessary to perform the STR-PCR analysis. Furthermore, the STR-PCR analysis can only be performed following FACS analysis and sorting, which makes it impossible to trace the individual phenotypes of host-derived T cells. This ist in contrast to methods used by other authors in recent studies examining TRMs in humans as part of HLA-mismatched solid organ transplantations (de Leur et al., 2019; Pallett et al., 2020; Snyder et al., 2019). In these studies, FACS analyses were performed using mABs against specific HLA surface molecules to simultaneously investigate the genetic origins and phenotypes of the cells, which was not possible in this study because many donors and recipients were HLA-identical. However, this approach is restricted on HLA-mismatched transplantations, and whether this selection process affects the behaviors and functions of T cells remains unclear. The fact that HLA mismatching can directly influence the outcomes of organ transplantation supports the assumption that HLA-mismatching does have an effect on the cells, representing a major disadvantage of this study model (Miller, Kiberd, Alwayn, Odutayo, & Tennankore, 2017).

The same applies to models in which, after sex-mismatched transplantations, the cellular origins were determined through the optical detection of gonosomes using fluorescence *in situ* hybridization (FISH) (Bartolomé-Casado et al., 2019; Divito et al., 2020; Haniffa et al., 2009; Strobl et al., 2020). For instance, sex-mismatched heart and kidney transplantations could be shown to be associated with higher rates of graft failures and poorer outcomes (Khush, Kubo, & Desai, 2012; Opelz, Süsal, Ruhenstroth, & Döhler, 2010). Studies have also indicated an influence of sex mismatching on the course of allo-HSCT (Wang et al., 2018; H. Zhang, 2020). Alteration of physiological immune dynamics is further probable when only rejected organs are analyzed, as performed by de Leur et al. (2019). In the present study, the risk of possible bias was mini-

mized by the STR-PCR analysis used, in which patients could be included independently of the stem cell donor.

5.1.5 Flow cytometry as a tool for immune monitoring

In the current study, obtained cells were analyzed and sorted by flow cytometry. The simultaneous measurement of T cells from blood and skin allowed for the dynamic shifts in T cells between these two compartments to be studied. Although it was not possible to completely trace which of the previously flow cytometrically analyzed cells were recipient-derived, the comparison between blood and skin T cell populations allowed for the formation of hypotheses regarding the course of TRM development and phenotype.

Another sophisticated approach that is often applied to study TRMs is the use of imaging-based technologies that have been developed in recent years (Beura, Mitchell, et al., 2018; Gebhardt et al., 2011; X. Jiang et al., 2012; Schenkel et al., 2013; Strobl et al., 2020). At the moment, however, these technologies continue to be technically limited by issues such as antibody compatibility and instrument sensitivity. In comparison to flow cytometry, considerably fewer parameters can be recorded simultaneously (Masopust & Soerens, 2019; Schenkel & Masopust, 2014) which can be a major obstacle when attempting to phenotype TRMs and represents a significant advantage for flow cytometry. The advantage of some imaging technologies is the ability to preserve spatial and anatomical information, which is not possible with flow cytometry; however, this advantage is limited by the fact that only small regions can be examined in two dimensions. Furthermore, applying microscopy the selection of the regions and the evaluation are subjective. Extrapolated information from these small regions may be inaccurate and biased. Although these imaging-based techniques may offer significant opportunities for the future of TRM research, these methods are currently technically limited and not yet as well-established as flow cytometry, which may significantly reduce the validity and significance of studies that rely on these methods for TRM phenotyping.

5.2 Results discussion

5.2.1 Clinical impacts of TRMs

Data on the treatment and clinical conditions of the examined patients were collected by reviewing clinical records, including general patient characteristics, treatment regimens, and the incidence of GvHD or post-transplantation infections. No significant differences between patients with and without STCC regarding their clinical outcome could be determined, which may be due to the low number of cases analyzed.

The role played by TRMs during transplantation has been discussed by numerous authors (Nicosia, Fairchild, & Valujskikh, 2020). However, in most cases, these studies examined solid organ transplantations and extrapolated these findings to the context of allo-HSCT. Various studies using animal models have examined the possible influence of TRMs on infections, rejections, and general clinical outcomes and have reached contradictory conclusions (Win et al., 2009; Y. Zhang et al., 2012). In humans, a possible correlation between donor-derived TRMs found in grafts and increased graft tolerance was described for visceral transplants (J. Zuber et al., 2015). Snyder et al. (2019) demonstrated in their study of human lung transplantation, that donor-derived TRMs were associated with lower rates of acute cellular rejection (Snyder et al., 2019).

In the same study, Snyder et al. (2019) described that the frequency of donor-derived $CD4^+$ TRMs was correlated with a lower risk of lung infection after transplantation (Snyder et al., 2019). Interestingly, after strong chemotherapy regimens that induce severe cytopenia, bacterial or fungal infections are initially relatively more frequent than infections of viral origin (Schuster et al., 2017; Sung et al., 2009; Turtle, Swanson, Fujii, Estey, & Riddell, 2009). TRMs, which survive chemotherapy and continue to provide viral protection, despite the low numbers of T cells in the blood, could explain this difference in vulnerability.

In an approach to study GvHD after allo-HSCT in humans, Haniffa et al. (2009) investigated the dynamics of skin APCs after transplantation. Using FISH analysis following sex-mismatched transplantations, they observed that one lineage of host-derived macrophages resided in the tissue for several months after transplantation. In contrast, two other APC populations were rapidly replaced by donor-derived cells. Recent studies described that infiltrating donor-derived APCs were predominant in skin tissue during aGVHD (Divito et al.,

2020; Jardine et al., 2020). Therefore, it would be conceivable that interaction of donor APCs with host T cells contribute to GvHD development.

Focusing on the potential role of host TRM in the pathogenesis of GvHD after allo-HSCT, reports using animal models have indicated that both host- and donor-derived TRMs can promote GvHD (Kong et al., 2021; Tkachev et al., 2021). Recently, several reports investigated skin T cells in human patients after allo-HSCT (de Almeida et al., 2022; Divito et al., 2020; Strobl et al., 2020). A population of host-derived T cells was described to proliferate and secrete inflammatory cytokines in close proximity to donor-derived T cells, suggesting that GvHD is due to a direct interaction of reactive T cells (Divito et al., 2020; Strobl et al., 2020). However, this was not confirmed in a recent report by de Almeida et al. (2022) who observed decreased IFN-γ expression levels in host-derived skin TRM in aGvHD skin lesions compared to donor-derived skin T cells. Remarkably, history of GvHD was associated with a lower percentage of host-derived T cells in the skin. In line with the present study, no significant association between the clinical course of GvHD and the presence of host T cells could be found (de Almeida et al., 2022; Divito et al., 2020). Additionally, GvHD occurrence was detected in complete absence of host-derived TRM. Considering these data, it seems unlikely that TRMs represent a key component in the pathogenesis of GvHD. However, the exact role of host T cells remains unclear and the investigation of larger patient numbers is necessary to draw more reliable clinical conclusions.

In summary, TRMs might influence clinical outcomes, including the occurrence of GvHD and the (re)activation of viruses and other infections. Further investigation could offer numerous opportunities to optimize transplantation procedures and to reduce severe complications.

5.2.2 Potential mechanisms for TRM survival

The analyses performed in the present study clearly demonstrated that chimerism developed in several patients and potential chimerism might exist in several additional patients. Overall, these results suggested that several TRMs were able to survive the conditioning therapy used to destroy hematopoietic stem cells and circulating immune cells, which contradicts a basic assumption in allo-HSCT that the conditioning therapy depletes all T cells of the stem cell recipient. None of the patients who received TBI conditioning regimens presented STCC,

which suggested that no differences in radiation sensitivity exist between TRMs and circulating T cells. Therefore, reduced susceptibility to cytotoxic drugs may explain TRM survival under chemotherapeutic regimens, although the number of cases available for study may be too limited to obtain any significant results. A study performed in 2005 reported that bone marrow resident T cells survived chemotherapy based on mafosfamide treatment in the context of autologous HSCT, which was confirmed *in vitro* (Casorati et al., 2005).

Skin-resident TRMs may survive chemotherapy applications because chemotherapeutic agents enter the skin at lower concentrations than those present in the blood, resulting in a reduced cytotoxic effect in the skin. This effect may vary based on inter-individual differences in the pharmacodynamics of different drugs (Levy, 1998). In plasma, drug concentrations can vary among patients. Therefore, estimating the sizes of concentration differences between the skin and blood may be difficult, although spectrophotometric methods could be useful for evaluating these differences.

Turtle et al. (2009) have provided evidence for a possible mechanism through which specific T cell populations might become more resistant to cytotoxic drugs (Turtle et al., 2009). They identified a distinct population of circulating memory T cells that express ATP-binding cassette (ABC) transporters, similar to stem cells or malignant cells. ABC transporters are ATP-dependent efflux pumps that are capable of transporting various substances out of the cell, including chemotherapy drugs, such as daunorubicin (Gottesman, Fojo, & Bates, 2002). Recently, TRMs in human tissues other than skin were shown to express similar transporters (Boddupalli, Nair, et al., 2016; Kumar, Kratchmarov, et al., 2018). To test this possibility of chemoresistance as an explanation for the persistence of skin TRM after allo-HSCT, de Almeida et al. (2022) conducted efflux assays using the fluorescent marker rhodamine-123, which is a substrate of ABC-efflux pumps (Chaudhary & Roninson, 1991; Gottesman et al., 2002; Turtle et al., 2009). However, neither in these experiments increased drug efflux from skin T cells, nor in Gene Set Enrichment Analyses (GSEA), applied to transcriptomic data from TRMs, increased expression of corresponding transporters could be detected. Therefore, it seems likely that other mechanisms were responsible for the survival of skin TRMs (de Almeida et al., 2022).

TRMs have also been reported to express the pro-survival protein B cell lymphoma 2 (BCL-2), which could explain the increased survival of TRMs com-

pared with circulating T cells (Mackay et al., 2013; L. M. Wakim et al., 2010). Furthermore, chemotherapeutic drugs, many of which target the replicative machinery of cells, may be less effective in TRMs due to a generally reduced division rate compared with that in circulating cells. Authors observed significantly slower homeostatic proliferation for TRMs compared with circulating T cells (Gebhardt et al., 2009). Additionally, T cells that survived chemotherapy in the study reported by Turtle et al. showed reduced susceptibility due to low proliferation (Turtle et al., 2009). de Almeida et al. (2022) tested the *in situ*-proliferation of skin T cells of patients after allo-HSCT by analyzing expression of Ki-67 which is associated with cell proliferation. They observed significantly decreased Ki-67 expression in skin T cells, possibly resulting in protection from chemotherapy. In addition, analysis of cell cycle distribution using single cell transcriptomic data revealed host-derived skin TRM to be non-proliferating, suggesting that detected skin TRM rather survived individually than having expanded *in-situ* after transplantation (de Almeida et al., 2022). In summary, several factors may support TRM survival following chemotherapy. However, in this study, many patients were identified who did not harbor host-derived skin T cells, suggesting potential individual differences in the response of TRMs to chemotherapy, which is discussed in the following sections.

5.2.3 Individual patient characteristics as major challenge for TRM research

STCC was identified in several patients, including two patients who had undergone allo-HSCT more than two years prior to the analysis, demonstrating the unique longevity of TRMs compared with other T cell populations. However, a high fraction of the patients did not show any STCC. Determining when patients without detected STCC lost host-derived TRMs is difficult based on the results obtained here. Either these cells did not survive chemotherapy, or they were replaced by new donor-derived T cells within the first months after allo-HSCT, which may be more likely since the vast majority of patients for whom STR-PCR analysis was possible shortly after allo-HSCT showed STCC. This supports the hypothesis that pre-existing TRMs decrease or are being replaced by new TRMs over time, which was observed by several authors studying TRMs in various organs and experimental models including human transplantation models (de Leur et al., 2019; Slütter et al., 2017; Wu et al., 2014). Other authors, in

contrast, described rather stable TRM populations in multiple sites (Bartolomé-Casado et al., 2019; Pallett et al., 2020; Snyder et al., 2019; Julien Zuber et al., 2016). Park et al. stated that pre-existing TRMs are not displaced by new TRMs formed by circulating cells but, instead, continue to exist in parallel (S. L. Park et al., 2018). However, they only considered periods of approximately 30 days after transplantation in mice, during which the formation of new TRMs was actively triggered under sterile conditions.

These controlled conditions drastically differ from the conditions examined in the present study. In the patients, severe lymphopenia was induced by the conditioning therapy, which was strongly reflected in the cell numbers identified in the skin, indicating that the chemotherapy regimen affected TRMs to a certain extent. Therefore, the numbers of TRMs might be sufficiently reduced in many patients after transplantation that these populations can vanish without individual stimulating influences. Interestingly, in a study with a design similar to the present study, the authors described stable levels of host derived skin T cells in a majority of allo-HSCT patients over the following months and years (Strobl et al., 2020). However, the origin of the cells was analyzed via X/Y-FISH which limited the study to the analysis of small tissue regions with low cell numbers and to patients undergoing sex-mismatched allo-HSCT. In addition, samples were partly taken from active GvHD lesions shortly after transplantation, making it difficult to draw conclusions about long-term residency. Nevertheless, these results obtained from skin samples taken from the upper arm might indicate that existence, maintenance and dynamic of TRM may differ even within one organ like the human skin depending on their individual location. As described previously, individual variations in the drug concentrations present in patient skin may also influence the severity of damage to the TRM population.

Potentially stimulating influences for continuation of TRM populations could include inflammation or renewed antigen contact after transplantation. TRMs have demonstrated proliferation capabilities (S. L. Park et al., 2018) which does not require long-term antigen contact, as short antigen contact and unspecific tissue inflammation have been demonstrated to serve as sufficient proliferation triggers (Casey et al., 2012). Previous inflammation in the skin in the areas examined for TRM populations could, therefore, strongly influence individual differences. Therefore, future research should more strongly consider individual patient history in the context of past tissue inflammation. Within this

framework, previously inflamed skin regions could be compared against healthy skin regions from the same patient. However, this analysis would involve an enormous amount of effort and require highly detailed patient documentation. Furthermore, multiple biopsies would have to be obtained from distinct areas, which represents a significant difficulty. However, such future investigations are necessary to determine the applicability of this inflammation-based hypothesis.

Clonality analysis and TCR sequencing represent promising approaches for obtaining information regarding the history of host-derived T cells and can reveal whether recipient cells were derived from single surviving TRMs that proliferated after transplantation or whether they represent distinct surviving clones. (de Almeida et al., 2022; Gaide et al., 2015; Mahe, Pugh, & Kamel-Reid, 2018; Singh et al., 2019)

Overall, many authors have indicated that TRM research requires approaches that focus on individual and local aspects because TRM maintenance can differ depending on the organ, tissue factors, antigen specificity, and species being studied (Casey et al., 2012; Ho & Kupper, 2019; Mackay et al., 2013; Masopust & Soerens, 2019; Schenkel et al., 2016; Yang, Yu, Kalwani, Tseng, & Baltimore, 2011). To obtain clearer conclusions regarding the exact dynamics associated with TRM development after allo-HSCT, the study model described here offers several promising possibilities. The longitudinal and regular examination of patients over longer periods of time, starting before or shortly after transplantation, would allow the changes in the T cell populations to be assessed in greater detail.

5.2.4 Phenotyping of TRMs in human skin

Expression of CD45RA/CD45RO as naïve/memory markers and expression of putative residence markers CD69 and CD103 were detected by flow cytometry. Detection of CD45RA/RO revealed similar expression in T cells obtained from blood and skin of the patients after allo-HSCT. However, it was striking that while T helper subsets both in blood and skin were mainly $CD45RO^+$, $CD8^+$ T cells expressed CD45RA in a high frequency which has already been described by other authors (Heitger et al., 1997). High $CD8^+$ T cell numbers shortly after transplantation can be attributed to the fact that the recovery of T cells after allo-HSCT occurs via the two different pathways described in chapter 1.2.3 (Heitger et al., 1997; C. L. Mackall et al., 1993; Crystal L. Mackall & Gress, 1997). One

explanation for the high CD45RA expression of the $CD8^+$ T cell compartment could be given by TEMRA cells. These are almost exclusively $CD8^+$ TEM that re-express CD45RA despite prior activation and can be found in varying percentages in blood and tissues (Faint et al., 2001; Sallusto, Geginat, & Lanzavecchia, 2004; Sathaliyawala et al., 2013; Thome et al., 2014). Therefore, it cannot be excluded that a certain proportion of TRM in human skin also expresses CD45RA. The use of additional markers such as the lymph node homing receptor CCR7 could provide further information on naïveté or activation state.

Overall, particularly shortly after transplantation, a significantly higher number of cells from the skin expressed CD69 than expressed CD103. CD69 is considered to be one of the most reliable markers for TRMs (Kumar et al., 2017). However, in mouse experiments, TRMs that did not express CD69 were observed in numerous organs, including the lungs, pancreas, and thymus (S. L. Park, Mackay, & Gebhardt, 2016; Steinert et al., 2015; Takamura et al., 2016). Watanabe et al. (2015), who studied patients treated with alemtuzumab, an anti-CD52 antibody that depletes predominantly circulating T cells, observed that the vast majority of suspected skin TRMs expressed CD69, and also other authors assumed that CD69 is expressed in nearly all skin TRMs (Ho & Kupper, 2019). However, using their study design, Watanabe et al. could not exclude the possibility that non-resident cells may also express CD69, since circulating cells have been shown to express CD69 as an activation marker (David Masopust & Soerens, 2019; Shiow et al., 2006).

This might also have affected the present study, considering the high expression of CD69 in skin cells shortly after transplantation. Although the observed $CD69^+$ T cells might have represented host-derived TRMs, which would account for a larger proportion of cells in the skin observed under lymphopenic blood conditions, the high expression of CD69 exceeded the expected frequency based on STR-PCR analyses. This difference between the expected outcome and the actual outcome could be explained by newly recruited and formed donor-derived TRMs that expressed CD69. However, CD69 may be unstably expressed in a variety of cells and may not be a reliable marker for permanent residency. Overall, a majority of TRMs in human skin are likely to express CD69, but CD69 cannot be considered a specific marker for TRMs.

When examining CD103 expression, $CD4^+$ T cells were observed to express this marker more frequently than $CD8^+$ T cells, which contradicts many previous studies performed in mice that described CD103 as a marker for $CD8^+$ TRMs in the skin (Gebhardt et al., 2009; Mackay et al., 2013). In these studies, $CD4^+$ TRMs, similar to $CD8^+$ TRMs, primarily expressed CD69, but the expression of CD103 was variable, and a large proportion of the cells were $CD103^-$ (Bromley, Yan, Tomura, Kanagawa, & Luster, 2013; Gebhardt et al., 2011; Glennie et al., 2015; C. O. Park et al., 2018). Additionally, Watanabe et al. (2015) observed in their study of human skin that fewer than 20% of $CD4^+$ TRMs, which appeared to be resident, expressed CD103, whereas the proportion among $CD8^+$ TRMs was nearly 50%.

In the present study, a large proportion of the TRMs isolated from the two patients with STCC more than two years after transplantation did not reliably express CD103. In these patients, the STR-PCR analyses detected a significantly higher fraction of host-derived T cells, compared with the number of cells that expressed CD103. In addition, newly formed donor-derived TRMs may also have accounted for a fraction of the $CD103^+$ T cells isolated from the skin. These results indicated that a significant proportion of TRMs in the skin was likely to have been $CD103^-$.

Only a proportion of $CD8^+$ TRMs and an even smaller proportion of $CD4^+$ TRMs are likely to express CD103, based on the findings of the previous studies described above. Therefore, it is likely that a considerable population of $CD4^+CD103^-$ TRMs was present in the patients' skin. These results indicated that in patients after allo-HSCT, T helper cell populations are more likely to persist in the skin and possibly provide early immune protection and recovery, which is discussed further in the next section.

In recent studies in mice (Fonseca et al., 2020; Klicznik et al., 2019; Wijeyesinghe et al., 2021) and human (de Almeida et al., 2022), another interesting observation regarding the residency state of TRM was made. Upon TCR stimulation, TRM were shown to be able to downregulate CD69 and CD103 and rejoin the blood circulation. This finding suggested that expression of the supposed TRM markers CD69 and CD103 could be dynamically regulated, rather than representing a steady state.

Approaches based on next-generation sequencing (NGS) technologies are promising for obtaining detailed information regarding specific phenotypes

of human TRMs. TCR sequencing can be informative for identifying specific features of distinct T cell subsets and allows for the performance of clonality analyses among T cells (de Almeida et al., 2022; Gaide et al., 2015; Mahe et al., 2018). Clonality analyses have the potential to reveal if TRM found in the skin originate from single proliferating TRM or if they represent cells with individual migration history.

Recently, single-cell RNA sequencing (scRNA-seq) was first performed on T cells obtained from patients with STCC including patients selected on the basis of the present study (de Almeida et al., 2022; Strobl et al., 2020). Among other interesting observations, the scRNA-seq results revealed significant upregulation of the gene *LGALS3* and the increased expression of its encoded protein, Galectin-3. This protein contributes to cell adhesion both to other cells and to the extracellular matrix and could potentially be used as a phenotypic marker for TRM (Hsu, Chen, & Liu, 2009). These analyses demonstrate the major opportunities available when applying modern technologies, e.g. appraches based on the investigations of single-cell transcriptomes, for overcoming some of the current issues associated with TRM phenotyping and research.

5.2.5 Tissue-resident memory T helper cells

T cells isolated from different sites and patients were compared based on their surface marker expression. $CD4^+$ and $CD8^+$ T cells were examined to determine the expression of various T cell markers, such as CD45RO or CD45RA, and the putative residence markers CD69 and CD103. The results emphasized the role played by $CD4^+$ T cells as resident T cells. In contrast to cytotoxic T cells, T helper cells were present at increased frequencies in the skin compared to the blood of the examined patients, especially shortly after HSCT in patients with STCC. In addition, T helper cells isolated from the skin expressed CD45RO, as well as CD69 and CD103, which are surface molecules that are regularly used as TRM markers at increased frequencies. These results suggested that host-derived TRMs identified in the patient skin were $CD4^+$ rather than $CD8^+$ T cells. This was further supported by data obtained from scRNA-seq analyses described in the previous section (de Almeida et al., 2022).

The potential role of T helper cells in TRMs represents a significant finding because most TRM research has focused on $CD8^+$ TRMs. Many authors have limited their research to this more-characterized population and have sus-

pected that TRMs are more likely to be found among the $CD8^+$ population for a variety of reasons (Gebhardt et al., 2018; Mackay et al., 2013; Mueller & Mackay, 2016; Schenkel, Fraser, & Masopust, 2014). First, many experiments have been based on the outcomes of viral infections inducing $CD8^+$ cytotoxic T cells. Second, $CD4^+$ T cells are particularly enriched in the lymphatic vessels draining the skin, where $CD8^+$ T cells have been found at a reduced frequency (Yawalkar, Hunger, Pichler, Braathen, & Brand, 2000). Mackay et al. (2012) concluded that this reduced presence in the lymphatic vessels is an indication that $CD8^+$ T cells are more likely to be TRMs. Similarly, Jiang et al. (2012), who used a vaccinia virus-infected mouse model, examined $CD4^+$ T-cell-depleted mice to study the generation of $CD8^+$ TRMs in reaction to skin infection. In another key study on TRM research, Gebhardt et al. (2009) examined a mouse model in which the transplantation of HSV-1-infected ganglia and specific $CD8^+$ T cells was performed to identify resident T cells in nonlymphoid tissue. Two years later, the same research group used a similar mouse model and observed that $CD8^+$ T cells were primarily found in the epidermis, where they persisted for at least 10 weeks (Gebhardt et al., 2011). In contrast, $CD4^+$ T cells were found in increased numbers in the dermis and were found to have higher motility and circulation. A longer residence time was not described but was also not explicitly excluded.

Moreover, in initial studies examining human models, only $CD8^+$ HSV-specific T cells were examined (Jia Zhu et al., 2007, 2013). However, the results of other studies have indicated that $CD4^+$ T cells are predominantly found in large quantities in the human skin (Clark et al., 2006; Sathaliyawala et al., 2013). Contrary to the studies described above, the results obtained from the present study suggest that $CD4^+$ T cells are of particular relevance and must receive more focus in the context of TRM research. $CD4^+$ T cells represent a substantial fraction of skin T cells obtained from patients shortly after HSCT and are the most likely TRMs to survive conditioning therapy in some patients. The relevance of $CD4^+$ T cells for TRM populations in human skin has been further indicated by the results of Watanabe et al. (2015), who reported abundant $CD4^+$ T cells, particularly in the dermis, whereas $CD8^+$ T cells were more likely to be found in the epidermis.

Mouse models have indicated multiple functions that resident T helper cells can perform in tissue. T helper cells can recruit new $CD8^+$ T cells into the

tissue and maintain them as TRMs (Laidlaw et al., 2014; Nakanishi, Lu, Gerard, & Iwasaki, 2009), which could be particularly important for the patients examined in the present study, who are dependent on the rapid reconstruction of a functioning immune system, for which TRMs are essential. Resident T helper cells can also directly exercise effector functions and, thus, contribute to the defense against pathogens, such as bacteria, fungi, parasites or viruses including SARS-CoV-2 (Beura et al., 2019; Glennie et al., 2015; Iijima & Iwasaki, 2014b; Niessl et al., 2021; S. L. Park et al., 2018; Snyder et al., 2019; Teijaro et al., 2011). Intriguingly, in particular $CD4^+$ TRMs , have the capacity to rejoin the blood circulation upon stimulation, potentially providing an elegant mechanism to enable comprehensive immune protection via tissue-crosstalk (de Almeida et al., 2022; Klicznik et al., 2019; Wijeyesinghe et al., 2021). In addition, resident T helper cells have been shown to have an significant impact on the clinical outcome of patients with various malignancies (Beumer-Chuwonpad, Taggenbrock, Ngo, & van Gisbergen, 2021). On the other hand, associations between $CD4^+$ TRMs and numerous autoimmune diseases such as bronchial asthma, psoriasis, glomerulonephritis and Crohn's disease have been described (Hondowicz et al., 2016; Kleinschek et al., 2009; Krebs et al., 2020; Matos et al., 2017). In view of these various implications, future research on TRMs should place an increased focus on $CD4^+$ TRMs, which is likely to require new experimental approaches, one of which has been presented here.

5.3 Conclusion

TRMs have been explored mainly in animal models and there is only scarce data on their properties in human organs. Using the clinical situation of allo-HSCT, this study was able to investigate skin TRM in humans. Extensive immune monitoring of the skin was performed, allowing broader insight into T cell dynamics after allo-HSCT, which was previously limited to the blood compartment.

In several patients, TRMs survived allo-HSCT despite severe lymphopenia in blood and massive overall reduction of T cell numbers in skin after conditioning therapy. They were durable and persisted for months and even years after transplantation. No correlation was found between STCC and a specific treatment or incidence of infections and GvHD. Hence, it is unlikely that TRMs are essential for the pathogenesis of GvHD. FACS analysis of blood and skin T cells revealed a lack of specificity of alleged residency markers CD69 and CD103. Remarkably, the data suggest that $CD4^+$ T helper cells are a major contributor to the TRM population in human skin.

To date, it remains unclear on which exact factors TRM survival and long-term maintenance depend, and no significant clinical implications could be observed in this study. However, TRMs might influence the occurrence of complications and patient outcome after allo-HSCT, which needs be investigated in a larger sample. Providing more robust data on the clinical effect of TRMs may be useful to optimize allo-HSCT by developing new preventive and therapeutic options. Advanced bioinformatic approaches could be used in the context of the allo-HSCT model to provide new information for more reliable identification of TRM. Since previous research mainly focused on cytotoxic T cells, a more specific study of the T helper cell population may shed light on unexplored characteristics and functions of TRMs. Further investigation of this cell population will be essential for a comprehensive understanding of TRMs, and thus the entire human immune system.

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7 Publications related to this work

Parts of this thesis contributed to the publication

de Almeida GP, Lichtner P, Eckstein G, **Brinkschmidt T**, Chu CF, Sun S, Reinhard J, Mädler SC, Kloeppel M, Verbeek M, Zielinski CE. Human skin-resident host T cells can persist long term after allogeneic stem cell transplantation and maintain recirculation potential. *Science Immunology.* 2022 Jan 28;7(67):eabe2634. doi: 10.1126/sciimmunol.abe2634. Epub 2022 Jan 28. PMID: 35089814.

8 Index of abbreviations

ALL	Acute lymphatic leukemia
Allo-HSCT	Allogeneic hematopoietic stem cell transplantation
AML	Acute myeloid leukemia
APC	Antigen presenting cell
ATG	Anti-thymocyte globulin
Auto-HSCT	Autologous hematopoietic stem cell transplantation
CD	Cluster of differentiation
CyA	Cyclosporin A
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
FDE	Fixed drug eruption
FISH	Fluorescence *in situ* hybridization
G-CSF	Granulocyte-colony stimulating factor
GI	Gastrointestinal
GvHD (a/c)	Graft-versus-host disease (acute/chronic)
GvL	Graft-versus-leukemia effect
GvT	Graft-versus-tumor effect
Gy	Gray
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
LPS	Lipopolysaccharide
mAB	Monoclonal antibody
MACS	Magnetic-activated cell sorting
MDS	Myelodysplastic syndrome
MHC	Major histocompatibility complex
MMF	Mycophenolate mofetil
MMUD	Mismatched unrelated donor
MMY	Multiple myeloma
MPS	Myeloproliferative syndromes
MRD	Matched related donor
MTX	Methotrexate
MUD	Matched unrelated donor
NHL	Non-Hodkin-Lymphoma
NLT	Non-lymphatic tissue
OMF	Osteomyelofibrosis
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PI	Propidium iodide
SAA	Severe aplastic anemia
SCS	Single-cell suspension

SLO	Secondary lymphatic organs
STCC	Skin T cell chimerism
STR	Short tandem repeat
TBI	Total body irradiation
TCM	Central memory T cell
TCR	T cell receptor
TEFF	Effector T cell
TEM	Effector memory T cell
T_{reg}	Regulatory T cell
TRM	Tissue-resident memory T cell

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