Illuminating T-cell repertoires in health and disease by ultra-deep sequencing

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1. Summary

The adaptive immune system as part of the human immune system provides defense against invading pathogens and provides a highly specific immunological memory. One of the main effector cells of the adaptive immune system are the T-lymphocytes. Antigen recognition of pathogens by this cell type is mediated through antigen-specific receptors located on the lymphocyte surface. The high diversity of these receptors, which is required for a broad range of antigen recognition, is achieved by rearrangements of the variable (V), diversity (D) and joining (J) gene segments of the T-cell receptor (TCR) genes resulting in a de novo sequence that can be seen as a fingerprint of each individual T cell.

T-cell disorders such as lymphomas or mature T-cell leukemias originate from a single malignant transformed T-lymphoid cell. Thereby, all malignant T cells derived from one precursor cell and share the identical TCR rearrangement. Molecular analyses of multiplex PCR generated TCR sequences are frequently used to distinguish between such clonal cell populations of T-cell malignancies and non-clonal cell proliferations without existing lymphoma. Within the advent of high-throughput sequencing (HTS), the composition of TCR repertoires can now be analyzed in an unprecedented depth which enables new insights into the role of T cells in the immune system.

In this thesis a combined multiplex T-cell receptor beta (TCRβ) PCR and HTS approach was established to analyze TCRβ repertoires of two T-cell related topics to elucidate i) the importance of the TCRβ repertoire of donors in allogeneic stem cell transplantation and ii) the origin of double negative T cells (DNTS) in patients suffering from the autoimmune lymphoproliferative syndrome (ALPS). Analyses of i) revealed that the diversity of the donor derived TCR repertoire plays a decisive role for a successful transplantation and influences significantly the clinical course regarding reactivation of Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) in the recipient. Whereas analyses and tracking of ii) the TCRβ repertoire in an ALPS patient indicated for the first time that DNTs can also originate from the $CD4^+$ T-cell compartment and not exclusively from $CD8^+$ T-cells.

Taken together, the successfully established approach for TCRβ repertoire analysis by massive parallel sequencing of TCRβ amplicons after multiplex PCR is suitable for the determination of T-cell repertoire diversity as well as tracking of individual T-cell

rearrangements. It can be used in a broad field of clinical applications and might support clinical diagnostics or treatment decisions for patients suffering from T-cell malignancies.

2. Zusammenfassung

Das adaptive Immunsystem, welches einen Teil des menschlichen Immunsystems bildet, dient der Abwehr von eindringenden Pathogenen und bildet ein hochspezifisches immunologisches Gedächtnis aus. Eine der Haupteffektorzellen des adaptiven Immunsystems sind die T-Lymphozyten. Dieser Zelltyp erkennt pathogene Antigene über antigen-spezifische Rezeptoren auf seiner Zelloberfläche. Die enorme Diversität dieser Rezeptoren, die für eine breite Antigenerkennung benötigt wird, wird über die Umlagerung der variablen (V), diversitäts (D) sowie verbindenden (joining - J) Gensegmente des T-Zellrezeptors (TCR) ausgebildet. Die daraus resultierende de novo Sequenz kann als Fingerabdruck für die individuelle T-Zelle angesehen werden.

T-Zellerkrankungen wie Lymphome oder Leukämien entstehen aus einer einzelnen transformierten T-Lymphozytenzelle. Dabei können alle malignen Zellen von einer Vorläuferzelle abgeleitet werden und weisen dementsprechend eine identische TCR Umlagerung auf. Molekulare Analysen von multiplex PCR generierten TCR Sequenzen werden daher häufig verwendet, um zwischen solchen klonalen Zellpopulationen in T-Zellerkrankungen und nicht klonaler Zellproliferation ohne Lymphomanteil zu unterscheiden. Mit dem Aufkommen von Hochdurchsatz-Sequenziermethoden (HTS) ist es nun möglich das TCR Repertoire in einem bisher nicht möglichen Umfang zu sequenzieren, welches neue Einblicke in die Rolle der T-Zellen im Immunsystem ermöglicht.

In der vorliegenden Arbeit wurde ein kombinierter Multiplex-PCR HTS Ansatz etabliert, mit dem das T-Zellrezeptor beta (TCRβ) Repertoire für die folgenden zwei T-Zell bezogenen Themenbereiche erfasst werden sollte, um i) die Bedeutung des TCRβ Repertoires von Spendern bei allogener Stammzelltransplantation sowie ii) den Ursprung doppelt negativer T-Zellen (DNTs) in Patienten mit Autoimmun-lymphoproliferativen Syndrom (ALPS) zu klären. Unter i) durchgeführte Analysen zeigten, dass die Diversität des TCR Repertoires im Spender eine entscheidende Rolle für eine erfolgreiche Transplantation einnimmt und diese signifikant den klinischen Verlauf in Bezug auf die Reaktivierung des Epstein-Barr Virus (EBV) und des Cytomegalievirus (CMV) im Empfänger beeinflusst. Hingegen brachten Analyse und Tracking des TCRβ Repertoires in einem ALPS Patienten unter ii) zum ersten Mal Hinweise darauf, dass

DNTs sich ebenso aus $CD4^+$ T-Zellen und nicht ausschließlich aus $CD8^+$ T-Zellen entwickeln können.

Abschließend lässt sich festhalten, dass unsere erfolgreich etablierte Methode zur TCRβ Analyse mittels umfangreicher paralleler Sequenzierung von TCRβ Amplifikaten nach multiplex PCR für die Erhebung der Diversität des T-Zellrepertoires sowie zum Tracking von individuellen T-Zellumlagerungen geeignet ist. Damit kann diese Methode in einem umfangreichen Bereich bei klinischen Fragestellungen Anwendung finden und zur Unterstützung der klinischen Diagnostik sowie als Entscheidungshilfe für die Therapie von malignen T-Zellerkrankungen eingesetzt werden.

3. Introduction

The defense against intra- and extracellular pathogens as well as the elimination of apoptotic or malignant cells are the main functions of the human immune system. Consequently, two subsystems have been evolved to achieve these tasks: (i) the innate immune system and (ii) the adaptive (also known as acquired) immune system. Both subsystems have to interact with each other to ensure an effective immune response (Hoebe, *et al* 2004, Medzhitov 2001). The innate immune system operates rapid but non-specific as a first line of defense against pathogens in a generic way. Thereby, pathogen recognition by the innate immune system is mediated by a small set of germline encoded, invariant pattern-recognition receptors (PRRs), which have broad specificities for conserved and common structural motives of microorganisms (Janeway 1989, Medzhitov 2007). These targets of PRRs can be also designated as pathogen-associated molecular patterns (PAMPs) and are often components of the bacterial cell wall, such as lipopolysaccharide, peptidoglycan or lipoteichoic acids. However, this part of the immune system does not confer a long-lasting immunity against a special pathogen.

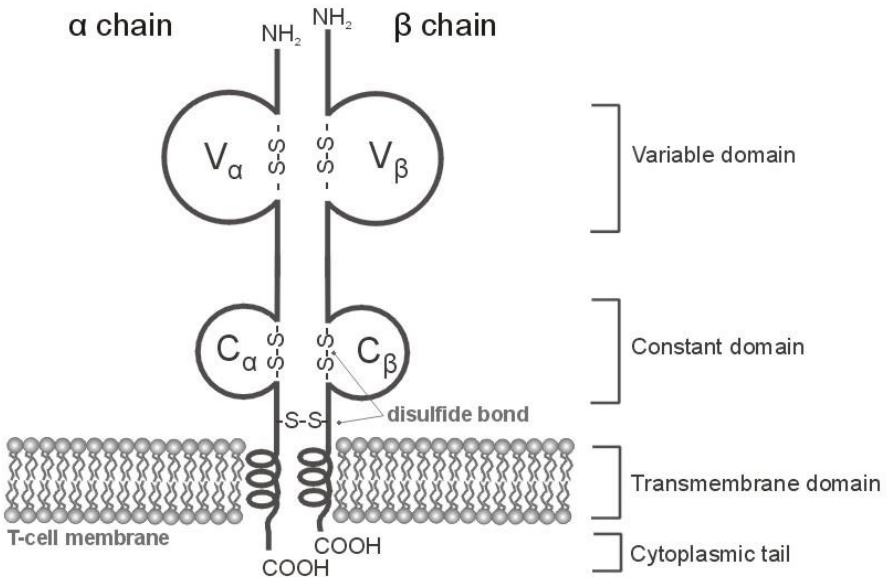
In contrast, the adaptive immune system is highly specific towards pathogens and affords an immunological memory. The two main effector cells of the adaptive immune system are B cells, which provide antibody-based immunity, and T cells, which provide cell-mediated immunity. Adaptive immune recognition of pathogens by these lymphocytes is mediated through antigen-specific receptors on their cell surface (Binz and Wigzell 1978, Nemazee 2006). The diverse repertoire of B- and T-cell receptors is generated by assembling of antigen encoding receptor genes and is further increased by additional mechanisms as non-templated nucleotide insertion or – in case of B cells – somatic hypermutations. These processes result in an immune repertoire with an extremely high diversity capable to recognize almost any antigen in a specific manner (Medzhitov 2007, Schatz, *et al* 1992). Analyses of the T-cell repertoire composition were part of the herein presented thesis and therefore its receptor type is described in detail in the following chapters.

3.1 T-cell receptors: composition and function

The T-cell receptor (TCR) is expressed on the surface of T lymphocytes and is responsible for antigen recognition by interaction with major histocompatibility complex (MHC) molecules

presenting the antigenic peptides. TCRs are heterodimeric membrane proteins comprising an alpha (α) and beta (β) chain in 95% of T cells, whereas 5% of T cells express a TCR which consists of a gamma (γ) and delta (δ) chain. This given α/β to γ/δ T-cell ratio might change in the course of T-cell disorders like lymphomas or leukemias and, furthermore, it could be even observed during bacterial infection (Born, *et al* 2006). The disulfide-linked TCR heterodimer is permanently anchored into the cell membrane of the T lymphocyte and is not secreted like immunoglobulins of B cell derived plasma cells. The large extracellular part of the TCR consists of two domains: the antigen-binding variable (V) and a constant (C) domain, which are followed by a transmembrane domain and a short cytoplasmic tail (**Figure 1**). TCRs are monovalent and by this mean able to bind only one antigen molecule at once. Under physiological conditions, TCRs recognize foreign antigen fragments only when those are presented on the surface of an antigen-presenting cell (APC) by MHC molecules (Davis and Bjorkman 1988). This interactive

Figure 1



Scheme of an α/β T-cell receptor that contains a variable (V), constant (C), transmembrane, and cytoplasmic domain. The α and β chain of the receptor are linked via disulfide bonds (S-S).

antigen recognition between TCR and MHC is known as "MHC restriction".

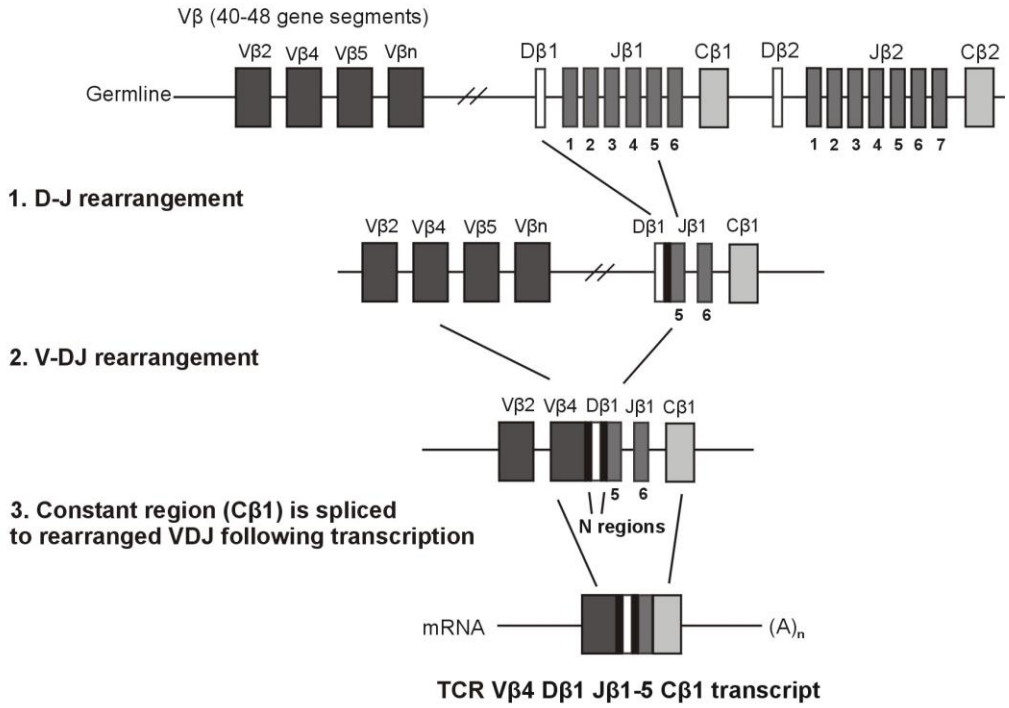
Two subpopulations of T cells can be identified: i) the $CD4^+$ T-cell lymphocytes – also referred as T helper cells – that recognize antigen fragments embedded in MHC class II molecules on the surface of APCs and ii) the $CD8^+$ T-cell lymphocytes – also referred as cytotoxic T cells – that interact for antigen recognition with MHC class I molecules (Meuer, *et al* 1982, Rudolph, *et al* 2006).

3.2 T-cell receptor diversity

The efficiency of T lymphocytes to recognize a vast number of pathogens as foreign antigens is primarily based on the high diversity of TCRs, which is mainly generated by somatic rearrangements of the T-cell genes along with the junctional variety at the recombination junctions.

During the development of T cells the genes that encode the two types of TCRs - α/β and γ/δ - undergo somatic rearrangements. Thereby, the TCRβ and TCRδ genes undergo recombination of the variable (V), diversity (D) and joining (J) gene segments whereas the TCRα and TCRγ genes are assembled by recombination of the V- and the J-segments only. In a first step of the VDJ recombination of the TCRβ and TCRδ genes, one of two Dβ or one of three Dδ segments is assembled with one J segment of 13 potential Jβ or 4 potential Jδ segments (**Figure 2**). Thereon, one of the functional 40-48 Vβ or 7-8 Vδ segments is combined with the already rearranged DJ-segments. The gene segments of the respective TCRα and TCRγ chains rearrange similarly. Due to the lack of a D-segment, one of the V segments (43-45 for TCRα; 4-6 for TCRγ) and one of the J segments (50 for TCRα; 5 for TCRγ) undergoes a one-step recombination only. The exact number of functional TCR genes depends on the haplotype of each individual (IMGT 2015, van Dongen, *et al* 2003). Throughout the V(D)J recombination random non-templated nucleotides are inserted at recombination junctions which results in a unique hypervariable complementarity-determining region 3 (CDR3) of the rearranged TCR forming the actual antigen binding site of the receptor. By this process more than 2.5×10^7 different α/β TCRs or 5×10^3 γ/δ TCRs could be generated (Arstila, *et al* 1999, van Dongen, *et al* 2003).

Figure 2



Structure and rearrangement of T-cell receptor genes using TCRβ as example. In the first step of the VDJ recombination, one of two diversity (Dβ) segments is assembled with one of 13 possible joining (Jβ) segments. Thereafter, one of 40-48 variable (Vβ) segments is combined to the already rearranged DJ-segments. During the rearrangement process non-templated nucleotides (N) are inserted at recombination junctions.

3.3 Analyses of T-cell receptor rearrangements

Malignancies like lymphomas and mature leukemias derive from the uncontrolled proliferation of a single transformed lymphoid cell. Thereby, all malignant cells derived from one precursor cell share the same characteristics and are defined clonal for this reason. Molecular analyses of TCR rearrangements are frequently used for diagnostics to discriminate between the occurrence of such monoclonal lymphoid cell populations as an indication for malignancy and polyclonal cell compositions in individuals without a lymphoproliferative disease (Langerak, *et al* 2007).

Initially, Southern blot analyses represented the gold standard for molecular clonality analysis. Thereby, clonal cell populations were displayed by accumulation of DNA fragments of identical length generated by digestion of genomic DNA with restriction enzymes and hybridization with specific radioactively labeled probes. Since this technique was very time consuming, required large amounts of high-quality DNA and hampered by limited sensitivity, PCR-based rearrangement analyses replaced the Southern blot approach especially for the analysis of formalin-fixed tissue samples (Langerak, *et al* 1997, Sandberg, *et al* 2005, van Dongen, *et al* 2003). The first PCR-based strategies for clonality testing were designed on the TCRγ only, due to its relative simple gene structure (Arber, *et al* 2001, Trainor, *et al* 1991). This TCRγ restriction, however, generated false negativity and false positivity resulting in the inability to accurately distinguish between monoclonal and polyclonal cell compositions (Langerak, *et al* 2012). Moreover, initial assays for the detection of TCRβ rearrangements by PCR were difficult to handle as well as to interpret, and thus lacked broad dissemination (Assaf, *et al* 2000). These drawbacks of the earliest PCR strategies provoked the design of novel TCR rearrangement detection arrays and nowadays the standardized protocols of the European BIOMED-2 network have become the benchmark technique for the analyses of clonality in B cells and T cells.

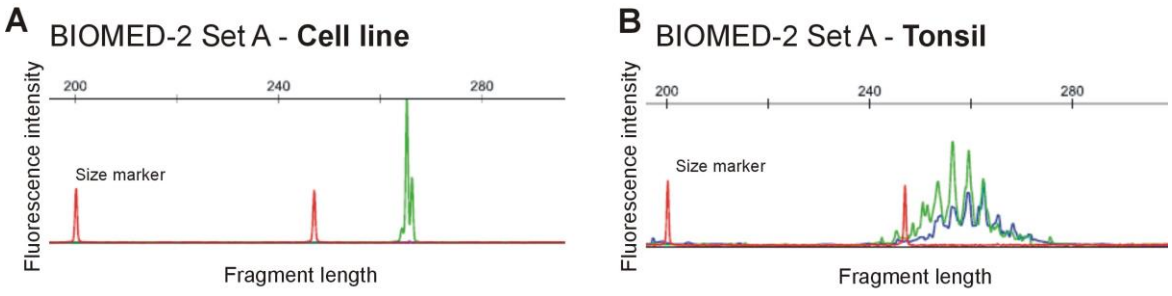
3.3.1 State of the art: BIOMED-2

At present, TCR analysis of suspected lymphocyte proliferations by standardized multiplex PCR analyses developed by the European BIOMED-2 consortium is the best practice for detection of clonality in clinical diagnostics (van Dongen, *et al* 2003). The detection of rearranged TCR genes is thereby assessed by different multiplex primer combinations for TCRβ and TCRγ. Primer dimerization of multiplex primers requires primer separation and results in two different multiplex primer combinations for TCRγ (Tube A and B) as well as in three different multiplex tubes for TCRβ (Tube A, B and C). In terms of TCRβ, Tube C was designed for the detection of incomplete DJ-rearrangements only. The combined usage of all TCRβ and TCRγ tubes enables detection of virtually all populations of TCR clones (van Krieken, *et al* 2007). In order to use formalin-fixed and paraffin-embedded (FFPE) material – in which DNA is highly fragmented due to the fixation procedure - amplicons generated by this approach were designed to be under the size of 300 base pairs (bp).

For discrimination between monoclonal cell populations with identical TCR rearrangements and polyclonal cell compositions with highly diverse CDR3, the separation of amplicons generated via the BIOMED-2 approach by high-resolution capillary electrophoresis (GeneScan) has proven to be particularly suitable (Droese, *et al* 2004, van Dongen, *et al* 2003). To this end, the single-stranded and fluorochrome-labeled TCR amplicons were size-separated based on their individual TCR CDR3 sequence. For monoclonal T-cell populations – as in the case of lymphoma cells carrying identical TCR CDR3 motives – all PCR products are of equal size and resulted in the rise of a single peak in the GeneScan (**Figure 3A**). In contrast, polyclonal TCR CDR3 sequences are presented as a Gaussian-like size distribution (**Figure 3B**). However, in clinical diagnostics the analyzed cases are frequently not explicit clonal or polyclonal. Several kinds of intermediate patterns exist, representing dominant peaks in a polyclonal background or giving more than three dominant products in the GeneScan profile, which is termed as oligoclonality (Langerak, *et al* 2012).

The main disadvantage of this technique is that solely the size distribution of the whole TCR repertoire is surveyed and that many different rearrangements with identical CDR3 size are summed up to one peak on the basis of their identical length. Sequencing of individual TCR rearrangements by Sanger sequencing will only be realizable without cloning if a significant clonality without extensive polyclonal T-cell background is present (Sanger, *et al* 1977).

Figure 3



GeneScan analysis of **(A)** a T-cell line as an example of monoclonal T-cell populations and **(B)** a tonsil illustrating the Gaussian-like amplicon distribution of a polyclonal cell composition.

3.3.2 On the rise: High-throughput sequencing

With the advent of high-throughput sequencing (HTS) the potential to analyze the TCR repertoire improved dramatically. The massive parallel sequencing of PCR products that have been created by TCR multiplex PCRs allows the detection of each single TCR rearrangement in a given heterogeneous mixture of T cells in a quantitative manner. After bioinformatics analysis of millions of sequence reads, a very deep and detailed insight in the composition of T-cell repertoires can be provided (Metzker 2010). This enables the determination of T-cell clonality, T-cell diversity and even tracking of individual T-cell populations in a variety of immunological contexts e.g. infections, vaccination or lymphocyte development (Calis and Rosenberg 2014).

3.3.2.1 Amplification and sequencing strategies

Several strategies for sequencing of TCR rearrangements based on genomic DNA (gDNA) or cDNA - after reverse transcription of mRNA - have been developed (Boyd, *et al* 2009, Freeman, *et al* 2009, Robins, *et al* 2009). In analysis of the TCR repertoire derived from cDNA the number of mRNA molecules does not necessarily reflect the real number of T cells. This is related to the fact that the TCR expression level in T cells is not identical, leading to a variant number of mRNA copies in different T cells. Thereby, the correct quantification of the analyzed T-cell populations might be affected (Robins 2013). Therefore, and because of its long-term stability gDNA is often the preferred starting material for sequencing applications (Woodsworth, *et al* 2013). In order to obtain a sufficient number of templates for sequencing, multiplex PCR approaches have been developed, utilizing mixtures of specific V- and J-Primer covering all V- and J-segments (Robins, *et al* 2009). Unfortunately, despite intensive adjustments of primer sets, the amplification efficiency of the various TCR targets cannot be perfectly balanced and still resultant biases can be observed. However, it has been recognized that biases in the amplification efficiency of TCRs are highly reproducible. For comparative studies, which relied on constant amplification conditions, the degree of PCR bias will be identical for every preparation and might therefore be disregarded to a certain degree (Woodsworth, *et al* 2013). Nevertheless, synthetic TCR targets have been employed that permit monitoring and further optimization of the unbalanced amplification (Carlson, *et al* 2013).

3.3.2.2 Sequencing technologies

For sequencing of generated TCR amplicon libraries several HTS platforms are available. At present, two benchmark platforms have been established: i) the MiSeq / HiSeq system produced by Illumina and ii) the Ion Torrent technology supported by LifeTechnologies (a Thermo Fisher scientific brand). Both systems differ in sequencing technology, read length, sequencing depth, and error frequency but share the advantages of being very time efficient and affordable (Calis and Rosenberg 2014, Robins 2013). The major drawback of the Ion Torrent technology is the high rate of deletions and/or insertions (also known as indels) from homopolymers, which present a large problem for TCR sequencing due to the arise of G nucleotide stretches in the sequence of the D-segment. Whereas the appearance of indels is relatively rare with the Illumina technology, the MiSeq (or equivalent the HiSeq) generates higher error rates with increasing read length. As these errors are position depended, they can be avoided by the use of paired-end sequencing. Thereby, a defined number of nucleotides is sequenced starting from both sides of the amplicon and the thereafter aligned sequence reads are of higher accuracy (Warren, *et al* 2011). Currently, none of the technologies is able to generate sufficient read length to sequence the entire TCR rearrangement. However, they are suitable to sequence the individual CDR3 motives and parts of the rearranged V- and J-segments, which is sufficient for an accurate assignment of the used segments. Irrespective of the sequencing technology but depending on the application type, the sequencing depth should be adjusted. Tracking of malignant low level T cells e.g. after leukemia treatment (also known as minimal residual disease – MRD) would need higher sequencing depth to detect really rare TCR clones. On the other hand, measurement of T-cell clonality in lymphomas needs significantly less sequence read-depth, due to the occurrence of dominant cell clones (Robins, *et al* 2012, Wu, *et al* 2012).

3.3.2.3 Data processing

HTS data of TCRs requires highly specialized bioinformatics tools and appropriate workflows due to the high biological diversity of TCR rearrangements and the tremendous size of sequencing output. However, sequence alignment of generated data with already published germ line structures can be realized for the V- and J-segment parts of the amplified TCR rearrangement. The real challenge for data processing is the accurate correction of the non-conserved, and highly individual CDR3 regions of the TCR.

Emerging errors in HTS data can be attributed to i) amplification errors due to inaccuracy of enzymes (polymerases) during PCR and ii) HTS-generated errors during sequencing (Nguyen, *et al* 2011). Based on the exponential amplification, errors that occur during PCR can be reconstructed as a phylogenetic tree (Robins 2013). Since no selection of these erroneous amplifications occurred their number increased continuously with every PCR cycle. However, the probability that two polymerase-based errors occurred in the same PCR cycle in a single amplicon is relatively low. Therefore, each individual sequence deviation in an amplicon can be regarded as a branch of the phylogenetic tree. For this reason, this kind of errors can be compensated by sequence clustering algorithms during data processing (Bolotin, *et al* 2012). In the clustering process low frequency TCRs with altered CDR3 sequence were summed up to the highly abundant TCR sequence, which might differ slightly in its rearranged sequence. This frequent TCR sequence which occurred with multiple copies presented the supposed sequence origin and could be also referred as the clonotype origin.

The frequency of sequencing errors is platform dependent. However, as long as any type of error is randomly and rare, a sufficient sequencing coverage of templates allowed adequate error correction similar to the phylogenetic tree (Bolotin, *et al* 2012, Robins 2013). Nowadays, improved accuracy of the sequencing technologies relieved the clustering process and thereby identification of the clonotype origin as well. Additionally, accuracy rates are reported for every sequenced base and this information can also be added to the clustering process (Bolotin, *et al* 2012, Warren, *et al* 2011). However, there has to be a strict balance in data processing and error correction, otherwise overcorrection might affect shifts in TCR repertoires and the resulting data will not reflect the existing T-cell diversity or clonotype frequencies. A permanent validation of results for plausibility should therefore be performed continuously.

3.4 T cells and stem cell transplantation

For a variety of hematological malignancies, including lymphoid or myeloid malignancies, the transplantation of hematopoietic stem cells represents one potential curative therapy. In this process, the immune system of concerned patients is destructed by radiation and/or chemotherapy before transplantation. The subsequent transplantation of multipotent hematopoietic stem cells might be autologous – when the patient's own stem cells are transferred – or allogeneic – in case of donor derived stem cells. Allogeneic hematopoietic stem cell transplantation (aHSCT) is

applied commonly whereby, in most instances, mobilization of hematopoietic stem cells in human leukocyte antigen (HLA) compatible donors is achieved through administration of granulocyte-colony stimulating factor (G-CSF). Therefore, G-CSF mobilized peripheral blood stem cells (PBSC) are nowadays the predominant source of stem cells used for aHSCT and have replaced bone marrow as the graft source (Hopman and DiPersio 2014). In comparison to the previously used bone marrow transplantation (BMT) the clinical advantage of G-CSF mobilized aHSCT includes accelerated engraftment and a shortened neutropenic period (Anderlini 2009, Pan, *et al* 1996).

However, success rates of aHSCT are compromised by the following three major factors: i) relapse of the underlying malignancy, ii) acute and also chronic graft versus host disease (GvHD), and iii) infectious complications. While GvHD can be eliminated by simple T-cell depletion of the graft, such a lymphocyte deficiency would predispose patients to cancer relapse and infections (Nikolich-Zugich, *et al* 2004, van Heijst, *et al* 2013). Prior to aHSCT, patients receive chemotherapy-based conditioning often including irradiation, and even if the PBSC graft is not T-cell depleted the reconstruction of the T-cell compartment in the recipient can take months or years and tends to remain incomplete (Fallen, *et al* 2003, Seggewiss and Einsele 2010, Storek, *et al* 2008). Once complete engraftment of the donor cells is achieved in absence of recipient hematopoiesis the transplanted donor T-cell repertoire is solely responsible for reconstruction of the T-cell compartment. Notwithstanding, a delayed reconstruction of the T-cell compartment and a restricted TCR repertoire diversity are associated with increased reactivation of latent viruses such as Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (Feuchtinger, *et al* 2005, Maury, *et al* 2001, Nikolich-Zugich, *et al* 2004). Therefore, strategies that improve the reconstruction of the T-cell diversity might reduce transplant associated mortality (Goldberg, *et al* 2007).

3.5 Purpose and aims

TCR repertoire pattern recognition by the standardized BIOMED-2 assay is nowadays the best practice for detection of clonal T-cell proliferation and thereby the standard diagnostics tool for lymphomas and mature leukemias. However, with the advent of HTS, analyses of the TCR repertoire in an unprecedented depth were enabled. Instead of overall TCR repertoire size-

patterns, heterogeneous mixtures of T cells can be sequenced in parallel facilitating the quantitative detection of individual TCR rearrangements simultaneously.

On the basis of this technical development, we intended to set up a method that allowed the analyses of T cells based on sequencing of their TCRβ rearrangements. Therefore, the herein presented thesis aimed i) the establishment and validation of a TCRβ multiplex approach, ii) the determination of T-cell repertoire diversity as well as iii) tracking of individual T-cell rearrangements in a clinical context. In particular, the impact of the donor derived T-cell repertoire diversity on the clinical outcome of the respective stem cell recipients after aHSCT was on special interest.

4. List of publications

The publications enclosed in this thesis and the therein performed contents are listed below.

4.1 Publication 1

Donor CD4 T-cell diversity determines virus reactivation in patients after HLA matched allogeneic stem cell transplantation

Ritter J., Seitz V., Balzer H., Gary R., Lenze D., Moi S., Pasemann S., Seegebarth A., Wurdack M., Hennig S., Gerbitz A., Hummel M.

Am J Transplant. Epub 2015 Apr 14.
The original article is online available at:
http://onlinelibrary.wiley.com/doi/10.1111/ajt.13241/pdf

4.1.1 Synopsis

The allogeneic transplantation of multipotent hematopoietic stem cells represents an effective immunotherapeutic treatment that can provide complete remission for patients suffering from hematological malignancies like leukemias. Prior to transplantation, the recipient's hematopoietic cells were destructed by chemotherapy and/or radiation. Therefore, when full donor chimerism in recipients is achieved, the donor hematopoiesis is the only source for immune cells after allogeneic hematopoietic stem cell transplantation (aHSCT) in the recipient. At present, peripheral blood stem cells (PBSC) that were mobilized via granulocyte colony-stimulating factor (G-CSF) are the predominant source of stem cells used for transplantations (Hopman and DiPersio 2014). However, beside relapse of the underlying malignancy, infectious complications compromise the success of aHSCT. It has been shown that a delayed reconstitution of the T-cell compartment after transplantation can be associated with an increased risk of reactivations of latent viruses (Epstein-Barr virus (EBV) and Cytomegalovirus (CMV)) (Feuchtinger, *et al* 2005, Nikolich-Zugich, *et al* 2004, van Heijst, *et al* 2013). Due to the fact that the transplanted T cells appear to be one of the major factors which affect the T-cell reconstitution in recipients, we hypothesized that the diversity of the donor T-cell repertoire determines the later reactivation of viruses and therefore the clinical outcome of the recipient. To test this hypothesis, we

investigated the diversity of donor derived T cells by analyzing their T-cell receptor beta (TCRβ) gene rearrangements via combined multiplex PCR and subsequent high throughput sequencing (HTS) of the generated PCR products. TCRβ rearrangements were amplified from flow cytometry sorted $CD4^+$ and $CD8^+$ T cells from the peripheral blood of 23 allogeneic stem cell donors before G-CSF mobilization and on the day of stem cell apheresis. After HTS of TCRβ amplicons we demonstrated that irrespective of G-CSF administration $CD4^+$ T cells revealed much higher receptor diversity in comparison to $CD8^+$ T cells. Moreover, we showed that the TCRβ diversity in $CD8^+$ T cells decreased with increasing donor age, an observation which was recently confirmed by Britanova and colleagues (Britanova, *et al* 2014). However, the most important finding was achieved by correlating the donor derived T-cell repertoires with the clinical outcome of the corresponding recipients. The results imply that a higher TCRβ diversity in $CD4^+$ T cells after G-CSF administration is associated with a lower reactivation rate of CMV and EBV. This finding could exclusively be observed for $CD4^+$ T cells after G-CSF treatment. Neither in CD8+ T cells irrespective of G-CSF administration nor in $CD4^+$ T cells before G-CSF mobilization a potentially protective affect could be verified. Taken together, our method for the analysis of TCRβ repertoires illustrated the importance of the composition of transplanted $CD4^+$ T cells for the control of latent viruses in recipients after aHSCT.

4.1.2 Contribution

I Conception	Design and optimization of simultaneous DNA and RNA extraction from sorted T cells	100%
	Development and optimization of a two-step TCRβ multiplex PCR for HTS	100%
	Workflow design of bioinformatics analysis	50%
II Execution	Assembling of T cell data for bioinformatics pipeline (data for V-segment assignment etc.)	100%
	Workflow optimization and pipeline output verification	75%
	Processing of samples for HTS analysis	80%
	Analysis of generated T-cell data	100%

III Reporting	Statistical analysis and figure preparation	95%
	Manuscript preparation and data interpretation	75%

4.2 Publication 2

Abnormally differentiated CD4$^+$ or CD8$^+$ T cells with phenotypic and genetic features of double negative T cells in human Fas deficiency

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4.2.1 Synopsis

The autoimmune lymphoproliferative syndrome (ALPS) is characterized by a defective regulation of lymphocyte homeostasis caused by abnormalities in apoptosis. This disorder results in lymphadenopathy, splenomegaly, as well as increased risks for lymphomas or autoimmune diseases (Fisher, *et al* 1995). Clinical manifestation includes expansion of double negative T cells (DNTs) developing alpha/beta T-cell receptors but lost the expression of CD4 and CD8 on the cell surface. In two thirds of ALPS patients, a genetic defect in the *FAS* gene has been identified (Fisher, *et al* 1995, Straus, *et al* 2001). Members of the FAS protein family are involved in lymphocyte apoptosis triggering a cascade that results into cell death (Lenardo, *et al* 1999). ALPS as a disease and the accompanied relevance of the FAS pathway were first characterized in the early 90s (Sneller, *et al* 1992). Since then enormous progress of diagnosis and treatment of this syndrome have been made, however, many aspects of this disease still remain unknown and at this time the only curative therapy for ALPS patients is the transplantation of hematopoietic stem cells (Shah, *et al* 2014).

Given that DNTs are progenitor cells of mature differentiated CD4$^+$ and CD8$^+$ T cells, we herein addressed the question if i) the mutation in the *FAS* gene might alter the differentiation of T cells at an early stage or if ii) single positive T cells (SPTs) that are destined for FAS-mediated apoptosis downregulate their co-receptors at a single differentiation state and become double-

negative again. To contribute to the clarification of this question, sequencing of TCRβ repertoires in DNTs as well as in $CD4^+$ and $CD8^+$ T-cell compartments of one ALPS patient were performed to figure the role of *FAS* gene mutation in the human T-cell differentiation and DNT ontogeny out. Flow cytrometric sorting and genetic analyses (which were performed in the collaborating laboratories) demonstrated similar phenotypic and transcriptional characteristics between ALPS DNTs and terminally differentiated effector memory T cells (TEMRA) that occur among $CD4^+$ and $CD8^+$ T cells. This provides for the first time that human DNTs can also differentiate from $CD4^+$ T cells. So far, current studies indicate that *FAS* mutant DNTs originate from chronically activated $CD8^+$ T cells only (Bristeau-Leprince, *et al* 2008, Giese and Davidson 1995). The finding that DNTs might also arise from $CD4^+$ T cells was further confirmed by the HTS analysis of the individual TCRβ rearrangements. Thereby, significant fractions of identical CDR3 TCRβ sequences were not only detected in the DNTs and $CD8^+$ TEMRA cells of an ALPS patient, also in the $CD4^+$ TEMRA cells and DNTs of the same patient a distinct overlap of TCRβ rearrangements could be shown. Despite the slightly higher overlap of CDR3 TCRβ sequences between $CD4^+$ T cells and DNTs, the relative contribution of $CD4^+$ vs $CD8^+$ T cells to DNTs remains unknown and might vary among patients. Taken together, the employment of our HTS approach for TCRβ analysis supported the finding that *FAS* mutated DNTs can also originate from $CD4^+$ T cells in ALPS patients. Furthermore, the findings of the genetic and sequencing analysis provide novel evidence for a role of FAS in early stages at T-cell differentiation.

4.2.2 Contribution

I Conception	Design and optimization of DNA extraction for limited numbers of T cells	100%
	Concept preparation for TEMRA cell sorting/analysis	60%
II Execution	Processing of samples for HTS analysis	100%
	Sequencing of TCRβ libraries (MiSeq)	100%
	Analysis and interpretation of T-cell data	90%
III Reporting	Preparation of "material and methods" as well as "results" for T-cell analysis (including figure preparation)	100%

5. Discussion

The detection of clonality by analysis of the T-cell receptor (TCR) gene rearrangements has become an important and adjuvant tool in the clinical diagnostics of lymphoproliferations. The rearrangements of the V(D)J genes of the TCRs are generated at early stage of T-cell development. During the underlying rearrangement process in which additional random non-templated nucleotides are inserted at recombination junctions, more than 2.5 x 10^7 different α/β TCRs could be generated (Arstila, *et al* 1999, Dik, *et al* 2005, Langerak, *et al* 2012). As a consequence of this highly diverse repertoire, each T cell displays an individual T-cell receptor molecule and the chance that two different lymphocytes with identical TCRβ rearrangements develop coincidentally is highly unlikely. However, since hematological malignancies such as lymphomas or mature leukemias are clonal diseases that derive from the uncontrolled proliferation of a single transformed lymphoid cell, all resultant tumor cells carry therefore the identical TCR rearrangement. Initially, for the detection of such clonal lymphocyte populations Southern blot analysis was employed. Since this technique is very time consuming and requires large amounts of high-quality DNA, it was rapidly replaced when PCR-based detection systems were established. The first PCR-based analyses focused due to its relative simple gene structure on the amplification of the TCRγ (Arber, *et al* 2001, Trainor, *et al* 1991). This restriction in clonality analysis to TCRγ resulted in the lack to accurately recognize all possible clonal T-cell populations thus bearing the risk of causing false negative results (Langerak, *et al* 2007). Therefore, at the end of the 90s, the European BIOMED-2 network started with the redesign and optimization of PCR primers and protocols for T-cell as well as B-cell clonality assessment and nowadays the standardized multiplex PCR protocols of this consortium have become the gold standard for clonality analysis (Langerak, *et al* 2012, van Dongen, *et al* 2003). For the distinction between monoclonal lymphocyte populations carrying identical TCR rearrangements and polyclonal cell compositions with highly diverse TCR sequences, fluorescence-labeled amplicons generated by the BIOMED-2 protocols were separated by high-resolution capillary electrophoresis (GeneScan) based on their length. However, the information provided by TCR size distribution is limited. This is caused by the simple size discrimination of amplicons, whereby different gene rearrangements with identical hypervariable complementarity-determining region 3 (CDR3) sequence sizes are summed up and are presented as one peak in the

electropherogram of the GeneScan analysis. Therefore, no sequence information is given by this approach and direct sequencing of clonal TCR rearrangements is only possible in the case of pronounced dominancy of a respective PCR product without significant polyclonal background derived from additional present polyclonal T cells. However, by the application of the advanced high-throughput sequencing (HTS) it is now possible to sequence very heterogeneous mixtures of TCR rearrangements derived from multiplex TCR PCR including the detection of all individual CDR3 sequences in a quantitative manner. Due to this enormous technical progress, several strategies were developed in the recent years by combining TCR multiplex PCR and HTS to study TCR repertoires (Freeman, *et al* 2009, Robins, *et al* 2009, Wang, *et al* 2010). Following this development, we established an optimized procedure for the analysis of T-cell repertoires and applied this approach to unravel the T-cell diversity in healthy and diseased conditions. In addition to the development and optimization of a multiplex TCRβ PCR, this includes the establishment of the entire HTS procedure using Illumina HiSeq and MiSeq technology as well as the co-development of the bioinformatics analysis of the generated data. This very complex approach was intensively validated (see 5.1) before applying it to patient samples (see 5.2 and 5.3).

5.1 Validation and interpretation of TCRβ HTS analysis

Validation of the combined TCRβ multiplex PCR and HTS assay was performed with flow cytometry sorted $CD4^+$ and $CD8^+$ T cells isolated from the peripheral blood of six healthy control donors as described in the first publication (Ritter, *et al* 2015). After amplifying the TCRβ repertoire by multiplex PCR and sequencing the amplicons on the Illumina HiSeq platform we were able to demonstrate a Gaussian-like distribution of the CDR3 length of all six individuals, which is comparable to the CDR3 pattern generated by conventional capillary electrophoresis (GeneScan) (van Dongen, *et al* 2003). This indicates that the T-cell receptor pattern could be replicated by our HTS approach.

Detailed analysis of the TCRβ sequences in our control samples (approximately 4.5 million reads per sample) revealed a usage of the Vβ and Jβ gene segments that was consistent with published findings (Robins, *et al* 2010). Analyzing the number of different clonotypes (number of sequence cluster with identical TCRβ rearrangement) which were amplified from 100 ng DNA as starting material, the $CD4^+$ T-cell compartment displayed higher numbers of

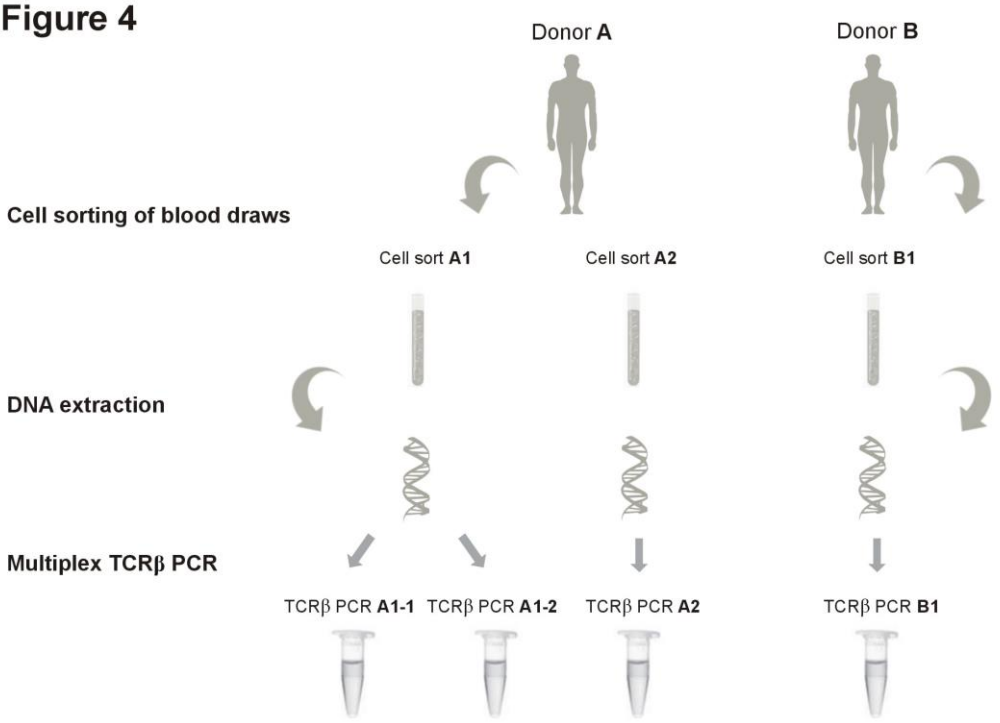
different clonotypes in comparison to $CD8^+$ T cells. In line with this, our analysis identified more pronounced clonotypes in $CD8^+$ T cells. As a consequence, the analyzed $CD4^+$ T cell fractions showed an overall higher T-cell diversity as compared to the $CD8^+$ T cells. This observation is confirmed by recently published data and support the reliability of our approach (van Heijst, *et al* 2013).

To get a much deeper insight into the TCR composition generated by HTS we performed a comparison of TCR repertoires of i) different DNA aliquots from the same DNA preparation of the identical T-cell sort of one donor, ii) different T-cell sorts of one individual at the same time point, and iii) sorted T cells of different donors (**Figure 4**). By comparing the two DNA aliquots of the same $CD4^+$ T cell sort of one individual we found that 4.1% of the TCRβ CDR3 sequences were identical. The percentage of overlapping CDR3 sequences was slightly lower (3.2%) when the TCRβ CDR3 input DNA derived from two different $CD4^+$ T-cell sorts of one individual. As a consequence of the lower T-cell diversity in the $CD8^+$ T-cell compartment, the percentage of overlapping TCRβ CDR3 sequences was respectively higher leading to 13.7% and 9.2% of identical TCRβ in the two DNA aliquots and both T-cell sorts of the same individual. On the contrary, almost no overlap between the TCRβ rearrangements of the $CD4^+$ or $CD8^+$ T cells of different individuals (0.03% and 0.19%) could be found after bioinformatics analysis of the HTS generated data.

The limited overlap of the TCRβ sequences from the same individual and/or even the same DNA preparation might be unexpected at first sight, however, considering the enormous T-cell diversity in healthy individuals this low re-occurrence of identical TCRβ rearrangements in the analyzed fractions is comprehensible. Since 100 ng of input DNA were used for each TCRβ HTS analyses and taken into account that each single lymphocyte contains approximately 7 pg of DNA, the maximum diversity of the generated output cannot exceed approximately 14.500 individual TCRβ sequences. Thus, sequencing very small fractions of the entire T-cell repertoire of healthy individuals that consist of approximately 1.5×10^{12} T cells, only those T-cell rearrangements occurring at higher frequencies are identifiable in two independent analyses. This is the reason why only minor overlaps of identical TCRβ sequences were presented in aliquots of the same DNA preparation. In accordance, the identified overlap in $CD4^+$ T cells is lower between two DNA aliquots or T-cell sorts of the identical individual as compared to the $CD8^+$ T-cell compartment.

Despite the fact of using only small fractions of the entire T-cell system, our data clearly indicate that this TCRβ HTS approach is representative enough to reflect the T-cell repertoire in individuals and enables an estimation of the given T-cell diversity.

Figure 4



Design of TCRβ HTS validation analysis for comparison of the re-occurrence of identical TCRβ rearrangements. The overlap of identical TCRβ sequences in different aliquots from the same T-cell sort (TCRβ PCR A1-1 and TCRβ PCR A1-2) was nearly identical as the overlap of rearrangements in the analyses of two different T-cell sorts of one individual at the same time point (TCRβ PCR A1-1 and TCRβ PCR A2). Due to the higher diversity in the $CD4^+$ T-cell compartment, higher overlaps could be observed in $CD8^+$ T cells. In contrast, almost no overlap could be verified between the T-cell repertoires of two different individual (donor A and donor B).

5.2 T-cell receptor diversity and stem cell transplantation

The transplantation of allogeneic hematopoietic stem cells represents a potential curative treatment for patients suffering from hematological malignancies. To this end, the hematopoietic cells of the recipients were eradicated prior to stem cell transplantation using chemotherapy and/or radiation. In consequence, the transferred hematopoietic cells of the donor represent the only source for immune cells after full donor chimerism in recipients is achieved. Granulocyte colony-stimulating factor (G-CSF) mobilized peripheral blood stem cells (PBSCs) have currently replaced bone marrow as the major graft source (Hopman and DiPersio 2014). The success of allogeneic hematopoietic stem cell transplantation (aHSCT) is however compromised by relapse of the primary malignancy and infectious complications.

There is evidence that a delayed reconstitution of T cells after aHSCT is associated with an increased risk for reactivations of latent viruses such as Cytomegalovirus (CMV) and Epstein-Barr virus (EBV) (Feuchtinger, *et al* 2005, Nikolich-Zugich, *et al* 2004, van Heijst, *et al* 2013). Since the composition of the transplanted donor-derived T cells appears to be an important factor for successful T-cell repertoire reconstitution in the recipient, we wanted to elucidate the impact of the transplanted T-cell diversity for the clinical outcome of the recipient. For this purpose, the T-cell diversity of flow cytometric sorted $CD4^+$ and $CD8^+$ T cells of 23 allogeneic stem cell donors before G-CSF administration and on the day of stem cell apheresis were monitored by our TCRβ HTS approach. The analyses of the $CD4^+$ and $CD8^+$ T-cell compartments confirmed the higher TCRβ diversity in $CD4^+$ T cells that was already observed in our validation experiments (see 5.1). However, the overall TCRβ diversity of the $CD4^+$ and $CD8^+$ T-cell compartments was not affected by the G-CSF administration. In line, no significant changes neither in the usage of the Vβ and Jβ segments nor in the CDR3 length distribution of the analyzed TCRβ sequences of the $CD4^+$ and $CD8^+$ T-cell fractions could be demonstrated under G-CSF mobilization. Despite the G-CSF induced increase of overall numbers of T-cells in the stem cell graft, no correlating increase of T-cell diversity was observed in the analyzed T-cell fractions on the day of apheresis. In addition, we were able to elucidate that the TCRβ repertoire diversity in the donor derived $CD8^+$ T cells is reduced by increased donor age which was also significant irrespective of G-SCF administration. The finding of a restricted T-cell repertoire in elderly patients is supported by recently published HTS data showing that reduced TCRβ repertoire diversity in the peripheral blood is age-related and already significantly reduced in healthy donors at the age of 40

(Britanova, *et al* 2014). The age-related decrease of T-cell diversity is broadly accepted as it was monitored before via oligonucleotide hybridization assays and spectratyping analysis (Kilpatrick, *et al* 2008, Naylor, *et al* 2005). In contrast to the recently published HTS data by Britanova and colleagues, the previous studies that were based on methods with lower resolution suggested that a decrease in T-cell diversity will not occur before a patient reaches the age of 75 (Britanova, *et al* 2014, Naylor, *et al* 2005, Pfister, *et al* 2006).

To unravel the impact of the transplanted T-cell repertoire of the donor for the clinical outcome of the stem cell recipient, we compared the diversity of TCRβ repertoires of $CD4^+$ and $CD8^+$ T cells of 23 donors after G-CSF administration with various clinical outcomes of the corresponding recipients. Thereby, no statistically significant correlations were detectable comparing the incidence of acute graft versus host disease (aGvHD), relapse rates or survival of the recipients with the donor-derived $CD4^+$ or $CD8^+$ T-cell diversity. However, the correlation of the donor T-cell repertoire with reactivation of latent viruses in recipients revealed a highly interesting observation: a lower diversity in the $CD4^+$ T-cell compartment of the donor after G-CSF administration was related to the reactivation of CMV and/or EBV in the respective recipient. Whereas it remains debatable to which extend the observed correlation between the higher EBV reactivation in recipients and the restricted donor-derived $CD4^+$ T-cell diversity might be a side effect of the concurrent CMV reactivation. Notwithstanding, our findings of higher virus reactivation rates in recipients that receive a restricted T-cell repertoire during transplantation is complemented by the work of van Heijst and colleagues that described also the association of CMV or EBV infection with lower TCRβ repertoire diversity (van Heijst, *et al* 2013). In contrast, their HTS data comprise only the T-cell repertoire of the recipients and do not cover the corresponding donors. Nevertheless, since a delayed reconstitution of T cells after aHSCT is known to be associated with a significant increase for reactivations of latent viruses, we identified for the first time the importance of the donor-derived $CD4^+$ T-cell diversity for the control of latent viruses after aHSCT (Feuchtinger, *et al* 2005, Nikolich-Zugich, *et al* 2004, Ritter, *et al* 2015).

5.3 Tracking of T-cell receptor beta sequences

Repertoire analyses of TCRβ rearrangements and the accompanied elevation of T-cell diversity represents only one possible application of the herein presented approach. In addition, sequencing

of TCRβ motives enables the identification of already published "public" clonotypes and/or the tracking of individual T-cell rearrangements. The tracking of individual clonotypes is based on the fact that the TCRβ CDR3 sequence of a T cell is highly unique due to the individual rearrangement in the VDJ recombination process. This unique immune receptor nucleotide sequence can therefore be seen as the fingerprint of the T cell, which represents a molecular tag of a T-cell clone (Robins 2013). By using these molecular tags the development of individual T cells can be tracked over time, between different tissues as well as in different cell compositions (DeWitt, *et al* 2015, Dziubianau, *et al* 2013, Morris, *et al* 2015).

One of the first clinical applications that benefits from clonotype tracking was the detection of minimal residual disease (MRD) in patients affected with leukemias (Logan, *et al* 2011, Wu, *et al* 2012). Since mature T-cell leukemias derive from the uncontrolled proliferation of a single transformed lymphoid cell, all corresponding malignant cells bear the identical TCRβ rearrangement as the progenitor cell. After identification of the unique TCRβ gene rearrangement sequence in a diagnostic bone marrow or blood sample of a patient, this malignant clonotype rearrangement can be used as a molecular tag for cancer cells enabling the tracking of residual malignant low frequent T cells after treatment. HTS of TCRβ sequences was shown to detect residual leukemic cells with high accuracy and sensitivity of up to one cancer cell in 10^6 non-malignant cells (Logan, *et al* 2014). Nevertheless, the tracking of T-cell clonotypes is not limited to one sequence at once – moreover, it is possible to track multiple clones simultaneously by sequencing the complete TCRβ repertoire of samples. By application of this simultaneous clonotype tracking, we herein analyzed T cells of ALPS patients to receive a better impression on the effect of *FAS* gene mutation in the human T-cell differentiation and DNT ontogeny (Rensing-Ehl, *et al* 2014).

ALPS patients suffer from a defective regulation of lymphocyte homeostasis that is caused by abnormalities in apoptosis and two thirds of diagnosed ALPS patients harbor a genetic defect in the lymphocyte apoptosis regulating *FAS* gene (Fisher, *et al* 1995, Straus, *et al* 2001). In addition, ALPS patients exhibit expansion of CD4 and CD8 double negative T cells (DNTs) but carry an alpha/beta T-cell receptor on their cell surface. Since DNTs are also progenitor cells of mature differentiated $CD4^+$ and $CD8^+$ T cells, we addressed the question by analyzing the TCRβ repertoire in different T-cell subsets if i) the mutation in the *FAS* gene might alter the differentiation of T cells at an early stage or if ii) single positive T cells (SPTs; $CD4^+$ or $CD8^+$ T

cells) triggered for FAS-mediated apoptosis are able to downregulate their co-receptors at a single differentiation state and become double-negative again. To this end, the TCRβ repertoires in DNTs as well as in $CD4^+$ and $CD8^+$ terminally differentiated effector memory T cells (TEMRA) of one ALPS patient were investigated by our HTS approach. Simultaneous tracking of TCRβ sequences that originate from DNTs revealed identical T-cell clones in $CD8^+$ TEMRA cells. This finding is in perfect harmony with previous studies, which postulate that *FAS* mutant DNTs originate from chronically activated $CD8^+$ T cells (Bristeau-Leprince, *et al* 2008, Giese and Davidson 1995). Moreover, we could demonstrate that DNT derived TCRβ rearrangements were also present in $CD4^+$ TEMRA cells. Thereby, simultaneous tracking of multiple clonotypes was employed to provide for the first time the evidence that DNTs in patients bearing a *FAS* mutation can also differentiate from $CD4^+$ T cells and not from $CD8^+$ T cells only.

5.4 Conclusion

The availability of high throughput sequencing (HTS) has revolutionized the methodical spectrum to unravel the composition of the immune system by enabling simultaneous detection of multiple individual T-cell receptor (TCR) rearrangements in heterogeneous lymphocyte populations in a quantitative manner. To date, the applications for this approach can be assigned into three major categories: i) detection of T-cell clonality, ii) assessment of T-cell diversity, and iii) tracking of individual and/or public TCR clonotypes. Following this classification two of these analyses types were applied in the herein presented thesis: the T-cell diversity in stem cell donors was assessed to determine the importance of the donor derived TCRβ repertoire in autologous hematopoietic stem cell transplantation (aHSCT) and the tracking of individual clonotypes was performed to elucidate the origin of double negative T cells (DNTs) in patients with autoimmune lymphoproliferative syndroms (ALPS). Thereby, we were able to identify the decisive role of the diversity of the donor derived TCRβ repertoire for the success of transplantation regarding the reactivation of viruses in the corresponding recipients (Ritter, *et al* 2015). Furthermore, tracking of individual clonotypes in ALPS patients indicated for the first time that DNTs can also originate from $CD4^+$ T cells (Rensing-Ehl, *et al* 2014). Beyond the work of this thesis, additional applications for the detailed analysis of T-cell repertoires by HTS are the identification as well as the usage of virus specific TCRs preventing re-activation of latent virus infections, which might increase treatment success in aHSCT patients. Moreover, in oncology,

the detection of tumor-infiltrating T cells in patients suffering from cancer will help to support targeted tumor therapies. However, to provide comparable studies and to accomplish the clinical implementation of TCR HTS analysis standardized protocols have to be established (Georgiou, *et al* 2014, van Dongen, *et al* 2015).

Next steps in TCR analyses need to include HTS of both, the heterodimeric heavy and light (alpha (α) and beta (β)) receptor chains simultaneously, which is of great importance for functional studies. To date, the proof of concept for the combined TCRα/β sequencing was provided by single cell emulsion PCR where amplification products of both chains were linked with a chimeric primer through bridge PCR (Turchaninova, *et al* 2013). This strategy is currently low-throughput but has the potential for high-throughput application. Plenty of creative applications are being on their way, therefore, feasible solutions are expected in the near future to solve this problem (Robins 2013).

HTS techniques and applications have grown rapidly and certainly have not yet reached their limits. With the essential establishment of this method in routine diagnostics, this technique offers the potential to drive the personalized medicine a major step forward in a short time frame.

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7. Appendix

7.1 Abbrevations

aHSCT	allogeneic hematopoietic stem cell transplantation
ALPS	autoimmune lymphoproliferative syndrome
APC	antigen-presenting cell
BMT	bone marrow transplantation
CD	cluster of differentiation
cDNA	complementary desoxyribonucleic acid
CDR3	hypervariable complementarity-determining region 3
CMV	Cytomegalovirus
D gene segment	diversity gene segment of the T-cell receptor
DNT	double negative T cell
EBV	Epstein-Barr virus
G-CSF	granulocyte colony-stimulating factor
gDNA	genomic desoxyribonucleic acid
GvHD	graft versus host disease
HLA	human leukocyte antigen
HTS	high-throughput sequencing
J gene segment	joining gene segment of the T-cell receptor
MHC	major histocompatibility complex
MRD	minimal residual disease
PAMP	pathogen-associated molecular pattern
PBSC	peripheral blood stem cell

PCR	polymerase chain reaction
PRR	pattern-recognition receptor
SPT	single positive T cell
TCR	T-cell receptor
TEMRA	terminally differentiated effector memory T cell
V gene segment	variable gene segment of the T-cell receptor

7.2 Curriculum Vitae

For reasons of privacy protection, personal data are not included in the electronic version of the thesis.

Publications

Ritter J., Seitz V., Balzer H., Gary R., Lenze D., Moi S., Pasemann S., Seegebarth A., Wurdack M., Hennig S., Gerbitz A., Hummel M.

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*T-cell receptor deep sequencing analysis of EBV specific T-cells before and after adoptive transfer in a patient after allogeneic stem cell******

European Society for Blood and Marrow Transplantation (EBMT) 2014; Istanbul, Turkey

*****Awarded as best science poster by the nature publishing group poster award

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T-cell spectrum analysis in celiac disease patients: a deep sequencing approach

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Deutsche Gesellschaft für Hämatologie und Medizinische Onkologie (DGHO) 2012; Stuttgart, Germany

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7.4 Selbständigkeitserklärung

Hiermit erkläre ich, dass ich die vorgelegte Arbeit selbst verfasst und keine weiteren als die aufgeführten Quellen sowie Hilfsmittel in Anspruch genommen habe. Die Dissertation wurde in dieser oder anderer Form keiner anderen Prüfungsbehörde vorgelegt.

Berlin, Juli 2015 Julia-Marie Ritter