T cell receptor next-generation sequencing reveals cancer-associated repertoire metrics and reconstitution after chemotherapy in patients with hematological and solid tumors

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ABSTRACT
The dynamics of immunoaging and the onset of immunoparesis in healthy individuals and cancer patients has been controversially discussed. Moreover, the role of chemotherapy on T cell regeneration needs further elucidation in light of novel immunotherapies that have become standard of care for many elderly cancer patients. We used next-generation immunosequencing to study T cell receptor (TCR) repertoire metrics on 346 blood samples from healthy individuals and cancer patients producing a dataset with around 8.8 million TCR reads. This analysis showed that decline of T cell diversity and increase in T cell clonality is a continuous process beginning in healthy individuals over 40 years of age. Untreated patients with both hematological and solid tumors showed blood TCR repertoires with significantly lower diversity and higher clonality as compared to healthy individuals across all decades. Loss in T cell diversity was essentially driven by a loss in richness in aging healthy individuals, while in cancer patients a loss in repertoire evenness was an additional contributing factor. Interestingly, chemotherapy did not impair the regeneration of blood TCR repertoire diversity to pre-treatment age-specific levels. Surprisingly, even patients over the age of 70 years receiving highly T cell toxic therapies reestablished their pre-treatment T cell diversity suggesting rebound thymic activity rather than recovery of T cell counts by peripheral expansion only. Taken together, these data suggest that human TCR repertoire metrics gradually deteriorate in the aging individual, but age-specific TCR metrics are restored after T cell depleting therapy even in elderly cancer patients.

Introduction
A decrease in immune function over time is thought to explain increasing rates of infection, autoimmunity and cancer with age. T cells are traditionally connected with this concept, since the thymus that generates the naïve T cell repertoire from bone marrow derived hematopoietic stem cells undergoes a visible decline from childhood to adulthood. This thymic involution is associated with a disorganization of the thymic tissue architecture, a decrease of the thymic stroma and its replacement by adipose tissue. Due to this visible, age-dependent decline, thymus-dependent T cell recovery has been assumed to be severely limited in adults. However, age-associated T cell immunoparesis is not only restricted to thymic involution since it also involves aging of hematopoietic stem cells, lymphoid progenitors and mature lymphocytes in secondary lymphoid organs.

T cell reconstitution is a critical feature of the recovery of the adaptive immune response and has two main components: thymic output of new T cells and peripheral homeostatic expansion of preexisting T cells. To explore immune repertoire metrics over time, extrapolations from animal models are not useful since – despite many immunological principles being shared across species – the size of the T cell system and the lifespan of typical animal models are fundamentally different from the human system. Studies on immunoaging and immune reconstitution in humans gave inconsistent results partly due to the use of methods that should be interpreted with caution, such as T cell receptor excision circle (TREC) measurements. In 2014, a next-generation sequencing (NGS) study by Britanova et al. has analyzed an extensive set of 39 healthy individuals setting the benchmark for studies on age-dependent TCR repertoire immunodynamics. As opposed to previous studies using less precise technologies, this group found a linear decrease of T cell diversity over time with significant reduction already apparent at age 40.

Unlike in the healthy population, it is still a matter of debate if cancer patients are able to restore a functional TCR repertoire after cytotoxic and notably often T cell toxic
therapy. In this comprehensive analysis of 346 TCR repertoires we found clear evidence for premature immunooaging in cancer patients independent of their treatment. To our surprise, however, we found that even elderly patients undergoing T cell toxic therapies largely reconstituted their age-specific TCR repertoire arguing strongly in favor of the hypothesis that thymic output may be reactivated driven by treatment induced lymphopenia.

Methods

Study approval

Informed consent was obtained from all patients and healthy donors (HD) for the use of their peripheral blood (PB) as approved by the ethics commission Hamburg (Ethikkommission der Ärztekammer Hamburg, Germany, project number PV4767). The study has been performed in accordance with the declaration of Helsinki of 1975.

Patients and samples

We included a cohort of 218 cancer patients, 94 of which with hematological cancers (hemC), 124 with solid cancers (solC) and 95 healthy donors (HD) as a reference cohort. In all investigated age groups (≤30, 31 to 40, 41 to 50, 51 to 60, 61 to 70 and ≥70) we included a minimum of 3 individuals. The analyses presented in Figures 5 and 6 include two different subcohorts: (i) matched patients according to age and tumor type who were untreated or received chemotherapy (labeled as „samples from different age-matched patients“). (ii) patients who had paired samples taken prior to and after chemotherapy (labeled as „paired samples from pre-post chemotherapy“). Patients with blood involvement by their disease were excluded from the analysis. Information on patient and sample characteristics are summarized in Supplementary Table 1.

Multiplex PCR of T cell receptor beta (TRB) repertoire for Illumina targeted next-generation sequencing (NGS)

The rearranged TRB receptor sequence containing the entire V, D and J gene segments was amplified using a mixed primer TRBA/B pool and a touch-down PCR protocol from peripheral blood (PB) genomic DNA. In two consecutive PCR reactions, amplicons were tagged with Illumina adapters and indices as previously described. PCR reactions were performed using Phusion HS II (Thermo Fisher Scientific Inc., Germany). Amplicons were purified after agarose gel electrophoresis using the NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Germany). Before being subjected to NGS, the concentration and quality of the amplicons/libraries was determined using Qubit (QIAGEN, Germany) and Agilent 2100 Bioanalyzer (Agilent technologies, Germany), respectively.

Illumina next-generation sequencing (NGS)

NGS and de-multiplexing was performed on an Illumina MiSeq sequencer (600-cycle, single indexed, paired-end run). Analysis of the TRB locus was computed using the MiXCR analysis tool V2.12 and V3.0.5. Each unique complementarity-
determining region 3 (CDR3) nucleotide sequence was defined as one clone and all clones of one sample/timepoint were defined as one TCR repertoire. Only productive sequences with a read count $\geq 2$ were included in the analysis.

Calculation of repertoire metrics

For this work, a number of indices reflecting broad repertoire metrics were used:

The Shannon index ($H$) is a commonly used measure of diversity,\(^{18}\) which can be calculated as follows:

$$H = \sum_{i=1}^{S} p_i \log_2 p_i$$

(1)

where $S$ is the number of species/clone (richness) and $p_i$ is the proportion of each clone within the repertoire. $p_i = n_i/N$, $n_i$ = read count of each individual clone and $N$ = the sum of all reads in the repertoire.

Since the Shannon index is a diversity measure that weighs small and large clones relatively evenly, we plotted diversity curves with alpha-modulated sensitivity for the relatively rare clones (alpha-parameterized diversity.\(^{19}\) As $\alpha$ increases, high frequency clones are weighed more. We generated diversity profile curves for $\alpha = 0$ to $\alpha = 5$, in steps of 0.2 using the R script kindly provided by Dr. V. Greiff of University of Oslo, Institute of Clinical Medicine.

Evenness is calculated from $H$ and $H_{\text{max}}$ (evenness = $\frac{H}{H_{\text{max}}}$) with $H_{\text{max}}$ being the maximal possible value of $H$, if every clone in the repertoire was present at the same frequency.

Figure 2. Age-dependent TCR repertoire clonality in healthy individuals and patients with cancer. Clonality index with mean±SD of PB TCR repertoire of HD, solC and hemC patients, plotted according to age group (a). Mean clonal space distribution of PB TCR repertoire in HD, solC patients and hemC patients per age group (b). Clone sizes are defined as hyperexpanded (0.01 < $x$ ≤ 1), large (0.001 < $x$ ≤ 0.01), medium (1E-04 < $x$ ≤ 0.001), small (1E-05 < $x$ ≤ 1E-04). Number of individuals per age group: HD/solC/hemC, ≤30: 17/5/9, 31–40: 14/8/3, 41–50: 15/14/8, 51–60: 16/28/15, 61–70: 16/23/4, ≥70: 17/29/37. Statistical test: unpaired, two-sided t-test between two subgroups, Pearson correlation between age and clonality index.
The clonality index is the reciprocal value of evenness (clonality = 1 – evenness). It assumes a value between 1 and 0, with 1 being a repertoire consisting of only one clone and 0 being a repertoire of maximal evenness.

**Generation probability calculation**

We investigated the generation probability of the T cell clones in our paired patient samples before and after chemotherapy treatment using the IGoR algorithm. All values are depicted on a square root scale for plotting purposes. T cell clones with low generation probability are presumed to be peripherally expanded by antigen pressure, whereas T cell clones exhibiting a high generation probability accumulate by random V(D) J recombination. Therefore, a T cell repertoire, which has seen antigens over a lifetime is expected to have a lower mean generation probability compared to a newly formed T cell repertoire (e.g. after lymphodepleting chemotherapy).

**Data analysis**

Analyses were carried out and data plotting was performed using R (version 3.4.4) and the package tcR as well as GraphPad Prism 7 (San Diego, CA). A P value of <0.05 was considered statistically significant.

**Data availability**

The datasets generated for this study can be found in the European Nucleotide Archive (ENA). ID: PRJEB33490
Results

Broad immune metrics in healthy individuals vs. cancer patients

A cohort of 85 untreated patients with hematological cancers (hemC) and 108 patients with solid tumors (solC) was subjected to peripheral blood T cell repertoire profiling by NGS and their T cell spaces were compared to 95 healthy individuals (HD). To account for differences in the immune repertoire due to age differences, we compared subcohorts of patients (hemC and solC, respectively) to age-matched subcohorts of HDs. Untreated patients with hematological (but non-T-cell) cancers showed significantly higher clonality and lower blood T cell diversity ($p < .0001$) compared to HDs (Figure 1a,c). This was expected since these patients had infiltration of primary and/or secondary lymphoid organs potentially impairing emigration of hematopoietic precursors to the thymus and/or peripheral T cell expansion. Yet, also...
untreated patients with solid malignancies showed significa-
cantly more clonal ($p = .0067$) and less diverse ($p < .0001$) repertoires compared to their respective age-matched sub-
group of HDs (Figure 1b,d). Taken together, HDs showed
more diverse and more evenly distributed peripheral blood
TCR repertoires as compared to patients with hematological
or solid cancers.

**Age-dependent immune metrics in healthy individuals
and cancer patients**

Patients and HDs were binned in to age groups spanning ten
years ranging from below 30 to over 70 years of age. As rough
estimates of age-dependent repertoire metrics, T cell clonality
and diversity were calculated (Figures 2a and 3a). In patients
with cancer, the increase in repertoire clonality started at
earlier age groups as compared to HDs (Figure 2a). Mean
clonal space distributions showed that in elderly HDs up to an
age of 60 years, small clones still make up approximately 15%
of the T cell space while the repertoire fraction of small T cell
clones is neglectable in cancer patients over 30 years of age
(Figure 2b). In line with previously published data, healthy
donors showed a clear contraction of their T cell repertoires
with increasing age as indicated by the decreased diversity,
beginning approximately beyond age 40 (correlation coeffi-
cient $R^2 = 0.27$, $p < .0001$, Figure 3a,b). For patients with solid
or hematological malignancies a similar age-dependent
decline of T cell repertoire diversity was observed, however,
at much steeper slope (correlation coefficient solid cancer
$R^2 = 0.08$, $p = .003$, hematological cancer $R^2 = 0.13$,
$p < .0001$, Figure 3a,b). We dissected the single parameters
of diversity (richness and evenness) to better understand how
they account for the decline in diversity over age (Figure 4).
The decreased diversity in healthy aging appeared to be most
explained by a loss of richness since the repertoires stayed
relatively even over time (Figure 4a,b). In the aging cancer
patient, however, both losses in richness and evenness con-
tributed to the loss of T cell diversity (Figure 4c,d).
Reconstitution of age-specific T cell repertoire metrics after chemotherapy

Next, we investigated T cell regenerative potential after chemotherapy in patients with cancer and a median age of 60 years. In these patients, disease- and age-specific T cell repertoire differences were already discernible before treatment initiation. We investigated a cohort of cancer patients (predominantly with solid tumors) after various types of chemotherapy and compared them with a control cohort of disease- and age-matched untreated cancer patients (Figures 5a and 6a). Interestingly, there were no significant differences between treated and untreated patients in terms of T cell repertoire diversity or clonality (Figures 5a and 6a). In addition, we wished to assess repertoire changes through chemotherapy in individual patients (paired subcohort) over time. This analysis – carried out in patients with chronic lymphocytic leukemia (CLL) receiving fludarabine- or bendamustine-based therapy and in brain tumor patients receiving temozolomide-based therapy – suggested that the T cell repertoire largely recovered to the age-specific pre-treatment diversity/clonality, even in elderly patients undergoing T cell toxic treatment (Figures 5b and 6b). Only in the fludarabine-treated cohort of hematological patients with CLL a trend towards higher clonality after treatment was recognizable (Figure 6b), but diversity measures remained stable (Figure 5b). Taking together both cohorts, no drastic changes in diversity (Figure 5c,d) or clonality (Figure 6c) were apparent after chemotherapy.

Rebound thymic activity rather than peripheral T cell expansion leads to reconstitution of the T cell repertoire after chemotherapy

Reconstituted T cell repertoire diversity and evenness after highly hematotoxic

Figure 6. Effect of chemotherapy on T cell clonality in cancer patients. Clonality index with mean ±SD of PB TCR repertoire of untreated and chemotherapy treated age-matched soC (n=5) and hemC (n=16) patient samples (a) and of paired soC (n=12) and hemC (n=17) patient samples before and after chemotherapy (b). Mean clonal space distribution of PB TCR repertoire of untreated and chemotherapy treated samples from soC patients (n=5+12) and hemC patients (n=16+17) (c). Clone sizes are defined as hyperexpanded (0.01 < x ≤ 1), large (0.001 < x ≤ 0.01), medium (1E-04 < x ≤ 0.001), small (1E-05 < x ≤ 1E-04). Statistical test: unpaired, two-sided t-test.
therapies was due to rebound thymic activity rather than peripheral T cell expansion. To confirm this further, we subjected these repertoires to an algorithm, which calculates the generation probability for each recombined receptor sequence found in the productive repertoire. Since TCR repertoires shaped over time by clonal peripheral expansion contain higher numbers of clones with lower generation probabilities (selected by specific antigens), we hypothesized that rebound thymic output after chemotherapy may lead to repertoires shifted towards clones with higher generation probabilities. Generally, we observed lower generation probabilities in T cells of untreated cancer patients as compared to healthy subjects, pointing to more peripheral expansion of clones in the cancer patients. Moreover, in line with our hypothesis, we observed a clear shift (hemC: \( p = 1.3E^{-15} \), solC: \( p < 2.2E^{-16} \)) towards an increased median generation probability over the treatment interval in the cancer patient cohorts (Figure 8). This data suggested that hematotoxic chemotherapies eradicate T cell clones in the periphery followed by repertoire reconstitution through rebound thymic output.

**Discussion**

Age-, disease- and treatment-associated impairment of T cell immunity in cancer patients is clinically relevant as it determines susceptibility to infection (especially viral reactivations and opportunistic infections) as well as anti-tumor immune control. It may also be a relevant factor that influences response to immune-activating antibodies that have become one of the mainstays of systemic cancer treatment over the
past years. NGS immunosequencing is an advanced technology for T cell repertoire metrics analysis with the potential to precisely assess repertoire changes in immunoaging, cancer-associated immunosuppression or post-treatment immune reconstitution. It allows simultaneous identification of tens of thousands to millions of T cell receptor (TCR) rearrangements from a single tissue sample. In this way, this technology makes it possible to characterize large repertoires in depth and at high throughput, to monitor repertoires over time and to integrate all information to derive quantitative and reliable repertoire metrics such as clonality and diversity indices or clonal space distribution.

Here, we report the first study applying this approach to an unprecedented number of peripheral blood samples derived from chemotherapy-treated and -untreated patients with solid and hematological malignancies as well as healthy control subjects. The NGS data deposited with this manuscript may serve as the so far most extensive publically accessible database on T cell immune repertoires and may be exploited by the scientific community for further analyzes. Bioinformatic analyzes presented in this manuscript may be condensed to three essential findings: i) the T cell space qualitatively deteriorates with age as evidenced by loss of diversity and increasing clonality, ii) the age-dependent impairment in T cell metrics is more pronounced in patients with cancer than in healthy subjects and iii) age-specific repertoire metrics can be fully restored after lymphotoxic treatment even in elderly patients with cancer. These findings open up interesting new perspectives on immunoaging, carcinogenesis and immune reconstitution in cancer patients as well as they may influence the way we think about treatment algorithms of immunological and cytotoxic cancer treatment. One essential aspect of this (which is in contrast to previously published work\textsuperscript{23,24}) is our NGS data suggest there is a significant contribution by the thymus to immune reconstitution after T cell toxic chemotherapy in adults, even in the age group above 70 years. The full recovery of T cell diversity after treatment in this age group was rather unexpected and – while acknowledging that this data does not allow to draw any conclusions about immunological memory – it suggests that we may not have

![Figure 8. Generation probability of TCR repertoires.](image-url)
to fear drastic and uncompensated diversity losses through chemotherapy that would endanger the success of subsequent administered immune-stimulating antibodies in the elderly population.

Moreover, we were surprised to see clear-cut signs of premature immunoaging in treatment-naive cancer patients. Of note, the considerably impaired diversity and clonality indices in cancer patients were not a confounding result of infiltration of lymphatic organs with subsequent displacement of lymphopoiesis since also patients with solid tumors without infiltration of lymphatic organs showed the same impairment. Clonality is not the counterpart of diversity in that increases in clonality do not necessarily reduce the overall diversity of the repertoire. Therefore, while increased blood T cell clonality in patients with cancer may simply reflect ongoing anti-tumor immune responses, the significantly lower age-adjusted diversity in these T cell repertoires may also point to pre-existing T cell repertoire defects that could be seen not only as a consequence, but also as a potential basis favoring carcinogenesis.

As TCR sequencing becomes more and more available to the scientific community and a growing number of research questions in the field of autoimmunity, vaccination and infection are addressed, our data may add a new level of evidence for age-specific biases in TCR repertoires. Studies on T cell repertoires need to be aware of these biases described in our study and therefore need to rigorously work with age-matched cohorts.

Taken together, our work gives valuable insight into immunoaging in health and disease and provides a reference NGS dataset of TCR repertoires across all age groups that may serve the scientific community for future studies.

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Disclosure statement

Author LF is employed by ENPICOM B.V. All other authors declare no competing interests.

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