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Quantitative Analysis of T Cell Receptor Diversity in Clinical Samples of Human Peripheral Blood

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Abstract

The analysis of T cell receptor diversity provides a clinically relevant and sensitive marker of repertoire loss, gain, or skewing. Spectratyping is a broadly utilized technique to measure global TCR diversity by the analysis of the lengths of CDR3 fragments in each V β family. However the common use of large numbers of T cells to obtain a global view of TCR V β CDR3 diversity has restricted spectratyping analyses when limited T-cell numbers are available in clinical setting, such as following transplant regimens.

We here demonstrate that one hundred thousand T cells are sufficient to obtain a robust, highly reproducible measure of the global TCR V β repertoire diversity among twenty V β families in human peripheral blood. We also show that use of lower cell number results not in a dwindling of observed diversity but rather in non-reproducible patterns in replicate spectratypes. Finally, we report here a simple to use but sensitive method to quantify repertoire divergence in patient samples by comparison to a standard repertoire profile we generated from fifteen normal donors. We provide examples using this method to statistically evaluate the changes in the global TCR V β repertoire diversity that may take place during T subset immune reconstitution after hematopoietic stem cell transplantation or after immune modulating therapies.

Keywords

TCR V β CDR3 repertoire diversity in human peripheral blood; spectratype; immune reconstitution; hematopoietic stem cell transplantation

1. Introduction

The T-cell receptor (TCR) repertoire diversity reflects both the capacity of the thymus to generate naïve T cells and the cumulative responses of T cells to antigenic challenges. The process of VDJ rearrangement during thymopoiesis results in the expression of a unique TCR in each T cell, hence populations of newly generated naïve T cells express a broad receptor repertoire. After T cells enter the periphery, however, clonal expansions of antigen-activated T cells can result in skewed or even oligoclonal repertoires (Stamenkovic et al.,

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1988; Moebius et al., 1990; Ferradini et al., 1993; Pantaleo et al., 1997; Boucher et al., 2007). Severe depletion of T cells, such as that produced by HIV or hematopoietic transplantation regimens can reduce repertoire diversity, both by loss of naïve populations and antigen-specific expansions (Connors et al., 1997; Gorochov et al., 1998; Killian et al., 2004; Hakim et al., 2005; Malhotra et al., 2006; Yin et al., 2009). Repertoire diversity can subsequently be restored in depleted or antigen-skewed T cell populations by renewed thymopoiesis or by preferential expansion of naïve cells (Roux et al., 2000; Franco et al., 2002; Sarzotti et al., 2003; Hakim et al., 2005; Hudson et al., 2007; Sportès et al., 2008; Markert et al., 2009; Yin et al., 2009).

Several methodologies have been used to analyze TCR diversity, ranging from flow cytometric or PCR assessment of the relative usage of different V β families (Muraro et al., 2000; Muraro et al., 2005; Ochsenreither et al., 2008), to exhaustive sequencing (Warren et al., 2011). Spectratyping, the most broadly utilized technique, measures TCR repertoire diversity based on variation in the lengths of RT-PCR products generated from the CDR3 region in each TCR V β family (Choi et al., 1989; Genevee et al., 1992; Pannetier et al., 1993; Gorski et al., 1994; Even et al., 1995). The CDR3 region encompasses the area of high diversity created by nucleotide insertions/deletions at the junction of the V, D and J segments. Because of the random generation of a very large number of unique CDR3 regions, the spectratype of CDR3 fragment lengths will form a bell curve, a Gaussian-like distribution. Clonal T cell expansions or severe cytoreduction result in skewing of the proportional distributions of CDR3 of different lengths. Tracking responses driven by tumor, pathogen, or allo-antigens has identified oligoclonal expansions within the TCR repertoire (Guilherme et al., 2000; Friedman et al., 2001; Clarencio et al., 2006; Ochsenreither et al., 2008; Rosenblatt et al., 2010). Assessment of the recovery of TCR repertoire diversity after cytoreductive therapies, and associated recovery of immune competence (Messaoudi et al., 2004; Wynn et al., 2010), has required a more global assessment (Muraro et al., 2000; Hakim and Gress, 2002; Talvensaari et al., 2002a; Hakim et al., 2005). Spectratyping is a well-established technique and many scoring methods have been described (reviewed in (Miqueu et al., 2007)). The simplest, such as counting the numbers of peaks (taking the total or the average per V β family) have been useful in describing major differences in T cell receptor repertoire diversity. More complex methods involve converting the spectratype of each V β family into frequency distribution based on the proportion of each of the different CDR3 lengths (calculated as areas under the curve). Using this form of analysis, spectratypes have been compared in pairs, to identify clonal expansions. Several modifications in these protocols have been published to date including several methods of statistical analysis (Bercovici et al., 2000; Collette and Six, 2002; Hori et al., 2002; Killian et al., 2002; Miqueu et al., 2007).

Standard spectratyping methodology examining global diversity of TCR repertoire in human peripheral blood, however, has been based on large numbers of cells (Kochenderfer et al., 2002; Yamanaka et al., 2010; Ma et al., 2011) and, due to the absence of standardized methods of analyzing and reporting the data, the interpretation of the data generated has remained an issue. The limited T-cell numbers available in lymphopenic patients following transplant regimens have restricted spectratyping studies of immune system reconstitution. To develop a rigorous tool for assessment of the CDR3 diversity available in the peripheral blood, we wanted to determine the minimal number of T cells that reproducibly encompass proportional CDR3 diversity available in the peripheral blood. We report that we have statistically validated that 1×10^5 T-cells will provide reproducible spectratypes of TCR V β CDR3 fragments reflecting proportional representation of TCR V β CDR3 diversity available in the human peripheral blood. In addition we demonstrate that quantitative assessments of global repertoire diversity are possible by comparing spectratype divergence to a standard based on the proportional distribution of CDR3 fragment lengths from a group of normal

donors and that these techniques can generate sophisticated analyses of the limiting numbers of T cells available from patients. Finally, we demonstrate that all this can be achieved using commonly used reagents, techniques and software in the laboratories.

2. Materials and Methods

2.1. Cell collection

Clinical samples were obtained with patient consent under IRB approved protocols. Peripheral blood mononuclear cells (PBMC) were isolated from blood samples obtained from patients and normal donors, by centrifugation over lymphocyte separation medium (Organon-Technica, Durham, NC). Samples were either processed immediately for sorting or were cryopreserved in cell freezing medium (Invitrogen).

2.2. Cell Sorting

T-cell subpopulations were sorted as CD3+CD8+ and CD3+CD4+ T-cells on a FACS Vantage SE (Becton Dickinson Immunocytometry Systems, San Jose, CA) as >99% pure. CD3+CD8+ T cell subsets were further defined as CD28+CD45RA+ (naïve), CD28+CD45RA – (memory) and CD28 CD45RA+/- (effector) T cells.

2.3. RNA Isolation

Cell were lysed in Trizol (Invitrogen) and stored at -80°C . Glycogen (UltraPure Glycogen 20 $\mu\text{g}/\mu\text{l}$. Invitrogen) and of tRNA (Cat# R5636 Sigma) were added as carriers to enhance recovery of RNA from small numbers of cells. An equal volume of 2-propanol alcohol and 1 ml of 75% ethyl alcohol were used to precipitate and wash the RNA.

2.4. cDNA Synthesis

Immediately following RNA isolation, all of the RNA was converted to cDNA using oligo(dT) primer for reverse transcription (First Strand cDNA kit; Roche Diagnostics Corp) according to the method recommended by the company. Briefly, a master mix was prepared that contained, 5mM MgCl_2 , 1mM dNTP mix, 1.6 μg oligo(dT) primer, 50 units RNase inhibitor, 20 units AMV Reverse Transcriptase and 1X reaction buffer. 20 μl of the master mix was transferred to the tube containing the freshly isolated RNA pellet. Reaction was transferred to 200 μl PCR tube, incubated at 25°C for 10 minutes, 42°C for 60 minutes, followed by 99°C for 5 minutes (ABI PCR system 9700). The cDNA product was either used immediately for V β specific PCR or frozen at -20°C for later use.

2.5. V β specific PCR

Five μl cDNA, out of the 20 μl cDNA product, was used to amplify 20 separate PCR reactions, each containing one human V β family specific primer coupled with a constant region (BC) primer (Supplemental Table #1). We have also tested using all 20 μl cDNA and observed similar results. A 30 cycle PCR was performed (GeneAmp PCR System 9700. Applied Biosystems) using the following conditions: 0.7 μM each of unlabeled V β specific and constant primers, 0.75 units cloned pfu DNA polymerase (Stratagene, LaJolla, CA), 1X buffer provided with polymerase and 200 μM dNTP mix, final reaction volume of 30 μl . Temperature settings were: hot start with 2 minutes at 95°C , followed by 30 cycles of 95°C 30 seconds, 55°C 45 seconds, 72°C 1 minute, and followed by one hold at 72°C for 10 minutes. The product was used immediately for run off PCR or frozen at -20°C for future use.

2.6. Semi-nested runoff

A 10 cycle semi nested runoff PCR reaction was then performed to label the V β specific PCR product using the same temperature settings as above. Runoff PCR conditions were, 2 μ l of V β specific PCR product (out of 30 μ l) generated in the step above, 2 μ M final concentration of 5'/6-FAM labeled common V β run off primer (IDT, Coralville, IA) to run the assay on ABI 3031 XL DNA sequencer, or 0.2 μ M final concentration of 5'/Cy5 labeled run off primer (IDT, Coralville, IA) to run the assay on "OpenGene" DNA sequencer (Visible Genetics), and 0.15U cloned pfu DNA polymerase, 200 μ M dNTP mix, in 1X reaction buffer provided with the polymerase, in a final volume of 10 μ l. (Supplemental Table #1)

2.7. CDR3 fragment length assessment

We used two different electrophoresis systems, the ABI 3031 XL capillary based electrophoresis DNA sequencer and the Visible Genetics OpenGene gel electrophoresis sequencer system, to run the labeled PCR product with identical results. For the ABI 3031 XL DNA sequencer 1 μ l of runoff reaction was mixed with 9 μ l loading buffer, consisting of Hi-Di Formamide (4311320 Applied Biosystems) and known size standards GeneScan-500 [ROX] Red (A) (cat# 401734 Applied Biosystems) mixed in 1:20 ratio. The reactions were heated at 95°C for 2 minutes and transferred on ice immediately, then electrophoresed. GeneMapper v3.7 software (Applied Biosystems) was used to analyze the data.

For the Visible Genetics OpenGene automated sequence system, 0.5 μ l of run-off reaction product labeled with 5' Cy5, was mixed with 1.5 μ l loading buffer containing Cy 5.5 labeled size markers (Visible Genetics, Toronto, Canada), heated at 80°C for 2 min and transferred to the ice immediately, and loaded to a 6% acrylamide-urea sequencing gel ("Surefill"; Visible Genetics). Gels were electrophoresed for 30 minutes on the OpenGene automated sequencer (Visible Genetics) using 1X TE buffer, gel temperature 50°C, 1500 Volt and laser power at 20%. Data were electronically collected and processed using "GeneObjects" software (Visible Genetics). We have observed that an electropherogram of TCR V β amplicons from naive T cells of a normal young individual shows 8 to 13 peaks, 3 bp apart and distributed in an approximate Gaussian shape. Therefore, amplicon peaks were used in our analysis if they occurred at multiples at 3 base pair separation, with or without gaps with the expected fragment length. The calculated "area under the peak" represents the quantity of labeled V β fragment PCR product of a defined length measured by the detector. The sum of the peak areas of up to thirteen fragments were assessed for each V β family. This data was used to calculate the total area for each V β family, to calculate the proportional representation of each size fragment in each V β family, and to calculate the "divergence score" (DS).

2.8. Normal Donor Standards

The spectratypes of 1X10⁵ CD3⁺ CD4⁺ and CD3⁺ CD8⁺ T-cells from each of 15 normal donors were aligned by base pair length in each V β family. The proportional area for each of possible 13 size fragments was calculated relative to the sum of the areas of all peaks in that V β family. The average proportional areas of each size fragment in each V β family established a normal donor standard for CD4⁺ and CD8⁺ T cells (Supplemental Table #2 and #3).

2.9. Statistical Analysis

To assess agreement among CDR3 profiles generated from three separately processed cell samples, we quantified the divergence of each fragment of each V β family as the absolute difference in the proportional area of the test sample from its counterpart normal donor

standard and summed the values for each peak in the profile to establish a “divergence score” (DS). The absolute values of the observed differences of proportion of each of the 13 CDR3 fragments for each V β family were added together to generate the Total Divergence Score “TDS” for a given V β family (supplemental Table #4). Spectratype reproducibility among triplicates was tested using the “Intraclass Correlation Coefficients” (ICCs) procedure (supplemental Table #5). CD4 and CD8 T cells were separately analyzed with corresponding normal donor standards. (supplemental Table #4). Spectratype reproducibility among triplicates was tested using the “Intraclass Correlation Coefficients” (ICCs) procedure.

To determine whether the difference in the divergence observed at two time points, for example at pre and post intervention, was statistically significant, the TDS of each of 20 V β families at two time points were analyzed using nonparametric Wilcoxon analysis (2-tailed) (Supplemental Table #5). Statistical analysis was performed using MedCalc software Version 11.2.1. Mariakerke, Belgium.

3. Results

3.1. Determination of factors critical to analysis of minimal T cell populations

We first optimized our spectratype procedure to ensure that we could obtain reproducible data from small cell samples. Since quantitative RNA recovery was assumed to be an important parameter we added glycogen as a carrier to maximize RNA recovery from small number of cells. We also tested two sets of published primer sequences for spectratyping (Puisieux et al., 1994; Currier and Robinson, 2001), and chose a primer set that worked best using our conditions for RT-PCR (Supplemental Table # 1). To determine the minimal number of T cells needed to generate reproducible spectratypes, we prepared three separate samples of 1×10^6 , 1×10^5 , 1×10^4 and 1×10^3 sorted CD3⁺CD8⁺ T-cells from a normal donor and processed and analyzed each sample separately. The proportional CDR3 fragment length distribution from the samples of 1×10^6 and 1×10^5 cells demonstrated very similar and reproducible spectratypes (Fig.1a). Spectratypes prepared from 1×10^3 or 1×10^4 CD8⁺ T cells, however, demonstrated a visible breakdown of the spectratype pattern and the replicate samples had significant variation in the proportional distribution and even in the number of peaks (Fig.1a). When the proportional distribution of fragment lengths was compared to that found in a Normal Donor Standard (NDS), the total divergence scores (TDS) among the three replicates were similar on 1×10^6 and 1×10^5 cells were 31.61 ± 2.6 and 35.93 ± 3.36 SD respectively. Much higher TDS (56.24 ± 9.71 and 68.09 ± 10.84 respectively) were observed for 10^4 and 10^3 cells (Fig.1b).

We did additional experiments to understand the basis for the variability observed in the 10^3 and 10^4 cell samples. The total quantity of cDNA used in the RT-PCR was not limiting. Using 1/10 or 1/100 of the cDNA generated from 1×10^6 or 1×10^5 CD8⁺ T cells produced relatively little variation in the spectratype pattern (Fig.1c). Furthermore, the amount of the PCR product needed for electrophoresis was not limiting. Dilutions of four-fold up to sixteen-fold of the V β 1 PCR labeled product resulted in highly reproducible spectratype patterns (Fig.1d). The peak height, that represents the amount of probe fluorescence, did decline with dilution, as did the total areas under the peaks, but the proportional distribution of areas under the peaks (as percentages of total area under the spectratype curve) did not change. This finding means that the PCR product can be diluted without loss of proportional area distribution, if necessary to avoid overloading the sequencer and chopping off the top of peaks. Therefore, the critical element for the reproducible data was the initial number of cells used as a source of RNA. The optimized protocol therefore provided a robust assay when assessing common V β families.

3.2. Assessment of reproducibility across 20 families of human TCR V β CDR3 repertoire

Having demonstrated that 1×10^5 T cells were adequate to produce reproducible spectratype patterns from common V β s, we then tested the reproducibility of spectratypes of 20 V β families, generated from 1×10^5 T-cells. We performed spectratype analysis on three replicate 1×10^5 cell aliquots from each of four normal donors, using one hundred thousand sorted CD4 $^+$ or CD8 $^+$ T-cells in each aliquot. All the aliquots were processed separately throughout the procedure, including cell lysis, isolation of RNA, generation of cDNA, V β -specific PCR, and electrophoresis. The spectratype histograms of all 20 V β families among the three aliquots were highly reproducible (Fig.2a). No evidence of random breakdown of spectral pattern or proportional representation of the fragment lengths was observed among the replicates.

The measurements of TDS for each of 20 V β families among triplicates (Supplemental Table #4) were tested for agreement and consistency using the Intraclass Correlation Coefficients (ICC) procedure (Weir, 2005), with each separately processed aliquot serving as an independent rater of the CDR3 spectratype. Our data showed close reproducibility of the proportional representation of the fragments, with average ICC of 0.9823 and 0.9818 for agreement and consistency respectively among measurements (Fig.2b). It was notable that both diverse and highly skewed spectratypes were reproducible. As has been previously noted (Ou et al., 2009), the fragment analysis does not always generate fully satisfactory spectratypes of all the V β families in all samples. The spectratypes of V β 20 and V β 22 in the individual shown in Fig 2 were not fully interpretable, however the patterns were well replicated; similarly, individual oligoclonal distributions as in V β 8 were consistent in all replicates.

3.3. Spectratype distribution in subsets of CD8 $^+$ cells

The sensitivity of the optimized procedure permits analysis subsets of small number of CD4 and CD8 subsets. We performed spectratype analysis on 1×10^5 total CD8 $^+$ T cells of a middle-aged adult patient after autologous peripheral blood stem cell transplant. The spectratypes of the TCR V β CDR3 fragments of total CD8 $^+$ T-cells appeared oligoclonal, despite the passage of 2 years from the time of transplant (Fig. 3A). We then sorted naïve (CD28 $^+$ CD45RA $^+$), memory (CD28 $^+$ CD45RA $^-$) and effector (CD28 $^-$ CD45RA $^{+/-}$) CD8 $^+$ T cells from the patient and analyzed the spectratypes using 100k of each of these subsets. Most V β families in the CD28 $^-$ effector population had oligoclonal spectratypes. The oligoclonal peaks in the effector subset aligned with dominant peaks in the total CD8 population. The effector population in this individual constituted more than 60% of the total CD8 $^+$ T cells. The spectratypes of sorted naïve and memory cells, in contrast, revealed the presence of significant repertoire diversity. When we numerically assessed the divergence scores for three V β families in the total T cells and sorted subpopulations (Fig.3b), the results numerically reflected the visually observed spectratype patterns.

Our procedure allowed us to quantify the repertoire diversity of naïve, memory and activated cells; the total divergence from the normal donor standard is highest in the activated effector CD8 $^+$ cells, with significantly lower divergence in the memory and naïve component of the CD8 $^+$ cells (Fig.3). This study suggested that some restoration of TCR diversity occurred in naïve and memory T cells but was masked by the oligoclonal nature of the predominant CD8 $^+$ effector cell population, which constituted 60% of CD8 $^+$ cells, when the total CD8 $^+$ population was analyzed.

3.4. Paired analysis of spectratype shifts

We also used this technique to obtain an overall quantitative assessment of repertoire diversity in a population of T cells across all of the V β families. This approach allowed us to

compare repertoire in T cells subsets collected before and after a therapeutic intervention. An overall measure of repertoire diversity can be determined by calculating the sums of the individual divergence scores from all of the V β families. Shifts in repertoire in an individual patient can be sensitively assessed by performing paired analysis by nonparametric Wilcoxon tests using the absolute divergence scores of each of the 20 V β families in the pre and post therapy T cell populations. A *P* value <0.05 indicates a statistically significant change in repertoire diversity between the baseline and post treatment samples.

As an example, we show the analysis of spectratypes of sorted CD4⁺ T-cells of a patient collected at baseline and after two weeks of IL-7 injections, a treatment that disproportionately expands naïve cells (Sportès et al., 2008). Spectratypes of many V β families appeared to have more of a bell curve distribution after two weeks of treatment as compared with the pretreatment ones (Fig.4a). The patient's total divergence scores when compared with the normal donor standard were lower in the post IL-7 treated sample than in the pretreatment sample (Fig.4b; full data in Supplemental Table #5). This data is consistent with a decline in the frequency of oligoclonal populations and an increase in overall repertoire diversity. To test the difference statistically, the absolute divergence of each V β family from the normal donor standard for CD4⁺ cells was calculated. Using Wilcoxon nonparametric paired analysis, the difference in the divergence scores between the pre and post IL-7 time points was determined to be statistically significant (*P* = 0.0042).

4. Discussion

This study was initiated in order to develop a sensitive assessment of global TCR V β repertoire diversity that could be applied in settings of limited T cell numbers, such as in lymphopenic patients or in sorted subsets of T cells. Although spectratyping is a well-established technology, no single standard has been accepted for analysis of spectratyping data (Miqueu et al., 2007). Studies of immune reconstitution of repertoire diversity have been based on broad qualitative categories of oligoclonal, skewed or polyclonal repertoire (Talvensaari et al., 2002b), relatively insensitive counts of peaks of CDR3 lengths in each spectratype (Soiffer et al., 2002) or arbitrary point scale scoring systems (Peggs et al., 2003). Gorochoff (1998) initially suggested comparing proportional distribution of the CDR3 lengths in spectratypes to an averaged standard generated from normal healthy individuals. Several groups have subsequently established their own control standards based on theoretical Gaussian distributions (Long et al., 2006) or comprised of the highly diverse T cells of cord blood (Peggs et al., 2003) or averaged standards from up to 35 normal donors (Killian et al., 2002; He et al., 2005). Skewed spectratypes have been statistically compared with Gaussian distributions using goodness of fit tests such as Kolmogorov-Smirnov, but such tests are limited by the fact that spectratypes are discrete and not continuous data. For comparisons involving repertoire changes that gradually approximate Gaussian distributions, such as repopulation of naïve cells after hematopoietic transplant or HAART therapy, these may nonetheless be very useful. Kurtosis and skewing statistics similarly are of limited value in characterizing or comparing antigen driven or cytoreduced reduced T cell populations, as these will often have a bimodal or multimodal spectratype, not merely a shifting of a single peak. Although T cells, especially CD8 cells, from normal healthy donors are individually divergent on multiple V β families, as more donor data sets were added to generate the standard, the averaged spectratype area distributions increasingly approximated the bell curves observed in sorted naïve cells or cord blood. We chose to generate our own standard from normal donors and to assess the divergence in proportional area peak by peak. This form of comparison of discrete distributions, involving calculation of a Hamming Distance of the match between the experimental and the standard distribution, supported non-parametric comparisons of multimodal distributions without any assumptions about the distributions of the individual V β family spectratypes. The average of

the TDS calculated for each V β family can serve as a global measure of repertoire diversity, similar to those reported by Long et al (2006) or Gorochov (1998) or, as we have shown here, paired comparisons of patient samples can be assessed by non-parametric statistics such as the Mann-Whitney U test.

One limitation of our work and that of other spectratyping-based models of repertoire is that there is an equal weighting given to all V β families, disregarding the number of genes included in a family or the overall proportion that a family contributes to the total repertoire. Semiquantitative PCR (Ochsenreither et al., 2008) or flow cytometric analyses of the relative frequency of each family have been incorporated into some analyses (Williams et al., 2002), but these require a significantly larger number of cells. It's worth noting that the total area under the curve of individual spectratypes cannot be used directly to calculate relative V β family frequency because the spectratyping PCR reactions are not quantitative.

Yet a rigorous analysis of the proportional distribution of CDR3 lengths relies upon the reproducibility of the spectratypes. While this can be accomplished using large numbers of T cells, T cell populations are often severely diminished in lymphopenic patients following hematopoietic stem cell transplantation. In this report we have demonstrated that 1×10^5 T cells are sufficient to generate reproducible spectratypes, a number readily available from clinical samples. We have shown that use of fewer than 1×10^5 T-cells resulted in a loss of reproducibility and a random breakdown of the proportional distribution of the CDR3 fragments. Further we showed that the loss of reproducibility was not due to limits in the amount of cDNA in the PCR process or the electrophoresis, but rather due to constraints on the initial T cell number utilized to isolate the RNA. The breakdown of the proportional distribution of the CDR3 fragments we observed when less than 1×10^5 T-cells were used to isolate the RNA could be due to an incomplete proportional representation of the available T cell TCR V β repertoire in a small sample. Indeed, such an increase in variability upon serial dilution has been used to assess the frequency of T cells expressing specific receptors (Hori et al., 2002).

The ability to assess global CDR3 repertoire diversity present in the human peripheral blood using only 1×10^5 T cells enhances the utility of spectratyping for studying clinical blood samples. As an example, we compared the spectratypes of 1×10^5 total CD8⁺ T-cells, and of sorted naïve, memory and effector CD8⁺ subsets collected from a middle-aged patient two years after autologous peripheral blood stem cell transplant. The overall CD8 population appeared to have a severely restricted repertoire, suggesting a failure to repopulate the periphery by the generation of new T cells. Comparison with the memory and naïve subsets demonstrated that the spectratype obtained from total CD8⁺ T cells was heavily influenced by the effector CD8⁺ compartment which comprised more than 60% of the total CD8 cells in this patient. Thus analysis of the total CD8 T cell population alone would have concealed the evidence of thymopoiesis driven recovery of a diverse repertoire in the naïve and memory compartments (Hakim et al, manuscript in preparation). The ability to examine the TCR V β spectratype in small numbers of T cells can be very useful tool for examining specific repertoire responses to an antigen, vaccine or infection.

As noted in this example, the divergence scores of spectratypes obtained from effector CD8⁺ T cells were significantly higher than those of naïve and memory cells and both the apparent diversity (visible in the spectratype) and the divergence scores were heavily influenced by the frequencies of naïve, memory and effector subsets. This difference was key to examining the effect of immune modulating therapies on overall repertoire diversity. We demonstrated that quantitative analysis of the divergence of each V β family from a normal donor standard provides a sensitive assessment of global shifts in repertoire diversity. Following a brief two-week IL-7 treatment, a therapeutic intervention that disproportionately expanded naïve

and central memory T cell populations at the expense of effector subsets, the post treatment repertoire could be demonstrated not only to differ from the initial state, but also to be closer to a normal diverse repertoire.

Spectratyping is a well-established technology that remains a valuable tool for examining both antigen driven peripheral expansion and thymic-dependent immune reconstitution. Our demonstration that 1×10^5 T cells from human peripheral blood is sufficient to assess TCR repertoire diversity will enhance the use of spectratyping to assess immune reconstitution in clinical studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Factors affecting reproducibility of spectratypes. (A) Cell number: Spectratypes of TCR V β 1 from three replicate samples of sorted CD8⁺ T cells aliquoted at 10^3 to 10^6 cells. (B) Total Divergence Scores (TDS) of each replicate. (C) cDNA quantity: Spectratypes prepared using up to one hundred fold less cDNA from 10^6 and 10^5 sorted CD8⁺ T cells to start TCR V β specific PCR. (d) PCR product quantity: Cy5.0 labeled V β 1 PCR product from 10^5 CD8⁺ T cells electrophoresed using 1, 1/4 and 1/16 of the usual loading amount..

Figure 2. Reproducible spectratypes of twenty Vβ families obtained from replicates of 10^5 sorted CD4⁺ T cells from a normal donor

(A) Spectratypes of all 20 Vβ families among three replicates. (B) Total divergence scores of Vβ families from three replicates and testing among replicates using Intraclass Correlation Coefficient (ICC) statistical procedure (Medcalc statistical software v. 11.2.1).

Figure 3. Spectratype analysis of the total CD8⁺ T-cells, and of sorted naïve, memory and activated CD8⁺ subsets collected from a middle-aged patient two years after autologous peripheral blood stem cell transplant

(A) Spectratypes of sorted total CD8⁺ and effector, memory and naïve CD8⁺ subsets. (B) TDS of spectratypes of three Vβ families from total CD8⁺ T-cells, and from sorted naïve, memory and activated CD8⁺ subsets.

Figure 4. Changes observed in the spectratype patterns before and after IL-7 treatment
 A. Spectratypes of Vβ1–Vβ6 prepared from 10^5 sorted CD4⁺ T cells from a middle age patient collected at baseline and after two weeks of IL-7 injections. (B) TDS of Vβ1–Vβ6.