Chapter 14

TCR ANALYSES

T-cell receptor CDR3 analysis: Molecular fingerprinting of the T-cell receptor repertoire

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Abstract: T-cells play a crucial role in immune surveillance against transformed cells and intracellular infections; they are involved in auto-immune reactions. They recognize their targets, i.e. MHC / peptide complexes, trough the T-cell receptor. TCR usage determines the molecular interaction of the immune system with biologically relevant MHC/peptide molecules. The TCR coding genes (variable, diversity and junctional) determine the molecular composition of the TCR alpha and beta heterodimer. The random association of the VDJ genes constitutes the complementarity determining region 3 (CDR3) responsible for antigen recognition and TCR specificity. The molecular composition of a T-cell population can be objectively defined by measuring the CDR3 region. Qualitative and quantitative comparisons of the TCR composition in different anatomic compartments, or longitudinally over time, allow to asses the entire TCR repertoire. This methodology can be supplemented with functional T-cell based assays and aids to objectively describe any alteration in the T-cell pool. TCR CDR3 analysis is useful in immunomonitoring, e.g. examining patients after BMT or solid organ transplantation, patients with HAART therapy, or patients receiving molecularly defined vaccines.

Key words: TCR, CDR3

1. PURPOSE AND VALIDITY OF MOLECULAR T-CELL ANALYSIS

T-cell responses can be described by combining three categories: i) Specificity and effector functions of a T-cell population, ii) the quantity of

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T-cell responses (i.e. number of responding T-cells within the CD4/CD8 population) and iii) the 'molecular quality' of T-cells, the molecular structure defined by the T-cell receptor (TCR) structure.

Several methods to measure T-cell responses are now available including evaluation of T-cell precursors using limiting dilution, the ELISPOT assay, *ex vivo* T-cell receptor (TCR) variable (v) segment analysis determined by flow cytometry and TCR-CDR3 length analysis (spectratyping) as well as identification of peptide-specific T-cells using MHC class I or MHC class II tetramers containing appropriate peptides. T-cell responses can be molecularly defined by i) identification of monoclonal T-cell receptors which are known to bind to a defined peptide target presented by the appropriate MHC molecule, ii) gauging the T-cell population as mono-, oligo- or polyclonal for a defined T-cell receptor (TCR) family in the CD4 or CD8 T-cell population. These data aid to

1. define and compare magnitudes of CD4+ or CD8+ T-cell responses over time in individual patients (either as result of progression/regression of a certain disease, or alternatively, alterations of the T-cell pool associated with treatment)
2. compare the molecular composition of TCR αβ or TCR γδ T-cells over time or in different anatomic compartments, or
3. to compare the TCR VA/VB (or TCR VD/VG) diversity with normal healthy controls.

This ‘molecular fingerprint’ of the TCR-repertoire can be corrected by the qualitative assessment of individual TCR VB families. TCR fingerprinting objectively describes the TCR composition in a given sample. This purely descriptive measurement can be complemented using other functional methodologies:

1. TCR analysis in individual T-cell subpopulations defined by differentiation and homing markers, or T-cells defined by cytokine production in response to the antigen of interest.
2. T-cells with defined MHC/peptide-specificity determined by tetramer-based sorting of T-lymphocytes.

1.1 Background: TCR – nomenclature and definition

The TCR nomenclature has been adapted to the latest issue "The T cell receptor *FactsBook*" (1). Four types of genes are involved in TCR synthesis: the variable (V), diversity (D), and joining (J) genes which code for the antigen / MHC binding sites and the constant (c) genes which encode the non-variable TCR polypeptide chain.

TCR locus: A group of TCR genes that are ordered and organized in the same chromosomal location in a given (i.e. human) species. The human genome encodes four TCR loci:
TRA (14q11.2)
TRB (7q34)
TRG (7p14)
TRD (14q11.2) (embedded within the A locus, see above)

Group: A group of TCR genes that share the same gene type (i.e. V, D, J or C), a group may contain functional TCR and TCR pseudogenes.
Subgroup: Set of genes that belong to the same group and share at least 75% identity at the nucleotide level (in the germline configuration for V, D or J), e.g. the TCR VB families 7.1. or 7.2. This is also of practical relevance, since some primers implemented for TCR-CDR3 analysis may encompass several subgroups, other RT-PCR primers are needed to cover individual TCR V genes.

1.2 Creating T-cell receptor diversity

Defining the TCR binding region: T-cell receptor fingerprinting measures the length of the complementarity determining region 3 (CDR3) (Figure 1). In most cases, rearrangement of the TCR loci results from deletional joining. The V genes are localized upstream from the J (or D and C) genes.

Two genes, the recombination activating gene 1 and 2 (RAG1 and RAG2) were identified in 1989 and 1990. RAG1 and RAG2 are transcribed in lymphocytes that show recombinase activity (i.e. B or T-cells). These genes are – with few exceptions- only active in thymic tissue pertaining to T-lymphocytes (2, 3) Junctional diversity is responsible for creation of the TCR diversity associated with antigen recognition (i.e. MHC/peptide complexes). The N-region diversity results from the deletion of nucleotides at the extremities of the coding V, D and J genes by activation of an exonuclease and the random addition of nucleotides by the terminal deoxynucleotidyl transferase (TdT). The enzyme preferentially adds G and C nucleotides at the junctions and is specifically transcribed during lymphocytic maturation. This region is determined complementarity determining region 3 (CDR3). In brief, the specificity of a T-cell resides in the CDR3 region, the length of this regions defines the diversity of the TCR repertoire. The CDR3 region in humans measures approximately 11 amino acid residues (i.e. 33 base pairs) and shows a Gauss-distribution: the most abundant transcript measures approximately 6 amino acid residues (see Figure 1).

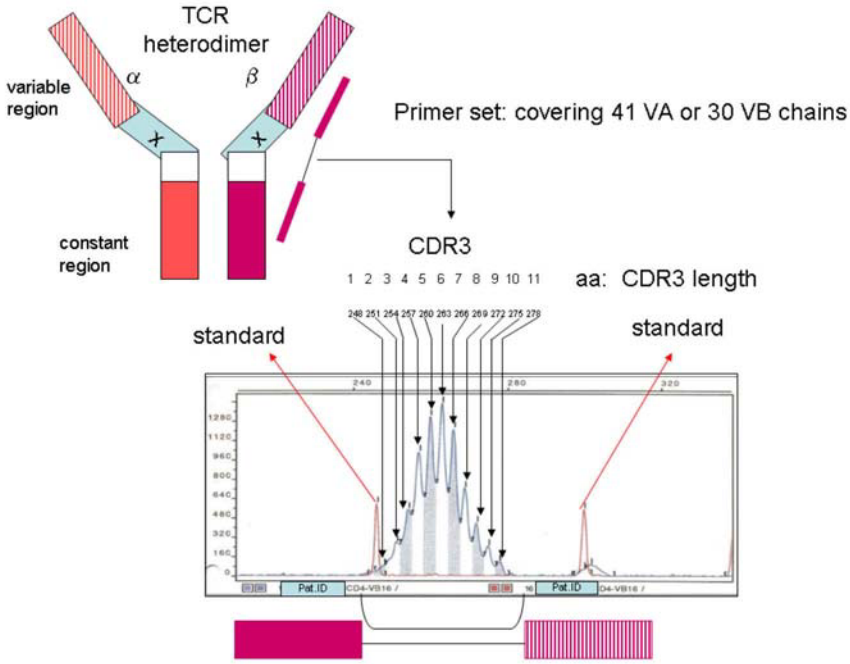


Figure 1. Determination of the TCR repertoire. A primer panel is used to amplify all possible TCR VB families using primers in the constant and in the respective variable region of the TCR. Example of the TCR CDR 3 analysis of the TCR VB16 family in CD4+ sorted T-cells. The major middle peak is 263 bp, there are 5 peaks on the left and right side respectively: a typical Gauss distribution of 11 aa coding for the CDR3 area. Note that each peak identifies 3 base pairs, coding for one amino acid residue. A single peak may suggest monoclonality. This has to be confirmed by DNA sequence analysis, since even a single peak (one amino acid residue) indicates that the length of the CDR3 region is 1 amino acid residue, however, different amino acid residues may be possible.

The CDR3 composition in a defined TCR VB "pool" may either be poly-, oligo- or monoclonal (see Figure 2). It is estimated that the theoretically possible TCR repertoire is not used in humans (4), but T-cells in human peripheral blood can carry up to 10^6 different TCR VB chains (5). Of note, most of the studies available today address the question of TCR diversity in the TCR VB chain, based on the presumption that each individual T-cell expresses only a single TCR VA chain paired with a single TCR VB chain. This is true for most T-lymphocytes in the peripheral circulation, although double TCR VB chains paired with a single TCR VA chain (and vice versa) have been reported (6, 7).

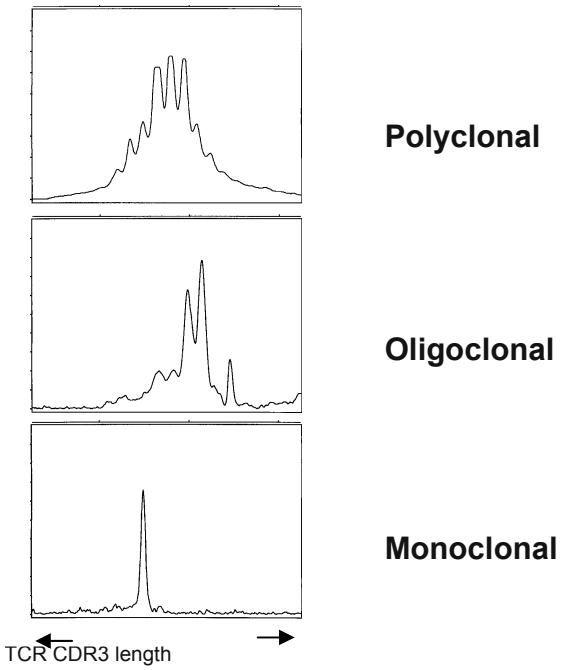


Figure 2. TCR CDR3 patterns aid to define the TCR composition.

Only a few studies addressed the molecular composition of gamma / delta T-cells which may also play a role in autoimmune disease, conferring resistance to infection or anti-tumor immune responses.

At this time, there is no indication that TCR genes can be somatically altered. TCR diversity is generated in the germline configuration and determines TCR specificity. Thus, the length of the TCR CDR3 regions describes in detail the TCR diversity identified by:

1. VB (VA/VG/VD) group or subgroup
2. Identification of the joining region
3. Measurement of the TCR CDR3 length
4. Molecular definition (sequencing) of the CDR3 region (if applicable)

2. METHODOLOGY

2.1 Determination of the TCR repertoire by DNA fragment analysis

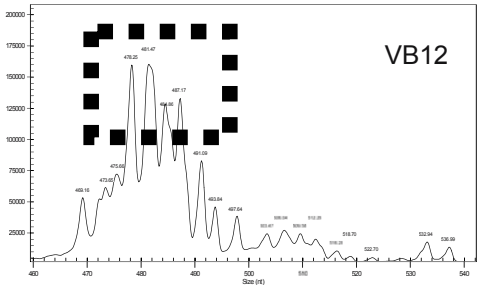
Cells are counted (absolute cell count) and a CD4 and CD8 determination is carried out to estimate the number of CD4 and CD8- positive T-cells in the sample. Most studies are performed using entire peripheral blood T-cells (PBL), this may not allow to detect monoclonal T-cell populations in CD4+ or CD8+ T-cell subsets, since e.g. a monoclonal TCR in a single VB family in the CD4+ T-cell population may be 'masked' by a polyclonal TCR in the CD8+ T-cell population in the same sample. In addition, the 'true' number of T-cells should be determined by combining an absolute cell count in the sample combined with a flow-cytometric analysis of TCR alpha/beta – positive T-cells. Some treatments or diseases may be associated with a skewed composition of the T-cell population: In general, most applications in TCR fingerprinting measure the composition of the TCR beta chain (in TCR αβ+ T-cells). An expansion of γδ+ TCR T-cells can neither be picked up by a lymphocyte count, nor by measuring CD3+ T-cells, since both αβ TCR and γδ TCR+ cells are CD3-positive.

The determination of the cell number is crucial since i) the same cell number should be analyzed in longitudinal studies, ii) it is associated with the sensitivity of the TCR fingerprinting assay: Each laboratory which uses this methodology should run appropriate controls to ensure that each TCR V-family is identified using the appropriate primer panel. Figure 3 shows that using a suboptimal cell number leads to 'false-positive monoclonality' in individual TCR variable families. This may be associated with the primer panel used for amplification of each TCR family (sensitivity) and the number of TCR transcripts in each TCR family.

Different ways to standardize samples are possible:

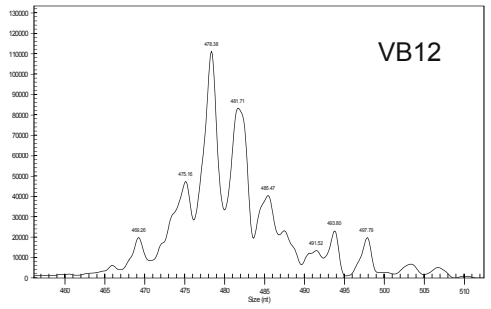
1. Standardization of the cell number either in PBL or in sorted CD4 and CD8+ T-cells.
2. Standardization of mRNA transcripts in samples using 'house-keeping genes' or genes shared by all TCR families (e.g. measuring the TCR constant chain).

If T-cells are sorted into CD4 and CD8+ T-cells, at least 90% purity should be obtained for the CD8+ T-cell population, and more than 80% of the CD4+ T-cell population. CD4+ T-cells may also include CD4+ dendritic cells or macrophages. Each laboratory should assess sensitivity and specificity of the TCR – analysis: this is crucial in order to avoid false positive/negative results and to ensure a high standard in monitoring TCR transcripts longitudinally in clinical trials.



CD8+ T-cells

0.5×10^5



0.25×10^5

"false" oligo- or monoclonality

Figure 3. 'False monoclonality'. Each laboratory should assess sensitivity and specificity of the TCR CDR3 analysis. Different CD8+ T-cell numbers amplified with the same TCR VB primers (e.g. VB12) show that insufficient mRNA transcripts lead to the impression of a false 'oligo- or monoclonal' T-cell population.

2.2 Determination of VB families by flow cytometry: T-cell quantification

The TCR CDR3 analysis allows to determine the structural composition, but does not address the quantity of each TCR VB, i.e. the absolute number of T-cells examined for TCR diversity. Several ways to gauge T-cells are possible: first, protein expression of TCR determined by flow cytometry (8). Up to 80% of the entire T-cell repertoire can be covered using a panel of TCR-specific mAbs. Alternatively, mRNA transcripts can be measured for each TCR VB family (4, 9). Both methodologies are possible for cell suspensions, but are not applicable for tissue sections. Thus, if the TCR-repertoire in PBL is compared to tissues, e.g. tumor sections, or lymph nodes, the latter approach using the standardization for TCR mRNA has to be performed. In addition, TCR cell surface expression determined by flow-cytometry may provide a different result as compared to TCR mRNA analysis: Activated T-cells may downregulate their TCRs, but enhance TCR

mRNA transcription upon activation (10, 11). At this point, TCR VB families should be quantified using either flow-cytometry or by measuring TCR VB mRNA transcripts, future studies will show if a combination of both techniques will provide biologically meaningful data in the context of immunomonitoring.

2.3 Qualitative analysis of the TCR repertoire

Each individual TCR variable CDR3 profile obtained from a TCR V-family can be depicted as a function of the CDR3 length: Each peak represents three base pairs (bp) coding for one amino acid (aa) residue. In general, in human healthy subjects, 9-11 aa can be identified in each CDR3 profile. The area of the entire CDR3 analysis is estimated as 100% for each TCR VA or VB family and the area under the curve for each individual CDR3 peak is expressed as the percentage of the entire CDR3 area. Briefly, total RNA is extracted and reverse transcribed into cDNA. Each individual TCR family can be amplified using a common primer in the constant region of the TCR supplemented with a primer specific for each TCR family (Figure 1). TCR VB-specific primers have been predominantly implemented in TCR CDR3 fingerprinting, TCR VA primers have also been reported and used in clinical studies (12, 13). At this point, 41 individual TCR VA and 30 VB families have been described (1). Positive amplicons are subjected to an elongation reaction using a dye-labeled primer which covering the constant region of the TCR which allows to amplify all TCR VB (or alternatively VA) families. The exact length of each product can be determined using a DNA-sequencer with an appropriate internal bp length standard labeled with a different dye as compared to the primer used in the elongation reaction. For instance, polyclonal, oligoclonal or monoclonal TCRs can be determined (Figure 2). Monoclonality has be confirmed by DNA sequence analysis, since a single peak in a given TCR V family states only that this population shares an identical length of the CDR3 region. It may be possible that a single peak with a defined CDR3 length codes for different amino acid residues, thus ‘monoclonality’ has to be confirmed by DNA sequence analysis.

2.4 Gauging the difference

TCR-CDR3 fingerprinting allows a snapshot of the TCR composition at a given time point. However, a comparative approach is often useful in clinical contexts, e.g. for patients undergoing immunotherapy. Individual TCR VA or VB repertoires may be depicted as a two-dimensional image (Figure 4) which reports the TCR CDR3 length for each individual TCR family.

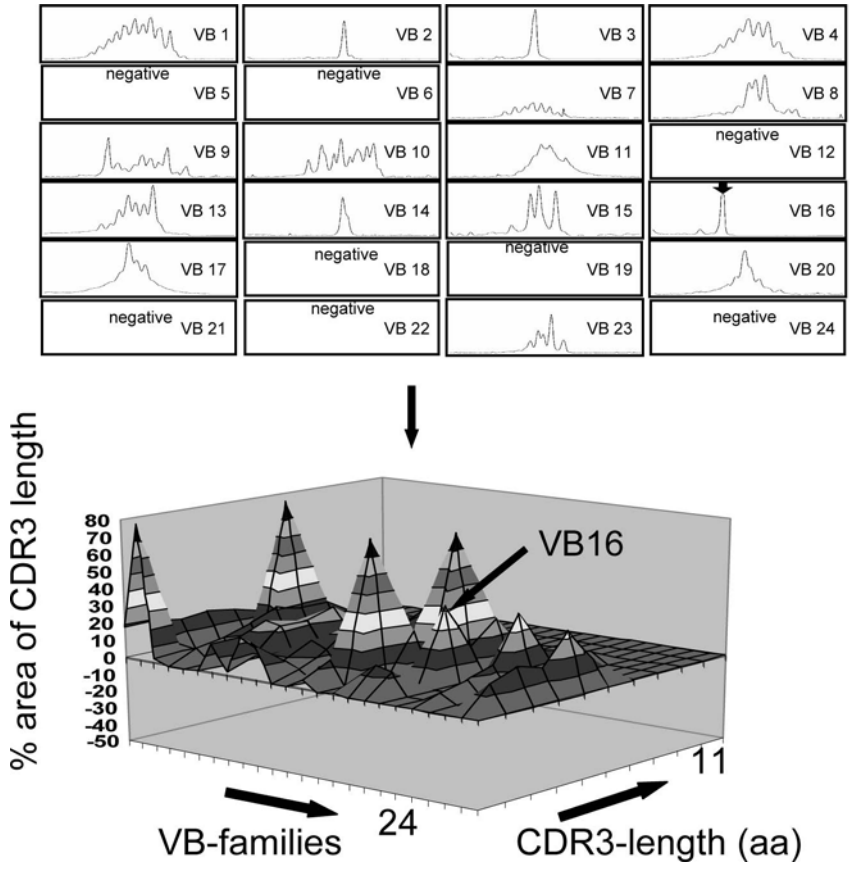


Figure 4. CDR3 length measurement. In normal healthy subjects, the CDR3 peak pattern represents a Gauss distribution. The data for each TCR family and the area under the curve for each TCR peak (i.e. 3 bp, 1 aa) are compiled. This CDR3 pattern may be depicted two-dimensionally, i.e. one picture for each TCR VA/VB family, or, alternatively, the entire TCR VA/VB repertoire may be combined in a single complex figure, thereby creating a TCR VA or TCR VB 'landscape' as a function of CDR3 length (as defined by the number of encoded aa) and the area under the curve for each individual CDR3 peak. For the sake of clarity, each 10% of the CDR3 peak is depicted in a different color. Two-dimensional (top) and three-dimensional (bottom) picture of the TCR repertoire analysis as defined by TCR VB spectratyping in a CD4+ TIL line from a patient with cervical cancer. The two-dimensional picture allows to analyze each VB family and the 'landscape' picture represents an overview of the TCR diversity. The single TCR VB16 chain (marked with the arrow) was found to be monoclonal and to recognize autologous tumor cells. See also Color Plate Section page 305.

Alternatively, TCR analysis can also be depicted as a 'landscape', i.e. as a function of the CDR3 length (z-axis), the VB family (x-axis) and the percentage of each individual CDR3 in a distinct VA or VB family (y-axis) (14, 15). This is possible using regular excel spreadsheet functions, or alternatively, using the Immunoscope® software (4, 16). This allows to describe the TCR repertoire at the time of evaluation. In order to compare either i) longitudinal differences (i.e. PBL or tissue samples collected at different time points) or ii) spatial differences (i.e. differences between a tumor sample and PBL or individual lymph node sections), the TCR CDR3 distribution pattern of individual VA or VB families can be used as a 'control' for other samples. This 'control' sample can be used from an individual patient (i.e. pre-, post-immunization, tumor/normal tissue) or a representative Gauss-distributed sample from either CD4+ or CD8+ PBL from healthy blood donors.

The difference at each CDR3 peak (for each VA or VB family) is calculated by comparing the area under the curve in the 'test' sample *versus* the control sample: Three possibilities can occur: i) the pattern is identical (a flat or 'smooth' area of the TCR landscape occurs), ii) some peaks are enhanced (resulting in 'over-representation' of certain CDR3 peaks which yields positive signals as +% perturbation (y-axis)), and iii) some peaks are diminished or ablated (resulting in 'under-representation' of CDR3 peaks, depicted as % perturbation (y-axis)). For sake of clarity, a certain percentage, (e.g. 10%) of over- or under-representation of the TCR CDR3 area (i.e. comparison of two samples) is depicted in a different color (Figure 5).

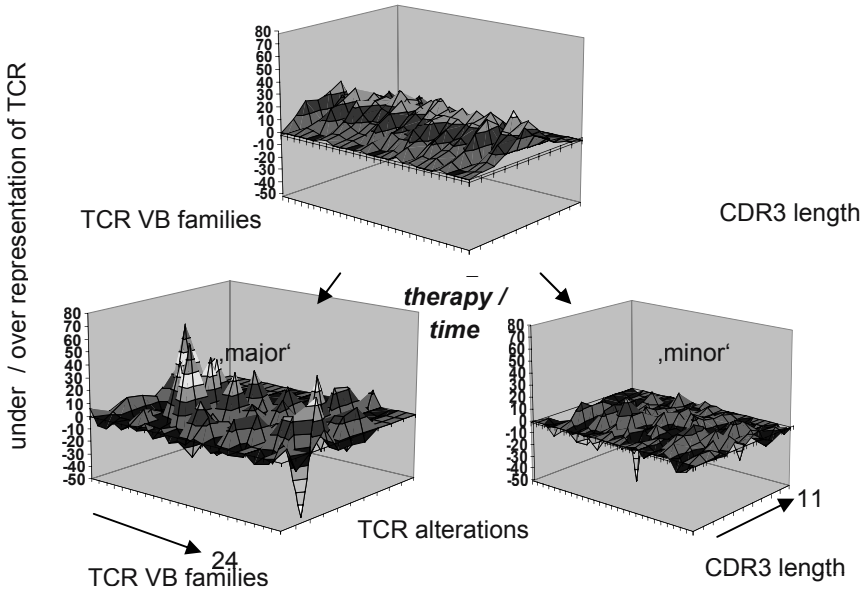


Figure 5. Visualizing the difference. TCR CDR3 patterns can be subtracted, i.e. each area under the CDR3 peak (e.g. CD4+ T-cells from PBL, top) is compared to the respective CDR3 peak areas of each individual TCR VA/VB family in the control sample. This results in over- or under-representation of individual TCR CDR3 peaks in each VA/VB family. 10% of difference are differentially colored. 'Major alterations' of the TCR repertoire (left, bottom) or a smooth surface (in blue) indicates no difference to the control sample (right, bottom). Differences can be time (longitudinal sampling of blood in an individual patient), spatial (e.g. tumor versus LN or PBL), or control samples from 'healthy subjects'.

This comparison allows the detailed description and comparison of the entire VA/VB repertoire. However, if the frequency of individual VB families is also available, this information can also be implemented in the comparison of the TCR VB chains, which would in this case include the comparative analysis of the quality (CDR3 length) and quantity (% frequency) of the TCR repertoire (Figure 6). Again, alterations in the quantitative composition of the TCR repertoire can be measured using flow-cytometry (8) or the RNA of each TCR family (9) using real-time PCR based quantification of individual TCR VB mRNA transcripts. The topology of TCR VB mRNA distribution displayed as a three-dimensional T-cell transcriptome has been coined TcLandscape (14, 17).

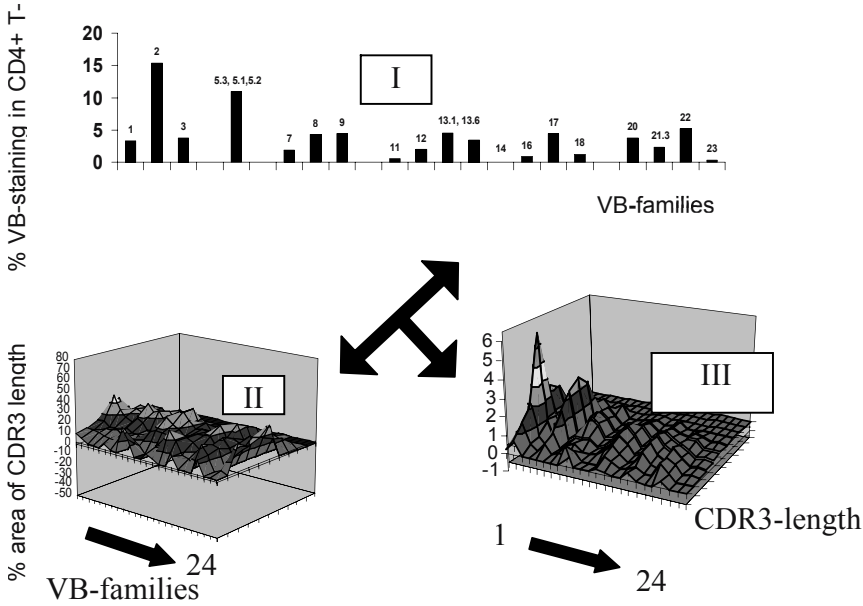


Figure 6. Gauging T-cell expansion. Qualitative and quantitative assessment of T-cells. Flow-cytometric determination of individual TCR VB families (I). TCR-VB-CDR3 analysis was performed (II). This landscape picture can be 'corrected' by implementing TCR VB frequencies of individual VB families as defined by flow cytometry (III). Note the difference between the 'quality' (II) and the 'quality-quantity' analysis (III). T-cells represent freshly harvested CD4+ TIL.

3. DATA INTERPRETATION

3.1 Emergence of TCR clonality and preferential usage of TCR chains

In general, healthy, ‘antigen-experienced’ individuals may show clonally altered, or even monoclonal TCRs. These ‘private’ alterations in the TCR repertoire have been reported in a number of apparently healthy individuals and identified in the CD8+ T-cell population, preferentially in the CD57+ T-

cell subset (18). Monoclonal or oligoclonal TCR VB families may occur in EBV or CMV-specific T-cell responses and reflect the immunological memory of previous encounters with antigens (19, 20). Some alterations can also be identified in CD4+ T-cells, presumably reacting to common infectious agents, e.g. CMV (21). Due to structural constraints of the TCR interaction with its nominal MHC/peptide ligand, some T-cell responses are characterized by a common usage of a TCR VB family, but not with a common CDR3 motif. For instance, the HLA-A2 restricted CD8+ T-cell response targeting the influenza matrix peptide M1 (aa 58-66) shows a preferential usage of the TCR VB17 family (22) which is polyclonal in nature. Other, preferential usage of certain TCR variable families may also be present in the human population. This is difficult to assess, since these studies depend either on the generation of *bona fide* T-cell clones directed against a single MHC/peptide complex, or alternatively on tetramer-sorted T-cells. For instance, the TCR VA2.1 chain appears to be frequently utilized in CD8+ T-cells recognizing the human HLA-A2 restricted melanoma – associated antigen Melan-A/MART-1 as determined by tetramer-sorting and T-cell cloning analysis (23, 24).

3.2 Clonal tracking of T-cells

Monoclonal expansion of T-cells may be indicative for antigen-driven process either in blood or in tissue. Since each monoclonal TCR can be molecularly defined (see above), a molecular probe specific for the respective CDR3 region can be designed and the individual T-cell clone can either be traced in different compartments or longitudinally over time in a patient. This methodology has been used to tag clonal T-cell populations in autoimmune myositis (25), or in patients with multiple sclerosis (MS) suggesting that expansion of certain TCR VB families may be associated with disease onset or progression (26, 27). Similarly, if a monoclonal T-cell response can be linked to MHC peptide recognition, it allows to visualize monoclonal TCRs with defined specificity *in situ*, e.g. in a patient with melanoma responding to peptide vaccination: An TCR VB16+ Melan-A/MART-1 reactive T-cell clone could be demonstrated in a vitiligo lesion associated with destruction of melanin-positive cells (i.e. melanoma cells and normal melanocytes) (28). Similar studies have been performed to track HPV-specific T-cells in patients with cervical cancer (29). Specific T-cells can be determined in tissue by using fluorochrome-labeled probes and real-time PCR (30) using the unique composition of the CDR3 region of a T-cell clone.

3.3 Clonal expansion and link to T-cell function

Antigen specific T-cells undergo a selection process driving T-cell proliferation. In patients with cancer, clonal expansions may be associated with tumor-surveillance. This has been demonstrated in patients with melanoma undergoing peptide vaccination: molecularly defined TCRs, present in PBL and in blood, were able to recognize autologous tumor cells (9). Indeed, some T-cell responses involved in tumor containment may be mediated by a few effective T-cell clones or even a single clonotypic TCR (31).

3.4 TCR perturbation and link to therapy

TCR CDR3 analysis has been determined in patients with severe combined immunodeficiency syndrome (SCID). Of interest, the appearance of CD45RO + T-cells has been associated with a skewed TCR repertoire, and low TREC levels (T-cell receptor excision circles). In contrast, the appearance of CD45RA+ T-cells was associated with high TREC levels, a marker for thymic output and a broadened TCR repertoire (32). Particularly in patients with HIV infection, the advent of highly active retroviral therapy (HAART) has changed the prognosis and outcome of HIV infection. Progression to AIDS, the clinical presentation with opportunistic infections is associated with perturbation of the TCR repertoire. HAART is able to restore the TCR repertoire. Thus, CDR3 analysis may represent a valuable marker to compare efficacy of novel treatment protocols, together with other markers, e.g. CD4 counts and viral load determination (16, 33).

HAART drastically reduces viral load and also anti-HIV directed CD8+ T-cell responses. In order to boost anti-HIV directed T-cell responses, HAART therapy may be interrupted to stimulate CD8+ anti-HIV specific T-cells. The monitoring of this critical state is crucial for clinical management and to determine the time point to restart HAART therapy. TCR CDR3 analysis may present an objective marker to study the TCR ‘perturbation’ in the structured stop (and start) of HAART therapy.

3.5 Transplantation

Allograft rejection is mediated by T-lymphocytes, the appreciation of the T-cell infiltrate into the transplant may represent an interesting prognostic marker, or alternatively, represent a surrogate marker for testing novel drugs preventing graft rejection. TCR CDR3 analysis has been studied in the context of allograft rejection and tolerance induction, e.g. in the setting of heart transplants (15) or in the context of allogeneic hematopoietic stem cell

transplantation (34), the detailed analysis of the TCR usage may also be helpful in the molecular definition of the effector cell population mediating either graft-versus-leukemia and graft-versus-host reactions (35). More recent studies suggest that TCR CDR3 analysis may represent a valuable tool to monitor GVHD after allogeneic stem cell transplantation (36).

3.6 Link to vaccination and infection

Dramatic clonal expansion of T-cells in human healthy subjects has been reported, most of them are possibly linked to common viral pathogens, e.g. EBV or CMV. Similarly to infection with influenza A, leading to expansion of the TCR VB17 family in HLA-A2 positive individuals, the anti-EBV response is apparently driving the TCR VB6 family if the infected individual carries the HLA-B8 allele. Recognition can be linked to the HLA-B8 restricted EBV-derived peptide FLRGRAYGL (37). Tetramer complexes and TCR CDR3 analysis may also be combined to determine it the TCR repertoire changes over time as a result of therapy or vaccination. For instance, if T-cells responding to a defined antigen exist prior to (therapeutic) vaccination, a tetramer – based analysis is not able to answer the question if antigen-specific T-cells induced by the vaccine are similar or different as compared to the pre-existing T-cells. Tetramer-guided sorting, followed by TCR CDR3 analysis is able to identify molecular differences in the TCR repertoire reacting to the nominal antigens used in the vaccine (38).

3.7 In search of a culprit

The pathogenesis of a number of diseases is still not very well understood. The exact mechanisms of containment of either transformed or virally infected cells have not been determined. In general, effective adaptive T-cell responses are desirable in these diseases. The flip side of the coin – in the context of cellular immune responses – is a strong T-cell response which mediates auto-immune disorders. Many parameters exist to measure disease activity in autoimmune diseases, but the magnitude of a cellular immune response is hard to assess, particularly if no molecular targets have been identified. Since TCR CDR3 analysis visualizes objectively every alteration in the TCR composition, it may be helpful to define new markers of disease activity in autoimmune diseases; it may also present a potential matrix to gauge immuno-suppressive effects of novel drugs. For instance, TCR diversity has been suggested as a readout in PBL from patient suffering from SLE (39), or in the synovial fluid from patients with RA (40) and TCR CDR3 analysis suggested that the T-cell infiltrates in patients with Crohn's disease are indicative of an antigen-driven process (41). Recently, TCR

CDR3 analysis supported the notion that T-cells play a major role in mediating inflammation in patients with MS (42, 43) or in patients with Guillain-Barre and Fisher syndrome (44) and shed light on the association of psoriatic lesions and joint inflammation (45) as well as a certain form of polymyositis and HTLV-infection (46). In addition, TCR CDR3 analysis may be helpful to appreciate the TCR repertoire of individuals with exposure to defined pathogens. For instance, TCR CDR3 analysis in HIV+ and HIV negative men, but with 'high-risk- exposure to the pathogen showed that – in general – high antigenic exposure is associated with a restricted T-cell repertoire (47). This underlines the need to select appropriate control populations if the TCR repertoire is compared to 'healthy controls'.

4. REPORTS

Comprehensive reports may include (for CD4 / CD8-sorted T-cells) the following data:
Data acquisition and data analysis.
Absolute cell count, CD4/CD8 ratio.
Sorting: CD4/CD8 absolute count after sorting.
After mRNA isolation and cDNA transcription:
Quality control of mRNA / cDNA.
TCR variable gene analysis (for individual VA, VB, VG or VD genes as requested).
TCR CDR3 analysis:
Synopsis of each TCR V family and potentially a three dimensional data report.
Quantification of individual TCR families either by flow-cytometry (see Figure 6) and/or measuring individual TCR mRNA transcripts.
A quality control report of the laboratory which addresses the sensitivity and specificity of the TCR CDR 3 analysis, including data for inter- and intra-assay variation.
Monoclonal TCRs should be confirmed by DNA sequence analysis. More than 2 monoclonal TCRs in a healthy individual: Repeat the entire panel, including flow-cytometry, if applicable. The presence or absence of each TCR variable family should be noted. The process of TCR CDR3 analysis is compiled in Figure 7.

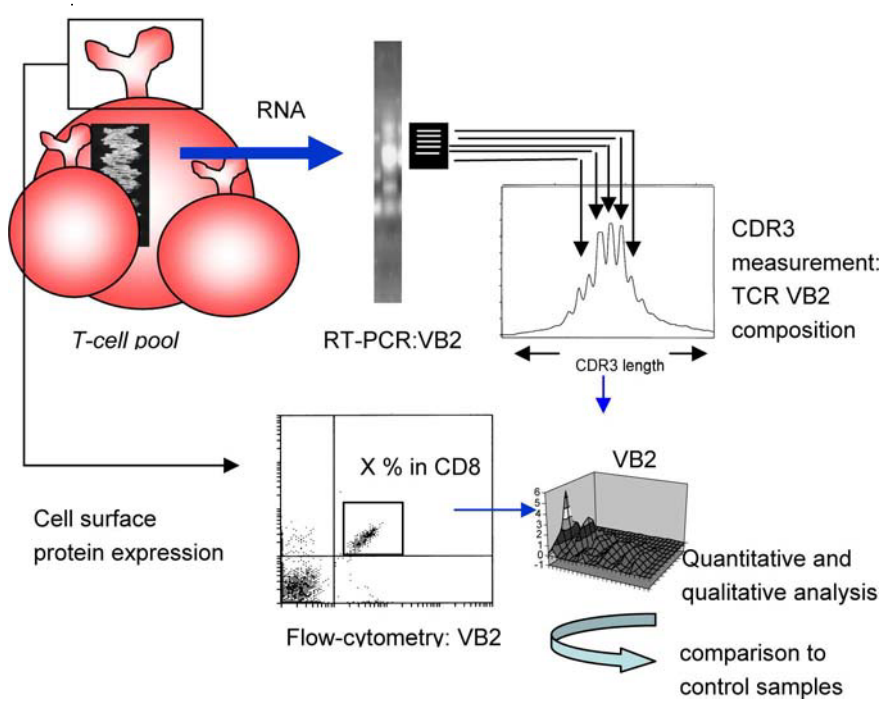


Figure 7. Compilation of TCR CDR3 fingerprinting: Amplification of each individual TCR V-family (e.g. here TCR VB2), run-off reaction and measuring the CDR3 length. T-cells may be quantified either by measuring the TCR VB mRNA content, or by flow-cytometry. Comparative analysis will aid to visualize differences in the T-cell pool, e.g. as response to therapy or any intervention affecting the cellular immune system.

If monoclonal TCR sequences are reported: report of the variable family, the joining region, D-region (for VB families) and the exact nucleotide and amino acid sequence of the CDR3 region responsible for TCR specificity.

If flow-cytometric analysis has been implemented to gauge the TCR repertoire:
List of each individual TCR VB family.
Differences in regard of quality (molecular composition) and quantity of each TCR family are reported as percent over- or under-representation as compared to

1. a control sample determined by the investigator in individual patients (i.e. prior or after therapy),
2. different anatomic compartments (e.g. TIL versus PBL), or
3. data from a normal healthy control population.

Selected reasons for false positive results (e.g.'false' monoclonality):
Insufficient number of T-cells may indicate monoclonality: higher cell 'input' will reveal the real picture (Figure 3).

False 'negative results:
Insufficient sorting/purity of T-cells, e.g. a monoclonal TCR in the CD8 population (e.g. in the TCR VB1 family) may be 'masked' by a polyclonal TCR population in the contaminating CD4+ T-cell population.
Poor mRNA isolation and quality in the target population / tissue of interest.
TCR mRNA 'turnover' may be associated with T-cell activation or resting state.

Specimens suitable for evaluation:
- Blood
- Fresh frozen tissue (not paraffin-embedded tissue, although CDR3 analysis has been performed using paraffin-embedded tissues (48)
- Biopsies
- Synovial fluid
- Cerebrospinal fluid
- Material from bronchoalveolar fluid

5. ASSESSMENT AND OUTLOOK

TCR CDR 3 analysis is a descriptive method to visualize the composition of the entire T-cell repertoire, which can be combined with other, functional readout assays of the cellular immune system. At this point, it represents a highly reproducible and high-throughput approach to gauge the T-cell repertoire in any tissue. Comparative analysis aids to compare the TCR diversity over time or in different anatomic compartments and aids to establish novel markers in the context of immunotherapy or disorders of the immune system. TCR analysis has been predominantly employed in human diseases. TCR CDR3 analysis can also be performed in murine disease models, or in non-human primates (49). The determination of the TCR repertoire may also be helpful in pre-clinical settings, i.e. in testing differences in protein or peptide composition, or vaccine formulations which affect the cellular immune response. Since T-cells are able to sense a single amino acid residue difference in antigenic peptides, the impact of subtle alterations can be reflected by differential expansion of TCR families (50). Potential indications for implementing TCR CDR3 analysis are listed in Table 1.

Table 1. Indications for TCR CDR3 fingerprinting.

Analysis	Advantage/comment(s)
Definition of the CD4+/CD8+ T-cell population in patients suffering from cancer, viral infection, or undergoing epitope-based vaccination.	This may be useful if no tetramer reagents are available, or if the entire CD4/CD8 T-cell population is examined for TCR diversity, particularly in studies addressing T-cell homeostasis.
Identification of T-cell malignancies.	Detection of monoclonal TCR transcripts and expansion of clonal T-cells.
Determination of superantigen effects on T-cells.	Expansion of certain polyclonal VB families.
Identification of individual expanded VB families in PBL or freshly harvested tumor infiltrating T-cells (TIL)	Individual VB families may be present in sufficient numbers to allow flow sorting. Such T-cells may represent effector cells directed against cancer cells or virus-associated antigens.
Longitudinal analysis of the quality and quantity of peripheral T-cell in patients undergoing T-cell 'reconstitution', e.g. during highly active anti-retroviral therapy (HAART), patients after bone marrow transplantation or patients with solid organ transplantation.	Gauging restoration of the CD4/CD8 T-cell compartment(s): Effective HAART-treatment is associated with a broad TCR-repertoire, graft monitoring after BMT, differentiation between graft versus tumor and graft versus host effects. Prognosis and assessment of tolerance in solid organ recipients.
Comparing TCR diversity within an individual patient in different anatomic compartments.	In order to compare the T-cell infiltrate in tumor (or any inflammatory) lesion, the T-cell infiltrate should be separated after CD4+/CD8+ staining in order to allow comparison with the TCR diversity within the CD4+ or CD8+ T-cell population.
Experimental: Identification of monoclonal TCRs in any tissue / anatomic compartment e.g. by laser-microdissection.	Identification of monoclonal TCRs, subsequent cloning into appropriate vector systems allows to generate TCR-transgenic T-cells which can be utilized to identify the target antigen of interest.
Experimental: Identification and tracking of clinically relevant T-cell subsets, e.g. suppressor (CD25+) T-cells, or memory T-cells mediating immune-surveillance.	'Tagging' T-cells allows following clinical relevant T-cell subsets associated with response/non-responsiveness to therapy defined by molecular marker analysis.
Pre-clinical analysis: Assessing differences in 'immunogenicity' of proteins, peptides or different antigen formulations	Alterations of the TCR repertoire in vitro may be helpful to gauge differences of antigens used for therapy. TCR CDR3 alterations may be combined with functional assays of the cellular immune system.

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