High throughput analysis of TCR-β rearrangement and gene expression in single T cells

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Analysis of T-cell receptor beta chain (TCR-β) rearrangement is essential to investigate T-cell responses in human autoimmune diseases, infection and cancer. Since the TCR-β locus contains 55 variable (V) region gene segments, multiple assays have been necessary to determine TCR-β rearrangements of individual T cells. We established a seminested rtPCR method for single T-cell analysis with two sets of degenerate primers covering 76 and 24% of the TCR-Vβ genes, respectively. The specificity of the approach was validated by screening cDNAs obtained from T-cell clones (TCC) with defined TCR-β rearrangement. We applied the method successfully to profile TCR-β rearrangement of single T cells sorted from body fluids or dissected tissue. Concomitant analysis of other gene transcripts allowed determining phenotype and function of TCR-β-defined single T cells. Our fast, cost-efficient and high throughput approach will facilitate studies on T-cell responses in human diseases.

T cells, the cellular arm of the acquired immune response, play a central role in many inflammatory diseases, autoimmunity and cancer. They recognize peptide antigens bound to HLA molecules by their T-cell antigen receptor (TCR), which is composed of heterodimeric glycoproteins (TCR-α and T-cell receptor beta chain (TCR-β)) generated by genomic rearrangement of variable (V), diversity (D), joining (J) and constant (C) region segments of each T-cell.[1] In response to antigenic challenge T cells undergo extensive clonal expansion resulting in a highly efficient antigen-specific immune response.[2–4] Analysis of the rearranged TCR genes, in particular the more polymorphic TCR-β chain provides a molecular fingerprint of each T-cell. Comparative analysis of rearranged TCR-β sequences clarifies whether T cells express a similar T-cell receptor or whether they are even derived from the same precursor cell. This approach allows identification and tracking of disease associated, *in vivo* expanded T cells.

However, the molecular analysis of rearranged TCR-β chains has been cumbersome because the TCR-β locus contains 55 different V segments.[5] Although antibodies covering more than 60% of the V TCR beta gene (TCRBV) repertoire are available for fast and reliable quantification of TCR-Vβ chain expression by flow cytometry, they do not allow analysis of TCR-β rearrangement at the molecular level.[6] To dissect the rearranged TCR-β segment sets of primers covering all V and J segments were established. This approach involving multiple PCR reactions was feasible to determine TCR-β rearrangement of T-cell clones (TCC). CDR3 spectratyping combined with sequencing was used to identify expanded TCC in polyclonal T-cell populations from blood or tissue.[7] However, the approach was not applicable for single cell analysis due to the large amount of cDNA required.[8–11] Other strategies were based on anchor or inverse PCR methods to determine TCR-β rearrangement, although they had a low sensitivity and did not allow analyzing a broad range of TCRBV genes or other transcripts at the level of single cells.[12–16] Early approaches to simplify TCR-β analysis using single degenerate consensus primers also failed due to the low sensitivity and specificity.[17,18] Alternatively, pools of primers were used in a multistep multiplex

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PCR approach at the genomic level.[9,19,20] This approach, due to the laborious procedure and high costs, was difficult to apply to large-scale TCR-β analysis. Furthermore, the genomic approach did not allow concomitant gene expression analysis of T cells. These limitations prohibited wide spread use of the technique for TCR-β analysis on the single cell level. Here, we describe a fast, cost-efficient rtPCR method to determine TCR-β rearrangement. This method simplifies TCR-β analysis to as less as one PCR reaction and allows concomitant analysis of gene expression profiling on individual T cells.

Materials and methods

Nomenclature

The nomenclature by Arden was used for the designation of TCR-Vβ chain usage of T cells. All TCR-Vβ chain sequences were extracted from the IMGT database (http://imgt.cines.fr/)).[21] For correspondence between both nomenclatures see http://imgt.cines.fr/textes/IMGTrepertoire/LocusGenes/nomenclatures/human/TRB/TRBV/Hu_TRBVnom.html.

Specimens

TCC were generated from cerebrospinal fluid (CSF) and blood of patients with inflammatory disease or brain tumors as described previously.[22] Sections of tissue from patients were used for laser capture microdissection (LCM). The study was approved by the Ethics committee of the University of Düsseldorf. Patients gave written informed consent.

Preparation of RNA and cDNA Synthesis of TCC

Approximately 2000 cells from each TCC were harvested for RNA isolation. RNA was isolated and reverse transcribed to cDNA by the RNeasy kit following the supplier's protocol (Qiagen, Superscript II; Invitrogen).

TCR-Vβ Analysis by Flow Cytometry and Conventional rtPCR

A set of anti-human TCR-Vβ monoclonal antibodies (mAb) was used to define the rearranged TCR-Vβ chains.[6] A total of 200 000 cells from each TCC were divided into seven aliquots and stained by seven mixtures of mAb separately: (1) Vβ5.1-FITC, Vβ3-FITC, Vβ17-FITC, Vβ12-PE, Vβ1-PE, Vβ3-PE 2, CD8-PerCP, CD4-APC (2) Vβ5.2-FITC, Vβ16-FITC, Vβ16-PE, Vβ2-PE, Vβ5.3-PE, CD8-PerCP, CD4-APC (3) Vβ8-FITC, Vβ7-FITC, Vβ8-PE, Vβ13.2-PE, CD8-PerCP, CD4-APC (4) Vβ11-FITC, Vβ14-FITC, Vβ9-PE, Vβ14-PE, CD8-PerCP, CD4-APC (5) Vβ13.6-FITC, Vβ22-FITC, Vβ13.1-PE, Vβ18-PE, CD8-PerCP, CD4-

APC (6) Vβ21.3-FITC, Vβ23-PE, Vβ20-PE, CD8-PerCP, CD4-APC and (7) isotype controls. Several PE- or FITC-labeled antibodies were combined in one staining, when the staining of the antibodies could be discriminated on the basis of the staining intensity. Further subtyping was performed when the combined staining did not provide clear results. Rearranged TCR-Vβ sequences were determined by rtPCR as described previously.[23]

Separating Cells from Brain Tumor

Brain tumor tissue was minced with scissors and then digested for 1 h at 37°C with 300 μg of collagenase/dispase (Boehringer, Mannhein) and 600 μg of DNase I (Boehringer, Mannheim) per ml in complete RPMI medium. The dissociated brain tissue was pelleted at 200 g for 10 min, resuspended in a 60% isotonic Percoll solution (Sigma), and overlaid with a 30% Percoll solution. Discontinuous gradients were centrifuged for 25 min at 1000 g. Brain-associated mononuclear cells were harvested from the 30/60% interphase and washed twice in complete RPMI medium before further analysis.

Sorting of Immune Cells

Peripheral blood mononuclear cells ($1 \times 10^6/200 \mu$l), glioma-derived mononuclear cells ($1 \times 10^6/200 \mu$l) and CSF cells ($5 \times 10^4/200 \mu$l) were resuspended in 100 μl PBS and stained with 10 μl of anti-human CD4-PE, CD8-APC and CD3-FITC mAb (Becton Dickinson) in a 5 ml Falcon tube, for 20 min at 4°C in the dark. The stained cells were washed twice with 4 ml of PBS and centrifuged for 4 min at 120 g. After removal of the supernatant the cell pellet was resuspended in 0.8 ml of PBS in a 5 ml Falcon tube and sorted by a FACSVantage SE (Becton Dickinson). Single T cells (CD4 + CD3 + or CD8 + CD3 +) were sorted into 96-well PCR plate containing 8 μl buffer, 0.57 μl 10%NP-40, 0.2 μl 10 × PBS, 0.57 μl random primer (300 μg/ml) (Pharmacia), 0.4 μl of 0.1 M DTT, 0.2 μl Rnasin (Promega), 0.27 μl Rnase inhibitor (Eppendorf) and 5.7 μl Rnase-free water (Ambion). After sorting, the plate was immediately put on dry ice. All samples were then stored at -80°C.

Laser Capture Microdissection of Tissue

Serial frozen sections (10 μm) were prepared from frozen brain tissue on slides for membrane-based LCM (Leica). Staining was performed with an enhancing and quick staining kit and anti-human CD8 mAb according to supplier's protocol (Dako). For every incubation step, RNaseOut inhibitor was used with the concentration of 400 U/ml. All solutions were prepared with DEPC-treated water. After dehydrating and air-drying steps, the sections

were visualized under the LCM (FW4000, Leica). Single CD8 + cells were selected and cut into PCR tubes containing buffer 7 μl of reverse transcription mix. The samples were then processed in the same way as the single T cells sorted by flow cytometry.

cDNA Synthesis from Single T Cells

To avoid DNA contamination, generation of cDNA and all rtPCR steps were performed in a separate room under a PCR workstation with a separate set of pipettes. Frozen samples were thawed on ice. Of reverse transcription mix, 7 μl (3 μl 5 × RT buffer (Invitrogen), 0.55 μl 25 mM dNTP (Invitrogen), 1.1 μl 0.1 M DTT, 0.2 μl Rnasin, 0.27 μl Rnase inhibitor, 0.2 μl reverse transcriptase (200 U/μl, Superscript III, Invitrogen) and 1.93 μl Rnase-free water) were added into each well. The final volume was 15 μl/well. Reverse transcription was performed on a GeneAmp PCR system 9700 (Perkin Elmer) 10 min at 25°C, 60 min at 55°C and 10 min at 70°C.

Designing of Degenerate Primer

In all, 55 TCR-Vβ chain sequences were listed in the IMGT database in 2003. With help of DNAMAN 5.2.10 sequence analysis software (Lynnon Corporation, Quebec, Canada), we aligned all sequences to identify regions with highest similarity. Two overlapping common motifs were identified (GTANN NACA and TGGTANNNNCAG) coding for amino acids (aa) 31, 41–44 of the TCR-Vβ chain (unique discontinues IMGT numbering). A total of 53 TCR-Vβ sequences were divided into 42 (motif 1) and 11 TCR-Vβ sequences (motif 2). Only TCR-Vβ10.1 and 16.1 did not fit with the two motifs. The following rules were then used to design primers: (1) 3′ primers were located in the high similarity regions (GTANNNACA or TGGTANNNNCAG) to cover all alleles; (2) G or C was added at the 5′ end of the primer to stabilize the degenerate primer during the annealing step of the PCR; (3) degenerate nucleotide codes were used for the positions that varied among different TCR-Vβ sequences; (4) to minimize the overall degeneracy, Inosine (I) was introduced at positions where 3 or 4 bases varied, because it forms hydrogen bonds with all four natural DNA bases (A, C, G and some what with T); (5) to further reduce degeneracy, in some cases, C-C, C-T, T-T and G-T mismatch was allowed, because G weakly binds T and pyrimidine–pyrimidine mismatch does not disrupt adjacent base-pairing.

The degenerate forward primer VP1 (GCIITK TIYTGGTAYMGACA) targets the V region (aa31, 39–44)[21] covering 42 TCR-Vβ chains, while VP2 (CTITKTWTTGGTAYCIKCAG) targets the same region with one nucleotide shift covering 11 additional TCR-Vβ chains. The primer for TCR-Vβ 16.1 and TCR-Vβ 10.1 which were not covered by the two degenerate primers, were devised separately (VP3,

TCR-Vβ 16.1: ATCTTTATTGGTATCGACGT, VP4, TCR-Vβ 10.1: ATGTTTACTGGTATCATAAG) and mixed with degenerate primer VP2 in the PCR reaction.

The specific reverse primer was located in the C region of TCR-β chain covering both C region alleles (CP1: GCACCTCCTTCCCATTCAC; aa 43–45.4 according to IMGT unique numbering for C-DOMAIN[24]). To improve the sensitivity of PCR, a nested reverse primer located upstream of the CP1 primer was designed (CP2: CACAGCGACCT CGGGTGG; located at aa 1–6).[24] All primers were designed by DNAMAN 5.2.10 and synthesized by MWG (Ebersberg, Germany).

PCR Amplification of cDNA from TCC or Single T Cells

PCR were carried out on a GeneAmp PCR system 9700 (ABI). One round of PCR was performed to amplify cDNA from TCC. Each 25 μl PCR reaction contained 1 μl cDNA, 200 μM dNTP (Invitrogen), 1 × buffer (Qiagen), 1.2 U Hotstar Taq DNA polymerase (Qiagen), and 200 nM specific primer CP1 and 2 μM degenerate primer VP1. The PCR condition were: one cycle of 94°C for 10 min followed by 40 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s with a final 10 min extension at 72°C and a 4°C hold. PCR products (5 μl) were run on 2% agarose gel. cDNA, which did not show amplification on the gel, were reamplifed in the same condition except that degenerate primer VP1 was replaced by VP2 degenerate primer (2 μM) mixed with two specific primer VP3 and VP4 for amplification of TCR-Vβ 10.1 and 16.1 (200 nM for each).

Seminested rtPCR was performed to amplify cDNA from single T cells. For the first round of PCR, each 25 μl PCR reaction contained 3 μl cDNA from a single T-cell, 200 μM dNTP, 1 × buffer, 1.2 U Hotstar Taq DNA polymerase, 200 nM specific primer CP1 and 2 μM degenerate primer VP1. The PCR condition were: one cycle of 94°C for 10 min followed by 50 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s with a final 10 min extension at 72°C and a 4°C hold. Of PCR products, 1 μl from first round of PCR were used as templates for second round of PCR, in addition, CP1 primer was replaced by CP2 nested primer. Annealing temperature was increased from 1°C to 51°C. Cycle number was reduced to 40 cycles. PCR product (5 μl) was run on 2% agarose gel. The cDNA, which did not show a PCR product on the gel, was reamplified in the same procedure except that degenerate primer VP1 was replaced by VP2 degenerate primer mixed with the two specific primer VP3 and VP4.

Sequence Analysis of PCR Products

PCR products were purified by PCR purification Kit (Qiagen). Sequencing reactions were performed

with purified PCR product and CP1 or CP2 primers by BigDye Terminator v3.1 Cycle Sequencing Kit (PE). After purified by DyeEx spin columns (Qiagen), samples were load on ABI PRISM® 310 DNA Sequencer. DNA sequences were analyzed by DNAMAN software and compared with TCR-β sequences of the IMGT database.

Gene Expression Quantification on Single Cell Level

In all, $3\,\mu$l cDNA from single T cells diluted with $6\,\mu$l water were mixed with $1\,\mu$l preformulated $20 \times$ mix which contains two unlabeled PCR primers and a FAM™ dye-labeled gene-specific (CD4 or CD8 gene) TaqMan® MGB probe (Assays-on-Demand, Applied Biosystems) and $10\,\mu$l $2 \times$ TaqMan® Universal PCR Master Mix (Applied Biosystems). Reaction systems ($20\,\mu$l) were run on ABI 7500 (Applied Biosystems) with 50 cycles and the C_t (threshold cycle) for CD8 or CD4 gene amplification was determined.

Results

Primer Design

To simplify the TCR-β rearrangement analysis, we developed an rtPCR method based on degenerate primers aimed to cover all TCR-β chains. In all, 55 TCR-Vβ chain sequences were aligned and searched for conserved areas (Figure 1). We identified a region
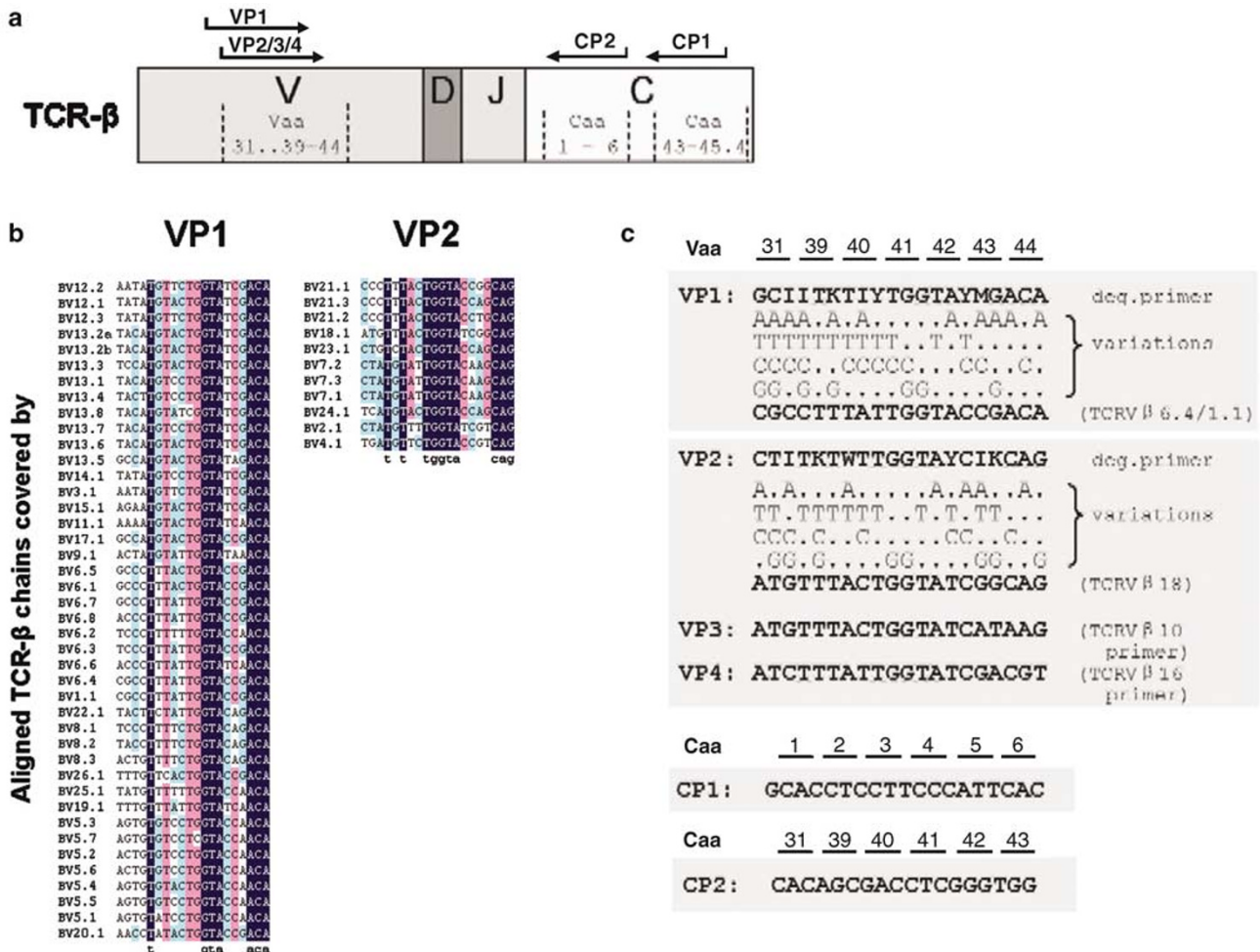

Figure 1 Primer design to cover all TCR-Vβ chains. (**a**) Structure of the rearranged TCR-β chain and localization of primers (based on the amino acid sequence (aa) of the variable (V) and constant (C) region according to the IMGT nomenclature). (**b**) All 55 TCR-Vβ sequences were aligned and a gene segment coding for V aa 31, 39–44, which matched in 9 of 20 positions for all TCR-Vβ chains was identified. The gap between 31 and 39–44 does not represent a gap in the primer sequence but is due to the unique IMGT numbering. Two degenerate primers VP1 and VP2 were designed which cover all TCR-Vβ chains except TCR-Vβ10.1 and 16.1. Displayed are all TCR-Vβ chains which are covered by primer VP1 and VP2. (**c**) The result of TCR-Vβ sequence alignment of all 55 TCR-Vβ chains is summarized. Sequence of degenerate primers VP1, VP2, VP3 and VP4 and all sequence variations introduced by TCR-Vβ chains and representative TCR-Vβ sequences covered by each degenerate primer are displayed. CP2, sequence of the constant region primer 2; CP1, sequence of the constant region primer 1; abbreviation for degenerate positions of the primers: W (A,T); M (A,C); Y (C,T); K (G,T); I (inosine).

with sequence homology among all TCR-Vβ genes except TCR-Vβ 10.1 and 16.1. This region was located in the center of the V segment gene (aa positions 31, 39–44 according to the IMGT nomenclature, http://imgt.cines.fr).[25] Two degenerate primers VP1 and VP2 were designed covering 76 and 24% of the 53 TCR-Vβ genes, respectively (Figure 1). For TCR-Vβ 10.1 and 16.1 two additional specific primers VP3 and VP4 were designed. The primers were combined with a primer located in the C region of the rearranged TCR-β gene (CP1). To increase the sensitivity of the assay for single cell analysis, we designed an additional nested primer in the C region (CP2).

Evaluation of Primer Sets

First, we investigated the specificity of the primer pairs. cDNAs were generated from more than 209 human TCC and oligoclonal T-cell cultures which had been defined for their TCR-Vβ expression by flow cytometry and conventional rtPCR amplification and sequencing. Of the cDNAs, 169 were
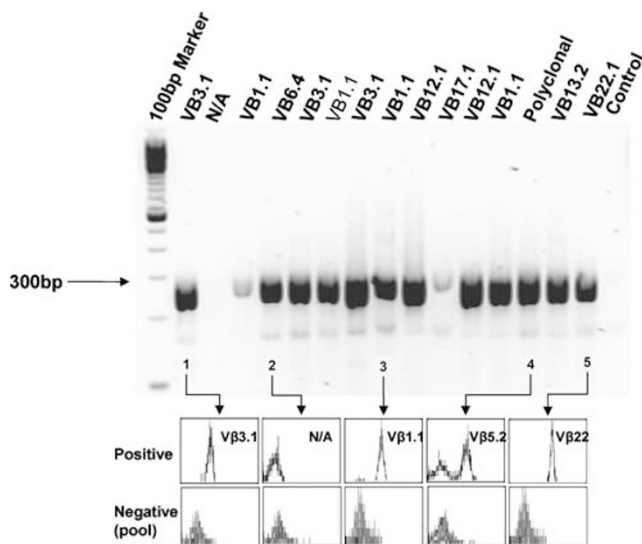
amplified by the VP1-C1 primer pair yielding PCR products and readable CDR3 and TCR-Vβ chains obtained by sequencing (80%). Negative samples were then amplified with the mix of VP2, VP3, VP4 and CP1 primers. PCR products were obtained in 18 TCCs and products successfully sequenced (11%). PCR products of 14 TCCs were obtained that did not allow successful sequencing of the CDR3 region (7%). No PCR product was obtained in eight TCCs most likely due to bad cDNA quality (4%). The obtained sequences were compared to the results of the conventional TCR-β analysis using TCR-Vβ family-specific rtPCR and flow cytometry. The results matched between these two methods when cDNA was derived from clonal T-cell populations (Figure 2). When the sample contained a polyclonal T-cell culture with more than one rearranged TCR-β chain, the amplification with the degenerate primer did not yield readable sequences (Figure 2). Overall, we identified all TCR-Jβ and most TCR-Vβ chains with this approach except the rare TCR-Vβ chains 11, 19, 24–30 which were not comprised in the 200 cDNAs analyzed (Table 1).

TCR-β Analysis of Single T Cells

Next, we applied the method to analyze the TCR-Vβ rearrangement of single T cells. Mononuclear cells separated from CSF, peripheral blood or tumor

Figure 2 Comparison of TCR-β analysis by flow cytometry and rtPCR. cDNA from 15 T-cell clones and T-cell lines which had been analyzed for their TCR-Vβ expression by flow cytometry and conventional rtPCR were amplified with the first set of degenerate primers (VP1-CP1). PCR products were visualized on an agarose gel (upper part) and sequenced. The identified TCR-Vβ sequences are displayed on top of the gel. Five representative antibody stainings of corresponding TCC are displayed below. The upper histogram displays the specific TCR-Vβ staining, the lower histogram shows all negative stainings using a mix of all other TCR-Vβ-specific antibodies. The results matched for three TCC expressing exclusively TCR-Vβ 3.1 (1), TCR-Vβ1.1 (3) and TCR-Vβ22 (5). A TCC expressing TCR-Vβ 6.4 did not stain with any of the antibodies because this Vβ is not recognized by the panel of available Abs (2). No readable sequence was obtained with a polyclonal T-cell line which stained partially with the mAb for TCR-Vβ 5.2. Polyclonal, sequence not readable due to polyclonality; control, no cDNA; N/A, specific antibody not available.

Table 1 TCR-Vβ sequences obtained by rtPCR of cDNAs generated from human TCC

TCRBV	TCRBV subtype
1	1S1[a]
2	2S1[b]
3	3S1[a]
4	4S1[b]
5	5S1[a], 5S2[a], 5S3[a], 5S5[a]
6	6S1[a], 6S2[a], 6S3[a], 6S4[a], 6S5[a], 6S6[a]
7	7S1[b]
8	8S1[a], 8S2[a], 8S3[a]
9	9S1[a]
10	10S1[b]
11	—
12	12S1[a], 12S2[a], 12S3[a]
13	13S1[a], 13S2a[a], 13S2b[b], 13S3[a], 13S5[a], 13S6[a], 13S7[a]
14	14S1[a]
15	15S1[a]
16	16S1[b]
17	17S1[a]
18	18S1[b]
19	—
20	20S1[a]
21	21S1[b], 21S3[b]
22	22S1[a]
23	23S1[b]

[a]Amplification by VP1-CP1 primer set.

[b]Amplification by VP2,3,4-CP1 primer set.

—, No TCC available expressing the particular TCR-Vβ chain. TCR-Vβ chains 24–30 were omitted due to their very low prevalence.

tissue were stained with anti-CD3, anti-CD8 and anti-CD4 mAb and single CD3 + CD4 + or CD3 + CD8 + T cells were sorted by flow cytometry. After generation of cDNA, a seminested two-step rtPCR was performed with the first degenerate primer set (VP1-CP1 for step 1, VP1-CP2 for step 2) (Figure 3a). Only samples which did not get amplified were further analyzed with the second set of primers (VP2,3,4-CP1 for step 1, VP2,3,4-CP2 for step 2). We obtained detectable PCR products from 25% of single T cells using the first and 1.5% using the second primer set (Table 2). All PCR products were sequenced to identify the rearranged TCR-β chain. The same analysis was performed with T cells isolated from tumor tissue, either separated by tissue digestion, Percoll centrifugation and cell sorting or by LCM from frozen brain tissue (Figure 3a and b). Similarly, we obtained PCR products suitable for sequencing from 22 to 25% of single T cells.
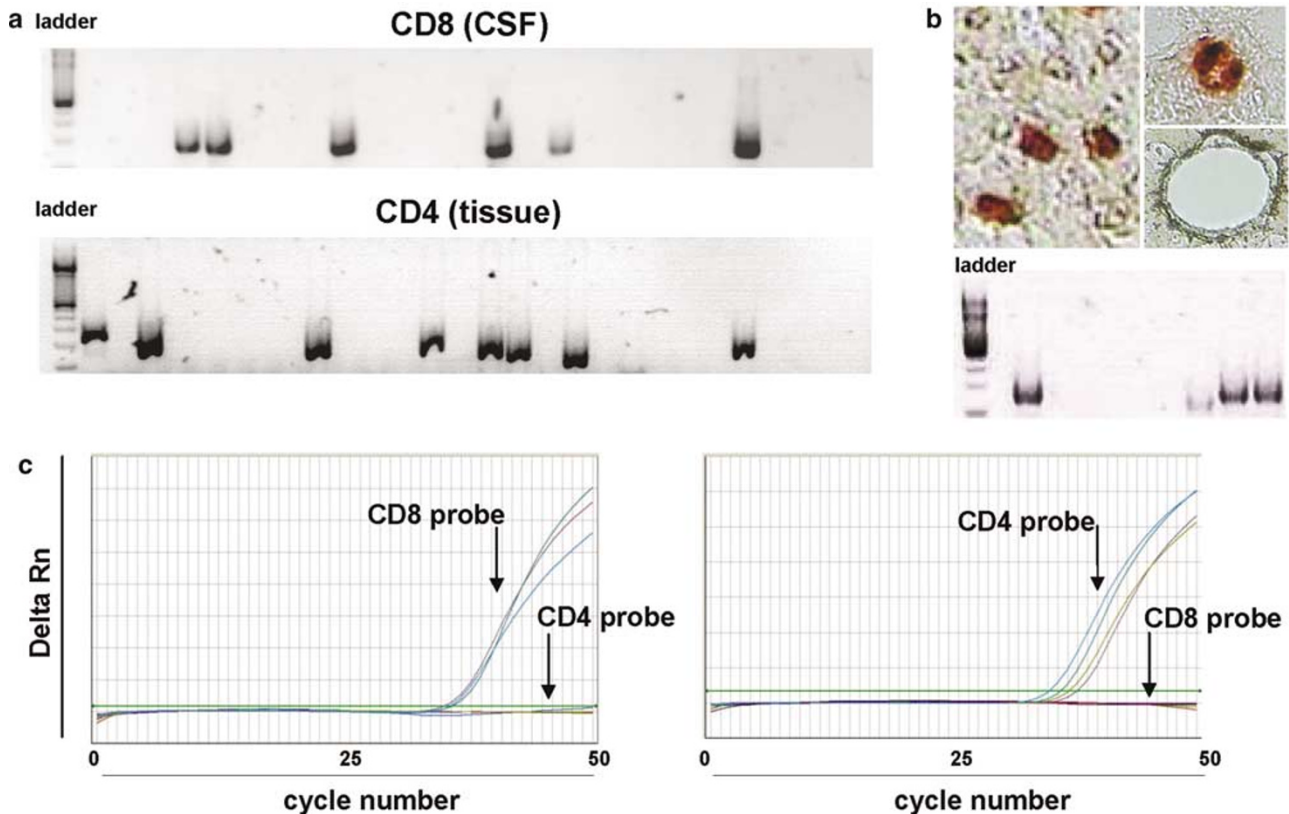

Figure 3 TCR-β chain identification and concomitant CD4 and CD8 expression analysis on single T cells isolated from cerebrospinal fluid and brain tissue. (**a**) cDNA from 24 single CD8 + T cells sorted from the CSF (upper graph) and 24 CD4 + T cells from tumor tissue (lower graph) were analyzed for the rearranged TCR-β chain by rtPCR using the VP1-CP1, VP1-CP2 primer pairs. (**b**) Staining for expression of CD8 + T cells in a brain tissue section (upper left). Positively stained CD8 + T cells were isolated from the tissue by LCM as shown for one representative CD8 + T-cell (before and after dissection; upper left) and analyzed for TCR-β expression. TCR-Vβ transcripts were amplified from single cells with theVP1-CP1, VP1-CP2 primer pair. PCR products from eight single cells are shown (lower graph). Sequencing of all four visible products allowed to define the CDR3 and TCR-Vβ sequence. (**c**) Concomitant analysis of CD4 and CD8 transcript expression in single CD3 + CD8 + (left) and CD3 + CD4 + (right) T cells isolated from tissue. All cells were defined for TCR-β chain rearrangement by amplification with the degenerate primers.

Table 2 Efficacy of single cell TCRVβ rtPCR

Source	Individual cells	TCRVβ chains amplified	Efficacy (%)
Single cells sorted from cerebrospinal fluid	192	51	26.6
Single cells sorted from brain tumor tissue	96	25	26
Single cells microdissected from brain tissue	55	12	21.8

The table displays the efficacy of the TCRVβ rtPCR amplification of cDNA from single cells either separated by cell sorting or laser capture microdissection.

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Concomitant Gene Expression Analysis on TCR-β Chain Defined T Cells

Our approach defines TCR-β rearrangement of single T cells infiltrating diseased tissues. It enables us to separate clonotypic (most likely disease associated) from polyclonal T cells (most likely nondisease associated) based on the TCR-β chain transcripts of each individual T-cell. Gene transcript analysis of tissue infiltrating clonotypic T cells can provide important insights into their function and phenotype. To test, whether gene expression analysis is possible in single T cells which have been defined for their TCR-β chain rearrangement, we performed a quantitative rtPCR for expression of CD4 and CD8. These receptors define the two major T-cell subsets CD4+ helper and CD8+ cytotoxic T cells. Single T cells were sorted according to the expression of CD4 or CD8. After determining the TCR-β chain rearrangement of these cells, gene transcript expression of CD4 and CD8 was analyzed. While CD4 transcripts were amplified from cDNA of most CD4+ T cells, no PCR products were obtained from CD8+ single cells. Similarly, CD8 transcripts were obtained from most CD8+ T cells, but none of the CD4+ T cells.

Discussion

T cells play a central role in the defence against infectious diseases and cancer but are also essential for the generation of autoimmunity.[3,4] T-cell responses are generated in the lymph nodes upon challenge with nonself antigens.[2] The few T cells that recognize the antigens with high affinity undergo extensive clonal expansion and differentiation. Armed antigen-specific T cells circulate through the body and accumulate and persist at sites of antigen exposure. While T cells with known epitope specificity can be identified and analyzed by immunological staining techniques (eg tetramer staining or intracellular cytokine release), this approach is not feasible when multiple target antigens exist or disease-associated antigens are unknown. This is particularly true for complex infectious diseases (eg most bacterial infections), diseases with yet unknown etiology (eg most autoimmune diseases) and cancer. In these diseases, TCR-β clonotype analysis at the single cell level permits identifying disease-associated T cells in the affected organ compartment. Identifying these cells provides a basis to decrypt their target antigens but also allows characterizing their phenotype and function by gene expression analysis in comparison to polyclonal T cells, which are most likely unspecifically recruited to the inflamed tissue.[26]

Several PCR-based methods for T-cell clonotype analysis are available, such as TCR-Vβ family PCR and inverse PCR, but they have low sensitivity and efficiency.[27] In addition, these methods, especially family TCR-Vβ family PCR-based method, are time consuming and require a large amount of cDNA. For example, at least 24 PCR reaction are needed for one TCC. CDR3 spectratyping technique combined with sequencing can identify clonotypic T cells in blood and tissue compartments[7] but this technique is not appropriate for large-scale screening of individual TCCs or single cell analysis.

We developed a new method which can determine rearranged TCR-β chain transcript on the single cell level using two sets of degenerate primers. We demonstrate that the majority of TCR-β chains can be amplified by this approach even on the single cell level. The efficacy of the approach at the single cell level is comparable to other methods which focus on the amplification of less complex rearranged BCR genes.[28,29] T cells isolated from diseased tissues cannot only be characterized for their TCR-β rearrangement but also for the expression of additional genes.

This approach not only significantly simplifies analysis of TCR-β expression of established TCC but also allows rapid and high throughput analysis of TCR-β expression in various compartments. Applying this strategy we can easily determine the level of T-cell clonality within inflamed organ tissues. After identification of dominant clonotypes, which likely represents the most specific and relevant compartment of the T-cell response, we can determine the gene expression profile of these cells in the diseased target organ. The approach provides an excellent tool to characterize the local T-cell response in tissues affected by infections, autoimmune diseases or cancer.

Acknowledgements

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