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Analysis of T-Cell Receptor Vβ Gene Repertoires after Immune Stimulation and in Malignancy by Use of Padlock Probes and Microarrays

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Background: **Detection of expanded T-cell clones, identified by their receptor (TCR) repertoires, can assist diagnosis and guide therapy in infectious, inflammatory, and autoimmune conditions as well as in tumor immunotherapy. Analysis of tumor-infiltrating lymphocytes often reveals preferential use of one or a few TCR Vβ genes, compared with peripheral blood, indicative of a clonal response against tumor antigens.**

Methods: **To simultaneously measure the relative expression of all Vβ gene families, we combined highly specific and sensitive oligonucleotide reagents, called padlock probes, with a microarray read-out format. T-Cell cDNA was combined with a pool of Vβ subfamily-specific padlock probes. Reacted probes were selectively amplified and the products hybridized to a microarray, from which the Vβ subfamily distribution in each sample could be determined relative to a control sample.**

Results: **In lymphocytes stimulated with the superantigen staphylococcal enterotoxin B, we detected expansions at the mRNA level of TCR subfamilies previously shown to respond to staphylococcal enterotoxin B. Expansions of the same Vβ families could also be detected by flow cytometry. In samples from two bladder cancer patients, we detected predominant representations of specific Vβ subfamilies in both tumor-infiltrating lymphocytes and in the draining lymph nodes, but not in non-tumor-draining lymph nodes or peripheral blood. Several expression profiles from draining lymph nodes in patients with malignant melanoma were divergent from profiles seen in non-tumor-draining lymph nodes.**

Conclusion: **Padlock probe-based parallel analysis of TCR Vβ gene distributions provides an efficient method for screening multiple samples for T-cell clonal expansions with reduced labor and time of analysis compared with traditional methods.**

According to the immunosurveillance hypothesis *(1)*, the immune system is continually sensitized against transformed cells and provides a first line of defense against tumor development. Solid malignant tumors are often infiltrated by T lymphocytes (TILs),[4] and extensive lymphocyte infiltrates have been associated with a more favorable prognosis in cancer patients *(2–5)*,

T cells recognize antigen-derived peptides bound to MHC molecules through T-cell receptors (TCRs), heterodimeric cell surface complexes composed of an α- and a β-chain. TCR diversity is created by imprecise somatic recombination of V and J segments for the α-chain and V, D, and J segments for the β-chain; insertion of junctional nucleotides at the V(D)J junctions; and finally by the combination of an α- and a β-chain in each T cell *(6)*. The human TCR β-chain locus is located on chromosome 7q34 and comprises 65 V segments and two clusters of D, J, and C segments *(7)*. The 46 functional V segments are grouped into 23 subfamilies, members of which share ≥75% sequence similarity, that are classified by standardized nomenclature *(8)*.

During an immune response, antigen-specific T cells undergo clonal expansion. At the cell population level, this can be detected as a skewed usage of TCR V gene

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[4] Nonstandard abbreviations: TIL, tumor-infiltrating lymphocyte; TCR, T-cell receptor for antigen; SEB, staphylococcal enterotoxin B; PBMC, peripheral blood mononuclear cell; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; PE, phycoerythrin; PerCp, peridinin chlorophyll-α protein; and SSC, standard saline citrate.

families. Vβ gene segments are the most commonly used markers of the T-cell repertoire. Assays for analyses of Vβ repertoires are typically based on PCR or antibody detection with flow cytometry *(9)*, but because of limited multiplexing ability *(10)* and the limited number of spectrally distinct fluorophores that can be combined, such assays must be performed in separate reactions for each V gene family. Parallel expression analyses using cDNA or oligonucleotide microarrays, on the other hand, may not generate reliable results because the high sequence similarity among subfamilies could lead to cross-hybridization of transcripts *(11–13)*. In addition, microarray expression and flow cytometry analyses require isolated cells in sufficient numbers, which are difficult to obtain when TILs are studied. In immunotherapy, for example, it is desirable to compare tumor-antigen-driven Vβ gene expression in TILs, tumor-draining and non-tumor-draining lymph nodes, and in peripheral blood. Accordingly, a considerable number of individual reactions must be performed, rendering the analysis laborious and costly, and requiring large amounts of sample.

We therefore developed an assay that uses padlock probes for simultaneous analysis of the entire Vβ gene family distribution in one sample. These probes are oligonucleotides whose 5′ and 3′ ends are designed to hybridize next to each other on a target sequence *(14)*. A DNA ligase can join the ends in a reaction capable of resolving minute sequence differences *(15, 16)*, thereby converting the probes to circular molecules. These can then be amplified by PCR, allowing detection of nucleic acid sequences present at very low copy numbers. In addition, the probes are sufficiently specific to detect single copy sequences in total human genomic DNA and are well suited for multiplex analysis because of their intramolecular target hybridization, which minimizes cross-reactions between different probe molecules *(17, 18)*. In the present study, we amplified circularized Vβ subfamily-specific padlock probes by PCR and identified the products by hybridization to microarrays, simultaneously revealing the relative abundance of each subfamily in relation to a reference sample. We evaluated the assay by analyzing Vβ gene expression in lymphocytes stimulated with the superantigen staphylococcal enterotoxin B (SEB) and by analyzing cDNA from patients with bladder cancer or malignant melanoma.

Materials and Methods

PADLOCK PROBES AND OLIGONUCLEOTIDES

All 96mer padlock probes (Table 1) were analyzed for secondary structures by use of the web-based mfold software (http://www.bioinfo.rpi.edu/applications/mfold/old/dna/) and were purchased 5′-phosphorylated from Thermo Hybaid. Oligonucleotide targets, amplification primers, and spot oligonucleotides were purchased from Thermo Hybaid and DNA Technology. Spot oligonucleotides were complementary to the tag sequences and had a spacer sequence [5′-NH_2-T$(CTT)_5$-3′] connected to their 5′ ends. Tag sequences were selected from the GeneFlex™ Tag Array collection (Affymetrix).

SEB STIMULATION

Peripheral blood mononuclear cells (PBMCs) from a healthy blood donor were obtained by density gradient centrifugation using Ficoll Hypaque (Amersham Biosciences), washed twice in phosphate-buffered saline, and then resuspended in RPMI 1640 (Invitrogen) supplemented with 100 mL/L human serum (Sigma-Aldrich), 2 mmol/L L-glutamine, 100 kIU/L penicillin, and 100 mg/L streptomycin. The cells were incubated at 37 °C in 5% CO_2 at 1–2 × 10^6 cells/mL. Cells were stimulated with 5 mg/L SEB (Sigma-Aldrich) for 72 h, washed, and incubated for an additional 5 days in the absence of superantigen. Cell concentration was maintained at 1–3 × 10^6 cells/mL throughout the culture period. Activation was assessed by visual examination and by flow cytometry using forward and side scatter characteristics. Samples for flow cytometry and quadruplicate samples for padlock probe analysis were obtained before cultures were supplemented with SEB and after 48 h, 72 h, 6 days, and 8 days. Four samples of RNA from each time point were collected for cDNA synthesis. Only two cDNA samples were prepared from the 48-h time point.

PATIENTS AND SPECIMENS

Tumor tissue and lymph nodes were obtained from patients undergoing surgery for malignant melanoma or urinary bladder cancer at the University Hospital of Uppsala, Sweden. The local ethics committee approved the study, and all patients gave informed consent.

Tumor-draining lymph nodes were identified by peritumoral injection of Patent Blue Dye (Guerbet) as described previously *(19, 20)*. Slices ~1 mm thick were obtained from primary tumors and from tumor-draining and non-tumor-draining lymph nodes. Specimens were processed immediately to minimize degradation of RNA.

PURIFICATION OF T CELLS

Single-cell suspensions of lymph node cells were obtained by grinding in a loose-fit glass homogenizer. Cell suspensions were washed twice in phosphate-buffered saline containing 50 mL/L fetal calf serum, 2 mmol/L EDTA, and 0.1 g/L sodium azide. TCR$\alpha\beta^+$ or $CD4^+$ cells were magnetically separated over MACS LS columns (Miltenyi Biotech), by positive selection using fluorescein isothiocyanate (FITC)-conjugated anti-TCRαβ or anti-CD4 antibody (Becton Dickinson), respectively, and anti-FITC Microbeads (Miltenyi Biotech). Alternatively, the cells were separated by negative selection with the $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech), according to the manufacturer's recommendations. Negative selection purities commonly reached 50–70%, but all TCR^+ cells were also

Table 1. Padlock probe, amplification primer, and array hybridization control sequences (5′–3′).[a]

Padlock probe	5′ Segment	General amplification sequence	Tag	3′ Segment
Vβ 1	agggaactgttgtgcgtaga	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*GTGCCTCGTTCTCGTGTAGA*	tctagttcagagtgcaagtc
Vβ 2	gtcaggcttgcatggttgat	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*GTTGACCGTTAGTTATGCGA*	ttactttcagagtggacaag
Vβ 3	tgtacccctcagtaaaatct	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*ATTTGACGAACGTATGCCGC*	attcttttctctagagacac
Vβ 4	tggatcgtcagggaagttca	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*GCGTGTGTGGACTCTCTCTA*	ttttgctatcgacttgacac
Vβ 5.1	aagtattcaaagaggaaatg	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTGGAATGTGACCGTGCTCT*	tgtctctctgtgtctcactg
Vβ 5.2	ctcagagctatagttatgga	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*GCACTAACTGGTCTGGGTCA*	aataaggcgttcacattcag
Vβ 6	tgactgcaaacaaccgatct	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTCACTAATCGTCTGCGGTG*	aaagatggatccctcaggcc
Vβ 7	tgttggggcatttaggtgat	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*ATCCGGTCTCATCGCTGAAT*	ttgaaggaataagtgagagc
Vβ 8	aacgttgttgttaaagtaaa	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*TAGGACTTGCGTCTCTGTGC*	ttccctgaatcatctatcgg
Vβ 9	gctttgtctggagatttagt	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CGAGTGCTCCGTGCGAAATA*	tgatgtgaagatttaagtga
Vβ 11	tttgagaacatttcagagtt	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*TAGCTCAGTTGTCCGGATCT*	tattttgtcattgcccatgg
Vβ 12	ttctcctttgccagtgtctt	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*TACCGCGCTTGGGACATGAT*	acactatagccatctgagac
Vβ 13	tgtaccctcaccaattgatt	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*GTCCtACATGGTGCTTCGTA*	tggatctctcctttggcagt
Vβ 14	gtatcttgggttctgggtta	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTGCCGCTGGGTCCAATATA*	ttttcagttactgtgatgag
Vβ 15	tcgagagacattgtatccaa	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*TGCGTCTATTTAGTGGAGCC*	gagaatttagcctgtgcctg
Vβ 16	catgtccagaaattgtgtca	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTGCCTGTGACTCGTGTATC*	tcgaaaccaataaagattat
Vβ 17	ttctgaaagtcatttattat	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*TGTCTATGTTTACAGCGGGC*	atccttcatctatatctcct
Vβ 18	tgcatctcagtcattcctcc	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTTCTGATGATCTATGCGCG*	actgtgtcctttcattgggc
Vβ 19	tatgtccttttttctggagta	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTGGACCTTAATCGTGTGCG*	ttgataccaataaacaaaag
Vβ 20	ctcagagctgatcaggccat	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*ATCTGTCCGACGCGAGATAT*	tctgagagattctggggcac
Vβ 21	ccagatataggattgcacca	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CGTTATCAACCTGGGTCCGA*	atcagtaaagggtagcatgg
Vβ 22	gtttccctctttttctatgat	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CAATCTAGTATCAGTGGCGC*	tatatcatttcagagtggct
Vβ 23	ctgagatttcattattataa	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CCGTGTGTGCAGAGCCTATT*	attgaatatttcagacttct
Vβ 24	aacctggtatcttgggttct	AGAGTGTACCGACCTCAGTAGCCGTGACTATCGACT	*CTGCCTGCTGTAGACTCGTA*	actagcttttcaaactgggt

PCR primers and hybridization control

	Sequence, 5′-3′
Forward primer	TAC TGA GGT CGG TAC ACT CT
Reverse primer	TAMRA[b]-AGT AGC CGT GAC TAT CGA CT
Hybridization oligonucleotide	TAMRA-TTT TTT GAC CGC GGA AGG GCT T
Hybridization control spot oligonucleotide	AAG CCC TTC AGC GGT CAA AAA A-$(TTC)_5$-T-NH_2

[a] For the padlock probes, probe ends hybridizing to second-strand cDNA of Vβ transcripts are in lower case, the general amplification segment is in upper case, and the tag sequences are in upper case italics.

[b] TAMRA, 6-carboxytetramethylrhodamine.

$CD4^+$ by fluorescence-activated cell sorting (FACS) analysis. Positively selected cell suspensions were >95% purity, as assessed by FACS analysis.

FLOW CYTOMETRIC ANALYSIS

PBMCs and lymph node cell suspensions at 1×10^5 cells/sample were washed in phosphate-buffered saline supplemented with 20 mL/L fetal calf serum and 0.5 g/L sodium azide, followed by staining with fluorophore-conjugated antibodies. The following antibodies were used: anti-TCR$\alpha\beta$ FITC, anti-CD4 FITC, anti-CD69 phycoerythrin (PE), anti-CD3 peridinin chlorophyll-α protein (PerCp), and anti-CD4 allophycocyanin (Becton Dickinson). Flow cytometric analysis of the Vβ repertoire was performed with the IOTest Beta Mark Kit (Beckman Coulter), according to the manufacturer's instructions. Briefly, 1×10^6 cells/sample were stained with a panel of 24 Vβ family-specific antibodies, combined in groups of 3 in eight tubes, one antibody being conjugated to FITC, another to PE, and the third to both FITC and PE. Costaining was performed with anti-CD3 PerCp (Becton Dickinson). A lymphocyte gate was established on the basis of forward and side scatter characteristics. The relative representation of a given Vβ family was expressed as the percentage of cells stained with the family-specific antibody among $CD3^+$ ($PerCP^+$) cells in the lymphocyte gate. Immunofluorescence was detected by flow cytometry (FACSCalibur; Becton Dickinson) and analyzed with the Cellquest software (Becton Dickinson).

RNA EXTRACTION AND cDNA SYNTHESIS

Cells were pelleted and lysed in 1 mL of TRIzol (Sigma-Aldrich), and total RNA was isolated according to the manufacturer's protocol. RNA concentrations and quality were determined on an Agilent 2100 Bioanalyzer (Agilent Technologies). We used 4 μg of RNA from SEB-stimulated PBMCs or total RNA from patient samples for

random hexamer (Gibco BRL)-primed double-stranded cDNA synthesis according to the manufacturer's protocol (Invitrogen). Samples were finally purified on a G-50 Sepharose Spin Column (Amersham Bioscience).

PADLOCK PROBE LIGATION, EXONUCLEASE TREATMENT, AND AMPLIFICATION

We combined 3 μL of ligation mixture with 7 μL of cDNA sample (H_2O in the background control reaction) for a final composition of 14 mM Tris-HCl (pH 8.3), 100 mM KCl, 7 mM $MgCl_2$, 0.35 mM NAD, 0.07 mL/L Triton X-100, 1.5 U of Ampligase (Epicentre), and 90 pM each padlock probe. Reaction mixtures were placed in a thermal cycler at 95 °C for 5 min and then cycled four times between 46 °C for 3 h, 60 °C for 10 min, and 95 °C for 2 min; after the cycling, they were kept at 8 °C until use. We added 10 μL of exonuclease mixture to the reactions for final concentrations of 67 mM Tris-HCl (pH 9.0), 50 mM KCl, 3.7 mm $MgCl_2$, 0.1 μg/μL bovine serum albumin, and 0.5 U/μL exonuclease I and exonuclease III (New England Biolabs). Reactions were incubated at 37 °C for 1 h, followed by 95 °C for 5 min. We then transferred 6 μL to 24 μL of PCR mixture for final concentrations of 30 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.9 mM $MgCl_2$, 200 μM deoxynucleotide triphosphates, 400 nM each PCR primer, and 33 mU/μL Platina Taq Polymerase (Invitrogen). Reactions were placed in a thermal cycler at 94 °C for 2 min and then cycled 25 times between 95 °C for 30 s and 56 °C for 10 s.

OLIGONUCLEOTIDE ARRAYS AND HYBRIDIZATION

Microarray slides and reusable silica masks were prepared as described by Banér et al. *(18)*. For hybridization, 30-μL PCR reactions were combined with 20 μL of 5× standard saline citrate (SSC), 1 mL/L Triton X-100, 11 mmol/L EDTA, and 0.1 nmol/L Hyb oligonucleotide (Table 1) as hybridization control. Samples were heated at 95 °C for 1 min and immediately placed on ice. We then transferred 40 μL to individual subarrays and incubated the hybridization cassette at 55 °C for 1.5 h. The silica mask was removed in a 0.02× SSC washing solution, and the slide was transferred to a 0.02× SSC wash for 10 min, followed by 10 s in 1 mmol/L $MgCl_2$, and was finally dried with pressurized air. Microarrays were scanned in a Genepix 4000B (Axon Instruments), and images were analyzed with QuantArray 2.0 software (GSI Lumonics).

Local background on the microarray was subtracted from recorded signals, and a mean was calculated from triplicate spots. Negative results were set to zero. Sample signals ($S_{V\beta x}$) were compared with the corresponding background signals ($bc_{V\beta x}$; padlock probe ligation reaction with no sample added) and set to zero if they were lower than $2(bc_{V\beta x})$. The representation of each Vβ subfamily was finally calculated as $S_{V\beta x}/\Sigma(S_{V\beta x} \ldots S_{V\beta n})$. All calculations were made in an Excel spreadsheet using a macro.

Results

PADLOCK PROBE DESIGN AND EVALUATION OF ASSAY PERFORMANCE

We designed padlock probes for 23 Vβ subfamilies, including two probes for the Vβ5 family (Table 1), positioning the ligation site within or in immediate proximity of previously described Vβ-specific PCR primer sequences *(21–23)*. We excluded the Vβ10-family-specific probe from the panel because the single member of that family has been reported to be a pseudogene *(8)*. We chose the sense strand as target, which allowed ligation either templated by RNA *(16)* or by second-strand cDNA. The segment connecting the target-complementary arms of each padlock probe contained sequences recognized by two general amplification primers, along with a specific sequence assigned to a Vβ subfamily (referred to as subfamily-specific tag sequence; Fig. 1A). The experimental procedure is described in Fig. 1B. A pool of all padlock probes was combined with a cDNA sample and ligase.

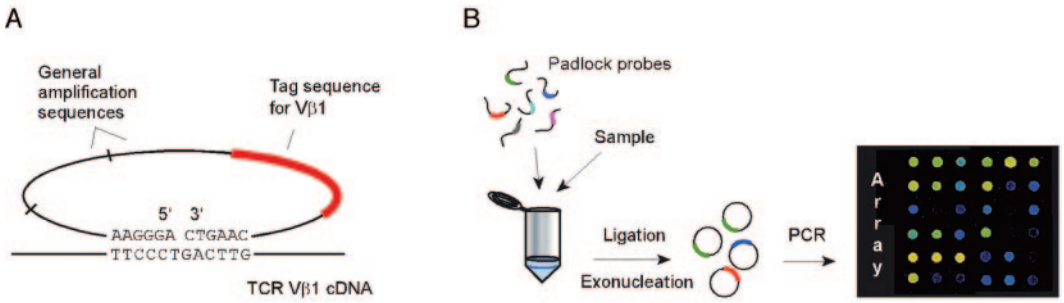

Fig. 1. Parallel analysis of TCR Vβ family expression by padlock probes.

(*A*), each subfamily-specific probe has target-complementary sequences at both the 5′ and 3′ ends, and the segment connecting these includes a general amplification sequence and a tag sequence (*red*) assigned to the subfamily. When the probe ends hybridize to a target sequence, the ends are brought in close proximity, allowing a DNA ligase to join them, converting the probe to a circular molecule. The illustration exemplifies a padlock probe for the Vβ1 subfamily hybridizing to TCR Vβ1 cDNA. For simplicity, only parts of the hybridizing sequences are shown (see Table 1). (*B*), schematic workflow of the assay. A pool of 24 padlock probes, each with a subfamily-specific tag sequence (color coded), is combined with a cDNA sample along with a ligase. After ligation, unreacted probes are degraded by exonuclease treatment, followed by PCR amplification of the circularized probes with a general primer pair, one of which is fluorescently labeled. The PCR products are applied to a microarray, where the tag sequences hybridize to complementary oligonucleotides deposited at predefined positions. Finally, the array is scanned for simultaneous readout of the Vβ distribution in the sample.

After ligation and exonucleolytic degradation of unreacted probes, all circularized padlock probes were amplified in parallel by the same PCR primer pair, one of which was fluorescently labeled. Amplification products were then hybridized to a microarray slide printed with oligonucleotides complementary to the subfamily-specific tag sequences and analyzed in a fluorescence scanner.

We investigated the repeatability of the method by assaying a pool of synthetic oligonucleotide targets several times. These data were used to determine thresholds for experimental variation of each individual Vβ subfamily. We defined a change in Vβ representation as significant if it deviated by more than 3 SD from the corresponding representation obtained with the oligonucleotide targets (data not shown). Repeated analysis of the same samples on different occasions gave very similar results, demonstrating the robustness of the method. The amount and quality of RNA from patient samples were determined on an Agilent Bioanalyzer before cDNA synthesis. Most of the patient samples contained RNA in the range of nanograms per microliter, which seemed to be the lower detection limit of the assay. We estimate that the ligation reactions required cDNA from ~50 ng of total RNA to generate reliable microarray signals (data not shown).

Analysis of SEB-stimulated PBMCs reveals expected changes in Vβ distribution

The TCRs also bind a class of microbial proteins, known as superantigens, that stimulate T cells bearing particular Vβ elements *(24)*. To demonstrate the ability of the method to detect changes in a Vβ distribution, we incubated PBMCs from healthy individuals with SEB, after which the superantigen was removed and cultures were continued for an additional 5 days. The Vβ repertoire was analyzed before stimulation and after 48 h, 72 h, 6 days, and 8 days. A fivefold increase in cell numbers was evident at 48 h, followed by a plateau and a slight decrease at day 8 (data not shown). The results of flow cytometry analysis at each time point is presented in Fig. 2A. We observed a decrease in the percentage of cells in the $CD3^+$ population expressing Vβ3, Vβ12, Vβ14, and Vβ17 at 48 and 72 h, followed by a dramatic increase in the same Vβ families at day 6, consistent with data from the literature *(23, 25)*. Measurements of Vβ distributions with the set of 24 padlock probes revealed that SEB stimulation significantly increased the representations of Vβ3, Vβ12, Vβ14, and Vβ17 (Fig. 2B, left) by 48 h. We observed a minor expansion of Vβ15, which has also previously been reported to increase on stimulation by SEB, whereas the representation of Vβ20 decreased

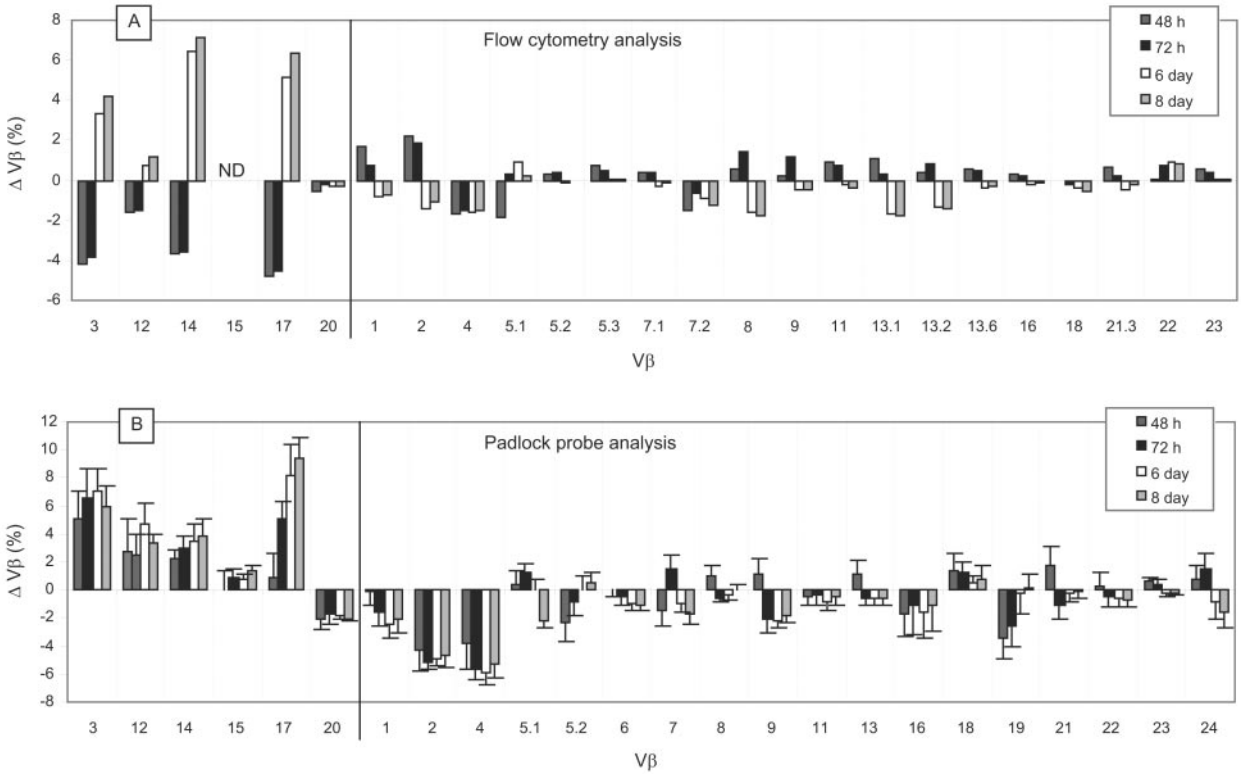

Fig. 2. SEB stimulation of T cells in vitro.

PBMCs were collected from a healthy donor and incubated in the presence of SEB for 48 h (■), 72 h (■), 6 days (□), and 8 days (▨). (*A*), flow cytometric analysis of $CD3^+$ cells. Representations of TCR Vβs are expressed as $\Delta V\beta$ ($V\beta_{SEB} - V\beta_{unstimulated}$). Cells expected to respond to SEB are shown on the *left*, separated by a *vertical line*. *ND*, no antibodies were available. (*B*), padlock probe-based analysis of Vβ distributions expressed as $\Delta V\beta$ ($V\beta_{SEB} - V\beta_{unstimulated}$). Vβ families expected to respond to SEB are shown on the *left*, separated by a *vertical line*. *Error bars* indicate SD for four samples, except for 48 h, for which two samples were analyzed (see *Materials and Methods*).

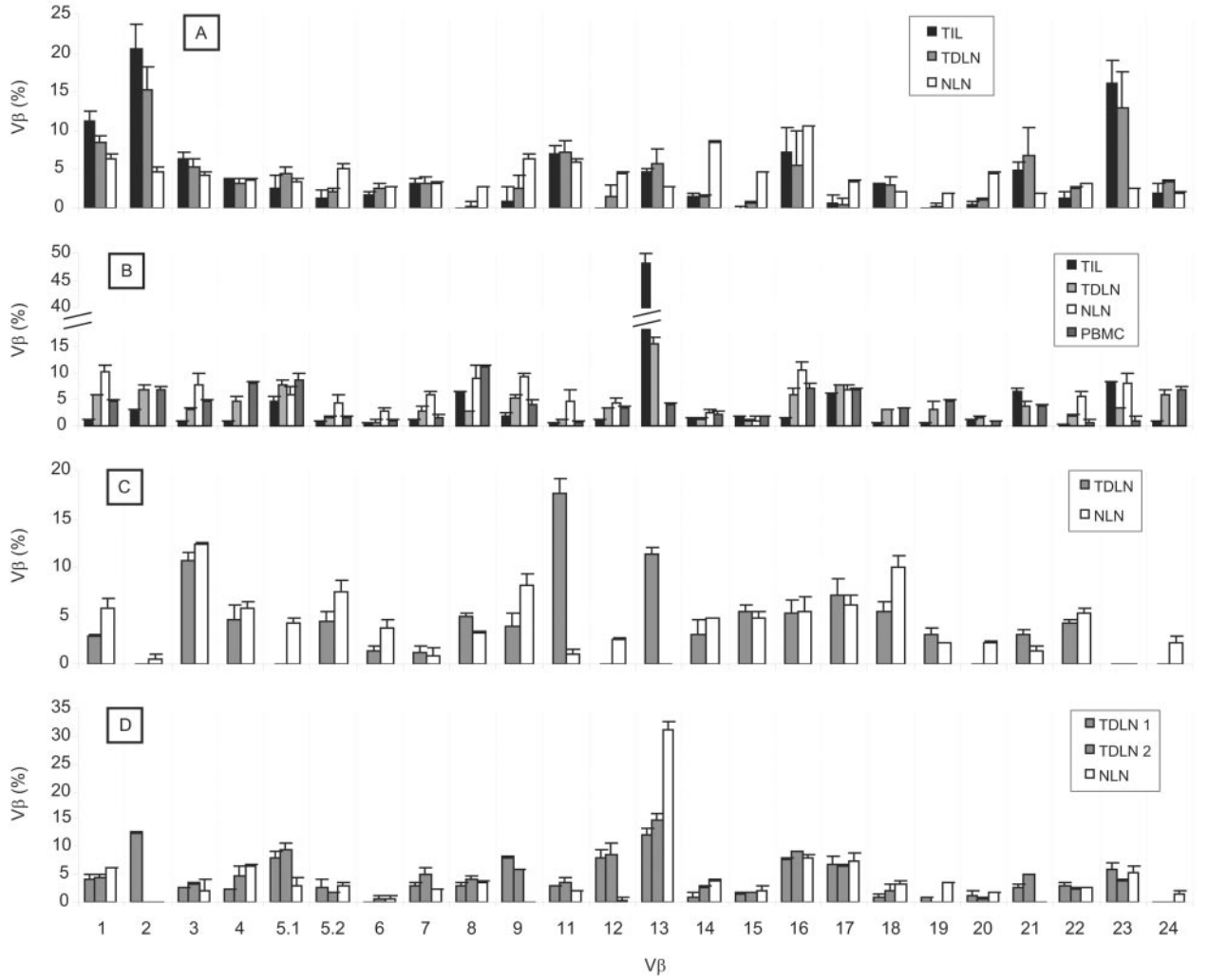

Fig. 3. Expression of Vβ families in samples from tumor patients.

(*A*), Vβ repertoire in TCR$\alpha\beta^+$ cells from a patient with squamous cell carcinoma of the bladder. ■, TILs; ▨, tumor-draining lymph nodes (*TDLN*); □, non-tumor-draining lymph nodes (*NLN*). (*B*), distribution of Vβ subfamilies in CD4$^+$ cells from a patient with adenocarcinoma of the bladder. ■, TILs; □, non-tumor-draining lymph nodes; ▨, tumor-draining lymph nodes; ■, PBMCs. (*C* and *D*), Vβ repertoires in CD4$^+$ cells from two patients with previously resected malignant melanoma. Symbols for tumor-draining (*TDLN*) and non-tumor-draining lymph nodes (*NLN*) are as in *A*. *Columns* represent the mean of two measurements; *error bars* indicate maximum values.

slightly, in contrast to its previously reported response to SEB. Flow cytometry data confirmed that no expansion of Vβ20 had occurred. In addition, Vβ18 representation increased slightly, whereas representation of the remaining Vβ gene families did not increase significantly during the investigated time period (Fig. 2B, right).

Vβ REPRESENTATIONS IN PATIENTS WITH MALIGNANT MELANOMA OR URINARY BLADDER CANCER

cDNA was prepared from total $\alpha\beta$ T cells or CD4$^+$ Th cells from tumors, and from tumor-draining and non-tumor-draining lymph nodes of patients undergoing surgery for malignant melanoma or urinary bladder cancer. In one patient with a squamous cell carcinoma of the bladder, the tumor-draining lymph node contained metastatic tumor cells. Predominant Vβ2 and Vβ23 representation, along with minor overrepresentations of Vβ13 and Vβ21, was detected among TILs and in the draining lymph node compared with the corresponding Vβ families in a non-tumor-draining lymph node (Fig. 3A). In the second patient, pathology examination revealed an adenocarcinoma confined to the bladder wall. In the TILs, Vβ13 dominated the expression profile, and the same subfamily dominated expression in the draining lymph node. On the other hand, Vβ13 expression was not detectable in a non-tumor-draining lymph node compared with the corresponding Vβ subfamilies in peripheral blood (Fig. 3B). Two patients with malignant melanoma, from whom the primary tumors had been removed on previous occasions, underwent extended lymph node dissection. The expression profile of a draining lymph node from one of these patients (Vβ7, Vβ11, and Vβ13) differed from that of the corresponding non-tumor-draining lymph node (Fig. 3C). Interestingly, two tumor-draining lymph nodes in the second patient had similar Vβ profiles, with strong representation of Vβ9 and Vβ12

and a much less prominent signal from Vβ13, compared with a non-tumor-draining lymph node (Fig. 3D).

Discussion

TCR Vβ-gene usage in selected T-cell populations can be used to detect and monitor T-cell activation and clonal expansions. We present here a method for high-resolution, sensitive screening of the expressed Vβ distribution in a sample by padlock probing with microarray-based read-out. The assay principle is straightforward, comprising a few reaction steps in a single vessel. Each reaction is internally controlled, requiring small amounts of sample, and the cost per analysis is reduced in proportion to the number of assays. Alterations in Vβ family distributions are easily detected when compared with a reference sample, such as unstimulated cells or PBMCs. The method is less well suited for absolute quantification of Vβ family expression in the present format because variations in the performance of individual probes might bias the result. For absolute quantifications, a defined reference sample, e.g., genomic non-T-cell DNA, could be used for normalization.

The method enables detection of specific Vβ expansions at the mRNA level, as demonstrated by the experiment in which SEB-cultured cells showed significant increases of Vβ families previously reported to be up-regulated by SEB *(23, 25)*. The Vβ distributions in the same samples were also analyzed by flow cytometry with a panel of TCR Vβ-specific monoclonal antibodies. The pattern of initial decrease and subsequent increase in cell surface production of SEB-specific TCRs, as well as the increased mRNA expression as early as 48 h, is consistent with previously reported dynamics of TCR production. On antigen binding, TCRs are internalized and degraded, whereas transcription of the corresponding mRNAs increases and is maintained at a high rate several days after stimulation *(26, 27)*. The experiment thus demonstrates that our method accurately reflects the current transcriptional status of the sample cells.

The assay was further applied to samples from cancer patients, demonstrating its ability to screen for clonal expansions in small biopsies obtained in a routine clinical setting. The finding of commonly expanded Vβ gene families in TILs and tumor-draining lymph node cells from a patient with bladder cancer (Fig. 3A) and their absence from non-tumor-draining lymph nodes, when compared with PBMCs, suggests an ongoing tumor-antigen-directed immune response. Analysis by flow cytometry at this point is technically difficult because of the limited cell numbers obtained from the biopsies.

In the past decade, the molecular basis of immunologic recognition of tumors has been elucidated, and numerous tumor antigens have been identified *(28)*. Many cancer immunotherapy strategies are now being tested in clinical trials *(29)*, with the common goal to activate tumor-reactive T cells, which are considered as the main effector cells of immune-mediated tumor regression. In a recent report, 13 patients with metastatic melanoma received autologous in vitro-expanded tumor-reactive T cells. The fate of the adoptively transferred T cells was investigated by flow cytometric analysis of the TCR repertoire in peripheral blood and immunohistochemistry of tumor specimens with a panel of Vβ-specific antibodies *(30)*. However, for routine clinical trials on this order of magnitude, higher throughput laboratory assays are necessary. In addition, analysis of TCR repertoires is valuable in diagnosing and monitoring T-cell malignancies *(31)*, in investigations of autoimmune conditions *(32)*, and for assessing T-cell repopulation after hematopoietic stem cell transplantation *(33)*.

In summary, padlock probe-based parallel analysis of TCR Vβ gene distributions facilitates rapid screening for clonal expansions at a reasonable cost and with reduced sample consumption because all Vβ gene families are analyzed in the same reaction. In addition, the parallel strategy allows the probe set to be conveniently expanded at any time *(17)*, for example, to analyze the representation of all members of the Vα, Vβ, Vγ, and Vδ families, without altering the assay procedure. The method could be of great benefit in clinical immunotherapy trials and in other settings in which large-scale or high-throughput analysis of TCR Vβ expression is of interest.

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