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ORIGINAL ARTICLE

Wilms' tumor protein 1 (WT1) peptide vaccination in AML patients: predominant TCR CDR3β sequence associated with remission in one patient is detectable in other vaccinated patients

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Abstract
Background Clinically effective T-cell responses can be elicited by single peptide vaccination with Wilms' tumor 1 (WT1) epitope 126–134 in patients with acute myeloid leukemia (AML). We recently showed that a predominant T-cell receptor (TCR) β chain was associated with vaccine-induced complete remission in an AML patient (patient 1). In this study, we address the question of whether this predominant clone or the accompanying $V\beta11$ restriction could be found in other AML patients vaccinated with the same WT1 peptide.
Materials and methods For assessment of $V\beta$ usage, cytotoxic T lymphocytes (CTLs) from four vaccinated patients were divided into specific and non-specific by epitope-specific enrichment. $V\beta$ families were quantified in both fractions using reverse transcribed quantitative PCR. $V\beta11$-positive 'complementary determining region 3' (CDR3) sequences were amplified from these samples, from bone marrow samples of 17 other vaccination patients, and from peripheral blood of six healthy controls, cloned and sequenced.
Results We observed a clear bias towards $V\beta11$ usage of the WT1-specific CTL populations in all four patients. The predominant CDR3β amino acid (AA) sequence of patient 1 was detected in two other patients. CDR3β loops with closely related AA sequences were only found in patient 1. There were no CDR3β AA sequences with side chains of identical chemical properties detected in any patient.
Conclusion We provide the first data addressing TCR $V\beta$ chain usage in WT1-specific T-cell populations after HLA A*0201-restricted single peptide vaccination. We demonstrate both shared $V\beta$ restriction and the sharing of a TCR β transcript with proven clinical impact in one patient.

Keywords Wilms' tumor protein 1 · Peptide vaccination · T cell receptor bias · Acute myeloid leukemia

Introduction

The transcription factor Wilms' tumor protein 1 (WT1) is a leukemia-associated antigen (LAA), which can be targeted by peptide vaccination resulting in T-cell responses in patients with acute myeloid leukemia (AML) [1–3]. WT1-specific T cells could not be detected in the peripheral blood of healthy controls ex vivo [4], but could be expanded in vitro from the T-cell repertoire of both healthy controls and patients with hematological malignancies (unpublished results [5, 6]). In phase I/II clinical trials involving vaccination with single HLA-restricted peptides, we and others have shown expansions of functional WT1-specific cytotoxic T cells (CTLs) in many patients,

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resulting in impressive clinical responses in some of these patients [1–3].

In general, both spontaneous and vaccine-induced T-cell responses result in T-cell receptor (TCR) bias due to selective pressure. Public T-cell clones are predominant T cell clones bearing similar TCR structures in multiple individuals, and have been described in the context of viral infection and autoimmunity. This concept includes both the usage of the same *variable* (*V*) region accompanied by shared short amino acid (AA) motifs in the respective 'complementarity determining region 3' (CDR3) in the α and/or the β chain as well as completely identical CDR loops [7–16]. The occurrence of shared or 'public' TCR structures may depend on the structure of the peptide-MHC I complex (pMHC I), the frequency of the TCR phenotype in the naïve repertoire, and its relative concentration in proportion to the reactive precursor pool [17–21]. Turner et al. [22] proposed the classification of this TCR bias into three types: type 1 bias is characterized by the selection of a single *V* chain or gene family but preserved CDR3 diversity, type 2 bias describes the preferred selection of conserved AA motifs within the CDR3 loops with or without restricted *V* usage, and type 3 bias is characterized by the predominance of identical AA sequences of all three CDR loops in one or both TCR chains.

A multitude of studies reported the absence of public CDR phenotypes in the context of melanoma-associated antigens [23–26] and, to our knowledge, no data have been published on shared TCR structures in the context of LAAs. Trautmann et al. [27] found a public anti-Melan-A/MART-1 CDR3α motif in four out of four melanoma patients, and a single shared AA motive in the CDR3β region was identified by Serana et al. [21] re-evaluating TCR sequences of CTL clones reactive against the melanocyte differentiation antigen Melan-A from 13 different publications. The only study directly proving the occurrence of public β chain sequences in the context of a tumor antigen is the work by Derre et al. [28], demonstrating shared CDR3β motifs in the CTL compartment against cancer-testis antigen NY-ESO-1 in at least three melanoma patients, accompanied with restriction of CDR3β length and *joining* (*J*) β gene usage.

In a previous study, we analyzed the T-cell repertoire of a patient (patient 1) with $WT1_{126–134}$ peptide vaccine-induced complete remission [29]. We detected a predominant clone of the highly restricted *V*β family TRBV11-2 (IMGT nomenclature [30]), which expanded both in blood and bone marrow (BM) after vaccination. Subsequently it decreased in both these compartments when the patient achieved complete remission. Relapse after 1 year was accompanied by a spontaneous increase of the clone in peripheral blood but not the BM. The aim of the current study was to determine whether a *V*β11 TCR bias could be detected within a population of patients treated in the same clinical trial and whether the predominant CDR3β AA sequence of patient 1 was detectable in other HLA.A2-positive patients who had received the same WT1 peptide vaccination.

Materials and methods

Patients and specimens

Samples from 20 patients with AML enrolled in a vaccination trial with the HLA A*0201-restricted peptide $WT1_{126–134}$ (RMFPNAPYL) [2] were analyzed. Two different types of samples were processed: (1) whole BM samples of 19 patients, (2) peripheral blood mononuclear cells (PBMCs) from four patients (patients 6, 8, 15, and 20). Additionally, PBMCs from six healthy controls were analyzed. Samples were drawn after informed consent of patients and healthy donors.

Epitope-specific enrichment of CTLs

Peripheral blood mononuclear cells were isolated by density gradient centrifugation using Ficoll-Hypaque (Biochrom, Cambridge, UK) and cryopreserved. CD8-positive cells were isolated from PBMCs as described [31]. To enrich epitope-specific CTLs from peripheral blood T cells, two different approaches were used: enrichment by tetramer staining (patients 6, 8, 15, 20) and by IFNγ secretion assay after incubation with the WT1 peptide (only patient 15) [29]. For tetramer enrichment, CTLs were stained with APC-labeled HLA A*0201 binding $WT1_{126–134}$ peptide tetramer and magnetically labeled with anti-APC MicroBeads (Miltenyi, Bergisch Gladbach, Germany). For selection by IFNγ secretion, CTLs were incubated for 6 h with 10 μg/ml of the $WT1_{126–134}$ peptide. CTLs were then labeled with IFNγ-Catch-Reagent (Miltenyi), and incubated while shaking for 5 min on ice and for additional 45 min at 37°C. Surface-bound IFNγ was then stained with anti-IFNγ-APC and magnetically labeled with anti-APC MicroBeads. Magnetic enrichment was performed using MACS columns (Miltenyi). Both the enriched and the depleted cell fractions were pelleted and resuspended in TRIzol for RNA extraction.

RNA extraction, cDNA synthesis

Total RNA from whole BM samples was extracted using acid guanidinium thiocyanat/phenol chloroform [32]. RNA from sorted purified CD8+ T cells and PBMCs was extracted using TRIzol (Invitrogen, Carlsbad, CA). Integrity was checked electrophoretically. Reverse transcription

was performed with Omniscript Reverse Transcriptase (Qiagen, Hilden, Germany). All BM samples were processed on separate days and independent of enriched CTL samples except the BM samples of patient 13 and 14, which were processed in parallel. Samples of cDNA were stored at −20°C.

Comparative quantification of *Vβ* families

Relative quantification of the expression of a single TCR *Vβ* chain was performed as previously described [33]. Briefly, quantitative reverse transcribed PCRs (qRT PCRs) were carried out with a universal reverse primer and TaqMan probe, both annealing at the constant segment of the *β* chain (*Cβ*) and 26 *Vβ* family-specific forward primers. The relative concentrations *P* (%) of the *Vβ* families of a sample were calculated regarding the slope and crossing point of the respective amplification reactions.

Differences of each family *i* between percentage of the enriched fraction P_{pos} and the depleted fraction P_{neg} of each patient were normalized by dividing through the standard deviation σ of each family as determined in a cohort of healthy donors [33] to avoid different weighting of *Vβ* alterations due to different physiological expression and variability as well as different amplification efficacies of the qRT PCR reactions (normalized difference $\Delta P'_i = \frac{P_{pos,i} - P_{neg,i}}{\sigma_i}$). For patient 15 the average values from both enrichment assays were used.

For statistical testing, normalized percentages P'_i were considered as independent. To address the question whether *Vβ*11 was significantly higher in the specific fractions of the four tested patients, a single one-tailed one-sample Wilcoxon signed ranks test was performed on $\Delta P'_{V\beta 11}$.

Cloning and sequencing of TCR *Vβ*11

Vβ-family 11 containing CDR3*β* regions were amplified from cDNA by conventional PCR using a forward primer annealing to 'framework region 3' and a reverse primer annealing to the constant chain *C* [33]. In two patients, additionally alternative primers were used (HBV21-2: AgA CTC CAC TCT CAA gAT CCA g, HBCrv-2: TCT CTg CTT CTg ATg gCT CA). All PCR reactions were set up in a template tamer (Qbiogene, QC, Canada) to prevent cross-contamination. PCR products of the right fragment size were excised out of agarose gels and purified using a QIA-EX II-kit (Qiagen). Fragments were cloned into a pCR2.1-TOPO vector and transformed into TOP10' using TOPO TA Cloning kit (Invitrogen). After linearization by *EcoR I* digestion, plasmids were sequenced commercially.

IMGT/V-quest tool [34] was used for identification of the *D* and *J* segment in each sequence. Alignment of the CDR3*β* AA sequences was performed with CLUSTAL 2.0.12 multiple sequence alignment tool. Similarities between the CDR3*β* sequences were identified calculating a phylogram using the neighbor joining (NJ) algorithm [35].

Deviation of the CDR3*β* lengths in patients 1, 6, and 15 were compared to the CDR3 repertoire of all other patients using aggregated means of the respective patients and the aggregated mean of the CDR3 lengths of all other patients. The latter did not deviate significantly from normal distribution according to Kolmogorov–Smirnov test. The mean CDR3 lengths of patient 1, 6, and 15 were tested separately against all other patients using a two-tailed one sample *t* test. Abundances of CDR3*β* sequences were compared using two-tailed Mann–Whitney *U* tests. In *t* tests and *U* tests, *P*-values <0.01 were considered significant in accordance to a Bonferroni correction for multiple testing.

Clone-specific qRT PCR

RT PCR quantification of the predominant *β* chain of patient 1 with primers/probes spanning the junctions between the *V*, *J*, and *C* segment as described [29] was applied to the BM samples of patient 1 as positive control as well as on BM samples of 15 other patients. These samples were identical with the samples used for cloning and sequencing.

Results

Restricted *Vβ* usage in the $WT1_{126–134}$-specific T-cell compartment

In our previous work we identified a *Vβ*11-positive predominant TCR *β* chain in the WT1-specific repertoire of an AML patient (patient 1) after peptide vaccination with corresponding *Vβ*-usage restriction [29]. To address the question of whether the predominant usage of *Vβ*11 in the specific CTL population of patient 1 could be found in other vaccinated HLA A*0201-positive patients, we selected four patients with clinical response to the vaccine and a high percentage of $WT1_{126–134}$-specific CTLs in peripheral blood (PB) and analyzed PB samples collected 2–6 months after vaccination start. Patient characteristics are given in Table 1. Two patients fulfilled the criteria for tetramer response at the time of sample collection; the two others had baseline T-cell responses before vaccination start as defined by Keilholz et al. [2] (data not shown). Comparative *Vβ* family quantification after epitope-specific enrichment was performed for five pairs of samples (enrichment by tetramer: patient 6, 8, 15, 20, enrichment by secretion assay: patient 15).

Comparing each single $V\beta$ family of the specific and the non-specific T-cell fractions of each sample, we found a strong bias in $V\beta$ usage of the WT1-specific CTL fraction towards $V\beta11$ in all four analyzed patients (Fig. 1a). Normalized percentages of $V\beta11$ were significantly higher in the specific CTL fractions ($P = 0.034$). In all patients, the normalized difference of $V\beta11$ was higher than the difference of any other $V\beta$ family, reflecting a pronounced

Table 1 Samples of peripheral T cells for epitope-specific enrichment: patient characteristics

Patient no.	Diagnosis	Clinical response[b]	No. of vac before sample collection	Months between vac start and sample collection	WT1.A2+ cells per CD3/CD8+ cells in PB (%)
6	sAML from MDS	SD 10 months	10	3	0.47
8	AML M5	SD 3 months	9	4	0.22
15[a]	AML M2	CR 41+ months	9	4	0.70
20	sAML from MDS	SD 5 months	4	2	2.11

AML acute myeloid leukemia, *CR* complete remission, *MDS* myelodysplastic syndrome, *sAML* secondary AML, *SD* stable disease, *vac* vaccination

[a] CTLs of this patient were enriched both by tetramer staining and IFNγ secretion

[b] Modified response criteria as proposed by Keilholz et al. [2]

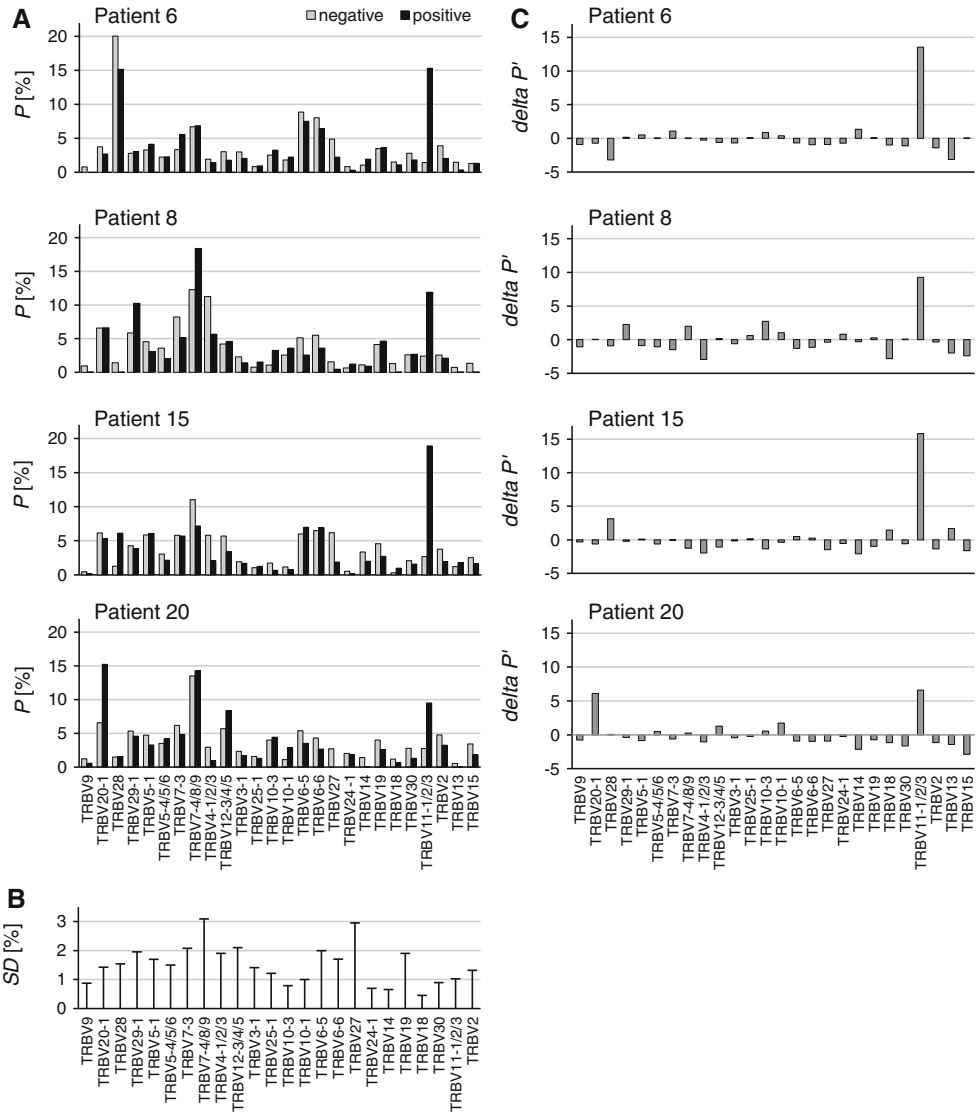

Fig. 1 Expression differences of the $V\beta$ families between the positive fractions of epitope-specific enrichment procedures and the negative (depleted) fractions of patients 6, 8, 15, and 20. **a** Relative expressions P of the quantified $V\beta$ families; **b** standard deviations of each family as determined in a set of 20 healthy controls; **c** normalized differences $\Delta P'$ calculated as described in methods. In patient 15, mean values from secretion assay and tetramer staining-based enrichment were used

type 1 bias in the CTL population specific for the vaccination epitope (Fig. 1c). In patient 15, $V\beta11$ displayed the highest normalized difference both after tetramer enrichment and IFNγ secretion assay.

The predominant CDR3β phenotype is detectable in two other patients

After the detection of this type 1 bias, we asked the question of whether the WT1-specific predominant β-CDR3 AA sequence of patient 1 could be detected in the $V\beta11$-positive TCR repertoire of other AML patients vaccinated with the identical single class I peptide. For this purpose, BM samples of 19 vaccination patients including patient 1 and PBMC samples of 6 healthy controls were processed for sequencing of $V\beta11$ containing TCR β chains. Cloning of $V\beta11$ sequences from BM was not successful in two patient samples. Patient characteristics of all others are given in Table 2. Of the 17 patients analyzed, 7 fulfilled the criteria for tetramer response in BM at the time of sample collection; 2 additional patients had baseline T-cell responses in BM before vaccination start as defined by Keilholz et al. [2] (data not shown). Furthermore, $V\beta11$ containing TCR β chains from both enrichment fractions of patients 6, 8, 15, and 20 were cloned and sequenced. Altogether 487 sequences translating to functional CDR3β loops were evaluable (232 BM samples with 7–19 sequences per patient, 178 sequences of sorted CTLs with 26–41 sequences per sample pair, and 77 from healthy controls with 7–18 sequences per donor, Supplementary material).

The predominant WT1-specific clonotype of patient 1 originally identified in a PBMC sample coded for a CDR3β loop of only nine-AA length with no detectable *D* segment (Fig. 2a). In the study reported here, this predominant genotype was detected in 14 out of 17 analyzed sequences of the BM sample of the respective patient; two of the three remaining sequences coded for CDR3 loops closely related to the predominant phenotype (Fig. 2b).

Table 2 Bone marrow samples: patient characteristics

Age (range, median)	22–83, 70
Gender (female/male)	9/8
Diagnoses	
AML (not classified)	1
AML FAB M1	2
AML FAB M2	3
AML FAB M4	3
AML FAB M5	1
AML FAB M7	1
sAML from MDS	5
MDS	1
Months between vaccination start and sample collection (range, median)	2–12, 7
Number of vaccination cycles before sample collection (range, median)	3–13, 9

AML acute myeloid leukemia, *FAB* French-American-British, *MDS* myelodysplastic syndrome, *sAML* secondary AML

Of all analyzed sequences, only 42 (9%) coded for a CRD3 loop of nine-AA length, with 22 of these containing the same *J* segment (TRBJ2-1). Shorter CDR3 loops were not detected in any of the samples. We found the predominant CDR3β phenotype in two other patients (patient 6, patient 15, Fig. 2b). Neither of these two patients had a significant CDR3β loop length restriction in $V\beta11$ towards shorter CDR3 loops as observed in patient 1 ($P < 0.001$). In all three cases, the AA sequence was coded by the identical near-germline recombination of non-mutated *V* and *J* with two random nucleotide additions at the *V*–*J* junction. This uniformity on the nucleotide level is due to the fact that in case of non-mutated *V* and *J* segments, there is only one possible combination of random nucleotides to generate an AA sequence with an acidic side chain at position 108 (IMGT [36], Fig. 2a).

We performed qRT PCR specific for the predominant clone of patient 1 in BM samples of patient 1 as positive control and of 15 other patients. None of the PCRs tested negative, but all 15 patients had normalized expressions two to four orders of magnitude lower than measured in the BM of patient 1 (Fig. 2c).

No other CDR3β sequences with similar chemical properties detected in other patients

To address the question of whether we could find CDR3β loops with physiochemically closely related properties, the 42 CDR3β loops with nine-AA length were aligned and clustered regarding their chemical properties, polar (hydrophilic) and non-polar (hydrophobic), with polar subdivided into neutral, basic, and acidic. Excluding the predominant phenotype of patient 1, 11 sequences were identified sharing the chemical properties of the predominant phenotype at least in 7 of the 9 AA positions. However, none had an acidic side chain at position 108, and only three CDR3 loops shared chemical properties in at least two AAs of the three central AAs of the loop with the predominant sequence (Fig. 3).

CDR3β sequences from the epitope-specific fractions are shared between different vaccinated patients

Evaluating the translated sequences of the CDR3β loops, we found 267 sequences appearing once, 49 sequences appearing twice, and altogether 25 with higher abundance up to 16 identical CDR3 loops in case of the predominant

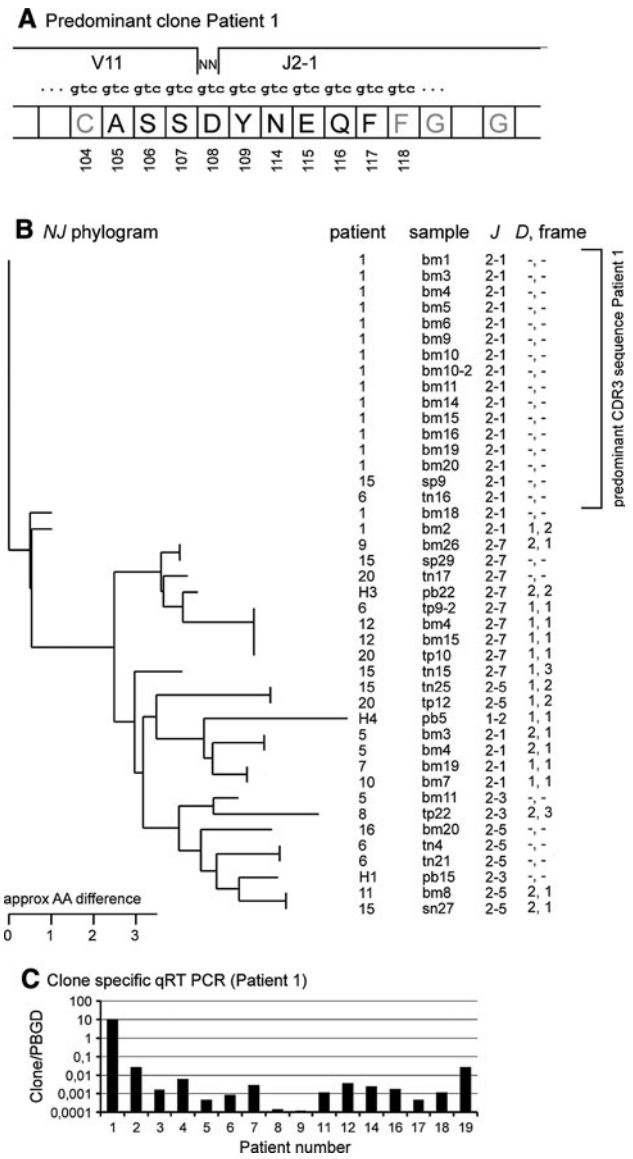

Fig. 2 Analysis of all clones coding for a CDR3β loop nine AAs in length. **a** Genotype and AA sequence of the predominant TCR *V*β chain observed in patient 1. *V*β11 and *J*β2-1 appear non-mutated; no recombined *D* is detectable. The two random nucleotides reflect the only combination resulting in an acidic side chain at position 108. **b** Neighbor joining (NJ) phylogram of all β CDR3 phenotypes of nine AA length. AA sequences identical to the predominant one were found in two other patients (patient 6, patient 15). Two sequences closely related to the predominant one were detected in patient 1. Further closely related sequences were not observed in either other patients or healthy controls (H1–H5). Sample nomenclature: *bm* bone marrow, *pb* peripheral blood, *sp/sn* secretion assay positive/negative, *tp/tn* tetramer positive/negative. **c** qRT PCR quantification of the predominant β chain of patient 1. Shown are means of duplicates from bone marrow samples of patient 1 as positive control and from bone marrow samples of 15 other vaccination patients. Data are normalized with prophobilinogen deaminase (PBGD)

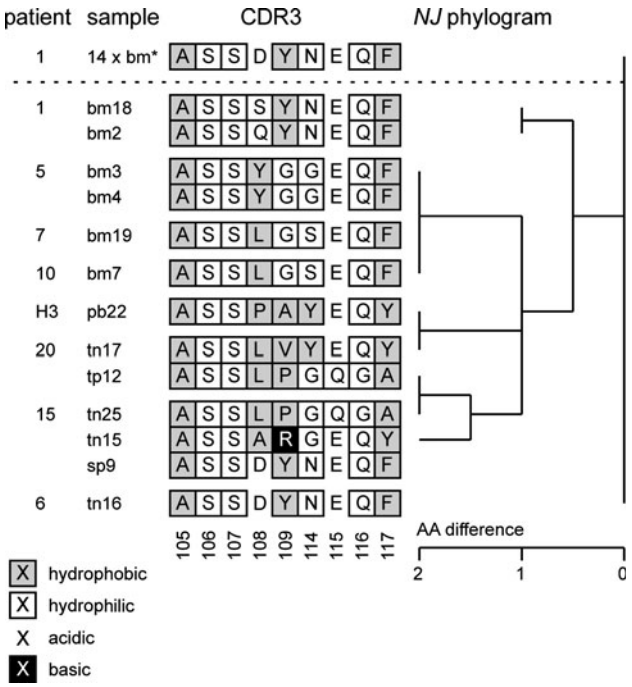

Fig. 3 Physiochemical properties of β-CDR3 loops nine AAs in length. Shown are the CDR3 loops with an AA sequence sharing the chemical properties polar (neutral, basic, acidic) and non-polar with the predominant CDR3 loop of patient 1(*) at least in seven of the nine AA positions. No other CDR3β AA sequence had an acidic side chain at position 108. Only three phenotypes shared chemical properties in at least two AAs of the three central AAs of the loop (IMGT position 108, 109, 114) with the predominant phenotype of patient 1. Sample nomenclature: see legend of Fig. 2

AA sequence of patient 1. In order to detect other shared specific CDR3 loops, we evaluated the sequences of patient 6, 8, 15, and 20. While observing six different CDR3β loops in the epitope-specific fraction of two different patients, none of the CDR3 sequences found in the depleted fraction were detected in a second patient. All shared AA sequences in the enriched fractions were detected in the BM of at least one additional patient.

Given the tetramer response in BM in half of the patients ([2] and data not shown) and the pronounced *V*β11 restriction observed in four out of four patients, we expected certain CDR3β sequences found in the specific CTL fractions to appear in the BM of other patients. All sequences obtained from negative fractions were compared with the sequences obtained from the enriched fractions. Sequences that were detected in both at least one negative and at least one positive fraction were not considered. Sequences from the specific fractions were found to have higher copy numbers in BM samples ($P < 0.001$). Because this result could be biased by the reduced diversity of the specific fractions, we considered only the number of different patients the CDR3 loops were detected in, which was higher for sequences obtained from the specific fractions compared to the ones from the depleted fractions ($P < 0.001$, Fig. 4).

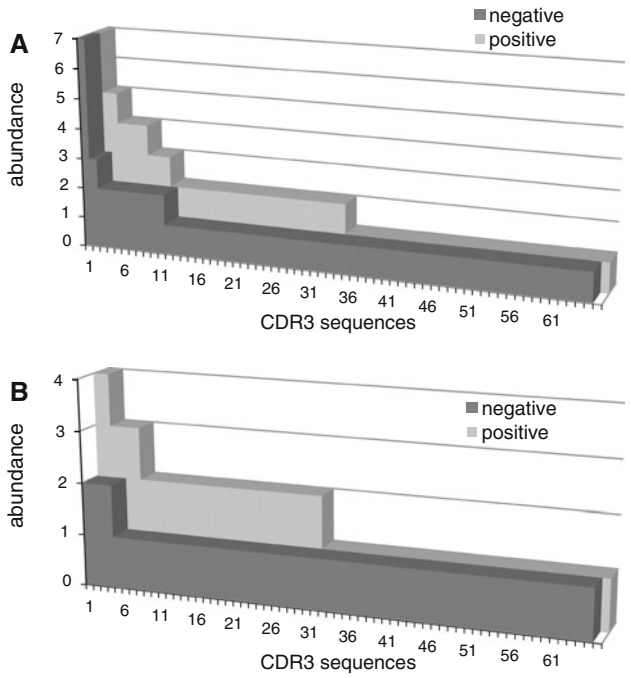

Fig. 4 CDR3β phenotype abundance. CDR3 sequences obtained from the positive fractions of epitope-specific enrichment procedures are compared to AA sequences from the negative fractions. Shown are the copy numbers of each CDR3β loop regarding all vaccinated patients. **a** Overall occurrence ($P < 0.001$); **b** number of patients in whom the identical β CDR3 loop could be detected ($P < 0.001$)

Discussion

In this work we analyzed the $WT1_{126–134}$-specific TCR β repertoire in AML patients who had received a vaccination with the respective peptide. In a previous work we reported a predominant vaccine-induced WT1-specific T-cell clone represented by a $V\beta11$-positive TCR β chain in one of these patients (patient 1) [29]. In that particular patient, the expansion of the mentioned T-cell clone was associated with an impressive clinical response. In the actual study, we analyzed T cells of four other patients vaccinated with the same WT1 peptide. Comparing the TCR $V\beta$ repertoire of the WT1-specific T cells with the non-specific repertoire, we observed a bias in the $V\beta$ usage of the WT1-specific CTLs towards the same $V\beta$ family 11 in all four patients. Subsequent sequencing the $V\beta11$-positive TCR β repertoire in BM of 17 vaccinated patients, we found the WT1-specific β chain of patient 1 in two other patients.

Characterizing the TCR repertoire in different cell compartments and subpopulations on a molecular level without generating T-cell clones has both its advantages and drawbacks. T-cell cloning always harbors the possibility of in vitro selection leading to T-cell clone sets not reliably reflecting the respective TCR repertoire in vivo. This bias can be circumvented by characterizing β transcripts directly ex vivo. On the other hand, analyzing cDNA pools makes it impossible to match corresponding α and β chains. It remains an open question whether the α/β chain pairing plays a critical role in context with inter-individual sharing of TCR features. However, published T-cell clones with public CDR3β motifs reactive against viral epitopes and autoantigens all were restricted to a single $V\alpha$ family [10, 14–16, 37]. The same $V\alpha$ restriction was observed in case of the NY-ESO-1 reactive CTL clones described by Derre [28]. Analyzing TCRs against Melan-A, Serana and colleagues did not address the α pairing in their meta-analysis, but re-evaluating the literature this publication was based on, we identified five α chains corresponding to the shared CDR3 motif in four different patients all containing the same $V\alpha$ family [27, 38]. It has been shown in a transgenic mice model that fixation of a single β chain results in selection of a single $V\alpha$ and $J\alpha$ with a highly restricted CDR3α sequence [39]. Estimating the human $\alpha\beta$ TCR diversity, Arstila and colleagues guessed that in the antigen experienced T-cell population, each α chain might pair with a unique β chain [40]. Considering these facts, it can be concluded that in case of a completely identical β chain being found in two different individuals, it has to be considered as almost certain that the respective TCRs would share the same specificity. Consequently, we decided to focus our repertoire study on the TCR β transcripts.

For MDS it has been shown that the oligoclonal expansion of WT1-specific T cells can result in detectable changes in the percentage of a few $V\beta$ families, and highly abnormal TCR $V\beta$ spectrotypes in the BM of MDS patients have been found to be associated with higher expression levels of WT1 [41, 42]. In the actual study, however, we did not address the question of a potential global TCR repertoire disturbance as a result of the WT1-peptide vaccination. Because of the low frequency of the specific cells and the likely event of a polyclonal peptide-specific CTL population, a vaccine-induced T-cell expansion would not have been detectable in a $V\beta$-family quantification of unsorted PBMCs [2]. Furthermore, it would have been impossible to distinguish between vaccine-induced T-cell responses and spontaneous expansions of T cells with other specificities. Because we wanted to analyze the T-cell fraction specific for the vaccine-peptide selectively, we performed an epitope-specific enrichment of the T cells prior to the $V\beta$ quantification step and separately compared the relative expression of each $V\beta$ family in the enriched and the depleted fraction of the same clinical specimen. This approach generated a reference data set (the non-specific fraction) for each specimen, which could be used for direct comparison and, as a consequence, enhanced the sensitivity of the assay for $V\beta$ usage restriction.

The occurrence of inter-individually shared immunodominant TCR V selection (type 1 bias) is a phenomenon

commonly observed in context with viral infection and autoreactivity. Although we could observe an impressive increase of the *Vβ*11 usage in the epitope-specific T-cell fraction of all four patients analyzed, the *Vβ*11-positive-specific CDR3 repertoires were polyclonal in all patients in spite of the detection of the predominant clonotype of patient 1 in two of them. This is in good accordance with the finding of Yokosuka et al. [39] demonstrating that in a system with a fixed α chain and random but *V*-restricted *β* chain usage, epitope reactivity could be detected in 35% of the pairings. Considering published shared clonotypes and our own unpublished data, it appears that shared *Vβ* restriction and CDR3*β* motifs are partially independent events rather than public *Vβ* restriction being an obligatory precondition for public CDR3*β* motifs [14, 43]. Accordingly, the shared Melan-A CDR3 motif identified by Serana et al. [43] was detected in clones containing seven different *Vβ* families. Furthermore, we found in the epitope-specific enriched fraction from BM of patient 8 a highly restricted *Vβ*29-1-positive CDR3*β* repertoire with a predominant clone with the same *J* usage and random nucleotides as in the predominant clone of patient 1 resulting in a six-AA consensus motif including the central acidic residue (type 2 bias, data not shown).

Vaccinating with a non-mutated self-antigen, one has to assume the stimulation of a negative selected population, i.e., a T-cell population with limited avidity. Self-antigens have been shown to elicit public *V* usage restriction and to select shared CDR3 motifs in context with autoimmune diseases [16]. Burrows showed negative selection of a shared TCR against an immunodominant EBV epitope resulting in the diversification of the remaining TCR repertoire with preserved inter-individual sharing. These data suggest that beyond a certain threshold, avidity may be a secondary factor effecting public TCR structures [14, 16]. It appears that the 'publicity' of a TCR depends on the number of patients under investigation and the percentage threshold of patients bearing a given feature [44]. Analyzing type-1-biased responses specific for a single pMHC as in our study should be a suitable condition for detecting low abundant sharing of non-dominant CDR3*β* loops because of the estimated limited diversity of the reactive repertoire. The significantly higher copy numbers and inter-individual sharing of sequences from the specific fractions observed in our patient population support the notion that a certain level of sharing of TCR structures happens even within polyclonal low avidity T-cell responses.

The grade of publicity seems to be, among others, dependent on precursor frequency, which is a function of both the possible number of recombinatorial events leading to the underlying nucleotide sequence and the number of transcripts coding for the same CDR3 phenotype [45]. The random deletion of an entire *D* segment has to be considered a highly improbable event [46]. Thus, in case of the predominant clonotype in patient 1, we must assume a direct *VJ* recombination limiting the number of random nucleotide combinations coding for the phenotype to a single one. It remains elusive whether or not the detection of a single copy of the predominant phenotype in two other patients has to be considered as subdominant expansion or reflection of an unusual high precursor frequency. Robins et al. [46] showed that the frequencies of the different CDR3 sequences in an individual could vary more than 10,000-fold, and a low number of random deletions and insertions as seen in the predominant clone of patient 1 was associated with high abundance. On the other hand, the restriction to a single pMHC corresponds with a small specific precursor pool and therefore reduced competition. Given the fact that clonal dominance is dependent not only of avidity but also of precursor competition, co-stimulatory context, and peripheral inhibition [21, 47–49], in the latter context the selection into the reactive T-cell pool appears to be likely as soon as the respective precursor is generated.

In conclusion, we provide the first data on the TCR repertoire of epitope-specific T cells in patients with AML undergoing single peptide vaccination with $WT1_{126–134}$ observing a type 1, type 2, and type 3 T-cell repertoire bias as defined by Turner [22]. We proved inter-individual sharing of *Vβ* family restriction in the WT1-specific cell compartment and could detect the predominant specific TCR *β* chain of one patient in two other patients after the start of the vaccination treatment. Further analyses are needed regarding the corresponding α chains and TCR avidities, but our observations lead to the conclusion that single epitope peptide vaccination against a WT1 epitope results in the selection of public TCR structures.

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References

1. Rezvani K, Yong AS, Mielke S, Savani BN, Musse L, Superata J, Jafarpour B, Boss C, Barrett AJ (2008) Leukemia-associated antigen-specific T-cell responses following combined PR1 and WT1 peptide vaccination in patients with myeloid malignancies. Blood 111(1):236–242. doi:10.1182/blood-2007-08-108241
2. Keilholz U, Letsch A, Busse A, Asemissen AM, Bauer S, Blau IW, Hofmann WK, Uharek L, Thiel E, Scheibenbogen C (2009) A clinical and immunologic phase 2 trial of Wilms tumor gene product 1 (WT1) peptide vaccination in patients with AML and MDS. Blood 113(26):6541–6548. doi:10.1182/blood-2009-02-202598

3. Oka Y, Tsuboi A, Murakami M, Hirai M, Tominaga N, Nakajima H, Elisseeva OA, Masuda T, Nakano A, Kawakami M, Oji Y, Ikegame K, Hosen N, Udaka K, Yasukawa M, Ogawa H, Kawase I, Sugiyama H (2003) Wilms tumor gene peptide-based immunotherapy for patients with overt leukemia from myelodysplastic syndrome (MDS) or MDS with myelofibrosis. Int J Hematol 78(1):56–61
4. Scheibenbogen C, Letsch A, Thiel E, Schmittel A, Mailaender V, Baerwolf S, Nagorsen D, Keilholz U (2002) CD8 T-cell responses to Wilms tumor gene product WT1 and proteinase 3 in patients with acute myeloid leukemia. Blood 100(6):2132–2137. doi:10.1182/blood-2002-01-0163
5. Ho WY, Nguyen HN, Wolfl M, Kuball J, Greenberg PD (2006) In vitro methods for generating CD8+ T-cell clones for immunotherapy from the naive repertoire. J Immunol Methods 310(1–2):40–52. doi:10.1016/j.jim.2005.11.023
6. Wolfl M, Kuball J, Ho WY, Nguyen H, Manley TJ, Bleakley M, Greenberg PD (2007) Activation-induced expression of CD137 permits detection, isolation, and expansion of the full repertoire of CD8+ T cells responding to antigen without requiring knowledge of epitope specificities. Blood 110(1):201–210. doi:10.1182/blood-2006-11-056168
7. Argaet VP, Schmidt CW, Burrows SR, Silins SL, Kurilla MG, Doolan DL, Suhrbier A, Moss DJ, Kieff E, Sculley TB, Misko IS (1994) Dominant selection of an invariant T cell antigen receptor in response to persistent infection by Epstein-Barr virus. J Exp Med 180(6):2335–2340
8. Babbe H, Roers A, Waisman A, Lassmann H, Goebels N, Hohlfeld R, Friese M, Schroder R, Deckert M, Schmidt S, Ravid R, Rajewsky K (2000) Clonal expansions of CD8(+) T cells dominate the T cell infiltrate in active multiple sclerosis lesions as shown by micromanipulation and single cell polymerase chain reaction. J Exp Med 192(3):393–404
9. Pantaleo G, Demarest JF, Soudeyns H, Graziosi C, Denis F, Adelsberger JW, Borrow P, Saag MS, Shaw GM, Sekaly RP et al (1994) Major expansion of CD8+ T cells with a predominant V beta usage during the primary immune response to HIV. Nature 370(6489):463–467. doi:10.1038/370463a0
10. Moss PA, Moots RJ, Rosenberg WM, Rowland-Jones SJ, Bodmer HC, McMichael AJ, Bell JI (1991) Extensive conservation of alpha and beta chains of the human T-cell antigen receptor recognizing HLA-A2 and influenza A matrix peptide. Proc Natl Acad Sci USA 88(20):8987–8990
11. Lehner PJ, Wang EC, Moss PA, Williams S, Platt K, Friedman SM, Bell JI, Borysiewicz LK (1995) Human HLA-A0201-restricted cytotoxic T lymphocyte recognition of influenza A is dominated by T cells bearing the V beta 17 gene segment. J Exp Med 181(1):79–91
12. Callan MF, Annels N, Steven N, Tan L, Wilson J, McMichael AJ, Rickinson AB (1998) T cell selection during the evolution of CD8+ T cell memory in vivo. Eur J Immunol 28(12):4382–4390. doi:10.1002/(SICI)1521-4141(199812)28:12<4382:AID-IMMU4382>3.0.CO;2-Z
13. Hong J, Zang YC, Tejada-Simon MV, Kozovska M, Li S, Singh RA, Yang D, Rivera VM, Killian JK, Zhang JZ (1999) A common TCR V-D-J sequence in V beta 13.1 T cells recognizing an immunodominant peptide of myelin basic protein in multiple sclerosis. J Immunol 163(6):3530–3538. doi:ji_v163n6p3530
14. Burrows SR, Silins SL, Moss DJ, Khanna R, Misko IS, Argaet VP (1995) T cell receptor repertoire for a viral epitope in humans is diversified by tolerance to a background major histocompatibility complex antigen. J Exp Med 182(6):1703–1715
15. Lim A, Trautmann L, Peyrat MA, Couedel C, Davodeau F, Romagne F, Kourilsky P, Bonneville M (2000) Frequent contribution of T cell clonotypes with public TCR features to the chronic response against a dominant EBV-derived epitope: application to direct detection of their molecular imprint on the human peripheral T cell repertoire. J Immunol 165(4):2001–2011. doi:ji_v165n4p2001
16. Kuwana M, Medsger TA Jr, Wright TM (1997) Highly restricted TCR-alpha beta usage by autoreactive human T cell clones specific for DNA topoisomerase I: recognition of an immunodominant epitope. J Immunol 158(1):485–491
17. Tynan FE, Burrows SR, Buckle AM, Clements CS, Borg NA, Miles JJ, Beddoe T, Whisstock JC, Wilce MC, Silins SL, Burrows JM, Kjer-Nielsen L, Kostenko L, Purcell AW, McCluskey J, Rossjohn J (2005) T cell receptor recognition of a 'super-bulged' major histocompatibility complex class I-bound peptide. Nat Immunol 6(11):1114–1122. doi:10.1038/ni1257
18. Kjer-Nielsen L, Clements CS, Brooks AG, Purcell AW, Fontes MR, McCluskey J, Rossjohn J (2002) The structure of HLA-B8 complexed to an immunodominant viral determinant: peptide-induced conformational changes and a mode of MHC class I dimerization. J Immunol 169(9):5153–5160
19. Turner SJ, Kedzierska K, Komodromou H, La Gruta NL, Dunstone MA, Webb AI, Webby R, Walden H, Xie W, McCluskey J, Purcell AW, Rossjohn J, Doherty PC (2005) Lack of prominent peptide-major histocompatibility complex features limits repertoire diversity in virus-specific CD8+ T cell populations. Nat Immunol 6(4):382–389. doi:10.1038/ni1175
20. Kedzierska K, Day EB, Pi J, Heard SB, Doherty PC, Turner SJ, Perlman S (2006) Quantification of repertoire diversity of influenza-specific epitopes with predominant public or private TCR usage. J Immunol 177(10):6705–6712. doi:177/10/6705
21. Kedl RM, Rees WA, Hildeman DA, Schaefer B, Mitchell T, Kappler J, Marrack P (2000) T cells compete for access to antigen-bearing antigen-presenting cells. J Exp Med 192(8):1105–1113
22. Turner SJ, Doherty PC, McCluskey J, Rossjohn J (2006) Structural determinants of T-cell receptor bias in immunity. Nat Rev Immunol 6(12):883–894. doi:10.1038/nri1977
23. Cole DJ, Wilson MC, Rivoltini L, Custer M, Nishimura MI (1997) T-cell receptor repertoire in matched MART-1 peptide-stimulated peripheral blood lymphocytes and tumor-infiltrating lymphocytes. Cancer Res 57(23):5320–5327
24. Dietrich PY, Walker PR, Quiquerez AL, Perrin G, Dutoit V, Lienard D, Guillaume P, Cerottini JC, Romero P, Valmori D (2001) Melanoma patients respond to a cytotoxic T lymphocyte-defined self-peptide with diverse and nonoverlapping T-cell receptor repertoires. Cancer Res 61(5):2047–2054
25. Pittet MJ, Gati A, Le Gal FA, Bioley G, Guillaume P, de Smedt M, Plum J, Speiser DE, Cerottini JC, Dietrich PY, Romero P, Zippelius A (2006) Ex vivo characterization of allo-MHC-restricted T cells specific for a single MHC-peptide complex. J Immunol 176(4):2330–2336. doi:176/4/2330
26. Sensi M, Traversari C, Radrizzani M, Salvi S, Maccalli C, Mortarini R, Rivoltini L, Farina C, Nicolini G, Wolfel T et al (1995) Cytotoxic T-lymphocyte clones from different patients display limited T-cell-receptor variable-region gene usage in HLA-A2-restricted recognition of the melanoma antigen Melan-A/MART-1. Proc Natl Acad Sci USA 92(12):5674–5678
27. Trautmann L, Labarriere N, Jotereau F, Karanikas V, Gervois N, Connerotte T, Coulie P, Bonneville M (2002) Dominant TCR V alpha usage by virus and tumor-reactive T cells with wide affinity ranges for their specific antigens. Eur J Immunol 32(11):3181–3190. doi:10.1002/1521-4141(200211)32:11<3181:AID-IMMU3181>3.0.CO;2-2
28. Derre L, Bruyninx M, Baumgaertner P, Ferber M, Schmid D, Leimgruber A, Zoete V, Romero P, Michielin O, Speiser DE, Rufer N (2008) Distinct sets of alphabeta TCRs confer similar recognition of tumor antigen NY-ESO-1157–165 by interacting with its central Met/Trp residues. Proc Natl Acad Sci USA 105(39):15010–15015. doi:10.1073/pnas.0807954105

29. Ochsenreither S, Fusi A, Busse A, Bauer S, Scheibenbogen C, Stather D, Thiel E, Keilholz U, Letsch A (2011) "Wilms Tumor Protein 1" (WT1) peptide vaccination-induced complete remission in a patient with acute myeloid leukemia is accompanied by the emergence of a predominant T-cell clone both in blood and bone marrow. J Immunother 34(1):85–91. doi:10.1097/CJI.0b013e3181f3cc5c
30. Giudicelli V, Chaume D, Lefranc MP (2005) IMGT/GENE-DB: a comprehensive database for human and mouse immunoglobulin and T cell receptor genes. Nucleic Acids Res 33 (Database issue):D256–D261. doi:10.1093/nar/gki010
31. Ochsenreither S, Fusi A, Busse A, Letsch A, Haase D, Thiel E, Scheibenbogen C, Keilholz U (2009) Long term presence of a single predominant tyrosinase-specific T-cell clone associated with disease control in a patient with metastatic melanoma. Int J Cancer. doi:10.1002/ijc.24939
32. Keilholz U, Golding-Lang P, Bechrakis NE, Max N, Letsch A, Schmittel A, Scheibenbogen C, Heufelder K, Egermont A, Thiel E (2004) Quantitative detection of circulating tumor cells in cutaneous and ocular melanoma and quality assessment by real-time reverse transcriptase-polymerase chain reaction. Clin Cancer Res 10:1605–1612
33. Ochsenreither S, Fusi A, Busse A, Nagorsen D, Schrama D, Becker J, Thiel E, Keilholz U (2008) Relative quantification of TCR Vbeta-chain families by real time PCR for identification of clonal T-cell populations. J Transl Med 6:34. doi:10.1186/1479-5876-6-34
34. Brochet X, Lefranc MP, Giudicelli V (2008) IMGT/V-QUEST: the highly customized and integrated system for IG and TR standardized V-J and V-D-J sequence analysis. Nucleic Acids Res 36 (Web Server issue):W503–W508. doi:10.1093/nar/gkn316
35. Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol 4(4):406–425
36. Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, Wu Y, Gemrot E, Brochet X, Lane J, Regnier L, Ehrenmann F, Lefranc G, Duroux P (2009) IMGT, the international ImMunoGeneTics information system. Nucleic Acids Res 37 (Database issue):D1006–D1012. doi:10.1093/nar/gkn838
37. Trautmann L, Rimbert M, Echasserieau K, Saulquin X, Neveu B, Dechanet J, Cerundolo V, Bonneville M (2005) Selection of T cell clones expressing high-affinity public TCRs within human cytomegalovirus-specific CD8 T cell responses. J Immunol 175(9):6123–6132. doi:175/9/6123
38. Vignard V, Lemercier B, Lim A, Pandolfino MC, Guilloux Y, Khammari A, Rabu C, Echasserieau K, Lang F, Gougeon ML, Dreno B, Jotereau F, Labarriere N (2005) Adoptive transfer of tumor-reactive Melan-A-specific CTL clones in melanoma patients is followed by increased frequencies of additional Melan-A-specific T cells. J Immunol 175(7):4797–4805. doi:175/7/4797
39. Yokosuka T, Takase K, Suzuki M, Nakagawa Y, Taki S, Takahashi H, Fujisawa T, Arase H, Saito T (2002) Predominant role of T cell receptor (TCR)-alpha chain in forming preimmune TCR repertoire revealed by clonal TCR reconstitution system. J Exp Med 195(8):991–1001
40. Arstila TP, Casrouge A, Baron V, Even J, Kanellopoulos J, Kourilsky P (1999) A direct estimate of the human alphabeta T cell receptor diversity. Science 286(5441):958–961. doi:7939
41. Campregher PV, Srivastava SK, Deeg HJ, Robins HS, Warren EH (2010) Abnormalities of the alphabeta T-cell receptor repertoire in advanced myelodysplastic syndrome. Exp Hematol 38(3):202–212. doi:10.1016/j.exphem.2009.12.004
42. Sloand EM, Melenhorst JJ, Tucker ZC, Pfannes L, Brenchley JM, Yong A, Visconte V, Wu C, Gostick E, Scheinberg P, Olnes MJ, Douek DC, Price DA, Barrett AJ, Young NS (2011) T-cell immune responses to Wilms tumor 1 protein in myelodysplasia responsive to immunosuppressive therapy. Blood 117(9):2691–2699. doi:10.1182/blood-2010-04-277921
43. Serana F, Sottini A, Caimi L, Palermo B, Natali PG, Nistico P, Imberti L (2009) Identification of a public CDR3 motif and a biased utilization of T-cell receptor V beta and J beta chains in HLA-A2/Melan-A-specific T-cell clonotypes of melanoma patients. J Transl Med 7:21. doi:10.1186/1479-5876-7-21
44. Venturi V, Price DA, Douek DC, Davenport MP (2008) The molecular basis for public T-cell responses? Nat Rev Immunol 8(3):231–238. doi:10.1038/nri2260
45. Venturi V, Kedzierska K, Price DA, Doherty PC, Douek DC, Turner SJ, Davenport MP (2006) Sharing of T cell receptors in antigen-specific responses is driven by convergent recombination. Proc Natl Acad Sci USA 103(49):18691–18696. doi:10.1073/pnas.0608907103
46. Robins HS, Campregher PV, Srivastava SK, Wacher A, Turtle CJ, Kahsai O, Riddell SR, Warren EH, Carlson CS (2009) Comprehensive assessment of T-cell receptor beta-chain diversity in alphabeta T cells. Blood 114(19):4099–4107. doi:10.1182/blood-2009-04-217604
47. Yoshinaga SK, Whoriskey JS, Khare SD, Sarmiento U, Guo J, Horan T, Shih G, Zhang M, Coccia MA, Kohno T, Tafuri-Bladt A, Brankow D, Campbell P, Chang D, Chiu L, Dai T, Duncan G, Elliott GS, Hui A, McCabe SM, Scully S, Shahinian A, Shaklee CL, Van G, Mak TW, Senaldi G (1999) T-cell co-stimulation through B7RP-1 and ICOS. Nature 402(6763):827–832. doi:10.1038/45582
48. de Souza AP, Bonorino C (2009) Tumor immunosuppressive environment: effects on tumor-specific and nontumor antigen immune responses. Expert Rev Anticancer Ther 9(9):1317–1332. doi:10.1586/era.09.88
49. Gaur U, Aggarwal BB (2003) Regulation of proliferation, survival and apoptosis by members of the TNF superfamily. Biochem Pharmacol 66(8):1403–1408. doi:S0006295203004908