Gamma/delta tumor infiltrating lymphocytes selectively infiltrate human renal cell carcinomas

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Abstract

Although γδ T cells are thought to participate in tumor immunosurveillance, little is known about their diversity in human cancers. Previously, we shown that γδ tumor infiltrating lymphocytes (TIL) may selectively infiltrate into human solid tumors and undergo activation. Here we compared γδ T cell receptor (TCR) repertoire and complementarity determining region (CDR3) size distribution in TIL, peripheral blood lymphocytes (PBL) and peritumoral tissue from 19 renal cell carcinoma (RCC) patients.

We first analyzed Vδ gene segment usage by flow cytometry (FCM) using monoclonal antibodies (anti- Vδ–1 and Vδ2) and PCR using a panel of Vδ family specific oligonucleotide primers (Vδ1-Vδ6). We observed a small, however consistent decrease of γδ T cells in TIL comparing to autologous PBL. FCM analysis of Vδ usage showed decrease of Vδ2 and increase of Vδ1 subpopulation in TIL. Further, we studied clonal diversity of each particular Vδ family by CDR3 size analysis using run off PCR. A detailed analysis of CDR3 size distribution revealed striking differences between γδ TIL, PBL and normal tissue. γδ T cells at tumor site were found to present clonal expansions over a more or less complex polyclonal background. Our data show selective infiltration of γδ T cells into tumor tissue.

Key words: *renal cell carcinoma, tumor infiltrating lymphocytes, T cell receptor repertoire, gamma/delta T cells, polymerase chain reaction, flow cytometry, tumor immunology, tumor, cancer.*

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Introduction

The stroma of certain types of human neoplasms are characteristically associated with intense lymphocytic infiltration. This include malignant melanoma, germinal cell tumors, medullary breast carcinoma and renal cell carcinoma (RCC) [1-5].

Although several authors indicate on significant association between degree of lymphocytic infiltration and prognosis for the patient [1-3, 5] these tumor infiltrating lymphocytes (TIL) appear to be functionally deficient and have only modest influence on tumor growth in vivo as it is evidenced from tumor progression [6-9].

However, their dormant state is reversible and several studies have shown that T cell clones or cell lines derived from TIL mediate specific anti-tumor functions upon culture with recombinant interleukin 2 (IL-2) [10-14]. Whereas, numerous observations indicate that host may spontaneously elicit antitumor response [for rev. see 15-18] however, it is still difficult to make this response more effective. Therefore, analysis of immunological mechanisms which may be involved in cessation of tumor growth and development is very relevant.

Specific recognition of tumor antigens is based on the interaction between the MHC-peptide complex and the monospecific T cell receptor (TCR) [19]. The latter is a heterodimeric protein composed of either the TCR α and β or TCR γ and δ chains [19]. Each TCR chain consists of a variable region which determines the antigen specificity,

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and a constant region. During T cell differentiation, unique variable region genes are created by recombination of variable (V), diversity (D), and joining (J) segments for the β and δ loci and V and J segments for the α and γ loci. Random combination of these segments as well as the pairing of the two chains generate combinatorial diversity, which is further increased by imprecise V-(D)-J joining and the additions of N and P nucleotides between V and D or D and J segments during the recombination process [19]. The V(D)J junction actually represents the complementarity determining region (CDR3), which plays a crucial role in antigen recognition [20].

It is postulated that during the course of an immune response, there is selective expansion of T cells sharing common TCR features. Following the observation that T cells in autoimmune lesions exhibit a limited set of TCR αβ cells, several investigators have attempted to identify biases in the TCR repertoire in TIL that might reflect an antigen driven expansions of potentially tumor reactive T cells [rev. in 21, 22].

However, while TCR αβ+ T cells have been analyzed extensively in a variety of tumors [23-29], the function and significance of γδ T cells in anti-tumor reactivity is less understood [30, 31]. In contrast to αβ T cells γδ T cells represent a minor population of peripheral blood T lymphocytes [32]. The majority of them do not express CD4 or CD8 molecules and often appear restricted to molecules other than classical MHC [33-35]. Due to the uniqueness of tissue distribution, TCR structure and cytotoxic functions, they are considered to recognize a different set of antigens and have distinct functions from those of TCR αβ cells [33-35].

In agreement with other reports [36-37], we observed that γδ T lymphocytes may selectively infiltrate into human malignant tumors [38]. While analyzing the phenotype of these TIL, we noticed that γδ T cells in tumor site are activated more than other CD3+ cells [39, 40]. In the present study we extend this observations on analysis of the TCR δ chain repertoire complexity based on the V-J junctional diversity. Here we compared CDR3 size heterogenity within Vδ gene families between γδ T cells from tumor, blood and normal renal tissue from renal cell carcinoma patients.

Materials and methods

Patients, blood and tissue samples

Fresh tumor, blood and peritumoral tissue samples were obtained after informed consent from 19 patients undergoing definitive surgery due to renal cell carcinoma. None of the patients had received preoperative anti-tumor therapy and no patient had any other obvious or declared clinical conditions. TIL and residual lymphocytes in unaffected renal tissue were isolated as described previously [39, 40]. In brief, tissue fragments were washed with normal saline and then were mechanically dissagregated to release the cells. The resultant suspensions were washed and centrifuged on Ficoll density gradient. Peripheral blood lymphocytes were prepared by centrifugation on Ficoll density gradient.

Flow cytometric analysis

Immunofluorescent staining and flow cytometric analysis (FCM) of γδ T cells from TIL and peripheral blood lymphocytes (PBL) were performed as described in detail elsewhere [39,40]. In this study the following monoclonal antibodies were used: FITC or PE – conjugated anti-CD3, anti-PAN γ/δ, which binds to all γδ T cells, anti γδ(-Vδ1) recognizing all γδ T cells except those expressing Vδ1, and anti-Vδ2 recognizing the Vδ2 product. Unrelated antibodies matched for the isotype were used as negative controls. All antibodies were purchased from Immunotech/Beckman Coulter, France.

RNA extraction and cDNA synthesis

Total RNA from 10^6 to 10^7 Ficoll purified cells was prepared by guanidinum thiocyanate-phenol-chloroform method [41]. This corresponds to a known amount of γδ T cells determined by FCM analysis. RNA was then reverse transcribed into cDNA in a reaction primed with oligo(dT) by using M-MLV reverse transcriptase (Boehringer Mannheim, Germany) as recommended by the manufacturer.

PCR amplification

Aliquots of the cDNA synthesis reaction (corresponding to an equivalent of 4 to 5 x 10^3 γδ T cells) were amplified in 50-µl reaction with one of the 6 Vδ specific primers and the Cδ primer (table 1) [22]. The final concentration of each primer was 0.5 µM, 0.2 mM each deoxynucleoside triphosphate, and 1.5 mM $MgCl_2$ in the Taq polymerase buffer (Perkin Elmer, CA). The amplification was performed with 2 U of Taq polymerase (Perkin Elmer, CA) on a DNA thermal cycler. The PCR cycle profile contained 40 cycles of denaturation at 92°C for 1.5 min, primers annealing at 56°C for 1 min, extension at 72°C for 1.5 min and one final polymerization step of 12 min. at 72°C.

Primer extension in run off reactions

Aliquots (2 µl) of the unlabeled 40-cycle Vδ-Cδ PCR products were subjected to a cycle of elongation (run-off) with a fluorophore-labeled Jδ1 specific primer (0.1 µM, final) (figure 1). The total volume was 10 µl, and the final concentration of deoxynuleoside triphosphates was 0.2 mM in the presence of 0.2 U of Taq polymerase.

Electrophoresis and fragment analysis

For the TCR Vδ repertoire analysis samples of the unlabeled PCR products were loaded on 2% agarose gel and after electrophoresis visualized by ethidium bromide staining. Separation and analysis of the runoff products were

Table 1. Primers for the TCR Vδ repertoire and CDR3 size distribution analysis

Primer	5'-3' sequence	Expected length	Remarks
Vδ1 forward	ACTCAAGCCCAGTCATCAGT	440	
Vδ2 forward	GAGTCATGTCAGCCATTGAG	470	
Vδ3 forward	ACAGCAGATCAGAAGCTGCA	300	
Vδ4 forward	CCAGTGATCCAAGTTATGGTC	380	
Vδ5 forward	CTGAAGGTCCTACATTCCTG	320	
Vδ6 forward	TATCATGGATTCCCAGCCTG	150	
Cδ reverse	GGATGGTTTGGTATGAGGCTG		
Jδ1 reverse	TTCCACAGTCACACGGGTTC		runoff PCR

performed using ALF DNA sequencer (Pharmacia Biotech, Sweden) and adopted DNA Fragment Manager V1.2 software (Pharmacia Biotech, Sweden). Dye -lybeled size standards were included in each electrophoresis run. This allows the precise determination of the sizes of the Vδ/Jδ run off DNA fragments.

DNA sequencing

Prior to sequencing analysis, the PCR products were purified using Wizard PCR Preps DNA Purification System (Promega, WI). Direct sequence analysis of PCR fragments was performed using dideoxy method with fluorescent Jδ1 primer and AutoCycle sequencing kit (Pharmacia Biotech, Sweden) and ALF DNA sequencer (Pharmacia Biotech, Sweden).

Results

To compare the proportion of γδ T cell subsets between the TIL and peripheral blood in RCC patients double staining was performed with anti-CD3 and specific γδ T-cell monoclonal antibodies. The γδ T cells were detected in all patients and accounted for only a small fraction of the peripheral blood lymphocytes and TIL. The level of this T cell subpopulation in patients PBL was similar to that observed in healthy individuals. Parallel analysis of matched PBL and TIL samples from the same patients revealed a small, however consistent decrease of γδ T cells in TIL (figure 2). Lower γδ T cell content among CD3+ cells was observed in 16 out of 19 TIL samples. The percentage of this cell subset in TIL was not affected either by tumor grade, stage or patient clinical performance status.

To assess proportion between two main subsets of γδ T cells, specific staining with anti γδ(-Vδ1) and anti Vδ2 mAbs was performed. As expected the Vδ2 subset was the main γδ subset in the peripheral blood of RCC patients and constituted on average 76% of total γδ T cells, whereas Vδ1 cells represented about 24%. Similar domination of the Vδ2 lymphocytes was also observed in TIL, however this subset

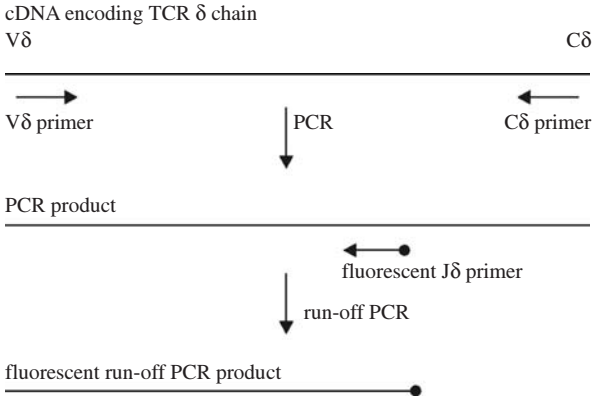

Fig. 1. Schematic representation of run-off PCR procedures. TCR δ sequences were amplified in PCR reaction with Vδ and Cδ specific primers. Each PCR reaction was re-amplified with single fluorescent Jδ primer followed by high resolution electrophoresis on a sequencing gel. Thus, PCR products differing in one nucleotide could be separated and visualized

slightly dropped to 68% of γδ + TIL and Vδ1 cells increased to 30% (figure 3).

In next set of experiments we tried to refine our analysis to the TCR Vδ repertoire and the CDR3 size distribution of Vδ-Jδ rearrangements.

Using PCR, TCR Vδ gene segment usage was analyzed in TIL, PBL and peritumoral tissue. The amplified material was revealed by agarose gel electrophoresis and ethidium bromide staining. PCR products giving a single band with the expected size were considered specific and indicative for a given gene segment usage. Reproducible results were obtained in all tumor and blood samples. However, in peritumoral, normal tissue samples amplifications of the TCR Vδ genes were not reproducible, probably because it contained too few lymphocytes.

In four out of 19 TIL samples we found clones that were not detected in PBL and in eight TIL samples some clones were absent, which were present in blood. It is noteworthy,

Table 2. Summary of CDR3 profiles. Numbers represent amount of cases displaying a particular type of distribution. P – policlonal distribution: above four distinct peaks; O – oligoclonal distribution: two-four peaks; M – single peak; PT – peritumoral tissue

	Vδ1			Vδ2			Vδ3			Vδ5		
	P	O	M	P	O	M	P	O	M	P	O	M
TIL	11	5	3	10	7	2	6	9	2	1	4	3
PBL	6	11	2	8	8	3	4	11	3	2	5	4
PT	1	4		1	2	1	1			1	2	

that the alterations in the TCR Vδ usage were observed only in minor γδ T-cell subpopulations i.e. Vδ3-Vδ6.

In summary, from both experiments it appears that Vδ2 and Vδ1 are the only dominating Vδ specificities infiltrating RCC. The other Vδ gene segments are represented to a much lesser extent or are totally absent.

To refine the analysis we further examined the CDR3 size distribution in detected clones. The Vδ-Cδ PCR products were copied in run off reactions with a nested Jδ1 fluorescent primer, followed by the determination of the size by electrophoresis on an automated DNA sequencer which allows the detection of clonal T cell expansion and/or deletions in vivo (figure 1) [43].

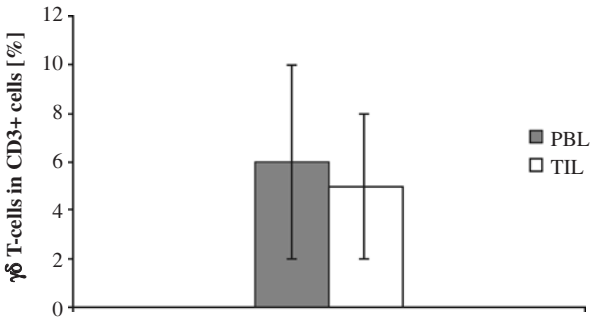

Fig. 2. γδ T-cell quantity in peripheral blood and tumor infiltrating lymphocytes in RCC patients. PBL and TIL samples were stained with pan-γδ T-cell and anti-CD3 antibodies and analyzed by flow cytometry. Data represents percentage of γδ T-cells in whole T-cell population

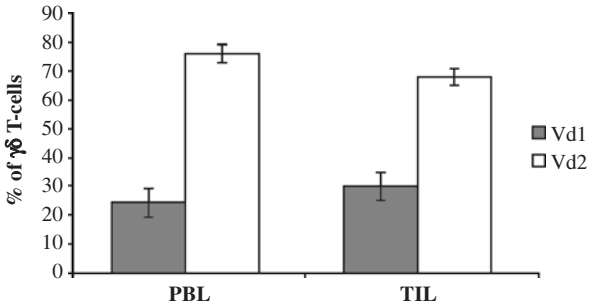

Fig. 3. Quantitative analysis of Vδ1 and Vδ2 subsets distribution among PBL and TIL in RCC patients. PBL and TIL samples were stained with anti-Vδ1, Vδ2 and pan-γδ T-cell antibodies and analyzed by flow cytometry. Data represents a percentage of a given cell sub-population among γδ T-cells

The representative results from PBL and tumor samples are shown in figure 4. These profiles, which reflected the CDR3 size diversity in a given Vδ subfamily, could be divided into three categories: a) multiple peaks in a nearly gausian distribution (such as Vδ1 in a TIL sample from patient 170), b) one single dominating peak (such Vδ1 in a TIL sample from patient 85), c) several dominant peaks (such as Vδ1 in PBL from patient 85) (figure 4A). In most patients (15 out of 19) the CDR3 size diversity in peripheral blood γδ T cells was restricted giving only a few or a single peak during analysis (see table 2 for results summary). This restriction was observed in all Vδ subfamilies analyzed. Similar to PBL pattern was seen in TIL except for Vδ1 where more than half cases displayed multiple peaks. The CDR3 profile did not correlate with the γδ cell count (data not shown).

We next compared CDR3 profiles between TIL, PBL and peritumoral tissue. Differences in the CDR3 profiles of Vδ genes were found in the majority of TIL preparations as compared to PBL or control tissue. The changes concerned appearance and/or deletions of particular clones.

In the Vδ1 subpopulation differences between TIL, PBL or control tissue were observed in eleven cases and in most cases were created (9/11) by clonal expansions as well as by deletions of clones present in blood or normal tissue. In four cases the CDR3 profile in TIL represented a single peak. In two cases the peaks had the same position as a dominant clone in PBL but in other two samples peak's position was shifted which may indicate on monoclonal expansion.

Similar changes in the CDR3 profiles were observed during analysis of Vδ2 subpopulation. Simultaneous expansions and deletions were found in five out of 16 compared cases. As a sole alteration deletions were present in four cases and expansions in two. A single peak suggesting a monoclonal expansion was seen in two cases.

Major differences in Vδ3 subpopulation were observed in eleven out of 17 TIL-PBL pairs. In seven cases occurred expansions along with deletions, in three TIL samples we observed only deletions and in one case an appearance of a new clone.

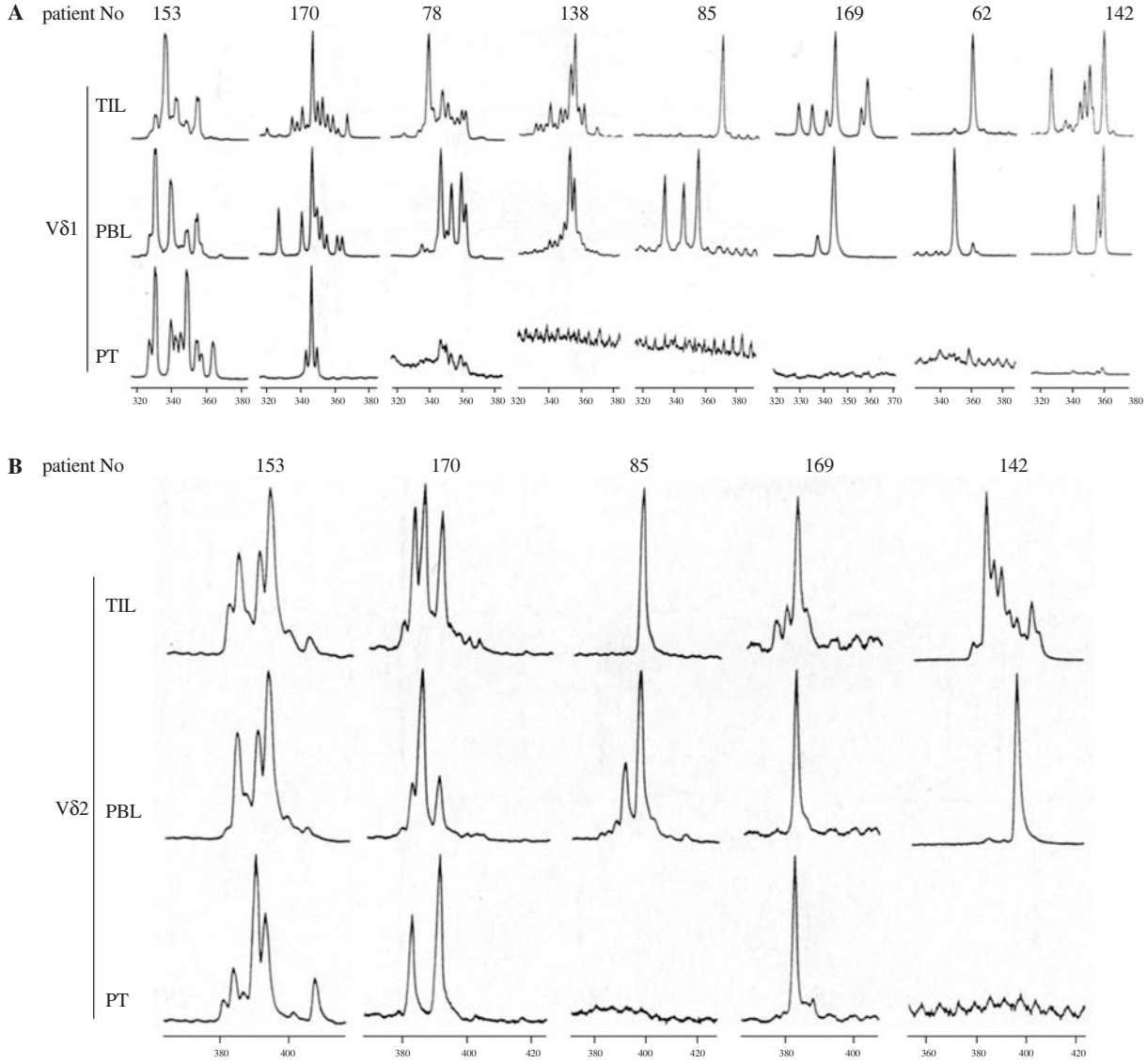

Fig. 4. Vδ-Jδ junction (CDR3) size distribution profiles of Vδ1 (4A), Vδ2 (4B), Vδ3 (4C) and Vδ5 (4D) transcripts in γδ T cells from TIL, PBL and peritumoral tissue (PT) from RCC patients. cDNA made from total RNA was subjected to PCR amplifications with Vδ-and Cδ- specific primers. The unlabeled amplification products were copied in run off reactions primed by fluorescent Jδ1 primer. The labeled aliquots were subjected to electrophoresis and analysis on an automated DNA sequencer. The patterns show the size and intensity distribution of in frame Vδ products. (X-axis: size of the PCR products reflecting CDR3 size; Y-axis: fluorescent intensity showing relative quantity of cells sharing the same CDR3 size.) Typical results are shown. Major selective Vδ1 clonal infiltration/expansion in TIL are visible in patient 153, 78, 85, 62, as well as Vδ2 in patient 142

In the Vδ5 subpopulation changes were found in six out of 8 cases. Expansions with deletions were observed in four cases, whereas in two other were only deletions.

In order to define more precisely the nature of freshly isolated γδ + TIL, the amplified Vδ-Cδ sequences of TIL samples giving a single peak (four Vδ1 and two Vδ2) were directly sequenced. Three cases (all Vδ1) exhibited a relatively clear sequencing pattern, confirming that a single peak reflects a single T cell clone (table 3). In the remaining TIL samples the sequence was unreadable indicating their polyclonal nature.

Discussion

It is assumed that γδ T cells may be involved in the immune response to tumor cells [30, 31]. However, the nature

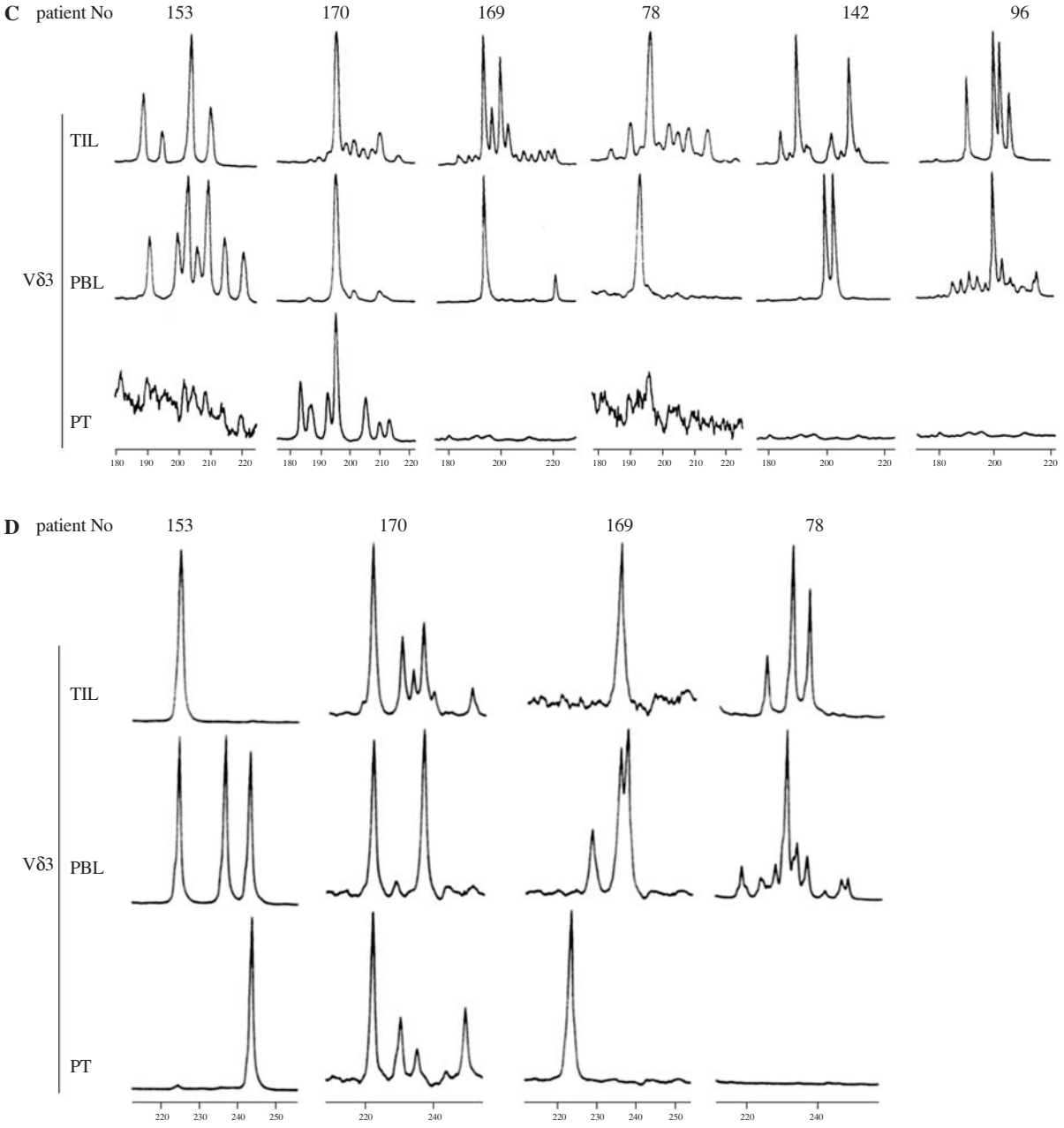

Fig. 4.

of the responding TCR repertoire is unknown. γδ T cells can recognize a large variety of antigens using only a small group of genes coding for the variable γ and δ regions of the TCR [32, 44]. This enormous diversity of γδ TCR is created by the usage of multiple D segments (δ chain) and extensive nucleotide additions and deletions at the V-(D)-J junctions [32, 44].

In this study we have analyzed the Vδ repertoire and CDR3 diversity of γδ T cells in tumor, paired PBL and peritumoral tissue from 19 RCC patients. We reasoned that analysis of the V-D-J junctions of γδ T cells infiltrating would provide insight into their activities in tumor tissue. Using flow cytometry, PCR and run off PCR we found that the repertoire of fresh γδ TIL from RCC is affected by the

Table 3. The V-D-J junction sequence of TCR δ from three TIL samples (from patient 62 – A, 85 – B and 148 – C respectively) which gave a single peak. Genomic germline sequences are underlined

A	
	Vδ1 Dδ2 Dδ3 Jδ1
5'	GTCTTGGGGAACCCTCGAGGCCCTTAATCTTTCATCACTGGGGATACGCCGAT
B	
	Vδ1 Dδ2 Dδ3 Jδ1
5'	GTCTTGGGGACCGGCAGACTTCTTCCAACAGTACTGGGGGATCAGGGACGGAAAGATATACCGAT
C	
	Vδ1 Dδ2 Jδ1
5'	GTCTTGGGGCAAGCGTTGTGGAATGCTTTTATACCGAT

tumor microenviroment. This was shown by the differences in the CDR3 size profiles between γδ T cells in TIL, blood or normal renal tissue.

Comparing the percentages of Vδ1 and Vδ2 subfamilies in TIL to those in PBL, we found a small decrease of Vδ2 and increase of Vδ1+ cells in TIL. However, it must be stressed out that analyzed T-cell populations accounted for about of 5% of T cell pool in TIL. Therefore, it is rather difficult to draw meaningful conclusions about significance of these findings in the perspective of antitumor response. Previous published observations demonstrated preferential homing of Vδ1+ cells into larynx cancer and expansion of Vδ1+ cells in the γδ + TIL cultures isolated from lung and kidney tumors [36, 37, 45, 46]. Unfortunately, studies based on in vitro TIL expansion could be negatively influenced by selective ex-vivo clonal T-cell growth and may not reflect in vivo conditions Although it was possible to detect high cytotoxic activity in these in vitro expanded clones, these studies showed γδ T-cell antitumor potential rather then their actual involvement in host anti-tumor response.

The analysis of the Vδ-Jδ junctions revealed that observed changes are not only quantitative but also qualitative. Decrease or even absence of clones being detected in PBL or peritumoral tissue along with clonal expansion of γδ TIL strongly point to their selected recruitment. Recent studies showed that tumor specific γδ T cell response may occur in animal models and in cancer patients [47-52]. In our previous study we found γδ TIL in an activated state which suggest that these cells may have recently encountered antigen or lymphokine stimulation [39]. Our results taken together illustrate that γδ TIL in RCC are not only activated but also selectively recruited. However, selective infiltration of T cells expressing a particular Vδ gene segment or having a particular CDR3 size does not necessarily prove a specific immune response to the autologous tumors. This raises the major issue of the selective homing and specificity of γδ T cells within TIL.

Dominant or expanded γδ T-cell clones might be without any known specificity. They might be randomly induced by local, inflammatory nonspecific factors and represent recently activated T cells. Therefore, their presence might result from TCR independent mechanisms such as unspecific tumor homing. Indeed, Kjaergaard and Shu using adoptive T-cell transfer demonstrated that activated T-cells were able to leave the bloodstream and infiltrate tumors regardless of their specificity [53]. In a tumor vaccine therapy model unspecific immunization or T-cell activation with a SEB superantigen also increased T-cell infiltration without any negative influence on tumor growth [54]. Increased expression of activation markers often observed on TIL might be a consequence of their prior activation outside tumor tissue and preferential extravassation instead of in situ stimulation by putative tumor antigens.

Alternatively, activation and expansion of γδ T cells in TIL might be antigen specific. The observed complexity of the responses may result from the involvement of multiple γδ clones in response to a single antigenic determinant or may reflect response to multiple antigens expressed at RCC tissue as it was shown for myelin basic protein or melanoma [55-57].

γδ TIL might specifically respond to ligands released by damaged or dead cells [58, 59]. Considering that cells damage as well as necrosis usually exist within tumors it is possible to speculate that some clones may recognize molecules liberated in cancerous tissue. Another antigen which γδ TIL might respond to is the heat shock protein (HSP) [60]. It has been shown that HSP may serve as a ligand of some type of γδ TCR as well as a molecule that can present antigenic peptide to γδ T cells [60, 61]. HSP is produced in large amounts under a variety of stress conditions including lymphokine activation, hypoxia, attack of reactive oxygen metabolites which may be present in cancer tissue [61]. Finally, γδ TIL might have been activated by specific interactions with yet unknown tumor antigens. In vitro and *in vivo* experiments show selective lysis of autologous tumor cells by recurrent γδ TIL from renal cancers, melanomas or selective expansion of these cells isolated from lung cancer upon culture with IL-2 [37, 40, 50, 52]. However, progressively growing tumors rarely contain

significant amount of tumor specific T-cells and their real significance in controlling tumor growth in vivo might be questioned [54]. This is contrasted to rejected tumors where nearly 50% of infiltrating T-cells are tumor specific as it was demonstrated by MHC-tetramer staining [54].

In summary, we report here the novel data on in vivo TCRδ diversity of γδ TIL from human RCC tumors. The experimental system used allowed us to perform a characterization of the V-D-J junction in TIL, PBL and peritumoral tissue and detection of selective γδ T cell infiltration into tumor site.

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