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γδ T cells response to *Mycobacterium tuberculosis* in pulmonary tuberculosis patients using preponderant complementary determinant region 3 sequence

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***Background & objectives*: The unique immunological functions of γδ T lymphocytes to contribute immunity against *Mycobacterium tuberculosis* attracted interest of researchers. However, little is known about the specificity of γδ T cell in tuberculosis patients and the lack of exact tuberculosis antigen recognized by γδ T cells limited its application. The analysis of complementary determinant region (CDR)3 sequence characteristic in γδ T cells of tuberculosis patients would contribute to understand the distribution specificity of γδ T cell. In present study, we investigated the diversity of the γ9/δ2 T cell immunorepertoire and analysed the specificity of the expressed CDR3 in pulmonary tuberculosis patients.**

***Methods*: The total RNA in peripheral blood mononuclear cell of 50 pulmonary tuberculosis patients and 10 healthy controls was extracted. The polymerase chain reaction was used to specifically amplify the CDR3 region of γ9 and δ2 chain. The PCR products were ligated into the pGEM-T easy vector. The plasmid DNA was sequenced using the ABI3700 and the T7 primer.**

***Results*: Our findings showed that predominant CDR3 sequence of δ2 chain in pulmonary tuberculosis patients was CACDTLVSTDKLIFGKG. The sequence specifically exists in almost all pulmonary tuberculosis patients. The conserved hydrophobic acid residue in 97 positions is present in the γδ T cell reactive to *M. tuberculosis*. The length of δ2 CDR3 in pulmonary tuberculosis patients has no relation with the disease progress.**

***Interpretation & conclusion*s: Our results suggest that γδ T cells appear to use CDR3 sequence to recognise *M. tuberculosis* antigen. γδ T cells reactive to *M. tuberculosis* were diverse and polyclonal.**

Key words CDR3 region - gammadelta T cells - predominant sequence - pulmonary tuberculosis

Tuberculosis (TB) has affected humanity since the beginning of the recorded time and is associated with poverty, malnutrition, overcrowding, and immuno-suppression[1]. In recent years, the vaccine of *M. tuberculosis* based on γδ T cells attracted attention of researchers, however, little is known about the structural basis of antigenic recognition by γδ T cell, and the lack of exact tuberculosis antigen recognized by γδ T cells limited its application.

γδ T cells are a distinct subset of $CD3^+$ T cell featuring T cell receptors (TCRs) that are encoded by Vγ and Vδ gene segments. In peripheral blood of healthy individuals, γδ T cells represent 2-10 per cent of total T cells, and of these, the majority express Vγ9Vδ2 TCRs. Studies in humans and animal models suggested that γδ T cells play an important role in immune response to *M. tuberculosis*[2]. In human, γδ T cell are present in increased proportions in peripheral blood of a fraction of tuberculosis patients[3]. γδ T cells could inhibit the proliferation of *M. tuberculosis* through the secretion of IFN-γ, but also participate in the anit-tuberculosis immune response elicited by other immune cells such as NK cells[4], dendritic cells[5] and $CD8^+$ αβ T cells[6]. Thus, the interaction net composed by many immune cells might play more important role in the infection of *M. tuberculosis*. Further studies are needed to define a precise subset of V γ9/δ2 T cells in immunity to *M. tuberculosis* infections as well as to find tuberculosis antigen recognized by the antigen-specific γδ T cells.

The antigen binding site of the γδ TCR is primarily formed from three complementary determinant regions (CDRs) contributed by each Vγ or Vδ domain. Both CDR1 and CDR2 regions are encoded by germline V genes, while the CDR3 region is formed by somatic rearrangement of V (D) and J fragments. Sequence diversity in antigen receptors is not evenly distributed among all six CDRs. The diversity is highly concentrated in one or two CDR3s[7]. To determine the characteristics of amino acid sequences of CDR3δ, Xu *et al*[8] cloned and sequenced Vδ2 cDNA from tumour infiltrated lymphocytes in rectal cancer and ovarian epithelial cancer. They used synthesized CDR3δ peptide as a probe to screen putative protein ligands in tumour protein extracts by affinity chromatography analysis and successfully identified a new antigen human mutS homolog 2 (hMSH2) that is recognized by human γδ TCR[8]. The analysis of CDR3 sequence characteristic in γδ T cells would contribute to understand the distribution specificity of γδ T cell in pulmonary tuberculosis patients. Predominant CDR3 sequence could also be used to construct the transfectant cell lines expressing *M. tuberculosis* specific γδ TCR, which could be applied to evaluate the importance of γδ CDR3 sequence[10,11] and identify new ligands for γδ TCR[12].

In the present study, we attempted to investigate the diversity of the γ9/δ2 T cell immunorepertoire by sequence analyses of the expressed CDR3 in pulmonary tuberculosis patients.

Material & Methods

Patients and study design: The study was performed on randomly selected 50 pulmonary tuberculosis patients (mean age, 45.3 ± 3.8, 28 men and 22 women) who had been admitted to the Beijing Tuberculosis and Thoracic Tumor Research Institute during 12 months (April 2008 - April 2009). Pulmonary tuberculosis was diagnosed by the clinical parameters: presence of cough/expectoration, chest X-ray showing infiltrated and / or cavities, a minimum of one positive sputum smear and culture result for acid-fast bacilli. The exclusion criteria were human immunodeficiency virus positivity, diabetes mellitus, pregnancy and immunological or autoimmune diseases (Table I). Ten healthy volunteers (mean age, 33 ± 1.6, 4 women and 6 men) were included as control group. Healthy subjects did not have any changes on X-ray and tuberculosis history or other underlying disease. Exclusion criteria for the healthy control group were smoking, medication, pregnancy and any abnormalities in renal and liver function tests. Permission for the study was obtained from the Clinical Ethics Committee of Institute of Pathogen Biology, Beijing, and all subjects were informed and gave their oral consent to participate.

The extraction of RNA and reverse transcription polymerase chain reaction: Total RNA was harvested

Table I. Characteristics of the pulmonary tuberculosis patients and healthy control

	Mean age Yr (range)	Male No. (%)	Other tuberculosis* No. (%)	Incidence time (months)	Treatment time (months)	Treatment program
Patients group (n=50)	45.3 ± 3.8 (15-80)	28 (56)	12 (24)	0-12	0-12	H,L,E,Z,V,R PAS,H,E,Z,Pa
Healthy control (n=10)	33 ± 1.6 (25-40)	6 (60)	0 (0)			

The treatment drugs: H, isoniazid; E, ethambutol; Z, pyrazinamide; PAS, pairs of amino acid; R, rifampicin; S, streptomycin; L, levofloxacin; V, rifapentine; AK, amikacin. Pa, cycloserine; *Patients having tuberculosis in other organs in addition to pulmonary. Incidence time denotes that the period from the clinical symptoms to receiving treatment

from peripheral blood mononuclear cells (PBMC) of the pulmonary tuberculosis patients and healthy subjects following the Qiagen RNeasy protocol (*http://www.qiagen.com*), including the optional DNase treatment. One microgram of total RNA was then converted into cDNA using reverse transcription system kit (Qiagen, China). First strand synthesis was primed by anchored oligo(dT) primers to generate a cDNA library representative of the entire cellular mRNA pool. Primer sequences complementary to upstream V regions and downstream C regions were used to amplify CDR3 regions. The primer sequence is from the report of Xu *et al*[8]. The primer was TCRγ9CDR3-up 5′-AATGTAGAGAAACAGGAC-3, TCRγ9CDR3-down ′5′-ATCTGTAATGATAAGCTTT-3′, TCRδ2CDR3-up 5'-GCACCATCAGAGAGAGATGAAGGG-3', TCRδ2CDR3-down 5'-AAACGGATGGTTTGGTATGAGGC-3'. The PCR products were separated on 1 per cent agarose/tris-acetate-EDTA (TAE) gels.

Cloning and sequencing of Vγ9 and Vδ2 chain: PCR products were purified by gel extraction using gel extraction kits (AXYGEN, China) according to the manufacturer's instructions. The purified PCR fragments were ligated into the pGEM-T easy vector (Invitrogen, USA) and the resulting plasmids were transfected by heat shock into DH5a competent *Escherichia coli* for propagation. Glycerol stocks were frozen to maintain the clones. Colonies were picked and grown overnight in 1-2 ml of Luria-Bertani broth containing ampicillin (50 mg/ml). Plasmids were purified using the DNeasy Miniprep kit (Qiagen, China). The plasmid DNA was sequenced using the ABI3700 and the T7 primer. Sequences were determined using the DNAman software analysis system[11]. CDR3 lengths were calculated as four less than the number of amino acids between the last conserved Cys residue in the V region to the conserved GXG or AXG motif in the joining segment as previously described[8].

Statistical analysis: Student's t-test was used for statistical evaluation of the data. $P<0.05$ was considered significant.

Results

Sequencing of the CDR3 regions confirm that γ9/δ2 T cells use prominent CDR3 sequence to recognize tuberculosis antigen: Antigen specificity is largely determined by the sequences encoded by the hypervariable CDR3 regions of the TCR. To study specificities of the γ9/δ2 T cell reactive to *M. tuberculosis*, the diversity of the Vγ9 and Vδ2 TCR CDR3 loops expressed by this population of T cells in pulmonary tuberculosis patients was analysed.

Table II. Vγ9 CDR3 gene sequence-deduced amino acid sequences from pulmonary tuberculosis patients and healthy control

		Vγ9	N/P	J	Frequency*
Patients group	1	*CALWE*	*VIS*	*ELGKKIKVFG*	*12/80(15)*
	2	*CALWE*	*W*	*ELGKKIKVFG*	*8/80(10)*
	3	CALWE	VNS	ELGKKIKVFG	4/80(5)
	4	CALWE	EQA	ELGKKIKVFG	2/80(3)
	5	CALWE	AGD	ELGKKIKVFG	2/80(3)
	6	CALWE	DK	ELGKKIKVFG	2/80(3)
	7	CALWE	ED	ELGKKIKVFG	2/80(3)
	8	CALWE	GKL	ELGKKIKVFG	2/80(3)
Healthy control	1	*CALWE*	*VIS*	*ELGKKIKVFG*	*4/40(10)*
	2	*CALWE*	*W*	*ELGKKIKVFG*	*4/40(10)*
	3	CALWE	DFS	ELGKKIKVFG	2/40(5)
	4	CALWE	VLR	ELGKKIKVFG	2/40(5)
	5	CALWE	KHL	ELGKKIKVFG	2/40(5)
	6	CALWE	LGSR	ELGKKIKVFG	2/40(5)
	7	CALW	FQDSF	ELGKKIKVFG	2/40(5)
	8	CALWE	APLDS	ELGKKIKVFG	2/40(5)
	9	CALWE	VRSL	ELGKKIKVFG	2/40(5)
	10	CALWE	NP	ELGKKIKVFG	2/40(5)

*Number of identical clones/total number of clones sequenced (percentage of clones with identical sequence). Not all the sequencing results were listed on the Table. In patients group, the sequence that the frequency was more than 3 was listed. And the sequence that the frequency was more than 5 in healthy group was listed, respectively.

Table III. Vδ2 CDR3 gene sequence-deduced amino acid sequences from pulmonary tuberculosis patients and healthy control

	Clone	v	N-D-N	J	97 position*	Frequency**
Patients group	1	*CACD*	*TLVS*	*TDKLIFGKG*	*L*	*26/80(33)*
	2	CACD	PLGEKY	TDKLIFGKG	*L*	10/80(13)
	3	CACD	ALAA	TDKLIFGKG	*L*	8/80(10)
	4	CACD	TVASRALGDN	TDKLIFGKG	*V*	8/80(10)
	5	CACD	TLGDTS	TDKLIFGKG	*L*	8/80(10)
	6	CACD	PVLGDTSY	TDKLIFGKG	*V*	6/80(7)
	7	CACD	TVIPPTGGIGC	TDKLIFGKG	*V*	4/80(5)
	8	CACD	ILGDTGSWD	TDKLIFGKG	*L*	2/80(3)
	9	CACD	GLLGDGRY	TDKLIFGKG	*L*	2/80(3)
	10	CACD	TRGAPN	TDKLIFGKG	*R*	2/80(3)
Healthy control	1	CACD	TVGSYVSTGE	TDKLIFGKG	*V*	3/40(7.5)
	2	CACD	TVGGNLRTED	TDKLIFGKG	*V*	3/40(7.5)
	3	CACD	TLRGNRRLEN	TDKLIFGKG	*L*	2/40(5)
	4	CACD	HVLGGPHGQG	TDKLIFGKG	*V*	2/40(5)
	5	CACD	FPSHTFHSTGGHT	TDKLIFGKG	P	2/40(5)
	6	CACD	TLLGDKY	TDKLIFGKG	*L*	2/40(5)

* The amino acid residue in 97 position. L, leucine; V, valine; P, praline; R, argine. ** Number of identical clones/total number of clones sequenced (percentage of clones with identical sequence). Not all the sequencing results were listed on the Table. In patients group, the sequence that the frequency was more than 3 was listed. And the sequence that the frequency was more than 5 in healthy group was listed, respectively.

The sequencing results are shown in Tables II and III. First the difference of Vγ9 CDR3 loops between pulmonary tuberculosis patients and healthy subjects was analysed. The sequence of clone 1 appeared 12 times among 80 sequenced Vγ9 chains. So, it was regarded as a predominant motif of Vγ9 chains in tuberculosis patients (Table II). But the sequence also appeared in healthy controls (clone 1). There was no significant difference for frequency of the sequence between patients and control groups indicating that the sequence was not specific for pulmonary tuberculosis patients.

According to previous report[10], the difference of δ2 TCR chain was most pronounced presumably because of the increased potential for diverse sequences due to the additional D gene segment rearrangements and nucleotide additions/substitution. The CDR3 regions contained conserved "CA" at the N-terminus and conserved "FGXG" at the C-terminus. In contrast, the inner regions of CDR3 were composed of variable sequences. Here, a common δ2 CDR3 sequence was found in PBMC of patients groups. The common sequence is "CACDTLVSTDKLIFGKG". No prominent CDR3 sequence was found in control groups and these sequences were not present in patients groups (Table III).

The length of δ2 CDR3 in patients and relation with the disease progress: Further, the difference of CDR3 length of δ2 chain was compared between pulmonary tuberculosis patient and healthy controls. The results were shown in the Fig. 1. From the point of CDR3 length distribution, γδ TCR seems to use shorter CDR3 sequence to recognize *M. tuberculosis* antigen, which is

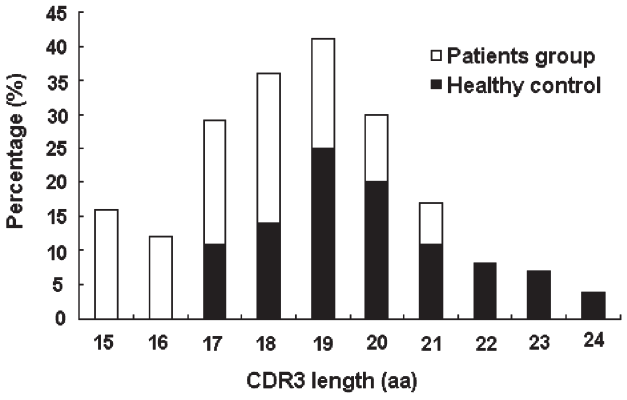

Fig. The δ2 chain CDR3 length distribution in pulmonary tuberculosis patients and healthy controls. For patient and healthy control groups, the percentage of a CDR3 length in total sequence results was calculated. The CDR3 length of δ2 chain of tuberculosis patients focused on 17 to 19 amino acid (aa) residues, while that of healthy controls concentrated on more than 20 amino acid residues.

different from that of healthy control. The CDR3 length of δ2 chain of tuberculosis patients focused on 17 to 19 amino acid residues, while that of healthy controls concentrated on more than 20 amino acid residue.

Conserved hydrophobic acid residue in 97 position in the γδ T cell reactive to M. tuberculosis: Our sequence analysis revealed that most Vδ2 T cell isolated from tuberculosis patients and healthy controls also carried a hydrophobic amino acid residue (isolecuine/leucine/valine) at conserved position 97 (Table III). The results suggested that the hydrophobic amino acid residue is not a prerequisite for γδ T cell reactive to *M. tuberculosis*.

Discussion

M. tuberculosis is an intracellular pathogen, and cell-mediated immunity plays a key role in the control of the bacterial replication and the subsequent protection against tuberculosis. It is becoming increasing recognized that γδ T cells, especially Vγ9/δ2 subset, are relevant for both innate and adaptive protective immunity against *M. tuberculosis*. Phosphate antigen of *M. tuberculosis* was regarded as main antigen recognized by γδ T cell. But Spencer *et al*[13] demonstrated that phosphoantigen-activated γ9/δ2 T cells display a restricted TCR diversity. Recently, protein antigen of *M. tuberculosis* recognized by γδ T cell has been identified[14,15]. These antigens could effectively activate γδ T cell, which could induce innate and adaptive immunity to *M. tuberculosis*. Meanwhile, γδ T cell could participate in the anit-tuberculosis immune response elicited by other immune cells. The interaction net composed by many immune cells might play an important role in the infection of *M. tuberculosis*.

The sequencing of CDR3 is a simple method to master the specificity of γδ T cells in disease and healthy condition[16]. Our results showed that predominant γ9 CDR3 sequence was not specific for tuberculosis patients, but predominant δ2 CDR3 sequence was specific. In recognizing antigen, δ chain seems to be more important than γ chain. Xu *et al*[8] demonstrated that the primary sequence of CDR3 in γδ TCR, especially CDR3δ, due to similarity to CDR3δ and VH CDR3 in gene composition, could serve as the key determinant for the specificity of antigen binding. Therefore, although predominant γ9 CDR3 sequence is not specific for tuberculosis patients, specific δ2 CDR3 sequence could represent the sequence specificity.

Our sequence results revealed that most Vδ2 T cell isolated from pulmonary tuberculosis patients carried a hydrophobic amino acid residue (isolecuine/leucine/valine) at conserved position 97. Crystallographic structure of Vγ9δ2 TCR demonstrated that the TCR have a pocket structure formed by CDRs of TCR γ and δ chains[17,18]. Almost all non-peptide antigen reactive clones have a conserved hydrophobic residue at position 97 of CDR3 δ with leucine, isoleucine and valine[19,20]. This conserved hydrophobic residue may interact with hydrophobic parts of antigenic molecules and contribute the antigen recognition of γδ T cells. Dacodeau *et al*[21] have reported that almost all phosphoantigen reactive clones carry a distinctive junctional motif containing strongly hydrophobic amino acids at position 97, which participate in the recognition of γδ TCR to antigen. But as per our previous data[11], the hydrophobic amino acid residue at position 97 plays little role in recognizing protein antigen. In the present study, our sequence results showed that healthy individual also carried hydrophobic amino acid residue at position 97. It seems that the role of CDR3δ97 in the recognition of tuberculosis antigen was not specific and needs to be further investigated.

Our analysis of CDR3 length suggested that γδ T cells reactive to *M. tuberculosis* were diverse and polyclonal. γδ TCR appears to use shorter CDR3 sequence to recognize *M. tuberculosis* antigen, which is different from that of healthy control. The CDR3 length of tuberculosis patients focuses on 17 to 19 amino acid residues, while that of healthy controls on more than 20 amino acid residue. Our results showed that γδ T cell response to *M. tuberculosis* in pulmonary tuberculosis patients using preponderant complementary determinant region 3 sequence, was diverse and polyclonal.

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