Characteristics of the V62 CDR3 Sequence of Peripheral γδ T Cells in Patients with Pulmonary Tuberculosis and Identification of a New Tuberculosis-Related Antigen Peptide

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Antigen-specific γδ T cells may play an important role in the immune response to Mycobacterium tuberculosis. However, little is known about the characteristics of the length distribution of the 62-chain complementarity determining region 3 (62 CDR3) of the γδ T-cell receptor (TCR) in patients with active pulmonary tuberculosis (TB) on a large scale. In addition, M. tuberculosis-activated γδ T cells potentially inhibit intracellular mycobacterial growth, but phosphoantigen-activated γδ T cells do not. Only a few M. tuberculosis-related antigen peptides or proteins that are recognized by γδ TCR have been identified. Twenty-four healthy donors (HDs) and 27 TB patients were included in the present study. The gene-scanning technique found that the 62 CDR3 length distribution patterns of γδ TCR in TB patients were perturbed, and each pattern included different predominant CDR3 sequences. The predominant 62 CDR3 sequences of γδ TCRs, which originated from TB patients and HD γδ T cells that were stimulated by M. tuberculosis heat resistance antigen (Mt-HAg), were used as probes to screen peptides recognized by γδ TCR using a phage display library. We identified four peptides that bound to the predominant 62 CDR3 fragments and showed homology to M. tuberculosis genes in a BLAST search. Notably, one peptide was related to M. tuberculosis H37Rv (QHIPKPP), and this fragment was confirmed as a ligand for the γδ TCR. Two fragments, Ag1 and Ag2, activated γδ T cells from HD or TB patients. In summary, the 62 CDR3 lineage of TB patients apparently drifts, and the predominant 62 CDR3 sequence that recognizes M. tuberculosis may exhibit specificity. The identified M. tuberculosis-related antigen peptides may be used as vaccines or adjuvants for protective immunity against M. tuberculosis.

Mycobacterium tuberculosis causes tuberculosis (TB), which is one of the most common serious chronic infectious diseases. TB is a threat to human health, especially with the increasing human immunodeficiency virus (HIV) pandemic and multidrug-resistant strains of M. tuberculosis (1). An estimated 8.6 million people developed TB in 2012, and 1.3 million people died from the disease, including 320,000 deaths in people infected with HIV (2). Different pathways and cell types interact to mediate the innate and adaptive immunity against M. tuberculosis (3). The γδ T-cell subset Vγ9Vδ2 cells was shown to play an important role in host immunity against M. tuberculosis in many studies (4–6). γδ T cells generally recognize antigens in a non-major histocompatibility complex (MHC)-restricted manner, which is similar to how B cells directly recognize a ligand or antigen. The human δ chain is composed of eight Vδ gene fragments, two Dδ gene fragments, three Jδ gene fragments, and one Cδ gene fragment, and these fragments are located on the long arm of the 14th chromosome (7). Varying numbers of nucleotides (3 to 24) randomly insert into the connection area of the rearranged T-cell receptor (TCR) Vδ and form a VNDNJ sequence with a highly variable complementarity determining region 3 (CDR3) (8). CDR3 length analyses indicated that the Ig heavy (H) and δ chains exhibit larger changes, but the light (L) and γ chains undergo smaller changes. The TCR δ chain is highly diverse in the connection area, which means that the δ chain plays an important role in antigen recognition. Adams et al. (9) reported that the CDR3 ring of the G8 γδ TCR in mice (Vγ4-1α1.3) primarily contacted T22 (major histocompatibility complex molecule Iib) within the crystalloid, and the δ chain played a key role in this interaction. The γ chains in CDR3 only played a minor role. The 62 CDR3 base sequence of the γδ TCR is very important in the antigen-recognition process. The TCR CDR3 length distribution patterns, which are stimulated by nonantigens in most healthy adults, exhibited completely random rearrangements and were polyclonal. However, a few specific TCR genes reacted and were amplified when certain antigens infected patients. Analyses of different individual CDR3 spectrum types may reveal the proliferation of one or several clones of the TCR family (10). Our previous study established methods for evaluating CDR3 fragment length polymorphisms in the TCR δ chain using M. tuberculosis heat resistance antigen (Mtb-HAg)-activated γδ T cells. Analyses of the characteristics of 62 CDR3 from γδ T cells of TB patients may identify the predominant CDR3 and the sequence that specifically reacts with TB. Furthermore, αβ T cells identify peptide or protein antigens that are related to M. tuberculosis (11–15), but little is known about the antigens recognized by γδ T cells, except for various phosphoantigens. These

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phosphoantigens may not inhibit intracellular mycobacterial growth (16). We identified a likely novel tuberculosis-related antigen peptide using predominant and specific CDR3 sequences.

Phage display technology is a biological technology used to screen functional peptides or protein fragments. Smith (17) was the first to insert an exogenous gene into phage IIH genes of the filamentous phage f1 to obtain a restricted phage that stably proliferated in vitro and produced a specific peptide in the form of a fusion protein on the phage surface. Xu et al. (18) identified 12 epitope peptides that bound and functionally activated γδ T cells in vitro using synthesized TCR s2 CDR3 peptides from tumor-infiltrating lymphocytes of ovarian epithelial carcinoma as specific probes to identify antigenic epitopes in a 12-epitope library. The researchers also successfully identified new ligands of γδ TCR using affinity chromatography and mass spectrometry (19). The latest studies identified novel mycobacterial protein antigens that were recognized by γδ T cells using cells transfected with the bacillus Calmette-Guerin (BCG)–specific γδ TCR or cells from pulmonary TB patients that predominantly expressed the γδ TCR CDR3 fragment (20-22). This study used the predominant sequences of δ2 CDR3 from TB patients and identified δ2 CDR3 sequences from stimulated γδ T cells using Mtb-HAg. We used synthesized δ2 CDR3 peptides as specific probes to identify new ligands for the recognized M. tuberculosis antigen-related epitopes using 7-peptide phage display. Phage enzyme-linked immunosorbent assay (ELISA) was used to select single CDR3β peptide-binding phage clones, and the ability of the identified peptides to activate γδ T cells was examined in vitro. Overall, M. tuberculosis antigen-related epitopes may be used as vaccines or adjuvants of M. tuberculosis in the diagnosis and treatment of TB, particularly in γδ T-cell immunity.

MATERIALS AND METHODS

Samples. Peripheral blood samples were collected from 24 students and workers at our school (average age, 26 years; range, 22 to 42 years) who were vaccinated with the BCG vaccine at birth or during childhood. This sample served as healthy controls. Active pulmonary TB patients (n = 27; average age, 42 years; range, 20 to 64 years) were recruited from The Infectious Disease Hospital of Bengbu, Anhui, China, based on clinical presentation, chest radiographs, and sputum samples that were positive for acid-fast TB. Twenty-one patients exhibited pulmonary TB. Twelve of these cases were first-onset patients, and 9 cases were retreatments. Two patients exhibited comorbid lung cancer, and 4 had tuberculous pleurisy. Extrapulmonary TB was observed in 2 cases. All patients tested negative for HIV infection. Peripheral blood samples from healthy donors and TB patients were obtained following informed consent. The Ethics Approval Committee of Bengbu Medical Institute approved this experiment.

Primer design. Primer sequences that were complementary to upstream V regions and downstream C regions were used to amplify the CDR3 regions, according to the literature (19, 23). The outside upstream primer was 5′-GCC ATT GAG TGT TGT CCT GAA CAC C3′, and it was located at the 208th base. The inside upstream primer (5′-GCA CCA TCA GAG GAT GAA GGG C3′) was located at the 448th base. Both primers shared a downstream primer (5′-AAA CCG ATG TGT TAT TAT GAG GCC C3′), which was located at the 8th base. This downstream primer was synthesized with or without a 6-carboxyfluorescein (FAM) fluorescent element (C8-FAM) at the 5′ end to mark PCR products and perform gene scanning. Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China) synthesized all primers.

RNA extraction and reverse transcription-PCR (RT-PCR). Total RNA was harvested from peripheral blood mononuclear cells (PBMCs) of pulmonary TB patients and healthy subjects using the TRizol reagent (catalog no. 15596-026; Invitrogen, Carlsbad, CA, USA). Total RNA was converted into cDNA using a reverse transcription kit (Fermentas, Canada). First-strand synthesis was primed using oligo(dT) primers according to the manufacturer’s instructions. We used nested PCR amplification to increase the sensitivity and specificity of PCR for the 62 CDR3 size spectratyping analyses. We first expanded the outside of the 82 CDR3 genes, and the products were added to the internal PCR as templates. The final PCR products were sent to the Chinese National Human Genome Center in Shanghai for spectratyping of the CDR3 gene fragment lengths using an ABI 3730 and Mapper 4.0 gene scan analysis software.

Cloning and sequencing of the V 62 CDR3 region. PCR products were purified using gel extraction kits (Generay, Shanghai, China). Purified DNA fragments were ligated into the pGEM-T easy vector (TaKaRa, Dalian, China), and the resulting plasmids were transfected by heat shock into competent DH5α Escherichia coli for propagation. Ampicillin resistance and the white-spot double-screening method were used to select positive colonies. Positive clones were identified using direct PCR amplification of bacterial suspensions, and the bacterial suspensions were sequenced using ABI3730 and M13 primers. Sequences were analyzed using DNASTar software.

Peptide synthesis. Four 82 CDR3 peptides were synthesized according to the length and cloning frequency of the 62 CDR3 genes in this and former experiments, including two predominate sequences that were derived from the TCR V82 in PBMCs of M. tuberculosis-infected patients (TB-Zh and TB-So) and other sequences from Mtb-HAg–stimulated healthy adults (Mt-N4 and Mt-N5) based on our previous work. Mtb-HAg was prepared according to the literature (24). The following peptides were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China): TB-Zh, CACDVHLGVTNDKLIHFGK; TB-So, CADTCDGDRD MTVLH; Mt-N4, CADTCDGDRD MTVLH; Mt-N5, CADTCDGDRD MTVLH. Two M. tuberculosis antigen-related epitopes, Ag1 and Ag2, which were bound to the 62 CDR3 peptide using the random peptide phage display technique, were also synthesized (see Table 4). The purity of each peptide was >85% based on high-performance liquid chromatography analyses.

Panning of δ2 CDR3 peptide-binding phage clones using a 7-mer random peptide phage display library. A 7-mer random peptide phage display library (New England Biolabs, Inc., Ipswich, MA, USA) was screened with 62 CDR3 peptides according to the manufacturer’s instructions. Briefly, a volume of 150 μl of 62 CDR3 synthetic peptide (100 μg/ml) was added to one well of a 96-well microtiter plate, incubated overnight at 4°C, and blocked with phosphate-buffered saline (PBS) containing 3% bovine serum albumin (BSA). Ten microliters of a phage library containing 2 × 1011 phages diluted in 100 μl of Tris-buffered saline–Tween 20 (TBST) buffer was added to one well of the coated plate for 60 min. The well was washed 6 times with this buffer containing 0.1% Tween 20, and specific bound phages were eluted with 0.2 M glycine HCl (pH 2.2) containing 1 mg/ml BSA. Bound phages were amplified by infecting Escherichia coli (ER2738 strain), which were incubated for 4.5 h at 37°C. The bacterial cells were removed using centrifugation, and polyethylene glycol (PEG) precipitation was used to purify the amplified phages. The number of blue plaques was counted and used to calculate an input volume that corresponded to 2 × 1011 phages. The second, third, and fourth rounds of screening were identical to the first round, except that the Tween 20 concentration was increased from the original 0.1% to 0.3%, 0.5%, and 0.5%, respectively. Forty-seven phage clones were obtained after the fourth round, and these clones were sent to Sangon Biotech (Shanghai) for sequencing.

Determination of the binding specificity of the panned single-colony phages and 62 CDR3 peptides. Individual phages were randomly selected after four rounds of panning, and positive phage clones were selected by phage ELISA using a horseradish peroxidase (HRP)-conjugated anti-M13 phage antibody (GE Healthcare). Briefly, a 96-well microtiter plate was coated with or without δ2 CDR3 peptides. The wells were
TABLE 1 Comparison of the number of δ2 CDR3 fragments (peaks), variation in gene fragment length, frequency, and length of the predominant fragments in PBMCs

<table>
<thead>
<tr>
<th>Group or variable</th>
<th>No. of peaks</th>
<th>Variation of fragment length (bp)</th>
<th>Frequency (×100%)</th>
<th>Length of predominant peak (bp)</th>
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</thead>
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<tr>
<td>TB patients (n = 27)</td>
<td>11.22 ± 2.74</td>
<td>25.11 ± 5.52</td>
<td>0.2755 ± 0.0619</td>
<td>143.35 ± 6.18</td>
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<td>Healthy donors (n = 24)</td>
<td>11.83 ± 2.18</td>
<td>19.87 ± 3.52</td>
<td>0.1974 ± 0.0353</td>
<td>143.51 ± 6.52</td>
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<tr>
<td>F</td>
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<td>6.629</td>
<td>5.699</td>
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<td>P</td>
<td>0.386</td>
<td>0.000^b</td>
<td>0.000^b</td>
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</table>

^a Values are means ± SD. ^b P < 0.001, compared to TB patients.

RESULTS
Increased frequency of the predominant δ2 CDR3 fragment peak in TB patients. Gene-scanning analysis of δ2 CDR3 fragments was performed in PBMCs from 27 TB patients and 24 healthy control subjects. The frequency of the predominant peaks of the δ2 CDR3 fragments in the TB group was significantly higher than that in the healthy group (0.2755 ± 0.0619 versus 0.1974 ± 0.0353; P < 0.05). The variation of δ2 CDR3 fragments in the TB group was significantly increased compared to the healthy group (25.11 ± 5.52 versus 19.87 ± 3.52; P < 0.05). However, there were no significant differences in the sizes of the predominant peaks or the number of peaks between the two groups (Table 1). These results indicated that the predominant δ2 CDR3 fragments tended to be oligoclonal in TB patients but polyclonal in the healthy subjects (Fig. 1).

Characteristics of δ2 CDR3 sequences in peripheral blood from four TB patients. Peaks of the PCR products of the δ2 CDR3 fragment-transfected colonies properly corresponded to the peaks of the RT-PCR products from PBMCs using gene scanning. The sequencing of δ2 CDR3-transfected clones (n = 73) revealed that the δ2 CDR3 regions contained two termini with conserved sequences, i.e., CACD and KLIFGKG, at the N and C termini, respectively, and the inner region exhibited diverse sequences, in which common motifs contained hydrophobic amino acid residues at position 97 (e.g., L, leucine; V, valine) (Table 2). TB patients appeared to use the TCR DJ3 fragments versus the δ2 TCR CDR3 sequencing from healthy-subject PBMCs in our previous work (data not shown). The δ2 TCR CDR3 sequences were not identical at the same position in the predominant peaks. The experimental results demonstrated that each TB patient exhibited a respective predominant sequence. There were no identical sequences in the predominant peaks of δ2 CDR3 fragments in TB patients.

Panning for M. tuberculosis antigen-related antigen epitopes bound to δ2 CDR3 peptides from the phage library. A 7-mer random peptide phage display library technique was used to identify epitopes that were recognized by the δ2 CDR3 peptide. The

FIG 1 TCRδ2 CDR3 length distribution of γδ T cells by gene scanning of four healthy subjects and four TB patients. In the normal pattern, δ2 CDR3 shows a Gaussian distribution without any spectratype expansion. In contrast, the TB patients exhibit predominant expansion.
<table>
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<tr>
<th>Source</th>
<th>Frequency&lt;sup&gt;a&lt;/sup&gt; (no. [%])</th>
<th>Vβ</th>
<th>N-D-N&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Jβ</th>
<th>Amino acid residue at position 97&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Irβ&lt;sup&gt;d&lt;/sup&gt;</th>
<th>CDR3 length (amino acids)</th>
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<td>L</td>
<td>DJ3</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> Number of identical clones/total number of clones sequenced (percentage of clones with identical sequence).

<sup>b</sup> N-D-N, TCR gene diversity region with nucleotide inserts on two sides.

<sup>c</sup> TCR DJ3 fragments used at high frequency in TB patients compared to healthy donors. In addition, the sequences were not completely the same at the same position in the predominant peaks of 82 CDR3 (statistic not shown).

<sup>d</sup> All present in the predominant sequences in four patients with pulmonary TB. We selected two from the former to synthesize.
following four δ2 CDR3 peptides were synthesized: TB-Zh, CAC DVLGVNTDKLIFGKG; TB-So, CACDTLGDRTDKLIFGKG; Mt-N4, CACDTGTTGGHYTDKLIFGKG; and Mt-N5, CACDTL WGIQGNTDKLIFGKG.

We used these peptides to pan for epitopes that are recognized by δ2 CDR3. The screening conditions in this experiment were made stricter by increasing the Tween 20 content. Positive phages were enriched in 4 rounds of panning. The input and output rates made stricter by increasing the Tween 20 content. Positive phages

### Table 3: The input and output of 7-peptide phage display library biopanning with δ2 CDR3 as a probe

<table>
<thead>
<tr>
<th>Round</th>
<th>TB-Zh (PFU)</th>
<th>TB-So (PFU)</th>
<th>Mt-N4 (PFU)</th>
<th>Mt-N5 (PFU)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.8 × 10^4</td>
<td>6.3 × 10^4</td>
<td>6.1 × 10^4</td>
<td>5.5 × 10^4</td>
</tr>
<tr>
<td>2</td>
<td>5.9 × 10^4</td>
<td>2.5 × 10^4</td>
<td>5.1 × 10^4</td>
<td>7.6 × 10^4</td>
</tr>
<tr>
<td>3</td>
<td>8.0 × 10^6</td>
<td>9.0 × 10^6</td>
<td>5.0 × 10^6</td>
<td>2.0 × 10^7</td>
</tr>
<tr>
<td>4</td>
<td>3.4 × 10^7</td>
<td>9.0 × 10^7</td>
<td>1.1 × 10^8</td>
<td>6.0 × 10^6</td>
</tr>
</tbody>
</table>

**DISCUSSION**

γδ T cells play an important role in the protective immunity against *M. tuberculosis* infection, but the exact mechanism has not been completely elucidated. TCR δ2 is essential for the recognition of specific antigens, and several attempts were made to characterize *M. tuberculosis*-associated γδ T cells. Rearrangement of the TCR δ2 is random in healthy individuals without antigen stimulation, but special T cells can be clonally activated and expanded by a foreign antigen, such as the *M. tuberculosis* purified protein derivative (PPD) RT23 and the *M. tuberculosis* protective 16-kDa antigen (25). The γδ TCR recognizes small molecules in a non-MHC-restricted manner (26), e.g., phosphate antigens, but these receptors also directly recognize peptide antigens, such as B cells. Spencer et al. (16) reported that human γδ T cells activated by BCG and phosphate antigen showed similar effecter cytokine production and cytolytic activity. However, BCG-activated γδ T cells inhibited intracellular *M. tuberculosis* growth, but the phosphate antigen-activated γδ T cells did not inhibit growth. Spectroscopic analysis showed that the CDR3 fragment of BCG-stimulated γδ T cells displayed more specific clones and an oligoclonal pattern or distorted Gaussian distribution, but phosphate antigen-activated γδ T cells showed more clones and a polyclonal pattern or normal Gaussian distribution, which indicated that BCG-stimulated γδ T cells exhibited a restricted antigen specificity (16).

Our previous work demonstrated that the fragment length of TCR δ2 CDR3 of γδ T cells that were activated and expanded by Mtb-HAg was longer than that prior to stimulation. This result suggests that Mtb-HAg-activated γδ T cells predominantly ex-

### Table 4: Amino acid sequences (29 motifs) of δ2 CDR3-specific peptides sequenced from 46 phage clones

<table>
<thead>
<tr>
<th>TB-Zh (no. of peptides) (n = 12)</th>
<th>TB-So (no. of peptides) (n = 10)</th>
<th>Mt-N4 (no. of peptides) (n = 12)</th>
<th>Mt-N5 (no. of peptides) (n = 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RATQLPQ (4)</td>
<td>SEISAST (2)</td>
<td>GWHHHPR (5)</td>
<td>SPRVGAT (2)</td>
</tr>
<tr>
<td>QHIPKPP (2)</td>
<td>ATKTRQP (2)</td>
<td>HKRPRNN (1)</td>
<td>ATKTRQP (1)</td>
</tr>
<tr>
<td>HRRPSRS (1)</td>
<td>LPVYHRL (1)</td>
<td>HRRPSRS (1)</td>
<td>HRRPSRS (1)</td>
</tr>
<tr>
<td>AFDTHTM (1)</td>
<td>YPTGLPL (1)</td>
<td>RRRPMAI (1)</td>
<td>AFDTHTM (1)</td>
</tr>
<tr>
<td>TPPEPAP (1)</td>
<td>RFRTTPA (1)</td>
<td>HGSTKRT (1)</td>
<td>SEISAST (1)</td>
</tr>
<tr>
<td>NQDVPFL (1)</td>
<td>ADHPPHH (1)</td>
<td>WNPKHKKH (1)</td>
<td>QHIPKPP (1)</td>
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<td>HRLRISP (1)</td>
<td>SRVRLGA (1)</td>
<td>WRQTRKD (1)</td>
<td>KRAPPTP (1)</td>
</tr>
<tr>
<td>GDTQRVA (1)</td>
<td>HRSHTTH (1)</td>
<td>GTTTTL (1)</td>
<td>YMLQPHSH (1)</td>
</tr>
</tbody>
</table>

* TB-Zh, TB-So, Mt-N4, and Mt-N5 represent nomination as synthesized peptides. Sequences of synthesized δ2 CDR3 were based on gene sequences of γδ T cells in TB patients and of the latter two peripheral γδ T cell clones of Mtb-HAg–stimulated adults (Mt-N4, Mt-N5).

* Present Ag1.

* Present Ag2.
press TCR δ2 CDR3 with antigen specificity. Spectratyping in the present study demonstrated that the TCR δ2 CDR3 fragment showed a Gaussian distribution in the healthy donors, but the frequency of the predominant peak of the δ2 CDR3 fragment in the TB patients was obviously higher than that of the healthy subjects. Xi et al. (20) found that the length of the δ2 CDR3 fragment in TB patients was shorter than that in healthy subjects, but our experiment did not reveal the same phenomenon.

Dominant TCR Vβ gene families are enriched in patients with active or latent TB (25, 27) when their PBMCs are cultivated with M. tuberculosis epitopes. However, the sequences of the dominant TCR Vβ2 gene family are not identical in TB patients. This finding suggests that specific T-cell clones are involved in immunity to M. tuberculosis. Our experiment demonstrated that the δ chain of each patient exhibited its own predominant peak, and the sequences were similar but not identical in TB patients. However, these sequence differences were attributed to chance. Xi et al. (20) found that a predominant CDR3 δ2 chain specifically existed in nearly all pulmonary TB patients. However, our sequencing results did not identify this specific sequence. Several possible reasons may explain this finding. First, different TB antigens might activate the same TCR δ-chain CDR3 region fragments in different individuals. Second, humans have different human leukocyte antigens (HLAs), which restrict the different M. tuberculosis antigens that οβ T cells recognize, so different T cells are clonally proliferated. However, whether γδ T cells are also influenced is unclear. Third, the pathogenicity of M. tuberculosis, molecular weight of the antigen, types of Iγ, scattered or additional antigen sites, and variability of the M. tuberculosis antigen composition could contribute to the diversity. Fourth, the different CDR3 sequences likely recognize antigens of the same size if their spatial structures are similar. Amino acid sequence analyses showed that the CDR3 region was diverse in different individuals, but similar amino acid sequences were also found. These results suggest that T cells against the same or similar antigens of M. tuberculosis in the same or different individuals are cloned and proliferate through a non-MHC-restricted manner. TB patients also tend to use the DJ3 fragment more than healthy donors, but clarification of the specific mechanisms requires further research. Finally, TB patients primarily use hydrophobic amino acids (isoleucine/leucine/valine) at the conserved 97th position when γδ T cells recognize phosphorylated antigens, which is consistent with the findings of Davodeau et al. (28).

Human γδ T cells recognize nonpeptide antigens, such as pyrophosphoantigens. Currently, only a few known TB protein antigens are recognized by γδ T cells (21, 22, 24). We used phage display technology, which can be used to screen antigen peptides and enzymes, to further investigate TB protein antigens. This study screened for antigen peptides. There are two types of antigen epitopes: οβ T cells recognize continuous antigens, and most B cells recognize a certain structure. γδ T cells recognize specific antigens through their TCR, and the CDR3 region specifically recognizes the antigen. However, whether free CDR3 short peptides can recognize a specific antigen and whether the TCR δ CDR3 recognizes a specific structure of an antigen is not clear. Generally, people focus on CD4+ T cells and an adjuvant for Th1 cell stimulation in research for a TB vaccine. We primarily research peptides from stimulated γδ T cells to analyze CDR3 sequences of γδ T cells in people with TB (11-15). We attempted to apply this technology to TB-related antigen peptides. The TB antigen specificity of γδ T cells might primarily depend on the flanking sequences of δ2 CDR3, which might rely on some key amino acids (18), e.g., those coded by the V and J genes, that may be little

### Table 5: BLAST displayed sequences of positive phage clones bound to Vβ 2-CDR3

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Accession no. (database)</th>
<th>Description</th>
<th>Identity (%)</th>
</tr>
</thead>
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<tr>
<td>RATQLPQ (Ag1)</td>
<td>WP_003411501.1 (NCBI Protein BLAST)</td>
<td>Hypothetical protein Rv2229c (M. tuberculosis H37Rv)</td>
<td>86</td>
</tr>
<tr>
<td>AFDTHTM (Ag2)</td>
<td>2A15_A (PDB)</td>
<td>Chain A, X-ray crystal structure of Rv0760 from M. tuberculosis at 1.68-Å resolution</td>
<td>100</td>
</tr>
<tr>
<td>HRRPSRS</td>
<td>2U0U_A (PDB)</td>
<td>Chain A, crystal structure of Cyp130 from M. tuberculosis in the ligand-free form (M. tuberculosis)</td>
<td>100</td>
</tr>
<tr>
<td>QHIPKPP</td>
<td>2VBW_B (PDB)</td>
<td>Chain B, feast-or-famine regulatory protein (Rv3291c) from M. tuberculosis complexed with l-phenylalanine (M. tuberculosis)</td>
<td>100</td>
</tr>
</tbody>
</table>

*PDB, Protein Data Bank.

Four sequences of enriched phage had a homologous sequence with a poisonous strain of M. tuberculosis.
related to the D gene, according to analyses of the TCR 82 CDR3 length and sequence. Therefore, we chose to synthesize four TCR 82 CDR3 peptides (two from TB patients and two from healthy donors using Mtb-HAg stimulation and cultivation). Several peptides were obtained using the phage 7-peptide library and TCR 82 CDR3 peptides, and we found four peptides that were relevant for M. tuberculosis in BLAST. Notably, one peptide was related to the feast-or-famine regulatory protein Rv3291c from M. tuberculosis, and we selected two peptides that could selectively stimulate γδ T cells from PBMCs from TB patients or healthy donors. We concluded that different healthy donors and TB patients exhibited different reactions to different M. tuberculosis-related peptides. There are several possible explanations for these differential reactions. First, we used the linear amino acid peptide, not the spatially structured protein. Therefore, the small amino acid peptide may not effectively activate the immune response, especially when the TB patient has a weakened immune system. Second, every sample has a different HLA, which may affect antigen recognition. Finally, antigen peptides from different patients may stimulate γδ T cells in specific patients. Further research is needed to determine whether the identified peptide antigens are the specific antigens in TB patients. A new TB-related antigen peptide would be used as a vaccine or adjuvant to M. tuberculosis. These antigen peptides may be combined with recombinant BCG ΔureC hly+ to costimulate γδ T cells (14). The results of the present study demonstrate that phage screening is a feasible strategy for quickly obtaining high-affinity epitope peptides. Furthermore, we confirmed that CDR3 δ is a key region for the recognition of a specific antigen, but it can also provide evidence for immunological treatment using γδ T cells.

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REFERENCES


