Identification of Human T Cell Receptor γδ-recognized Epitopes/Proteins via CDR3δ Peptide-based Immunobiochemical Strategy*\textsuperscript{S}

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Human T lymphocytes, bearing T cell receptor (TCR) γδ, play an important role in anti-tumor/microbe immune responses. However, few tumor antigens recognized by TCRγδ have been defined so far. To investigate antigenic epitopes/proteins recognized by γδT cells, we have established a new immunobiochemical strategy that uses complementarity-determining region 3 of TCR δ chain (CDR3δ) peptide-mediated epitope/protein-binding assays. CDR3δ peptides synthesized using the CDR3 region in TCR Vδ2 chain were validated for their binding specificity to target cells or tissues. These CDR3δ peptides were then employed as probes to pan putative epitopes in a 12-mer random peptide phage-displayed library and to identify putative protein ligands within tumor protein extracts by affinity chromatography and liquid chromatography/electrospray ionization-tandem mass spectrometry analysis. As a result, we have identified nine peptides and two proteins for TCRγδ, including human mutS homolog 2 (hMSH2) and heat shock protein (HSP) 60. All nine tested epitope peptides not only bind to γδT cells but also functionally activate γδT cells in vitro. Identification of HSP60 confirms the validity of this method as HSP60 is an identified ligand for TCRγδ. We show that hMSH2 is expressed on the surface of SKOV3 tumor cells, and cytotoxicity of Vδ2 γδT cells to SKOV3 cells was blocked by anti-hMSH2 antibody, suggesting that hMSH2 may be a new ligand for TCRγδ. Taken together, our findings provide a novel immunobiochemical strategy to identify epitopes/proteins recognized by γδT cells.

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\textsuperscript{3} The abbreviations used are: TCR, T cell receptor; BCR, B cell receptor; CDR, complementarity-determining region; CDR3δ, 3 in T cell receptor δ chain; CEPA, CDR3δ peptide-mediated epitope/protein-binding assay; FCM, flow cytometry; FCS, fetal calf serum; HBV, hepatitis B virus; hMSH2, human mutS homolog 2; HSP, heat shock protein; MHC, major histocompatibility complex; MICA/B, MHC class I chain-related A/B; OEC, ovarian epithelial carcinoma; PBMC, peripheral blood mononuclear cell; SPR, surface plasmon resonance; TIL, tumor-infiltrating lymphocyte; TLR, Toll-like receptor; ULBP, UL-16 binding protein; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; ELISA, enzyme-linked immunosorbent assay; FTIC, fluorescein isothiocyanate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; WT, wild type; LC/ESI/MS/MS, liquid chromatography/electrospray ionization-tandem mass spectrometry; TdR, thymidine; IL, interleukin.

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and Toll-like receptors (TLRs) are functionally alike, because these receptors recognize conserved molecules (7).

Although γδT cells only account for a small proportion in the human T lymphocyte pool, they play important roles in both anti-tumor and anti-microbe responses in innate immunity (8). Human γδT cells recognize stress-induced or malignancy/infection-related antigens. Such recognition is mediated through a non-MHC restricted manner in most cases. Among the known antigens for γδT cells, the most frequently identified ones are non-peptide antigens, such as pyrophosphoantigen (naturally occurring and synthetic antigens) recognized by T cells bearing Vγ9δ2 heterodimer TCR chain (9–11). In addition, classical MHC and MHC-like molecules can directly serve as ligands for γδT cells (12). MHC class I chain-related gene A (MICA) and B (MICB) are recognized by Vδ1 γδT cells (13, 14). UL-16 binding protein (ULBP) is a novel ligand for NKG2D receptor in humans (15). We found that RAET1E2, a soluble isoform of the UL16-binding protein RAET1E produced by tumor cells, inhibits NKG2D-mediated NK cytotoxicity (16). Because of the sequence and structure similarity of the extra-cellular domains between MICA and ULBPs, ULBPs are also considered as antigens for Vδ1 γδT cells (17). Heat shock proteins (HSP) are highly conserved among prokaryotes and eukaryotes and are known to be involved in various stress conditions. HSP had been implicated in γδT cell-mediated anti-tumor response (18, 19). Moreover, other protein antigens, such as ectopically expressed mitochondrial ATPase, have also been identified as ligands for TCRγδ (20).

However, a crucial issue regarding γδT cells is the paucity of the identified ligands for these cells. To date, only a few human TCRγδ-recognized protein antigens have been reported. To better understand γδT cell function, we have developed a new technical strategy to identify human TCRγδ-recognized epitopes/proteins by combining immunological and biochemical methods using the CDR3 peptide of T cell receptor δ2 chain (CDR3δ)-mediated epitope/protein-binding assays (CEPAs). This CDR3δ peptide-based immunobiochemical strategy was based on a hypothesis that the primary sequence of CDR3, especially CDR3δ, because of its similarity to CDR3β of TCRβ and Vγ1, CDR3 of BCR in gene composition, could serve as the key determinant of specificity in antigen binding by the TCRγδ. We have confirmed this hypothesis using in vitro binding assays. Synthesized TCR Vδ2 CDR3 peptides derived from tumor-infiltrating lymphocytes (TILs) in ovarian epithelial carcinoma (OEC) could specifically bind tumor cell lines and tissues, suggesting the determinant role of CDR3δ in antigen binding. Moreover, CDR3δ peptide-mediated binding specificity was blocked by preincubation with the same peptide, which decreased the cytotoxicity of γδT cells to OEC cells in vitro, further indicating such binding is specific (21). Based on our previous findings, we then used synthesized CDR3δ peptides as specific probes to identify putative TCRγδ-recognized antigenic epitopes in a peptide library and TCRγδ-recognized proteins in an affinity chromatography system. As shown in supplemental Fig. 1, CDR3δ peptide-based immunobiochemical strategy is technically composed of four steps as follows: selecting probes for epitopes/proteins, screening epitopes/proteins, identifying epitopes/proteins sequences, and validating epitopes/proteins functions. Based on the specific binding of CDR3δ peptide to its target, we developed a set of CEPAs, which contains eight assays running through all the four steps of our strategy, including the following: 1) CDR3δ peptide-mediated surface plasmon resonance (SPR) to determine interaction between CDR3δ peptides and target proteins; 2) CDR3δ peptide-mediated immunofluorescence assays to detect specific binding of CDR3δ peptides to target cells; 3) CDR3δ peptide-mediated enzyme immunoassays to detect specific binding of CDR3δ peptides to target proteins and tissues; 4) competitive test to confirm CDR3δ peptide sharing antigenic binding specificity with TCRγδ; 5) panning putative epitope peptides in a 12-mer random peptide phage-displayed library with immobilized CDR3δ peptides; 6) screening antigenic proteins in affinity chromatography with CDR3δ peptide as affinity molecule; 7) phage-ELISA to select single CDR3δ peptide-binding phage clone; and 8) CDR3δ peptide-mediated Western blotting to detect CDR3δ peptide-binding proteins.

With this strategy, we have identified seven tumor-related epitopes and two HBV infection-related epitopes, as well as two self-proteins, including HSP60 and human mutS homolog 2 (hMSH2). HSP60 has been identified as a ligand for γδT cells (18, 19), whereas hMSH2 is a DNA mismatch repair protein in the cell nucleolus (22). We show that hMSH2 is expressed on tumor cell membrane and recognized by Vδ2 γδT cells, suggesting that hMSH2 may be a novel ligand for TCRγδ. Taken together, our findings provide a novel strategy to identify epitopes/proteins for TCRγδ.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—The following four Vδ2 CDR3 peptides were synthesized, including three (OT1, OT2, and OT3) corresponding to the sequences derived from γδTIL in OEC and one CDR3 δ peptide HP1, whose sequence was derived from a peripheral γδT cell clone from an HBV-infected patient: OT1, CACDSHGPSRLMMEGGLLGTDKLIFGKG; OT2, CARKDLPIWNWIPRDKLIFGKG; OT3, CDFPSHTFHS-TGHHTDKLIFGKG; and HP1, CACDHPLGDTVRHDK-LIFGKG. Meanwhile, a structurally very similar peptide HP1m was synthesized with mutations of the first four amino acids and was employed as the control for HP1: HP1m, TQEHHPL-GDTRVHDKLIFGKG. The conformational preference of the CDR3δ peptides OT3 and HP1 was analyzed by molecular dynamics. The results suggested that both of them displayed loop-like conformations in water (supplemental Fig. 2 shows the Cα root mean square deviation of the CDR3δ peptides). The modeled structure of OT3 and HP1 is shown in Fig. 1A and Fig. 2A, respectively. Seven tumor-related epitope peptides, named as EP1 to EP7, and two HBV infection-related epitopes, EP8 and EP9, were also synthesized (Table 1). All peptides were biotinylated by adding a biotin tag at the N terminus of each peptide sequence for detection in the following assays. Peptides were synthesized by the Academy of Military Medical Sciences, China. The purity of each peptide was 85% in high performance liquid chromatography analysis.

**Cell Culture and γδT Cell Preparation**—A human OEC cell line SKOV3 was cultured in McCoy’s 5A medium (HyClone) supplemented with 10% fetal calf serum (FCS), a gift from
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Dr. Keng Shen (Department of Gynecology, Peking Union Medical College Hospital, Beijing, China). Another human OEC cell line HO8910 was established in our laboratory and cultured in RPMI 1640 medium (Invitrogen) with 10% FCS. A human uterine cervix cancer cell line (HeLa) and a human T lymphoma cell line (J.RT3-T3.5) deficient in both TCR α and β chains were obtained from the American Type Culture Collection (ATCC) and maintained in 10% FCS/Dulbecco’s modified Eagle’s medium (Invitrogen) and 10% FCS/RPMI 1640 medium (Invitrogen), respectively. Human liver cancer cell line HepG2 and an HBV whole genome-transfected human liver cancer cell line (2.2.15 cell) were obtained from Cell Bank at the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences, China, and cultured with 10% FCS/minimum Eagle’s medium/NEAA medium (Invitrogen) and 10% FCS/minimum Eagle’s medium (Invitrogen), respectively. Fresh PBMC separated from peripheral blood of healthy donors by density gradient centrifugation on Ficoll-Hypaque (GE Healthcare) were grown in RPMI 1640 medium (Invitrogen) with 10% FCS/IL-2 (200 units/ml) in 24-well culture plates with immobilized anti-pan-TCRγδ-monoclonal antibody (Immunotech). After 2 weeks of culture, the purity of γδT cells was >90% as assessed by flow cytometry analysis and ready for further use.

SPR Assay—SPR experiment was performed using an IAsys biosensor (Affinity Sensors). The surface of CMD chip was activated with 200 mM N-ethyl-N’-dimethylaminopropylcarbodiimide and 50 mM N-hydroxysuccinimide. 10 ng of HSP60 was coupled to the activated CMD chip. After immobilization, the surfaces were blocked with 1 mM ethanolamine (pH 8.5) and then washed with 1 mM formic acid to remove noncoupled proteins. OT3 peptide from low to high concentrations (0.25 to 2 mM) consecutively and HP1/HP1m peptides (0.5 mM) were employed for the binding assay at room temperature. BIAevaluation version 3.1 (Biacore AB) was used for data analysis.

FCM and Confocal Microscopy Assays—Immunofluorescence assays were applied to assess CDR3δ peptide/OT3 graft immunoglobulin (OT3-Ig), whose Vα CDR3 region of a human autoantibody (IgG) recognizing thyroglobulin was replaced with OT3 peptide sequence (21), epitope peptide-mediated binding activity to target cells (tumor cells/γδT cells), and the expression of hMSH2 on tumor cells. For FCM assay, cells were incubated with biotinylated CDR3δ/epitope peptides, OT3-Ig (mouse IgG), and rabbit anti-hMSH2 polyclonal antibody (Santa Cruz Biotechnology). FITC-conjugated streptavidin (Pierce) or FITC-conjugated goat anti-mouse/rabbit IgG antibody (Pierce) was then added and incubated for 30 min at 4 °C. Wide type immunoglobulin (WT-Ig) and rabbit IgG (Zhongshan, China) were used as controls for OT3-Ig and rabbit anti-hMSH2 polyclonal antibody. The cells were analyzed on a FACSsort flow cytometer (BD Biosciences). For confocal microscopy, cells were fixed on slides by 2% cold paraformaldehyde and in turn incubated with biotinylated synthesized HP1 peptide and with FITC-conjugated streptavidin. Cells incubated with biotinylated synthesized HP1m peptide instead of the HP1 peptide were used as controls. Slides were examined with a confocal laser microscope (LSM 510; Carl Zeiss). Immunohistochemistry and ELISA—Immunohistochemistry assay was used to evaluate CDR3δ peptide-mediated binding activity to target tissues. Briefly, formalin-fixed paraffin-embedded sections of HBV-infected liver tissues or normal tissues were deparaffinized and then boiled by microwave for antigen retrieval. After quenching with hydrogen peroxide, the sections were blocked with 5% goat serum. Biotinylated synthesized HP1 peptide (5 μg) was then added to the slides. The sections were subsequently incubated with horseradish peroxidase (HRP)-conjugated streptavidin (Zhongshan, China). Binding was visualized using diaminobenzidine (Sigma) as the substrate and observed under a light microscope. In CDR3δ-ELISA, 96-well plates were coated with proteins extracted from SKOV3 cells (10 μg/well) in 0.1 M NaHCO3 (pH 9.6), or formalin-fixed SKOV3 cells (4 × 105/well). After blocking with 3% bovine serum albumin solution, the plates were incubated with biotinylated CDR3δ peptides or OT3-Ig/WT-Ig. After reaction with HRP-conjugated streptavidin (Pierce) or HRP-conjugated goat anti-mouse IgG antibody (Pierce) and substrate (Sigma), the plates were then read on a microplate reader (Labsystem) at 450 nm.

MTT Colorimetric Test and CDR3δ Peptide Competitive Test—This test was used for evaluation of γδT cell cytotoxicity to tumor lines. Briefly, SKOV3 cells as target cells were seeded onto 96-well plates (1 × 105/well). For competitive testing, target cells were preincubated with CDR3δ peptides (0.1 mg/ml) or anti-hMSH2 polyclonal antibody (0.2 mg/ml) at 37 °C for 2 h, and equivalent rabbit IgGs (Zhongshan, China) were used as controls for anti-hMSH2 polyclonal antibody. Then γδT cells as effector cells were incubated with target cells for another 8 h (the γδT cells were preincubated with epitope peptides for 2 h in stimulating assay). MTT solution (5 mg/ml) was added to wells (15 μl/well) and incubated at 37 °C for 4 additional hours. The reaction was stopped by the addition of 100 μl of lysis buffer (10% SDS) to dissolve the tetrazolium crystals. The plate was examined in a Multiskan Microplate Reader (Thermo Labsystems, Finland) and the percentage of specific lysis was calculated by using Equation 1,

\[
\frac{T_A - ((E + T)_A - E_A)}{T_A} \times 100% \quad \text{(Eq. 1)}
\]

\(T_A\) = absorbance of target cells for control; \(E_A\) = absorbance of effector cells for control, and \((E + T)_A\) = absorbance of effector cells cultured with target cells. The absorbance was determined at 570 and 630 nm.

Panning of CDR3δ Peptide-binding Phage Clones in a 12-mer Random Peptide Phage-displayed Library—A 12-mer random peptide phage-displayed library (New England Biolabs) was screened by CDR3δ peptides as follows: a 96-well plate was coated with 100 μl/well coating buffer (containing 10 μg CDR3δ peptide) for 2 h at 37 °C and then blocked with PBS containing 3% bovine serum albumin overnight at 4 °C. The primary library solution was added to the wells (10 μl per well containing 1011 colony-forming units) and shaken gently at room temperature for 1 h. After thoroughly washing with PBS, 0.1% Tween 20, the CDR3δ peptide-binding phages were eluted by acidic buffer (0.2 M glycine-HCl (pH 2.2)) and neutralized by 1 M Tris-HCl (pH 9.1) immediately. In some cases, a high concentration of CDR3δ peptide (200 μg/ml) was used for compet-
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A new peptide immunoassay was developed to identify TCRγδ-recognized epitopes in biopanning. The excess active groups were coupled to CDR3 sequence using the ELISA. The positive phage clones were then selected by phage-ELISA using HRP-anti-phage antibody (Amersham Biosciences). The in-gel peptide binding experiments were performed to confirm the binding of OT3 peptide to SKOV3 cells. The in-vitro cytotoxicity of human γδT cells was measured by FCM. The results suggest that OT3 peptide and TCRγδ bind the same ligands on SKOV3 cells. Further investigation of CDR3 peptide binding specificity is needed.
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**FIGURE 1.** Synthesized CDR3δ peptide OT3 specifically bound tumor cell line SKOV3. A, representative structure of OT3 peptide in molecular dynamics simulation. Cα trace was shown with residues colored according to their types (blue for acidic amino acids, red for basic, yellow for polar, and gray for nonpolar residues). B, flow cytometry analysis of surface staining of tumor cell lines by OT3 peptide. Normal PBMC was used as control. Cells were stained with biotinylated (bio)-OT3 peptide and FITC-conjugated streptavidin. C and D, ELISA of OT3 peptide binding to SKOV3 cell and its protein extracts. SKOV3 cells (4 × 10^5/well) or protein extracts (10 µg/well) have been immobilized on ELISA plate. The bio-OT3 peptide was added with various concentrations, and HRP-conjugated streptavidin was added subsequently. Each assay was run in duplicate. *, p < 0.05; **, p < 0.01. A value of SKOV3 cell-coated wells detected with bio-OT3 (1 µg/well), when compared with that of mock wells; **, p < 0.01. A value of SKOV3 cell coated wells detected with bio-OT3 peptide (10 µg/well), when compared with that of mock wells; A value of SKOV3 protein extracts coated wells detected with bio-OT3 peptide (1 or 10 µg/well) compared with that of mock-wells. E and F, OT3 peptide competitive binds SKOV3 in vitro. MTT assay evaluating cytotoxicity inhibition of γδT cells against SKOV3 after OT3 peptide preincubation. PBMC-derived γδT cells with the purity more than 90% in FCM analysis were used as effector cells in MTT assay (E). OT3 peptide (10 µg/well) was incubated with SKOV3 cells for 2 h before adding γδT cells. Results are representative of three independent experiments (F). The A value of OT3 peptide added wells was compared with that of wells without OT3 (*, p < 0.05, ET ratio = 10:1; **, p < 0.01, ET ratio = 20:1, and ET = 40:1). ET stands for the ratio of effector cells to target cells. G, flow cytometry analysis of surface staining by OT3-graft Ig with tumor cells SKOV3 and HeLa (blue). WT-Ig was used as control (red). Results are representative of three independent experiments. H, ELISA of OT3-graft Ig to SKOV3 total protein extract. The proteins extracts have been coated on an ELISA plate (1 µg protein/well). The OT3-Ig and WT-Ig were added, respectively, to the plate in the same concentration (5 µg/ml). Each assay was run in duplicate.

infected liver tissues and cell line 2.2.15 containing the intact dimers of the HBV genome, respectively. HP1 peptide specifically bound 2.2.15 cells but not normal cells (PBMC) and other types of tumor cell lines, such as SKOV3 and HepG2 (Fig. 2B). In immunohistochemistry assay, HP1 peptide specifically bound hepatocarcinoma tissue (Fig. 2C). Strong fluorescent staining was detected on HBV-infected 2.2.15 cells after incubating with HP1 peptide in confocal microscopy assay (Fig. 2D). Consistent with the above results, ELISA shows the specific interaction of HP1 peptide and HBV-infected 2.2.15 total protein extract (Fig. 2E). Taken together, our data suggest that the binding of synthesized HP1 peptides to targets, either tissue, cell, or protein extract, is specific.

**Biopanning for Epitopes from Phage Library Using CDR3δ Peptides**—To identify epitopes recognized by CDR3δ peptides, a CDR3δ peptide-mediated biopanning of a 12-mer random peptide phage-displayed library was performed. The positive phage population was enriched in five rounds of panning, with output to input ratio about 1 × 10^3 (supplemental Table 1). Through the selection of phage-ELISA, 120 phage clones binding specifically to immobilized-CDR3δ peptides were sequenced. As shown in supplemental Table 2, the sequences of displaying peptide on positive phage clones revealed that these clones share several motifs. We chose seven OEC tumor-related sequences and two HBV infection-related sequences as candidate epitopes from the sequenced phages based on their high frequencies among all 120 sequences. These peptide epitopes were chemically synthesized (named EP1–9) for functional assays (Table 1).

**BLAST**—BLAST search was performed to identify human proteins containing motifs in the sequences of the CDR3δ peptide-bound phage displaying peptides. The BLAST results of the nine sequences that we chose as candi-
date epitopes are listed in supplemental Table 3. No exactly matched protein sequences were returned from BLAST analysis, which was based on primary sequence searching, and the best matching manner had a maximum of 7 uninterrupted amino acids matching all 12 amino acids. In addition, most proteins in BLAST results were from prokaryotes or insects and hypothetical proteins, consistent with a role of TCRγδ in innate immune recognition. The BLAST results of other CDR3 peptide-bound motifs were similar (data not shown).

Epitope Peptides Bind and Activate γδT Cells in Vitro—To test whether the synthesized putative epitope peptides specifically bind TCRγδ, we performed a binding assay using FCM. As shown in Fig. 3A, 65% of γδT cells showed a binding activity to biotinylated peptide EP7, whereas J.RT3-T3.5 cells, a T cell line deficient in TCR chains, had much lower binding percentage. All immobilized peptides EP1–6 were able to induce proliferation of γδT cells in PBMC to an average 16% in 2 weeks, markedly higher than negative control (2.5%). The percentages of expanded γδT cells in PBMC ranged from 7.8% (EP1 and EP5) to 32.3% (EP3) (Fig. 3B). We determined the sequences of CDR3 regions in both γδ9 and γδ2 chains of EP-expanded γδT cells and found that EP-expanded TCRγδ cells were polyclonal, but with some common motifs in the CDR3 region (supplemental Table 5). The proliferation of γδT cells induced by soluble peptides EP3–5 further confirmed such stimulating activities of epitope peptides. Significant proliferation of γδT cells to peptides EP3/4 (Fig. 3C) and EP5 (Fig. 3D) was observed in a dose-dependent manner. Peptide EP6 induced the cytotoxicity of γδT cells to SKOV3 in a dose-dependent manner (Fig. 3E). Taken together, these data demonstrate that these epitope peptides not only bind to but also activate γδT cells. Similarly, HBV infection-related epitope peptides EP8 and EP9 had similar

FIGURE 2. HBV infection-related CDR3γδ peptide HP1 specifically bound HBV-infected tissues/cells/cell extracts, and HBV infection-related epitope peptides stimulated γδT cells in vitro. A, representative structure of HP1 peptide in the molecular dynamics simulation. C-w trace was shown with residues colored according to their type (the same as in Fig. 1A). B, flow cytometry analysis of surface staining by HP1 peptide to PBMC, tumor cell lines (SKOV3 and HepG2), and HBV-infected cells 2.2.15 (red). For cells with HP1m peptide, a structurally very similar peptide to HP1 with mutations of the first four amino acids, staining served as negative controls (green). Blue line shows the staining with PBS. C, immunohistochemistry staining of hepatocarcinoma tissues. The same tissues were stained with biotinylated HP1 peptide, HP1m peptide, and PBS as shown from left to right. Staining was visualized using diaminobenzidine as the substrate (brown), ×200). D, confocal microscopy analysis of the staining of HP1 peptide on HBV-infected cells 2.2.15. Binding of biotinylated HP1 peptide was analyzed by confocal microscopy after staining with FITC-conjugated streptavidin. As a control, control peptide HP1m and PBS instead of HP1 peptide was used. E, HP1 peptide specifically bound 2.2.15 protein extracts in ELISA, **, p < 0.01. A value of 2.2.15 cell protein extract-coated wells detected with HP1 peptide, when compared with all controls, including blank, HepG2 protein extracts, and HP1m peptide. F, immobilized EP8 and EP9 could induce expansion of γδT cells from PBMC. We used panel 1, RPMI 1640 medium mock as negative control; panel 2, immobilized 1 μg of anti-pan-TCRγδ monoclonal antibody as positive control; panels 3 and 4, immobilized 10 μg of epitope peptides EP8, EP9 respectively. G, EP8 and EP9 could induce proliferation of resting γδT cells at 10 μg/ml in [3H]ThdR incorporation assay.
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**TABLE 1**

Amino acid sequences of synthesized epitope peptides

| No. | Amino acid sequences | CDR3 probe | Bianning manner* | Frequency of predominant cloneb | Property         |
|-----|----------------------|------------|------------------|---------------------------------|-----------------|
| EP1 | WPHMMPHPFKVK         | OT3        | NS               | 9/51                            | Tumor related   |
| EP2 | PLLPMHPMKVSH         | OT1        | NS               | 1/35                            | Tumor related   |
| EP3 | KPPTQRRRRRQTM        | OT2        | NS               | 2/10                            | Tumor related   |
| EP4 | KPTFRLHRHRHRR       | OT1        | NS               | 4/35                            | Tumor related   |
| EP5 | YPRHMAGHYSVPN       | OT3 (OT1)  | NS               | 18/51 (10/35)                   | Tumor related   |
| EP6 | FIHHYWTTRRPS        | OT3        | S                | 9/51                            | Tumor related   |
| EP7 | WHHPWMPYPPGRV       | RT3        | S                | 6/11                            | Tumor related   |
| EP8 | HFPMLWRSDRSH        | HP1        | NS               | 5/13                            | HBV infection-related |
| EP9 | HNMMQGKTFSA         | HP1        | NS               | 2/13                            | HBV infection-related |

a There are two bianning manners including nonspecific (NS) and specific (S). NS is nonspecific elution by acidic buffer, and S stands for specific elution by high dose of corresponding probe.

b Frequency of predominant clone expressed same peptide in all tested phage clones was listed in supplemental Table 2. For example, 9/51 means this peptide appears 9 times in all 51 sequenced phage clones.

* EP2 sequence (PLLPMHPMKVSH) was found in one clone of all 35 tested phage clones with varied sequences of peptides, 10 of which shared conserved motif LPMHPM.

DISCUSSION

In this study, we demonstrate a novel strategy to identify epitopes/proteins recognized by γδT cells by combining immunological and biochemical methods. With this strategy, we identified nine γδT cell-reactive epitopes and two antigenic proteins, HSP60 and hMSH2. Another putative antigenic protein (pyruvate kinase 3 isofrom 1) showed possible sequence characteristics as a ligand for TCRγδ according to MS analysis, although the validation proceedings continue. Therefore, our method provides a novel and specific high throughput system to identify epitopes/proteins recognized by γδT cells.

This strategy includes four independent but closely related traditional steps, including selecting probe for epitopes/proteins, screening epitopes/proteins, identifying epitope/protein sequences, and validating epitope/protein functions. The binding specificity of synthetic CDR3 peptides to target molecules forms a principal base for a set of CEPAs.

In selecting probes for γδT cell-recognized epitopes/proteins, we used four assays to verify the specificity of these peptides, including CDR3 peptide-mediated SPR, enzyme immunoassay, immunofluorescence assays, and peptide competition tests. Biotin-streptavidin affinity system makes an amplification signal, which includes enzyme immunoassays as well as immunofluorescence assays. These results were consistent with the nonbiotinylated CDR3 peptide in the SPR assay, indicating the CDR3 peptides were specific and could be used as selecting probes. Moreover, data from the CDR3 peptide competitive test further confirmed the specificity of CDR3 peptide for their targets. Although CDR3 peptide showed good binding activities to targets, there were some shortcomings. A linear peptide with less than 30 amino acid residues would have some unspecific binding. To circumvent this, we constructed and expressed a CDR3-graft antibody, whose CDR3 sequences in heavy chains were replaced with CDR3, the CDR3-graft antibody showed the similar binding specificity with CDR3 peptides. In summary, the tested CDR3 peptides showed specific binding activities to target proteins, cells, and tissues and can serve as probes for screening epitopes/proteins.

CDR3 peptides were employed in two different ways: pan-ning a 12-mer random peptide phage-displayed library and
binding by affinity chromatography. One issue is whether we can obtain possible epitope peptides in the 12-mer library. It is reported that T22, an identified protein recognized by murine TCRαβ in T9253 cells, formed a TCRαβ-recognized epitope 15–18 amino acid residues in length (24). Therefore, the 12-mer random peptide phage-displayed library might cover most parts of the TCRαβ-recognized epitopes, suggesting that the strategy is feasible. We used four CDR3α peptide probes in this study. As a result, we found that CDR3α peptide had sufficient affinity to its bound proteins in the CDR3α peptide-mediated affinity chromatography, which was confirmed by the enriched collection of eluted proteins from SKOV3 cell total proteins with an elution peak. Therefore, our data suggest that CDR3α peptide-mediated affinity chromatography is useful for isolation of ligand for TCRγδ. Because the principle of this technique is based on interaction between the ligand-binding side (such as CDR3) and the receptor-binding side (such as epitope), the peptides from the ligand-binding side in other immune receptors, such as NKG2D, TLR, and BCR, might also be tested for their ligand proteins in a similar manner.

We have characterized nine peptides as putative epitopes, among which seven were tumor-related and two were HBV infection-related. BLAST search revealed that most matched proteins were conserved proteins of prokaryotes. We did not find any related human proteins in BLAST matching these putative epitope peptides, suggesting the interaction between TCRγδ and ligands, epitopes may have three-dimensional conformation rather than linear structures. Moreover, we used LC-ESI MS/MS to identify the sequences of putative antigenic proteins after CDR3α peptide-mediated Western blotting. We found that 3 proteins among 54 are considered to be possible ligands for TCRγδ, including pyruvate kinase 3 isoform 1, hMSH2, and HSP60. An important feature of our strategy was that the proteomic technique was used in combination with the immunological/biochemical method. On the one hand, the specificity of the immunological/biochemical method provides reliable support to the proteomic analysis, resulting in a high probability of successfully identifying TCRγδ recognized proteins. On the other hand, proteomic technique brings an obvious advantage to this strategy, i.e. rapid and high throughput.

In validating epitopes/proteins functions, we tested the binding of putative epitopes to γδT cells through a series of experiments. These CDR3α-bound epitope peptides were able to bind γδT cells and could trigger their proliferation and cytotoxicity to target cells in vitro.

**FIGURE 3.** Tumor-related epitope peptides bound and stimulated γδT cells in vitro. A, flow cytometry analysis of γδT cells surface staining by biotinylated epitope peptide EP7. The same staining of J.RT3-T3.5 cells was performed as control group. B, expansion of γδT cells from PBMC by immobilized epitope peptides. Panel 1, RPMI 1640 medium was added as negative control; panel 2, 1 μg of anti-pan-TCRγδ monoclonal antibody was immobilized as positive control; panels 3–8, 10 μg of epitope peptides EP1–6 were immobilized, respectively. The figure shows phycoerythrin (PE)-anti-TCRγδ and FITC-anti-TCRγδ double staining after culturing for 2 weeks. C and D, epitope peptides EP3, EP4, and EP5 triggered the proliferation of resting γδT cells in [3H]TdR incorporation assay. The counts/min value showed the activation status. E, MTT assay to evaluate the change of cytolytic activity of the γδT cells against SKOV3 on the binding of EP6. γδT cells expanded from PBMC as mentioned above were used as effector cells, and SKOV3 were target cells. Different doses of EP6 were incubated with γδT cells for 2 h before adding to target cells. Results are representative of three independent experiments.
The finding of HSP60 as a ligand for TCRγδ with our strategy is sufficient and powerful evidence for the validity of the CDR3δ peptide-based immunobiochemical strategy. HSPs were first discovered in cellular response to hyperthermia stress (25). As molecular chaperones, HSPs were first known to play critical roles in protein folding (26). Moreover, it was found that stressful conditions up-regulate the expression of HSPs, and HSPs are involved in immune responses to various pathogens and tumors (27, 28). HSPs have been identified as target antigens of tumor cells for γδT cells (18, 19). The γδT cells of oral cancer patients were able to lyse tumor cells of the same origin via recognition of HSP60 on the surface of oral tumor cells (29). The cell surface expression of HSP70 on heat-stressed tumor cells but not on unstimulated tumor cells increases their susceptibility to lysis by Vγ9δ2 T cell clones derived from autologous blood lymphocytes, and both the anti-Vδ2 monoclonal antibody and anti-HSP70 monoclonal antibody inhibit the responses (30). Our SPR analysis confirmed again the binding of synthesized CDR3δ peptide to HSP60 (supplemental Fig. 3). Besides, hMSH2 was identified.

Human mutS homolog 2 is an important component in the DNA mismatch repair pathway. Germ line mutations in hMSH2 account for ~98% of hereditary nonpolyposis colorectal cancers. Mutations in the hMSH2 gene are also associated with a greatly increased risk of sporadic colorectal cancer (22). In addition, changed expression of MSH2 RNA and/or protein has been reported in various malignancies, including ovarian, prostate, breast, lung, and renal cell carcinoma (32–36). MSH2 may exert its role in pathogenesis and progression of cancer because of its functions in cell proliferation and apoptosis (31). Although endogenous hMSH2 is located in the nuclei, through reverse transcription-PCR and sequence analysis, it was found that there seemed to be mutations in the mRNA of hMSH2 in SKOV3 cells (data not shown), and the FCM data indicate that a part of SKOV3 cells have surface expression of the mutational hMSH2, which implies a possible interaction of hMSH2 to TCRγδ. This hypothesis was confirmed by our cytotoxicity assay. The cytotoxicity of Vδ2 γδT cells, but not Vδ1 ones against SKOV3 cells,
could be blocked by anti-hMSH2 antibody. It suggests that ectopically expressed hMSH2 in malignant situation might become a novel ligand for V\textsubscript{8}T\textsubscript{6} T cells. The ectopic expression of hMSH2 in transformed cells may alert γδT cells of the transformed cells. In summary, we successfully obtained functional epitopes/proteins for TCRγδ through a novel strategy, based on the core technique CEPA, demonstrating that it is useful in identification of γδT cell-recognized epitopes/proteins.

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