Antigen Specificity of γδ T Cells Depends Primarily on the Flanking Sequences of CDR3δ*

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The structural basis that determines the specificity of γδ T cell receptor (TCR) recognition remains undefined. Our previous data show that the complementary determining region of human TCRδ (CDR3δ) is critical to ligand binding. Here we used linear and configurational approaches to examine the roles of V, N-D-N, or J regions in CDR3δ-mediated antigen recognition. Surprisingly, we found that the binding activities of CDR3δ from different γδ TCRs to their target tissues and ligands depend on the conserved flanking sequences (V and J) but not as much on the D region of CDR3δ fragment. We further defined the key residues in the V and J regions of CDR3δ fragments, including the cysteine residue in the V fragment and the leucine residue in the J fragment that determine their ligand binding specificity. Our results demonstrate that TCRδ primarily uses conserved flanking regions to bind ligands. This finding may provide an explanation for the limited number of γδ TCR ligands that have as yet been identified.

Extensive studies suggest that γδ T cells play important roles in host defense against microbial infections, monitoring of tumorigenesis, immunoregulation, and development of autoimmunity (1–3). However, little is known about the structural basis of antigenic recognition by γδ T cell receptor (TCR) because of the limited identified specific ligands for γδ TCR and the lack of structural information revealing how γδ TCR might interact with such ligands.

The crystallographic structure of a murine γδ TCR in complex with major histocompatibility complex class (MHC) Ib T22 (4, 5) showed that the CDR loops of γδ TCR, predominantly germline-encoded residues of the complementary determining region of human TCRδ (CDR3δ), are in direct contact with T22, suggesting that the primary sequence of CDR3 in γδ TCR, especially CDR3δ, serves as a key determinant for the specificity of antigen recognition. Our recent finding that CDR3δ peptide mimics human γδ TCR binding to tumor cells and tissues is consistent with the role of CDR3δ in γδ TCR recognition (6).

Based on this finding, we used synthesized CDR3δ peptide as a probe to screen putative protein ligands in tumor protein extracts by affinity chromatography analysis. With this novel strategy, we have successfully identified seven tumor-related epitopes, two hepatitis B virus (HBV) infection-related antigenic epitopes, and two self proteins including heat shock protein (HSP) 60 and human mutS homolog 2 (hMSH2) that are recognized by human γδ TCR (7). These results further support that the primary sequence of CDR3δ in γδ TCR determine the specificity of antigen binding.

CDR3δ is composed of fragments derived from V, N-D-N, and J gene segments. The flanking sequences composed of V and J fragment is conserved while N-D-N region is diverse. The diversity of γδ TCRs is supposedly higher than that of TCRαβ due to the link of D gene fragment and the insertion of nucleotide acids (8). However, the number of identified antigenic ligands recognized by γδ TCR remains very limited. It has been demonstrated that γδ TCR recognizes some protein antigens and small phosphate or amine-containing compounds, including nonclassical MHC class I molecule T22 and T10 in mice (9), UL-16-binding protein (ULBP) (10) and mitochondrial F1-ATPase in humans (11). Nevertheless, important questions regarding γδ TCR recognition remain to be addressed. For example, given the seemingly high diversity of γδ TCR, why have only limited antigenic ligands been identified? What are the contributions of individual fragments of CDR3δ to antigen recognition? In αβ TCR, a single mutation in D gene fragment (12) abolishes its antigenic recognition, whereas the contribution of the different fragments in γδ TCR recognition remain unknown. Answers to these questions will shed important insights to antigen recognition of γδ T cells.

In this study, we investigated the contribution of individual fragments of CDR3δ in antigen recognition. We mutated V, N-D-N, or J fragments of a V82 TCR CDR3 sequence (OT3) in peptide and engineered γδ TCR. We found that the conserved
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flanking regions of CDR3δ play a critical role in antigenic binding to OEC cells/tissues or hMSH2 protein, a new ligand for γδTCR we found recently (7). Furthermore, we have identified the cysteine residue in V fragment and the leucine residue in J fragment as critical residues in the binding activity of γδ TCR. These results demonstrate that TCRδ chain uses the conserved flanking regions to recognize their antigens, suggesting that ligands for γδ TCR may also be conserved and limited in number.

EXPERIMENTAL PROCEDURES

Cell Lines and Human Tissue Specimens—Various tumor cell lines including HO8910, 803, Hela, HepG2, K562, and J.RT3-T3.5 cell were obtained from the American Type Culture Collection (ATCC). The human ovarian tumor cell line SKOV3 was a gift from Dr. Keng Shen (Department of Gynecology, The Peking Union Medical College Hospital, China). PBMCs were obtained from peripheral blood of healthy donors by density gradient centrifugation on Ficoll-Hypaque (Amersham Biosciences). Fresh tumors and normal tissue specimens were obtained from the Peking Union Medical College Hospital. All of the tissue specimens from patients diagnosed by standard histopathological and immunohistochemical assay were collected prior to treatment with chemotherapy, radiotherapy, or Chinese traditional medical therapy. All human studies were carried out according to proven guidelines by PUMC.

RT-PCR and Sequence Analysis—Total RNA was isolated from the tumor infiltrating lymphocytes (TILs) of the ovarian epithelial carcinoma (OEC) tissues with TRIzol reagent (Promega). cDNA was synthesized using oligo-dT (Promega) as primer and moloney murine leukemia virus reverse transcriptase (Promega) in the reverse transcription reaction and amplified by PCR using Vδ- and Cδ-specific primers as follows. The primers were TCRδV2: 5'-GCA CCA TCA GAG AGA -3'; TCRδC 5'-AAA CGG ATG GTT TGG -3'. Amplified cDNA was cloned into the pGEM-T easy vector (Promega) and sequenced with the ABI automatic sequencer 3770.

Construction of Cell Lines Expressing γδ TCR—A full-length γ or δ chain was amplified from PBMC cDNA using primers containing Kpn-I and Xho-I restriction sites. Primers encoding the entire γ9 and δ2 CDR3 sequence were used to construct the first half and the second half of the γ9 and δ2 chain. The two overlapping PCR products were then used in a third PCR reaction to recreate the full-length γ9 and δ2 chain whose CDR3 region was the OT3 nucleic acid sequence. The full-length TCR chain was digested with Kpn-I and XhoI and cloned into pREP7 and pREP9 expression vectors containing either hygromycin or neomycin resistance. The J.RT3-T3.5 cells (1.2 × 10^6) were transfected with 20 μg each of pREP7-γ9 plasmid and pREP9-δ2 plasmid by electroporation at 260 V and 975 μF using the Bio-Rad Gene-Pulser. After 48 h, the cells transfected with both vectors were selected by hygromycin and neomycin for 4 weeks. The resulting cell lines were then validated for expression of γδ TCR using reverse transcription polymerase chain reaction and FACS analysis.

Peptide Synthesis and Protein Expression—Peptides including wild-type CDR3δ (OT3) and its mutants were synthesized in the peptide synthesis facility of the Academy of Military Medical Sciences, China. Peptide OT3Vm, OT3Dm, and OT3Jm were respectively synthesized with random arrangement amino acid sequence in V, N-D-N, and J fragment of OT3. MAB peptide derived from melanoma Ag recognized by T cells-1 (MART)-specific αβ T cells, as a negative peptide control, was synthesized (see Table 2). The purity of synthesized peptides was more than 90% in HPLC analysis. The synthesized peptides were all labeled with a biotin at their N terminus. The engineered chimeric protein and its mutants, containing the extracellular domains of the human γ9 and δ2 TCR chains fused to the hinge region, CH2 and CH3 domains of human IgG1, heavy chain, were expressed by the Sino Biological Inc. The CDR3δ sequence of the chimeric protein is concordant with the OT3 peptide and its V/D/J mutants. The proteins were named as γ9/δ2(OT3)-Fc, γ9/δ2(OT3)Vm-Fc, γ9/δ2(OT3)-Dm-Fc, and γ9/δ2(OT3)Jm-Fc, respectively.

Sequence Mutagenesis of V/D/J and Site-directed Mutation in CDR3δ(OT3)—We constructed the full-length δ2 chain containing different mutations of OT3 according to the previous amino acid sequence. For the mutant D and J fragment, overlapping PCR was used to construct the full-length δ2 chain. For the mutant V fragment and site-directed mutation, the full-length δ2 chain whose CDR3 region was the OT3 nucleic acid sequence was subcloned into pCDNA3.1. The plasmid of pCDNA3.1-δ2 chain was amplified with mutant-specific primer by pfu DNA polymerase. The PCR products were visualized on 0.8% TAE agarose gels and then purified using silica columns according to the manufacturer’s instructions. The phosphate base was added to the 5'-end of the product using the T4 polynucleotide kinase under the action of ATP. After looping, the plasmid was transformed into DH5α Escherichia coli. Once the sequence was confirmed, the constructs were digested with Kpn-I and XhoI and cloned into pREP9 expression vectors. We co-transfected each of them together with an identical pREP7-γ9 chain into J.RT-T3.5 cells, and various transfected cells with stable expression of γδ TCR were evaluated by function assay.

Stimulation of J.RT3-T3.5 Transfectants—The transfected cells expressing different γδ TCR were preincubated with 10 ng/ml phorbol myristate acetate (PMA) at room temperature for 30 min. PMA treatment was essential for TCR-mediated activation in this system. After extensive washing with RPMI 1640, the cells were plated into 24-well plates at 1 × 10^6 in the presence of tumor cell protein extracts, heat shock protein (HSP)70, or iso-butylamine (IBA). After 24 h, the supernatants or cells were harvested. The level of IL-2 in supernatants was detected using human IL-2 ELISA kit (B.D. Company) according to the manufacturer’s instructions. Meanwhile, the RNA of cells was extracted to analyze the level of IL-2 by real-time PCR. For experiments in which the responses for different mutated TCR constructs were compared, surface γδ TCR expression levels on all transfected cells were measured by FACS analysis to ensure there was no significant difference. Cells were stained within 48 h of their use in the stimulation assay.

Enzyme-linked Immunosorbent Assay—96-well plates were coated with 0.5 μg of the N-terminal fragment of hMSH2 pro-
tein (MNS) in 0.1 M NaHCO₃ (pH 9.6). After blocking with 5% bovine serum albumin, the plates were incubated with biotin-conjugated CDR3δ (OT3) peptide or γ9δ2 (OT3)-Fc protein and its variants for 1 h at room temperature. The plates were developed using HRP-conjugated streptavidin (Pierce) or HRP-conjugated goat anti-human IgG antibody (Sigma) and substrate (Sigma) and read on a microplate reader at 450 nm (Labsystem).

**Flow Cytometry**—Cells were incubated with biotin-conjugated CDR3δ(OT3) peptide or their V/D/J mutants or the γ9δ2 (OT3)-Fc protein and its V/D/J mutants (30 min, 4 °C). FITC-conjugated streptavidin (Pierce) or FITC-conjugated goat anti-human IgG antibody was then added and incubated (30 min, 4 °C). The cells were analyzed on a flow cytometer (Becton Dickinson).

**Confocal Microscopy**—Cells were fixed on slides by 2% cold paraformaldehyde and in turn incubated with biotin-conjugated synthesized CDR3δ(OT3) peptide and their V/D/J mutant peptide, or the γ9δ2(OT3)-Fc protein and its V/D/J variants. FITC-conjugated streptavidin or FITC-conjugated goat anti-human IgG antibody (Pierce) was then added and incubated (30 min, 4 °C). Controls included phosphate-buffered saline or wild-type Ig as the primary antibody. Slides were incubated (30 min, 4 °C). The cells were analyzed on a flow cytometer (Zeiss).

**Immunohistochemistry**—Formalin-fixed paraffin-embedded sections of tumor tissues were deparaffinized and then boiled by microwave for antigen retrieval. After quenching with peroxide, the sections were blocked with 5% goat serum. Then, biotin-conjugated synthesized peptides (0.5 μg) or V/D/J mutants and γ9δ2(OT3)-Fc protein or V/D/J mutants were added to the slides. The sections were incubated with HRP-conjugated streptavidin or HRP-conjugated goat anti-human IgG antibody. Binding was visualized using diaminobenzidine (Sigma) as the substrate and observed under microscope.

**Surface Plasmon Resonance (SPR)**—SPR studies were carried out with a BIAcore 3000 instrument at 25 °C using HBS-EP running buffer (BIAcore, Sweden). Protein MNS was diluted to 30 μg/ml in 10 mM sodium acetate, pH 4.0, and immobilized on a CM5 chip using EDC/NHS according to the manufacturer’s instructions. The amount of immobilized protein was about 6000 resonance units. OT3 or its V/D/J mutant peptide and γ9δ2(OT3)-Fc protein or its V/D/J mutant protein were flowed as analyte at different concentrations. The recorded sensograms were analyzed using BIAnalysis software (Biacore Life Sciences). The data thus obtained were globally fit using a 1:1 binding model to calculate the dissociation constants (KD).

**RESULTS**

**Specific Binding of CDR3δ Peptide to Antigens for γδ TCR Depends on Its Flanking Sequences**—We previously identified a gene sequence of CDR3δ in Vδ2 from TILs in OEC (6) and named its synthesized peptide as OT3. CDR3δ is formed by somatic rearrangement of V, N-D-N, and J fragments. V and J genes encode the conserved flanking sequences of CDR3δ, while N-D-N genes encode inner diverse regions (Fig. 1A). To determine which fragment of CDR3δ is the key determinant for antigen recognition, we generated OT3 peptide mutants by replacing V, N-D-N, or J fragments of OT3 with randomly arranged amino acid sequences of the same length termed peptide OT3Vm, OT3Dm, and OT3Jm, respectively. The binding of these synthesized peptide mutants to target cells and tissues in vitro was examined. As shown in Fig. 1, B and C, in contrast to the staining by wild-type OT3 peptide, the percentages of SKOV3, H08910, or Hela tumor cell lines stained by peptides OT3Vm and OT3Jm were dramatically reduced whereas the percentage of positive cells stained by peptide OT3Dm was only modestly reduced. Similar binding activities of these OT3 peptides to OEC specimens were also observed (Fig. 1D).

We further examined the interaction of CDR3δ peptides with an identified ligand for γδ TCR, hMSH2 protein. As shown in Fig. 1E, the binding activity of peptides OT3Vm and OT3Jm to the N-terminal fragment of hMSH2 protein (MNS) was dramatically reduced while that of OT3Dm was not significantly affected. Furthermore, SPR analysis showed dramatic decreases in binding affinity of peptides OT3Vm and OT3Jm with MNS when compared with peptide OT3. In contrast, the peptides OT3Dm retained its strong binding to MNS (Fig. 1F). These data strongly suggest that V and J fragments are the primary determinants for the binding capacity of CDR3δ peptide to target tumor cells/tissues and ligand, while the D fragment only contributes minimally to such binding.

**Cell Surface-expressed γδ TCR Binding to Tumor Cells Depends on the V and J Fragment of CDR3δ**—The binding of various OT3 peptides to different target cell tissues or antigen ligand needs to be further validated in the context of intact γδ TCR recognition. Therefore, we generated T cell lines expressing different γδ TCRs containing OT3 or its mutants. We selected a γ9 chain that paired with the δ2 chain. We first analyzed the CDR3 sequence of the Vγ9 chain in TIL of OEC (Table 1). No predominant motif was observed among these sequences. Nevertheless, the sequence of clone 9 appeared three times among 20 sequenced Vγ9 chains. Therefore, we used the full-length γ9 chain containing the CDR3 region from clone 9 to pair with various δ2 chains containing different CDR3. These results from RT-PCR and FACS demonstrated the successful generation of J/RT3-T3.5 transfectants expressing γδ TCR with OT3 or its mutants.

To test the antigen recognition capability of these cells expressing γδ TCRs, we stimulated the transfected cells with tumor antigens. After stimulation with protein extracts from tumor cells, the production of IL-2 by the cell lines was detected by ELISA. As shown in Fig. 2A, compared with cells expressing γδ TCR with wild-type OT3, those expressing γδ TCR with OT3Vm or OT3Jm produced much less IL-2 after the cells were stimulated with protein extracts from different tumor cells and recombinant HSP70 protein. Importantly, cells expressing γδ TCR with OT3Dm secreted similar amounts of IL-2 to that secreted by cells expressing γδ TCR with wild-type OT3. These IL-2 secretion results were further confirmed by quantitative analysis of IL-2 mRNA in the stimulated cells (Fig. 2B). These results suggest that mutations in the V and J but not D regions affect γδ TCR antigen recognition capability.

**Cell Surface-expressed γδ TCRs Bind to Tumor Cells through Cysteine in the V or Leucine in the J Fragment of CDR3δ**—To determine the key positions of antigen recognition in the V and
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FIGURE 1. Specific binding of CDR3δ2 peptide to tumor target cells or tissues and ligand for γδ TCR depends on its flanking sequences. A, schematic of flanking sequences in CDR3δ2. B, FACS analysis of the binding through wild-type OT3 peptide or its V/D/J mutants to tumor cells. Peptide OT3Vm, OT3Dm, or OT3Jm represents the peptide mutant in the V, D, or J fragment of a CDR3δ2 sequence (OT3), respectively. After incubation with biotin-labeled synthetic peptides, tested cells were stained with FITC-conjugated streptavidin and then analyzed by FACS. Data shown are one of three independent experiments yielding similar results. C, confocal microscopy analysis of binding via wild-type OT3 peptide and its V/D/J mutants to tumor cells. After incubation with biotin-labeled synthetic peptides, tested cells were stained with FITC-conjugated streptavidin and then analyzed by laser-scanning confocal microscopy. Confocal images are one representative of two independent experiments. Bars, 50 μm. D, immunohistochemistry analysis of the binding activities of wild-type OT3 peptide or its V/D/J mutants to specimens (OEC and normal ovarian specimens). Synthesized Vδ3 CDR3 peptide MAB was used as a negative control. Binding was visualized using diaminobenzidine as the substrate (brown (×200). E, ELISA of the binding by wild type OT3 peptide and its V/D/J mutants to the N-terminal end protein of hMSH2 (MNS, 1–329 amino acids). 96-well plates were coated with 0.5 μg of MNS in 0.1 M NaHCO3 (pH 9.6). After blocking with 5% bovine serum albumin, the plates were incubated with biotin-conjugated CDR3δ2(OT3) peptide or its V/D/J mutants for 1 h at room temperature. The plates were developed using HRP-conjugated streptavidin (Pierce) and substrate (Sigma) and read on a microplate reader at 450 nm. Data are shown as mean of OD ± S.D. of three independent experiments.

TABLE 1

Vγ9 CDR3 gene sequence-deduced amino acid sequences from γδTIL in OEC

| Clone | TCRγV | N | J | Number |
|-------|-------|---|---|--------|
| 1     | CALWE | ALGSR | WFKIAFE(Fγy1.1) | 2 |
| 2     | CALWE | ALGSR | QELGKKIKVF(Gγy1.2) | 2 |
| 3     | CALW  | AG    | ELGKKIKVF(Gγy1.2) | 1 |
| 4     | CALW  | DVPVR | ELGKKIKVF(Gγy1.2) | 1 |
| 5     | CALW  | KVHL  | ELGKKIKVF(Gγy1.2) | 1 |
| 6     | CALW  | VI    | ELGKKIKVF(Gγy1.2) | 1 |
| 7     | CALW  | NSK   | ELGKKIKVF(Gγy1.2) | 1 |
| 8     | CALW  | FP    | ELGKKIKVF(Gγy1.2) | 1 |
| 9     | CALW  | W     | ELGKKIKVF(Gγy1.2) | 1 |
| 10    | CALW  | VL    | ELGKKIKVF(Gγy1.2) | 1 |
| 11    | CALW  | VYTSR | ELGKKIKVF(Gγy1.2) | 1 |
| 12    | CALW  | AG    | ELGKKIKVF(Gγy1.2) | 2 |
| 13    | CALW  | DK    | LGKKIKVF(Gγy1.2) | 1 |
| 14    | CALW  | VQ    | LGKKIKVF(Gγy1.2) | 1 |

J fragment, we mutated each amino acid of the V and J fragment of OT3 (Table 2). After the transfected cells were stimulated with whole protein extracts of SKOV3 cells (OEC cell line), the production of IL-2 was compared. Mutations of cysteine in the V fragment or leucine in the J fragment dramatically decreased IL-2 production of the tested cells (Fig. 3A). We further investigated whether these residues are required for binding to non-peptide antigens by γδ T cells. As shown in Fig. 3B, the cysteine in the V or leucine in the J fragment of CDR3δ2 did not affect the binding of γδ TCR to non-peptides, suggesting that both residues are indispensable in binding activity of γδ TCR to peptide antigen.

Engineered Soluble γδ TCRs Critically Depend on the V and J Fragment of CDR3δ2 for Binding to Tumor Cells/Tissues and Ligands—We used a third approach to examine the interaction between γδ TCR and its target cells/tissues or protein ligands. We engineered chimeric proteins composed of the extracellular domains of human γδ and δ2 chains containing OT3 or its V/D/J mutants fused to the hinge region, CH2 and CH3 domains of human IgG1 heavy chain and expressed the fusion protein in eukaryocytes. We examined the binding capacity of these soluble γδ TCR to tumor cells by FACS and confocal microscopy analyses. In contrast to the high percentages of SKOV3 cells (90%) and other tumor cells (60–65%) stained positive with γ9/δ2(OT3)-Fc fusion protein, both fusion proteins with V or J mutation in CDR3δ2 stained a significantly lower proportion of the tumor cells (from 9 to 58%) in FACS analysis (Fig. 4A). Furthermore, confocal microscopy analysis showed dramatic decreases in fluorescent intensities stained by Vm and Jm fusion proteins when compared with wild-type OT3 fusion protein (Fig. 4B). In both analyses, the Dm fusion protein retained its strong binding to these tumor cells (Fig. 4, A and B). Importantly, similar binding activities of these g9/d2(OT3)-Fc fusion proteins to OEC specimens
were also observed (Fig. 4C). In addition, these engineered soluble γδ TCR proteins also interacted with an MNS in ways similar to those displayed in tumor cell binding (Fig. 4, D and E). Taken together, these results further support that V and J but not D regions in CDR3δ (OT3) critically contribute to ligand recognition.

**DISCUSSION**

Although γδ TCRs have been identified for more than 20 years, their known ligands are still very limited in number. It is not clear why this is the case. Our data here may provide a possible explanation: γδ TCRs critically depend on their conserved regions to recognize ligands, and the limited diversity in these regions correspond to a limited number of γδ T cell ligands.

We have applied three approaches to determine the functional importance of the individual fragment of CDR3δ. First, we used wild-type CDR3δ2 and its V/D/J mutant peptides to examine their ligand binding capabilities. Mutation in the V or J fragment dramatically impaired their staining capability to tumor cells or tissues, as well as hMSH2, a ligand for γ9/δ2 TCR. Mutation in N-D-N region did not affect its binding to the same target. Second, we generated cells which expressed γδ TCRs containing wild-type CDR3δ2 or its V/D/J mutants and examined their antigen recognition capability. Mutation in the V or J fragment impaired the binding capability of transfected cells expressing γδ TCR, while mutation in the N-D-N fragment did not have this effect. Third, we examined γδ TCR recognition using engineered chimeric proteins containing the extracellular domains of the human γ9 and δ2 chains with the CDR3δ2 or its V/D/J mutants. Again, the results suggest that the V and J but not D region contribute critically to γδ TCR recognition. We also identified cysteine in the V fragment or leucine in the J fragment is the critical determinant in γδ TCR antigen recognition of antigen. Taken together, this evidence strongly suggests that the conserved flanking sequences of the CDR3δ domain play a key role in determining antigen binding activity.

The results of Adams et al. (13) demonstrated that the D gene segment is responsible for binding to T22 antigen for G8 γδ T cells. Our results showed that the V/J segments of TCR δ are responsible for binding to γδ TCR. The contradiction may be explained by two differences: first, the antigen recognized by γδ TCR derived from human and mice was different. T22 was the antigen originating from mouse γδ TCR and the OT3 sequence recognizing the human antigen. Second, the length of γδ TCR CDR3 from human and mice was different. The length of mice γδ TCR CDR3 was shorter than that of human. So our conclusion was that for tumor peptide antigen, V/J segments of TCR δ, but not the D gene segment, are responsible for binding to γδ TCR.
Our results have revealed an important aspect of γδ TCR antigen recognition. All heterodimers of αβ TCR, γδ TCR, and BCR are products of rearranging genetic segments. At the first glance, these three receptors are similar in structure and genetic composition. However, the mechanisms of diversity formation are quite different. Because of a large number of gene segments, the diversity of antibodies relates to a large extent on V, D, and J gene rearrangement (14). By contrast, there are only a few V, (D), and J segments encoding the αβ TCR, so the use of V segments and N region addition greatly increase the available diversity of αβ TCR (15). There are even fewer V, D, and J segments that encode the γδ TCR. The potential diversity of γδ TCR comes primarily from the use of Dδ segments in all their reading frames, and N region addition at three different positions.

Comparing to $10^{15}$ for the αβ TCR repertoire, the total potential repertoire of γδ TCR is about $10^{18}$ of junctional diversity; however, to date only a handful of ligands for γδ TCR have been identified. It appears paradoxical that a huge potential repertoire of γδ TCR only recognizes a few ligands unless the potential repertoire is not utilized for the generation of specificity. Alternatively, the N-D-N fragment in γδ TCR may not play a critical role in determining antigen specificity. Our data clearly support the second possibility. The flanking sequences encoded by V and J fragments are more important in antigen recognition, since the mutation of V and J portion of the CDR3δ almost completely abolished their binding to tumor cells or tissues, while mutation of N-D-N residues minimally affected γδ TCR antigen recognition. Based on our results, we postulate that the flanking sequences may act as “rough-adjusters” for antigen recognition, while the highly diverse fragment of N-D-N may serve to fine-tune the affinity of γδ TCR to certain antigens. Our finding supports the notion that the conserved fragment within CDR3δ and the flanking sequences form a broad specificity to “rough-see” the molecules induced by stressful stimuli such as tumorigenesis or infection by recognizing that limited epitopes existed in these molecules. With evolution, such a “rough-see” way connects with “fine-see” way of αβ T cells and B cells by delivery of co-stimulator signals. Both αβ T cells and B cells are capable of distinguishing precisely a tiny and unique difference (epitope) in a given antigen from those of numerous foreign antigens. This very narrow specificity is based on the high diversities of antigen-receptor repertoires.

γδ TCR recognition may behave more similar to pattern recognition by innate immune cells. Macrophages and DCs can recognize non-self structures such as pathogen-associated molecular patterns (PAMPs) by pattern recognition receptor (PRR) such as Toll-like receptors (TLRs) (16). More recent studies strongly emphasize the innate features and functions of γδ T cells, including the participation in wound healing (17), tissue repair (18), and the ability to present antigen (19–20). Previously, we have used CDR3 peptide as probe to pan twelve

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**FIGURE 4. Binding activity of engineered soluble γδ TCR with CDR3δ2 (OT3) or V/D/J mutant to tumor cells or tissues and hMSH2 protein.** A, flow cytometric analysis of the binding by γδ2(OT3)-Fc protein and its V/D/J mutants including γδ2(Vm-Fc), γδ2(Dm-Fc), and γδ2(Jm-Fc) to tumor cells. After incubation with the protein, tested cells were stained with FITC-conjugated goat anti-human heavy chain and then analyzed by flow cytometry. Data are one of three independent experiments yielding similar results. B, confocal microscopy analysis of the binding by γδ2(OT3)-Fc protein and its V/D/J mutants to tumor cells. After incubation with the fusion protein, tested cells were stained with FITC-conjugated goat anti-human heavy chain and then analyzed by laser-scanning confocal microscopy. Confocal images are one representative of two independent experiments. C, immunohistochemistry analysis of the binding activities of γδ2(OT3)-Fc protein and its V/D/J mutants to tumor cells or tissues. Brown ammonium is visualized using diaminobenzidine as the substrate (brown) (×100). D, ELISA analysis of the binding activities of γδ2(OT3)-Fc protein and its V/D/J mutants to MNS. Data are mean of OD ± S.D. of two independent experiments. E, SPR analysis of the binding affinity by γδ2(OT3)-Fc protein and its V/D/J mutants to MNS. γδ2(OT3)-Fc protein and its V/D/J mutants were injected at concentration of 1:1 serial solution from 3000 nM to 187.5 nM, 2500 nM to 156.2 nM, 1200 nM to 150 nM, and 1000 nM to 125 nM, respectively. Data are representative of two independent experiments.
peptide libraries. Nine peptides as putative epitopes were identified, among which seven were tumor-related and two were HBV infection-related. BLAST searches revealed that most matched proteins were conserved proteins of prokaryotes (7). Results suggest the possibility that γδ TCR recognizes some conserved molecules. During the recognition, the flanking sequences within CDR3δ, the conserved fragment of γδ TCR, might recognize readily their ligands via a pattern recognition model. Our finding may provide an explanation for the limited number of γδ TCR ligands that have as yet been identified.

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