

# Flanking V and J Sequences of Complementary Determining Region 3 of T Cell Receptor (TCR) $\delta$ 1 (CDR3 $\delta$ 1) Determine the Structure and Function of TCR $\gamma$ 4 $\delta$ 1\*

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The  $\gamma\delta$  T cell receptor (TCR) differs from immunoglobulin and  $\alpha\beta$  TCR in its overall binding mode. In human, genes  $\delta$ 1,  $\delta$ 2, and  $\delta$ 3 are used for TCR $\delta$  chains. Previously, we have studied antigen binding determinants of TCR $\delta$ 2 derived from dominant  $\gamma\delta$  T cells residing in peripheral blood. In this study we have investigated the critical determinants for antigen recognition and TCR function in TCR $\delta$ 1 originated from gastric tumor-infiltrating  $\gamma\delta$  T lymphocytes using three independent experimental strategies including complementary determining region 3 (CDR3) of TCR $\delta$ 1 (CDR3 $\delta$ 1)-peptide mediated binding, CDR3 $\delta$ 1-grafted TCR fusion protein-mediated binding, and TCR $\gamma$ 4 $\delta$ 1- and mutant-expressing cell-mediated binding. All three approaches consistently showed that the conserved flanking V and J sequences but not the diverse D segment in CDR3 $\delta$ 1 determine the antigen binding. Most importantly, we found that mutations in the V and J regions of CDR3 $\delta$ 1 also abolish the assembly of TCR and TCR-CD3 complexes in TCR $\gamma$ 4 $\delta$ 1-transduced J.RT3-T3.5 cells. Together with our previous studies on CDR3 $\delta$ 2 binding, our finding suggests that both human TCR $\delta$ 1 and TCR $\delta$ 2 recognize antigen predominately via flanking V and J regions. These results indicate that TCR $\gamma\delta$  recognizes antigens using conserved parts in their CDR3, which provides an explanation for a diverse repertoire of  $\gamma\delta$ TCRs only recognizing a limited number of antigens.

T lymphocytes can be divided into two distinct subsets,  $\alpha\beta$  and  $\gamma\delta$  T cells, based on the type of T cell receptors (TCR)<sup>2</sup> expressed. The  $\alpha\beta$  T cells mainly recognize peptide antigens bound to class I or class II major histocompatibility complex (MHC) molecules, whereas  $\gamma\delta$  T cells mount immune

responses directly to nonpeptide antigens and superantigens (1). Extensive studies suggest that  $\gamma\delta$  T cells may function in many aspects of immunity including infection, tumor immunity, tissue homeostasis, and immune regulation (2–4). In humans,  $\gamma\delta$  T cells use three main V $\delta$  ( $\delta$ 1,  $\delta$ 2,  $\delta$ 3) and various V $\gamma$  genes to make their functional TCRs (5, 6). Most of  $\gamma\delta$  T cells in peripheral blood express V $\delta$ 2 chain paired with V $\gamma$ 9 chain, whose TCRs are dedicated to the recognition of nonpeptide phosphoantigens, alkylamines, and synthetic aminobisphosphonates (7). Whereas in other tissues  $\gamma\delta$  T cells express TCRs primarily made of predominant V $\delta$ 1 or a few V $\delta$ 3 chains paired with a diverse array of V $\gamma$  chains. V $\delta$ 1 T cells reside mainly in mucosal and epithelium tissue and recognize MHC-related molecules such as MHC class I chain-related gene A (MICA) and MICB as well as several other ligands (8, 9).

The structural basis of antigen recognition of  $\alpha\beta$  TCR is well defined. However, the parallel information for  $\gamma\delta$  TCR is largely lacking due to the fact that only a limited number of  $\gamma\delta$ TCR-specific ligands have been identified so far. Although  $\alpha\beta$  TCRs often use all six complementary determining region (CDR) loops in their recognition of peptide-MHC complexes, the crystallographic structure study showed that predominantly germ line-encoded residues of the CDR3 of human TCR $\delta$  (CDR3 $\delta$ ) are responsible for most of the interactions between the G8  $\gamma\delta$  TCR and MHC class Ib T22 protein in mice (10). Moreover, the residues involved in the recognition interface are derived predominantly from germ line-encoded D $\delta$  segment. In addition, recently studies have shown that mutations in all CDR loops of human V $\gamma$ 9 $\delta$ 2 TCR could alter the antigen binding, suggesting that the recognition of prenyl pyrophosphates by V $\gamma$ 9 $\delta$ 2 TCR is dependent on all its CDRs (11).

CDR3 $\delta$  is composed of V, N-D-N, and J gene segments. The flanking V and J sequences are conserved, whereas N-D-N region is diverse as the result of VDJ recombination and insertion of N nucleotides (12, 13). Our groups have successfully identified two self-proteins including heat shock protein 60 and human mutS homolog 2 (hMSH2) as TCR $\delta$ 2 ligands using affinity chromatography analysis with a synthetic CDR3 $\delta$ 2 peptide as the probe and confirmed their recognition by TCR $\delta$ 2-expressing cells in peripheral blood (14). Furthermore, we also demonstrated that the direct antigen recognition role of CDR3 $\delta$ 2 sequences in TCR $\gamma$ 9 $\delta$ 2 derived from ovarian epithelial carcinoma (OEC) (15). We have identified that the flanking V and J regions of human CDR3 $\delta$ 2 play a critical role in ligand

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<sup>2</sup> The abbreviations used are: TCR, T cell receptor; hMSH2, human mutS homolog 2; CDR3 $\delta$ , complementary determining region 3 of human TCR $\delta$ ; CDR3 $\delta$ 1/2, CDR3 of human TCR $\delta$ 1/2; TIL, tumor infiltrating lymphocyte; OEC, ovarian epithelial carcinoma; GTM, one master sequence of CDR3 $\delta$ 1 representing four cases of gastric tumor  $\gamma\delta$ TILs; SPR, surface plasmon resonance; MICA, MHC class I chain-related gene A.

## Flanking V and J Sequences of CDR3 $\delta$ 1 Determine TCR $\gamma$ 4 $\delta$ 1

**TABLE 1**

Amino acid sequences of GTM and its mutants in V, N-D-N, and J region of CDR3 $\delta$ 1

Peptide	V	N-D-N	J
GTM	CA	FLPHA	DKLIFGKG
GTMVm	FL	FLPHA	DKLIFGKG
GTMDm	CA	YRDEK	DKLIFGKG
GTMJm	CA	FLPHA	PASNCWTH

binding, as targeting for OEC cells/tissues and hMSH2 protein (16).

Compared to V $\delta$ 2, V $\delta$ 1 T cells differ in V $\delta$  gene usage, paired  $\gamma$  chains (17, 18), resident tissue (19, 20), and antigen specificity (8, 21–23). It remains unclear if the V $\delta$ 1 subset utilizes a distinct antigen recognition mechanism and the nature of the contribution of CDR3 $\delta$ 1 domain to the antigen recognition. In this study we aim to answer these questions by investigating the involvement of flanking V and J sequences of CDR3 $\delta$ 1 in antigen recognition using a specific V $\gamma$ 4 $\delta$ 1 TCR-derived from gastric tumor infiltrating  $\gamma\delta$ T lymphocytes. Three independent but complimentary strategies, including utilizing synthetic CDR3 $\delta$ 1 peptides, CDR3 $\delta$ 1-grafted TCR fusion proteins, and cells expressing TCR $\gamma$ 4 $\delta$ 1, were employed in the present study to investigate the structural basis of specific CDR3 $\delta$ 1 binding to tumor antigens. We found that the conserved flanking V and J sequences of CDR3 $\delta$ 1 are critical for antigen binding as well as the assembly of functional TCR $\gamma$ 4 $\delta$ 1 complex.

### EXPERIMENTAL PROCEDURES

**Cell Lines and Tumor Tissues**—Various tumor cell lines including BGC823, G401, GLC-82, HT29, SKOV3, Daudi, and J.RT3-T3.5 were obtained from American Type Culture Collection (ATCC). Fresh tumor tissues and tissue specimens were obtained from the Peking Union Medical College Hospital. Tumor tissues were used or processed within 2 h after surgery.

**Generation of  $\gamma\delta$  T Cell-enriched Tumor-infiltrating Lymphocyte (TIL)**—The tissues were rinsed with RPMI 1640 media containing cidomycin, penicillin, and streptomycin and cut into pieces. After washed twice, the tissue pieces were added to a 24-well plate coated by anti-TCR $\gamma\delta$  antibody (Beckman Coulter) for 2 h and cultured in RPMI 1640 media containing 10% FCS supplemented with L-glutamine and 2-mercaptethanol and 400 units/ml IL-2 for generation of  $\gamma\delta$  T cell-enriched cells that were spontaneously released from tumor tissues and expanded again by immobilized anti-TCR $\gamma\delta$  antibody for 2 or 3 weeks. After immunofluorescent analysis in flow cytometer (BD Biosciences), the expanded  $\gamma\delta$  T cell-enriched TILs were used for experiments.

**cDNA Cloning and Mutagenesis of Human CDR3 $\delta$ 1**—RNA was isolated from TILs of gastric carcinoma tissues (TRIzol reagent, Promega) followed by cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Promega) and oligo-dT (Promega). First, the V region of  $\delta$ 1 was amplified using V $\delta$ 1- and C $\delta$ -specific primers and cloned into the pGEM-T easy vector (Promega) for sequence analysis with the ABI automatic sequencer 3770. We chose one master sequence representing four cases of gastric tumor  $\gamma\delta$ TILs (termed GTM) for mutagenesis. Synthetic peptides with engineered mutations in the V, N-D-N, and J regions are named as GTMvm,

GTMDm, and GTMJm, respectively (Table 1). We then amplified dominant full-length  $\delta$ 1 chain with GTM as its CDR3 $\delta$ 1 and  $\gamma$ 4 chain in one case of gastric tumor-derived  $\gamma\delta$ TIL that paired with  $\delta$ 1 chain. For protein and transfectant mutagenesis, the full-length  $\delta$ 1 and  $\gamma$ 4 were cloned from gastric carcinoma  $\gamma\delta$ TIL first, and different mutants of CDR3 $\delta$ 1 were constructed by overlapping PCR. The full-length  $\delta$ 1 mutants, called as  $\delta$ 1(GTMvm),  $\delta$ 1(GTMDm),  $\delta$ 1(GTMJm),  $\delta$ 1(GTMVAm),  $\delta$ 1(GTMDAm), and  $\delta$ 1(GTMJAm), were constructed respectively.

**CDR3 $\delta$ 1 Peptide Synthesis**—Peptides including GTM and its mutant variants were synthesized in the peptide synthesis facility of the Academy of Military Medical Sciences, China. The CH3 sequence, one epitope sequence of another CDR3 $\delta$ 2 derived from OEC was synthesized for a negative peptide control (WPHNWPHFKVK). The purity of each synthetic peptide was >90% as shown by HPLC analysis. The synthesized peptides were totally labeled with a biotin at their N terminus (16).

**Chimeric Protein and MICA Protein Expression**—The engineered chimeric protein and its mutants containing the extracellular domains of the human TCR $\gamma$ 4 and TCR $\delta$ 1 chains fused to the hinge region, CH2 and CH3 domains of human IgG1 heavy chain, were expressed by the Sino Biological Inc. (16). The CDR3 $\delta$  sequence of the chimeric protein is concordant with the GTM peptide and its V/D/J mutants. Proteins  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc,  $\gamma$ 4-Fc/ $\delta$ 1(GTMvm)-Fc,  $\gamma$ 4-Fc/ $\delta$ 1(GTMDm)-Fc, and  $\gamma$ 4-Fc/ $\delta$ 1(GTMJm)-Fc were purified and verified by SDS-PAGE and Western blot, respectively. The pET42a-MICA<sub>1–3 $\alpha$</sub>  containing the extracellular domains of MICA was cloned in our laboratory, and the recombinant MICA<sub>1–3 $\alpha$</sub>  protein was expressed in *Escherichia coli* BL21. Purified recombinant MICA<sub>1–3 $\alpha$</sub>  was verified by SDS-PAGE and Western blotting.

**Construction of TCR $\gamma$ 4 $\delta$ 1-expressing Cell Line**—Three lentiviral vectors, pWPXL- $\delta$ 1 (GTM)-GFP, pWPXL- $\gamma$ 4, and pWPXL- $\delta$ 1(GTM)-IRES- $\gamma$ 4, were constructed, inserting  $\delta$ 1 or  $\gamma$ 4 into pWPXL (Addgene). In pWPXL- $\delta$ 1 (GTM)-GFP vector,  $\delta$ 1 chain was inserted immediately in the upstream of GFP. There was no termination code in the downstream primer so that a  $\delta$ 1-GFP gene was transcribed. pWPXL- $\gamma$ 4 was constructed in the same way, but there was a termination code in the downstream primer so that only  $\gamma$ 4 was transcribed. To create bicistronic pWPXL- $\delta$ 1(GTM)-IRES- $\gamma$ 4,  $\delta$ 1 and  $\gamma$ 4 chain were cloned into pIRES (Clontech) separately and then inserted into pWPXL between sites PmeI and SpeI. Similarly, other mutant vectors were constructed as well. The full-length V $\delta$ 1 chain and full-length V $\gamma$ 4 chain were amplified with Phusion<sup>®</sup> High Fidelity DNA polymerase (Finnzymes Oy). All constructs were verified by DNA sequence analysis. Lentiviral vectors were prepared by the transient transfection of 293T cells using a Lipofection-based method of cotransducing lentiviral gene transfer plasmid pWPXL, the helper plasmid psPAX2, and envelope plasmid pMD2.G (14, 24). In brief, the 293T cells were seeded in a 60-mm plate at  $1 \times 10^6$  cells and incubated overnight. The cells were cotransfected with pWPXL, psPAX2, and pMD2.G plasmids in accordance with the method of Lipofection. 48 h after transfection, the viral supernatants were collected, centrifuged, and used for infecting J.RT3-T3.5 cells (24).

TCR $\gamma$ 4 $\delta$ 1-transduced J.RT3-T3.5 cells were validated for  $\gamma$  $\delta$  TCR expression by immunofluorescent analysis in flow cytometer and Western blot.

**Immunofluorescent Analysis by Flow Cytometry**—To determine the binding of peptides or proteins with various tumor cell lines, tested cells were incubated with biotin-conjugated GTM peptide,  $\gamma$ 4-Fc/ $\delta$ 1 (GTM)-Fc protein, or their V/D/J mutants for 30 min at 4 °C. Fluorescein isothiocyanate (FITC)-conjugated streptavidin (Pierce) or FITC-conjugated goat anti-human IgG antibody was then added and incubated for 30 min at 4 °C. The cells were analyzed on a flow cytometer (BD Biosciences). Controls included CH3 peptide shown above or wild type human IgG-Fc. The expression of fusion protein  $\delta$ 1-GFP on J.RT3-T3.5 cells was determined by immunofluorescent analysis directly. To determine the expression of TCR $\gamma$ 4 $\delta$ 1 on J.RT3-T3.5 cells, cells were stained with phycoerythrin (PE)- or FITC-conjugated antibodies and the corresponding isotype controls (Beckman). Immunofluorescence was measured by Accuri C6 Flow Cytometer and analyzed by CFlow Software. Cell sorting was conducted on a FACS ARIA cell sorter. Specific anti-human antibodies including anti-TCR $\gamma$  $\delta$  antibody, anti-CD3, and isotype controls were purchased from Beckman Coulter. Anti-V $\delta$  and anti-actin antibodies for Western blot were purchased from Santa Cruz Biotechnologies.

**Confocal Microscopy**—Cells were plated on plastic sheets overnight and fixed on slides with 4% cold paraformaldehyde. Then fixed cells were incubated with biotin-conjugated GTM peptide or the  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc protein and their V/D/J mutants followed by incubating with FITC-conjugated streptavidin or goat anti-human IgG antibody (Pierce). Controls included CH3 peptide or human IgG-Fc as the primary antibody. Slides were examined with a confocal laser microscope (LSM 510; Carl Zeiss) (16).

**Immunohistochemistry**—Formalin-fixed paraffin-embedded sections of tumor tissues were deparaffinized and then boiled in microwave for antigen retrieval. After quenching with peroxide, the sections were blocked with 5% goat serum. Biotin-conjugated peptides or  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc protein or their mutants were then 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 microscopically analyzed (16).

**Surface Plasmon Resonance (SPR)**—SPR studies were carried out with a BIAcore 3000 instrument at 25 °C using HBS-EP running buffer (BIAcore). Protein MICA was diluted to 30  $\mu$ g/ml in 10 mM sodium acetate (pH 5.0) and immobilized on a CM5 chip using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide/N-hydroxysulfosuccinimide according to the manufacturer's instructions. The amount of immobilized protein was about 8000 resonance units. GTM peptide or  $\gamma$ 4/ $\delta$ 1 (GTM)-Fc protein or their mutants were flowed as the analyte at different concentrations. The recorded sensograms were analyzed using BIAevaluation software (Biacore Life Sciences). The data thus obtained were globally fit using a 1:1 binding model to calculate the dissociation constants ( $K_D$ ) (16).

**Enzyme-linked Immunosorbent Assay (ELISA)**—The 96-well plates were coated with N-terminal fragment of MICA protein

(2  $\mu$ g/ml) in 0.1 M NaHCO<sub>3</sub> (pH 9.6) at 4 °C overnight. After blocking with 5% bovine serum albumin, the plates were incubated with biotin-conjugated GTM peptide or  $\gamma$ 4-Fc/ $\delta$ 1 (GTM)-Fc protein and their mutants for 1 h at 37 °C. 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) (16).

**Western Blot**—The chimeric proteins were separated by SDS-PAGE and electrically transferred to a nitrocellulose membrane. The murine monoclonal anti-human V $\delta$  and goat polyclonal anti-human V $\gamma$  (Santa Cruz) were used as primary antibodies and HRP-conjugated anti-mouse, and HRP-conjugated anti-goat antibodies were employed as the secondary antibody. HRP-conjugated anti-human IgG Fc (Sigma) was used to detect Fc directly. Chemiluminescent HRP substrate (Pierce) was added, and the blot was exposed to x-ray film for an appropriate duration. The protein extract of transfected cells was analyzed by Western blot using murine monoclonal anti-human V $\delta$ 1 and anti-actin primary antibodies (Santa Cruz) and HRP-conjugated anti-mouse IgG as the secondary antibody.

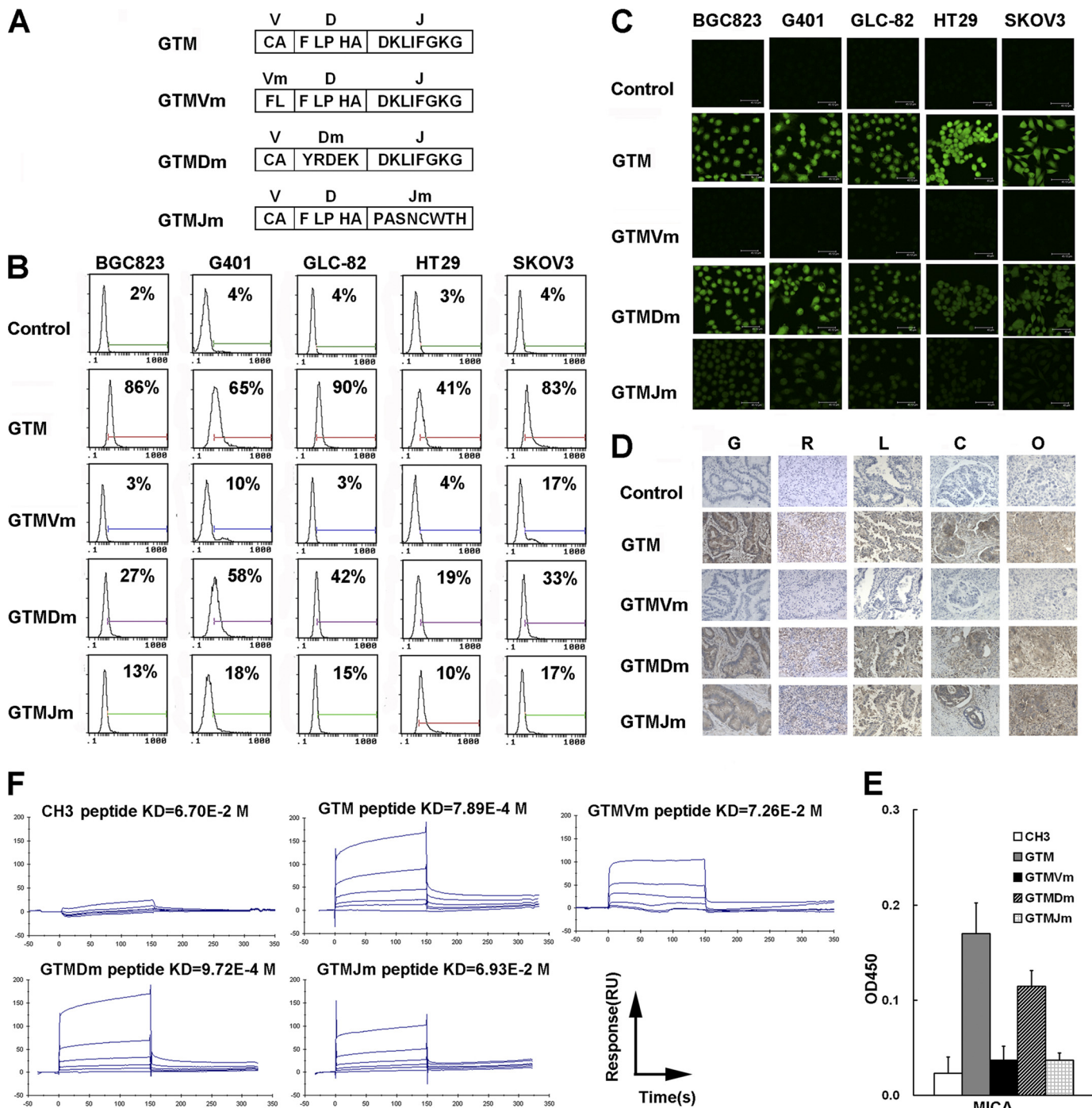
**Cytotoxicity Assay**—Daudi cells as target cells were added to the 96-well plates at a density of  $3 \times 10^4$  per well. Effector cells were incubated with anti- $\gamma$  $\delta$ TCR antibody, isotype IgG1 antibody, or without anything for 1 h at 4 °C and then added to the plate at effector/target ratios of 1.25:1, 2.5:1, 5:1, and 10:1, respectively, and each condition was plated in triplicate. There were four control groups: maximal cpm release group, volume corrected group, background group, and spontaneous cpm release group. We detected the cytotoxicity assay according to a cytotox 96 nonradioactive cytotoxicity assay reagents kit (Promega) instruction (25, 26).

## RESULTS

### *Binding of CDR3 $\delta$ 1 Peptide to Target Tumor Cells/Tissues and TCR $\gamma$ $\delta$ -specific Antigen MICA Depends on Its Flanking V and J Sequences*

We recently obtained a dominant  $\delta$ 1 chain from gastric tumor-derived  $\gamma$  $\delta$ TILs isolated from four patients and a matching  $\gamma$ 4 chain from one patient. We named this CDR3 $\delta$ 1 sequence as GTM. CDR3 $\delta$ 1 consists of the conserved flanking V and J segments as well as diverse N-D-N (D) sequences (Fig. 1A). To determine which segment in CDR3 $\delta$ 1 determines the specific antigen recognition, we synthesized GTM peptide based on the sequence of isolated  $\gamma$  $\delta$ TILs and the mutant peptides by replacing V, D, or J segments of GTM with randomly arranged amino acid sequences of the same length. The latter are termed as GTM<sub>V</sub>m, GTM<sub>D</sub>m, and GTM<sub>J</sub>m, respectively (Fig. 1A). Binding of these synthetic wild type and mutant peptides to target cells and tissues *in vitro* was examined by flow cytometry and microscopy, respectively. As shown in Fig. 1, B and C, in contrast to the staining with wild type GTM peptide, tumor cell lines BGC823, G401, GLC-82, HT29, and SKOV3 all showed dramatic reduction in binding to the mutant peptides GTM<sub>V</sub>m and GTM<sub>J</sub>m, whereas their binding to mutant peptide GTM<sub>D</sub>m with mutations in the inner N-D-N region was only slightly affected. Similar binding profiles of these peptides to primary tumor specimens were also observed (Fig. 1D). Our data suggest that the conserved flanking V and J

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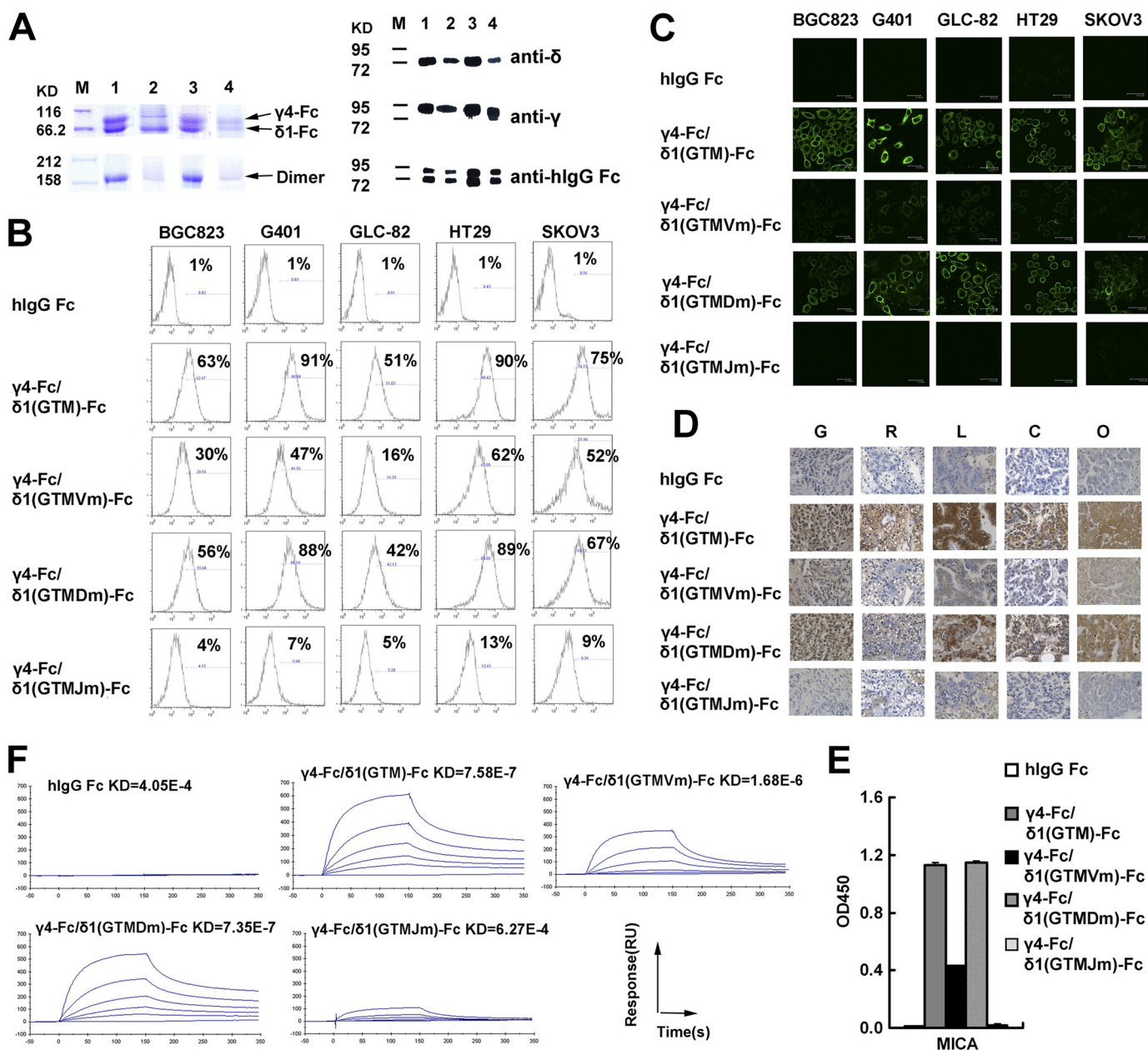


**FIGURE 1. Binding activity of CDR3 $\delta$ 1 and its mutant peptides to tumor cells/tissues or ligand MICA.** *A*, shown are schema of the amino acid sequences of GTM and its V/D/J mutants. *GTM*, *GTMVm*, *GTMDm*, and *GTMJm* represent the wild type and mutant peptides in V, D, and J region of a CDR3 $\delta$ 1, respectively. *B*, shown are flow cytometry profiles of the binding of GTM or its mutant peptides to various tumor cell lines BGC823, G401, GLC-82, HT29, and SKOV3. After incubation with biotinylated peptides, tested cells were stained with FITC-streptavidin and then analyzed by flow cytometry. Data are representative of three independent experiments. *C*, shown are confocal images of the binding of GTM peptide or its mutant peptides to various tumor cells. After incubation with the peptides, tested cells were stained with FITC-streptavidin and then analyzed by laser scanning confocal microscopy. Confocal images are one representative of at least three independent experiments. *D*, binding of GTM peptide and its mutants to primary tumor specimens was analyzed by immunohistochemistry. CH3 peptide was served as negative control. Binding was visualized using diaminobenzidine as the substrate (*brown*) ( $\times 100$ ). *G*, gastric cancer; *R*, renal carcinoma; *L*, lung carcinoma; *C*, colon carcinoma; *O*, ovarian cancer. *E*, binding of GTM peptide and its mutants to MICA was analyzed by ELISA. Data shown are the mean of three independent experiments. *F*, binding of GTM peptide and its mutants to MICA was analyzed by SPR. The  $K_D$  values are shown in the figure.

sequences but not the diverse D regions are critical for the binding to target tumor cells.

To further demonstrate that the peptides indeed interacted with TCR $\gamma\delta$ 1-specific antigen on those tumor cells, we next examined the interaction of GTM peptide and its mutants with

known TCR $\gamma\delta$ 1 reactive self-antigen MICA protein (27, 28). As shown in Fig. 1*E* by SPR and in Fig. 1*F* by ELISA, the binding of mutant peptides GTMVm and GTMJm to the extracellular segment of MICA was significantly reduced, whereas that of GTMDm was not overtly affected. These data suggest that

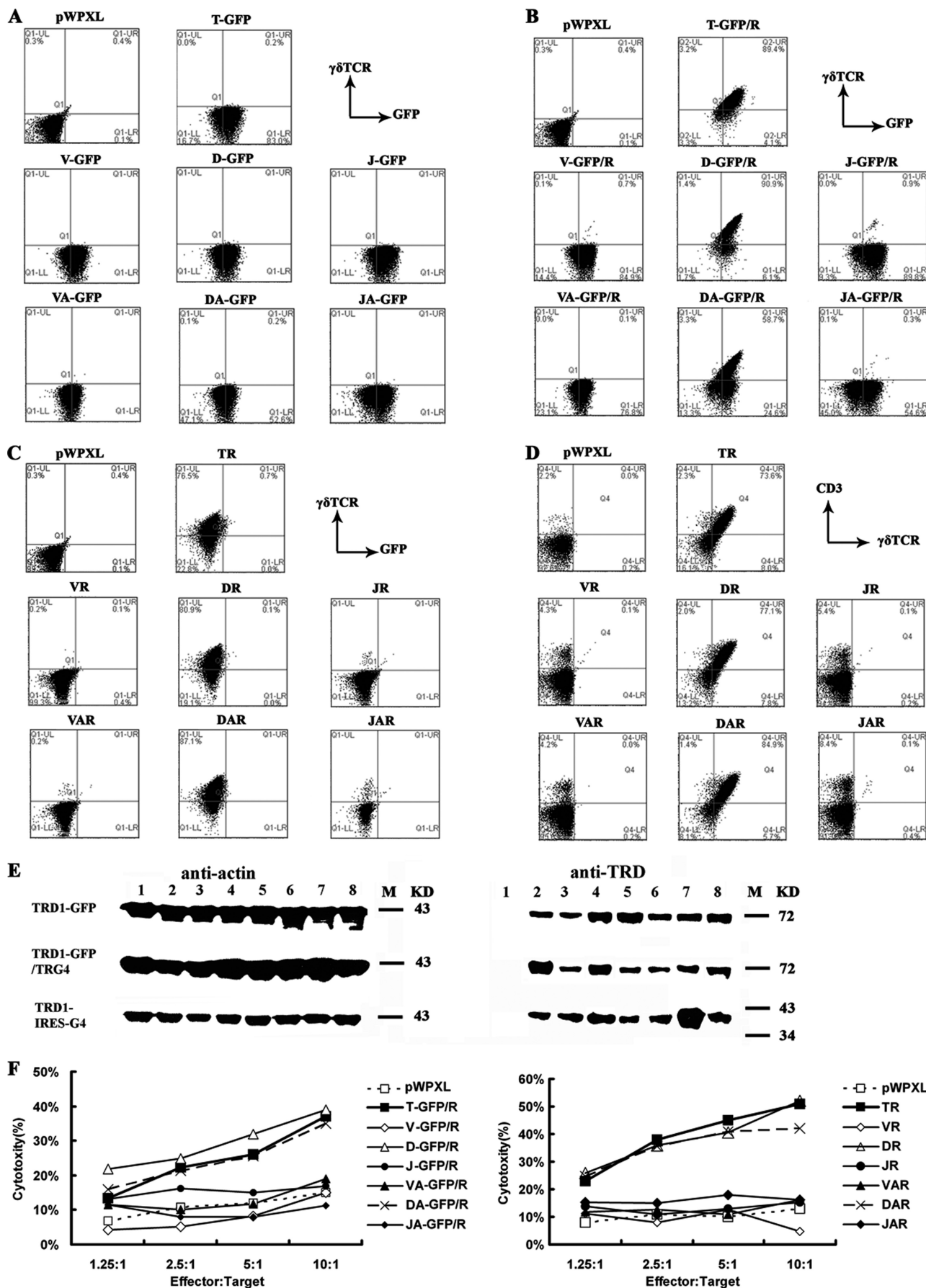


**FIGURE 2. Specific binding of TCR  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc fusion protein and its mutants to tumor cells or tissues and antigen MICA.** *A*, the expression of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc fusion protein and its mutants was analyzed by SDS-PAGE and Western blotting. Two subunits with different  $M_r$  of the four fusion proteins are shown by reduced PAGE (upper left). TCR $\gamma$ 4-Fc chain (upper bands) ran slightly higher than TCR $\delta$ 1-Fc (lower bands). Intact  $\gamma$ 4-Fc/ $\delta$ 1-Fc dimmers are shown by non-reduced PAGE (lower left). Lane 1,  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc; lane 2,  $\gamma$ 4-Fc/ $\delta$ 1(GTMVm)-Fc; lane 3,  $\gamma$ 4-Fc/ $\delta$ 1(GTMDm)-Fc; lane 4,  $\gamma$ 4-Fc/ $\delta$ 1(GTMJm)-Fc; lane M, protein marker. We further characterized the fusion proteins by Western blotting using anti-V $\delta$ , anti-V $\gamma$ , and anti-human IgG Fc as primary antibody (right panels). *B*, binding of TCR $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc fusion protein and its V, D, and J mutants to tumor cells was analyzed by flow cytometry. After incubation with fusion proteins, tested cells were stained with FITC-conjugated goat anti-human IgG and then analyzed by flow cytometry. Data are representative of three independent experiments. *C*, binding of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc and its V, D, and J mutants to tumor cells was analyzed by confocal microscopy. After incubation with fusion proteins, cells were stained with FITC-conjugated goat anti-human IgG and followed by laser scanning confocal microscopic analysis. Confocal images are representative of three independent experiments. *D*, binding of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc protein and its V, D, and J mutants to primary tumor specimens was analyzed by immunohistochemistry. Human IgG Fc was used as a negative control. Binding was visualized using diaminobenzidine as the substrate (brown) ( $\times 100$ ). G, gastric cancer; R, renal carcinoma; L, lung carcinoma; C, colon carcinoma; O, ovarian cancer. *E*, shown is ELISA analysis of the binding of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc and its mutants to antigen MICA. Data shown are the mean of three independent experiments. *F*, shown is SPR analysis of the binding of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc and its mutants to MICA antigen. The  $K_D$  values are shown in the figure.

GTM binding to the extracellular domain of MICA is specific, and the V and J segments are the primary determinants for the binding, whereas the sequence in the D region only contributes to the binding minimally. Our data also demonstrate that the differential binding of GTM or its mutant peptides to target tumor cells in Fig. 1, *A* and *B*, was likely through specific  $\gamma\delta$ TCR/antigen interaction.

*Engineered Soluble CDR3 $\delta$ 1 Fragment Is Critically Dependent on Its V and J Sequences for the Antigen Binding*—To further validate the binding of CDR3 $\delta$ 1 peptide GTM or its mutants to its antigen in the context of intact TCR $\gamma\delta$ , we engineered chimeric proteins composed of the extracellular domains of  $\gamma$ 4 and  $\delta$ 1 containing GTM or its V, D, J mutant sequences fused to the hinge region, CH2, and CH3 domains of human IgG1 heavy

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chain. We expressed the fusion proteins ( $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc,  $\gamma$ 4-Fc/ $\delta$ 1(GTMVm)-Fc,  $\gamma$ 4-Fc/ $\delta$ 1(GTMDm)-Fc, and  $\gamma$ 4-Fc/ $\delta$ 1(GTMJm)-Fc) in *E. coli*. (Fig. 1A). We examined the binding capacity of these recombinant  $\gamma$ 4-Fc/ $\delta$ 1-Fc fusion proteins to tumor cells followed by flow cytometry and confocal microscopy. As shown in Fig. 2, the binding activity of  $\gamma$ 4-Fc/ $\delta$ 1(GTMVm)-Fc or  $\gamma$ 4-Fc/ $\delta$ 1(GTMJm)-Fc but not  $\gamma$ 4-Fc/ $\delta$ 1(GTMDm)-Fc was significantly compromised compared with that of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc on various tumor cell lines (Fig. 2, B and C) and primary tumor specimens (Fig. 2D), further supporting that the conserved flanking V and J domains of CDR3 $\delta$ 1 play critical roles in antigen binding. Similar results were also observed from the direct binding of  $\gamma$ 4-Fc/ $\delta$ 1(GTM)-Fc or its mutant fusion proteins to antigen MICA in SPR and ELISA assays (Fig. 2, E and F).

*The V and J Sequences of CDR3 $\delta$ 1 Are Critical for Formation and Function of TCR $\gamma\delta$ 1 in Transduced J.RT3-T3.5 Cells*—To further substantiate the roles of the V and J segments of CDR3 $\delta$ 1 of  $\gamma\delta$ 1TILs in antigen recognition at cellular level, we lentivirally transduced the wild type or mutant  $\delta$ 1 chains along with the full-length  $\gamma$ 4 into J.RT3-T3.5 cells to establish stable cell lines expressing different CDR3 $\delta$ 1-grafted TCR dimers on the cell surface. Flow cytometry analysis demonstrated that TCR $\gamma\delta$  expression on J.RT3-T3.5 cells cotransduced with pWPXL- $\gamma$ 4 and pWPXL- $\delta$ 1(GTM) (termed T-GFP/R) or pWPXL- $\gamma$ 4 and pWPXL- $\delta$ 1(GTMDm) (termed D-GFP/R) was readily detectable (Fig. 3A). However, no  $\gamma\delta$ TCR expression was found on the surface of cells transduced with pWPXL- $\gamma$ 4 and pWPXL- $\delta$ 1(GTMVm) (termed V-GFP/R) or pWPXL- $\gamma$ 4 and pWPXL- $\delta$ 1(GTMJm) (termed J-GFP/R) (Fig. 3B). These data suggest that mutations in the V and J regions of CDR3 $\delta$ 1 are disruptive to the assembly of  $\gamma\delta$ TCR on cell surface. To further confirm this finding, we randomly substituted amino acids in the V, N-D-N, and J regions of CDR3 $\delta$ 1 with alanines by mutagenesis (termed VA-GFP, DA-GFP, and JA-GFP, respectively) to eliminate the impact of different amino acids. As shown in Fig. 3B, only cells transduced with DA-GFP/R but not with VA-GFP/R and JA-GFP/R, exhibited TCR expression, consistent with the results from D-GFP/R, V-GFP/R, and J-GFP/R. To further rule out a possibility of imbalanced expression of  $\gamma$ 4 and  $\delta$ 1 chains by two different co-expression constructs, we engineered bicistronic pWPXL- $\delta$ 1-IRES- $\gamma$ 4 constructs expressing both  $\gamma$ 4 chain and wild type (termed TR) or mutant  $\delta$ 1 chain (termed VR, DR, JR, VAR, DAR, and JAR). Upon the lentiviral transduction, we only observed wild type or D region mutant construct-derived TCR expression on the surface of J.RT3-T3.5

cells, whereas constructs with mutations in the V or J region resulted no detectable TCR surface expression (Fig. 3, B and C), further indicating the importance of the V and J segments in the assembly of TCR $\gamma\delta$ 1. In conclusion, our data demonstrated that the V and J regions of CDR3  $\delta$ 1 play critical roles in antigen binding as well as TCR $\gamma\delta$  assembly.

Although Western analysis confirmed the cellular expression of  $\delta$ 1 and its mutant proteins in the transduced cells (Fig. 3E), we found that J.RT3-T3.5 cells had no detectable surface expression of TCR when transduced solely with the construct pWPXL- $\delta$ 1 (GTM) (T-GFP) or its mutants in the absence of  $\gamma$ 4 chain (Fig. 3A), suggesting an absolute dependence of  $\gamma$ 4 chain for the surface expression of  $\delta$ 1 chain. It is also known that the TCR-CD3 complex can only be transported to the cell surface after all required subunits of the complex are completely assembled (29). We hypothesized that the detection of CD3 surface expression on cells transduced with TR or its mutants was another surrogate measure for TCR surface expression. Indeed, cells transduced with TR or its mutants in D region (DR and DAR) showed robust CD3 expression, whereas cells expressing mutants in the V and J regions (VR, VAR, JR, and JAR) had little detectable CD3 expression (Fig. 3D). These results show the indispensable role of the V and J sequences in the assembly of functional TCR and TCR-CD3 super complex.

Finally, we tested whether mutations in the V and J regions would impact the functionality of  $\gamma\delta$  T cells. However, TCR $\gamma\delta$ 1 used in our study was obtained from primary  $\gamma\delta$ TILs with yet to be determined antigen specificity. Despite the fact that Burkitt's lymphoma cell line Daudi has been used as the target cell for measuring  $\gamma\delta$ 2 T cell cytotoxicity (30), it has been reported that some  $\gamma\delta$ 1 T cells may also exhibit reactivity toward Daudi cells (31, 32). We, therefore, examined the cytotoxic effect of J.RT3-T3.5 cells transduced with TCR $\gamma\delta$ 1 or its mutants on Daudi cells. Cells transduced with pWPXL or V-GFP/R, J-GFP/R, VA-GFP/R, and JA-GFP/R showed certain cytolytic effect on Daudi cells (Fig. 3F, left). However, T-GFP/R-, D-GFP/R-, and DA-GFP/R-expressing J.RT3-T3.5 cells exhibited promoted cytolytic activity toward Daudi cells (Fig. 3F, left). Furthermore, the enhanced Daudi cell lytic activity of J.RT3-T3.5 cells were readily blocked by anti-TCR $\gamma\delta$  antibody, suggesting that the lytic activity is mediated by TCR $\gamma\delta$  (data not shown). We also found that J.RT3-T3.5 cells transduced with TR and its mutants exhibited similar Daudi cells cytotoxicity (Fig. 3F, right). Taken together, the V and J but not D regions are also critical for the cytotoxic function of  $\gamma\delta$  T cells in our assay system.

**FIGURE 3. The surface expression and cytotoxic function of TCR $\gamma\delta$ 1 and its mutants.** A, expression of TCR $\gamma\delta$  and GFP in J.RT3-T3.5 transduced with T-GFP and its mutants was analyzed by flow cytometry. Phycoerythrin labeled anti-TCR $\gamma\delta$  antibody, which recognizes all subtypes of  $\gamma\delta$ T cells. B, shown is flow cytometry analysis of the surface expression of TCR $\gamma\delta$  in J.RT3-T3.5 transduced with T-GFP/R or its mutants. C, shown is flow cytometry analysis of the surface expression of TCR $\gamma\delta$  in J.RT3-T3.5 transduced with TR and its mutants. D, expression of TCR $\gamma\delta$  and CD3 in J.RT3-T3.5 transduced with TR and its mutants was analyzed by flow cytometry. E, Western analysis of the expression of TCR $\gamma\delta$  components in J.RT3-T3.5 cells is shown. 1, transduced with pWPXL (containing a GFP gene); 2, transduced with T-GFP or T-GFP/R or TR; 3, J.RT3-T3.5 transduced with V-GFP or V-GFP/R or VR, mutants in V fragment of CDR3; 4, J.RT3-T3.5 transduced with D-GFP or D-GFP/R or DR; 5, J.RT3-T3.5 transduced with J-GFP or J-GFP/R or JR; 6, J.RT3-T3.5 transduced with VA-GFP or VA-GFP/R or VAR; 7, J.RT3-T3.5 transduced with DA-GFP or DA-GFP/R or DAR; 8, J.RT3-T3.5 transduced with JA-GFP or JA-GFP/R or JAR. Anti-actin (left panel) or anti-V $\delta$  (right panel) were used as the primary antibodies. F, shown is cytotoxicity analysis of J.RT3-T3.5 transduced with TCR $\gamma\delta$ 1 and its mutants on Daudi cells. Left, J.RT3-T3.5 cells transduced with pWPXL, T-GFP/R and V/D/J mutants. right, J.RT3-T3.5 cells transduced with pWPXL, TR and V/D/J mutants. The cytotoxicity was determined using the LDH cytotoxicity detection kit. The ratios of effector:target cells were 1.25:1, 2.5:1, 5:1, and 10:1. Data are representative of three independent experiments.

### DISCUSSION

$\gamma\delta$  T cells together with  $\alpha\beta$  T cells and B cells are the only cells that use somatic rearrangement to generate a diverse antigen receptor repertoire. Compared with immunoglobulin and  $\alpha\beta$  TCRs,  $\gamma\delta$  TCRs have the most potential of CDR3 diversity generated by VDJ recombination (33). Despite the vast repertoire of TCR $\gamma\delta$ , only a few TCR $\gamma\delta$ -specific antigens have been identified by our group and others, including murine MHC class Ib molecules T10 and T22 (10), human MHC class I-like molecules MICA/B (28), an ATP synthetase F1-apolipoprotein A-I complex (23), and hMSH2 (14). Developmentally, it is known that T cells somatically rearrange their  $\delta$  locus before any other TCR chains at the CD4/CD8 double negative stage in the thymus, suggesting the critical role of TCR $\gamma\delta$  in lineage determination. Functionally,  $\gamma\delta$  T cells possess “innate-like” property due to their rapid antigen response and lack of MHC class I or II restriction. Therefore, understanding the structural determinants of TCR $\gamma\delta$  in antigen recognition is at the center of  $\gamma\delta$  T cell biology.

Previously, we have studied the ligand recognition of TCR $\gamma$ 9 $\delta$ 2 from the major subset of  $\gamma\delta$ T cells (11, 16). Here we further expanded our study to a dominant TCR $\gamma$ 4 $\delta$ 1 derived from gastric tumor TILs. We explore the functional importance of the individual V, D, or J segment of CDR3 $\delta$ 1 on antigen recognition using our well established strategies including the use of synthetic CDR3 $\delta$ 1 peptides and CDR3 $\delta$ 1-grafted TCR fusion proteins as well as cells with forced expression of TCR $\gamma$ 4 $\delta$ 1. Mutations of amino acid sequences in the V and J regions but not the D region of CDR3 $\delta$ 1 peptide significantly abolish the recognition of antigen on tumor cells/tissue as well as in the form of pure protein (MICA). Consistent results were also obtained from using CDR3 $\delta$ 1-grafted TCR fusion proteins, further confirming the hypothesis that the conserved flanking V and J regions of CDR3 $\delta$ 1 play a critical role in antigen binding to TCR $\gamma$ 4 $\delta$ 1, whereas the D sequence is dispensable for antigen recognition. These results are apparently in contradiction with the findings in mice that the D segment is responsible for binding to T22 antigen for G8  $\gamma\delta$ T cells (10). This may be explained by the difference of species and/or the length of CDR3 $\delta$ . Indeed, G8 sequence of mouse CDR3 $\delta$  is much longer than human GTM, indicating that a different folding and recognition mode may exist.

During the course of our studies, we unexpectedly observed that the conserved V and J sequences were also required for the assembly of functional TCR $\gamma$ 4 $\delta$ 1 on T cell surface, which is a prerequisite for the surface expression of CD3 $\gamma\epsilon$  and CD3 $\zeta\zeta$  in the form of CD3/TCR $\gamma$ 4 $\delta$ 1 multisubunit super complex necessary for differentiation, survival, and function of  $\gamma\delta$ T cells (34, 35). More specifically, our results showed that only wild type  $\delta$ 1 chain and its mutants in the D region, but not in the V or J regions, could be paired with  $\gamma$ 4 chain to form a TCR $\gamma\delta$ -CD3 complex that is readily detectable with anti-TCR $\gamma\delta$  or anti-CD3. Despite their robust intracellular protein expression, it seems that mutations in the V and J segments render the  $\delta$ 1 chain assembly incompetent, leading to the disruption of  $\gamma\delta$ TCR-CD3 surface expression. The unassembled polypeptide chains are likely to be sequestered in the endoplasmic retic-

ulum and subsequently degraded (29). Indeed, J.RT3-T3.5 cells, a CD3 negative Jurkat cell variant, became endogenous CD3-positive upon the forced expression of  $\delta$ 1 and  $\gamma$ 4 chains. In fact, a similar observation has been reported in the case of TCR $\alpha\beta$  expression (36). Our results support the notion that the V and J sequences also dictate the assembly of  $\gamma\delta$ TCR-CD3 and, therefore, the differentiation, survival, and function of T cells.

In our previous study we similarly found that the flanking V and J sequences, but not the D region of CDR3 $\delta$ 2, were critical in the antigen recognition. Together with our current data, it seems that this is a common feature of  $\gamma\delta$  T cells. Our collective data suggest that  $\gamma\delta$  T cell repertoire may be much smaller than we originally calculated based on the diversity of D segment. This apparently answers the lingering question of why only a handful of TCR $\gamma\delta$ -specific antigens have ever been identified 25 years after the first discovery of  $\gamma\delta$  T cells. On the other hand, the role of flanking V and J regions of TCR $\gamma$ 4 $\delta$ 1 in TCR assembly is unique to V $\gamma$ 4V $\delta$ 1 T cells as no TCR assembly disruption was observed when similar mutations were introduced into the same flanking regions of TCR $\gamma$ 9 $\delta$ 2 (16).

Various studies have shown that human Burkitt's lymphoma cell line Daudi is sensitive to  $\gamma\delta$  T lymphocytes and could be efficiently killed by V $\gamma$ 9V $\delta$ 2 T cells (20, 37, 38) and some V $\gamma$ 9V $\delta$ 1 T cells (31, 32). Here we show that V $\gamma$ 4V $\delta$ 1 T cells also have specificity toward Daudi cells. However, CDR3 $\delta$ 1 antigen MICA is not expressed by Daudi cells (data not shown), raising the question of how exactly different types of  $\gamma\delta$  T cells could recognize tumor cells in general. Of note, both  $\gamma$ 4 $\delta$ 1 T cells used in our current study and  $\gamma$ 9 $\delta$ 2 T cells in our previous study were isolated from TILs of different tumors. It is possible that  $\gamma\delta$  T cells use their TCR as a pattern recognition receptor to sense the stress-induced self-molecules and use other receptors to detect tumor-specific antigens given the fact that  $\gamma\delta$  T cells also express various NK cell receptors including NKG2D, NKG2A, and KIR (39). The ligation of both receptors in concert may lead to the engagement of the cytolytic effect on tumor cells. Indeed, a recent structure study reveals that both TCR3 $\delta$ 1 and NKG2D competitively bind to MICA with different binding modes, constituting the tumor recognition and killing mechanisms (40). However, definitive answers to this very important question remain to be substantiated at the functional level.

Given our similar findings in CDR3 $\delta$ 2, our results suggest that both human TCR $\delta$ 1 and TCR $\delta$ 2 recognize antigen predominantly via the flanking V and J region. Our data indicate that TCR $\gamma\delta$  recognizes antigens using conserved parts in their CDR3 and provide a reason for why the diverse repertoire of  $\gamma\delta$ TCR only could recognize a limited number of antigens. In another word,  $\gamma\delta$  T cells might use a limited TCR repertoire to perform the surveillance for the limited number of stress-induced antigens.

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