FLANKING V and J SEQUENCES OF COMPLEMENTARY DETERMING REGION 3 OF T CELL RECEPTOR (TCR) δ1 (CDR3δ1) DETERMINE THE STRUCTURE AND FUNCTION OF TCRγδ1*

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The γδ T cell receptor (TCR) differs from immunoglobulin and αβ TCR in its overall binding mode. In human, genes δ1, δ2 and δ3 are used for TCRδ chains. Previously, we have studied antigen binding determinants of TCRδ2 derived from dominant γδ T cells resided in peripheral blood. In this study, we investigated the critical determinants for antigen recognition and TCR function in TCRδ1 originated from gastric tumor infiltrating γδ T lymphocytes, using three independent experimental strategies including complementary determining region (CDR) 3 of TCRδ1 (CDR3δ1) peptide mediated binding, CDR3δ1-grafted TCR fusion protein mediated binding and TCRγδ1 and mutants-expressing cell mediated binding. All three approaches consistently showed that the conserved flanking V and J regions but not the diversified D segment in CDR3δ1 determine the antigen binding. Most importantly, we found that mutations in the V and J regions of CDR3δ1 also abolish the assembly of TCR and TCR/CD3 complexes in TCRγδ1-transduced J.RT3-T3.5 cells. Together with our previous studies on CDR3δ2 binding, our finding suggests that both human TCRδ1 and TCRδ2 recognize antigen predominately via flanking V and J region. These results indicate that TCRγδ recognizes antigens using conserved parts in their CDR3, which provides an explanation for a diverse repertoire of γδTCRs only recognizing a limited number of antigens.

T lymphocytes can be divided into two distinct subsets, αβ and γδ T cells, based on the type of T cell receptors (TCR) expressed. The αβ T cells mainly recognize peptide antigens bound to class I or class II major histocompatibility complex (MHC) molecules, while γδ T cells mount immune responses directly to nonpeptide antigens and superantigens (1). Extensive studies suggest that γδ T cells may function in many aspects of immunity including infection, tumor immunity, tissue homeostasis, immune regulation (2-4). In humans, γδ T cells use three main Vδ (δ1, δ2, δ3) and various Vγ genes to make their functional TCRs (5,6). Most of γδ T cells in peripheral blood express Vδ2 chain paired with Vγ9 chain, whose TCR are dedicated to the recognition of nonpeptide phosphoantigens, alkylamines and synthetic aminobisphosphonates (7). Whereas in other tissues, γδ T cells express TCRs primarily made of predominant Vδ1 or a few Vδ3 chains paired with a diverse array of Vγ chains. Vδ1 T cells reside mainly in mucosal and epithelium tissue and recognize MHC-related molecules,
such as MHC class I chain-related gene A (MICA), MICB, as well as several other ligands (8,9).

The structural basis of antigen recognition of αβ TCR is well defined. However, the parallel information for γδ TCR is largely lacking due to the fact that limited number of γδ TCR specific ligands have been identified so far. While αβ TCRs often use all six complementary determining region (CDR) loops in their recognition of peptide-MHC complexes, the crystallographic structure study showed that predominantly germline-encoded residues of the CDR3 of human TCRδ (CDR3δ) is responsible for most of the interactions between the G8 γδ TCR and MHC class Ib T22 protein in mice (10). Moreover, the residues involved in the recognition interface are derived predominantly from germline-encoded Dδ segment. In addition, recently studies have shown that mutations in all CDR loops of human Vγ9δ2 TCR could alter the antigen binding, suggesting that the recognition of prenyl pyrophosphates by Vγ9δ2 TCR is dependent on all its CDRs (11).

CDR3δ is composed of V, N-D-N, and J gene segments. The flanking V and J sequences are conserved, while 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 TCRδ2 ligands using affinity chromatography analysis with a synthetic CDR3δ2 peptide as the probe and confirmed their recognition by TCRδ2-expressing cells in peripheral blood (14). Furthermore, we also demonstrated the direct antigen recognition role of CDR3δ2 sequences in TCRγδ2 derived from ovarian epithelial carcinoma (OEC) (15). We identified that the flanking V and J regions of human CDR3δ2 play a critical role in ligand binding, as targeting for OEC cells/tissues and hMSH2 protein (16).

Comparing to Vδ2, Vδ1 T cells differ in Vδ gene usage, paired γ chains (17,18), resident tissue (19,20), and antigen specificity (8,21-23). It remains unclear if Vδ1 subset utilizes a distinct antigen recognition mechanism and what is the contribution of CDR3δ1 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 CDR3δ1 in antigen recognition using a specific Vγ4δ1 TCR derived from gastric tumor infiltrating γδ T lymphocytes.

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 hours after surgery.

Generation of γδ 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-TCRγδ antibody (Beckman Coulter) for 2 hours and cultured in RPMI-1640 media containing 10% FCS supplemented with L-glutamine and 2-mercaptethanol and 400 U/ml of IL-2 for
generation of γδ T cell-enriched cells, which were spontaneously released from tumor tissues and expanded again by immobilized anti-TCRγδ antibody for two or three weeks. After immunofluorescent analysis in flow cytometer (Becton Dickinson), the expanded γδ T cell-enriched TILs were used for experiments.

cDNA cloning and mutagenesis of human CDR3δ1- 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 δ1 was amplified using Vδ1 and Cδ 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 γδTILs (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 (Table1). We then amplified dominant full-length δ1 chain with GTM as its CDR3δ1 and γ4 chain in one case of gastric tumor derived γδTIL that paired with δ1 chain. For protein and transfectant mutagenesis, the full-length δ1 and γ4 were cloned from gastric carcinoma γδTIL first and different mutants of CDR3δ1 were constructed by over-lapping PCR. The full length δ1 mutants, called as δ1(GTM-Vm), δ1(GTM-Dm), δ1(GTM-Jm), δ1(GTM-VAm), δ1(GTM-DAm) and δ1(GTM-JAm), were constructed respectively.

CDR3δ1 peptide synthesis- Peptides including GTM and its mutant variants were synthesized in the peptide synthesis facility of the Academy of Military Medical Sciences, China. CH3 sequence, one sequence of another CDR3δ2 derived from ovarian epithelial carcinoma as a negative peptide control, was synthesized CH3 sequence, 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 TCRγ4 and TCRδ1 chains fused to the hinge region, CH2 and CH3 domains of human IgG1, heavy chain, were expressed by the Sino Biological Inc (16). The CDR3δ sequence of the chimeric protein is concordant with the GTM peptide and its V/D/J mutants. Proteins γ4-Fc/δ1(GTM)-Fc, γ4-Fc/δ1(GTM-Vm)-Fc, γ4-Fc/δ1(GTM-Dm)-Fc and γ4-Fc/δ1(GTM-Jm) -Fc were purified and verified by SDS-PAGE and Western Blot, respectively. The pET42a-MICA1-3α contains extracellular domains of MICA was cloned in our lab and the recombinant MICA1-3α protein expressed in E. coli BL21. Purified recombinant MICA1-3α was verified by SDS-PAGE and Western Blotting.

Construction of TCRγ4δ1-expressing cell line- Three lentiviral vectors, pWPXL (Addgene) and pIRES (Clontech). pWPXL-δ1 (GTM)-GFP vector were constructed, inserting δ1 chain into pWPXL. A GFP gene was inserted immediately after δ1 chain. There was no termination code in the downstream primer so that a δ1-GFP gene was transcribed. pWPXL-γ4 was constructed in the same way but there was a termination code in the downstream primer so that only γ4 was transcribed. In order to create bicistronic pWPXL-δ1(GTM)-IRES-γ4, δ1 and γ4 chain were cloned into pIRES separately and then inserted into pWPXL between sites Pme I and Spe I. Similarly, Other mutant vectors were constructed as well. The full-length Vδ1 chain and full-length Vγ4 chain were amplified with Phusion® High-Fidelity DNA Polymerase (Finnzymes Oy.). All constructs were verified by DNA sequence
Lentiviral vectors were prepared by the transient transfection of 293T cells using a Lipofectamine 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 60mm plate at 1×10^6 cells and incubated overnight. The cells were cotransfected with pWPXL, psPAX2 and pMD2.G plasmids in accordance with the method of Lipofectamine. 48 hours after transfection, the viral supernatants were collected, centrifuged and used for infecting J.RT3-T3.5 cells (24). TCRγδ1-transduced J.RT3-T3.5 cells were validated for γδ 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, γ4-Fc/δ1 (GTM)-Fc protein, or their V/D/J mutants for 30 min at 4°C. 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 (Beckton Dickinson). Controls included CH3 peptide shown above or wild-type hIgG-Fc. The expression of fusion protein δ1-GFP on J.RT3-T3.5 cells was determined by immunofluorescent analysis directly. To determine the expression of TCRγδ1 on J.RT3-T3.5 cells, cells were stained with phycoerythrin (PE) or fluorescein isothiocyanate (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-TCRγδ antibody, anti-CD3 and isotype controls were purchased from Beckman Coulter and BD Biosciences, respectively. Anti-Vδ 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 γ4-Fc/δ1(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 hIgG-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 γ4-Fc/δ1(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, Sweden). Protein MICA was diluted to 30 μg/ml in 10mM sodium acetate (pH 5.0), and immobilized on a CM5 chip using EDC/NHS according to the manufacturer’s instructions. The amount of immobilized protein was about 8000 resonance units. GTM peptide or γ4 /δ1 (GTM)-Fc protein or their mutants were flowed as 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).
Enzyme-linked immunosorbent assay (ELISA)- The 96-well plates were coated with N-terminal fragment of MICA protein (2 μg/ml) in 0.1 M NaHCO$_3$ (pH 9.6) at 4°C overnight. After blocking with 5% bovine serum albumin, the plates were incubated with biotin-conjugated GTM peptide or γ4-Fc/δ1 (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 Vδ and goat polyclonal anti-human Vγ (Santa Cruz) were used as primary antibodies and HRP-anti-mouse, and HRP-anti-goat antibodies were employed as the secondary antibody. HRP-anti-human IgG Fc (Sigma) was used to detect Fc directly. Chemiluminescent HRP substrate (Pierce) was added and the blot was exposed to an X-ray film for an appropriate duration. The protein extract of transfected cells was analyzed by Western Blot using murine monoclonal anti-human Vδ1 and anti-actin primary antibodies (Santa Cruz) and HRP-anti-mouse-Ig as the secondary antibody. Cytototoxicity assay- Daudi cells as target cells were added to the 96-well plates at a density of 3×10$^4$ per well. Effector cells were incubated with anti-γδTCR antibody, isotype IgG1 antibody or without anything for 1 hour at 4°C and then added to the plate at effector/target (E/T) ratios of 1.25:1, 2.5:1 and 5:1 respectively, each condition was plated in triplicates. 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 cytotox 96 non-radioactive cytotoxicity assay reagents kit (Promega, USA) instruction (25,26).

RESULTS

Binding of CDR3δ1 peptide to target tumor cells/tissues and TCRγδ-specific antigen MICA depends on its flanking V and J sequences. We recently obtained a dominant δ1 chain from gastric tumor derived γδTILs isolated from four patients and a matching γ4 chain from one patient. We named this CDR3δ1 sequence as GTM. CDR3δ1 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 CDR3δ1 determines the specific antigen recognition, we synthesized GTM peptide based on the sequence of isolated γδTILs 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 GTMVm, GTMDm, and GTMJm, respectively (Fig. 1A). Binding of these synthetic wildtype and mutant peptides to target cells and tissues in vitro was examined by flow cytometry and microscopy, respectively. As shown in Fig. 1B and 1C, in contrast to the staining with wildtype GTM peptide, tumor cell lines BGC823, G401, GLC-82, HT29 and SKOV3 all showed dramatic reduction in binding to the mutant peptides GTMVm and GTMJm whereas their binding to mutant peptide GTMDm 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 sequences but not the diverged D regions are critical for the binding to target tumor cells.

To further demonstrate that the peptides were indeed interacting with TCRγδ1-specific antigen
on those tumor cells, we next examined the interaction of GTM peptide and its mutants with known TCRγδ1 reactive self-antigen MICA protein (27,28). As shown in Fig. 1E by SPR and in Fig. 1F by ELISA, the binding of mutant peptides GTMVm and GTMJm to the extracellular segment of MICA was significantly reduced while that of GTMDm was not overtly affected. These data suggest that GTM binding to the extracellular domain of MICA is specific, and the V and J segments are the primary determinants for the binding while 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. 1A and 1B was likely through specific γδTCR/antigen interaction.

Engineered soluble CDR3δ1 fragment is critically dependent on its V and J sequences for the antigen binding. To further validate the binding of CDR3δ1 peptide GTM or its mutants to its antigen in the context of intact TCRγδ, we engineered chimeric proteins composed of the extracellular domains of γ4 and δ1 containing GTM or its V, D, J mutant sequences fused to the hinge region, CH2 and CH3 domains of human IgG1 heavy chain. We expressed the fusion proteins [γ4-Fc/δ1(GTM)-Fc, γ4-Fc/δ1(GTM-Vm)-Fc, γ4-Fc/δ1(GTM-Dm)-Fc, and γ4-Fc/δ1(GTM-Jm)-Fc] in E. coli. (Fig. 1A). We examined the binding capacity of these recombinant γ4-Fc/δ1-Fc fusion proteins to tumor cells followed by flow cytometry and confocal microscopy. As shown in Fig. 2, the binding activity of γ4-Fc/δ1 (GTM-Vm)-Fc or γ4-Fc/δ1(GTM-Jm)-Fc but not γ4-Fc/δ1(GTM-Dm)-Fc was significantly compromised comparing to that of γ4-Fc/δ1(GTM)-Fc on various tumor cell lines (Fig. 2B, Fig. 2C) and primary tumor specimens (Fig. 2D), further supporting that the conserved flanking V and J domains of CDR3δ1 play critical roles in antigen binding. Similar results were also observed from the direct binding of γ4-Fc/δ1(GTM)-Fc or its mutant fusion proteins to antigen MICA in SPR and ELISA assays (Fig. 2E and 2F).

The V and J sequences of CDR3δ1 are critical for formation and function of TCRγδ1 in transduced J.RT3-T3.5 cells. To further substantiate the roles of the V and J segments of CDR3δ1 of γδ1TILs in antigen recognition at cellular level, we lentivirally transduced the wildtype or mutant δ1 chains along with the full-length γ4 into J.RT3-T3.5 cells to establish stable cell lines expressing different CDR3δ1-grafted TCR dimers on the cell surface. Flow cytometry analysis demonstrated that TCRγδ expression on J.RT3-T3.5 cells cotransduced with pWPXL-γ4 and pWPXL-δ1(GTM) (termed T-GFP/R) or pWPXL-γ4 and pWPXL-δ1(GTM-Dm) (termed D-GFP/R) was readily detectable (Fig. 3A). However, no γδTCR expression was found on the surface of cells transduced with pWPXL-γ4 and pWPXL-δ1(GTM-Vm) (termed V-GFP/R) or pWPXL-γ4 and pWPXL-δ1(GTM-Jm) (termed J-GFP/R) (Fig. 3B). This data suggests that mutations in the V and J regions of CDR3δ1 are disruptive to the assembly of γδTCR on cell surface. To further confirm this finding, we randomly substituted amino acids in the V, N-D-N and J regions of CDR3δ1 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. 3C only cells transduced with DA-GFP but not with VA-GFP and JA-GFP exhibited TCR expression, consistent with the results from previous experiment (Fig. 3B). To further rule out a possibility of imbalanced expression of γ4 and δ1
chains by two different co-expression constructs, we engineered bicistronic pWPXL-δ1-IRES-γ4 constructs expressing both γ4 chain and wildtype or mutant δ1 chain. Upon the lentiviral transduction, we only observed wildtype or D region mutant constructs-derived TCR expression on the surface of J.RT3-T3.5 cells, while constructs with mutations in the V or J region resulted no detectable TCR surface expression (Fig. 3B, Fig. 3C), further indicating the importance of the V and J segments in the assembly of TCRγ4δ1. In conclusion, our data demonstrated that the V and J regions of CDR3 δ1 play critical roles in antigen binding as well as TCRγδ assembly.

Although Western analysis confirmed the cellular expression of δ1 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-δ1 (GTM) (T-GFP) or its mutants in the absence of γ4 chain (Fig. 3A), suggesting an absolute dependence of γ4 chain for the surface expression of δ1 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 while 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 γδ T cells. However, TCRγ4δ1 used in our study was obtained from primary γδTILs with yet to be determined antigen specificity. Despite the fact that Burkitt's lymphoma cell line Daudi have been used as target cells for measuring γ9δ2 T cell cytotoxicity (30), it has been reported that some γ4δ1 T cells may also exhibit reactivity towards Daudi cells (31, 32). We therefore examined cytotoxic effect of J.RT3-T3.5 cells transduced with TCRγ4δ1 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 towards Daudi cells (Fig. 3F, right). Furthermore, the enhanced Daudi cell lytic activity of J.RT3-T3.5 cells were readily blocked by anti-TCRγδ antibody, suggesting that the lytic activity is mediated by TCRγδ (data not shown). We also found that J.RT3-T3.5 cells transduced with TR and its mutants also exhibited similar Daudi cells cytotoxicity. Taken together, the V and J but not D regions are also critical for the cytotoxic function of γδ T cells in our assay system.

**DISCUSSION**

γδ T cells, together with αβ T cells and B cells are the only cells that use somatic rearrangement to generate a diverse antigen receptor repertoire. Compared to immunoglobulin and αβ TCRs, γδ TCRs have the most potential of CDR3 diversity generated by VDJ recombination (33). In spite of the vast repertoire of TCRγδ, only a few TCRγδ-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 (AS-ApoA-I)
complex (23) and hMSH2 (14). Developmentally, it is known that T cells somatically rearrange their δ locus before any other TCR chains at CD4/CD8 double negative stage in the thymus suggesting the critical role of TCRγδ in lineage determination. Functionally, γδ 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 TCRγδ in antigen recognition is at the center of γδ T cell biology.

Previously, we have studied the ligand recognition of TCRγ9δ2 from the major subset of γδT cells (11,16). Here we further expanded our study to a dominant TCRγ4δ1 derived from gastric tumor TILs. We explore the functional importance of the individual V, D, or J segment of CDR3δ1 on antigen recognition using our well-established strategies including the use of synthetic CDR3δ1 peptides, CDR3δ1-grafted TCR fusion proteins as well as cells with forced expression of TCRγ4δ1. Mutations of amino acid sequences in the V and J regions but not the D region of CDR3δ1 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 CDR3δ1-grafted TCR fusion proteins, further confirming the hypothesis that the conserved flanking V and J regions of CDR3δ play a critical role in antigen binding to TCRγ4δ1 while 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 γδT cells (10). This may be explained by the difference of species and/or the length of CDR3δ. Indeed, G8 sequence of mouse CDR3δ of is much longer than human GTM, indicating that 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 TCRγ4δ1 on T cell surface, which is a prerequisite for the surface expression of CD3γε and CD3ζζ in the form of CD3/TCRγ4δ1 multisubunit super complex necessary for differentiation, survival, and function of γδT cells (34,35). More specifically, our results showed that only wildtype δ1 chain and its mutants in the D region but not in the V or J regions could be paired with γ4 chain to form a TCRγδ/CD3 complex that is readily detectable with anti-TCRγδ or anti-CD3. Despite their robust intracellular protein expression, it seems that mutations in the V and J segments render the δ1 chain assembly incompetent, leading to the disruption of γδTCR/CDR3 surface expression. The unassembled polypeptide chains are likely to be sequestered in the endoplasmic reticulum 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 δ1 and γ4 chains. In fact, similar observation has been reported in the case of TCRαβ expression (36). Our results support the notion that the V and J sequences also dictate the assembly of γδTCR/CDR3 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 CDR3δ2 were critical in the antigen recognition. Together with our current data, it seems that this is a common feature of γδ T cells. Our collective data suggest that γδ 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 TCRγδ specific antigens have ever been identified 25 years after the first discovery of γδ T cells. On the other hand, the role of flanking V and J regions of TCRγ4δ1 in TCR
assembly is unique to Vγ4Vδ1 T cells since no TCR assembly disruption was observed when similar mutations were introduced into the same flanking regions of TCRγδ2 (16).

Various studies have shown that human Burkitt's lymphoma cell line Daudi is sensitive to γδ T lymphocytes and could be efficiently killed by Vγ9Vδ2 T cells (20, 37, 38) and some Vγ9Vδ1 T cells (31, 32). Here we show that Vγ4Vδ1 T cells also have specificity toward Daudi cells. However, CDR3δ1 antigen MICA is not expressed by Daudi cells (data not shown), raising the question of how exactly different types of γδ T cells could recognize tumor cells in general. Of note, both γ4δ1 T cells used in our current study and γ9δ2 T cells in our previous study were isolated from TILs of different tumors. It is possible that γδ 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 γδ 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, recent structure study reveals that both TCRδ1 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 CDR3δ2, our results suggest that both human TCRδ1 and TCRδ2 recognize antigen predominately via the flanking V and J region. Our data indicate that TCRγδ recognizes antigens using conserved parts in their CDR3 and provide a reason for why the diverse repertoire of γδTCR only could recognize a limited number of antigens. In another word, γδ T cells might use a limited TCR repertoire to perform the surveillance for the limited number of stress-induced antigens.

REFERENCES


**FOOTNOTES**

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The abbreviations used are: TCR, T cell receptors; hMSH2, human mutS homolog 2; CDR3δ, the complementary determining region 3 of human TCRδ; CDR3δ1/2, the complementary determining region 3 of human TCRδ1/2; GTM, γδ TIL, γδ Tumor Infiltrating Lymphocytes; SPR, Surface plasmon resonance; ELISA, Enzyme-linked immunosorbent assay

**FIGURE LEGENDS**

Fig. 1. Binding activity of CDR3δ1 and its mutant peptides to tumor cells/tissues or ligand MICA. A. Schema of the amino acid sequences of GTM and its V/D/J mutants. GTM, GTMVm, GTMDm and GTMJm represent the wildtype and mutant peptides in V, D and J region of a CDR3δ1, respectively. B. 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 incubated with biotinylated peptides, tested cells were stained with FITC-Streptavidin and then analyzed by flow cytometry. Data are representative of three independent experiments. C. Confocal images of the binding of GTM peptide or its mutant peptides to various tumor cells. After incubated 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 analyzed by immunohistochemistry. CH3 peptide was served as negative control. Binding was visualized using diaminobenzidine as the substrate (brown) (×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 analyzed by ELISA. Data shown are as mean of three independent experiments. F. Binding of GTM peptide and its mutants to MICA were analyzed by SPR. The KD values are shown in the figure.

Fig. 2. Specific binding of TCR γ4-Fc/δ1(GTM)-Fc fusion protein and its mutants to tumor cells or tissues and antigen MICA. A. The expression of γ4-Fc/δ1(GTM)-Fc fusion protein and its mutants
analyzed by SDS-PAGE and Western Blotting. Two subunits with different MW of the four fusion proteins are shown by Reduced PAGE (upper left). TCRγ4-Fc chain (upper bands) run slightly higher than TCRδ1-Fc (lower bands). Intact γ4-Fc/δ1 dimmers (lane 1 and 3) are shown by non-reduced PAGE (lower left). Lane 1, γ4-Fc/δ1(GTM)-Fc; Lane 2, γ4-Fc/δ1(GTMVm)-Fc; Lane 3, γ4-Fc/δ1(GTMDm)-Fc; Lane 4, γ4-Fc/δ1(GTMJm)-Fc; Lane M, protein marker. We further characterized the fusion proteins by Western Blotting using anti-Vδ, anti-Vγ, and anti-human IgG Fc as primary antibody (right panels). B. Binding of TCRγ4-Fc/δ1(GTM)-Fc fusion protein and its V, D, and J mutants to tumor cells analyzed by flow cytometry. After incubated with fusion proteins, tested cells were stained with FITC-goat-anti-human IgG and then analyzed by flow cytometry. Data are representative of three independent experiments. C. Binding of γ4-Fc/δ1(GTM)-Fc and its V, D, and J mutants to tumor cells analyzed by Confocal microscopy. After incubated with fusion proteins, cells were stained with FITC-goat-anti-human IgG and followed by laser scanning confocal microscopic analysis. Confocal images are representative of three independent experiments. D. Binding of γ4-Fc/δ1(GTM)-Fc protein and its V, D, and J mutants to primary tumor specimens analyzed by immunohistochemistry. Human IgG Fc was used as a negative control. Binding was visualized using diaminobenzidine as the substrate (brown) (×100). G, gastric cancer; R, renal carcinoma; L, lung carcinoma; C, colon carcinoma; O, ovarian cancer. E. ELISA analysis of the binding of γ4-Fc/δ1(GTM)-Fc and its mutants to antigen MICA. Data shown are mean of three independent experiments. F. SPR analysis of the binding of γ4-Fc/δ1(GTM)-Fc and its mutants to MICA antigen. The KD values are shown in the figure.
Table 1 Amino acid sequences of GTM and its mutants in V, N-D-N and J region of CDR3δ1

<table>
<thead>
<tr>
<th>Peptide</th>
<th>V</th>
<th>N-D-N</th>
<th>J</th>
</tr>
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<tbody>
<tr>
<td>GTM</td>
<td>CA</td>
<td>FLP HA</td>
<td>DKLIFGKG</td>
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<tr>
<td>GTM-Vm</td>
<td>FL</td>
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<td>DKLIFGKG</td>
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<td>GTM-Dm</td>
<td>CA</td>
<td>YR DEK</td>
<td>DKLIFGKG</td>
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<tr>
<td>GTM-Jm</td>
<td>CA</td>
<td>FLP HA</td>
<td>PASNCWTH</td>
</tr>
<tr>
<td>CH3</td>
<td></td>
<td></td>
<td></td>
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</tbody>
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GTM: γδ Tumor Infiltrating Lymphocytes, γδTIL. CH3 sequence is one sequence of another CDR3δ2 derived from ovarian epithelial carcinoma as a negative peptide control.
Fig. 1
Fig. 3
Flanking V and J sequences of complementary determining region 3 of T cell receptor (TCR) δ 1 (CDR3δ1) determining the structure and function of TCRγδ1

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