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Vδ1 T cell receptor binds specifically to MHC I chain related A: Molecular and biochemical evidences

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Abstract

Human MHC class I chain-related A (MICA) is a tumor-associated antigen that can be recognized by Vδ1 subset of tumor-infiltrating γδ T cells. We previously reported that immobilized recombinant MICA protein could induce the proliferation of tumor-infiltrating Vδ1 γδ T cells in vitro. But there has been no direct evidence showing the engagement of γδ T cell receptors (TCR) of the induced cells with MICA. In the current investigation, we show that MICA induces specific cytolytic activity of the expanded γδ T cells. We expressed the coupled V domains from the MICA-induced T cells as a single polypeptide chain VδVγ TCR (γδ scTCR). Such scTCR can specifically bind MICA of HeLa cells. Direct interaction of γδ scTCRs with in vitro expressed MICA was monitored using an IAsys biosensor. We found that the Vδ1 scTCR can specifically bind to immobilized MICA molecule and MICAα1α2 domains are responsible for the binding reaction.

Keywords: MHC class I chain-related A; Cytotoxicity; γδ T cell receptor; Single polypeptide chain γδ TCR; Biosensor

The human MHC class I-related A (MICA) is a three-domain (α1–3), β2m-independent molecule. MICA is distantly related to MHC class I, but is functionally distinct [1]. MICA has no role in the presentation of peptide antigens; instead, it may function as self-antigen that can be stress-induced [2,3].

Compared with αβ T cells, γδ T cells are a minor subset of the T cells in lymphoid organs and tissues such as spleen, lymph nodes, and peripheral blood. The human γδ T cells are composed of two main subsets: Vδ1 and Vδ2 T cells, which are differentially distributed in certain tissues [2,4]. Vδ2 T cells represent the majority of peripheral blood γδ T lymphocytes [5,6]. Vδ1 T cells reside mainly within epithelial tissues and epithelial tumors, where these cells might provide a first line of defense against infections or malignancies [1,6]. Consistent with their preferential distribution in epithelial tissues, Vδ1 T cells can interact with molecules expressed by epithelial cells. For example, some Vδ1 T cells recognize CD1c in a TCR-mediated pattern [7]. With regard to the recognition of MICA by T cells, there are some hints: (i) in tumors positive for MICA expression, the frequencies of Vδ1 γδ T cells are significantly higher than in those negative for MICA expression [3]. (ii) Further researches suggested that MICA is a tumor-associated antigen that can be recognized by tumor-infiltrating Vδ1 T cells (TILs). Numerous Vδ1 T cell lines and clones recognize the stress-inducible MICA molecules on the surface of epithelial cells [8]. (iii) The Vδ1 T cells reactive with MICA deliver cytotoxic activities, which can be blocked by monoclonal antibody (mAb) specific for Vδ1, although the blocking was partial [2]. (iv) Vδ1-Vγ TCR gene transfer and MICA tetramer binding studies demonstrate the interaction of Vδ1 TCR with MICA [9]. However, there has been no evidence for the direct recognition of MICA protein by Vδ1 TCR.

We previously reported that recombinant MICA could induce the proliferation of human ovarian epithelial carcinoma infiltrated Vδ1 T cell, and proliferated Vδ1 T cell has

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the cytolytic activity against MICA-positive tumor cell lines [10,11]. In the present study, we found that anti-MICA antibodies could block the cytolytic activity of Vδ1 T cells against MICA$^+$ cells. We sequenced TCR gene of MICA-expanded γδ T cells and produced the coupled γδ TCR V domain as a single polypeptide chain fragment of variable region (γδ scTCR). We observed that the γδ1 scTCRs can specifically bind MICA molecules on HeLa cells by an anti-MICA mAb blocking assay. Direct interaction of γδ scTCR with MICA was monitored using an IAsys biosensor. We showed that the scTCR proteins are functional fragment of the T cell receptor; Vδ1 scTCR corresponding to MICA-expanded Vδ1 γδ T cell TCR can bind to MICA protein, while Vδ2 or Vδ3 scTCR cannot. Lastly, we show that either the α1α2 domain of MICA protein or anti-MICAα1 or α2 mAb can completely inhibit the binding reaction of Vδ1 scTCR with MICA. Our studies suggest that Vδ1 TCR can specifically bind to MICA molecule and the binding reaction is MICAα1α2 domain dependent.

Materials and methods

Cell lines and human tissue specimens. Human ovarian tumor cell line SKOV3 was provided kindly by Professor Keng Shen from the Gynecological Department of the Peking Union Hospital. HeLa (carcinoma of cervix, epithelial phenotype) and SP2/0 (myeloma) cell lines are preserved in our laboratory. Fresh ovarian tumor tissues and normal ovarian tissues were obtained from Peking Union hospital during clinical operation with the consent of patients. All of the tumor specimens were ovarian epithelial carcinoma diagnosed according to standard histopathological and immunohistochemical criteria.

*Cloning and expression of rMICA*008α1–3 and α1α2 domains.* The construction of MICAα1–3 recombinant plasmid was reported previously [11]. By a similar way, the MICAα1α2 cDNA fragments were cloned into pET30 vector (Novagen). The recombinant plasmids were identified by sequencing and transformed into *Escherichia coli* BL21 (DE3).

Purification and concentration detection of rMICAs. Purification of rMICA proteins was described previously [11]. The yield of proteins was quantified by using BCA protein assay kit (Pierce).

Monoclonal antibodies. The following mAbs were used for flow cytometry and blocking experiments: δTCS1 (anti-TCR-δ1/Jδ1), anti-TCR-δ2, anti-pan-TCRγδ (Immunotech, BD), anti-TCR-γ9. 12B7 (anti-MICAα1), and 10C6 (anti-MICAα2) were produced in a routine procedure and examined by ELISA.

Expansion of Vδ1 γδ TILs in vitro. The expansion of Vδ1 γδ TIL in vitro is similar to that of γδ TILs expansion described previously [11,12], except MICAα1α2 was used as a substitute for MICAα1-3. Tissues cultured with immobilized anti-pan-γδ TCR mAbs (Immunotech, BD) were used as positive controls.

Flow cytometry. The phenotype of T cells was determined as reported previously by immunofluorescence assay [11], using two-color staining with the following labeled antibodies (Immunotech, BD): FITC-conjugated anti-γδ TCR (IgG1), phycoerythrin (PE)-conjugated anti-αβ TCR (IgG2b), and PE- or FITC conjugated isotype controls.

Cytotoxicity assay and monoclonal antibody inhibition. The colorimetric MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used to evaluate the cytolytic activity of the expanded Vδ1 γδ TILs in vitro as reported previously [11]. HeLa cells were incubated with 200 μg anti-MICA mAbs 10C6 or 12B7 at 37 °C for 90 min and then used as target cells for cytotoxicity assay.

RT-PCR analysis of MICA-expanded γδ T cell Vγ and Vδ gene. RNA was prepared from γδ T cells that expanded by immobilized rMICA protein. The cDNA of TCR Vγ and Vδ was obtained by reverse transcription-polymerase chain reaction (RT-PCR). A set of primers were synthesized according to Vγ and Vδ gene sequences published in GenBank and classified in IMGT (Table 1).

Gene scanning of the CDR3 length's diversity of MICA-expanded γδ T cell Vγ and Vδ genes. A semi-nested PCR was designed for the γδ T cell CDR3 length scanning. The reverse primers are identical to those in Table 1, but labeled with 5′-Fam fluorescent molecule. The forward primers were identical to FR2 in Vγ or FR3 in Vδ (Table 2). One microliter of gel-purified, diluted first PCR product was amplified for 30 cycles with nested primers and then was analyzed by using the ABI sequencer 377.

Sequencing of TCR Vγ and Vδ gene from MICA-expanded γδ T cell and ovarian tumor TILs. The RT-PCR products were subcloned into T easy vector and sequenced.

Construction of γδ scTCR. After sequencing, TCR Vδ1 and Vγ gene fragments were generated by PCR amplification. A flexible linker encoding 15-amino acid $(Gly4Ser)_3$ and flank sequences overlapping Vδ1 gene in 5′-end and Vγ primers in 3′-end was synthesized. The linker primer was used in separate PCRs to amplify the Vδ1 gene with the 5′ primer and Vγ gene with the 3′ primer, and an assembly reaction was followed to construct the γδ scTCR fragment. The γδ scTCR expression recombinant was constructed by inserting (*Nco*I/*Xho*I) γδ scTCR fragment into pET32 vector (Novagen). The scTCR fragment was ligated behind the thioredoxin gene and followed by a 6-histidine tag in pET32.

Induction, purification, and refolding of γδ scTCR protein. To induce expression of scTCR, the transformed *E. coli* strain DB21 (DE3) was

Table 1
The nucleotide sequence of primers used for PCR amplification of TCR Vγ and Vγ gene and γδ scTCR construction

Vγ1–4:	5′TCTTCCAACTTGGAAGGGAGAACG3′
Vγ5:	5′TCTTCCAACTTGGAAGGGGGAAC3′
Vγ8:	5′TCTTCCAACTTGGAAGGGAGAAC3′
Vγ9:	5′GCAGGTCACCTAGAGCAACCTC3′
Cγ (reverse):	5′GGAAGAAAAATAGTGGGCTTGGGG3′
Vδ1:	5′GCCCAGAAGGTTACTCAAGCCCAG3′
Vδ2:	5′GCCATTGAGTTGGTGCCTGAACAC3′
Vδ3:	5′TGTGACAAAGTAACCCAGAGTTCCCC3′
Cδ (reverse):	5′AAACGGATGGTTTGGTATGAGGC3′
Vγ1–4 linker:	5′ggcggctcgggcggtggcggatcgTCTTCCAACTTGGAAGGGAGAACG3′[a]
Vδ1–*Nco*I:	5′catgccatgGCCCAGAAGGTTACTCAAGCCCAG3′
Vδ2–*Nco*I:	5′catgccatgGCCATTGAGTTGGTGCCTGAACAC3′
Vδ3–*Nco*I:	5′catgccatggTGTGACAAAGTAACCCAGAGTTCCCC3′
Cγ–*Xho*I:	5′ccgctcgagGGAAGAAAAATAGTGGGCTTGGGG3′
Linker:	5′AGTCAGCCTCATACCAAACCATCCGTTTcaggtggaggaggttcaggtggaggcggctcgggcggtggcggatcg3′

[a] Primers used to construct scTCR, the lowercase letter indicates the *Nco*I/*Xho*I site or a linker sequence.

Table 2
The nucleotide sequence of primers used for Gene Scanning

Vγ2/4:	5′GATCTTGCTGAAGGAAGTAMCGGC3′
Vγ3:	5′GCGATCTTACTGTAACAAATACCTTC3′
Vγ8:	5′GTGATCTTCCTGTAGAAAATGCCG3′
Vγ9:	5′GAAGTCATACAGTTCCTGGTGTC3′
Vγc (reverse):	5′ FAM-GGAAGAAAAATAGTGGGCTTGGGG3′
Vδ1:	5′CAGCCTTACAGCTAGAAGATTCAGC3′
Vδ2:	5′ GCACCATCAGAGAGAGATGAAGGG3′
Vδ3:	5′TCACTTGGTGATCTCTCCAGTAAGG3′
Vδc (reverse):	5′ FAM-AAACGGATGGTTTGGTATGAGGC3′

grown in 2× YT containing ampicillin. After IPTG induction, the BL21 (DE3) host was harvested and sonicated on ice in short bursts. The inclusion bodies were washed several times in PBS and solubilized in 8 M urea/10 mM Na_2HPO_4·NaH_2PO_4/500 mM NaCl (pH7.4). The supernatant was loaded onto a nickelchelate column (Amersham Pharmacia Biotech) following the instructions of the manufacturer. The collected samples were checked by SDS–PAGE and quantified using BCA protein assay kit (Pierce).

ELISA (quality test for γδ scTCRs). To determine whether γδ scTCR retained a native conformation capable of binding to anti-TCR mAbs, an ELISA was conducted. For each test, 2, 4, and 8 μg of scTCR were incubated overnight in 96-well plates in a total volume of 100 μl. After blocking with 15% BSA in PBS, the anti-TCR mAbs δTCS1 (anti-TCR-δ1/Jδ1) and anti-TCR-δ2, which can bind the natural cell-expressed γδ TCR, were added to a final concentration of 0.5 μg/ml. Bound mAbs were detected using a HRP-labeled goat anti-mouse IgG antibody.

γδ scTCR as staining reagents. For FCM staining, the purified γδ scTCR proteins were labeled using FITC protein labeling kit (Pierce). About 1×10^6 cells were incubated with FITC-labeled γδ scTCR (300 μg/ml, 20 μl) for 60 min at 4 °C and then washed (with PBS containing 2% FBS) three times. For mAb-binding inhibition assays, the cells were incubated for 60 min at 4 °C with 10 μg/ml anti-MICA mAb, then washed one time and incubated with γδ scTCR under the same condition described above.

Binding of γδ scTCR to MICA measured by surface plasmon resonance. Direct interaction of γδ scTCR with MICA was monitored using an IAsys biosensor. The biosensor uses surface plasmon resonance to probe the refractive index change in a cuvette due to the binding of molecules to immobilized ligand. The CMD-chip surface of cuvette was activated using EDC/NHS [200 mM *N*-ethyl-*N*′-(dimethylaminopropyl)carbodiimide and 50 mM *N*-hydroxysuccinimide, affinity sensors] and thereafter rMICAα1–3 (p*I* = 6.33) was coupled to the activated CMD-chip in 10 mM acetate buffer (pH 5.7) at 50 μg/ml. After immobilization, the surface was blocked with 1 mM ethanolamine (pH 8.5) and then washed with 1 mM formic acid to remove away non-coupled protein. All binding was performed in PBS/T running buffer at room temperature (23–25 °C). After each binding experiment, the MICA surface was regenerated by washing with 1 mol/L M formic acid twice.

A gradient concentration of γδ scTCR protein from 2.5 to 0.16μM was obtained by a series dilution with PBS/T. Binding of γδ scTCR to MICA was measured by biosensor at different concentrations. Data were collected automatically by IAsys and analyzed with FASTplot and FASTfit software.

Results

Immobilized MICAα1α2 could induce expansion of Vδ1 γδ TILs in vitro

In our earlier report, we found that the TILs could be expanded by anti-pan TCR γδ mAbs or by the three-domain MICAα1α2α3 protein [11,12]. In our present study, a similar assay was conducted in order to determine whether the two-domain MICAα1α2 retains the function of full-length MICA to expend TILs. We found that the γδTILs from the ovarian tumor specimens were expanded by immobilized rMICAα1α2 or immobilized anti-pan-TCR γδ mAb in vitro (Figs. 1A–C.), and the majority of the MICA-expanded γδ TILs belonged to the Vδ1 subset as shown in Fig. 1D. In contrast, the majority of γδ T cells derived from PBMC that expanded by immobilized anti-TCR γδ mAb were of Vδ2/Vγ9 T cells (Fig. 1E), which indicated a differential distribution of γδ T cell subset.

MICA-expanded Vδ1 γδ TILs displayed specific cytolytic activities towards HeLa cells, which can be blocked by anti-MICA mAbs

The Vδ1 γδ TILs expanded from the ovarian tumor specimens by immobilized rMICA showed considerable cytotoxic activity against the $MICA^+$ HeLa cells. Moreover, the cytotoxicity of Vδ1 γδ TILs against HeLa cells was inhibited strongly if the tumor cells were preincubated with anti-MICA mAbs 12B7 and 10C6, which can specifically bind to MICAα1 and α2 domains, respectively. Compared with mAb 12B7, the mAb 10C6 displayed a much higher inhibitory activity. The Vδ2 γδ T cells expanded from PBMC also displayed strong cytolytic activities towards HeLa cells. But in the case of Vδ2 γδ T cells, no specific cytolytic inhibition was observed in the antibody blocking assays (Fig. 2).

Gene scanning and sequencing of MICA-expanded γδ T cell receptor Vγ and Vδ genes

Taking the mRNA from MICA-expanded γδ T cells as template and converting it to cDNA, we amplified the Vδ and Vγ genes. In order to scan the length of γδ TCR V gene, a series of semi-nestal PCRs was carried out using the primers listed in Table 2. The reverse primers were 5′-FAM-labeled for length scanning. As shown in Fig. 3, the CDR3 lengths of Vδ and Vγ were oligoclonally diverse, which indicated that the diversity of γδ TCR from MICA-expanded γδ T cells was highly restricted. The length distribution of Vδ and Vγ is also shown in Fig. 3.

Parallel to the gene scanning, the RT-PCR products of Vδ and Vγ genes from MICA-expanded γδ T cells were subcloned to T easy vector. We sequenced 4–6 individual clones from each V gene. The subcloned PCR products showed that all the TCR Vγ and Vδ transcripts, except one Vδ1 product, were in-frame (data not shown). The sequences and the length diversities of Vδ and Vγ correlated to the gene scanning results are shown in Table 3.

Expression and quality test of γδ scTCR proteins

In our expression vector employed to produce γδ scTCR recombinant protein, the Vδ region was joined

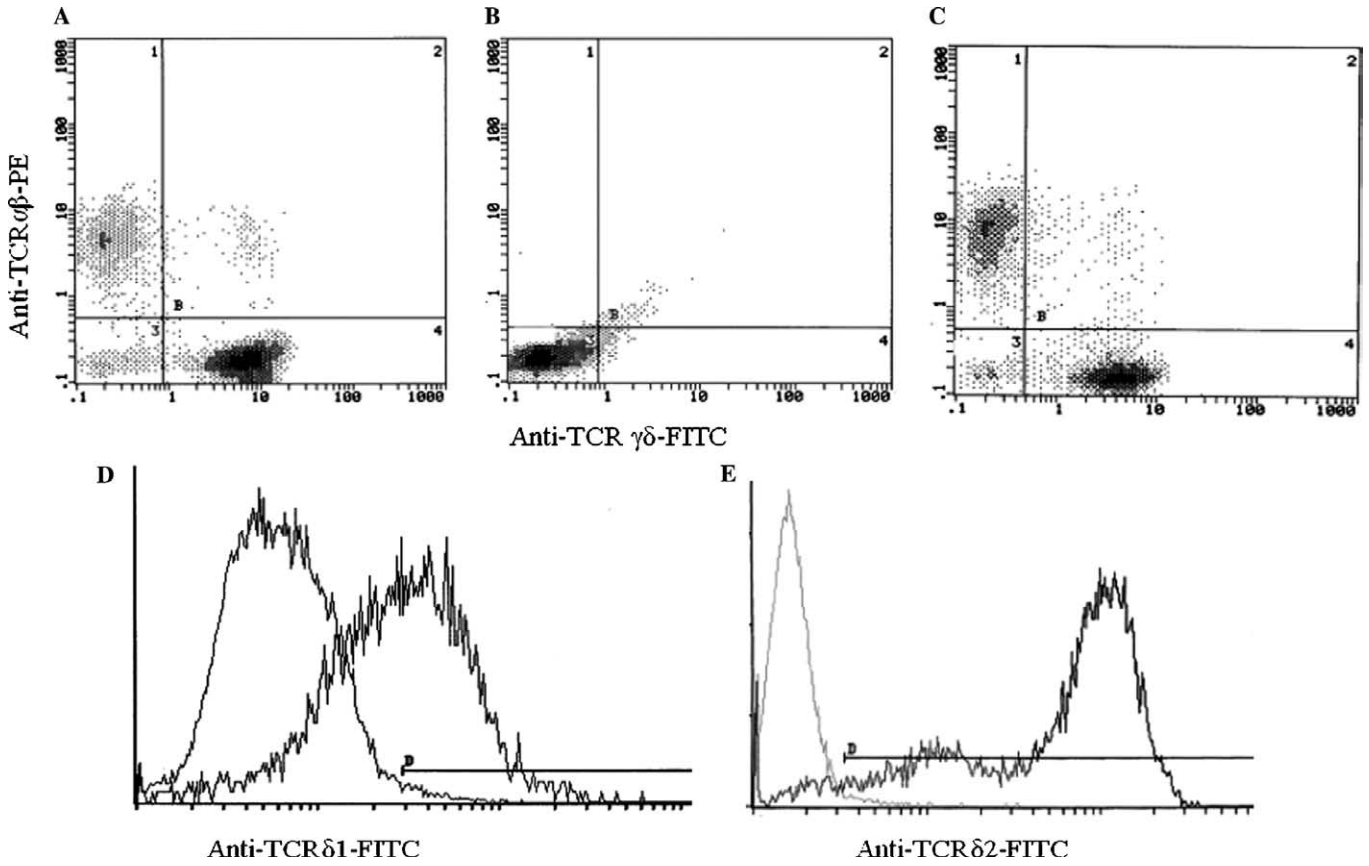

Fig. 1. Expansion of human γδT cells from ovarian TILs by stimulation of immobilized rMICAα1α2 in vitro. Two-color flow cytometry analysis showed the proportions of αβ and γδ T cells (A–C). (A) TILs expanded by immobilized MICA12; (B) negative control; (C) PBMC expanded by immobilized anti-TCR γδ mAb. (D) The cells were analyzed by biotin-conjugated anti-Vδ1 mAb and FITC-conjugated streptavidin. The percentage of Vδ1 T cells in this figure is 66%. (E) Anti-pan-TCR γδ expanded PBMCs, the Vδ2-positive subset percentage is 94%, stained with anti-Vδ2-FITC.

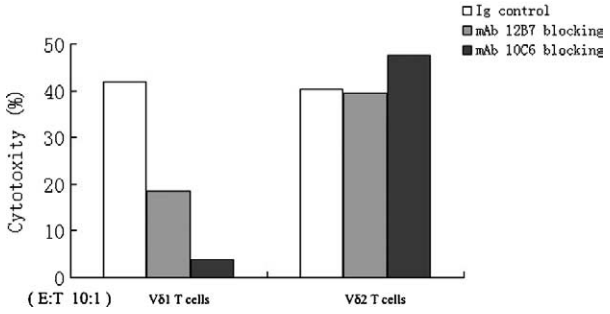

Fig. 2. Inhibition of γδ T cell cytotoxic function by mAbs. Cytotoxicity of MICA-expanded Vδ1 γδ T cells against $MICA^+$ HeLa cell line was inhibited by mAb 12B7 or 10C6. Cytotoxicity of anti-pan-γδ TCR-expanded Vδ2 γδ T cells against $MICA^+$ HeLa cell line could not be inhibited by either mAb 12B7 or 10C6. E:T, effector-to-target cell ratio.

to Vγ region by a 15-residue flexible peptide linker (Fig. 4A). Five Vδ1-Vγ scTCR (OTδ1-A2, 3, 4, 5, and 7) recombinants, two Vδ2-Vγ scTCR (OTδ1-D1, 2) and one Vδ3-Vγ scTCR (OTδ1-D3) were constructed. All the constructs were subcloned into the same site of pET32 expression vector system. The paired junctional sequences that were used in generating γδ scTCR recombinants are shown in Table 4. The γδ scTCR proteins thus produced were mainly in the inclusion bodies. After purification and renaturation, the final expression product of scTCR proteins was shown by SDS–PAGE (Fig. 4B). An ELISA was conducted to determine whether γδ scTCR retained the native conformation recognizable by anti-TCR mAbs. The anti-TCR mAbs δTCS1 (anti-TCR-δ1/Jδ1) and anti-TCR-δ2, which can bind the γδ TCRs of natural T cells, were used as primary antibody. The results showed that with 2, 4, and 8 μg scTCR coated, the anti-TCR mAbs specifically bind to γδ1 scTCR and γδ2 scTCR (Fig. 4C).

Vδ1 scTCR can specifically bind MICA

The rMICA (α1–3) protein was coupled to a CMD surface of biosensor chip (10 ng/mm^2). Interaction of γδ scTCRs with MICA was measured by surface plasmon resonance using IAsys biosensor. Two Vδ2 scTCRs and one Vδ3 scTCR protein were expressed and purified by a procedure identical to that of Vδ1/Vγ scTCRs and were used as control. As shown in Fig. 5A, Vδ1/Vγ scTCRs, but not Vδ2/Vγ scTCRs or Vδ3/Vγ scTCR, bind specifically to immobilized MICA. The binding kinetics was concentration-dependent: the response unit

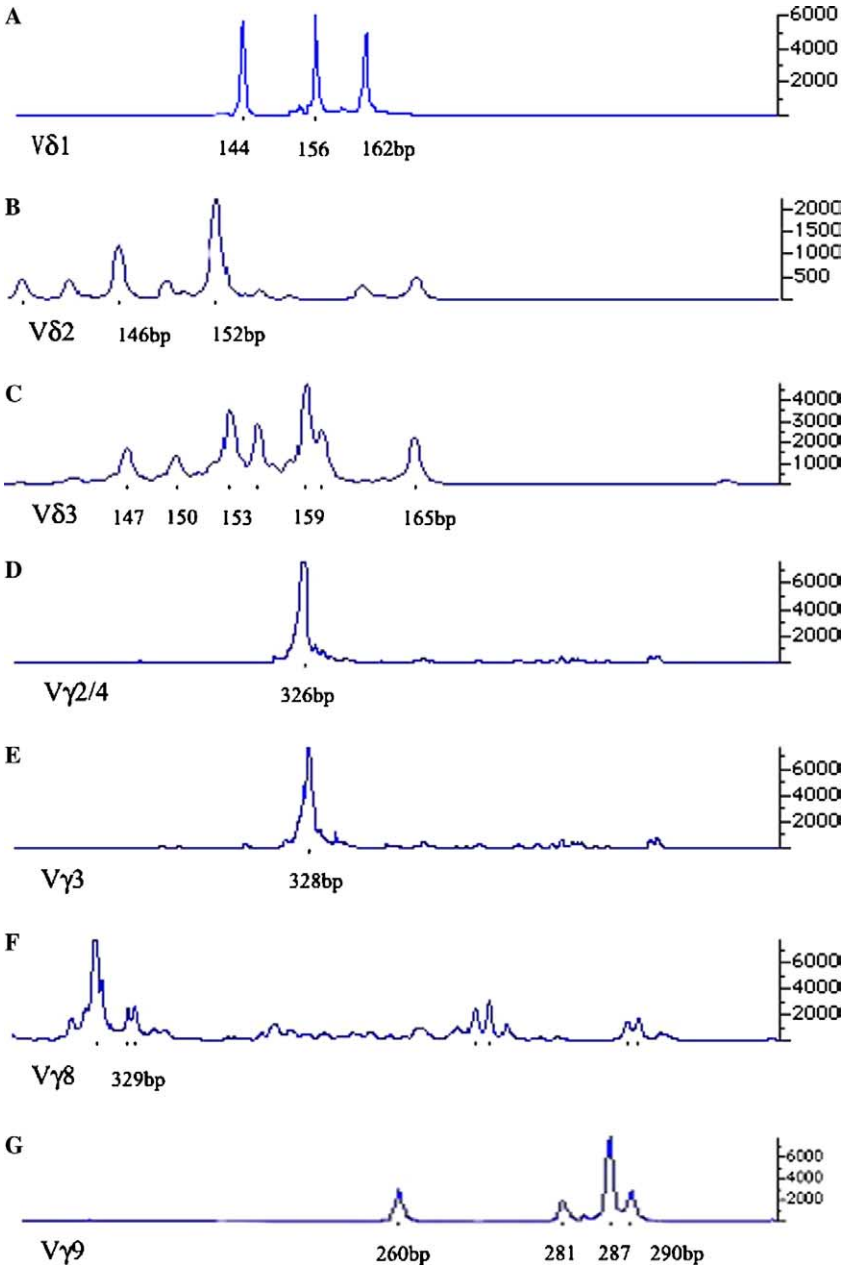

Fig. 3. Scanning of the CDR3 length diversity of MICA-expanded γδ T cell Vγ and Vδ. The CDR3 lengths of Vδ and Vγ oligoclonally diverse. The corresponding lengths are shown.

is higher with increased antigen concentration. Such results indicate a specific binding (Fig. 5B). Using IAsys FASTfit software, we analyzed the interaction data, the dissociation rates of two Vδ1/Vγ scTCR are 0.008 and 0.005 s^{-1}, and the association rates (K_a) are 2885 ± 58 and 2897 ± 83 L/ms for OTδ1-A5 and A7, respectively. The K_D values of OTδ1-A5 and A7 are 2.8 and 1.6 μmol, respectively.

Although we have detected a relatively higher affinity value than that of the most αβ TCR–ligand interactions, we wondered whether the affinity of Vδ1 scTCR with its MICA ligand might be lower in an FCM staining. We did find that Vδ1 scTCR stained the $MICA^+$ HeLa cells weakly. HeLa cells were used in this investigation due to its high level MICA expression on the cell surface [11]. The anti-MICA mAb blocking showed the staining was MICA-specific (Fig. 5E).

Following injection of the mixture of Vδ1 scTCR with an overdose of rMICAα1α2 protein, the binding of Vδ1 γδ scTCR with the coated rMICAα1–3 can be blocked, as shown in Fig. 5C. Moreover, prior to the injection of Vδ1 scTCR, the pretreatment of MICA-coated surface with either the anti-MICAα1 or α2 mAbs (12B7 or 10C6) can inhibit the binding (Fig. 5D). These results indicate that the binding of Vδ1 scTCR with MICA depends on MICAα1α2 domains.

Table 3
Junctional diversities (CDR3 region) of MICA-expanded γδ TCR

Name	Length[a] (bp)	Vδ1/Vγ	N(DN)	Jδ1/Jγ1/Jp	Frequency
Vδ1	144	LGE	FPGDTT	DKLIFGKGT	(2/6)
	156	LG	LGRTLHWGIP	DKLIFGKGT	(1/6)
	162	LGE	LILLMGGYPPGT	DKLIFGKGT	(2/6)
Vγ2	326	ATW	A	NYYKKLFGSGT	(2/4)
	335	ATWD	GPM	NYYKKLFGSGT	(1/4)
Vγ3	328	ATWD	RHN	KKLFGSGT	(2/2)
Vγ4	326	ATWD	R	YYKKLFGSGT	(4/4)
Vγ9	281	ALWE	EV	LGKKIKVFGPGT	(1/4)
	284	ALWEV	QE	LGKKIKVFGPGT	(1/4)
	287	ALW	KGEG	ELGKKIKVFGPGT	(1/4)
	287	ALWE	ARG	ELGKKIKVFGPGT	(1/4)

[a] Corresponding to gene scanning.

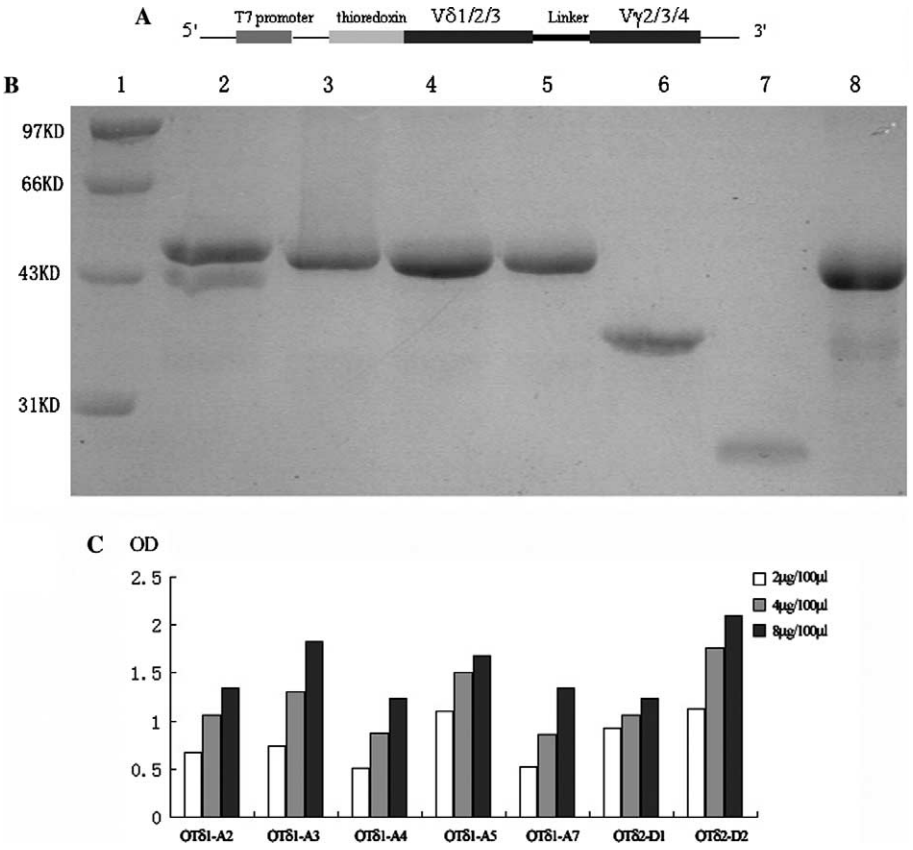

Fig. 4. γδ scTCR construction, SDS–PAGE analysis of γδ scTCR and MICA protein, and testing of γδ scTCRs by ELISA. (A) The γδ scTCRs were generated in an *E. coli* system using the pET32 vector that contained the thioredoxin gene followed by the Vδ and Vγ cDNAs of the MICA-expanded γδ T cells, flanked by a linker fragment. (B) SDS–PAGE gel, stained with Coomassie brilliant blue, showing the γδ scTCRs and MICA proteins used in this study; ~10 μg of protein/lane was loaded. Lane 1, marker; 2, OTδ1-A7; 3, OTδ1-A5; 4, OTδ1-A3; 5, OTδ2-D2; 6, MICAα1α2α3; 7, MICAα1α2; 8, OTδ1-A2. (C) The ELISA showing that different γδ scTCRs are recognized by mAbs δTCS1 (anti-TCR-δ1/Jδ1) or anti-TCR-δ2, which can bind the natural cell-expressed γδ TCR.

Discussion

It was reported about a total of 54 known human MICA alleles up to now. MICA*008 was the most common allele in the Caucasian and the Han populations in China [1,13]. It was used in our current study. HeLa cells were found to express high levels of MICA*008 and were selected to be the target cells in our study [14,15].

We previously reported that immobilized rMICAα1–3 could induce the expansion of human Vδ1 γδTILs that displayed MICA-dependent cytotoxicity against human epithelial carcinomas [11]. In the present work, we found that the immobilized rMICAα1α2 could also induce the expansion of human OEC-derived γδ TILs (Vδ1 phenotype) in vitro. This suggests that MICAα1 and α2 domains are the function-critical domains, which are responsible for

Table 4
The junctional sequence (CDR3 region) of γδ scTCR constructions

γδ scTCRs	Vδ	NDN	Jδ	Vγ	N	Jγ
OTδ1-A2	(Vδ1): LGE	LILLMGGYPPG	TDKLIFGKGT	(Vγ2): ATW	A	NYYKKLFGSGT
OTδ1-A3	(Vδ1): LGE	LILLMGGYPPG	TDKLIFGKGT	(Vγ3): ATWD	RHN	KKLFGSGT
OTδ1-A4	(Vδ1): LGE	LILLMGGYPPG	TDKLIFGKGT	(Vγ4): ATWD	R	YYKKLFGSGT
OTδ1-A5	(Vδ1): LGE	FPGDT	TDKLIFGKGT	(Vγ2): ATW	GH	KKLFGSGT
OTδ1-A7	(Vδ1): LGE	LGRTLHWGIP	DKLIFGKGT	(Vγ3): ATWD	RHN	KKLFGSGT
OTδ2-D1	(Vδ2): ACD	PLSLRYT	DKLIFGKGT	(Vγ3): ATWD	RHN	KKLFGSGT
OTδ2-D2	(Vδ2): ACD	PVNSQLLGDNIWAAQLFFGKGT		(Vγ2): ATW	ANYY	KKLFGSGT
OTδ3-D3	(Vδ3): A	LVPLKY	TDKLIFGKGT	(Vγ2): ATWD	GPM	NYYKKLFGSGT

stimulating the proliferation and activation of Vδ1 T cells. The cytotoxicity of expanded Vδ1 γδ TILs could be inhibited greatly if the tumor cells were preincubated with anti-MICA mAbs 12B7 (against MICAα1 domain) or 10C6 (against MICAα2 domain). Compared with 12B7, the inhibition of 10C6 was much higher, which indicated that MICAα2 domain was more critical than α1 domain in triggering the cytotoxicity response. Vδ2 T cells derived from PBMCs had similar cytotoxicity against tumor cells, but were in a MICA-independent way, because the anti-MICA mAbs cannot block its cytotoxicity. These results suggest that the two subsets of γδ T cells recognize distinct ligand molecules, through which to reach their cytotoxic activation against tumor cells. It was reported that MICA could deliver both the TCR-dependent stimulatory signal 1 and the NKG2D-dependent co-stimulatory signal 2 for a subset of Vδ1 γδ T cells [9,16]. Since both Vδ1 and Vδ2 γδ TILs express NKG2D [17], the variation of γδ TCR expression relating to MICA stimulation may be correlated to the different cytotoxicity properties.

Kinetic measurements of αβ TCRs binding to the peptide–MHC complexes have provided a great deal of insight into the mechanisms of αβ T cell activation. For the similar purpose, we produced a few soluble γδ scTCRs to perform the direct binding experiments. There have been several reports on *E. coli* produced αβ scTCR. It was reported that in contrast to the poor refolding property of most αβ scTCR produced in these systems, the thioredoxin fused scTCR protein exhibited much higher solubility and these scTCRs possess their expected functions [18]. Recently, the mammalian expression system was used in TCR research and produced successful applications [19–21].

By using surface plasmon resonance biosensor, we have generated the binding curves between the MICAα1–3-coupled surface and the Vδ1 scTCR. The association rate of the binding reaction we observed matched with those reported for αβ TCR and ligand pairs [22]. The dissociation rate found here was close to $K_{off} = 0.008\ s^{-1}$. This value is similar to the dissociation rate of the murine γδ TCR G8 and its antigen, non-classical MHC molecule T22, which was so far the only complete kinetic analysis performed on a γδ TCR/antigen interaction; but the order of magnitude is lower than the dissociation rates reported for most αβ TCR–peptide–MHC interactions. The dissociation constant (K_D) found here was about 2 μM, one order of magnitude higher than that of murine γδ TCR G8, which has a $K_D = 0.11$ μM; but in the range of most αβ TCR–ligand interactions, which had K_D values of about 1–10 μM [23].

Taking the MICA-γδ scTCR binding data and the sequence information of scTCR together, we found that the Vδ1 chain is most critical for the binding. Although with different binding affinities, all the Vδ1 scTCRs we constructed so far can bind to MICA, while the Vδ2 or Vδ3 scTCR failed to bind. In our constructions, the OTδ1-A2, A3, and A4 were composed of an identical Vδ1 chain and different Vγ chain (Vγ2/3/4). But it seemed that the substitution of the Vγ2, Vγ3 or Vγ4 chain had little influence on the binding capacity, indicating that Vγ chain of γδTCR had little contribution to MICA binding. The OTδ1-A3 and OTδ2-D1, which were composed of identical Vγ chain but different Vδ chain, had totally different binding affinity against MICA. It indicates the minor role of Vγ chain as well. The OTδ1-A3 and A7 were identical except the different Vδ1-CDR3 region, and they had slight difference in affinity with MICA, suggesting that the CDR3 region of Vδ1 chain had some influence on the binding affinity. In a recent report, Shin and Chien found a conserved motif in T22-specific γδ T cells, and sequence diversity around these conserved residues modulates T22-γδ TCR binding affinities, and the Vγ usage was less important for the T22 antigen specificity [24,25]. But in the MICA-γδ TCR interactions, we cannot rule out the possibility that non-CDR3 region in the Vδ1 chain drove the binding to MICA, for the relative insensitivity to Vδ1 junctional sequence variation.

The specific binding of Vδ1 γδ scTCR to MICA arises from the interaction of scTCR with MICAα1α2, because the soluble MICAα1α2 protein could block the binding and the blocking is dependent on the concentration of MICAα1α2, and lastly, mAbs specific against MICAα1 or α2 domain could inhibit the binding as well.

The present results support a dual-signal theory which explains the activation of γδT cells [9] is the dual signal theory claims that the interaction between TCRγδ and MICA might serve as the signal 1 for MICA induced γδ T cell activation, while NKG2D might provide the signal 2 when interacting with its own ligand [26,27]. However, it is still unclear about how the Vδ1 γδ T cells recognize $MICA^+$ cells. Groh and Spies have demonstrated that the Vδ1 γδ TCR can specifically bind MICA

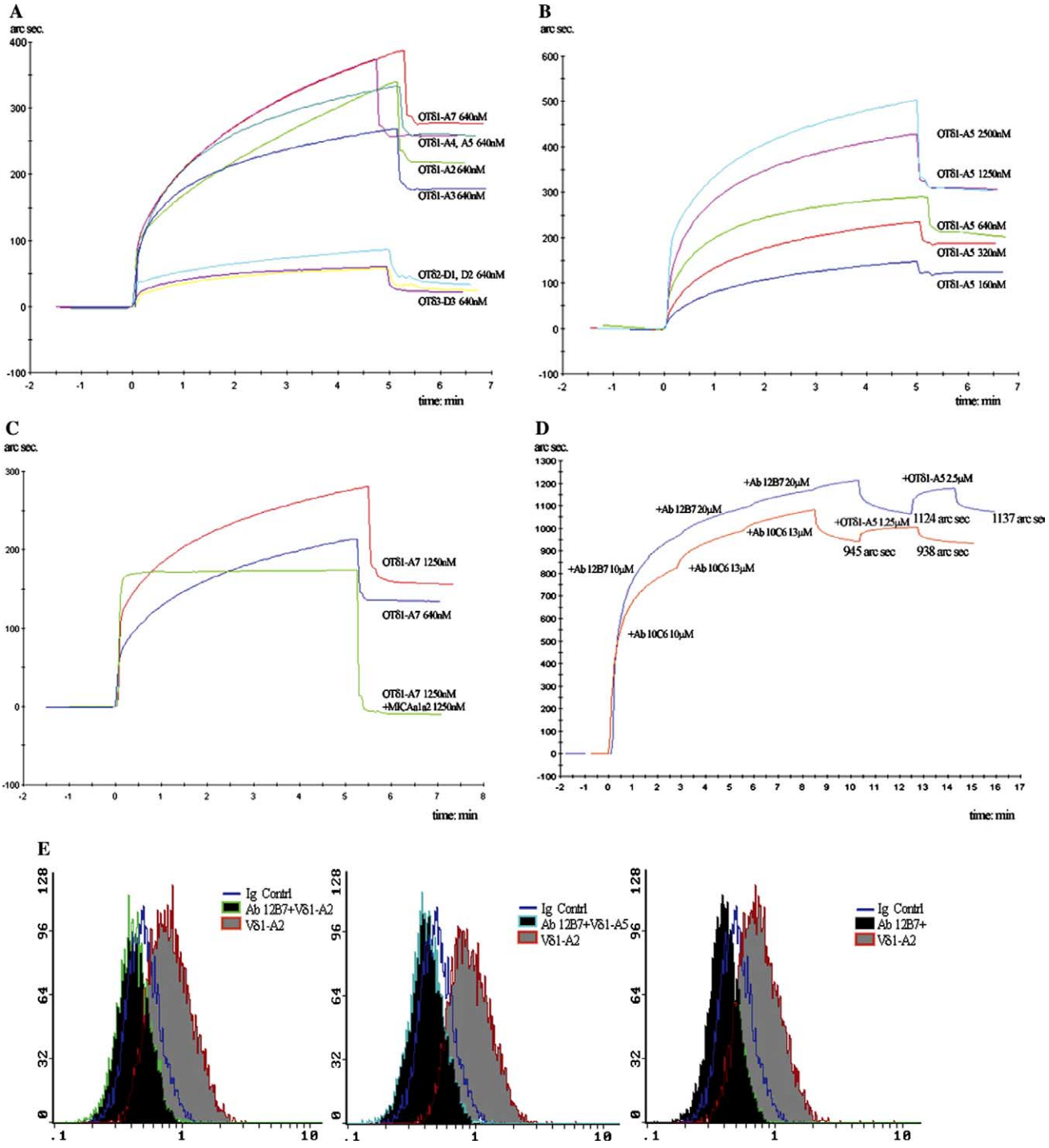

Fig. 5. Affinity measurements of Vδ1 scTCR binding to MICA. (A) The Vδ1/Vγ scTCR (OTδ1-A2, 3, 4, 5, and 7), but not the Vδ2/Vγ (OTδ1-D1, 2) or Vδ3/Vγ (OTδ1-D3) scTCR, binds specifically to immobilized MICA. All the Vδ/Vγ scTCRs were detected in 640 nM. (B) Binding curve profiles for kinetic analysis of a γδ scTCR protein (OTδ1-A5, in 2500–160 nM) association with and dissociation from immobilized MICA. (C) The competitive inhibition of rMICAα1α2 (1250 nM) to the binding of OTδ1-A7 (1250 nM) with rMICAα1–3. (D) The competitive inhibition of mAb 10C6 in different concentration to the binding of OTδ1-A5 (1250 nM) with rMICAα1–3. (E) Anti-MICA mAb-binding inhibition assays showing that different γδ scTCRs (OTδ1-A2, OTδ1-A5, and OTδ1-A7) are approximately equal in their ability to specifically recognize MICA on the surface of HeLa cells.

molecule using Vδ1 γδ TCR gene transfer and MICA tetramer binding assay [9]. Our results support the recognition of Vδ1 γδ TCR to MICA. But in our binding assay, the pairing of Vδ1 to Vγs is not so restricted as claimed by the previous report. Further detection is needed to explain the difference and the structural studies may be necessary to unravel the interaction between TCR of Vδ1 γδT cells and MICA.

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