

Imaging of T-cell receptor fused to CD3 ζ reveals enhanced expression and improved pairing in living cells

CHANGLI TAO^{1,2*}, HONGWEI SHAO^{2*}, YIN YUAN², HUI WANG², WENFENG ZHANG²,
WENLING ZHENG¹, WENLI MA¹ and SHULIN HUANG²

¹Institute of Genetic Engineering, Southern Medical University, Guangzhou, Guangdong 510515;

²Guangdong Province Key Laboratory for Biotechnology Drug Candidates, School of Life Science and Biopharmaceutics, Guangdong Pharmaceutical University, Guangzhou, Guangdong 510006, P.R. China

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Abstract. T cell receptor (TCR) gene adoptive therapy is a promising clinical approach for the treatment of malignant tumors and viral diseases. However, the effectiveness of this strategy is hampered by the generation of mixed TCR heterodimers comprising both exogenous and endogenous TCR chains (i.e., mispairing of TCR chains). In the present study, we constructed genetically encoded reporters fused to a pair of fluorescent proteins [enhanced cyan fluorescent protein (ECFP)/enhanced yellow fluorescent protein (EYFP)] to monitor the expression of TCR $\alpha\zeta\beta\zeta$ and pairing between TCR $\alpha\zeta$ and TCR $\beta\zeta$. We demonstrate that these reporters provide accurate images of TCR $\alpha\zeta\beta\zeta$ expression, which is markedly stronger with evident microclusters accumulated at the plasma membrane compared to wild-type (wt)TCR. Using fluorescence resonance energy transfer (FRET) analysis, we demonstrate that, in addition to the improved pairing, the expression and assembly of TCR $\alpha\zeta\beta\zeta$ are independent of endogenous CD3 subunits. These results suggest that the fusion genes, TCR $\alpha\zeta$ and TCR $\beta\zeta$, coupled to ECFP and EYFP, respectively can effectively monitor the expression and interaction in cells. Our data suggest a novel strategy with which it is possible to effectively express and

pair TCR $\alpha\zeta\beta\zeta$, thus making TCR gene adoptive therapy more effective.

Introduction

Antigen-specific TCR gene transfer enables the instantaneous generation of defined T cell immunity and TCR gene-modified T cells are fully functional *in vitro* and in murine models (1-6). The retroviral transfer of a MART1-specific TCR for adoptive T cell therapy first used in clinical trials on melanoma patients demonstrated the feasibility of TCR gene therapy (7,8). However, several factors currently limit the efficacy and hamper the application of TCR gene immunotherapy. One of these critical issues is that the introduced TCR α and β chain can potentially assemble with endogenous TCR chains (i.e., TCR mispairing), which not only reduces the expression of the desired TCR pair, but can create a new TCR with unknown specificity that can potentially cause autoimmunity and off-target toxicity (9,10).

A number of strategies have been investigated to prevent the mixed TCR dimer formation. Examples of such strategies are the replacement of the C domains of the TCR α and β chains by the corresponding murine domains (11,12), introduction of an additional inter-chain disulfide bond between the constant domains of TCR α and β chains (13,14), the inversion of amino acid residues in the constant region of the TCR α and β chains that form the TCR interface (15), and the use of single-chain TCR (scTCR) chimeras, including three-domain TCRs that contain other signaling domains, such as CD3 ζ or Fc ϵ RI γ (V α V β C β CD3 ζ) (16-18). However, the full extent to which these strategies can prevent TCR chain mispairing is unclear.

In a recent study, we have isolated a TCR (V α 12 and V β 7) from tumor infiltrating lymphocytes (TILs) of patients (HLA-A²⁺ and AFP⁺) with hepatocellular carcinoma (19). We demonstrated that T cells derived from central memory cells modified by tumor-specific TCR gene transfer were more effective than T cells derived from CD8⁺ T cells modified by TCR gene transfer in inducing CTL activity and effector cytokine secretion. However, wild-type (wt)TCR α 12 and β 7 expression in transduced human T cells was lower than the levels of endogenous TCR chains, mispairing with the endogenous TCR.

Correspondence to: Professor Shulin Huang, Guangdong Province Key Laboratory for Biotechnology Drug Candidates, School of Life Science and Biopharmaceutics, Guangdong Pharmaceutical University, 280 E. Road, Guangzhou Higher Education Mega Center, Guangzhou, Guangdong 510006, P.R. China
E-mail: shulhuang@sina.com

Professor Wenli Ma, Institute of Genetic Engineering, Southern Medical University, 1838 Guangzhou Avenue North, Guangzhou, Guangdong 510515, P.R. China
E-mail: wenli668@gmail.com

*Contributed equally

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In the present study, we generated a chimeric TCR $\alpha\zeta\beta\zeta$ which was modified by fusing the original constant domains downstream of the extracellular cysteine of wtTCR α and β chains to complete human CD3 ζ . Subsequently, we constructed genetically encoded reporters coupled with a pair of fluorescent proteins to monitor the expression of TCR $\alpha\zeta\beta\zeta$ and pairing between TCR $\alpha\zeta$ and TCR $\beta\zeta$ using confocal laser scanning microscopy (CLSM) in living cells (Jurkat and BEL-7402 cells). We demonstrate that these reporters provide accurate images of TCR $\alpha\zeta\beta\zeta$ expression and pairing. Of note, we observed that the expression of TCR $\alpha\zeta\beta\zeta$ was markedly stronger and with evident microclusters accumulated at the plasma membrane compared to wtTCR $\alpha\beta$. Fluorescence resonance energy transfer (FRET) imaging analysis of the T cells and non-T cells revealed that the expression of TCR $\alpha\zeta\beta\zeta$ does not need the engagement of CD3 subunits. Using these reporters, we demonstrate that in addition to enhanced pairing, TCR $\alpha\zeta\beta\zeta$ expression is greater in T cells or non-T cells without CD3 subunits. Taken together, these results suggest the reporters we used are useful in studying the expression/pairing of TCR $\alpha\zeta\beta\zeta$, which may be an effective approach to preventing mispairing in TCR gene therapy.

Materials and methods

Cells, genes and reagents. Jurkat/E6-1 (ATCC TIB-152) cells were cultured in RPMI-1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA), human embryonic kidney cells (HEK293) and human hepatocellular carcinoma cells (BEL-7402; maintained in our laboratory) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco/Invitrogen). All cultures were supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen), 100 U/ml streptomycin and 100 U/ml penicillin. The TCR α 12 and β 7 chains were isolated from TILs of patients (HLA-A²⁺ and AFP⁺) with hepatocellular carcinoma by our laboratory as previously described (19). CD3 ζ chains were isolated from peripheral blood mononuclear cells (PBMCs) of a healthy donor (GenBank accession no. NM_000734.3). The pair of fluorescent proteins [enhanced cyan fluorescent protein (ECFP)/enhanced yellow fluorescent protein (EYFP)] was kindly provided by Dr G. Zhang (Guangzhou University of Chinese Medicine, Guangzhou, China). Monoclonal antibodies (mAbs) used for flow cytometry included FITC-conjugated anti-TCRV α 12.1 mAb (Pierce Biotechnology, Rockford, IL, USA) and PE-conjugated anti-TCRV β 7.1 (Beckman Coulter, Brea, CA, USA).

Splice overlap extension (SOE) PCR. The strategy for the tripartite DNA fragment fusion by SOE PCR is shown in Fig. 2A. Variable fragments for generating TCR $\alpha\zeta$ -ECFP (including TCR α , CD3 ζ -a and EYFP) and TCR $\beta\zeta$ -EYFP (including TCR β , CD3 ζ -b and ECFP) were amplified using a set of forward primers and reverse primers. The first step of SOE PCR reactions was performed with 100 ng (3 fragments were mixed with an equimolar ratio) of template without primers, 10X Buffer, 2 mM dNTPs, 25 mM MgSO₄, 0.5 U KOD-Plus-Neo Polymerase in a 25 μ l reaction volume. The PCR cycling conditions were as follows: initial denaturation at 94°C for 2 min, followed by 5 cycles at 94°C for 30 sec, at 57°C for 30 sec and at 68°C for 1.5 min and completed with a final extension at 68°C for 7 min.

This initial PCRs generate overlapping gene segments that are then used as template DNA for the second step of SOE PCR to create a full-length product. Therefore, another 25 μ l reaction mixture (containing 10X Buffer, 2 mM dNTPs, 25 mM MgSO₄, 0.5 KOD-Plus-Neo Polymerase and 1 μ M forward primer P1/P1' and reverse primer P6/P6') was added to the first reaction mixture for the second step of SOE PCR. The reaction conditions were initial denaturation at 94°C for 2 min, followed by 30 cycles at 94°C for 30 sec, at 62°C for 30 sec, and at 68°C for 1.5 min and a final extension at 68°C for 7 min, 2 μ l of the amplicons were analyzed by agarose gel electrophoresis (1.0%) (Fig. 2B and C). Primer sequences for the amplification of variable regions and fusion chains are presented in Table I. The wtTCR α -ECFP and wtTCR β -EYFP fusing sequences were generated using the same methods.

Vector construction. To construct a bicistronic vector expressing both the TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP chains, the fusion sequences TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP were cloned into the original pIRES2-EGFP vector (Clontech Laboratories, Inc., Palo Alto, CA, USA). The TCR $\alpha\zeta$ -ECFP fusion gene was inserted into the *Bst*XI/*Not*I restriction site of the pIRES2-EGFP vector (Clontech Laboratories), located downstream of an internal ribosomal entry site (IRES) sequence of the plasmid to replace the EGFP gene, then the TRB ζ -EYFP fusion gene was inserted into the *Nhe*I/*Sal*I restriction site, located upstream of IRES. The other bicistronic vector expressing both non-modified wtTCR α -ECFP and wtTCR β -EYFP was constructed in a manner similar to the above-mentioned procedure. The single vectors expressing TCR α -ECFP or TCR β -EYFP which were used to remove the spectral bleed-through (SBT) contamination in the FRET images were constructed as previously described (20). The identity of all constructs was verified by direct sequencing.

Production of adenoviral particles and the transduction of TCR gene into Jurkat cells. We used the Ad5F35 chimeric adenoviral vector (21) which contained the Ad35 fiber knob incorporated into an Ad5 capsid as the packaging vector, and the whole wtTCR $\alpha\beta$ cassette (TCR β -IRES-TCR α) and TCR $\alpha\zeta\beta\zeta$ cassette (TCR $\beta\zeta$ -IRES-TCR $\alpha\zeta$) was cloned into the shuttle plasmid (pDC315) (Fig. 4A). Adenoviral particles were produced by co-transfection of the packaging cells, HEK293, with Ad5F35 vector and recombinant pDC315, containing wtTCR $\alpha\beta$ or TCR $\alpha\zeta\beta\zeta$. The virus-containing supernatants were filtered through 0.45- μ m filters. Adenoviral particle titers were determined by the 50% tissue culture infectious dose (TCID₅₀) method and the supernatants were directly used for the infection of the target Jurkat T cells. For Jurkat T cell transduction, 6-well plates were seeded with Jurkat cells at 1x10⁶ cells/well in 2 ml of RPMI 1640 medium supplemented with 2% FBS, cultured for 35 min, and then transduced with MOI 100 PFU of wtTCR $\alpha\beta$ or TCR $\alpha\zeta\beta\zeta$ viral supernatants. Following culture for 24 h at 37°C with 5% CO₂, the culture medium was replaced with RPMI 1640 medium supplemented with 10% FBS. After a further 48-h culture period, the cells were analyzed for TCR expression by flow cytometry.

Flow cytometry. The TCR-transduced Jurkat T cells (5x10⁵) were analyzed for transgene expression by flow cytometry using

Table I. Primers for amplifying 6 gene fragments (regular PCR and SOE PCR).

Fragments	Primers	Sequences (5'→3')
TCR α	P1	ACGCCACAACCTTGGCCACCATGATATCCTTGAGAGTT
	P2	CAGCAGGCCAAAGCTCTGTGGGCTGGGGAAGAAGGTGT
CD3 ζ -a	P3	ACACCTTCTTCCCCAGCCCACAGAGCTTTGGCCTGCTG
	P4	TCGCCCTTGCTCACCATGCGAGGGGGCAGGGCCTG
ECFP	P5	CAGGCCCTGCCCCCTCGCATGGTGAGCAAGGGCG
	P6	AGTGC GGCCGCTTACTTGTACAGCTCGTCCAT
TCR β	P1'	ATAGCTAGCGCCACCATGGGCTGCAGGCTGCTCTG
	P2'	ATCCAGCAGGCCAAAGCTCTGTGCTCTACCCAGGC
CD3 ζ -b	P3'	GCCTGGGGTAGAGCACAGAGCTTTGGCCTGCTGGAT
	P4'	TCGCCCTTGCTCACCATGCGAGGGGGCAGGGCCTG
EYFP	P5'	CAGGCCCTGCCCCCTCGCATGGTGAGCAAGGGCG
	P6'	CGGCGTCGACTTACTTGTACAGCTCGTC

Restriction enzyme sites are underlined.

FITC-conjugated anti-TCRV α 12 mAb and PE-conjugated anti-TCRV β 7 mAbs. The cells were incubated with the mAbs on ice for 30 min. Subsequently, the Jurkat cells were washed twice with PBS and centrifuged for 10 min at 1000 x g, and the cell pellet was resuspended and fixed with 2% paraformaldehyde before taking measurements on an Epics XL flow cytometer (Beckman Coulter). The samples were analyzed using EXPO32 software (Beckman Coulter) and are displayed as dotplots.

Imaging by CLSM. The recombinant vectors, pIRES-TCR β -EYFP/TCR α -ECFP and pIRES-TCR β ζ -EYFP/TCR α ζ -ECFP, were transfected into the Jurkat and BEL-7402 cells using Lipofectamine LTX/PLUS (Invitrogen) according to the manufacturer's instructions. The cells transfected with pIRES-TCR α -ECFP and pIRES-TCR β -EYFP were used as controls to remove the SBT contamination in FRET analysis. After 24 h of transfection, the Jurkat cells grown on glass-bottom dishes were washed twice with PBS and immobilized with 0.05% low metling agarose for 15 min and the BEL-7402 cells were washed twice with PBS solution. Confocal images of the cells were acquired using an Olympus FluoView 1000 confocal laser scanning microscope with FV10-ASW 1.7 software (Olympus, Tokyo, Japan). The FRET donor (TCR α -ECFP or TCR α ζ -ECFP) was excited with a 458 nm Ar-laser and the acceptor (TCR β -EYFP or TCR β ζ -EYFP) was excited with a 515 nm Ar-laser. The cells were scanned from 475 to 585 nm with a 10 nm step-size and 20 nm band-width to obtain original images.

FRET analysis. FRET efficiency was analyzed using the sensitized acceptor emission (SE) method. FRET occurs when 2 fluorophores (donor and acceptor) have sufficiently large spectral overlap which results in the energy of the donor fluorescence transferring to the acceptor. The spectral overlap between donor and acceptor fluorophores also causes FRET signal contamination, termed SBT. It is important to remove

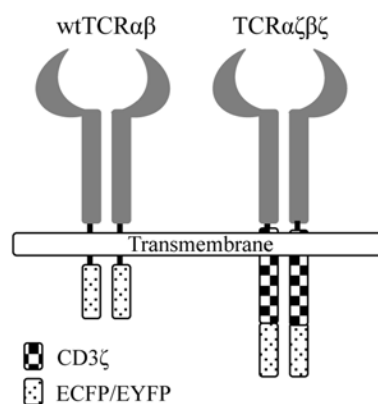


Figure 1. Schematic representation of the wild-type (wt)TCR $\alpha\beta$ and modified TCR $\alpha\zeta\beta\zeta$ variants used in the present study. wtTCR $\alpha\beta$ was isolated from tumor infiltrating lymphocytes (TILs) of patients, the α chain was fused with enhanced cyan fluorescent protein (ECFP) and the β chain was fused with enhanced yellow fluorescent protein (EYFP). TCR $\alpha\zeta\beta\zeta$ was modified by the replacement of the extracellular and transmembrane domain with CD3 ζ chains, and also fused to ECFP and EYFP.

SBT in FRET efficiency measurements. The pFRET images were corrected as shown in the equation and as previously described (22): $pFRET = uFRET - DSBT - ASBT$, where $uFRET$ is uncorrected FRET, $ASBT$ is the acceptor spectral bleed-through signal, and $DSBT$ is the donor spectral bleed-through signal. The final FRET efficiency equation was calculated as $E = 1 - I_{DA} / \{I_{DA} + pFRET \times [(\psi_{dd}/\psi_{aa}) \times (Q_d/Q_a)]\}$, where I_{DA} is the intensity of the donor in the presence of the acceptor, ψ_{dd} and ψ_{aa} are the collection efficiency in the donor and acceptor channel, and Q_d and Q_a are the quantum yield of the donor and acceptor, respectively (22,23).

Statistical analysis. Mean FRET efficiency was calculated from multiple (n=6) cell images in each group and 6 random regions of interest (ROI) in each cell image. Statistical analysis

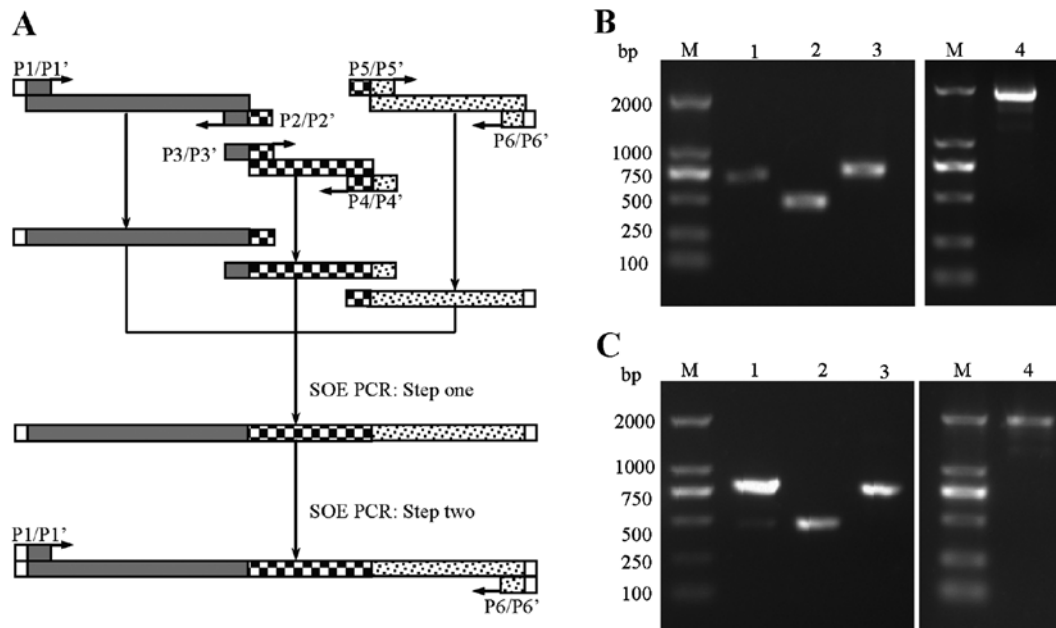


Figure 2. Generation of the fusion sequences TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP by splice overlap extension (SOE) PCR. (A) Schematic diagram of the strategy for the tripartite DNA fragment fusion. (B) PCR products. Left panel: lane M, molecular weight marker DL2000 (Takara); lane 1, TCR β fragment (810 bp); lane 2, CD3 ζ -b fragment (458 bp); lane 3, EYFP fragment (731 bp). Right panel: lane 4, TCR $\beta\zeta$ -EYFP fusion (1953 bp). (C) PCR products. Left panel: lane 1, TCR α fragment (681 bp); lane 2, CD3 ζ -a fragment (463 bp); lane 3, ECFP fragment (738 bp). Right panel: lane 4, TCR $\alpha\zeta$ -EYFP fusion (1826 bp).

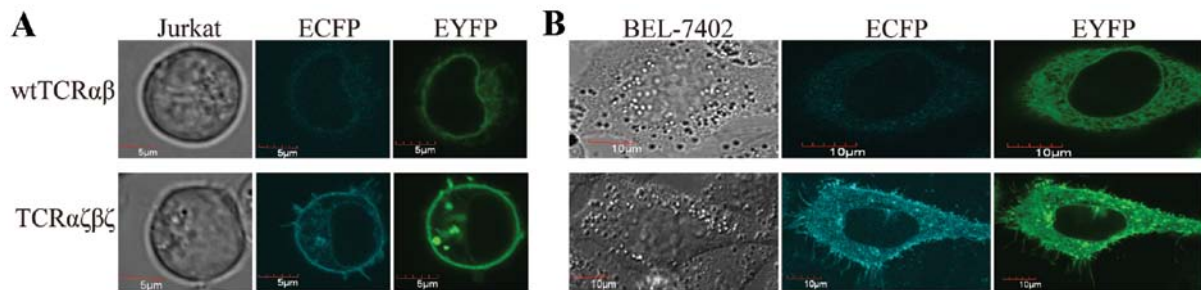


Figure 3. Expression of wild-type (wt)TCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ in living cells. (A) Jurkat cells and (B) BEL-7402 cells expressing wtTCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ were monitored using CLSM with FV10-ASW 1.7 software. Shown are the images in the ECFP and EYFP channels. The scale bars in Jurkat cells are 5 μ m, and in BEL-7402 cells are 10 μ m. All images shown are representative of at least 3 independent experiments. ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein.

was performed using two-way ANOVA with a Bonferroni's multiple comparisons test using Graphpad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA). Differences with P-values <0.05 were considered statistically significant. Data are expressed as the means \pm SD.

Results

TCR $\alpha\zeta\beta\zeta$ generation and location on the surface of cells. We designed a modified TCR in which the extracellular and transmembrane domain of TCR α or β chains were exchanged for CD3 ζ chains at a structurally favorable position. To better observe the expression of the modified TCR $\alpha\zeta\beta\zeta$ in living cells, we fused the C terminus of TCR $\alpha\zeta$ and TCR $\beta\zeta$ chains to a pair of cyan and yellow fluorescent proteins, ECFP and EYFP, respectively (Fig. 1). The fusion genes, TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP, were obtained by SOE PCR (Fig. 2B and C). To compare the relative levels of surface expression

with wtTCR $\alpha\beta$, we also fused the C terminus of the wtTCR α and wtTCR β chains to a pair of ECFP and EYFP fluorescent proteins (Fig. 1). The fusion genes were then cloned into the pIRES2-EGFP vector.

We first examined whether the TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP fusion chains are expressed on the surface of the cells. We transfected the recombinant plasmids into Jurkat cells, as well as BEL-7402 cells, and 24 h after transfection, the fluorescence observation by CLSM revealed that the introduced fusion genes, TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP, were located at the plasma membrane of the cells and were effectively expressed in T cells and non-T cells (Fig. 3).

TCR $\alpha\zeta\beta\zeta$ enhanced surface expression in T cells and non-T cells. We observed a very interesting phenomenon, namely that in the TCR $\alpha\zeta\beta\zeta$ -transduced cells, but not in the TCR $\alpha\beta$ -transduced cells, microclusters were clearly evident at the plasma membrane and the cilia of the plasma membrane

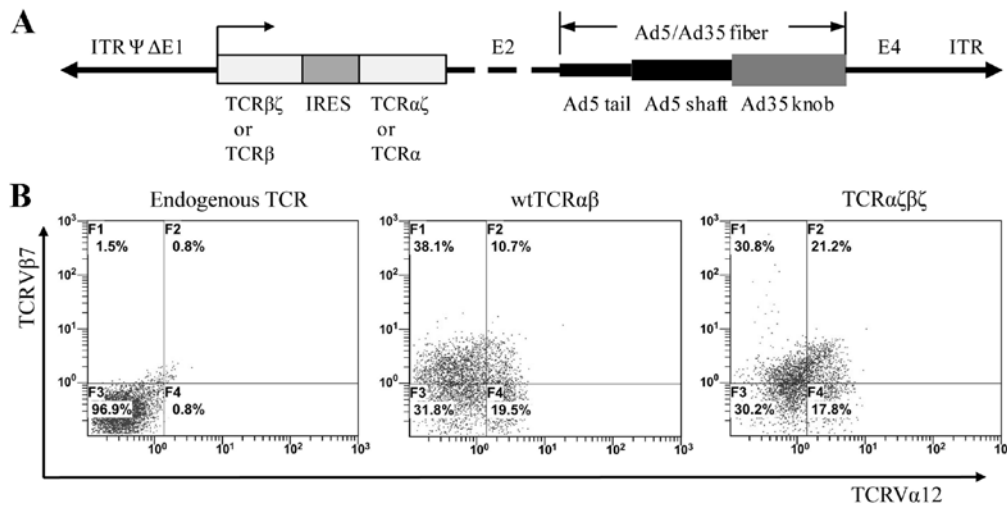


Figure 4. TCR $\alpha\zeta\beta\zeta$ shows enhanced surface expression on Jurkat cells compared to wild-type (wt)TCR $\alpha\beta$. (A) Schematic diagram of recombinant wtTCR $\alpha\beta$ and TCR $\beta\zeta\alpha\zeta$ adenovirus. (B) Jurkat cells transduced with wtTCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ stained with anti-TCR V α 12.1 mAb^{FITC} and anti-V β 7 mAb^{PE} were analyzed by FACS.

(Fig. 3). This observation indicated that TCR $\alpha\zeta\beta\zeta$ expression was stronger in the Jurkat cells and BEL-7402 cells compared to the expression of wtTCR $\alpha\beta$. To confirm this result, we inserted the wtTCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ genes separately into the shuttle plasmid, pDC315, to produce Ad5F35 adenovirus (Fig. 4A). Ad5F35 adenovirus which contained the Ad35 fiber knob incorporated into an Ad5 capsid was able to effectively transduce human T cells. The wtTCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ adenoviral vectors were introduced into the Jurkat T cells followed by FACS analysis. Double immunofluorescent staining with anti-TCRV α 12 mAb^{FITC} and anti-V β 7 mAb^{PE} showed that the Jurkat cells transduced with wtTCR $\alpha\beta$ displayed little surface co-expression (up to 10.7%). By contrast, there was a clear increased surface coexpression (up to 21.2%) in the TCR $\alpha\zeta\beta\zeta$ -transduced Jurkat cells (Fig. 4B). These data are in accordance with the imaging evidence showing the enhanced surface expression of TCR $\alpha\zeta\beta\zeta$ in the cells. These results demonstrated that the FRET reporter, TCR $\alpha\zeta$ -ECFP/TCR $\beta\zeta$ -EYFP, can be effectively used to express TCR $\alpha\zeta\beta\zeta$ may thus be used for monitoring the expression and interaction of TCR $\alpha\zeta\beta\zeta$.

Highly preferred pairing between TCR $\alpha\zeta$ and $\beta\zeta$, but not wtTCR α and β in Jurkat cells. To investigate the interaction of TCR $\alpha\zeta$ and $\beta\zeta$ in T cells, we used Jurkat cells (clone E6-1) expressing endogenous TCR as a recipient TCR cell model. We hypothesized that the introduced wtTCR α and β chains or TCR $\alpha\zeta$ and $\beta\zeta$ chains composing heterodimers would result in FRET efficiency between the donor (ECFP) and acceptor (EYFP) fluorescent proteins. Once the introduced TCR $\alpha\zeta$ and $\beta\zeta$ chains paired with endogenous TCR β and α chains, it would fail to detect FRET between the mispaired TCR (Fig. 5C). We observed that the TCR $\alpha\zeta\beta\zeta$ displayed a higher FRET signal compared to wtTCR $\alpha\beta$ in Jurkat cells. (Fig. 5A). Six independent cell images and 6 ROI in each cell image were selected for FRET efficiency analysis in each group. The results from statistical analysis showed that the average FRET efficiency between TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP (16.7 \pm 2.5%) was significantly increased compared to wtTCR α -ECFP and

wtTCR β -EYFP (7.9 \pm 1.3%) in the Jurkat cells ($P < 0.0001$) (Fig. 5D). These data suggested that the fusion of wtTCR α 12 and β 7 with CD3 ζ improved pairing.

TCR $\alpha\zeta\beta\zeta$ assembles independently of CD3 subunits in T cells and non-T cells. To better characterize the highly preferred pairing of TCR $\alpha\zeta$ and $\beta\zeta$, we selected BEL-7402 cells which are deficient in TCR and CD3 molecules as the next recipient cell model to investigate the interaction of TCR $\alpha\zeta\beta\zeta$ and endogenous CD3 subunits. We hypothesized that since there was no endogenous TCR in BEL-7402 cells, the pairing of introduced wtTCR α and β or TCR $\alpha\zeta$ and $\beta\zeta$ would not be interfered with and would result in the same FRET signal. Unexpectedly, we observed that TCR $\alpha\zeta\beta\zeta$ still displayed a higher FRET signal compared to wtTCR $\alpha\beta$ in BEL-7402 cells (Fig. 5B). The average FRET efficiency between wtTCR α and β chains (10.5 \pm 1.0%) was significantly reduced compared to the TCR $\alpha\zeta$ and $\beta\zeta$ chains (17.1 \pm 2.1%) in BEL-7402 cells ($P < 0.0001$) (Fig. 5D). The results indicated that the assemble of wtTCR α and β chains was impaired in the absence of CD3 subunits, in spite of no endogenous TCR in the BEL-7402 cells. Of note, we found that the average FRET efficiency between TCR $\alpha\zeta$ -ECFP and TCR $\beta\zeta$ -EYFP in the BEL-7402 cells (17.1 \pm 2.1%) was not reduced compared to the Jurkat cells (16.7 \pm 2.5%) ($P > 0.05$) (Fig. 5D). These results demonstrated that the expression and assembly of TCR $\alpha\zeta\beta\zeta$ was not compromised even without the CD3 $\gamma\epsilon$, $\delta\epsilon$ and $\zeta\zeta$ signaling dimers in the BEL-7402 cells.

Discussion

In the present study, we generated several ECFP and EYFP fusions as a pair of reporters that allow the monitoring of the expression and pairing between TCR $\alpha\zeta$ and TCR $\beta\zeta$ in living cells. First, we used a wtTCR $\alpha\beta$ (V α 12 and V β 7) isolated from TILs of patients to complete human CD3 ζ molecule to generate a pair of chimeric TCR $\alpha\zeta$ and TCR $\beta\zeta$, then ECFP fused to TCR $\alpha\zeta$, and EYFP fused to TCR $\beta\zeta$ via overlap PCR,

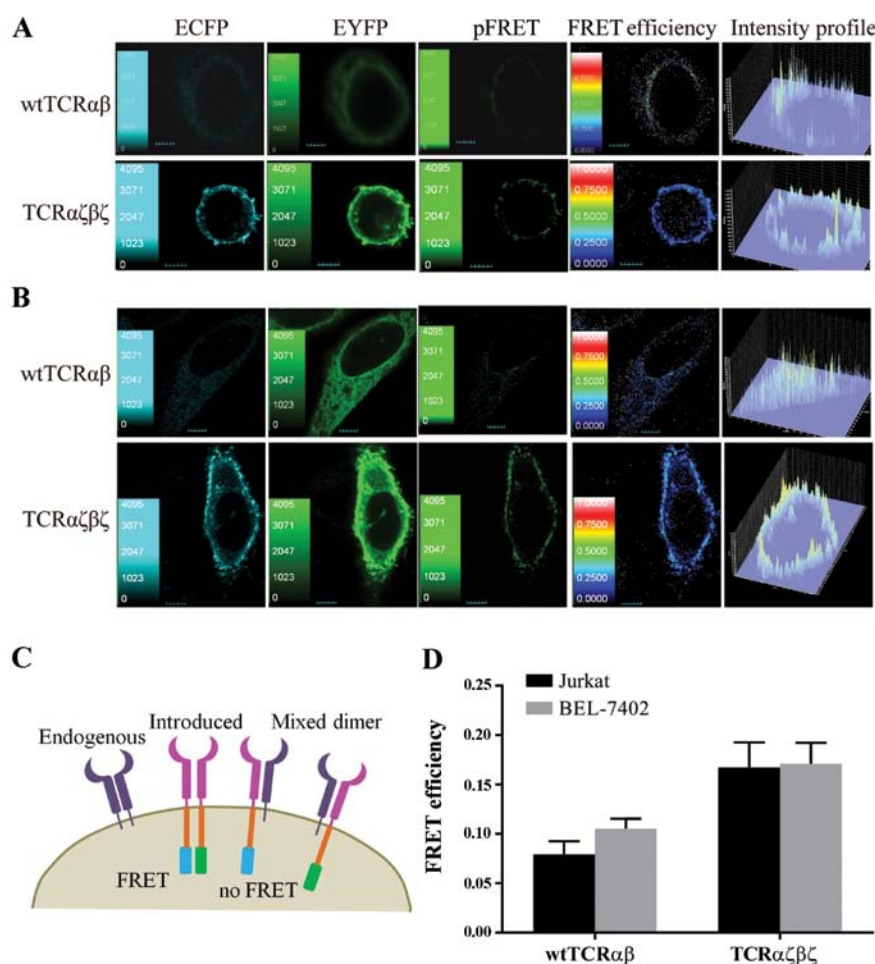


Figure 5. FRET efficiencies of wild-type (wt)TCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ in living cells. Confocal images of (A) Jurkat cells and (B) BEL-7402 cells expressing wtTCR $\alpha\beta$ or TCR $\alpha\zeta\beta\zeta$ in the ECFP and EYFP channels were captured by CLSM using FV10-ASW 1.7 software, and the pFRET, FRET efficiency and intensity profile were calculated by the sensitized acceptor emission (SE) method. The continuous color scale (from black to white) represents the FRET efficiency (from 0 to 1). The scale bars in Jurkat cells are 5 μm , and in BEL-7402 cells are 10 μm . (C) Schematic diagram of pairing of TCR $\alpha\zeta\beta\zeta$ chains in T cells. (D) Six independent cell images in each group and 6 random regions of interest (ROI) in each cell image were selected for the statistical analysis of FRET efficiency. Data are expressed as the means \pm SD ($P < 0.05$). Data represents one of 6 independent experiments with similar results. ECFP, enhanced cyan fluorescent protein; EYFP, enhanced yellow fluorescent protein.

respectively. This suggested strategy using these reporters offers several advantages over conventional immunofluorescent staining using specific antibodies: it provides accurate imaging of modified TCR $\alpha\beta\zeta$ expression in Jurkat T cells, and can be used in combination with FRET methods to study the interaction of TCR $\alpha\zeta$ and TCR $\beta\zeta$ in living cells.

Using these reporters, we observed that the expression of the introduced TCR $\alpha\zeta\beta\zeta$ was enhanced when compared to the non-modified wtTCR $\alpha\beta$ in Jurkat cells, which was in line with our FACS analysis. Our results showed that the fusion gene, TCR $\alpha\zeta\beta\zeta$, coupled with ECFP/EYFP was able to be expressed normally on the surface of cells. Furthermore, we found that wtTCR $\alpha\beta$ and TCR $\alpha\zeta\beta\zeta$ were expressed not only in Jurkat cells but also in non-T cells (BEL-7402). Of note, we observed that the TCR $\alpha\zeta\beta\zeta$ -transduced cells, but not the wtTCR $\alpha\beta$ -transduced cells showed evident microclusters at the plasma membrane and cilia of the plasma membrane. These phenomena indicated that the surface expression of TCR $\alpha\zeta\beta\zeta$ was markedly enhanced due to the fusion CD3 ζ molecules, whereas the expression of wtTCR $\alpha\beta$ was impaired in the absence of CD3 molecules.

The fluorescence images of the reporters were then subjected to FRET analysis which allowed the detection of the interaction of TCR $\alpha\beta$ in the living cells. The FRET efficiencies between wtTCR α -ECFP and β -EYFP or TCR $\alpha\zeta$ -ECFP and $\beta\zeta$ -EYFP in the Jurkat cells revealed that the introduction of TCR $\alpha\zeta\beta\zeta$ improved pairing compared to the wtTCR $\alpha\beta$ majority of which mispaired with endogenous TCR. The FRET efficiency of TCR $\alpha\zeta$ and $\beta\zeta$ in the Jurkat and BEL-7402 cells did not show any significant differences, suggesting that TCR $\alpha\zeta\beta\zeta$ may be expressed/or assembled in BEL-7402 cells without endogenous CD3 subunits. The assembly of the TCR-CD3 complex is in part determined by the transmembrane charges, the positive charges at the transmembrane regions of TCR α interacting with the negative charges at the CD3 $\delta\epsilon$ and CD3 $\zeta\zeta$ dimers, while TCR β interacts with CD3 $\gamma\epsilon$ (24-26). In the TCR $\alpha\zeta$ and $\beta\zeta$ chains, the extracellular connecting peptide motif, transmembrane and intracellular domains of TCR α and β are replaced by CD3 ζ . Thereby, CD3 $\gamma\epsilon$ or CD3 $\delta\epsilon$ cannot be associated with the modified TCR $\alpha\zeta\beta\zeta$, which explains why TCR $\alpha\zeta\beta\zeta$ can be expressed independently of CD3 components.

In conclusion, using the strategy of fluorescent proteins fused to the TCR $\alpha\zeta$ and $\beta\zeta$ chains we monitored and measured the expression and interaction of TCR $\alpha\zeta$ and TCR $\beta\zeta$ in living cells accurately. The ECFP and EYFP fusion reporters revealed that the TCR $\alpha\beta$ chains fused to CD3 ζ enhanced the expression and prevented mispairing, paving the way to potential immunological studies dealing with TCR mispairing.

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