OPEN

Functional analysis of the antigen binding sites on the MTB/HIV-1 peptide bispecific T-cell receptor complementarity determining region 3α

Chao-Ying Zhou, Rui-Ning Wang, Wen-Ting He, Dong-Rong Luo, Si-Rui Yuan, Qian Wen, Sheng-Feng Hu, Xin-Ying Zhou and Li Ma

Objective: *Mycobacterium tuberculosis*/human immunodeficiency virus (MTB/HIV) coinfection has become an urgent problem in the field of prevention and control of infectious diseases in recent years. Adoptive cellular immunotherapy using antigen-specific T-cell receptor (TCR) engineered T cells which recognize the specific antigen artificially may have tremendous potential in anti-MTB/HIV coinfection. We have previously successfully identified a MTB $Ag85B_{199–207}$ and HIV-1 $Env_{120–128}$ peptide-bispecific TCR screened out from peripheral blood mononuclear cells of a HLA-A*0201^{+} healthy individual and have further studied that how residues on the predicted complementarity determining region (CDR) 3 of the β chain contribute to the bispecific TCR contact with the peptide-MHC. However, it is not clear which amino acids in the predicted CDR3α of the bispecific TCR play a crucial role in ligand recognition.

Methods: The variants in the CDR3α of the bispecific TCR were generated using alanine substitution. We then evaluated the immune effects of the five variants on T-cell recognition upon encounter with the MTB or HIV-1 antigen.

Results: Mutation of two amino acids (E112A, Y115A) in CDR3α of the bispecific TCR caused a markedly diminished T-cell response to antigen, whereas mutation of the other three amino acids (S113A, P114A, S116A) resulted in completely eliminated response.

Conclusion: This study demonstrates that Ser^{113}, Pro^{114} and Ser^{116} in CDR3α of the bispecific TCR are especially important for antigen recognition. These results will pave the way for the future development of an improved high-affinity bispecific TCR for use in adoptive cellular immunotherapy for MTB/HIV coinfected patients.

AIDS 2023, **37**:33–42

Keywords: adoptive cellular immunotherapy, alanine substitution, bispecific T-cell receptor, complementarity determining region 3α, *Mycobacterium tuberculosis*/HIV coinfection

Introduction

Mycobacterium tuberculosis/human immunodeficiency virus (MTB/HIV) coinfection has become an urgent problem in the field of prevention and control of tuberculosis (TB) and HIV/AIDS in recent years. Tuberculosis is a common opportunistic infectious disease of HIV/AIDS and also the most common cause of death of HIV/AIDS. Similarly, HIV infection is an important factor in the development of active TB [1]. In 2020, an estimated 9.9 million people fell ill with TB, and among them, 8% were HIV-positive; there were an estimated 1.5 million TB deaths, among which about 214 000 deaths were HIV-positive people [2]. Although MTB/HIV

Institute of Molecular Immunology, School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou 510515, China.

Correspondence to Li Ma, Institute of Molecular Immunology, School of Laboratory Medicine and Biotechnology, Southern Medical University, Guangzhou 510515, China.

Tel: +86 20 61648322; fax: +86 20 61648322; e-mail: mali_61648322@smu.edu.cn

Received: 7 July 2022; revised: 22 September 2022; accepted: 13 October 2022.

DOI:10.1097/QAD.0000000000003408

ISSN 0269-9370

coinfected patients can be effectively treated, there are still many problems such as severe drug toxicities and adverse effects, susceptibility to drug resistance, the immune reconstitution inflammatory syndrome and so on [3]. Hence, there is an urgent need to develop new therapies for MTB/HIV coinfection.

The immune mechanism against MTB/HIV coinfection is mainly T-cell-mediated immunity. However, it is quite obvious that the immune system is disordered in MTB/HIV coinfected individuals, which mainly reflects for the significantly reduced number of effector T cells and the obviously decreased secretion of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α) and perforin [4]. Thus it can be seen that adoptive transfer of lots of effector T cells to MTB/HIV coinfected patients is a quite effective method. At present, the application of antigen-specific T-cell receptor (TCR) gene modified T cells in the immunotherapy of T-cell immune-related diseases such as bacterial and viral infections and tumors has made great progress and become a hot spot in the field [5–7]. This shows that adoptive immunotherapy of antigen-specific TCR gene modified T cells has bright prospects for the treatment of MTB/HIV coinfection.

As is known to all, T-cell recognition of foreign antigens associated with MHC proteins is achieved through the TCR [8]. T-cell receptors engage both peptide and MHC molecule via their six complementarity determining regions (CDRs) loops which play a leading role in the specificity of TCR recognition of antigens, three from the Vα domain and three from the Vβ domain [9]. Among them, the CDR3 region which is the most variable is the critical determining factor of T-cell antigen recognition specificity and mediates T-cell diversity [10]. Explanation of the role of TCR residues in antigen binding as well as T-cell activation mainly include structural research on the alteration of residues within the CDRs loops of the α and β chains. Generally, alanine substitution is a common method for mutation analysis. The murine class II-restricted D5 TCR [11,12] and the murine class I-restricted 2C TCR [13] have been extensively studied to determine the role of individual CDR residues on TCR affinity and functional activity using alanine substitution.

Previously, we have successfully identified a MTB $Ag85B_{199-207}$ and HIV-1 $Env_{120-128}$ peptide-bispecific TCR screened out from peripheral blood mononuclear cells (PBMCs) of a HLA-A*0201$^+$ healthy individual and have further studied that how residues on the predicted CDR3 of the β chain contribute to the bispecific TCR contact with the peptide-MHC [14,15]. However, it is not clear which residues in the predicted CDR3α of the bispecific TCR play a crucial role in ligand recognition. Here, we extend the study to the bispecific TCR α chain using alanine substitution.

Materials and methods

Cells

Peripheral blood mononuclear cells were from a HLA-A*0201$^+$ healthy volunteer with informed consent. The research had been carried out in accordance with the World Medical Association Declaration of Helsinki and was approved by the ethics committee of the Southern Medical University. Monocyte-derived dendritic cells (DCs) and CD8$^+$ T cells were generated as previously described [15]. The 293T, T2 and J.RT3-T3.5 (kindly provided by Dr Wei He, Peking Union Medical College, Beijing, China) cells were routinely cultured.

Generation of variant T-cell receptors

The wild-type (WT) bispecific TCR has generated as described in previous study [15]. Briefly, for generating the variant TCR α-chains, the 5'-end β15-fu-SGSG-P2A-α17 fragment was amplified from the WT plasmid pHAGE-β15-fu-SGSG-P2A-α17-IRES-IZsGreen using the forward primer P1 and the reverse primer P3, P5, P7, P9, or P11, and the 3'-end β15-fu-SGSG-P2A-α17 fragment was generated using the forward primer P4, P6, P8, P10, or P12 and the reverse primer P2. The corresponding fragments were then mixed and joined by carrying out a PCR using P1 and P2 primers to produce the variants. The DNA fragments containing the variant TCRs were then digested and inserted into the pHAGE-fullEF1a-MCS-IZsGreen lentiviral vector at XbaI and BamHI sites. All of the primers (P1–P12) are outlined in Table 1.

Recombinant lentivirus production

The 293T were transfected with the lentivirus triad of plasmids pHAGE, psPAX2 and pMD2.G using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). Lentiviral supernatant was collected, filtered and ultracentrifuged. Pellets were gently resuspended and aliquoted and should be frozen at −80°C. Viral titers were determined and calculated.

Genetic modification of T cells

Before transduction, CD8$^+$ cells were seeded in a 24-well plate (Nunc, Roskilde, Denmark). The lentiviral particles containing 8 μg/ml polybrene was added. Cells were transduced by 3 cycles of spin-infection at a 24-h interval.

Detection of exogenous T-cell receptors

After transduction, CD8$^+$ cells were harvested to eliminate the dead cells using the Dead Cell Removal Kit (Miltenyi Biotec) and detect the expression of GFP. The exogenous TCRs were determined by flow cytometry using APC-Cy7-conjugated anti-CD8$^+$ monoclonal antibody (Biolegend, San Diego, California, USA), PE-conjugated $Ag85B_{199-207}$/HLA-A*0201 dextramer (Immudex, Copenhagen, Denmark) and APC-conjugated $Env_{120-128}$/HLA-A*0201 dextramer (Immudex). Furthermore, RNA was isolated from CD8$^+$ cells and reverse transcription was carried out. Primer sequences are summarized in Table 1.

Table 1. Primer sequences.

Primer	Sequence (5′-3′)	Length (bp)
P1	Kozak[a] GCTCTAGA**GCCAGG**ATGGCCTCCC	24
P2	CGGGATCCTCAGCTGGACCACAGCCGCAGCGTCATGAGC	39
P3	CCTGAATAGGGACT**GG**CTGCACAG[b]	24
P4	CTGTGCAG**CC**AGTCCCTATTCAGG	24
P5	CCTCCTGAATAGGG**GGC**CTCTGCAC	25
P6	GTGCAGAG**GCC**CCCTATTCAGGAGGAG	27
P7	CTCCTCCTGAATAGG**C**ACTCTCTGCACAG	29
P8	CTGTGCAGAGAGT**G**CCTATTCAGGAGGAG	29
P9	CTCCTGA**GGC**GGGACTCTCTGCAC	24
P10	GTGCAGAGAGTCCC**GCC**TCAGG	22
P11	CTCCTCC**G**G**C**ATAGGGACTCTCTGCAC	27
P12	GTGCAGAGAGTCCCTAT**G**C**C**GGAGG	25
GAPDH forward	GGATATTGTTGCCATCAATGACC	23
GAPDH reverse	AGCCTTCTCCATGGTGGTGAAGA	23
β15-fuSGSGP2A-α17 forward	TCCTGTCTGCCACCATCCTCTAT	23
β15-fuSGSGP2A-α17 reverse	CAGCCACAAAAACAGGAACGA	21

GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
[a]Kozak sequence (GCCAGG), a nucleotide sequence located in the 5′ untranslated mRNA region that allows ribosomes to recognize the initiator codon, was underlined.
[b]Mutated nucleotides were bold and labeled underlined simultaneously.

Measurement of cytokine release

$CD8^+$ cells were tested for reactivity in cytokine-release assays using ELISA kits [IFN-γ, TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF); ExCell Bio, Shanghai, China; granzyme B (GrB); eBioscience, San Diego, California, USA]. T2 were pulsed with HLA-A*0201-restricted MTB $Ag85B_{199-207}$ (KLVANNTRL), HIV-1 $Env_{120-128}$ (KLTPLCVTL) or CMV $pp65_{495-503}$ peptide (NLVPMVATV) (Proimmune, Oxford, UK) at indicated concentrations for 3 h at 37°C. Stimulator cells and responder cells were cocultured in a 96-well U-bottom plate (Nunc) for 24 h, except IFN-γ, which was detected after 18 h of incubation. In some groups, DCs were transfected with the pV1J.ns-tPA-Ag85B (gifted by Dr Kris Huygen in Pasteur Institute of Brussels, Brussels, Belgium) or the pCAGGS-Env plasmid (gifted by Dr James M. Binley in Torrey Pines Institute for Molecular Studies, San Diego, California, USA) respectively using Lipofectamine 2000 Transduction Reagent (Invitrogen, Carlsbad, California, USA).

Assessment of T-cell proliferation

T-cell proliferation was detected using Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan). Briefly, $CD8^+$ cells were cocultured with T2 loaded with 10 μg/ml corresponding peptides in a 96-well U-bottom plate. After 2, 4, or 7 days of coculture, the supernatants were transferred into empty wells and CCK-8 solutions were added. The plate was incubated for 4 h at 37°C. The optical absorbance at 450 nm was then measured with a microplate reader (Thermo Scientific Varioskan Flash; Thermo Fisher Scientific Inc.).

Intracellular cytokine staining

Intracellular cytokine staining was performed to assess the level of IFN-γ produced by T cells and was performed as previously described [15].

EuTDA cytotoxicity assays

Cytotoxicity assays were done to evaluate the cytotoxic activity of $CD8^+$ cells using a DELFIA europium-2,2′:6′,2″-terpyridine-6,6″-dicarboxylic acid (EuTDA) cytotoxicity kit (Perkin-Elmer Life Sciences, Norwalk, Connecticut, USA) according to the instructions. Percentage cytotoxicity was calculated by the following formula: [(experimental release - spontaneous release) / (maximum release - spontaneous release)] × 100.

Determination of CD69 in J.RT3-T3.5 cells

Before transduction, J.RT3-T3.5 cells were seeded in six-well plates (Nunc) and then incubated with concentrated lentivirus. After 3 days of transduction, cells were pooled and cocultured with peptide-pulsed T2. Eighteen hours later, cells were collected, washed, and stained with APC-labeled anti-CD69 antibody (eBioscience). Data were rapidly analyzed by flow cytometry.

Statistical analysis

All statistical analyses were performed using the SPSS version 17.0 for windows (SPSS, Chicago, Illinois, USA). A one-way ANOVA and multiple comparisons tests (least significant difference or Dunnett's T3) were used to compare the differences between the experimental groups. *P*-values were two-sided. Differences with $P < 0.05$ were considered significant.

Results

Bispecific T-cell receptor CDR3α variants construction

The pHAGE-fullEF1a-MCS-IZsGreen lentiviral vector with expression cassettes consisting of TCR α17 and β15 genes was constructed (Fig. 1a). The bispecific TCR

expression in this vector is driven by the long terminal repeat, and α- and β-chains are expressed as a single open reading frame using the furin (RAKR)-linker (SGSG)-P2A peptide sequence (Fig. 1b). Generally, ribosomes skip the synthesis of the glycyl-prolyl peptide bond at the C-terminus of the P2A peptide, leading to the equimolar cleavage between β- and α-chain [16]. The furin cleavage site could remove the P2A peptide remaining on the TCR β chain, and the SGSG linker has been demonstrated to efficiently synthesize functional TCRs compared to the 2A sequence only [17]. To promote preferential pairing and improve cell surface expression of the exogenous TCR, nine critical amino acids (AAs) in the TCR α and β C regions were replaced by murine counterparts as described previously [15]. The amino acid residues 112–116 (ESPYS) of the TCR CDR3α were targeted for alanine substitution, which designated E112A, S113A, P114A, Y115A and S116A, respectively (Fig. 1c). Using the primers listed in Table 1, these variants containing single amino acid substitution were produced.

Exogenous T-cell receptors expression of engineered T cells

After transduction GFP fluorescence was monitored in empty vector transductant and the WT and variant TCRs transductants (Fig. 2a). MHC dextramers were then used to detect the expression of exogenous TCRs and evaluate TCRs affinity. Cells expressing the WT TCR had a higher proportion of double MTB/HIV-1 dextramers-positive cells (15%) within the GFP-positive population than other groups ($P < 0.05$). Similar levels of double-dextramers staining were found on E112A and Y115A groups, which exhibited a remarkable increase compared with empty vector transductant respectively ($P < 0.05$). No enhanced double-dextramers staining was observed between the S113A and empty vector transductant ($P > 0.05$), as well as P114A and S116A transductants (Fig. 2B, C). Transcription level of exogenous TCR genes was further examined. The electrophoretic band of a segment of β15-fu-SGSG-P2A-α17 was clearly observed in the WT and variant TCRs groups, while

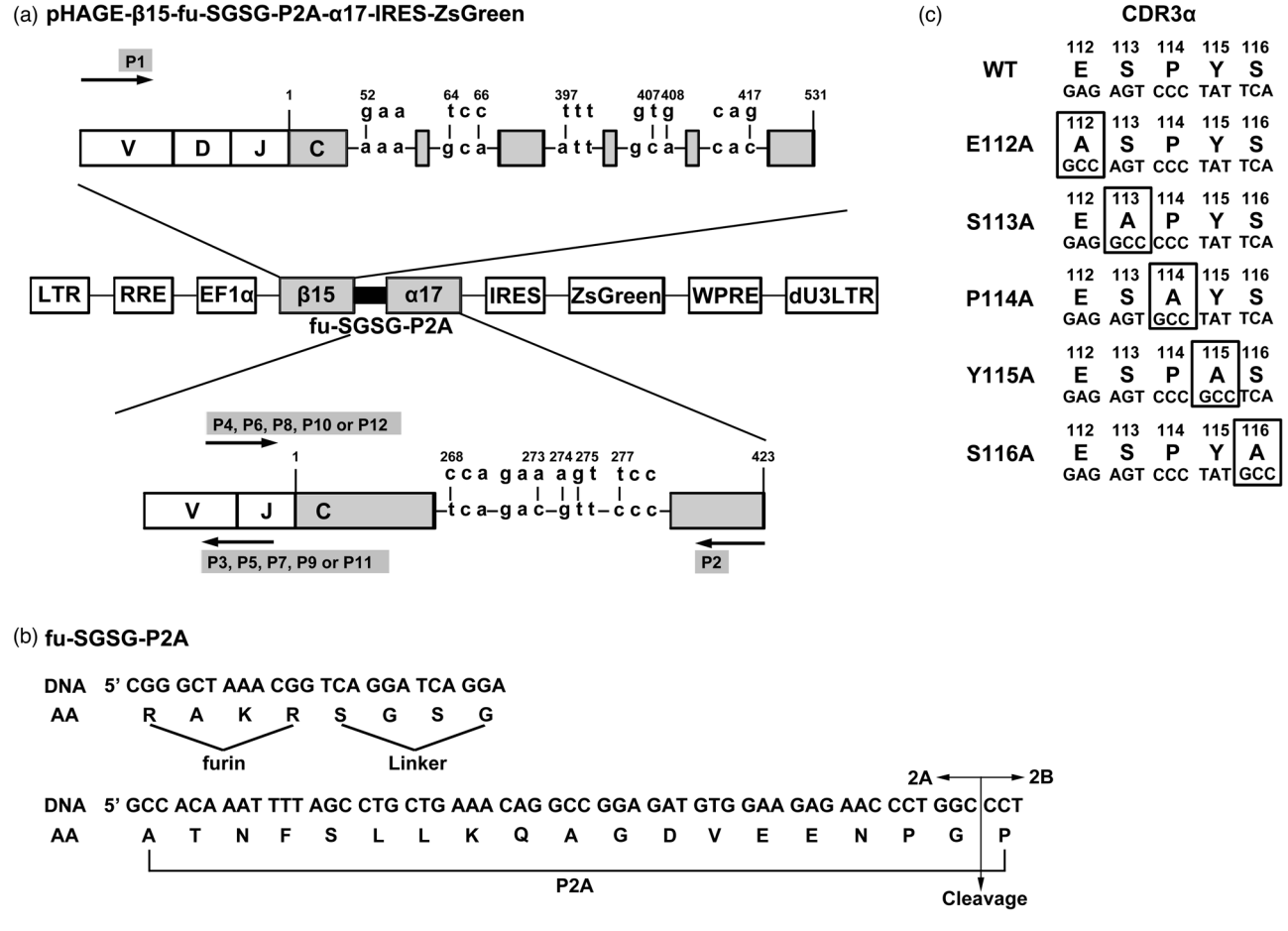

Fig. 1. Generation of alanine substitution of the bispecific TCR CDR3α. (a) Schematic illustration of the pHAGE-fullEF1a-MCS-IZsGreen lentiviral vector encoding the variant bispecific TCRs. TCR β and α chains were linked with furin-SGSG-P2A. Nine critical AAs in the C regions of β15 and α17 were replaced by their murine counterparts. (b) The sequences of the furin-SGSG-P2A. (c) Alanine substitution at the CDR3 region of the bispecific TCR α chain. AA, the amino acid sequence; DNA, the DNA sequence; WT, wild-type.

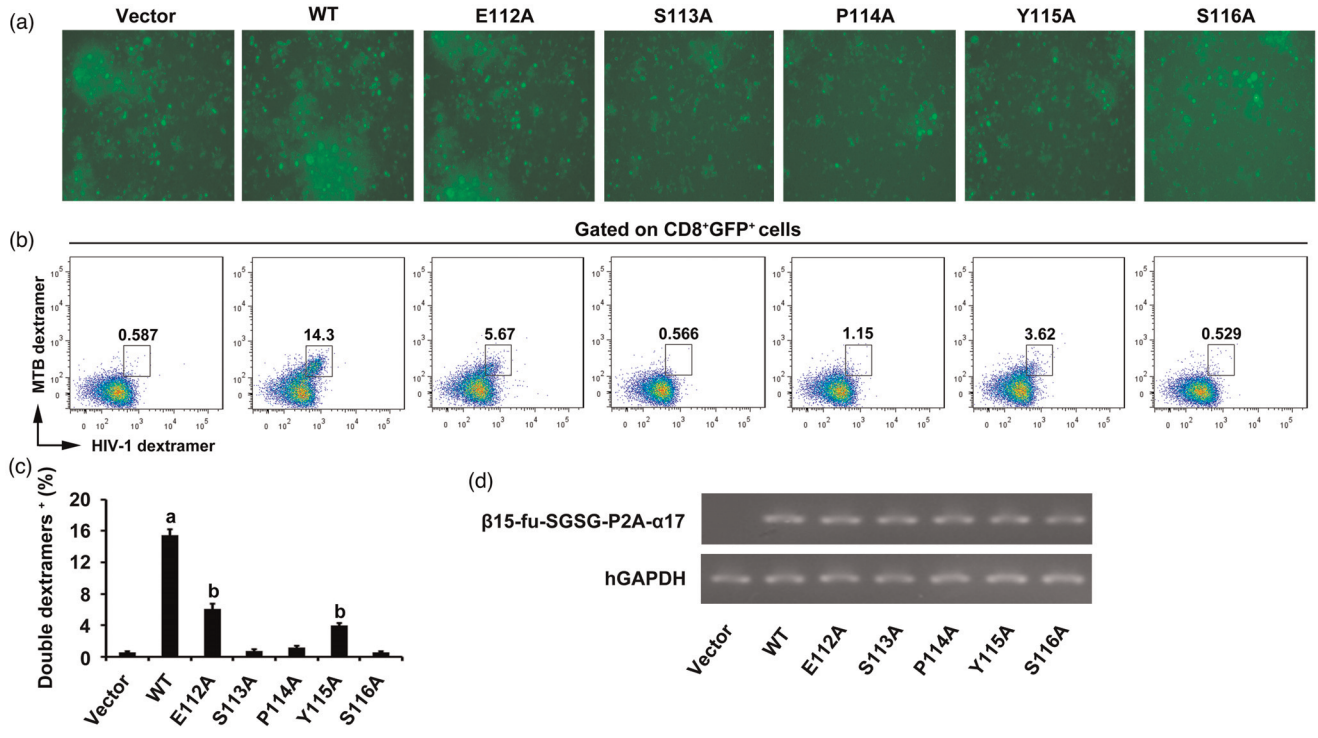

Fig. 2. Variant bispecific TCRs expression of transduced CD8$^+$ T cells. (a) Fluorescence micrograph of CD8$^+$ T-cells engineered with variant bispecific TCRs. (b) Dextramers staining of CD8$^+$ T cells transduced with variant bispecific TCRs. Cells were stained with APC-Cy7-conjugated anti-CD8 monoclonal antibody, PE-conjugated Ag85B$_{199-207}$/HLA-A*0201 dextramer and APC-conjugated Env$_{120-128}$/HLA-A*0201 dextramer. The data were analyzed within the CD8$^+$GFP$^+$ population. (c) Comparison of the frequency of CD8$^+$GFP$^+$double dextramers$^+$ T-cells among the variant bispecific TCRs modified groups. (d) The PCR products of a segment of β15-fu-SGSG-P2A-α17 were analyzed on an agarose gel. $^aP < 0.05$ compared to other six groups. $^bP < 0.05$ compared to Vector. Vector, transduced with empty vector only carrying the GFP gene; MTB dextramer: PE-conjugated Ag85B$_{199-207}$/HLA-A*0201 dextramer; HIV-1 dextramer: APC-conjugated Env$_{120-128}$/HLA-A*0201 dextramer.

it was not discovered in the empty vector transductant (Fig. 2D).

Cytokine release by T cells

To examine the effects of the TCR CDR3α alanine substitution on T-cell recognition, the levels of cytokines were determined. The CD8$^+$ T cells expressing the WT TCR generated higher levels of IFN-γ, TNF-α and GM-CSF than other groups ($P < 0.05$) when stimulated by either MTB Ag85B$_{199-207}$ or HIV-1 Env$_{120-128}$ peptide-loaded T2 cells. Compared with untransduced or empty vector transduced group, the E112A or Y115A TCR gene modified CD8$^+$ cells when cocultured with T2 cells pulsed with MTB or HIV-1 peptide showed an obvious increase in IFN-γ, TNF-α and GM-CSF secretion ($P < 0.05$). In marked contrast, the CD8$^+$ cells that were transduced with the S113A, P114A or S116A TCR constructs failed to produce noticeable levels of cytokines in response to MTB or HIV-1 peptide-exposed T2 cells compared with control groups (untransduced or empty vector transduced group, $P > 0.05$) (Fig. 3a–c).

To further appraise the recognition of the several TCR variants, engineered CD8$^+$ T cells were subjected to cocultivation experiment with different concentration of MTB or HIV-1 peptide-pulsed T2 cells. Variant TCRs engineered T cells specifically released cytokines upon encounter with the MTB or HIV-1 peptide in a dose-dependent manner (Fig. 3d, e). CD8$^+$ T cells transduced with the WT TCR as well as E112A and Y115A TCR identified T2 cells loaded with as little as 0.01 μg/ml MTB or HIV-1 peptide, reflecting that these TCRs were comparatively high-avidity receptors. However, coculti-vation of CD8$^+$ T cells expressing the E112A or Y115A TCR with peptides-pulsed T2 cells secreted lower levels of cytokines compared with the WT group. In sharp contrast, specific release of cytokines was not observed when the S113A, P114A or S116A TCR modified T cells as well as control T cells were cocultured with different concentration of peptides-loaded T2 cells.

Additionally, the specific IFN-γ secretion of engineered CD8$^+$ T cells was measured by intracellular cytokine staining ulteriorly. The percentage of cells within the GFP$^+$ double dextramers$^+$ population that produced IFN-γ in response to MTB or HIV-1 peptide was about 41%-48% in the case of WT TCR expressing cells, whereas a background level of IFN-γ was secreted in the

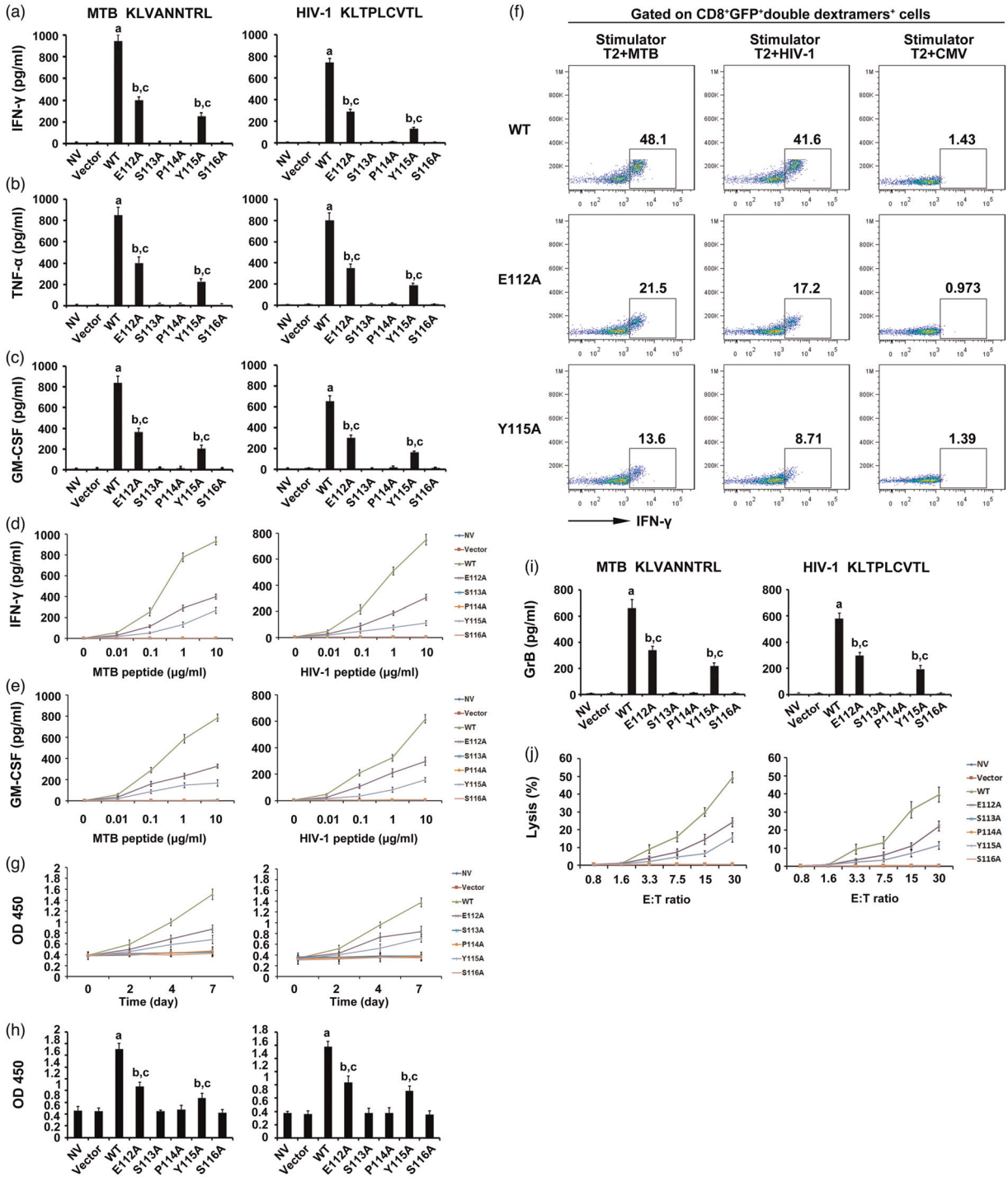

Fig. 3. Immune response of variant bispecific TCRs transduced CD8$^+$ T cells. (a–c) The releases of IFN-γ, TNF-α and GM-CSF in response to T2 cells loaded with 10 μg/ml of MTB Ag85B$_{199–207}$ (KLVANNTRL) (left panel) or HIV-1 Env$_{120–128}$ (KLTPLCVTL) (right panel) were measured by ELISA. (d, e) Peptide titration was measured as the IFN-γ and GM-CSF production after coculture with peptide-loaded T2 cells. (f) Intracellular cytokine staining for IFN-γ of variant bispecific TCRs engineered CD8$^+$ T cells. The WT, E112A or Y115A TCR transduced CD8$^+$ T cells were co-incubation with MTB Ag85B$_{199–207}$, HIV-1 Env$_{120–128}$ or control peptide CMV pp65$_{495–503}$-pulsed T2 cells. Cells were gated for CD8$^+$GFP$^+$double dextramers$^+$ cells and then analyzed for intracellular IFN-γ. (g, h) Proliferation of CD8$^+$ T cells after coculture with peptide-pulsed T2 cells was measured by CCK-8. (i) ELISA was used to detect the GrB level of CD8$^+$ T cells which were co-incubation with 10 μg/ml of MTB Ag85B$_{199–207}$ peptide or HIV-1 Env$_{120–128}$ peptide-pulsed T2. (j) DELFIA assay was used to measure the direct cytotoxicity on T2. NV: no vector. Vector: transduced with empty vector only carrying the GFP gene. MTB peptide: Ag85B$_{199–207}$ peptide; HIV-1 peptide: Env$_{120–128}$ peptide. $^aP < 0.05$ compared to other seven groups. $^bP < 0.05$ compared to NV. $^cP < 0.05$ compared to vector.

control T cells when cocultured with the irrelevant CMV $pp65_{495-503}$ peptide-pulsed T2 cells. In contrast, the E112A or Y115A variant TCR modified T cells when exposed to MTB or HIV-1 peptide-loaded T2 cells showed an apparent decrease in IFN-γ secretion compared with the WT TCR transductant (Fig. 3f).

Antigen-specific proliferation of T cells

We performed a cell proliferation assay to detect the antigen-specific proliferation of the variant TCRs modified T cells (Fig. 3g, h). When cocultured with T2 cells pulsed with MTB or HIV-1 peptide, the WT TCR engineered $CD8^+$ T cells proliferated more extensively as measured by OD450 than other groups at 2, 4 and 7 days post-stimulation, particularly at day 7 ($P < 0.05$). The E112A and Y115A transductants proliferated remarkably greater than untransduced and empty vector transduced cells after exposure to MTB or HIV-1 peptide-loaded T2 cells, especially on day 7 ($P < 0.05$). In stark contrast, the S113A, P114A and S116A transductants as well as control groups exhibited a background level of proliferation at every time ($P > 0.05$).

Lysis of T cells

The GrB release and EuTDA cytotoxicity assays were done to assess the abilities of $CD8^+$ T cells expressing the WT or variant TCRs to mediate specific T2 cells lysis. The WT TCR transductant when cocultured with MTB or HIV-1 peptide-pulsed T2 cells secreted dramatically higher level of GrB than other groups ($P < 0.05$). The E112A and Y115A transductants when exposed to MTB or HIV-1 peptide-loaded T2 cells had higher GrB secretion than control groups ($P < 0.05$), while the S113A, P114A and S116A transductants did not ($P > 0.05$) (Fig. 3i). As expected, the antigen-specific lysis mediated by $CD8^+$ T cells was similar with GrB (Fig. 3j).

Evaluation of the function of engineered $CD8^+$ T cells against endogenous peptides

We evaluated the abilities of variant TCRs engineered $CD8^+$ T cells to recognize naturally processed epitopes from MTB and HIV presented by autologous DCs (Fig. 4a–d). The WT TCR transductant which were incubated with DCs transfected with the Ag85B-expressing plasmid pV1J.ns-tPA-Ag85B or the Env-expressing plasmid pCAGGS-Env exerted appreciably higher levels of IFN-γ and TNF-α secretion and lytic function when compared with other groups ($P < 0.05$). In agreement with expectations, the E112A and Y115A transductants when exposed to the endogenously presented antigens by DCs revealed noticeably enhanced activities compared with control groups ($P < 0.05$), while the S113A, P114A and S116A transductants did not ($P > 0.05$).

T-cell activation in engineered J.RT3-T3.5 cells

Given that J.RT3-T3.5 cells are a TCR negative T-cell line, we used them to further value the properties of variant TCRs. According to expectations, the WT TCR transduced J.RT3-T3.5 cells when cocultured with MTB or HIV-1 peptide-pulsed T2 cells produced significantly higher level of CD69 than other groups. The expression of CD69 of the E112A and Y115A transductants responded to both peptides showed a remarkable increase in contrast to those in the empty vector transduced cells, while the CD69 expression of the S113A, P114A and S116A transductants did not. However, no distinct differences in CD69 expression were found in all groups after exposure to the irrelevant CMV $pp65_{495-503}$ peptide-pulsed T2 cells (Fig. 4e).

Discussion

In this report, alanine substitution variants were generated in the predicted CDR3α of the bispecific TCR which recognizes both the HLA-A*0201-restricted MTB $Ag85B_{199-207}$ peptide and HIV-1 $Env_{120-128}$ peptide, to identify the substituted TCRs with changed levels of antigen specific activity in TCRs engineered T cells. As is known to all, alanine mutagenesis is a widely used method in the determination of the functions of TCR residues since alanine which is the smallest chiral amino acid can eliminate the side chain beyond the β carbon and yet does not alter the main-chain conformation nor does it impose extreme electrostatic or steric effects [18]. It is worth noting that MTB and HIV-1 dextramers staining showed that the variant TCRs presenting reduced antigen-specific reactivity still retained the ligand recognition (Fig. 2b), which means that the overall conformations of the variant TCRs were not altered. Of course, it cannot be ruled out that alanine mutation can also change protein conformation as described in the molecular Bax mutants study [19]. In reality, each mutant bispecific TCR generated was expressed efficiently on the surface of J.RT3-T3.5 cells, which fail to express CD3 molecular and TCR α/β heterodimer. As the J.RT3-T3.5 cells can express CD69 when cocultured with the MTB or HIV-1 peptide-pulsed T2 cells (Fig. 4e), the mutant TCR/CD3 complexes which were expressed by these cells were functional. It suggests that alanine mutation of CDR3α in the bispecific TCR, which does not alter either association with the normal TCR β chain or signal transduction of the TCR/CD3 complex, affects contact with antigen, rather than disrupting the conformation of the bispecific TCR.

Our results prove that the bispecific TCR residues in the predicted CDR3α loop are extremely important in the recognition of ligand. Transduction of $CD8^+$ T cells with the bispecific TCR CDR3α substitution constructs of alanine for serine at position 113 and 116, as well as proline at position 114 resulted in nearly disappeared recognition of peptides-loaded T2 cells respectively. Substitution of alanine for glutamic acid at residue 112 or tyrosine at residue 115 led to seriously decreased but not

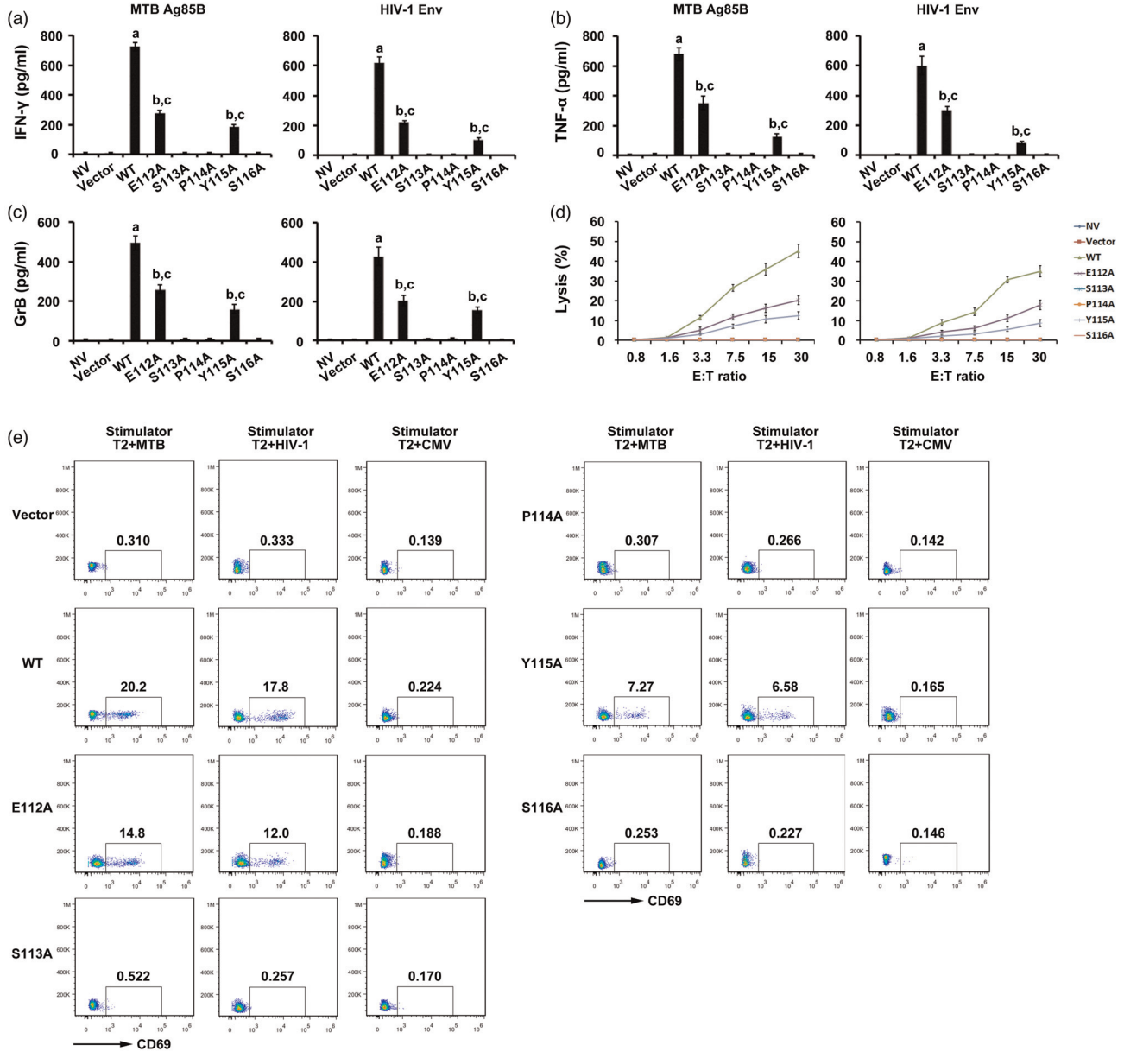

Fig. 4. Effects of variant bispecific TCRs on T cells activity in response to endogenous antigens stimulation and measurement of CD69 surface expression of engineered J.RT3-T3.5 cells. Dendritic cells were transfected with the pV1J.ns-tPA-Ag85B plasmid or the pCAGGS-Env plasmid and then were co-incubation with $CD8^+$ T cells. The IFN-γ (a), TNF-α (b) and GrB (c) levels of T cells were analyzed by ELISA. (d) DELFIA assay was used to detect the lytic function of T cells. (e) The J.RT3-T3.5 cells were co-incubation with $Ag85B_{199-207}$, $Env_{120-128}$, or control peptide CMV $pp65_{495-503}$-pulsed T2 and were analyzed within the GFP-positive population. NV, no vector. Vector: transduced with empty vector only carrying the GFP gene. MTB Ag85B: the Ag85B-expressing plasmid pV1J.ns-tPA-Ag85B; HIV-1 Env: the Env-expressing plasmid pCAGGS-Env. CMV, $pp65_{495-503}$ peptide; HIV-1, $Env_{120-128}$ peptide; MTB, $Ag85B_{199-207}$ peptide. [a]$P < 0.05$ compared to other seven groups. [b]$P < 0.05$ compared to NV. [c]$P < 0.05$ compared to Vector.

abolished reactivity in transduced T cells. With strength from above, the serine and proline residues in the CDR3 loop of the bispecific TCR α-chain seem to be especially vital for ligand recognition. When substitution of alanine for serine, a single side chain hydroxyl group was removed, resulting in the abolished antigen activity, which demonstrated that just a small structural change can have a major impact on antigen recognition. Proline is a special amino acid as it has a secondary amino group and contains a pyrrole ring, which causes proline in a protein to impart a rigid protein structure. For that very reason, proline has been shown to play a crucial part in determining the structure of proteins [20] and could affect T-cell recognition in the 1G4 TCR [21]. Glutamic

acid at position 112 which has a negative charge could stick to a positively charged amino acid or the positively charged antigen in peptide-MHC complex to form a salt bridge which has a serious effect on high specificity and affinity of protein antigen-antibody interactions [22]. Substitution of alanine for glutamic acid is likely to thus disrupt the formation of salt bridges. The aromatic amino acid Tyr^{115} was speculated to have a great influence on recognition of MTB and HIV-1 peptide antigens by the bispecific TCR. A study has reported that the interactions of aromatic groups in amino acids, including non-bonded stacking of the aromatic groups of amino acid pairs and interactions between carbonyl oxygens and aromatic side chains, have been demonstrated to impart plenty of stability to the protein structure [23]. In addition, aromatic interactions have been shown to play an important role in antigen recognition due to the high degree of solvent accessibility of aromatic residues at antibody binding sites [24].

Although all of our results including our previous study have obviously indicated the significance of multiple residues in the predicted CDR3α and CDR3β regions of the bispecific TCR in identifying antigen/MHC complexes, we cannot currently rule out the possibility that other regions of the TCR are involved in ligand recognition. For instance, the role of residues within the predicted antigen binding loops αl, α2, βl, and β2 in antigen recognition has also been extensively studied. Kasibhatla S's work has clearly showed that residues in each of these loops of the class II MHC molecular I-A^d-restricted D5 TCR can participate in antigen recognition simultaneously [11]. Chimeric TCR with substitutions in N-terminal halves of V segments spanning loops α1 and α2 [25], or TCR with substitutions in individual residues in loop β1 [26] have also demonstrated that these loops play a crucial part in antigenic peptide recognition. Another well documented example is that amino acid substitutions in the CDR2β region of the 1G4, DMF4 and DMF5 TCRs respectively dramatically enhanced $CD4^+$ T-cell antigen-specific reactivity [21]. Alanine substitutions of CDR residues in the 2C TCR indicated that CDR1 and CDR2 regions of the α and β chains were the major contributors to the binding energy of the 2C TCR for the cognate QL9/L^d antigen [27]. A murine class II-restricted TCR has also been extensively studied to determine the role of individual CDR1 residues on TCR affinity and functional activity using amino acid substitutions, resulting in a >100-fold increase in the affinity of the TCR for the cognate peptide/MHC complex that did not seem to change the specificity of the cognate antigen of T cells transfected with these variant TCRs [28].

In conclusion, this study demonstrates that the Ser^{113}, Pro^{114} and Ser^{116} in CDR3α of the bispecific TCR are especially important for antigen recognition. These results will pave the way for the future development of an improved high-affinity bispecific TCR for use in adoptive cellular immunotherapy for MTB/HIV coinfected patients.

Acknowledgements

We thank Dr Kris Huygen (Pasteur Institute of Brussels, Brussels, Belgium) for providing the pV1J.ns-tPA-Ag85B plasmid. We also thank Dr James M. Binley (Torrey Pines Institute for Molecular Studies, San Diego, California, USA) for providing the pCAGGS-Env plasmid.

Funding: This work was supported by the National Natural Science Foundation of China (grant numbers 82072242, 82272344), Basic and Applied Basic Research Foundation of Guangdong Province (grant numbers 2021A1515010933, 2022A1515012109), Science and Technology Program of Guangzhou (grant number 202201010837, 202201010984), and Natural Science Foundation of Guangdong Province (grant number 2018030310486).

Author contribution: C.Y.Z. conceived of the study, participated in the cell experiments, substantially contributed to molecular biology studies and immunoassays, performed the statistical analysis and drafted the manuscript. R.N.W. and W.T.H. participated in cell and virus infection experiments. D.R.L. and S.R.Y. participated in molecular biology studies. Q.W., S.F.H. and X.Y. Z. participated in research design. L.M. conceived of the study, and participated in its design and coordination and revised the manuscript critically. All authors read and approved the final manuscript.

Conflicts of interest

There are no conflicts of interest.

References

1. Qiu B, Wu Z, Tao B, Li Z, Song H, Tian D, *et al*. **Risk factors for types of recurrent tuberculosis (reactivation versus reinfection): a global systematic review and meta-analysis.** *Int J Infect Dis* 2022; **116**:14–20.
2. WHO. *Global tuberculosis report 2021*. Geneva, Switzerland: WHO; 2021.
3. Sharan R, Bucsan AN, Ganatra S, Paiardini M, Mohan M, Mehra S, *et al*. **Chronic immune activation in TB/HIV co-infection.** *Trends Microbiol* 2020; **28**:619–632.
4. Bell LCK, Noursadeghi M. **Pathogenesis of HIV-1 and *Mycobacterium tuberculosis* co-infection.** *Nat Rev Microbiol* 2018; **16**:80–90.
5. Carpenter SM, Nunes-Alves C, Booty MG, Way SS, Behar SM. **A higher activation threshold of memory CD8+ T cells has a fitness cost that is modified by TCR affinity during tuberculosis.** *PLoS Pathog* 2016; **12**:e1005380.
6. Yang H, Buisson S, Bossi G, Wallace Z, Hancock G, So C, *et al*. **Elimination of latently HIV-infected cells from antiretroviral therapy-suppressed subjects by engineered immune-mobilizing T-cell receptors.** *Mol Ther* 2016; **24**:1913–1925.
7. Spear TT, Callender GG, Roszkowski JJ, Moxley KM, Simms PE, Foley KC, *et al*. **TCR gene-modified T cells can efficiently treat established hepatitis C-associated hepatocellular carcinoma tumors.** *Cancer Immunol Immunother* 2016; **65**:293–304.

8. Birnbaum ME, Mendoza JL, Sethi DK, Dong S, Glanville J, Dobbins J, *et al*. **Deconstructing the peptide-MHC specificity of T cell recognition.** *Cell* 2014; **157**:1073–1087.
9. Mariuzza RA, Agnihotri P, Orban J. **The structural basis of T-cell receptor (TCR) activation: an enduring enigma.** *J Biol Chem* 2020; **295**:914–925.
10. Yang J, He J, Lu H, Wei L, Li S, Wang B, *et al*. **Molecular features of the complementarity determining region 3 motif of the T cell population and subsets in the blood of patients with chronic severe hepatitis B.** *J Transl Med* 2011; **9**:210.
11. Kasibhatla S, Nalefski EA, Rao A. **Simultaneous involvement of all six predicted antigen binding loops of the T cell receptor in recognition of the MHC/antigenic peptide complex.** *J Immunol* 1993; **151**:3140–3151.
12. Nalefski EA, Kasibhatla S, Rao A. **Functional analysis of the antigen binding site on the T cell receptor alpha chain.** *J Exp Med* 1992; **175**:1553–1563.
13. Manning TC, Schlueter CJ, Brodnicki TC, Parke EA, Speir JA, Garcia KC, *et al*. **Alanine scanning mutagenesis of an alphabeta T cell receptor: mapping the energy of antigen recognition.** *Immunity* 1998; **8**:413–425.
14. Zhou CY, Wen Q, Chen XJ, Wang RN, He WT, Zhang SM, *et al*. **Human CD8(+) T cells transduced with an additional receptor bispecific for both *Mycobacterium tuberculosis* and HIV-1 recognize both epitopes.** *J Cell Mol Med* 2016; **20**:1984–1998.
15. Zhou CY, Wang RN, Wen Q, He WT, Zhang SM, Du XL, *et al*. **Alanine mutagenesis in the complementarity determining region 3 of the MTB and HIV-1 peptide-bispecific T cell receptor beta chain affects ligand recognition.** *Front Immunol* 2017; **8**:983.
16. Kim JH, Lee SR, Li LH, Park HJ, Park JH, Lee KY, *et al*. **High cleavage efficiency of a 2A peptide derived from porcine teschovirus-1 in human cell lines, zebrafish and mice.** *PLoS One* 2011; **6**:e18556.
17. Yang S, Cohen CJ, Peng PD, Zhao Y, Cassard L, Yu Z, *et al*. **Development of optimal bicistronic lentiviral vectors facilitates high-level TCR gene expression and robust tumor cell recognition.** *Gene Ther* 2008; **15**:1411–1423.
18. Lefevre F, Remy MH, Masson JM. **Alanine-stretch scanning mutagenesis: a simple and efficient method to probe protein structure and function.** *Nucleic Acids Res* 1997; **25**:447–448.
19. Wang Q, Sun SY, Khuri F, Curran WJ, Deng X. **Mono- or double-site phosphorylation distinctly regulates the proapoptotic function of Bax.** *PLoS One* 2010; **5**:e13393.
20. Vakilian M. **A review on the effect of prolyl isomerization on immune response aberration and hypersensitivity reactions: a unifying hypothesis.** *Clin Immunol* 2021; **234**:108896.
21. Robbins PF, Li YF, El-Gamil M, Zhao Y, Wargo JA, Zheng Z, *et al*. **Single and dual amino acid substitutions in TCR CDRs can enhance antigen-specific T cell functions.** *J Immunol* 2008; **180**:6116–6131.
22. Gully BS, Venugopal H, Fulcher AJ, Fu Z, Li J, Deuss FA, *et al*. **The cryo-EM structure of the endocytic receptor DEC-205.** *J Biol Chem* 2021; **296**:100127.
23. Bissantz C, Kuhn B, Stahl M. **A medicinal chemist's guide to molecular interactions.** *J Med Chem* 2010; **53**:5061–5084.
24. Padlan EA. **On the nature of antibody combining sites: unusual structural features that may confer on these sites an enhanced capacity for binding ligands.** *Proteins* 1990; **7**:112–124.
25. Hong SC, Chelouche A, Lin RH, Shaywitz D, Braunstein NS, Glimcher L, *et al*. **An MHC interaction site maps to the amino-terminal half of the T cell receptor alpha chain variable domain.** *Cell* 1992; **69**:999–1009.
26. White J, Pullen A, Choi K, Marrack P, Kappler JW. **Antigen recognition properties of mutant V beta 3+ T cell receptors are consistent with an immunoglobulin-like structure for the receptor.** *J Exp Med* 1993; **177**:119–125.
27. Manning TC, Parke EA, Teyton L, Kranz DM. **Effects of complementarity determining region mutations on the affinity of an alpha/beta T cell receptor: measuring the energy associated with CD4/CD8 repertoire skewing.** *J Exp Med* 1999; **189**:461–470.
28. Weber KS, Donermeyer DL, Allen PM, Kranz DM. **Class II-restricted T cell receptor engineered in vitro for higher affinity retains peptide specificity and function.** *Proc Natl Acad Sci USA* 2005; **102**:19033–19038.