T Cell Receptor Fused to CD3ζ: Transmembrane Domain of CD3ζ Prevents TCR Mis-Pairing, Whereas Complete CD3ζ Directs Functional TCR Expression

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Abstract: TCR gene therapy represents a feasible and promising treatment for patients with cancer and virus infections. Currently, this treatment rationale is hampered by diluted surface expression of the TCR transgene and generation of potentially self reactive T-cells, both a direct consequence of mis-pairing with endogenous TCR chains. As we reported previously (Gene Ther 16:1369, 2000; J Immunol 180:7736, 2008), TCR mis-pairing can be successfully addressed by a TCR:CD3ζ fusion protein (i.e., TCR:ζ). Here, we set out to minimize the content of CD3ζ in TCR:ζ, specific for MAGE-A1/HLA-A1, without compromising TCR pairing and function. Domain-exchange and 3D-modeling strategies defined a set of minimal TCR:ζ variants, which, together with a murinized and cysteine-modified TCR (TCR:mu+cys), were tested for functional TCR expression and TCR pairing. Our data with Jurkat T cells show that the CD3ζ transmembrane domain is important for cell-surface expression, whereas the CD3ζ intracellular domain is crucial for T-cell activation. Notably, inability of TCR:ζ to mis-pair was not observed for TCR:mu+cys, which depended exclusively on the transmembrane domain of CD3ζ and could not be recapitulated by a limited number of structurally defined CD3ζ transmembrane amino acids. The extracellular CD3ζ domain was dispensable for TCR:ζ's ability to prevent TCR mis-pairing, bind pMHC and mediate NFAT activation. In primary human T cells, however, minimal TCR:ζ without CD3ζ's extracellular domain but not TCR:ζ nor TCR:mu+cys revealed compromised cell surface expression and T cell function. Taken together, our study demonstrates that CD3ζ's transmembrane domain dictates TCR:ζ's inability to TCR mis-pair, but only TCR coupled to complete CD3ζ and not its minimal variants were functionally expressed in primary T cells.

Keywords: 3D-modeling, CD3ζ, domain exchange, gene therapy, melanoma, minimal TCR:ζ, TCR mis-pairing, T cell receptor.

INTRODUCTION

T cell receptor (TCR) gene therapy is based on retroviral transduction of T cells with tumor or virus-specific TCRαβ transgenes. Clinical TCR gene therapy trials proved feasible and applicable toward multiple tumor types, such as metastatic melanoma, colorectal carcinoma and synovial sarcoma [1-4]. This first series of studies with TCR-engineered T cells, although showing variable clinical responses in limited numbers of patients, demonstrates that responses generally lag behind those observed with non gene-engineered T cells [5-9]. In addition, in case of TCRs directed against MART-1, gp100 or CEA, but not the cancer testis antigen NY-ESO-1, treatment resulted in on-target toxicity, i.e., severe inflammation of healthy tissue expressing the target antigen [1, 2, 4].

The efficacy and safety of clinical TCR gene transfer may be further enhanced by strategies that address TCR mis-pairing. TCR mis-pairing is a recognized phenomenon of TCR gene transfer, and defined by the incorrect pairing of TCRα or TCRβ transgenes with endogenous TCRβ or TCRα chains, respectively. TCR mis-pairing leads to the formation of unknown TCR specificities, which dilute the surface-expression of the therapeutic TCRαβ heterodimer and can potentially result in off-target toxicity. Although, there is no clinical evidence for TCR mis-pairing-induced autoreactivity, preclinical data derived from a mouse model demonstrate that TCR mis-pairing can lead to graft-versus-host disease [10]. Strategies that prevent TCR mis-pairing are therefore expected to improve T cell avidity by increasing the level of cell surface expression of therapeutic TCRαβ heterodimer and at the same time reduce potential off-target toxicity, reviewed in Govers *et al*. [11]. These strategies include the murinization of TCRα and –β constant domains [12], introduction of additional cysteine residues in TCRα and –β to form an extra disulfide bridge [13], the exchange of structurally important amino acids between TCRα and –β [14], or the replacement of parts of TCRα and –β constant domains by complete human CD3ζ (TCR:ζ) [15].

In previous studies, TCR:ζ has been extensively characterized regarding its ability to address TCR mis-pairing and

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functionally retarget T cells toward several tumor and virus antigens, such as MAGE-1/HLA-A1, gp100/HLA-A2, BMFL-1/HLA-A2, JC/HLA-A2 and EBNA-4/HLA-A11 [15-20]. Notably, TCR:ζ's surface expression, which is enhanced when compared to wt TCR, and its ability to form immune synapses are independent of endogenous CD3 proteins. This receptor, possibly as a consequence of conformational changes, results in enlarged synapse sizes in TCR-transduced T cells [18, 20]. Despite these unique properties, TCR:ζ and wt TCR do not differ with respect to the molecular 'make-up' of immune synapses and their ability to mediate antigen-specific T cell functions.

In the current study, we set out to minimize the content of CD3ζ present in TCR:ζ, specific for MAGE-A1/HLA-A1, without compromising the pairing and functional properties of TCR:ζ, and with the intent to potentially decrease the immunogenicity of this receptor. In analogy to a study by Sommermeyer and colleagues, who defined a limited number of amino acids that preserved the benefits of murinized TCRs [21], the present effort would define those domains or amino acids of CD3ζ responsible for improved TCR pairing and function. To generate minimal TCR:ζ variants we applied a domain-exchange as well as a 3-dimensional (3D) modeling strategy, and tested variants for TCR pairing and functional expression. We observed that the CD3ζ transmembrane domain, and not a limited number of structurally defined amino acids, is critical for TCR:ζ's surface expression and its inability to mis-pair with endogenous TCR chains, whereas the intracellular CD3ζ domain is critical for T cell activation. A minimal TCR:ζ variant that lacked the extracellular CD3ζ domain was best at preserving both TCR pairing and function in Jurkat T cells, but was not functionally expressed in primary T cells.

MATERIALS AND METHODS

Cells and Reagents

Jurkat T cell clone 19 [18], which expresses MelA/HLA-A2 (MelA) TCR, and EBV-transformed B cell blasts (APD) was cultured in RPMI 1640 medium (BioWhittaker, Verviers, Belgium) containing 10% Fetal Bovine Serum (FBS, Stonehouse, Gloucestershire, UK), streptomycin (100 μg/ml) and penicillin (100 U/ml) (Jurkat T cell medium). The human embryonic kidney cell line 293T, the packaging cell line Phoenix-A, and melanoma cell line Mel2A were cultured in DMEM medium (BioWhittaker) supplemented with 10% FBS, 2 mM L-glutamine, non-essential amino acids and antibiotics. T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere [22] and cultured in HEPES buffered RPMI 1640 medium supplemented with 10% Human Serum, 2 mM L-glutamine, streptomycin and penicillin. Monoclonal Abs included: FITC- and non-conjugated anti-TCR-Vα19 mAb (Pierce Biotechnology, Inc., Rockford, IL); PE- and non-conjugated anti-TCR Vβ27 mAb (BD Biosciences, San Jose, USA); PE- and non-conjugated anti-TCR-Vβ9 mAb (BD Biosciences and Coulter-Immunotech, Marseille, France, respectively); PE-conjugated anti-CD107a mAb (BD biosciences); APC- and non-conjugated anti-CD3ε mAb (OKT3) (BD Biosciences and Coulter-Immunotech, respectively); Cy5- and non-conjugated Rabbit-anti-Mouse (RαM) IgG Fab (Jackson

ImmunoResearch, Suffolk, UK). Peptide/MHC (pMHC) monomers included: MAGE-1 (M1: EADPTGHSY)/HLA-A*0101; and Melan-A (MelA: ELAGIGILTV)/HLA-A*0201 biotinylated peptide/MHC monomers (Sanquin, Amsterdam, Netherlands), and these pMHC monomers were tetramerized as described previously [23]. Other reagents included: Retronectin (human fibronectin fragments CH-296, Takara Shuzo Co. Ltd., Otsu, Japan); PMA (Sigma-Aldrich, St. Louis, USA); PHA (Remel Europe, Dartford, England); golgistop (BD biosciences) and streptavidin-PE (BD biosciences).

Cloning of Minimal TCR:ζ Variants

We have generated a panel of 11 TCRs, as schematically represented in (Fig. **1**), that are specific for MAGE-A1/HLA-A1 (M1/A1) and have the TCR-V gene usage TRAV19/J39/C and TRBV9/D2/J2-3/C2 (with TCR-V(D)J gene nomenclature according to http://imgt.cines.fr), originally derived from CTL clone MZ2-82/30 as described previously [15]**.** Control TCRs (n=3) included wt TCR [18], TCR:ζ [15, 18] and a murinized plus cysteine-modified TCR (i.e., TCR:mu+cys), the latter designed according to Cohen and colleagues [24]. TCR:mu+cys was generated *via* overlap PCR to fuse together M1/A1 TCR-V and murine TCR-C domains (Vα-mCα and Vβ-mCβ2), which were ligated in pBullet vectors [15] *via SalI-XhoI* (TCRα) and *NcoI-XhoI* (TCRβ). Cysteine mutations (TCRα T189, and TCRα S191) were generated using QuickChange Site-Directed Mutagenesis Kit (Fynnzymes, Espoo, Finland) according to the manufacturer's instructions. The minimal TCR:ζ variants (n=8) were generated *via* either one of the two approaches. First, six variants were made using a domain-exchange strategy in which extracellular (ec), transmembrane (tm) or intracellular (ic) domains of CD3ζ, and combinations of these domains, were exchanged for corresponding TCR domains. These six variants were named as follows: TCR:ζ Δec; TCR:ζ Δtm; TCR:ζ Δic; TCR:ζ Δec+ic; TCR:ζ Δtm+ic; and TCR:ζ Δec+tm (Δ indicates lack of CD3ζ domain(s), and replacement by corresponding TCR domain(s)). The exact boundaries of ec, tm and ic domains of CD3ζ, TCRα and TCRβ are provided in the legend to (Fig. **1A**). These variants were generally constructed by overlap PCR and gene synthesis, as described in detail in *Supplementary Methods*. Second, two additional TCR:ζ variants were made using a 3D-modeling strategy in which a limited number of amino acids of TCR tm were exchanged for CD3ζ tm amino acids at structurally favorable positions. These two variants were named as follows: TCR tmζ1 and 2, with the exact tm sequences provided by (Fig. **1B**). The modeling software to design these variants, and their construction, generally by gene synthesis, is described in detail in *Supplementary Methods*. All TCR constructs made (n=11) were sequence verified (Service XS, Leiden, Netherlands).

Retroviral TCR Gene Transfer into T Cells

TCR cDNAs were used to transduce Jurkat T cell clone 19 as well as human PBMC. To this end, Moloney Murine Leukemia retroviruses were produced by a co-culture of the packaging cells 293T and Phoenix-A following calcium-phosphate transfections [16, 18]. Packaging cells were trans-
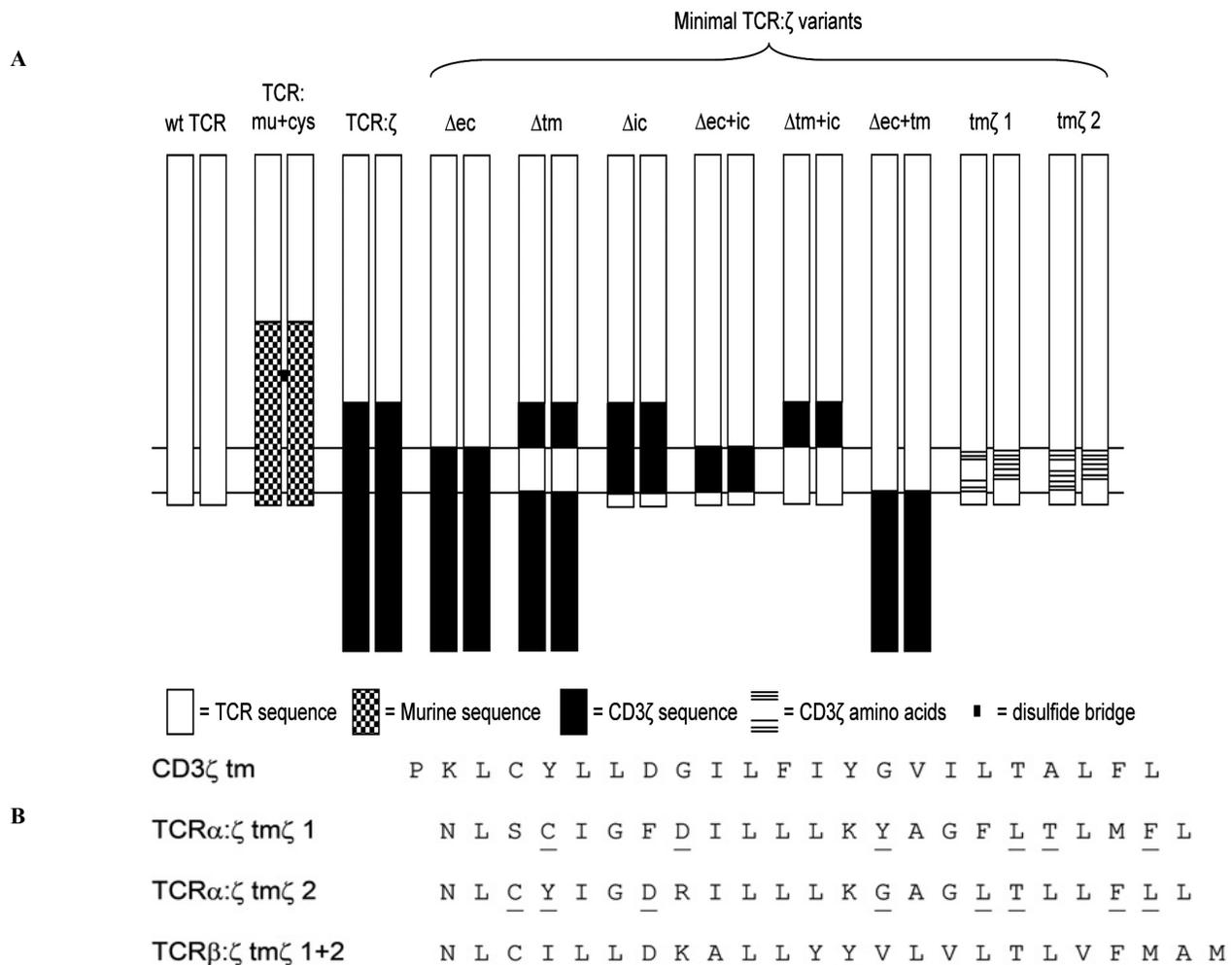
Fig. (1). Scheme of minimal TCR:ζ variants. (**A**) Minimal TCR:ζ constructs were modified *via* replacement of either extracellular (Δec), transmembrane (Δtm), intracellular (Δic), extra and intracellular (Δec+ic), transmembrane and intracellular (Δtm+ic) or extracellular and transmembrane CD3ζ domains (Δec+tm) (black) by corresponding TCRα and TCRβ domains (white). Boundaries for the CD3ζ/TCRαβ ec, tm and ic domains were defined as follows. CD3ζ (genbank accession number: CAI21380.1) ec, tm and ic: nt 73-90 (aa 25-30); nt 91-153 (aa 31-51); and nt 154-489 (aa 52-163). TCRα [48] ec, tm and ic: nt 703-765 (aa 235-255); nt 766-825 (aa 256-275); and nt 826-840 (aa 276-280). TCRβ [48] ec, tm and ic: nt 790-846 (aa 264-282); nt 847-912 (aa 283-304); and nt 913-933 (aa 305-311). In addition, two minimal TCR:ζ constructs were designed in which defined amino acids of tm CD3ζ were transplanted onto structurally favourable positions in TCRα and TCRβ tm domains, and were termed minimal TCR:tmζ1 and 2 (See Fig. (**1B**)). Control TCRs include: wt TCR, TCR:ζ and a TCR containing murine constant domains and additional cysteines (TCR:mu+cys, as described in [24]). (**B**) Transmembrane amino acids of CD3ζ and minimal TCR:ζ variants TCRα:tmζ1, TCRα:tmζ2, and TCRβ:tmζ (CD3ζ amino acids underlined).

fected with TCR cDNAs, pHIT60 MLV GAG/POL, and VSV-G envelope plasmids for Jurkat T cell transductions, or with TCR cDNAs, pHIT60 MLV GAG/POL, and pCOLT-GALV-envelope encoding plasmids for human PBMC transductions. The transduction procedure used was optimized for human T cells and described previously [25].

Flow Cytometry and FACsort

TCR-transduced T cells (1×10^4 cells) were monitored by flow cytometry for surface expression of transgenic TCR using FITC-conjugated anti-TCR-Vα19, PE-conjugated anti-TCR-Vβ9 mAbs and/or R-PE-conjugated M1/A1 tetramer; endogenous TCR (in case of Jurkat T cells) using PE-conjugated anti-TCR-Vβ27 mAb and/or R-PE-conjugated MelanA/A2 tetramer; and endogenous CD3 using APC-conjugated anti-CD3ε mAb. After T cells were washed, they were incubated with mAbs for 30 min on ice, or 15 min at RT for pMHC tetramers. Next, T cells were washed and fixed with 1% PFA (Brunschwig, Amsterdam, the Netherlands) before measurements on a FACSCalibur dual-laser flow cytometer (Beckton Dickinson, Alphen a/d Rijn, the Netherlands). Samples were analyzed using BD Cellquest software and displayed as dotplots or histograms. Enrichment of M1/A1 TCR-expressing Jurkat Clone 19 T cells was performed by two-color Fluorescent Activated Cell Sorting (FACS) following staining with FITC conjugated anti-TCR-Vα19 and PE conjugated anti-TCR-Vβ9 mAbs.

Flow Cytometry-Based FRET

PE-labeled mAbs were used as donor and Cy5-labeled RαM antibody as acceptor and used in the following donor/acceptor combinations (as described in [18]): anti-TCR-

Vb27^{PE} + anti-TCR-Vα19/ GαM^{Cy5} and anti-TCR-Vb9^{PE} + anti-CD3ε (OKT3)/GαM^{Cy5}. Staining was performed sequentially, with extensive washing steps in between, using the following order of staining steps: first non-conjugated mAbs, second Cy5-labeled Abs, and last PE-labeled donor Abs. Fluorescence intensities of emissions at 570 nm (donor channel, excitation at 488nm), 670 nm (acceptor channel, excitation at 635), and over 670 (FRET channel, excitation at 488 nm) were measured and collected on a FACSCalibur. Data were analyzed with the FLEX software on a per-cell basis [26].

NFAT Reporter Gene Assay

Gaussia-Luciferase reporter gene under the control of 6 NFAT response elements, a minimal IL-2 promotor, and a TATA box (in short: GLuc-NFAT(RE)6) was used to quantify TCR-mediated stimulation. Gluc-NFAT(RE)6 was generated by digesting a plasmid containing NFAT-6-luc [27] with *Nco*I-*Hin*DIII and ligating this fragment (containing the 6 NFAT response elements, minimal IL-2 promotor and TATA box), together with a *Eco*RI-*Nco*I linker, in *Eco*RI-*Hin*DIII digested pGluc-basic vector (New England Biolabs, Ipswich, USA). 5×10^6 Jurkat TCR-transduced T cells were transiently nucleofected with an Amaxa nucleofector (AmaxaBiosystems, Cologne, Germany) according to previous optimizations [28, 29]. Briefly, T cells were resuspended in 100 μl supplemented buffer V to which 5 μg GLuc-NFAT(RE)6 was added and pulsed with the Nucleofector set at program C-16. Next, T cells were immediately transferred to 2.5 ml warm Jurkat T-cell medium in T25 flasks for O/N recovery at 37°C and 5% CO_2. Non-tissue culture-treated 96-well plates were coated with non-conjugated mIg Ab, anti-TCR-Vβ9 mAb (100 ng) or biotinylated M1/A1 or JC/A2 monomers (titrated from 126 nM down to 4 nM). The latter added to streptavidin-coated plates O/N at 4°C. Twenty hours post-transfection, 0.2×10^6 T cells at a concentration of 1×10^6/ml were transferred to each well in the 96-well plates and stimulated for 6 hours at 37°C and 5% CO_2. Subsequently, 25 μl supernatant was transferred to 96 wellplate read-out plates (Corning incorporated, Costar assay plate, Lowell, USA) and placed in a luminometer. Next, 50 μl assay buffer was added (Gluc substrate; New England Biolabs, Ipswich, USA). Light units indicative of the Gaussia Luciferase-mediated enzymatic transition of coelenterazine into coelenteramide were measured according to the manufacturer's instructions (Mediators, Vienna, Austria) and expressed (in RLU) relative to wt TCR luciferase activities (100% for each concentration)

CD107a Mobilization Assay

TCR-transduced human PBMC (1×10^6) were resuspended in 0.15 ml T cell medium, 0.5 μl Golgistop (BD biosciences), and 100 μl anti-CD107a-PE (BD biosciences). Mel2A and APD target cells (1×10^6) were resuspended in 0.25 ml T cell medium (± 10 μM M1 peptide). Next, 50 μl target cells and 50 μl T cells were mixed in a tissue culture treated 96-well plates (Greiner bio-one) and incubated for 2 h at 37°C and 5% CO_2. After the cells were washed, they were stained with anti-Vα19-FITC and anti-CD3ε-APC for 30 minutes at 4°C in the dark. Next, cells were washed and fixed with 1% PFA and measured on a FACS Calibur dual-laser flow cytometer. T cells gated for viability (FSC and SSC) and positive for CD3ε and TCR-Vα19, were assessed for surface expression of CD107a.

Statistical Analyses

Differences among TCRs in various assays were tested with student's t-tests (unpaired; two-tailed) using Graphpad Prism 4 software. Differences with p values <0.05 were considered significant.
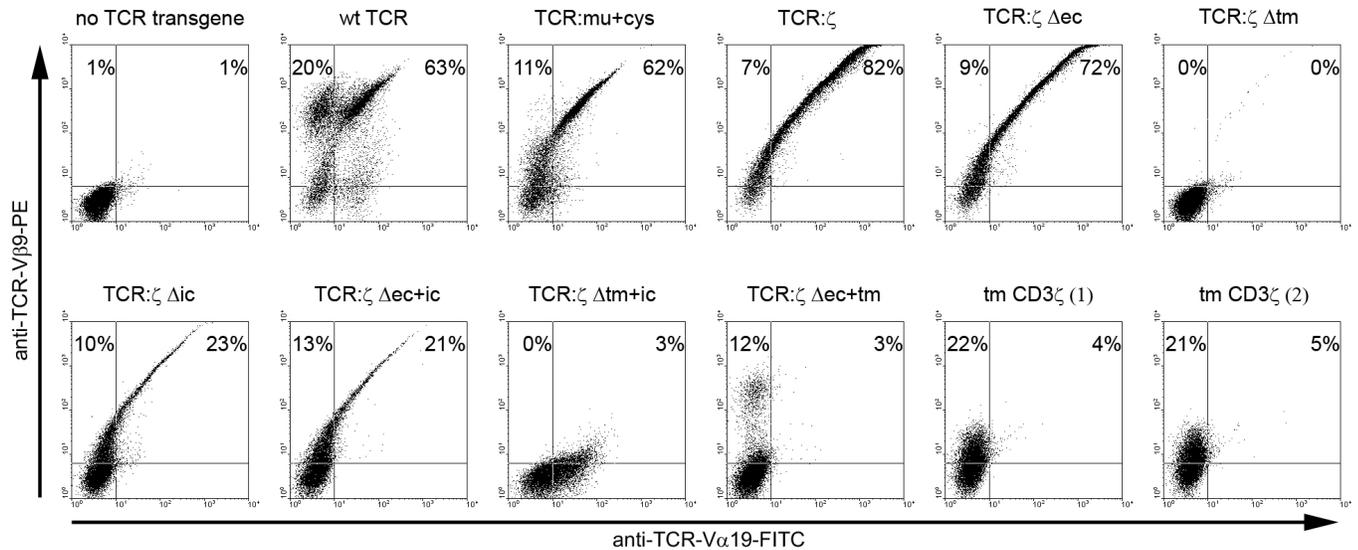
RESULTS

CD3ζ Transmembrane Domain is Required for Surface Expression of TCR:ζ

Jurkat T cells containing MelA/A2 TCR were used as recipient cells for a panel of 6 M1/A1 minimal TCR:ζ's, each incorporating a different but minimal of ec, tm and/or ic CD3ζ domains (see Fig. (**1**) for details). These dual-TCR Jurkat T cells were assessed for surface expression of transgenic TCR by flow-cytometry. Minimal TCR:ζ Δec, Δic, Δec+ic, all containing the CD3ζ tm domain, revealed a surface expression pattern like that of parental TCR:ζ with a typical diagonal, high mean fluorescence intensity (MFI) and absence of single TCR-chain positive cells (see Fig. (**2A**); [18]). TCR:ζ Δic and Δec+ic revealed lower levels of surface expression than TCR:ζ Δec or TCR:ζ. In contrast, TCR:ζ Δtm, Δtm+ic and Δec+tm transduced Jurkat T cells showed no surface expression of either or both TCRα:ζ and TCRβ:ζ chains (Fig. **2A**). Further analysis of these latter TCRs revealed that mRNA, but not intracellular proteins were consistently present, suggesting that not gene transcription but more likely protein translation and/or transportation to the cell surface were hampered (Supplementary Fig. **1**). Notably, the TCR:mu+cys, a murinized and cysteine-modified TCR used as a control TCR, shows an extended diagonal surface expression pattern and no or less single TCR-chain positive T cells than wt TCR, similar to TCR:ζ or its CD3ζ tm-containing variants (Fig. **2A**) and suggestive for high preferential TCR pairing.
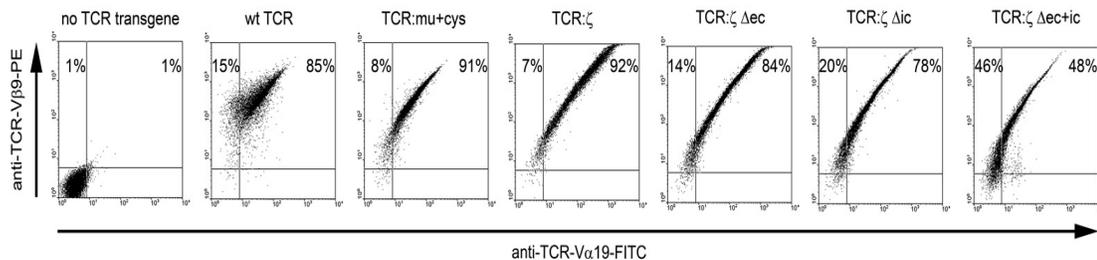
CD3ζ Transmembrane Domain Critically Determines the Inability of TCR:ζ to Associate With Endogenous CD3 and TCR Chains

The minimal TCR:ζ variants were assessed for their inability to associate with endogenous CD3, considered a unique characteristic of TCR:ζ, using conventional flow cytometry and flow cytometric Fluorescence Resonance Energy Transfer (FRET). Flow cytometry analyses after double-staining for TCRβ and CD3ε revealed diagonal dot-plots in Jurkat T cells expressing wt TCR and TCR:mu+cys, but not TCR:ζ or any of its minimal TCR variants (Fig. **3A**). These data suggest on one hand a CD3-independence of minimal TCR:ζ variants that contain CD3ζ tm, similar to the reported CD3-independence of TCR:ζ, and confirm on the other hand a CD3-dependence of wt TCR and TCR:mu+cys [11]. The lack of competition for CD3-proteins by TCR:ζ Δec, Δic, Δec+ic was also reflected in the expression of endogenous TCR. TCR:ζ or these variants did not alter the MFI of endogenous TCRβ, whereas wt TCR or TCR:mu+cys approximately halved the MFI of endogenous TCRβ (data not shown). Subsequent studies focused only on the three

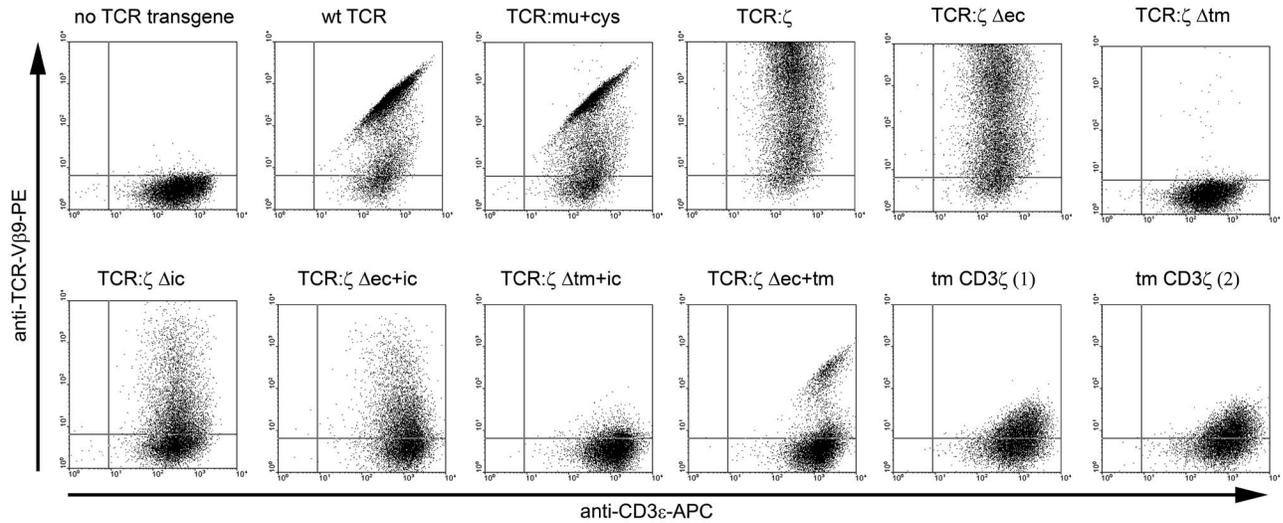**Fig. (2). Minimal TCR:ζ Δec, Δic and Δec+ic express at T cell surface.** Jurkat T cells expressing MelA/A2 TCR (Jurkat cl. 19) were transduced with one of the following M1/A1 TCRs: minimal TCR:ζ Δec, Δtm, Δic, Δec+ic, Δtm+ic, Δec+tm, TCR tmζ 1, tmζ 2, TCR:ζ, wt TCR, TCR:mu+cys, or no TCR transgene. (**A**) Surface expression levels of transgenic MA1/A1 TCRs were measured *via* flow cytometry using anti-TCR-Vα19[FITC] and anti-TCR-Vβ9[PE] mAbs. Representative dotplots out of 5 individual measurements are displayed. See Supplementary Fig. **1**. for extended analysis of intracellular protein and mRNA expression of those minimal TCR:ζ variants that did not show surface expression of both TCR-Vα19 and TCR-Vβ9, i.e., minimal TCR:ζ Δtm, Δec+tm, Δtm+ic, TCR:tmζ 1 and tmζ 2. (**B**) TCR surface expression of Jurkat T cell lines transduced with minimal TCR:ζ Δec, Δic and Δec+ic or controls wt TCR, TCR:ζ and TCR:mu+cys after FACSort with anti-TCR-Vα19[FITC] and anti-TCR-Vβ9[PE] mAbs. Representative dotplots out of 5 individual measurements are displayed and percentages of stained T cells in upper left and upper right quadrants are indicated. (**C**) Mean Fluorescence Intensities or percentages (both + SEM) of TCRαβ in upper right quadrants in (**B**), n=6-11 independent measurements (statistically significant differences in comparison to wt TCR are calculated with student's t-tests; p-values indicated in Fig).
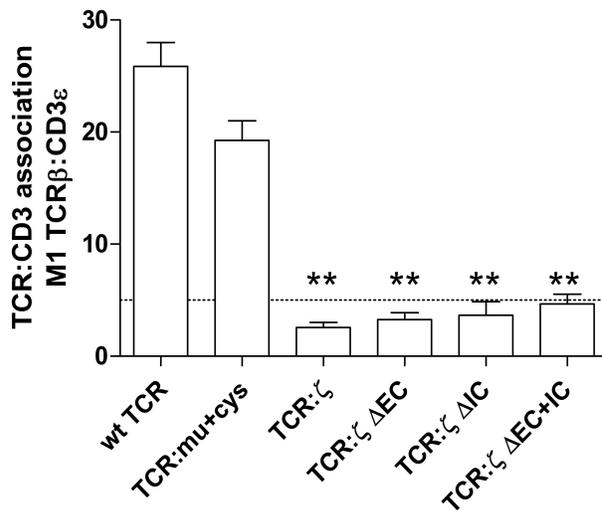
minimal TCR:ζ variants that were expressed on the cell surface. Flow cytometric FRET confirmed lack of association between TCR:ζ or its minimal variants, but not wt TCR or TCR:mu+cys, and CD3ε (Fig. **3B**). Flow cytometric FRET was also applied to address the extent TCRs were prone to

TCR mis-pairing. Using antibodies specific for endogenous TCR-Vβ27 (PE-fluorochrome, donor) and the TCR-Vα19-transgene (Cy5-fluorochrome, acceptor), we observed no FRET signals above background for TCR:ζ Δec, Δic, or Δec+ic variants, similar to parental TCR:ζ, indicating that
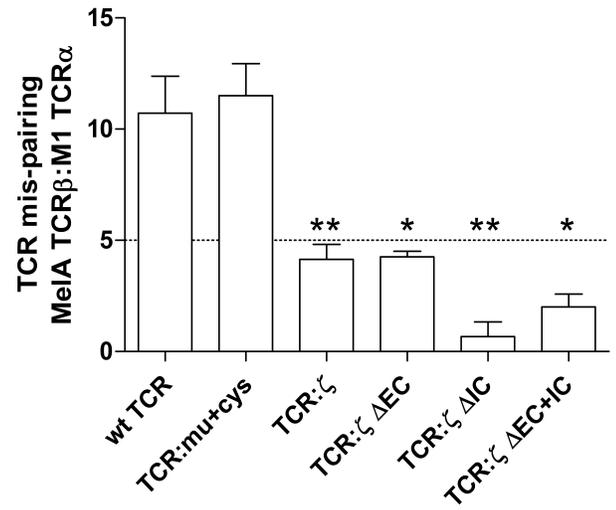
A

B

C

Fig. (3). Minimal TCR:ζ Δec, Δic and Δec+ic show neither CD3 association nor TCR mis-pairing. (A) Jurkat T cells expressing minimal TCR:ζ Δec, Δic and Δec+ic, TCR:ζ, wt TCR, TCR:mu+cys transgenes were tested for surface expression of transgenic TCR and CD3ε *via* flow cytometry using anti-TCR-Vβ1^{PE} and anti-CD3ε^{APC} antibodies. Representative examples out of 5 individual measurements are displayed. **(B)** CD3ε association and **(C)** TCR mis-pairing were determined of minimal TCR:ζ Δec, Δic and Δec+ic and control TCRs using Fluorescence Resonance Energy Transfer (FRET) between anti-Vβ9^{PE}->anti-CD3ε-RαM^{Cy5} and anti-Vβ27^{PE}->anti-Vα19-RαM^{Cy5} mAbs, respectively. Please note that FRET was measured using TCR-transduced but non-sorted Jurkat T cells after gating on M1/A1-specific TCR expressing cells. Dotted line represents the level of background signal (5%) and bars represent mean FRET values + SEM, n=4 independent measurements (statistically significant differences in comparison to wt TCR are calculated with student's t-tests; * = $p<0.05$; ** = $p<0.005$).

these variants preserve TCR:ζ's ability to successfully address TCR mis-pairing (Fig. **3C**). Notably, when using a sensitive methodology such as FRET, TCR:mu+cys TCRα are mis-paired with endogenous TCRβ to the same extent as wt TCRα. Additional proof for the absence or presence of TCR mis-pairing came from single TCR chain transductions of Jurkat T cells. Supplementary Fig. **2** demonstrates significant cell surface expression of a TCRαβ heterodimer (indicative of TCR mis-pairing) upon transduction with single chains (either TCRα or -β) of wt TCR or TCR:mu+cys, but not minimal TCR:ζ variants or TCR:ζ.

Next, we attempted to identify a limited number of CD3ζ tm amino acids important for TCR:ζ's surface expression and inability to mis-pair. A 3D-modeling strategy was applied to define a set of CD3ζ transmembranal amino acids that was subsequently transplanted onto favorable positions in the transmembrane domain of TCR chains. This resulted in TCR:ζ variants tmζ1 and -tmζ2 which were retrovirally introduced in Jurkat T cells. Flow cytometry revealed no cell surface expression of minimal TCR:tmζ1 or -2 heterodimers on these dual-TCR Jurkat T cells (Fig. **2A**). TCRα:tmζ1 and -2 chains were not able to complex with TCRβ:tmζ or

endogenous TCRβ. Also, TCRβ:tmζ showed moderate levels of surface expression through mis-pairing with the endogenous TCRα. Analysis of mRNA and intracellular protein suggested difficulties in protein transportation to the cell surface of particularly both TCRα-chains (Supplementary Fig. **1**). Thus, we were unable to attribute strong CD3-independent cell surface expression and prevention of TCR mis-pairing to a subset of CD3ζ transmembranal amino acids.

Minimal TCR:ζ Δec Performs best at Preserving the Ability of TCR:ζ to Bind pMHC and Activate NFAT

To study TCR-mediated functions, we have FACSorted Jurkat TCR-transductants with TCRα and -β mAbs to enhance and equalize surface expression levels of the different TCR formats (Fig. **2B**). Enrichment resulted in similar surface expression levels of TCRs (range between 74 - 92%), except for TCR:ζ Δec+ic which fell behind (48%) (Fig. **2C**). MFIs were within the same range for TCR:ζ Δec, Δic and parental TCR:ζ (TCR:ζ Δec: 163 and 1145; TCR:ζ Δic: 133 and 998; and TCR:ζ: 242 and 1646 for TCRα and -β chains, respectively), and were lowered for TCR:ζ Δec+ic (76 and 578) (Fig. **2C**). In fact, MFIs of TCR:ζ Δec+ic were within the same range as those for wt TCR and TCR:mu+cys (wt TCR: 64 and 633; TCR:mu+cys: 66 and 540). Please note that levels of surface expression of TCR:ζ and its variants do not take into account TCR stainings in upper left quadrants of flow cytometry dot plots. Since these stainings extend the diagonal of flow cytometry dotplots and are *not* due to TCR mis-pairing (Fig. **3C**), levels of surface expression, as put in Fig. **2C**, may underestimate total surface expression levels of TCR:ζ and its variants.

Subsequently, transgenic TCRs were standardized for average cell surface expression levels (with % surface expression of wt TCR (see (Fig. **2C**)) set to 1.0) and compared

for their ability to recognize and bind pMHC. The TCR:ζ Δec transgene revealed a slightly lower percentage of pMHC binding compared to the TCR:ζ transgene, detected at all measured concentrations, although differences reached no statistical significance (Fig. **4A**). Wild-type TCR, TCR:mu+cys, and TCR:ζ transduced Jurkat T cells showed the highest percentages of pMHC positive populations (Fig. **4A**). Again, when analyzing the MFIs of pMHC binding, we noted that Jurkat T cells expressing TCR:ζ Δec or parental TCR:ζ were the two T cell lines with the highest TCR expression levels (Figs. **4B** and **2C**). Although TCR:ζ Δec shows a pMHC binding approximately half of that of parental TCR:ζ, both show a significantly increased MFI compared to wt TCR (TCR:ζ vs wt TCR: $p<0.05$ at all concentrations; and TCR:ζ Δec vs wt TCR: $p<0.05$ at 4 out of 6 concentrations). TCR:ζ Δic and Δec+ic variants showed a negligible pMHC binding, whereas TCR:mu+cys showed a pMHC binding that was slightly lower than wt TCR.

Next, we measured antigen-specific T cell activation by employing a Gaussia Luciferase reporter assay based on six response elements of Nuclear Factor of Activated T cells (NFAT), a transcription factor that is a key step in T cell activation. With transgenic TCRs being standardized for average cell surface expression levels (Fig. **2C**), we observed that TCR:ζ Δec and parental TCR:ζ proved to be most potent in activating Jurkat T cells by inducing the highest level of luminescence, which was significantly higher when compared to wt TCR (Fig. **5**). TCR:ζ Δic and Δec+ic variants, however, did not mediate T cell activation, even though these TCRs were able to bind pMHC. To further investigate the potency of TCR:ζ Δic and Δec+ic to induce intracellular T cell signaling, we stimulated Jurkat T cells with anti-TCR-Vβ9 mAbs and demonstrated that these two TCRs, both lacking the intracellular CD3ζ immunoreceptor tyrosine-
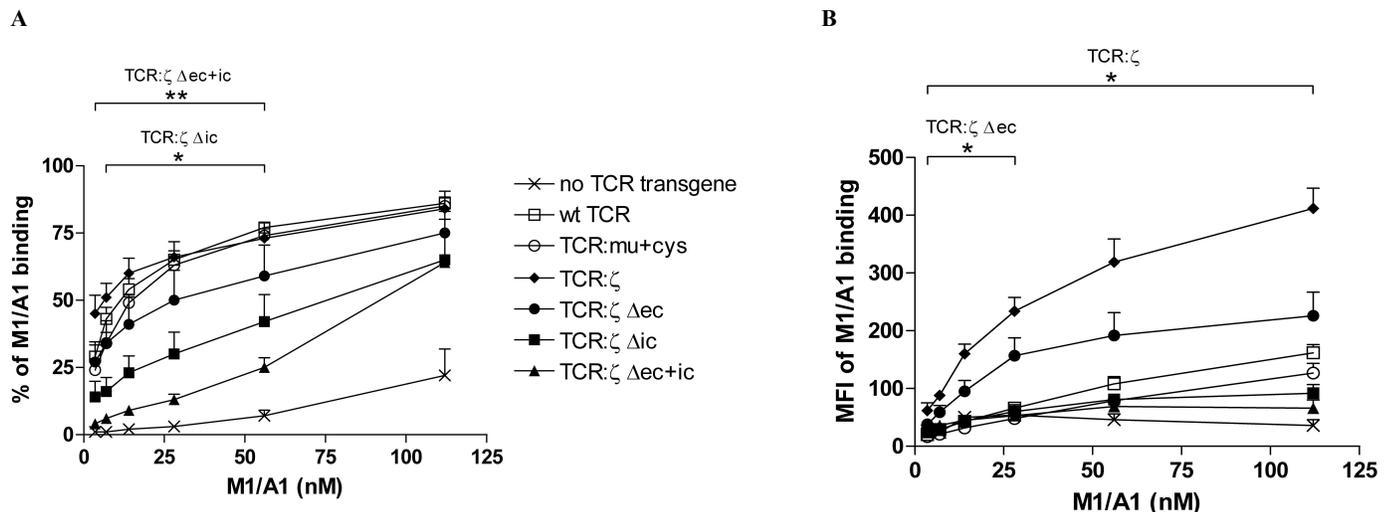

Fig. (4). T cells transduced with minimal TCR:ζ Δec, Δic or Δec+ic show decreased ability to bind pMHC, which is least compromised for TCR:ζ Δec. Jurkat T cells expressing minimal TCR:ζ Δec, Δic and Δec+ic, TCR:ζ, wt TCR, TCR:mu+cys transgenes or no TCR transgene were tested for their ability to bind M1/A1 tetramer PE complexes. The amount of M1/A1-tetramer used to stain 0.5×10^6 cells (30 min, RT) was titrated from 112 nM down to 3.5 nM. Percentages (**A**) and MFIs (**B**) of pMHC binding by T cells were measured *via* flow cytometry. Percentages of pMHC binding were standardized for average cell surface expression levels (with % surface expression of wt TCR (see Fig. (**2C**)), set to 1.0). Curves represent mean percentages of MFI or pMHC binding + SEM, n=4 independent measurements (statistically significant differences in comparison to wt TCR are calculated with student's t-tests; * = $p<0.05$; ** = $p<0.005$).
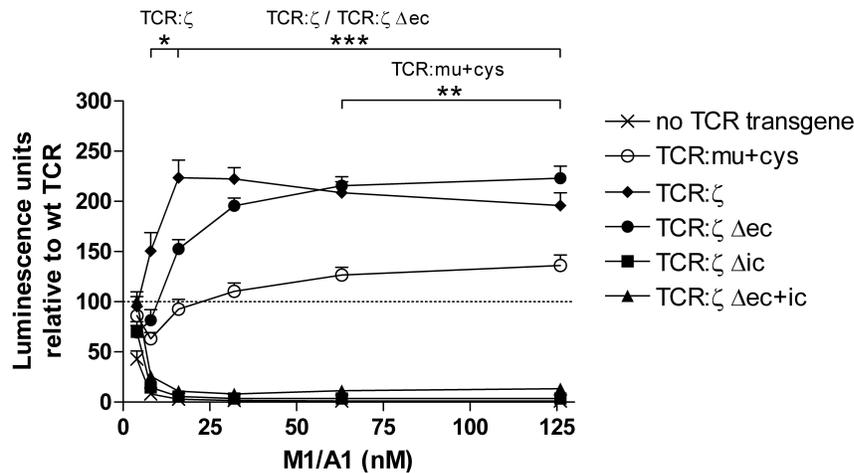
Fig. (5). T cells transduced with minimal TCR:ζ Δec show a potent antigen-specific NFAT response. Jurkat T cells expressing minimal TCR:ζ Δec, Δic and Δec+ic, TCR:ζ, wt TCR, TCR:mu+cys transgenes or no TCR transgene were tested for their ability to mediate activation of Nuclear Factor of Activated T cells (NFAT). TCR-transduced Jurkat T cells were nucleofected with a Gaussia Luciferase reporter construct under control of 6 NFAT response elements, and stimulated for 6h with pMHC monomers. The concentration of monomers was titrated from 126 nM down to 4 nM. Luciferase activities of wt TCR T cells for 4, 8, 16, 32, 63, and 126 nM pMHC were: 588926, 670147, 656821, 444174, 181790, and 37084 relative luminescence units (RLU), respectively, and were all set at 100% (dotted line). Luciferase activities of TCR-transduced T cells were standardized for average cell surface expression levels (as explained in legend to Fig. **4A**). Curves represent mean luminescence units + SEM, n=6-14 independent measurements (statistically significant differences in comparison to wt TCR are calculated with student's t-tests; * = $p<0.05$, *** = $p<0.0005$).

based activation motifs (ITAMs), were not able to mediate T cell activation (Supplementary Fig. **3**). Stimulations of the endogenous TCRβ with anti-TCR-Vβ27 mAb did not show reduced NFAT activation upon transduction with TCR:ζ Δec, Δic or Δec+ic, in line with parental TCR:ζ [18] (data not shown). In contrast, stimulation with anti-TCR-Vβ27 mAb did show significantly reduced NFAT activation upon transduction with wt TCR or TCR:mu+cys, a result of downregulated surface expression of CD3-dependent TCRs in dual TCR T cells.

Minimal TCR:ζ Δec Shows a Compromised Surface Expression and Function in Primary Human T Cells

The next step was to test the minimal TCR:ζ variant that performed best in Jurkat T cells with respect to surface expression, absence of TCR mis-pairing, pMHC binding and activation of NFAT, i.e., TCR:ζ Δec, in primary human T cells. Anti-CD3 mAb-activated human PBMC were retrovirally transduced with TCR:ζ Δec, TCR:ζ, wt TCR and TCR:mu+cys. To enhance the functional expression of TCR:ζ Δec, we codon optimized CD3ζ Δec and aligned the two chains in a TCRβ-2A-TCRα configuration in a pMP71 retroviral vector [30-33]. Surface expression of TCR transgenes and pMHC binding were assessed by flow-cytometry in cultures of TCR-engineered T cells (> 90 % CD8-positive T cells). Please note that, for reasons explained above, percentages of surface expression displayed in the upper right quadrants of flow cytometry dotplots may provide an underestimation of the actual levels of surface expression of TCR:ζ and TCR:ζ Δec (with and without codon optimization) (Fig. **6A**). TCR:ζ Δec revealed a weak surface expression and pMHC binding, which was, unexpectedly, not improved when using TCR:opt.ζ Δec in a β-2A-α configuration in pMP71 (Figs. **6A,B**). Notably, the surface expression levels of TCR:ζ and TCR:mu+cys were higher when com-

pared to wt TCR (Fig. **6A**). However, when looking at the pMHC binding, only TCR:ζ, and not TCR:mu+cys, demonstrated enhanced performance when compared to wt TCR (Fig. **6B**).

Finally, primary human T cells expressing TCR transgenes were stimulated with antigen-positive and negative target cells, after which CD107a mobilization on the cell surface was measured within the TCR-Vα19 positive T cell population as a measure for cytotoxicity. TCR:ζ Δec, whether or not with codon optimized CD3ζ and in an optimal vector cassette, mediated negligible antigen-specific responses in contrast to TCR:ζ ($p<0.005$, Fig. **6C**). Both TCR:ζ and TCR:mu+cys show a higher percentage of CD107a positive cells than T cells with wt TCR transgenes.

DISCUSSION

In this study, we generated novel MAGE-A1-specific TCRs fused to partial rather than complete human CD3ζ in an effort to identify a minimal TCR:ζ that preserved the TCR pairing and functional properties of TCR:ζ. To this end, a panel of 8 minimal TCR:ζ's (see Fig. (**1**) for details) was tested for surface expression, association with endogenous CD3 and TCR chains, pMHC binding and TCR transgene-mediated functions in Jurkat T cells and primary human T cells. Our observations revealed that for TCR:ζ and its variants: (a) intact CD3ζ transmembrane (tm) domain critically determines surface expression and inability to associate with endogenous CD3 and TCR chains in Jurkat T cells; (b) both CD3ζ extracellular (ec) and intracellular (ic) domains are dispensable for pMHC binding, whereas only CD3ζ ec domain is dispensable for T cell mediated signaling in Jurkat T cells; and (c) the combination of CD3ζ ec, tm and ic domains is required for surface expression and T cell function in primary human T cells.
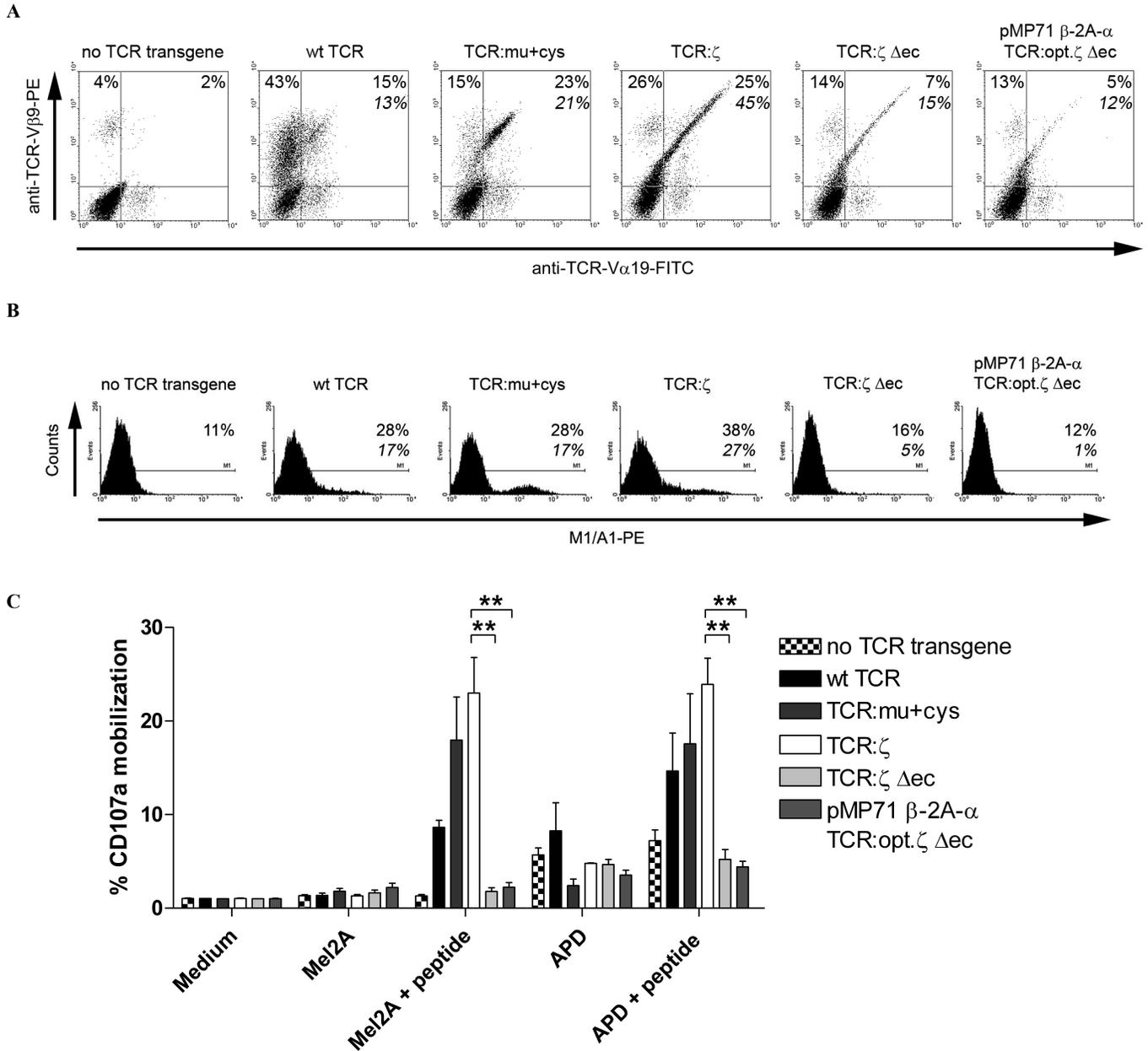
Fig. (6). Minimal TCR:ζ Δec demonstrates compromised surface expression and antigen-specific function in primary human T cells. Primary human T cells were transduced with one of the following M1/A1 TCRs: TCR:ζ Δec, pMP71 β-2A-α TCR:opt.ζ Δec, TCR:ζ, wt TCR, TCR:mu+cys, or no TCR. (**A**) Surface expression of transgenic TCR was measured *via* flow cytometry. Anti-TCR-Vα19[FITC] and anti-TCR-Vβ9[PE] antibodies were used to stain TCR-transduced T cells. Representative examples out of 5 individual measurements of two healthy donors are displayed and percentages of stained T cells in upper left and upper right quadrants are indicated. Percentages indicated in italics are either percentages of upper right quadrants (wt TCR, TCR:mu+cys) or the sum of percentages of upper left and upper right quadrants (TCR:ζ, TCR:ζ Δec, TCR:opt.ζ Δec), corrected for the corresponding percentage(s) of Mock T cells. These percentages (in italics) may better represent the actual levels of surface expression of TCR:ζ and its variants (see Result section for details). (**B**) pMHC binding by transgenic TCR was measured *via* flow cytometry. M1/A1-tetramer-PE complexes (15 nM) were used to stain TCR-transduced T cells, and percentages of positive cells in histograms are indicated. Representative examples out of 5 individual measurements of two healthy donors are displayed and percentages of stained T cells in selected histogram region are indicated (in italics: corrected for pMHC binding observed by Mock T cells). (**C**) CD107a-mobilization to cell surface of TCR-transduced T cells. TCR-transduced T cells were stimulation for 2h with medium, Mel2A or APD (both M1 negative, HLA-A1 positive), or Mel2A and APD loaded with 10 µM M1 peptide, after which T cells were analyzed for CD107a expression by flow cytometry (see Materials and Methods section for details). Bars represent mean CD107a values + SEM, 2 independent measurements of two healthy donors (statistically significant differences are calculated with student's t-tests; ** = p<0.005). Please note that at the time of flow cytometry analyses, TCR-engineered primary human T cells contained > 90 % CD8-positive T cells.

In the first series of experiments, we have analyzed 6 minimal TCR:ζ variants, in which the ec, tm and/or ic domain(s) of CD3ζ were omitted (and replaced by corresponding TCR domain(s)). Cell surface expression analysis revealed that not all minimal TCR:ζ variants resulted in detection of a M1/A1 TCR heterodimer. In case the CD3ζ tm domain was absent (i.e., TCR:ζ Δtm, Δtm+ic, Δec+tm), TCRα:ζ and -β:ζ transgenes were not properly expressed as a TCR heterodimer (Fig. **2A**). In fact, TCR chains of these minimal TCR:ζ variants showed aberrant protein expression or transport to the cell surface (Supplementary Figs. **1B**, **C**). TCR:ζ and its variants that were expressed (i.e., TCR:ζ Δec, Δic, and Δec+ic,), show similarly strong levels of cell surface expression (TCR:ζ Δec+ic to a lesser extent; Fig. **2**) and an inability to associate with CD3ε (Fig. **3A**, **B**). Functional assays, however, identified two groups of TCRs: (1) TCR:ζ and TCR:ζ Δec; and (2) TCR:ζ Δic and TCR:ζ Δec+ic. The latter group of TCRs, in contrast to the first group, revealed lowered to negligible binding of pMHC (Figs. **4A**, **B**) and an inability to induce NFAT activity upon stimulation with either pMHC (Fig. **5**) or anti-TCR-Vβ9 mAb (Supplementary Fig. **3**). Our finding that TCR:ζ variants that lack the CD3ζ ic domain showed compromised pMHC binding is unexpected. Studies with other antigen-specific receptors argue that lack of, or not having access to, ITAM-bearing CD3ζ ic domain results in enhanced surface expression and pMHC binding. For example, inhibition of protein tyrosine kinases demonstrated that early TCR-mediated signaling normally provides a negative feedback loop that facilitates pMHC-induced TCR down-regulation and T cell apoptosis [34, 35]. Moreover, studies with Chimeric Antibody Receptors coupled to CD3ζ (i.e., CAR:ζ) showed that CD3ζ ITAMs directly contribute to loss of transgene expression and enhanced sensitivity to apoptosis [36]. In contrast, TCR:ζ is different from other receptors since it does not dimerize with endogenous TCR/CD3 complexes [18] and may signal differently [37]. Moreover, recent studies into immune synapses suggested that TCR:ζ has a distinct conformation [20], and we propose that conformational changes due to extensive deletions, such as Δic and Δec+ic, cause the observed decrease in pMHC binding. The observation that TCR:ζ Δic and Δec+ic can not mediate activation of NFAT, even upon stimulation with anti-TCR mAb, points out that TCR-mediated signaling either requires a modified TCR that contains ITAMs (i.e., TCR:ζ or minimal TCR:ζ Δec) or a (modified or wt) TCR that is able to recruit endogenous and ITAM-containing CD3 molecules.

In addition to the shared inability to associate with CD3ε, minimal TCR:ζ Δec, Δic, and Δec+ic and the parental TCR:ζ, all containing an intact CD3ζ tm domain, showed no association with endogenous TCR chains (Fig. **3C** and Supplementary Fig. **2**). Notably, flow cytometry of TCR:ζ Δtm+ic and TCR:ζ Δec+tm showed cells that were single positive for either TCR-Vα19 or -Vβ9, respectively (Fig. **2A**), indicating that CD3ζ ec or ic domains do not completely prevent these TCRs from mis-pairing. Our finding that prevention of TCR mis-pairing, and consequently enhanced pairing between TCRα:ζ and -β:ζ, is governed by the CD3ζ tm domain is consistent with a report by Rutledge and colleagues [38], in which the CD3ζ tm domain was used to induce dimerization of monomeric proteins, such as the IL-2Rα chain.

In the next series of experiments, we have transplanted a limited set of CD3ζ tm amino acids onto TCRα and TCRβ with the intent to preserve surface expression and inability to TCR mis-pair and, at the same time, retain structural and spatial requirements to associate with endogenous CD3 chains (i.e., TCR:tmζ1 and -2, (see Fig. **1**)). This 'transplantation set' of amino acids was identified through 3D modeling and included the ones defined by Call and colleagues to be critically involved in CD3ζ homodimerization (i.e., C2, D6, L9, Y12, T17 and F20) [39]. We observed that TCR:tmζ heterodimers did not express at the cell surface (Fig. **2A**). In fact, TCRα:tmζ1 and -2 chains were not able to complex with TCRβ:tmζ nor endogenous TCRβ, and TCRβ:tmζ showed moderate levels of surface expression through mis-pairing with the endogenous TCRα. These data suggest that TCR:ζ properties related to the presence of an intact CD3ζ tm domain, such as enhanced cell surface expression and inability to associate with endogenous CD3 and TCR chains, cannot be attributed to a limited number of individual CD3ζ tm amino acids.

TCR:ζ's pairing and functional properties were best preserved in minimal TCR:ζ Δec. This TCR variant retains high surface expression levels (Fig. **2**), prevents pairing with endogenous CD3 and TCR chains (Fig. **3**), binds pMHC (although binding is somewhat reduced when compared to TCR:ζ, (Fig. **4**), and potently activates NFAT (Fig. **5**). Interestingly, recent confocal microscopy studies demonstrated that minimal TCR:ζ Δec is able to form immunological synapses with similar sizes as those formed by parental TCR:ζ [20]. In minimal TCR:ζ Δec there is only a single artificial boundary, i.e., the one between TCR ec and CD3ζ tm sequences, potentially preventing or diminishing humoral and/or cellular immunogenicity. Collectively, our observations warranted testing of minimal TCR:ζ Δec in primary human T cells. In bulk populations of human PBMC (non-sorted for either TCR or CD8 expression), we observed that the difference in surface expression between minimal TCR:ζ Δec and parental TCR:ζ was more pronounced when compared to Jurkat T cells (Fig. **6A**). Also, minimal TCR:ζ Δec showed lower pMHC binding and induced negligible CD107a mobilization to the cell surface when compared to TCR:ζ (Figs. **6B**, **C**), which most likely was accounted for by the low level of surface expression. These observations, perhaps unexpectedly, were not different when using minimal TCR:ζ Δec in an optimal vector, an optimal TCR cassette and with codon optimized CD3ζ tm+ic domains. Apparently, the absence of the membrane-proximal CD3ζ ec domain in minimal TCR:ζ Δec results in a stringent decrease in functional TCR expression. Interestingly, single chain (sc) Fv [40] and scTCR ([15], and data not shown) that do contain CD3ζ ec coupled to either CD3ζ tm+ic, CD4 tm + Fc(ε)RIγ ic, or Fc(ε)RIγ tm+ic, have indeed demonstrated significant surface expression in human T cells. In minimal TCR:ζ Δec, six CD3ζ ec amino acids (GDLDPK) were replaced by either TCRα (DVKLVEKSFETDTNLNFQNLS) or TCRβ (GFTSESYQQGVLSATILYE) ec amino acids (that cover the connecting-peptide motifs CPMα and CPMβ, respectively). The CPMα and CPMβ are reported to interact with tetracysteine motifs in the membrane-proximal stalk regions of CD3δε and CD3γε dimers, and as such contribute to TCR/CD3 complex formation and T cell activation [41,

42]. In addition, a defective or mutated CPMα is compromised with respect to its association with CD8, translation of antigen-specific stimulation into phosphorylation of Lck, Fyn, and ZAP70, and production of IL-2 [41, 43, 44]. We observed that the presence of CPMα and -β in minimal TCR:ζ Δec appears not sufficient to induce TCR/CD3 complex formation (Figs. **3A**, **B**), suggesting a more critical role for intact TCR tm for association with CD3 chains [45]. The extent to which the presence of CPMα and -β in minimal TCR:ζ Δec improved pMHC binding in T cells is difficult to assess due to poor expression levels in primary human T cells (Fig. **6A**) and non-specificity of pMHC binding in CD8α-transduced Jurkat T cells (data not shown). Nevertheless, earlier findings proved that M1/A1-specific TCR:ζ, not containing CPMα or –β, were able to associate with and depend on CD8α for ligand binding [20, 46], and may suggest a non-dominant role of CPM with respect to TCR:CD8α association.

An alternative TCR format that is designed to address TCR mis-pairing, in addition to TCR:ζ, is TCR:mu+cys. In TCR:mu+cys two separate strategies have been combined: murinization of the TCR-C domain and introduction of cysteine amino acids at structurally favorable positions to allow formation of an additional disulfide bridge, which together result in enhanced functional expression [24, 47] (reviewed in [11]). Our studies with M1/A1-specific TCR:mu+cys, taken along as a control TCR in the present paper, confirmed this notion to some extent. In Jurkat T cells, TCR:mu+cys, when compared to wt TCR, showed similar levels of surface expression and pMHC binding, and somewhat enhanced levels of antigen-specific NFAT activation (Figs. **2A**, **4** and **5**), whereas in primary human T cells, TCR:mu+cys showed enhanced levels of surface expression and similar levels of pMHC binding and antigen-specific CD107a mobilization to the cell surface (Fig. **6**). However, we did observe that TCR:mu+cys mediates a significantly enhanced antigen-specific IFNγ response (data not shown). These findings, although not fully in accordance with previous reports and potentially unique to the TCR-V regions of the M1/A1 TCR [24, 47], generally argue that TCR:mu+cys improved functional TCR expression. Expectedly, enhanced functional TCR expression was related to enhanced preferential pairing between the two modified TCR:mu+cys chains, which was suggested by the flow cytometric absence of single TCRα or TCRβ positive cells (Fig. **2A**). Unexpectedly, however, flow cytometric FRET (Fig. **3B**) and single TCR chain transductions (Supplementary Fig. **2**) clearly demonstrated that TCR:mu+cys mis-paired with endogenous TCR chains to the same extent as wt TCR. Also *in vivo*, murine TCRs with a cysteine modification did not fully prevent the pathology related to TCR mis-pairing, i.e., TCR transfer-induced Graft versus Host disease [10]. We therefore propose that from a safety point of view, TCR:ζ but not TCR:mu+cys provides a better alternative to wt TCR.

In conclusion, our studies showed that CD3ζ domains separate various properties of TCR:ζ, i.e., the CD3ζ tm domain determines surface expression and lack of association with endogenous CD3 and TCR chains, whereas CD3ζ ic domain contributes to T cell signaling. Functional expression of TCR:ζ in primary human T cells, however, required the complete rather than minimized content of CD3ζ.

CONFLICT OF INTEREST

None declared.

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