SUPPLEMENTARY METHODS

Cloning of Minimal TCR:ζ Variants

TCR:ζ variants generated by a domain exchange strategy or 3D-modeling strategy were acquired through cloning or gene synthesis. Exact amino acid constitutions of PCR, digestion, or gene synthesis products are indicated below. Primer sequences will be provided upon request.

TCR:ζ Δec: TCR chains (TCRα: nucleotide (nt) 1-702; TCRβ: nt 1-789) were fused together with CD3ζ tm+ic domains (CD3ζ: nt 91-489) (started from 1st methionine). PCR products were digested with *Nco*I-*Xho*I (TCRα:ζ) and *Xho*I- *Xho*I (TCRβ:ζ) and ligated in digested wt TCR pBullet vectors. For experiments in primary human T cells, the TCR:ζ Δec chains were put in a pMP71 vector in a TCRβ-2A-TCRα configuration. Overlap PCR fused together both TCR ec domains to codon optimized CD3ζ tm+ic domains (the latter domains from Geneart, Regensburg, Germany), after which PCR products were digested with *Not*I-*Mlu*I (TCRβ) and *Mfe*I-*Eco*RI (TCRα) and sequentially ligated in digested pMP71 vector.

TCR:ζ Δtm: TCR chains were partially constructed by hybridized oligomers (oligomers ordered via Eurogentec, Maastricht, Netherlands), digested with *Bam*HI-*Sac*I, that covered nt: CD3ζ: 76-90; TCRα: 766-825; CD3ζ: 154-220 or CD3ζ: 76-90; TCRβ: 847-912; CD3ζ: 154-220. The additional CD3ζ sequence (nt 221-489) was acquired through a *Sac*I-*Xho*I digestion of TCRα:ζ, after which both fragments were ligated in *Bam*HI-*Xho*I digested TCRα:ζ pBullet vector. To generate TCRβ:ζ Δtm, an additional *Nco*I-*Bam*HI fragment of TCRβ:ζ was required (to circumvent digestion of an internal *Xho*I site) and all fragments were ligated into a *Nco*I-*Xho*I digested pBullet vector.

TCR:ζ Δic: TCR chains were obtained via *Nco*I-*Bam*HI digests of parental TCR:ζ (covering nt TCRα: 1-702; CD3ζ: 73-75 or TCRβ: 1-789; CD3ζ: 73-75) and hybridized oligomers (Eurogentec), the latter digested with *Bam*HI-*Xho*I (CD3ζ: 76-153; TCRα: 826-840 or CD3ζ: 76-153; TCRβ: 913-933). Variants were finalized by ligation in *Nco*I-*Xho*I digested pBullet vectors.

TCR:ζ Δec+ic: TCR chains were obtained via hybridized primers (Eurogentec) (covering nt TCRα: 724-765; CD3ζ: 91-153; TCRα: 276-280 or CD3ζ: 108-153; TCRβ: 913-933), digested with *Hin*DIII-*Xho*I (TCRα) or *Bfu*A1-*Xho*I (TCRβ). For TCRβ a NcoI-BfuA1 digestion fragment from TCRβ:ζ was acquired to circumvent an internal *Xho*I site, and fragments were ligated in digested wt TCR pBullet vectors.

TCR:ζ Δtm+ic: TCRα was generated via gene synthesis (Geneart) (nt TCRα: 1-702; CD3ζ: 73-90; TCRα: 826-840) and ligated via *Nco*I-*Xho*I in a pBullet vector. TCRβ was obtained via a *Nco*I-*Bam*HI digestion of TCRβ:ζ Δic (nt TCRβ: 1-789; CD3ζ: 73-75), a *Bam*HI-*Xho*I fragment from hybridized primers (Eurogentec) (CD3ζ: 76-90; TCRβ: 847-933), and ligation of these fragments in a pBullet vector.

TCR:ζ Δec+tm: TCRα was obtained via *Bsp*I-*Xho*I digestion of TCRα:ζ Δtm and ligation of this fragment in a pBullet vector already containing wt TCRα. TCRβ was generated by *Nco*I digestion of wt TCRβ and ligation of this fragment in a pBullet containing TCRβ:ζ Δtm.

TCR:tmζ1 and 2: YASARA (www.yasara.org, [1]) was used to build a model of the interacting transmembranal amino acids in the TCR/CD3 complex. The structure of the CD3ζζ transmembrane domain has been resolved by NMR and was introduced as such in our model (PDB file 2hac [2]). The other CD3 transmembrane helices were modelled based on the large CD3ζ helix. Predicted CD3 helices were used to identify transmembrane amino acids of CD3ζ important for interaction with the other helices in the complex. These amino acids were transplanted onto TCRα and TCRβ chains, preferentially replacing amino acids defined to mediate TCRα-TCRβ but not TCRα-CD3δε or TCRβ-CD3γε associations. This 3D-modelling exercise revealed two modified TCRα and one TCRβ tm domain(s) (see Fig. **1B**). For both TCRα chains the tm domain with small flanking regions was generated through gene synthesis (Geneart), digested with *Hin*DIII and *Xho*I, and ligated in pBullet vector with wt TCR. CD3ζ amino acids substitute TCRα amino acids at positions 256, 260, 266, 270, 271, and 274 for variant tmζ1, and positions 255, 256, 259, 266, 269, 270, 273, and 274 for variant tmζ2. The TCRβ chain (combined with either TCRα chain) was generated through gene synthesis (Geneart) and ligated in a pBullet vector [3] using *Sal*I and *Not*I. CD3ζ amino acids substitute TCRβ amino acids at positions 282, 256, 289, 292, 296, 297, and 300.

Intracellular Flow Cytometry

TCR-transduced T cells ($1x10^4$ cells) were monitored by flow cytometry for intracellular expression of transgenic TCR using FITC-conjugated anti-TCR-Vα19 and PE-conjugated anti-TCR-Vβ9 mAbs. After T cells were washed, they were permeabilized with the permeabilization kit (BD Biosciences) for 10 minutes at room temperature (RT) and washed again. T cells were stained with TCR-Vα19 and TCR-Vβ9 mAbs for 20 minutes on ice. Next, T cells were washed and measured on a FACS Calibur dual-laser flow cytometer.

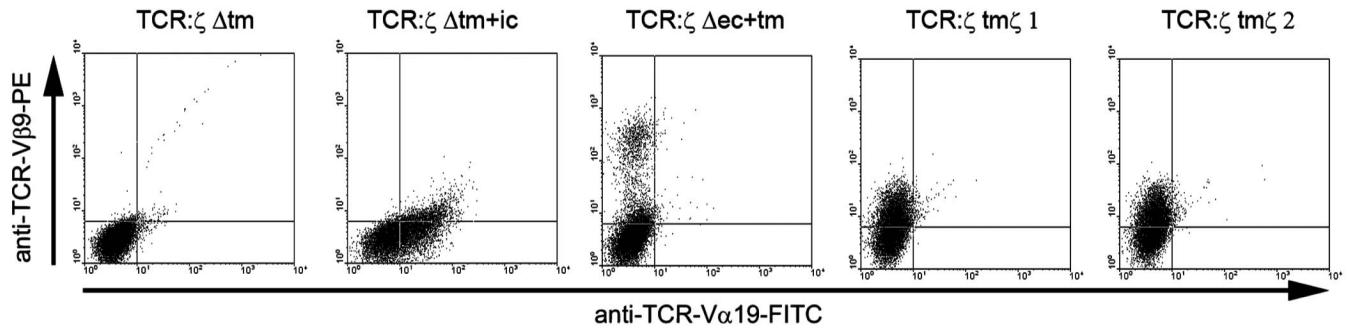
mRNA Isolation and RT-PCR

mRNA was isolated by means of TRIzol (Invitrogen). $1x10^6$ cells were lyzed in 1 ml TRIzol and incubated for 5 minutes at RT. The lysate was mixed with 0,2 ml Chloroform (Sigma-Aldrich), incubated for 2 minutes at RT, and subsequently centri-

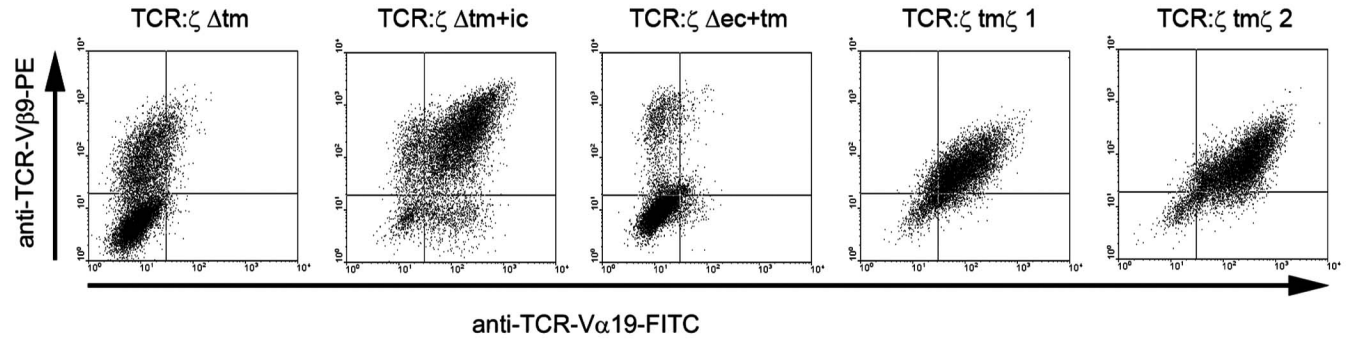
1875-0370/11 2011 Bentham Open

fuged for 15 minutes at 12,000g and 4°C. The aqueous phase was acquired, supplemented with 0,5 ml isopropanol (Sigma-Aldrich) and 1 µl glycogen (Roche, Woerden, the Netherlands), and centrifuged for 10 minutes at 12,000g and 4°C. The pellet was resuspended in 1 ml ethanol (Merck, New Jersey, USA), mixed, and centrifuged for 5 minutes at 7,500g and 4°C. The pellet was reconstituted in RNase-free water. For cDNA synthesis 5 µg mRNA was mixed with 0.5 ng oligo-dT primers (Invitrogen), 0.05 ng random hexamer primers (Promega) and water to 12 µl. The mixture was heated to 70°C for 10 minutes and quickly cooled on ice. 1st strand buffer (Invitrogen), 0.5 mM dNTPs (Promega), 0.005 M DTT (Invitrogen), and RNasin (Promega) was added to the mixture before incubation at 42°C for 2 minutes. Superscript III RTase (Invitrogen) was added to the mixture and placed at 42°C for 50 minutes, followed by 10 minutes at 70°C. The subsequent PCR was performed with transgene and GAPDH specific primers, sequences of which will be provided upon request.

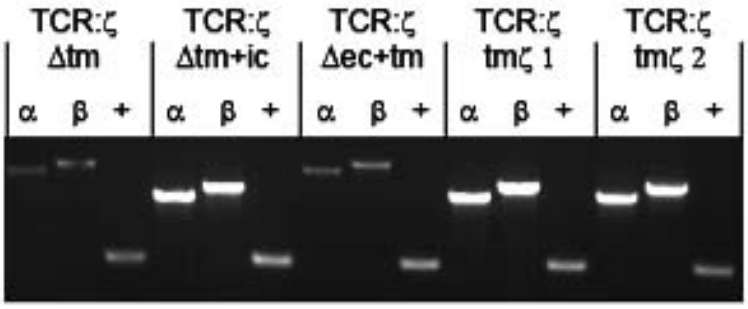
A



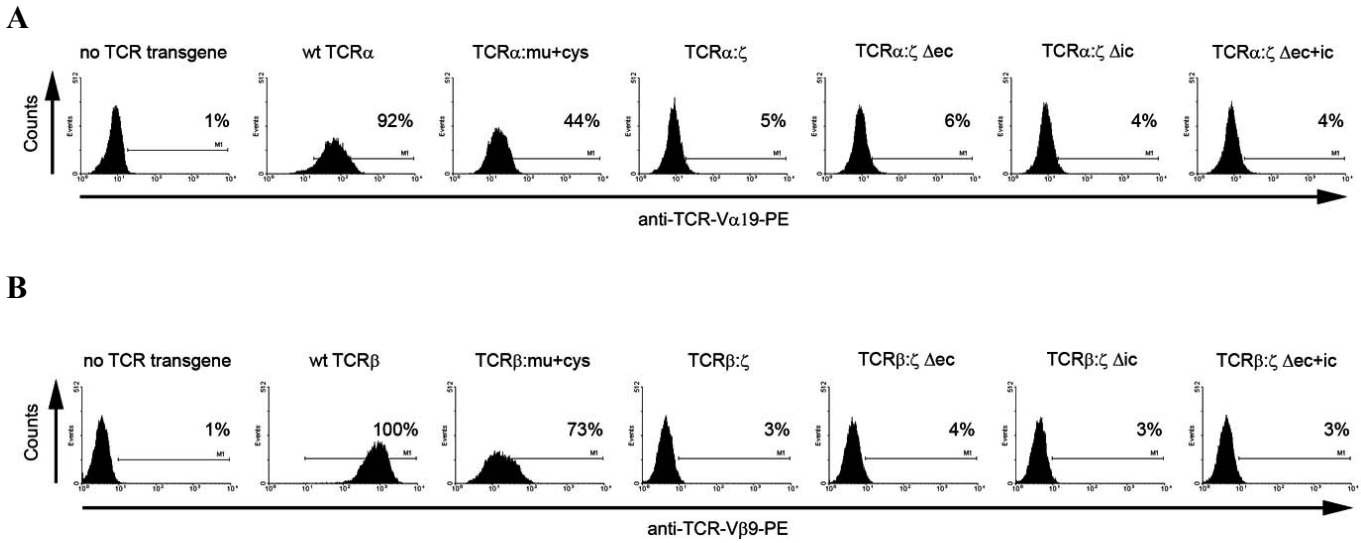
B



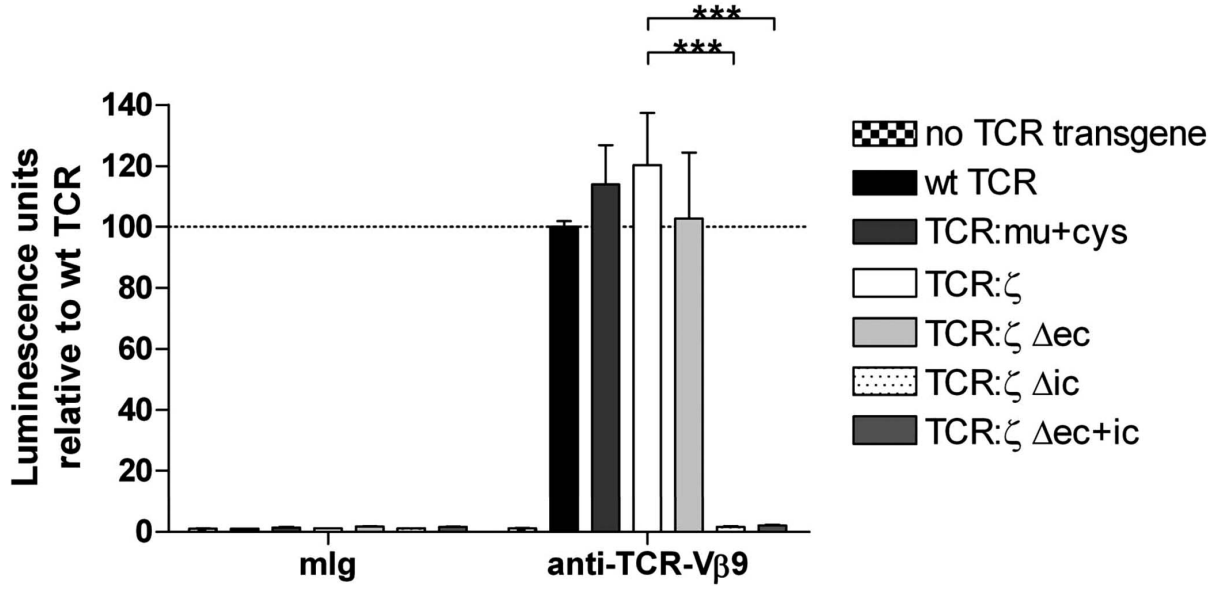
C



Supplementary Fig. (1). Minimal TCR:ζ variants without a complete CD3ζ transmembrane domain show aberrant protein production or transportation to cell surface. Jurkat T cells expressing MelA/A2 TCR were transduced with one of the following M1/A1 TCRs (those that were not surface-expressed, see Fig. **2A**): minimal TCR:ζ Δtm, Δtm+ic, Δec+tm, TCR tmζ 1 or tmζ 2. Surface (**A**) and intracellular (**B**) expressions of transgenic TCR 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. T cells were permeabilized with the permeabilization 2 kit before mAb staining for intracellular transgenic TCR expression measurements. (**C**) mRNA expression of TCR transgenes (TCRα = α; TCRβ = β) and GAPDH (+, positive control). mRNA was isolated by means of TRIzol after which cDNA synthesis was performed with superscript III reverse transcriptase. PCR was performed with TCR transgene and GAPDH specific primers, and products were loaded on an agarose gel. TCRα and TCRβ transgenes vary in size between 795 nt – 1164 nt and 891 nt – 1248 nt, respectively, due to altering TCR and CD3 domain compositions. GAPDH primers amplify a sequence of 319 nt. Data of one out of two independent experiments with similar results are shown. See *Supplementary Methods* for details.



Supplementary Fig. (2). Single TCRα or TCRβ chains of wt TCR and TCR:mu+cys, but not TCR:ζ Δec, Δic, Δec+ic or parental TCR:ζ, express at the surface of T cells. Jurkat T cells expressing MelA/A2 TCR were transduced with either the TCRα-chain (**A**) or the TCRβ-chain (**B**) of minimal TCR:ζ Δec, Δic, Δec+ic, parental TCR:ζ, wt TCR , TCR:mu+cys or no TCR transgene. Surface expression of transgenic TCR was detected via flow cytometry using anti-TCR-Vα19^{FITC} or anti-TCR-Vβ9^{PE} mAbs and presented as histograms. Percentages of TCRα or TCRβ expression are indicated. Representative examples out of 2 individual measurements are displayed.



Supplementary Fig. (3). TCR:ζ Δic and Δec+ic show no NFAT activation upon stimulation with anti-TCR mAb. Jurkat T cells expressing minimal TCR:ζ Δec, Δic, Δec+ic, parental TCR:ζ, wt TCR**,** TCR:mu+cys 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 6 hours by anti-TCR-Vβ9 mAb or control mIg. Luciferase activities of wt TCR for TCR-Vβ9 mAb stimulation was 1069540 relative luminescence units, which was set to 100% (dotted line). Bars represent mean luminescence units + SEM of 15 to 27 independent measurements. (Statistical significance is based on student's t-test; *** = $p<0.0005$).

REFERENCES

[1] Krieger E, Koraimann G, Vriend G. Increasing the precision of comparative models with YASARA NOVA--a self-parameterizing force field. Proteins 2002; 47: 393-402.

[2] Call ME, Schnell JR, Xu C, Lutz RA, Chou JJ, Wucherpfennig KW. The structure of the zetazeta transmembrane dimer reveals features essential for its assembly with the T cell receptor. Cell 2006; 127: 355-68.

[3] Willemsen RA, Weijtens ME, Ronteltap C, Eshhar Z, Gratama JW, Chames P, et al. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. Gene Ther 2000; 7: 1369-77.