

RESEARCH ARTICLE

A bicistronic vector backbone for rapid seamless cloning and chimerization of $\alpha\beta$ T-cell receptor sequences

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Abstract

To facilitate preclinical testing of T-cell receptors (TCRs) derived from tumor-reactive T-cell clones it is necessary to develop convenient and rapid cloning strategies for the generation of TCR expression constructs. Herein, we describe a pDONRTM221 vector backbone allowing to generate GatewayTM compatible entry clones encoding optimized bicistronic $\alpha\beta$ TCR constructs. It harbors P2A-linked TCR constant regions and head-to-head-oriented recognition sites of the Type IIS restriction enzymes BsmBI and BsaI for seamless cloning of the TCR α and TCR β V(D)J regions, respectively. Additional well-established TCR optimizations were incorporated to enhance TCR functionality. This included replacing of the human $\alpha\beta$ TCR constant regions with their codon-optimized murine counterparts for chimerization, addition of a second interchain disulfide bond and arrangement of the TCR chains in the order β -P2A- α . We exemplified the utility of our vector backbone by cloning and functional testing of three melanoma-reactive TCRs in primary human T cells.

Introduction

Adoptive transfer of *in vitro* expanded tumor infiltrating lymphocytes (TIL) naturally expressing cancer-reactive T-cell receptors (TCR) has yielded promising results in metastatic melanoma [1] and in epithelial cancers [2]. However, this treatment option is confined only to patients, in whom the expansion of functional TIL is successful [3]. This limitation can be overcome by identification and cloning of the $\alpha\beta$ TCR of tumor-reactive T-cell clones to generate $\alpha\beta$ TCR-transduced blood-derived T cells for adoptive therapy [4]. $\alpha\beta$ TCR expression constructs often share optimizations that favor expression of transgenic TCRs and reduce putative mispairing with endogenous α and β TCR chains. These, amongst others, comprise expression of the α and β TCR chains from the same promoter by linking the individual genes with a 2A

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element [5], arrangement in the order β -2A- α [5, 6], replacement of the human constant domains with their murine counterparts (chimerization) [7] and introduction of a second disulfide bond into the $\alpha\beta$ TCR constant domain [8, 9].

As of yet, we employed the MultiSite Gateway™ cloning system [10] to assemble bicistronic $\alpha\beta$ TCR constructs and integrate the optimizations described above. This strategy required about three weeks of hands-on time, mainly due to the need to remove cloning scars between the joined DNA fragments. Here we suggest a TCR-cloning strategy relying on a single universal vector backbone designed to incorporate TCR-specific V(D)J sequences and to generate chimerized and optimized $\alpha\beta$ TCR chains preferentially pairing with each other. The method described herein follows the Golden Gate cloning approach that capitalizes on the unique feature of Type IIS restriction enzymes [11]. These restriction enzymes cut outside of their recognition site which allows to freely choose the resulting overhangs for seamless and directional cloning. Using this approach, we could assemble chimerized and optimized $\alpha\beta$ TCR constructs in two to four days and generate TCR expression vectors in four to six days. We validated our approach with three melanoma-reactive $\alpha\beta$ TCRs retrovirally transduced into primary human T cells. Compared to our previous TCR cloning strategy, we could greatly reduce hands-on time while retaining high cloning efficiency.

Material and methods

Cell lines

293FT cells (kindly provided by Thomas Kindler, University Cancer Center Mainz, Germany) and Phoenix amphotropic packaging cells were cultivated in DMEM. COS-7 cells and the previously described human melanoma cell lines SK29-MEL.1 [12] and D05 [13] were cultured in RPMI 1640. Cell-culture media were supplemented with 10% heat-inactivated FCS (Sigma Aldrich, St. Louis, MO, USA) and 1% Penicillin/Streptomycin (Sigma Aldrich) unless stated otherwise.

T-cell culture

Transgenic T cells were freshly generated by retroviral transduction. T cells were cultured in Panserin (PAN-Biotech, Aidenbach, Germany) supplemented with 10% heat-inactivated human serum (kindly provided by the blood bank of the University Medical Center Mainz), 1% Penicillin/Streptomycin (Sigma Aldrich), rhIL-2 (250 U/mL—600 U/mL; Novartis, Basel, Switzerland). Transgenic T cells were once stimulated with anti-CD3/CD28 beads (Thermo Fisher Scientific, Waltham, MA, USA) on the day after transduction and were then weekly restimulated with irradiated (10,000 Gy) antigen-expressing tumor cells at a stimulator-to-T cell ratio of 1:5. Transgenic T cells were additionally selected with puromycin (1 μ g/mL; Sigma Aldrich).

Cloning and vectors

PCR reactions were set up using the Q5 High-fidelity DNA Polymerase or the *Taq* DNA Polymerase (both NEB, Ipswich, MA, USA). Restriction enzymes (BsmBI, BsaI) and the T4 ligase with appropriate buffers were from NEB and Thermo Fisher Scientific. Where indicated, vectors and PCR products were purified with the Monarch Gel Extraction kit (NEB) or with PCR Clean-up columns (Macherey Nagel, Dueren, Germany). Plasmid preparations were done with the Plasmid kits from Qiagen (Hilden, Germany). 10-beta competent *E. coli* (NEB) were used for plasmid transformation.

The Gateway™ compatible entry vector pDONR™221 (Thermo Fisher Scientific) was modified by standard molecular cloning techniques as described in the results section. The pMXs-IRES-Puro retroviral expression vector (Cell Biolabs, San Diego, CA, USA) served as destination vector. Following the Gateway™ converter system (Thermo Fisher Scientific) the pMXs-IRES-S-Puro vector was converted to a Gateway™ compatible destination vector by insertion of the chloramphenicol resistance gene and the *ccdB* gene flanked by *attR* recognition sites at the multiple cloning site using the XhoI restriction site. In the final destination vector (pMXs-IRES-puro-DEST) the transgene is connected to the puromycin resistance gene by an IRES sequence which allows expression from the same retroviral promoter. Entry vectors were recombined into the final destination vector performing the LR clonase (Thermo Fisher Scientific) reaction.

The retroviral pMP71-PRE plasmid harboring the codon optimized (GeneArt) and chimerized (codon optimized murine constant regions) anti-CDK4^{R24C} TCR derived from the HLA-A*02 restricted T-cell clone 14/35 has previously been described by others [14].

The Firefly luciferase (Fluc) expression plasmid pLenti-EF1a-Pac-T2A-Fluc was generated by replacing the Gaussia luciferase (Gluc) of the pLenti-EF1a-Pac-T2A-Gluc plasmid [15] (kindly provided by Preet Chaudhary, University of Southern California, CA, USA) with the Firefly Luciferase *hluc*⁺ (Promega, Waltham, MA, USA) via Gibson Assembly (NEB).

Sequencing was performed using the services of Eurofins (Luxembourg, Luxembourg). Sequence analyses were made using Geneious software (Biomatters, Auckland, New Zealand, Version 8.1.9).

Production of retroviral particles

Retroviral particles were generated as previously described [16]. In brief, Phoenix amphotropic retroviral packaging cells were cotransfected with the helper plasmids pCOLT-GALV and pHIT60 (both 5 μ g per culture dish) as well as the respective pMXs expression vector (10 μ g per culture dish) using FuGENE6 Transfection Reagent (Promega, Madison, WI, USA) according to the manufacturer's instructions. The next day, medium was replaced with T-cell culture medium and supernatant was harvested after additional incubation for 16 h by pelleting cellular debris.

Retroviral transduction of CD4⁺ and CD8⁺ T cells

CD3⁺ T cells were purified from PBMC using the CD3⁺ selection kit (Miltenyi Biotec, Bergisch Gladbach, Germany). PBMC from healthy donors were provided by the blood bank of our institution according to the guidelines of the local ethics committee (Ethics committee of the Medical Association of Rhineland-Palatinate, Mainz, Germany). All donors gave written informed consent. Transduction of PBMC with retroviral vectors was exclusively performed within a genetic engineering facility (registration number: B 11.110) in accordance with the German Genetic Engineering Act. For retroviral transduction freshly prepared CD3⁺ T cells were activated for 2 days with an anti-CD3 antibody (30 ng/mL; clone: OKT3; hybridoma obtained from ATCC, Manassas, VA, USA) and rhIL-2 (600 U/mL). Subsequently, CD3⁺ T cells were spinofected with virus particles (90 min at 2000 rpm) in the presence of Polybrene (4 μ g/mL; Sigma Aldrich) and rhIL-2 (600 U/mL), and were further cultivated for 22 h or were left untreated. Thereafter, transgenic T cells were cultured as described above. Where indicated CD4⁺ and CD8⁺ T cells were isolated from transgenic CD3⁺ T cells using the CD4⁺ and CD8⁺ selection kit (Miltenyi Biotec), respectively.

Flow cytometry

Cells were surface-stained with the following fluorescent-conjugated antibodies: Anti-V β 1-PE (clone BL37.2, 5 μ l), anti-V β 4-PE (clone WJF24, 5 μ l), anti-CD8-FITC (clone B9.11, 2 μ l), anti-

CD8-PE (clone B9.11, 2 μ l) and IgG-PE/IgG-FITC (clone 679.1Mc7, 4 μ l) was from Beckman Coulter (Brea, CA, USA) and anti-murine TCR-FITC (clone CL075F, 1 μ l) from OriGene (Rockville, MD, USA). Flow cytometry was performed on a FACS Canto II (BD Bioscience, San José, CA, USA) and data was analyzed using FlowJo (BD Bioscience, Version 8.0).

IFN γ -ELISpot assay

IFN γ -ELISpot-assays were performed as described previously [17]. In brief, 293T or COS-7 cells (20,000 cells/well) were transfected with cDNA encoding HLA-A*02:01. Where indicated, cells were cotransfected with cDNA encoding full length CDK4 harboring the R24C mutation or encoding full-length NY-ESO-1. Transfection was performed directly on ELISpot plates (Millipore, Burlington, MA, USA) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Twenty-four hours thereafter transfectants were additionally pulsed with the peptide ACDPHSGHFV (CDK4^{R24C}) or SLLMWITQC (NY-ESO-1), where indicated (both synthesized by Dr. Jan-Wouter Drijfhout, University Medical Center Leiden, The Netherlands). Transgenic T cells (5,000–10,000 TCR⁺ cells/well) were then added to (peptide pulsed) transfectants or to freshly seeded melanoma cells (50,000 cells/well). Where indicated, cells were cultured in the presence of the HLA-A*02 blocking antibody MA2.1 at a concentration of 200 μ g/mL (clone: MA2.1; hybridoma obtained from ATCC). After 20–24 h incubation ELISpot plates were developed and IFN γ spots were visualized with an ImmunoSpot analyzer (Cellular Technology Limited, Cleveland, OH, USA).

Cytotoxicity assay

Cytotoxicity of effector T-cells was evaluated performing a bioluminescence-based lysis assay as recently described [18, 19]. To this end SK29-MEL.1 or D05 melanoma cells stably expressing Firefly luciferase (Fluc) were generated by lentiviral transduction. Melanoma cells (10,000 cells/well) were cocultured with effector cells at the indicated effector-to-target ratio in the presence of 0.15 mg/mL D-Luciferin (Biosynth, St. Gallen, Switzerland). Eighteen hours later, relative luminescence units (RLU) were determined using a FluoStar Omega plate reader (BMG Labtech, Ortenberg, Germany) and a 10 s integration time. Spontaneous cell death was measured in wells containing target cells only, maximum cell death was induced by exposure of target cells to Digitonin (Sigma) at a concentration of 30 μ g/mL. Lysis was calculated using the following equation: $\text{lysis [\%]} = 100 \cdot ((\text{spontaneous RLU} - \text{test unit RLU}) / (\text{spontaneous RLU} - \text{maximum RLU}))$.

Results

Structure of the seamless-cloning vector backbone

In order to accelerate generation of $\alpha\beta$ TCR expression constructs, we designed a Gateway[™] compatible pDONR[™]221 entry vector backbone (VBB) for seamless cloning and chimerization of variable $\alpha\beta$ TCR sequences (summarized in Fig 1). The VBB harbors a bicistronic TCR-expression cassette lacking the TCR α - and TCR β -variable sequences. The TCR-expression cassette comprises the murine TCR α - and TCR β -chain constant regions (mTRAC and mTRBC) separated by the ribosomal skipping element P2A and arranged in the order β -P2A- α . mTRAC and mTRBC are codon-optimized and harbor the previously described T48C and S57C mutations, respectively, resulting in an additional interchain disulfide bond [9]. Following the Golden Gate cloning strategy [11], two different Type IIS restriction enzyme recognition sites were placed each in head-to-head orientation at the 5' end of the mTRBC region (BsaI) and at the P2A-mTRAC junction (BsmBI). Upon plasmid cleavage with BsaI or BsmBI

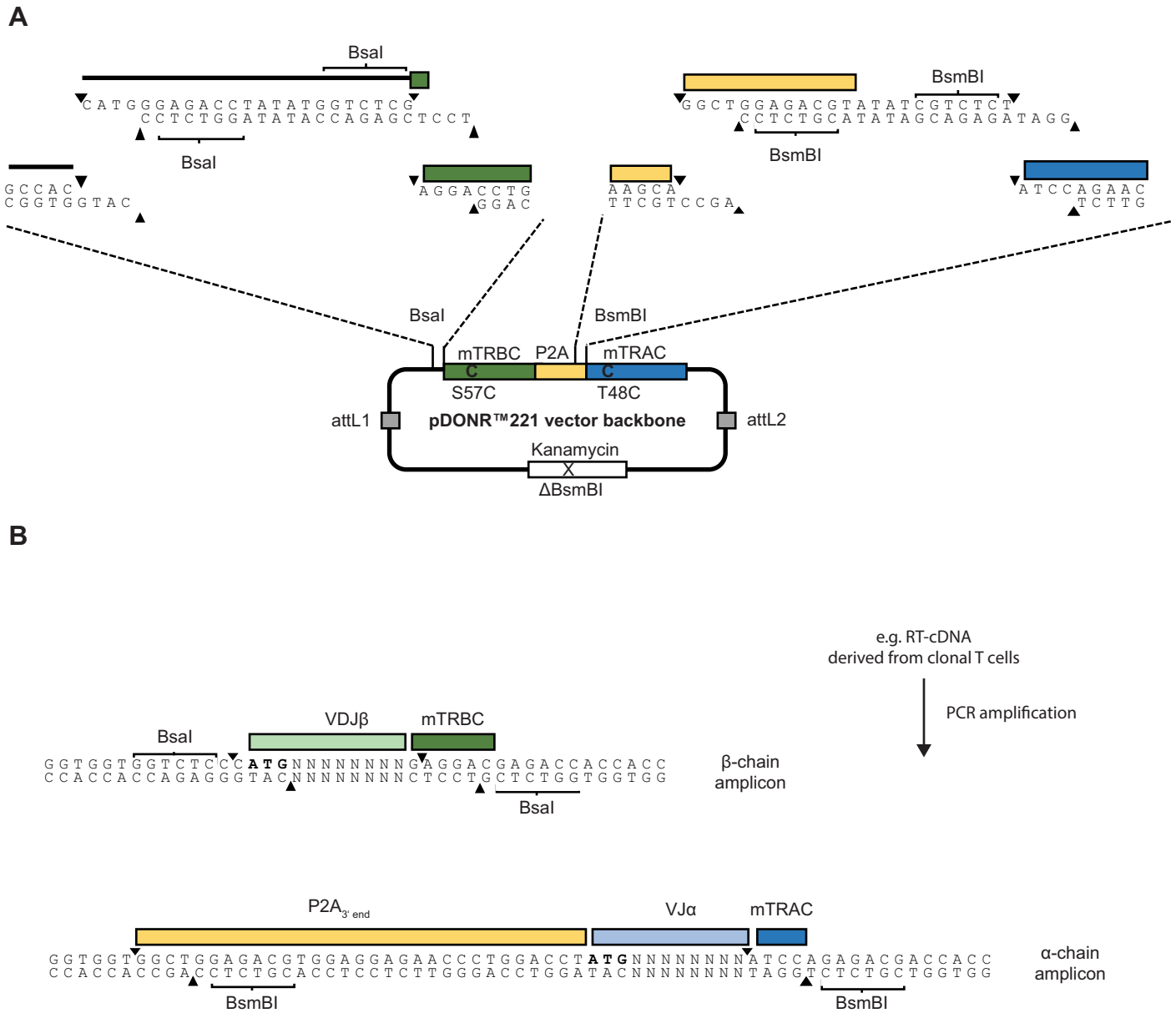


Fig 1. Schematic depiction of the vector backbone for generation of chimerized $\alpha\beta$ TCR constructs by BsmBI and BsaI restriction. (A) The VBB represents a pDONRTM221 vector harboring the invariable elements of a chimerized bicistronic $\alpha\beta$ TCR construct comprising the murine TCR beta and alpha chain constant regions (mTRBC and mTRAC) separated by a P2A element. In addition, two BsaI and BsmBI restriction sites each, are placed in head-to-head orientation at the vector/mTRBC and the P2A/mTRAC junctions, respectively, leading to unique overhangs upon cleavage. A naturally occurring BsmBI-restriction site in the Kanamycin resistance gene has been removed by site-directed mutation. (B) The TCR α chain VJ (VJ α) and the TCR β chain VDJ (VDJ β) regions of the TCR of choice are flanked during PCR amplification with BsmBI- or BsaI-restriction sites, respectively, to generate appropriate overhangs. PCR fragments are then restricted and ligated into the vector backbone yielding the full-length chimerized $\alpha\beta$ TCR construct. Spikes indicate cleavage sites of the Type IIS restriction enzymes required for generation of homologous overhangs and removal of non-coding or redundant sequences.

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non-compatible overhangs are generated (Fig 1A). These unique overhangs were then used to directionally and scarlessly insert PCR-amplified TCR α - and TCR β -variable sequences to assemble full length chimerized TCR (cTCR) constructs. To this end the antigen-specific VJ α and VDJ β regions are flanked during PCR amplification with BsmBI and BsaI restriction sites, respectively (Fig 1B). Overhangs are designed to allow precise and seamless ligation of the VJ α

Table 1. Primer used to generate TCR α and TCR β V(D)J fragments.

V(D)J	Primer	Sequence 5'-3'
TCR α	BsmBI.alpha.for	GGTGGT <u>GGCT</u> GGAGACGTGGAGGAGAACCCTGGACCT NNNNNN.CTCTGC. . .
	BsmBI.alpha.rev	GGTGGT <u>CGTCTCT</u> GGAT NNNNNN. . .
TCR β	BsaI.beta.for	GGTGGTGGT <u>TCC</u> CATG NNNNNN. . .
	BsaI.beta.rev	GGTGGTGGT <u>TCG</u> TCCTC NNNNNN..

Type IIS restriction enzyme recognition sites are underlined, unique overhangs generated upon cleavage are shown in boldface type and TCR-specific sequences are indicated by N.

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fragment at the P2A/mTRAC junction and of the VDJ β fragment at the 5' end of the mTRBC region of the VBB. Of note, it is crucial to reconstruct the P2A element via primer design since the 3' end of P2A is excised upon BsmBI restriction of the VBB due to a naturally occurring BsmBI restriction site within the P2A element. Core primer sequences used to amplify VJ α and VDJ β fragments are given in [Table 1](#).

The resulting bicistronic chimerized $\alpha\beta$ TCR construct can then be subcloned into any Gateway™ compatible expression vector performing the Gateway™ LR clonase reaction for further testing.

Assembly of bicistronic chimerized $\alpha\beta$ TCR constructs

We employed our VBB to either assemble $\alpha\beta$ TCR expression constructs *de novo* using as starting material RT-cDNA transcribed from clonal T cells—the respective TCR sequence had been identified following the methods described by Boria et al. [20] and Birkholz et al. [21]—or we re-constructed $\alpha\beta$ TCRs that had been previously cloned but e.g. had not been chimerized. Specific primer used to assemble all cTCR expression constructs in this study along with specification of the starting material (RT-cDNA or plasmid DNA) are given in [S1 Table](#).

In initial experiments, $\alpha\beta$ TCR constructs were generated in a two-step process summarized in [Fig 2](#). In the first step the cTCR α chain was assembled ([Fig 2A](#)). VJ α fragments were flanked with BsmBI recognition sites by PCR, column purified, digested by BsmBI and were again column purified. We then ligated the VJ α fragments into BsmBI restricted and gel eluted VBB, transformed the recombined plasmid and verified successful assembly of the cTCR α chain by colony PCR and Sanger sequencing of positive clones. [S2 Table](#) summarizes primer used to identify positive transformants via colony PCR and primer used for sequencing. In the second step we assembled the cTCR β chain following a similar procedure ([Fig 2B](#)). After successful assembly, the full-length cTCR construct was then subcloned into the Gateway™ compatible expression vector pMX-IRES-puro-DEST that had been generated as described in the Material and Methods section ([Fig 2C](#)). Using this approach cloning of the final cTCR expression vector was usually achieved within only six days. An exemplary result is shown in [Fig 3](#) showing assembly of a cTCR expression vector using as template plasmid DNA harboring a non-optimized $\alpha\beta$ TCR derived from the tumor-reactive T-cell clone 55/74. Assembly of both the cTCR α and cTCR β chain was highly efficient with 6/6 and 5/5 positive clones, respectively, as determined by colony PCR ([Fig 3A and 3B](#)). As expected subcloning of the full-length cTCR construct into the Gateway™ compatible pMXs-IRES-puro-DEST expression vector was also highly efficient ([Fig 3C](#)). Using this two-step approach, we successfully constructed eight different cTCRs using as starting material either RT-cDNA (n = 2) or plasmid DNA (n = 6). The average efficiency (n = 8) for assembly of the cTCR α chain and cTCR β chain as determined by colony PCR was 98% and 84%, respectively ([S3 Table](#)).

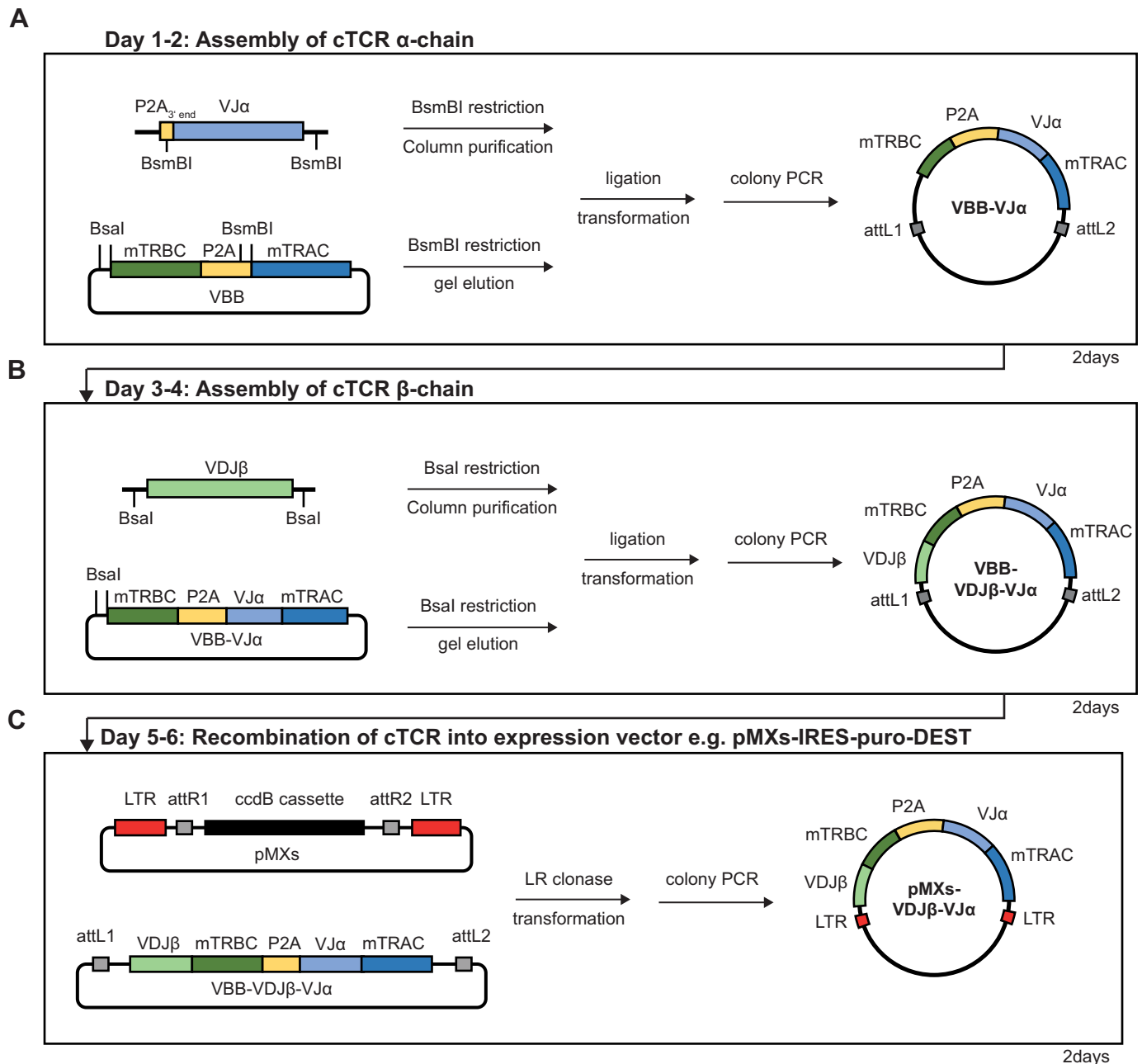


Fig 2. Workflow for generation of cTCR expression constructs using the VBB. (A) Day 1–2: The VJ α fragment was generated by PCR, column purified, restricted by BsmBI and again column purified. VBB was restricted by BsmBI and gel eluted. Purified VBB and VJ α fragments were ligated and the recombined plasmid was transformed. Colony PCR was used to identify clones carrying the insert (VBB-VJ α). (B) Days 3–4: VDJ β fragment was generated by PCR, column purified, restricted by BsaI and again column purified. Plasmid from VBB-VJ α was prepared, restricted by BsaI and gel eluted. Purified VBB-VJ α and VDJ β fragments were ligated and the recombined plasmid was transformed. Colony PCR was used to identify clones carrying the insert (VBB-VDJ β -VJ α). Days 5–6: Plasmid from VBB-VDJ β -VJ α was prepared and recombined into a Gateway compatible expression vector e.g. pMXs-IRES-puro-DEST performing the LR-clonase reaction. Positive clones (pMXs-VDJ β -VJ α) were identified by colony PCR.

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In an effort to further reduce hands-on time and material usage we combined BsaI and BsmBI restriction of both the VBB and the column-purified V(D)J-encoding PCR products as well as ligation into the vector backbone in a one-step, one-vessel restriction/ligation reaction. An example of successful *de novo* assembly of cTCR expression constructs from RT-cDNA

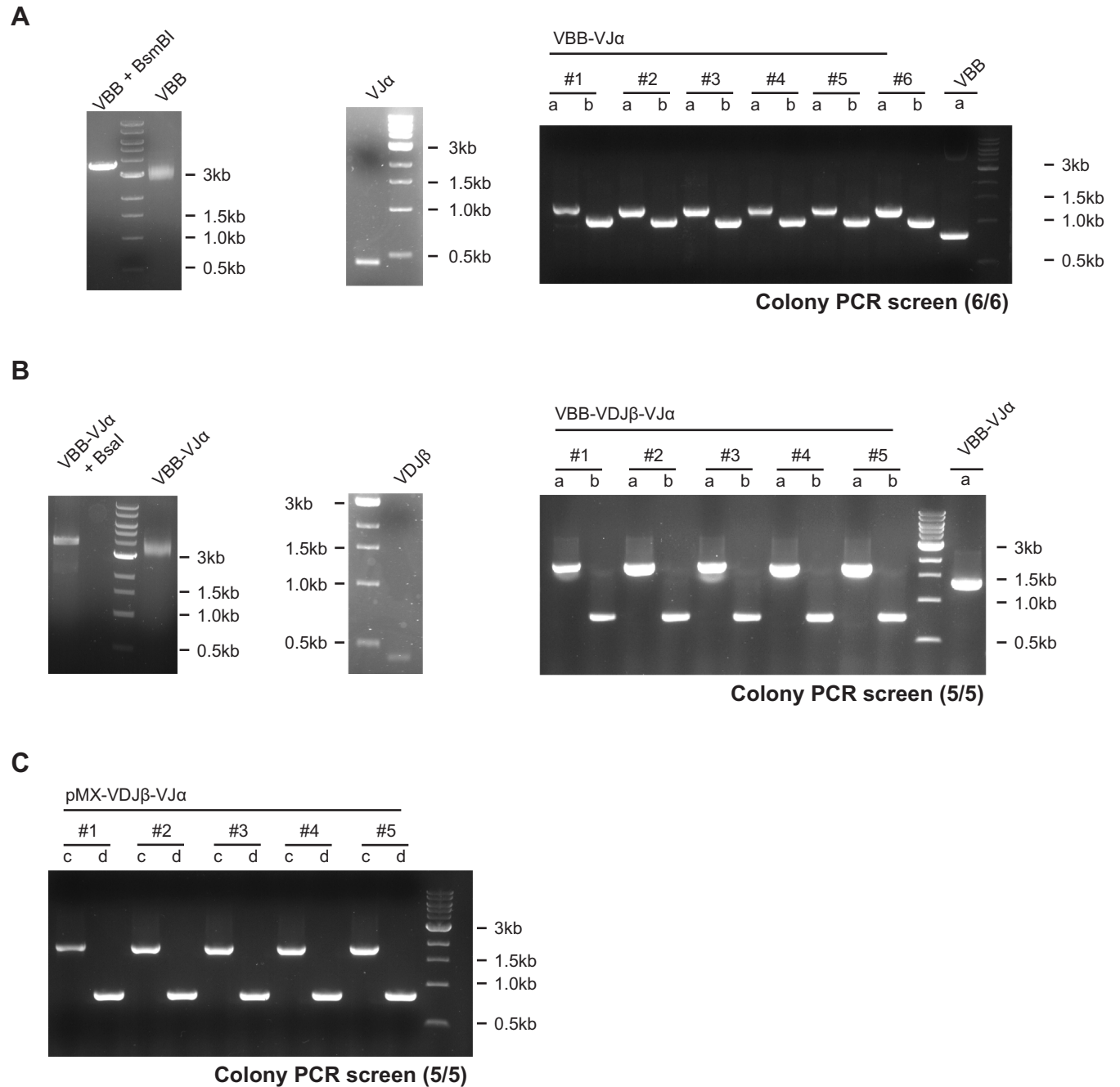


Fig 3. Assembly of a cTCR derived from T-cell clone 55/74. Workflow as shown in Fig 2. (A) Left: The VBB was restricted by BsmBI and gel purified. Uncut VBB served as control. Middle: The VJ α fragment was amplified by PCR, loaded on a gel to verify correct amplification, column purified and restricted by BsmBI and column purified. Right: Colony PCR was used to screen VBB-VJ α positive clones using primer pairs a (M13.for, mTRAC.p670.rev) and b (M13.for, mTRBC.p618.rev). VBB served as control. (B) Left: The VBB was restricted by BsaI and gel purified. Uncut VBB-VJ α served as control. Middle: The VDj β fragment was amplified by PCR using the primer given in S1 Table, loaded on a gel to verify correct size, restricted by BsaI and column purified. Right: Colony PCR screen used to identify VBB-VDj β -VJ α positive clones using primer pairs a and b. VBB-VJ α served as control. (C) LR-clonase reaction was performed to recombine the cTCR construct into our pMXs expression vector. Colony PCR screen used to identify pMXs-VDj β -VJ α clones with primer pairs c (pMXs.for, mTRAC.p670.rev) and d (pMXs.for, mTRBC.p618.rev).

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derived from the melanoma reactive T-cell clones 2C/406 and 5C/169 using the one-step approach is shown in [S1 Fig](#). From the respective 8 analyzed colonies 3 (5C/169) and 8 (2C/406) colonies were screened positive for the full-length cTCR construct corresponding to a cloning efficiency of 38% and 100% respectively. The average cloning efficiency of the one-step approach ($n = 6$) was 46% as summarized in [S3 Table](#). Of note, the lowest cloning efficiency was observed in the presence of additional Type IIS restriction enzyme recognition sites within the V(D)J-encoding PCR products resulting in only 9% (T-cell clone 4/134) and 15% (T-cell clone 4/76) positive colonies when performing the one-step approach. Collectively these results suggest that although the one-step approach allows to generate a full-length cTCR construct within only 2 days (and the final expression vector within 4 days) the cloning efficiency is drastically reduced as compared to the standard two-step approach as shown in [Fig 2](#).

Generation of transgenic T cells expressing cTCRs using the seamless-cloning vector backbone

To functionally validate our $\alpha\beta$ TCR cloning vector backbone we chose the well-characterized CD8⁺ T-cell clone 14/35 directed against mutant CDK4 (R24C) that has originally been established via an autologous mixed lymphocyte-tumor culture (MLTC) using the CDK4^{R24C}-positive melanoma-cell line SK29-MEL.1 as stimulator of blood-derived T cells [12]. Its HLA-A*02-restricted $\alpha\beta$ TCR has been cloned by others into the retroviral expression vector pMP71-PRE [14]. In addition, we cloned two $\alpha\beta$ TCR *de novo* using as starting material RT-cDNA transcribed from RNA derived from two HLA-A*02 restricted NY-ESO-1 specific CD8⁺ T cell clones. The respective T cell clones 4/76 and 4/134 had been established in our group via MLTCs using the HLA-A*02⁺ NY-ESO-1⁺ melanoma cell line D05 as stimulator of blood-derived T cells and recognized the same NY-ESO-1 peptide (SLLMWITQC). Using the primer given in [S1 Table](#) and the pMP71-PRE plasmid harboring the anti-CDK4^{R24C}/A2 $\alpha\beta$ TCR construct or RT-cDNA derived from the T cell clones 4/76 or 4/134 as template we PCR-amplified the respective TCR α and TCR β V(D)J regions and generated pDONR[™]221 entry clones as described above. Subsequently, the final bicistronic $\alpha\beta$ TCR constructs were recombined into the retroviral expression vector pMXs-IRES-Puro-DEST by performing the Gateway[™] LR-clonase reaction.

After assembly of the respective retroviral expression constructs, we transduced the anti-NY-ESO-1/A2 cTCRs 4/76 and 4/134 and the anti-CDK4^{R24C}/A2 cTCR 14/35 into CD3⁺ T cells. After short term expansion in the presence of puromycin, CD4⁺ and CD8⁺ populations were enriched to high purity (above 95%) by MACS isolation. As shown in [Fig 4A](#) we observed high expression levels of all three cTCRs in both CD4⁺ and CD8⁺ T cells. Expression of cTCRs was verified with both a mAb specific for the V β chain of the respective cTCR and a mAb recognizing the murine TCR constant region, which gave similar results. As shown in [Fig 4B](#) puromycin selection was crucial in order to obtain high expression levels of cTCRs. Of note, additional cTCRs ($n = 9$) cloned in this study had been successfully expressed in primary T cells with an average expression level of 80% on day 4–17 after transduction ([S4 Table](#)).

Next, we analyzed specificity and functionality of CD4⁺ and CD8⁺ T cells transduced with the anti-NY-ESO-1/A2 cTCRs 4/76 and 4/134 or the anti-CDK4^{R24C}/A2 cTCR 14/35 in 24 h IFN γ -ELISpot assays. In a first step, redirected CD4⁺ and CD8⁺ T cells were incubated with 293T or COS-7 cells transfected with HLA-A*02 and loaded with titrated amounts of the NY-ESO-1 peptide (SLLMWITQC) or the CDK4^{R24C} peptide (ACDPHSGHFV), respectively ([Fig 5A](#)). Analysis of the anti-NY-ESO-1 specific cTCRs and the anti-CDK4^{R24C} cTCR revealed a similar dose dependent functionality in both CD4⁺ and CD8⁺ T cells. To further analyze recognition of endogenously processed antigen, 293T or COS-7 cells were transfected

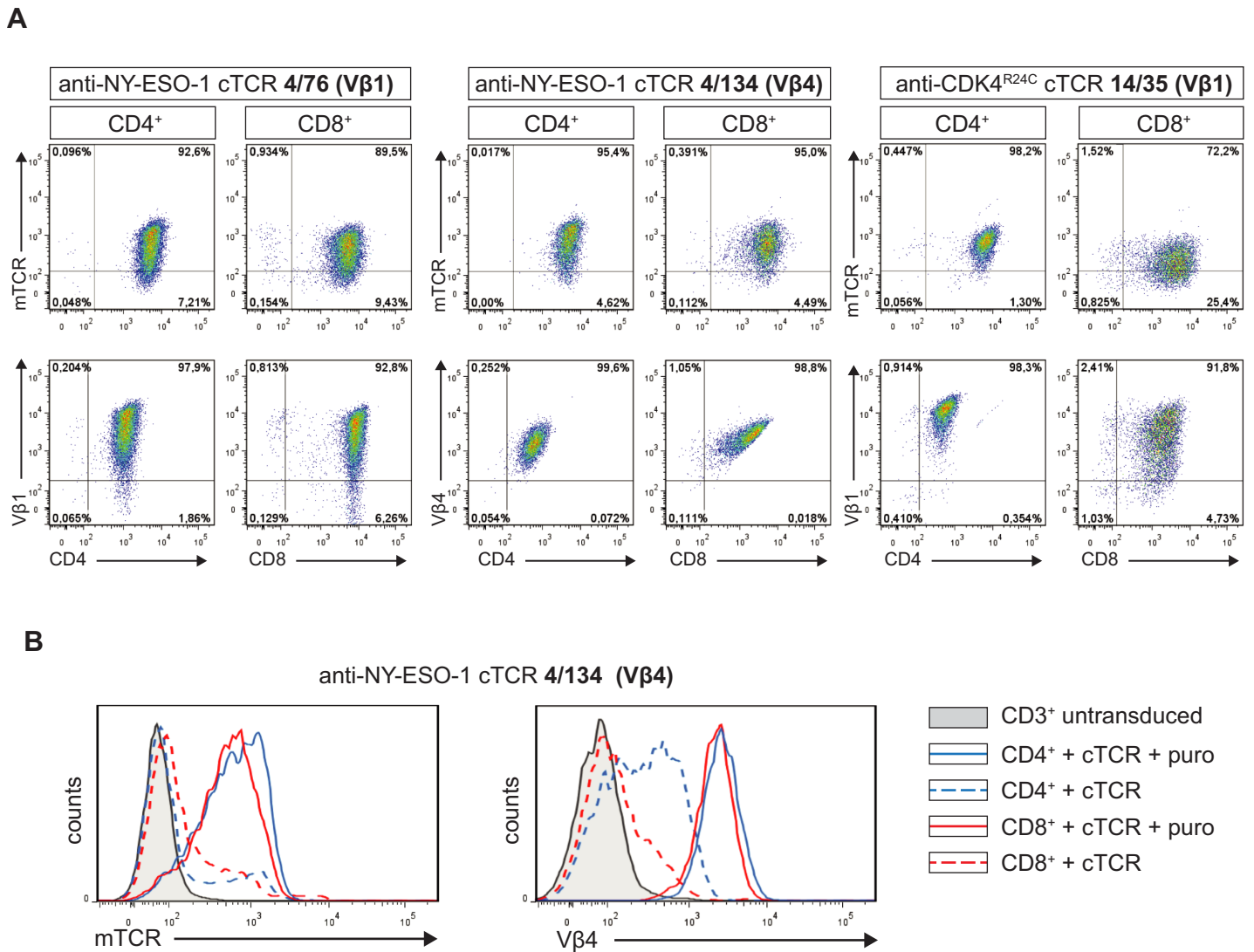


Fig 4. Expression of cTCRs in CD4⁺ and CD8⁺ T cells. (A) CD4⁺ and CD8⁺ T cells were retrovirally transduced with the indicated cTCR and expanded in the presence of puromycin. Flow cytometry analysis depicting expression of the indicated cTCR in CD4⁺ and CD8⁺ T cells. (B) CD4⁺ and CD8⁺ T cells were retrovirally transduced with the anti-NY-ESO-1/A2 cTCRs 4/134 and were expanded in the presence or absence of puromycin. Flow cytometry analysis showing expression of cTCR in CD4⁺ and CD8⁺ T cells as compared to untransduced CD3⁺ T cells. (A-B) Antibodies directed against the murine TCR constant region (mTCR) or against the variable region (Vβ) of the respective cTCR were used.

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with HLA-A*02 and were cotransfected with titrated amounts of NY-ESO-1 or CDK4^{R24C} cDNA, respectively (Fig 5B). Interestingly, CD8⁺ 4/134 cTCR-transduced T cells recognized NY-ESO-1 cDNA transfected targets while no response was observed with CD4⁺ T cells expressing the same cTCR. In contrast, both CD4⁺ and CD8⁺ T cells transduced with the 14/35-cTCR or with the 4/76-cTCR displayed similar responses against antigen-cDNA transfected target cells. These results were also mirrored when using as targets HLA-A*02⁺ melanoma cell lines that naturally expressed NY-ESO-1 (D05) or harbored the CDK4^{R24C} mutation (SK29-Mel.1). The response of 4/76 cTCR-transduced T cells against D05 cells and of 14/35 cTCR-transduced T cells against SK29-MEL.1 was similar for CD4⁺ and CD8⁺ T cells (Fig 5C). In contrast CD4⁺ 4/134 cTCR-transduced T cells did not recognize D05 cells despite clear

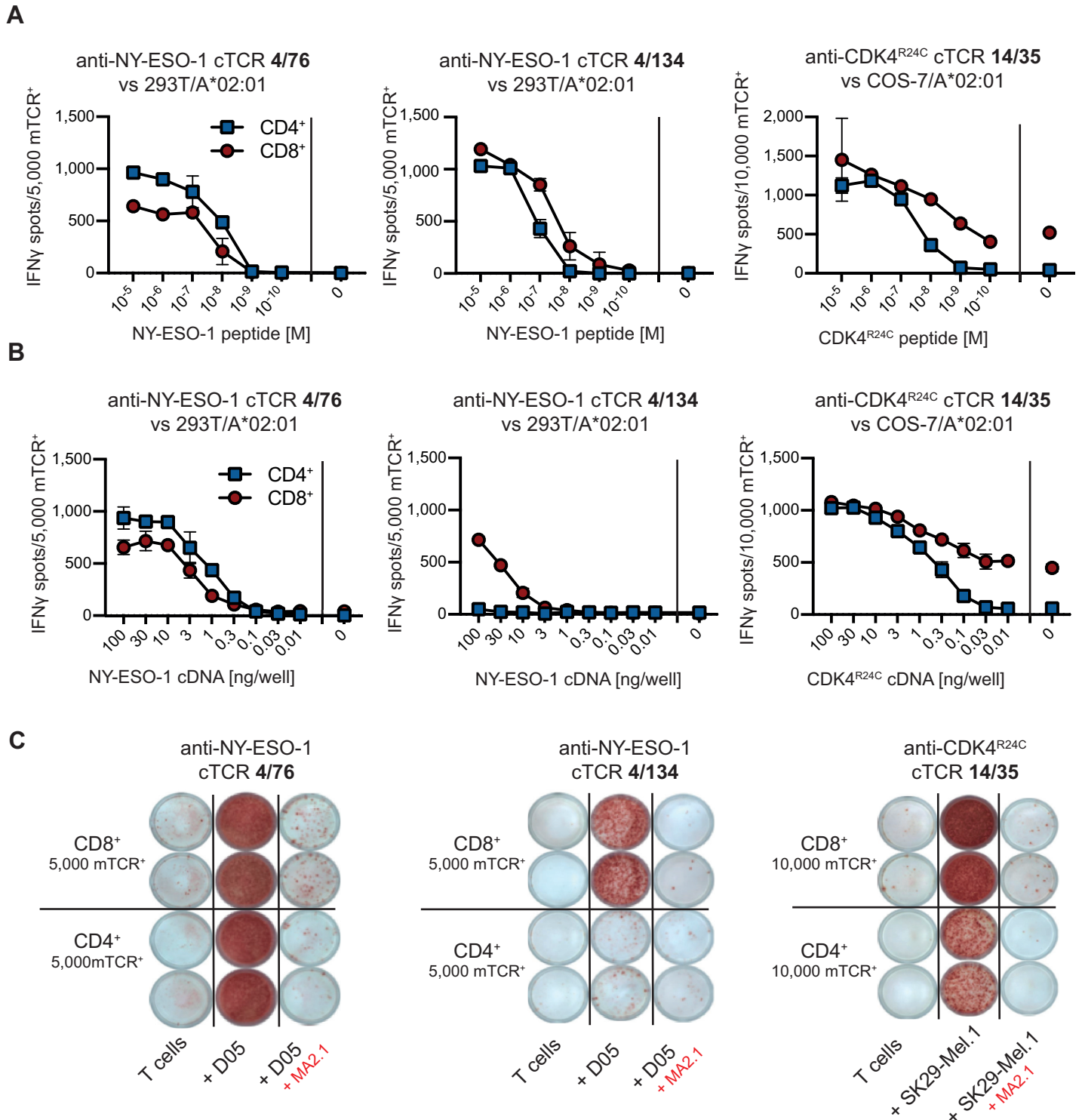


Fig 5. cTCR⁺ CD4⁺ and CD8⁺ T cells specifically recognize antigen expressing target cells. IFN γ production of CD4⁺ and CD8⁺ T cell transduced with the indicated cTCR as determined in 24 h ELISpot assays. cTCR expression was analyzed prior to testing and results were used to correct for the amount of cTCR⁺ cells within different T cell populations applied to functional tests. (A-C) IFN γ production after incubation of T cells with HEK-293T or COS-7 cells transiently transfected with HLA-A*02:01 and (A) loaded with titrated amounts of the indicated peptide or (B) cotransfected with titrated amounts of cDNA encoding the indicated antigen. (C) IFN γ production after incubation of T cells with either no target cells or with NY-ESO-1⁺ HLA-A*02⁺ D05 cells or CDK4^{R24C}⁺ HLA-A*02⁺ SK29-MEL.1 cells in the presence or absence of the HLA-A*02 blocking antibody MA2.1. Data points represent mean values of doublets \pm standard deviation.

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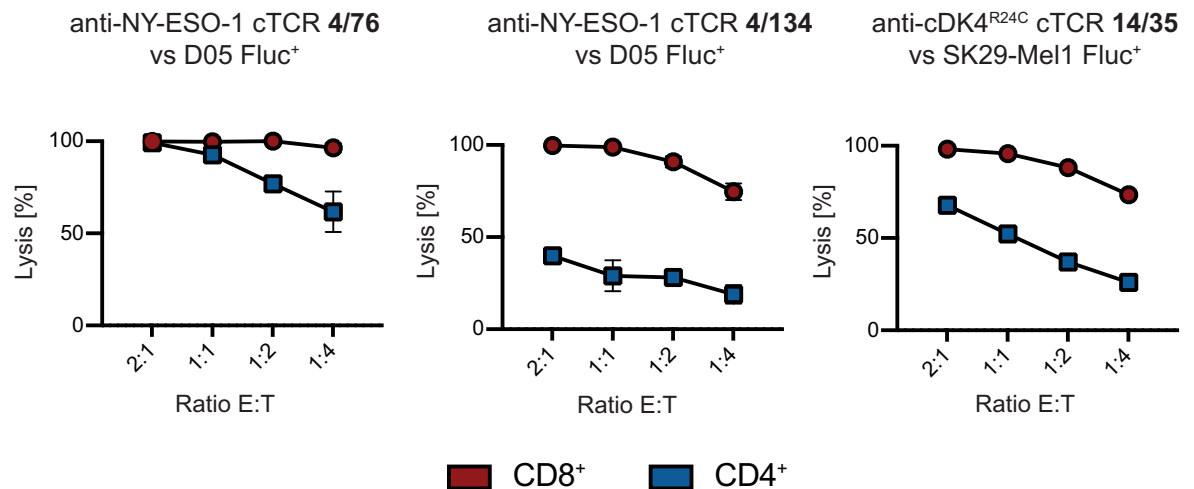


Fig 6. Lytic activity of cTCR⁺ CD4⁺ and CD8⁺ T cells. Cytotoxicity of T cells transduced with the indicated cTCR as determined by a 18 h-bioluminescence assay. cTCR expression was analyzed prior to testing and results were used to correct for the amount of cTCR⁺ cells within different T cell populations applied to functional tests. Target cells were either Firefly luciferase expressing NY-ESO-1⁺ HLA-A*02⁺ D05 cells or CDK4^{R24C} HLA-A*02⁺ SK29-Mel.1 cells as indicated. Data points represent mean values of triplicates \pm standard deviation.

<https://doi.org/10.1371/journal.pone.0238875.g006>

recognition by the respective CD8⁺ population. Addition of an HLA-A*02 blocking antibody (MA2.1) confirmed MHC I-restriction of target recognition.

Finally, we analyzed whether cTCR-transgenic T cells also elicit target cell lysis in a bioluminescence-based cytotoxicity assay (Fig 6). D05 and SK29-MEL.1 cells stably expressing Firefly Luciferase were cocultured with redirected CD4⁺ or CD8⁺ transgenic T cells. Analysis of the bioluminescence signal after 18 h essentially mirrored the results shown before. Redirected CD8⁺ T cells showed strong cytotoxicity against antigen positive target cells. CD4⁺ T cells transduced with the same cTCRs were also able to eliminate target cells but to a much lower extent. Of note, and as expected from the results shown before, cytotoxicity of CD4⁺ T cell transduced with the 4/134 cTCR against NY-ESO-1⁺ HLA-A*02⁺ D05 melanoma cells was considerably lower as compared to CD4⁺ T cell transduced with the 4/76 cTCR.

Collectively, we cloned seven $\alpha\beta$ TCR *de novo* using as starting material RT-cDNA transcribed from as little as 30 ng of RNA derived from clonal T cells. The respective TCR variable regions had been identified following the methods described by Boria et al. [20] and Birkholz et al. [21] as already stated above. Furthermore, we re-constructed six $\alpha\beta$ TCRs that had been previously cloned but had not been chimerized or had been cloned in the order α -P2A- β . Compared to the parental T-cell clones or the non-optimized $\alpha\beta$ TCR we observed no loss of function and specificity (not shown).

Discussion

We report on a vector backbone for the generation of $\alpha\beta$ TCR constructs. It harbors the uniform components of a bicistronic chimerized $\alpha\beta$ TCR construct. TCR-specific V(D)J-regions are ligated into this backbone using Type IIS restriction enzymes to assemble full length $\alpha\beta$ TCR chains. This approach is based on the Golden Gate cloning method [11]. A similar strategy has been applied by others for the same purpose [22, 23], but the vector backbone presented herein displays several unique and distinct features of additional value.

Hu et al. generated full-length TCR constructs by Golden Gate cloning, but ligated only the hypervariable CDR3 region into components of an extensive vector backbone library,

members of which each encode one of the different TCR α and β variable sequences [22]. In contrast, our TCR cloning approach is based on a single, uniform vector backbone, which harbors only the TCR constant domains. Although this requires to ligate the complete, and compared to the sole CDR3 region, considerably longer TCR V(D)J sequences into the vector backbone, there is no need to generate and maintain a plasmid library covering all TCR α and β variable sequences. More similar to our approach, Coren et al. generated vector backbones encoding either human or macaque TCR constant regions with head-to-head oriented recognition sites for the Type IIS restriction enzyme BbsI at the vector/TRAC and the P2A/TRBC junctions [23]. TCR α and β variable sequences were flanked with BbsI recognition sites and appropriate overhangs during PCR amplification, and were seamlessly ligated into the vector backbone for assembly of human/macaque $\alpha\beta$ TCR constructs [23]. While the vector backbones of both groups represent final expression vectors, we first assembled pDONR[™]221 entry vectors and, in a second step, recombined the final $\alpha\beta$ TCR construct into a Gateway[™] compatible expression vector. Although this adds an additional cloning step, our approach integrates the convenient Gateway[™] cloning system therefore allowing to use different (Gateway[™] compatible) expression vectors. Of note, several different expression vectors used to study TCR-based therapy have already been converted to Gateway[™] compatibility [24, 25].

Another distinctive feature of our backbone plasmid is the usage of two different Type IIS restriction enzymes, BsmBI and BsaI, for the assembly of the TCR α and β chain, respectively. This offers advantages when variable TCR regions of interest harbor a BsmBI or BsaI restriction site. E.g. if a naturally occurring BsaI restriction site would be present in the TCR α VJ-sequence of interest, the two-step cloning procedure would be chosen to start with BsaI-mediated assembly of the TCR β chain (and vice versa). This is particularly important considering gel-purification of the premature construct after assembly of the first TCR chain and subsequent restriction to circumvent purification of two fragments. When the same Type IIS restriction site (BsmBI or BsaI) is present in both subcloned TCR α and β fragments we would suggest to remove at least one restriction sites e.g. by site-directed mutation or in fact purify two vector backbone fragments.

When performing our standard cTCR assembly approach (summarized in Fig 2) we did observe a very high cloning efficiency that was considerably lower when combining both restriction/ligation of both TCR α and β chains as shown in S1 Fig. This difference might be a result of either the purification steps implemented in our standard protocol or could be due to more efficient ligation of only two fragments when assembling the TCR α and β chains separately. Interestingly, in the presence of internal Type IIS restriction sites in the subcloned fragments, we observed a drastically reduced cloning efficiency when performing restriction/ligation in the same vessel. This is presumably due to continuous restriction and relegation of the assembled vector as also observed by others [11, 23]. A strategy to circumvent this problem is performing restriction and ligation steps sequentially in the same vessel, e.g., by heat inactivation of the restriction enzyme [11].

Our vector backbone integrates many of the optimizations known to enhance transgenic TCR expression and functionality, i.e. (i) codon optimization, (ii) β -P2A- α order, (iii) an additional interchain disulfide bond and (iv) chimerization. Codon optimization is known to positively regulate transcription [26], while the β -P2A- α order is considered to avoid premature degradation of the TCR α chain [5]. Chimerization of TCRs [7] and addition of an interchain disulfide bond [8, 9] have been shown to greatly enhance T-cell reactivity, which was mainly attributed to enhanced TCR expression as a result of preferential pairing of modified TCRs in the presence of endogenous human TCRs. Moreover, it has been shown that chimerization (alike minimal murinization) of TCRs not only increases transgenic TCR expression with regard to percentage and mean fluorescence intensity (MFI) but also leads to enhanced TCR/

CD3 stability [7, 27]. It was suggested that this, independently from TCR expression, enhances transgenic TCR functionality although the underlying mechanisms are not yet fully understood [27]. The TCR modifications provided by our backbone vector are especially useful to prevent mispairing with endogenous TCR chains. Autoreactivity of mispaired TCR is a major safety concern in current clinical trials on TCR transfer [28]. Chimerization of transgenic TCRs is one of the safety measures applied, although induction of xenogenic immune reactions is a concern when using murine sequences. However, in a pivotal human trial conducted with fully murine TCRs, cancer regression was observed and no adverse events were attributed to the murine sequences [29]. Although immune responses against murine TCR components have been detected, no impact on clinical outcome has been described [30]. Nevertheless, it has been suggested that a minimal murinization may reduce the risk of immunogenicity [27, 31].

Our functional data confirmed that the vector backbone can be reliably used to express cTCRs in primary T cells and that redirected T cells are functional in a clear HLA-restricted and antigen-specific manner. Functionality of the MHC I-dependent cTCRs analyzed in this study was not restricted to transgenic CD8⁺ T cells but was also observed upon expression in CD4⁺ T cells. This is in line with several other reports showing that CD4⁺ T cells transduced with MHC I-restricted TCRs recognize the respective peptide/MHC I complex and display cytotoxic activity both in vitro [32] as well as in vivo [33]. The ability of CD4⁺ T cells transduced with an MHC I-restricted TCR to circumvent the CD8-coreceptor may resemble a measure of TCR avidity [32]. Along this line another study described CD8-independent function only upon transfer of a highly peptide/MHC I-avid TCR while low avidity MHC-I restricted TCRs did not function in CD4⁺ T cells [34]. Our functional data on the A*02/NY-ESO-1-specific cTCRs derived from T cell clone 4/76 and 4/134 thus suggest a higher avidity of the TCR derived from clone 4/76. In addition, these results implicate the CD8 molecule as an enhancer of functional avidity of MHC I-restricted TCRs as also suggested by others [35].

In conclusion, the TCR-cloning strategy described herein allows to rapidly generate optimized $\alpha\beta$ TCR expression constructs. The TCR optimizations integrated in the vector backbone are known to enhance transgenic TCR expression and functionality in part by preventing formation of mispaired TCRs. The vector backbone presented herein might therefore represent a useful tool for the preclinical investigation of human $\alpha\beta$ TCRs in particular when using primary T cells expressing endogenous TCRs as effector cells. Collectively our results demonstrate the utility and versatility of our vector backbone for rapid cloning and simultaneous optimization of functional $\alpha\beta$ TCR.

Supporting information

S1 Table. Primer used to amplify VJ α and VDJ β fragments of all cTCR constructs generated in this study. Antigen specificity and HLA-restriction of the respective TCR is given where known. ^aT cell clones 5C/169 and 2C/406 express an identical TCR and are derived from the same MLTC. ^bThe sequence 5' of the BsaI restriction site of primer BsaI_BC44.22_VDJ β .rev has been modified to prevent hairpin formation. Template during PCR was either plasmid DNA or RT-cDNA derived from clonal T cells. nd = not determined. (XLSX)

S2 Table. Primer sequences for colony PCR and Sanger sequencing. (XLSX)

S3 Table. Cloning efficiencies using the standard two-step and the one-step cloning approach. Number of positive clones were determined by colony PCR. SD = Standard

deviation.
(XLSX)

S4 Table. Transduction efficiencies. Summary of all transgenic T cells that were generated by retroviral transduction of TCRs assembled using the seamless-cloning approach. Shown are the percentage of mTCR⁺ clones among the indicated transduced T-cell population as determined by FACS analysis. SD = Standard deviation.
(XLSX)

S1 Fig. Assembly of cTCR expression constructs using the one-step cloning approach. (A) Workflow of the one-step cTCR assembly approach. Unrestricted, column purified VJ α and VDJ β PCR fragments, the unrestricted vector backbone, BsaI and BsmBI, T4 ligase and T4 ligase buffer were mixed and 30 cycles of restriction/ligation were performed (5 min at 37°C, 5 min at 16°C). (B) VJ α and VDJ β fragments were amplified from RT-cDNA derived from the indicated T cell clones using the primer given in S1 Table, loaded on a gel to verify correct amplification and were column purified. Then one-step assembly was performed and recombinant plasmids were transformed. (C) Colony PCR was used to identify VBB-VDJ β -VJ α positive clones using primer pairs a (M13.for, mTRAC.p670.rev) and b (M13.for, mTRBC.p618.rev). (C) LR-clonase reaction was performed to recombine the cTCR construct into our pMXs-IRES-puro-DEST expression vector. Colony PCR screen was used to identify pMXs-VDJ β -VJ α clones using primer pairs c (pMXs.for, mTRAC.p670.rev) and d (pMXs.for, mTRBC.p618.rev).
(EPS)

S1 Raw images.
(PDF)

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