

Generation of TGFβR2(-1) neoantigen-specific HLA-DR4-restricted T cell receptors for cancer therapy

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ABSTRACT

Background Adoptive transfer of patient's T cells, engineered to express a T cell receptor (TCR) with defined novel antigen specificity, is a convenient form of cancer therapy. In most cases, major histocompatibility complex (MHC) I-restricted TCRs are expressed in CD8⁺ T cells and the development of CD4⁺ T cells engineered to express an MHC II-restricted TCR lags behind. Critical is the choice of the target antigen, whether the epitope is efficiently processed and binds with high affinity to MHC molecules. A mutation in the transforming growth factor β receptor 2 (TGFβR2(-1)) gene creates a frameshift peptide caused by the deletion of one adenine (-1) within a microsatellite sequence. This somatic mutation is recurrent in microsatellite instable colorectal and gastric cancers and, therefore, is a truly tumor-specific antigen detected in many patients.

Methods ABabDR4 mice, which express a diverse human TCR repertoire restricted to human MHC II molecule HLA-DRA/DRB1*0401 (HLA-DR4), were immunized with the TGFβR2(-1) peptide and TGFβR2(-1)-specific TCRs were isolated from responding CD4⁺ T cells. The TGFβR2(-1)-specific TCRs were expressed in human CD4⁺ T cells and their potency and safety profile were assessed by co-cultures and other functional assays.

Results We demonstrated that TGFβR2(-1) neoantigen is immunogenic and elicited CD4⁺ T cell responses in ABabDR4 mice. When expressed in human CD4⁺ T cells, the HLA-DR4 restricted TGFβR2(-1)-specific TCRs induced IFNγ expression at low TGFβR2(-1) peptide amounts. The TGFβR2(-1)-specific TCRs recognized HLA-DR4⁺ lymphoblastoid cells, which endogenously processed and presented the neoantigen, and colorectal cancer cell lines SW48 and HCT116 naturally expressing the TGFβR2(-1) mutation. No MHC II alloreactivity or cross-reactivity to peptides with a similar TCR-recognition motif were observed, indicating the safety of the TCRs.

Conclusions The data suggest that HLA-DR4-restricted TCRs specific for the TGFβR2(-1) recurrent neoantigen can be valuable candidates for adoptive T cell therapy of a sizeable number of patients with cancer.

BACKGROUND

Adoptive T cell therapy (ATT) with T cell receptor (TCR)-engineered T cells has become a promising option for cancer treatment. Clinical trials with TCR-engineered T cells showed objective responses¹ but also

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Recurrent somatic mutations leading to targetable neoantigens are rare. The few described are presented by major histocompatibility complex (MHC) I molecules to CD8⁺ T cells. Case studies showed tumor regression with tumor-infiltrating lymphocytes containing neoantigen-specific CD4⁺ T cells. The TGFβR2(-1) mutation is recurrent in colorectal cancer (CRC) with microsatellite instability. CD8⁺ T cell responses specific for the TGFβR2(-1) neoantigen have been shown. No MHC II-restricted T cell receptor (TCRs) specific for TGFβR2(-1) have been described.

WHAT THIS STUDY ADDS

⇒ We demonstrated that TGFβR2(-1) neoantigen is immunogenic and processed and presented in HLA-DR4-restricted manner. Two TCRs, were generated, which recognized naturally expressed TGFβR2(-1) neoantigen in CRC lines.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our finding and safety assays suggest the potential clinical translation for the treatment of patients with tumors bearing the TGFβR2(-1) mutation. TGFβR2(-1) is prevalent in different individuals and may enable application of the therapy to a broad number of patients with cancer.

led to fatal toxicity when T cells were not truly tumor-specific.²⁻⁴ Most human tumor-specific neoantigens without a viral etiology are peptides encoded by nonsynonymous somatic cancer-specific mutations (substitutions, insertions, deletions, gene fusions, and frameshifts) absent in normal cells of the host.⁵⁻⁸ Targeting neoantigens by ATT is advantageous over other targets allowing specific elimination of cancer cells without on-target off-tumor toxicity in normal, non-tumor tissues. The occurrence of neoantigens depends on mutational load (number of somatic mutations per Mb of coding DNA).⁹ Mutational load is particularly high in microsatellite instable (MSI) colorectal cancers (CRC).¹⁰ MSI in CRC is a condition of genetic



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hypermethylability in repetitive DNA motifs (microsatellites) that results from an impaired DNA mismatch repair system (MMR).^{11 12} This MMR deficiency in MSI-CRC is caused by an epigenetic silencing (hypermethylation) of the MLH1 gene promoter or by germline mutation of DNA mismatch repair genes MLH1, MSH2, MSH6, PMS2 (disease known as Lynch syndrome), followed by somatic inactivation of the second allele.^{13 14} MSI occurs in 15% of all CRC cases. The genetic alterations lead to accumulation of point mutations (insertions or deletions) in DNA microsatellites and, if occurring in coding regions, they give rise to frameshift variants encoding novel, often truncated peptides. Those frameshift peptides are potential sources of immunogenic neoantigens.¹⁵ One such frameshift mutation was described within the transforming growth factor β receptor 2 (TGF β R2) that is present in 77% of MSI-CRC patients and almost 72% of Lynch syndrome patients.^{16 17} The presence of a truly tumor-specific neoantigen shared by many patients is rare and, therefore, targeting such a shared neoantigen allows potentially more patient to benefit from ATT. The TGF β R2(-1) frameshift results from a deletion of one nucleotide in a microsatellite consisting of 10 adenines, a poly(A)10 tract of the gene at nucleotide bases 709–718.¹⁸ Within the TGF β R2(-1) frameshift region an HLA-A2-restricted epitope has been described¹⁹ and TCRs against it have been generated as potential candidates for ATT.^{20 21} We describe herein a HLA-DRA/DRB1*04:01 (HLA-DR4)-restricted epitope, which we used to generate neoantigen-specific TCRs functional on CD4⁺ T cells as potential therapeutics. Early studies already emphasized the CD4⁺ T cell importance in antitumor immunity.^{22 23} Appreciated for their role in helping antigen-specific CD8⁺ T cells, they proved to be capable of tumor elimination also on their own. It has been demonstrated that tumor-specific CD4⁺ T cells can effectively mediate tumor regression being directly cytotoxic or eliminating tumors indirectly by bystander killing through stroma cell targeting.^{24–27} CD4⁺ and CD8⁺ T cells synergized in tumor eradication. Therefore, both CD4⁺ T cells and CD8⁺ T cells needed to be tumor-specific.^{28 29} Taken together, studies underline that CD4⁺ T cells are capable to induce tumor regression.

In this study, we generated TGF β R2(-1)-specific TCRs using ABabDR4 mice that express a diverse human TCR $\alpha\beta$ repertoire restricted to HLA-DR4.^{30–33} In these mice, the TGF β R2(-1) neoantigen is foreign, so the mice are not tolerant to it and may serve as a convenient source for TCR isolation. We showed that the TGF β R2(-1) neoantigen is endogenously processed and presented by HLA-DR4 molecules and that the HLA-DR4-restricted TGF β R2(-1)-specific TCRs are functional and recognize naturally expressed mutant TGF β R2(-1) in CRC cell lines with no detectable off-target reactivity. The TCRs specific for a frequent recurrent mutation may be worthwhile to be tested in the clinic.

MATERIALS AND METHODS

Cell lines and culture

The human CRC cell lines HCT116³⁴ and SW48³⁵ were obtained from Prof G. Willimsky and SW48 from Prof. U. Stein, Berlin, respectively. Cell lines SW48-DR4, HCT116-DR4 and K562-DR4 were generated by retroviral transduction with HLA-DRA and HLA-DRB1*04:01 (HLA-DR4) cDNAs fused by the self-cleaving element P2A. The HLA-DR4 DNA construct was introduced into MP71_IRES_GFP vector using restriction sites NotI and Sall. For generation of BSM-TGF β R2(-1) cell line (naturally expressing HLA-DR4⁺), the TGF β R2(-1) minigene was first synthesized (GeneArt). Next, using restriction sites PmlI introduced via PCR, the TGF β R2(-1) minigene was cloned into the MP71_mCh vector.³⁶ The BSM-TGF β R2(-1) cell line (HLA-DR4⁺) was generated by retroviral transduction of the TGF β R2(-1)_MP71_mCh vector.³⁶ The human melanoma cell line FM3 (HLA-DR4⁺) was provided by the European Searchable Tumor Cell Bank and Database. All cell lines, including the lymphoblastoid cell line (LCL) panel (EBV-transformed lymphoblastoid B cell lines), were maintained in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS; PAN Biotech), 1 \times antibiotic-antimycotic, 1 mM sodium pyruvate and 100 μ M non-essential amino acids (all reagents were purchased from Life Technologies). The CRC cell lines' medium was additionally supplemented with gentamicin (Life Technologies). The packaging cell line 293GP-GLV (GALV cells)³⁷ producing retroviral particles was cultured in Dulbecco's modified Eagles medium supplemented with 10% FCS and 1 \times antibiotic-antimycotic. The primary human peripheral blood leukocyte (PBL)-derived T cells were kept in RPMI-1640 medium supplemented with 1 mM HEPES, 10% heat-inactivated FCS, 1 \times antibiotic-antimycotic, 1 mM sodium pyruvate and optionally with 300 U/mL or 30 U/mL IL-2. The murine T cells medium was additionally supplemented with 50 μ M 2-mercaptoethanol.

Immunization of mice

TCRs were generated in transgenic ABabDR4 mice expressing a diverse human TCR $\alpha\beta$ repertoire restricted to human major histocompatibility complex (MHC) class II molecule HLA-DR4.^{31 33} All animal experiments were approved and conducted according to national guidelines (Landesamt für Gesundheit und Soziales, Berlin, Germany). All mice were kept at the animal facility of the Max-Delbrück Center for Molecular Medicine under SPF (specific-pathogen-free) conditions. ABabDR4 mice were immunized at an age of 8–12 weeks using the TGF β R2(-1) peptide (VALMSAMTTSSSQKN, GenScript). The 80 μ g of peptide dissolved in 100 μ L PBS were added to 50 μ g of oligonucleotide CpG (CpG 1826, MOLBIOL) and 100 μ L of incomplete Freund's adjuvant (IFA, Sigma-Aldrich). A volume of 200 μ L emulsion was injected subcutaneously into mice. Immunizations were repeated at 4-week intervals.

Intracellular cytokine staining

One week after the last immunization, the peripheral blood of immunized ABabDR4 mice was analyzed for the presence of responding CD4⁺ T cells. The cell fraction after lysing red blood cells with ACK buffer (150 mM NH₄Cl, 1 mM KHCO₃, 100 nM Na₂EDTA) was stimulated with TGFβR2(-1)-specific HLA-DR4-restricted peptide (VALMSAMTTSSSQKN, GenScript) at 1 μM, no peptide as negative control or 20 μL containing 8×10⁵ CD3/CD28 Dynabeads T activator (Invitrogen) as positive control. After 2 hours, protein transport inhibitor Brefeldin A (BD Golgie Plug) was added and intracellular IFNγ staining was performed after 13 hours of incubation at 37°C. After Fc blocking (anti-mouse CD16/32, BioLegend), cells were fixed using BD Cytfix/Cytoperm KIT (BD Bioscience) and stained with 50 μg/mL of anti-CD3, -CD4 and -IFNγ antibodies (BioLegend) for 30 min at 4°C, then analyzed by flow cytometry.

IFNγ secretion assay

Ten days after the last immunization, mice were sacrificed and splenocytes were isolated. Next, after 4 hours of restimulation with TGFβR2(-1)-specific HLA-DR4-restricted peptide at 1 μM, cells producing IFNγ were stained using IFN-γ Secretion Assay KIT (Miltenyi Biotec). Positive CD4⁺ T cells producing IFNγ were collected by FACS into lysate buffer RTL Plus (RNeasy Micro Kit, QIAGEN) for TCRs isolation.

TCR isolation

From sorted CD4⁺ T cells, RNA was isolated (RNeasy Micro Kit, QIAGEN). This was followed by synthesis of cDNA and 5' RACE PCR (SMARTer RACE cDNA Amplification Kit, Clontech) with 0.5 μM primers specific for the constant region of either TCRα (5'-CGGCCACTTTCAGGAG GAGGATTCGGAAC-3') or TCRβ (5'-CCGTAGA ACTTGACAGCGGAAGTGG-3') chain. Each reaction was performed in volumes of 50 μL with 0.5 μL Phusion High-Fidelity DNA polymerase (New England Biolabs), 10 mM dNTPs, 1× Universal Primer Mix (10×, UPM, Clontech). Thermocycler conditions were as follows: 2 min at 98°C, 5 cycles of 98°C for 30s and 72°C for 45s, 5 cycles of 98°C for 30s, 68°C for 30s and 72°C for 45s, 25 cycles of 98°C for 20s, 68°C for 20s and 72°C for 45s, final elongation at 72°C for 5min. The RACE PCR products were purified from an electrophoresis gel and cloned into TOPO vectors (Zero Blunt TOPO PCR cloning Kit, Invitrogen) to transform TOP10 OneShot competent *Escherichia coli* (Invitrogen). Depending on clonality, around 50 clones per TCR chain were sequenced and further analyzed.

Generation of TCR transgene cassettes in MP71 vector

Isolated TCRα and TCRβ chains contained human constant regions. To reduce potential cross pairing with endogenous TCRs, we exchanged the human constant regions for the mouse counterparts.³⁸ To this purpose, overlapping extension PCR (OE-PCR) was performed from two PCR products. The first PCR murine constant

region was amplified from an already murinized TCR-3600 vector.²⁹ Primers alpha_ms_fwd and huTCR3600_rvs were used to amplify murine alpha constant region. Primers beta_ms_fwd and TCR3600_beta_EcoRI_rvs were used to amplify murine constant beta region. The second PCR included TCRα/β variable gene amplification using primers specific for each TRAV/TRBV with V(D)J_alpha_rvs and VDJ_beta_rvs, respectively (all primers listed below). The third OE-PCR was the final one to combine variable regions of each TCRα/β with a constant murine region. Such full length single TCRα and TCRβ transgenes were cloned using NotI and EcoRI restriction sites into MP71 vector for combinatorial expression of different TCRα + TCRβ and to identify functional TCRαβ pairs.³⁶ Functional TCRα + TCRβ pairs were synthesized with Homo Sapiens codon optimization (GeneArt) for better expression in human PBLs. Chains TCRβ with TCRα were linked with the porcine teschovirus-1-derived self-cleaving peptide P2A.³⁹ The synthesized expression cassettes encoded two TCRs: TCR1414_1 or TCR1414_2.

alpha_ms_fwd 5'-ATATCCAGAACCCCGAGCCTGCCCG TGTACC-3'

huTCR3600_rvs 5'-CAGGAATTCTCATCAGCTGG ACCAC-3'

beta_ms_fwd 5'-GGATCTGAGAAACGTGACCCCCCA AAGGT-3'

TCR3600_beta_EcoRI_rvs 5'-ACTGAATTCTCAGCTG TTCTTCTTCTTGACCATGG-3'

V(D)J_alpha_rvs 5'-AGCTGGTACACGGCAGGCTC GGGGTTCTG-3'

TRAV21_Not1 5'-ATTGCGGCCGCCATGGAGAC CCTCTTGGGCC-3'

TRAV22 Not1 5'-ATTGCGGCCGCCATGAAGAGGATA TTGGGAGC-3'

TRAV12-3_Not1 5'-GCATTGCGGCCGCCATGATG AAATCCTTGAGAGTTTTAC-3'

VDJ_beta_rvs 5'-ACCTTGGGGGGGGTCACTTTCTC AGATCC-3'

TRBV28_Not1 5'-ATTGCGGCCGCCATGGGAAT CAGGCTCCTCTG-3'

TRBV12-4_Not1 5'-ATTGCGGCCGCCATGGGCTC CTGGACCC-3'

TRBV18_Not1 5'-TATGCGGCCGCCATGGACAC CAGAGTACTCTGC-3'

Transfection of GALV packaging cells

To produce amphotropic TCR-bearing retroviral particles, 293GP-GLV (GALV) cells were used.³⁷ Virus supernatant was produced by transfecting GALV cells with the retroviral vector MP71³⁶ containing TCR expression cassettes. For transduction of HCT116 and SW48 cell lines, GALV supernatants were prepared with vector MP71 containing HLA-DR4_GFP, GFP, TGFβR2(-1)_mCh or mCh. GALV cells seeded at 7×10⁵ per well in 6-well plates were transfected with 3 μg of plasmid DNA using either lipofectamine 2000 reagent (Thermo Fisher Scientific) or 18 μg of DNA when calcium chloride (Sigma Aldrich) method was used.⁴⁰ Forty-eight hours and 72 hours after

transfection, 3 mL of virus supernatant were harvested, filtrated (0.45 µm pore size) and used directly for transduction or stored at -80°C.

Transduction of primary T cells

Human primary T cells were transduced with TCRs after CD8⁺ cell depletion from PBLs using MACS anti-CD8 MicroBeads human and LD separation column (Miltenyi Biotec, cat. nr 130-045-201). One million CD8⁺ cell-depleted PBLs per well were seeded in 24-well plates (non-tissue culture, Thermo), previously coated with 5 µg/mL anti-CD3 (OKT3, BD Pharmingen) and 1 µg/mL anti-CD28 antibodies (CD28.2, BD Pharmingen) in 1 ml T cell medium supplemented with 300 U/mL IL-2. After 2 days, activated T cells were transduced with 1 mL retroviral supernatant supplemented with 4 µg/mL protamine sulfate (Sigma-Aldrich) and spinoculated for 90 min at 32°C and 800g. A second transduction was performed the next day on RetroNectin-coated 6-well plates (25 µg/mL in PBS), precoated with virus particles for 2 hour at 32°C and 2000g. PBLs were transferred into virus-coated 6-well-plates and spinoculated for 30 min at 800g. Transduced PBLs were expanded for 7 days in T cell medium supplemented with 300 U/mL IL-2. For the next 3–5 days, the cells were transferred into low IL-2 medium (30 U/mL). After this resting phase, transduced PBLs were used for co-culture assays or were frozen and stored in liquid nitrogen. For transduction of single TCR α or β chain combinations, virus supernatants were mixed 1:1 in 1 ml volume.

Co-culture experiments

Co-culture of human T cells transduced with different non-codon optimized TCRα + TCRβ combinations were incubated with BM14 cells (HLA-DR4⁺) as target cells at ratio 1:5, where 1×10⁴ transduced CD4⁺ T cells were seeded with 5×10⁴ BM14 target cells in round bottom 96-well plates for 16–18 hours. Transduction efficiencies were in the range of ~30%. Co-culture of human T cells transduced with codon optimized TCRs were incubated with target cells (BSM, BM14, SW48, HCT116 or panel of LCLs) at a ratio 1:1. Therefore, 1×10⁴ transduced CD4⁺ T cells were seeded with 1×10⁴ target cells in round bottom 96-well plates for 20–24 hours. Transduction efficiencies were in the range of ~60%. As positive control, 50 ng/mL of Phorbol 12-myristate 13-acetate and 5 µg/mL Ionomycin were added to T cells alone. Negative controls were transduced T cells alone seeded in medium. TGFβR2(-1) peptide (VALMSAMTTSSSQKN, GenScript) was added at 10⁻⁶ M unless indicated differently. After incubation, supernatant was collected and IL-2 or IFNγ concentrations were measured by enzyme-linked immunosorbent assay (ELISA; BD OptEIA). For alanine scan experiments, each individual amino acid of the TGFβR1(-1) peptide was replaced by alanine (GeneScript, purity >85%). Peptides were loaded onto BSM cells at 10⁻⁷ or 10⁻⁹ M concentrations and co-cultured with TCR-transduced CD4⁺ T cells. For peptide cross-reactivity experiments, each of 15

ordered peptides (GeneScript, purity 75%–98%) were loaded onto K562-DR4 cells at 10⁻⁶ M concentration and co-cultured with TCR-transduced CD4⁺ T cells.

ELISA

ELISA microwells were coated with 50 µL/well of capture antibody appropriately diluted in coating buffer and incubated overnight at 4°C. Next day, wells were washed three times with wash buffer. After that, plates were blocked with 100 µL/well assay diluent (10% FCS in PBS) and incubated at room temperature (RT) for 1 hour. Next, wells were washed three times with wash buffer. A 50 µL of prepared standards, controls and samples was distributed into appropriate wells. If needed, samples were diluted in assay diluent. Plates were sealed and incubated for at least 2 hours at RT. Then, wells were washed as before, but with five total washes. A volume of 50 µL of working detector (detection antibody+streptavidin-horseradish peroxidase (SAv-HRP) reagent) was added to each well and plates were incubated for 1 hour at RT. After the last wash (8 times), a 50 µL of substrate solution was added to each well and incubated for about 5 min at RT in the dark. Next, a 25 µL stop solution was added to each well. Absorbance at 450 nm and 570 nm was then determined.

Expression profile of CRC cell lines

Cancer cell lines were lysed and RNA was isolated using RNeasy Plus Micro Kit (QIAGEN). Next, reverse transcription RT-PCR was performed using SuperScript IV First-Strand Synthesis System (Thermo Fisher Scientific). Obtained cDNA product was then used for amplification of 159–1240 bp and 60–1066 bp fragments (transcript variants 1 and 2) of the TGFβR2 gene using gene specific primers TGFβR2 1k product_fwd 5'-ACTCCTGTGCAGCTTCCCTCGGC-3' and TGFβR2 1k product_rvs 5'-GGCCTTATAGACCTCAGCAAAGCGACC-3'. Amplified PCR products were visualized on 1% agarose gel, bands were cut out and DNA was purified using ZymoClean Gel DNA Recovery Kit (Zymo Research). Finally, the DNA was sequenced using Sanger sequencing (Eurofins).

Flow cytometry

The following antibodies (BioLegend) were used for staining at 1:100 dilutions unless stated otherwise: anti-mouse CD4-FITC (RM4-5), anti-mouse CD4-BV421 (L3T4, BD Pharmingen), anti-mouse CD3-PE (145–2C11), anti-mouse CD3-APC (145–2C11), anti-mouse IFNγ-BV421 (XMG1.2), anti-mouse IFNγ-PE (XMG1.2), anti-mTCRβ-chain-APC (H57-597), anti-human CD3-APC (SK7) and anti-human CD4-BV421 (OKT4). Magnetic beads anti-human CD8 (Microbeads 130-045-201) were used for CD8 depletion at 1:250 dilution (Miltenyi Biotec). FACSaria II (BD Bioscience) was used for sorting responsive CD4⁺ T cells, FACSaria F (BD Bioscience) was used to sort transduced cell lines. For analysis of cells, LSR-Fortessa was used (BD Bioscience).

Frameshift peptide sequence: SLVRLSSCVPALMSAMTTSSSQKNITPAILTCC				
NetMHCIIpan 3.2			NetMHCIIpan 4.0	IEDB recommended 2.22
epitope sequence	peptide core	IC50 (nM)	rank	IC50 (nM)
CVPVALMSAMTTSSS	MSAMTTSSS	47.2	21	51.0
VPVALMSAMTTSSSQ	MSAMTTSSS	33.2	8.70	50.0
PVALMSAMTTSSSQK	MSAMTTSSS	24.7	3.60	49.0
VALMSAMTTSSSQKN	MSAMTTSSS	25.2	3.50	49.0
ALMSAMTTSSSQKNI	MSAMTTSSS	30.3	9.40	67.0
LMSAMTTSSSQKNIT	MSAMTTSSS	43.4	25	153.0

Figure 1 Peptide:MHC class II binding affinities of TGF β R2(-1) frameshift-derived epitopes for HLA-DRB1*04:01 allele predicted by NetMHCIIpan 3.2, NetMHCIIpan 4.0 and IEDB recommended 2.22. Sequence highlighted in bold letters is the one chosen for further experiments (called TGF β R2(-1) peptide). Predictions are given as IC50 (nM) units or rank (the lower the number, the higher the affinity). Peptides predicted as <50 nM are considered as high, <500 nM as intermediate and <5000 nM as low affinity. MHC, major histocompatibility complex; IEDB, Immune Epitope Database.

RESULTS

Isolation of TGF β R2(-1)-reactive TCRs restricted to HLA-DR4

The TGF β R2(-1) frameshift peptide is an ideal target, as it arises from a somatic cancer-specific mutation, representing a truly tumor-specific neoantigen absent in normal cells. In silico pMHC binding prediction (Immune Epitope Database (IEDB) recommended 2.22) suggested a high binding affinity of a VALMSAMTTSSSQKN region of the TGF β R2(-1) frameshift peptide to HLA-DR4 (predicted IC50 of 49 nM) (figure 1). This epitope's core is located in the center of the frameshift peptide. For this putative epitope, TCRs were generated in ABabDR4 mice, which express a diverse human TCR $\alpha\beta$ repertoire restricted to HLA-DR4. The TGF β R2(-1) frameshift peptide does not occur in the mouse genome based on nucleotide BLAST library search and, thus, it is a foreign antigen. Following immunization of ABabDR4 mice with the TGF β R2(-1) peptide VALMSAMTTSSSQKN, specific CD4⁺ T cells were observed after short in vitro stimulation of peripheral blood-derived T cells with the TGF β R2(-1) peptide, measured by intracellular IFN γ staining (figure 2A). To isolate TGF β R2(-1)-reactive TCRs, we collected splenocytes from the most responsive mouse (nr. 1414) on day 10 after third immunization (figure 2B). Following splenocytes restimulation in vitro for 4 hours with 1 μ M TGF β R2(-1) peptide, we isolated 6000 responding CD4⁺ T cells, which reflected 1.2% of CD3⁺ lymphocytes, by use of the mouse IFN γ secretion assay (figure 2C).

Three dominant TCR α and five dominant TCR β chains were identified in collected antigen-specific CD4⁺ T cells (figure 3A). Combinatorial expression of each out of the 3 TCR α with each of the 5 TCR β chains was performed, resulting in 15 different TCR $\alpha\beta$ combinations that were transduced into human peripheral blood leucocytes (PBLs)-derived T cells (figure 3B). To reduce cross pairing of transduced TCR $\alpha\beta$ with endogenous TCR $\alpha\beta$ chains, their constant regions were murinized.⁴¹ T cells with all 15 TCR $\alpha\beta$ combinations were used for co-culture with HLA-DR4 expressing BM14 cells, loaded with the TGF β R2(-1) frameshift peptide. Specific TCR pairs 1A/1B and 2A/3B were identified as reflected by the high IFN γ amount measured in the supernatant by ELISA (figure 3C). Functional TCR $\alpha\beta$ chain combinations were cloned as codon optimized versions into one expression vector, linked by P2A element for equal molarity

expression³⁹, TGF β R2(-1)-specific TCR pairs TCR 1A/1B and TCR 2A/3B were called TCR1414_1 and TCR1414_2, respectively.

Peptide sensitivity

One important feature of a TCR is its sensitivity to peptide amount at which the TCR still recognizes the pMHC complex. To analyze peptide sensitivity, human CD4⁺ T cells transduced with TCR1414_1 or TCR1414_2 were co-cultured with BSM (HLA-DR4⁺) cells loaded with different concentrations of the TGF β R2(-1) peptide. As a readout, IFN γ concentration was measured by ELISA. Both investigated TCRs showed recognition up to 10⁻⁹ M TGF β R2(-1) peptide with EC50 values ~2,65 nM and 6 nM for TCR1414_1 and TCR1414_2, respectively (figure 4).

TGF β R2(-1) neoantigen is naturally processed and presented

Immunization of ABabDR4 mice with TGF β R2(-1) peptide VALMSAMTTSSSQKN shows its immunogenicity but does not reveal, if the whole frameshift peptide SLVRLSSCVPALMSAMTTSSSQKNITPAILTCC is processed by endogenous endosomal-lysosomal antigen-processing compartments and presented on HLA-DR4 molecules. To address the question, TCR1414_1- and TCR1414_2-transduced CD4⁺ T cells (figure 5A) were co-cultured with antigen presenting BSM cells transduced with a TGF β R2(-1) minigene encoding the 47 amino acids long TGF β R2(-1) frameshift peptide along with mCherry (mCh) as a reporter gene (figure 5B). As a readout, IFN γ and IL-2 concentrations were measured in the supernatant of the co-cultures by ELISA (figure 5C). Results from figure 5C show that both TCRs (TCR1414_1 and TCR1414_2) recognize the BSM-TGF β R2(-1) cell line endogenously expressing the full length TGF β R2(-1) frameshift region, as measured by IFN γ and IL-2 production. This demonstrates that the TGF β R2(-1) minigene has been processed and presented on the surface of BSM-TGF β R2(-1) cells. Control BSM-mCh cells were not recognized, unless loaded with the TGF β R2(-1) frameshift peptide.

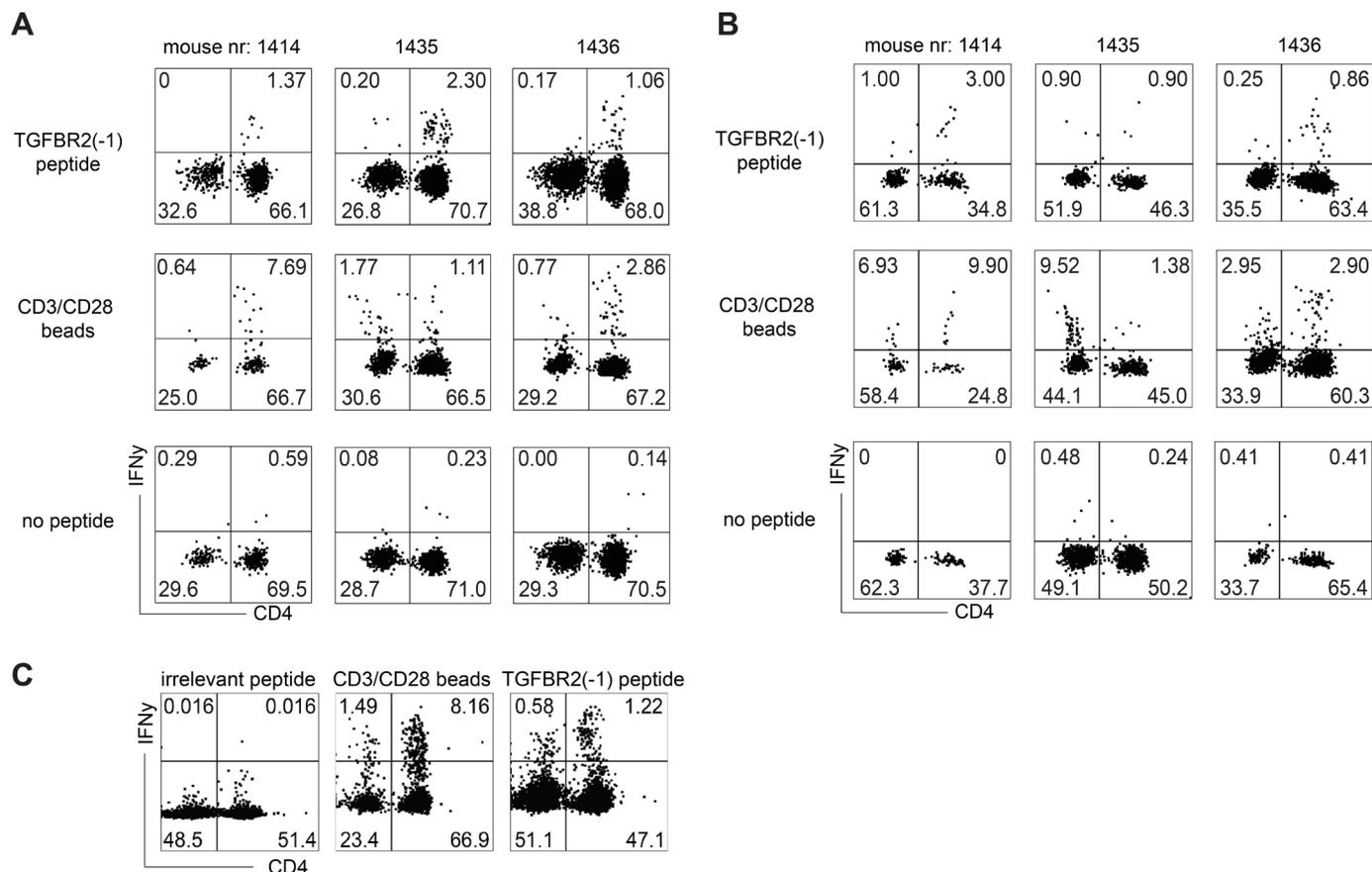


Figure 2 Generation of TGFβR2(-1)-reactive TCRs in ABabDR4 mice. (A, B) Intracellular cytokine staining (ICS) of PBLs from ABabDR4 mice 7 days after second (A) or third (B) TGFβR2(-1) peptide (VALMSAMTTSSSQKN) immunization. Isolated PBLs were stimulated with 10^{-6} M TGFβR2(-1) peptide, CD3/CD28 beads were used as positive control and no peptide as negative control. After 2 hours, brefeldin A was added to the cultures and ICS was carried out after o/n incubation. Cells are gated on single CD3⁺ lymphocytes. Numbers in gates are population size given in percentage. (C) Sorting of TGFβR2(-1)-reactive IFN γ -producing CD4⁺ T cells from spleen of ABabDR4 mouse nr 1414 at day 10 after third TGFβR2(-1) peptide immunization. Restimulated splenocytes in vitro for 4 hours with TGFβR2(-1) peptide, CD3/CD28 beads or irrelevant peptide as controls were labeled using mouse IFN- γ Secretion Assay KIT. Labeled cells were sorted by flow cytometry. In total, 6000 IFN γ -producing CD4⁺ TGFβR2(-1)-reactive T cells (1.22%) were collected. Cells are gated on single CD3⁺ lymphocytes. Numbers in gates refer to population size given in percentage.

Recognition of TGFβR2(-1) neoantigen naturally expressed in CRC cell lines

Because the mutation is most common in CRC, we focused on analysis of two CRC cell lines, SW48 and HCT116, reported to naturally express the TGFβR2(-1) frameshift mutation.⁴² To confirm the deletion of one adenine in the TGFβR2 gene, which causes the frameshift peptide containing the TGFβR2(-1) neoantigen, we performed RT-PCR with both CRC cell lines, amplifying ~1Kb fragment of the TGFβR2 gene. Electrophoresis revealed two bands since PCR primers were designed to cover both splicing variants of the TGFβR2 gene (figure 6A). Sequencing of the PCR fragments showed the presence of the mutation (lack of one adenine), reflected in only nine adenines being present in both CRC cell lines SW48 and HCT116 (figure 6A). TGFβR2 wild type (10 adenines microsatellite) in FM3 melanoma cells was used as control. Next, we checked if the TGFβR2(-1)-reactive TCR1414_1 and TCR1414_2 can recognize CRC cell lines SW48 and HCT116 that naturally express the TGFβR2(-1)

neoantigen. Therefore, TCR1414_1- and TCR1414_2-transduced human CD4⁺ T cells were co-cultured with SW48 and HCT116 cells transduced to express HLA-DR4, due to the absence of this MHC II gene in both CRC cell lines (figure 6B). SW48-DR4 and HCT116-DR4 cells with natural expression levels of the TGFβR2(-1) frameshift peptide were recognized by both, TCR1414_1- and TCR1414_2-transduced human CD4⁺ T cells, as reflected by IFN γ release (figure 6C).

TGFβR2(-1)-specific TCRs show no HLA alloreactivity

ABabDR4 mice used for generation of TGFβR2(-1)-specific TCRs lack other human MHC molecules except for HLA-DR4 and, therefore, there is a potential risk of alloreactivity of the TCRs to other human HLAs. To address this question, we transduced human CD4⁺ T cells with TCR1414_1 or TCR1414_2 and co-cultured them with a panel of 16 Epstein-Barr virus-transformed B-LCL expressing different MHC I and MHC II molecules (online supplemental table 1). This LCL panel covers

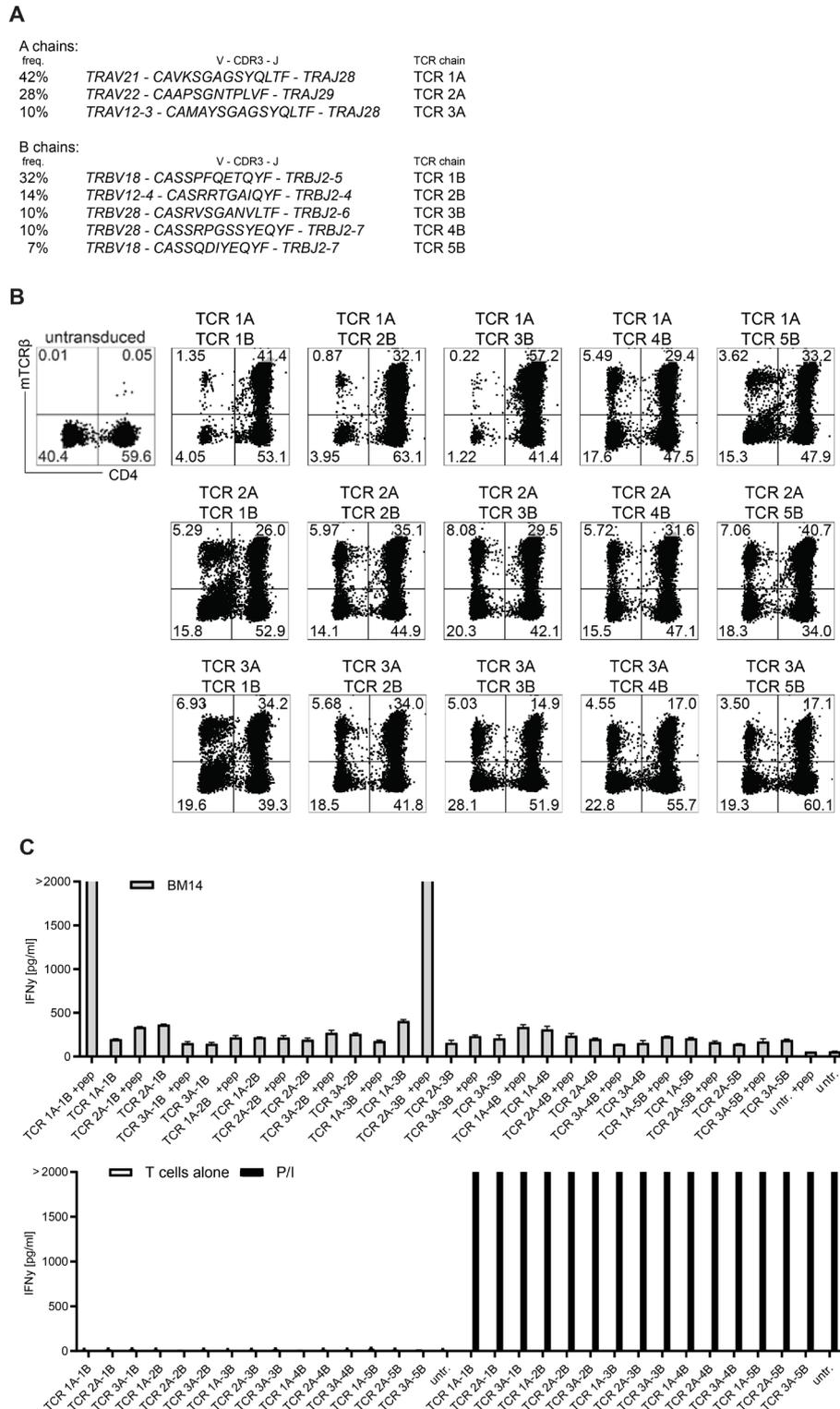


Figure 3 Identification of TGFβR2(-1)-specific TCR-α and TCR-β chain combinations. (A) Frequencies of CDR3 regions and V/J gene segments of TCR α-chains and β-chains. After RACE-PCR from responding CD4⁺ T cells, the amplified PCR product of each chain was separately cloned and around 50 clones for each TCRα and TCRβ were sequenced and their frequencies are presented in percentages. (B) Combinatorial expression of TCRα and TCRβ genes in human CD4⁺ T cells with given transduction efficiency in percentages, stained with antibodies specific for mouse TCRβ constant region (mTCRβ) and human CD4. Cells are gated on CD3⁺ lymphocytes. (C) Identification of functional pairs TCR 1A/1B and TCR 2A/3B. Human CD4⁺ T cells transduced with 15 ABAbDR4-derived TCR combinations were co-cultured with HLA-DR4⁺ BM14 cells loaded with 10⁻⁶ M TGFβR2(-1) peptide (+pep). After 16 hours, IFNγ was measured in the supernatant. As negative control, T cells alone were used and as positive control, T cells stimulated with PMA/Ionomycin (P/I). Intra-assay duplicates with SD are displayed. Similar data were obtained with HLA-DR4⁺ BSM and K562-DR4 cells (data not shown). PMA, phorbol 12-myristate 13-acetate; TCR, T cell receptor.

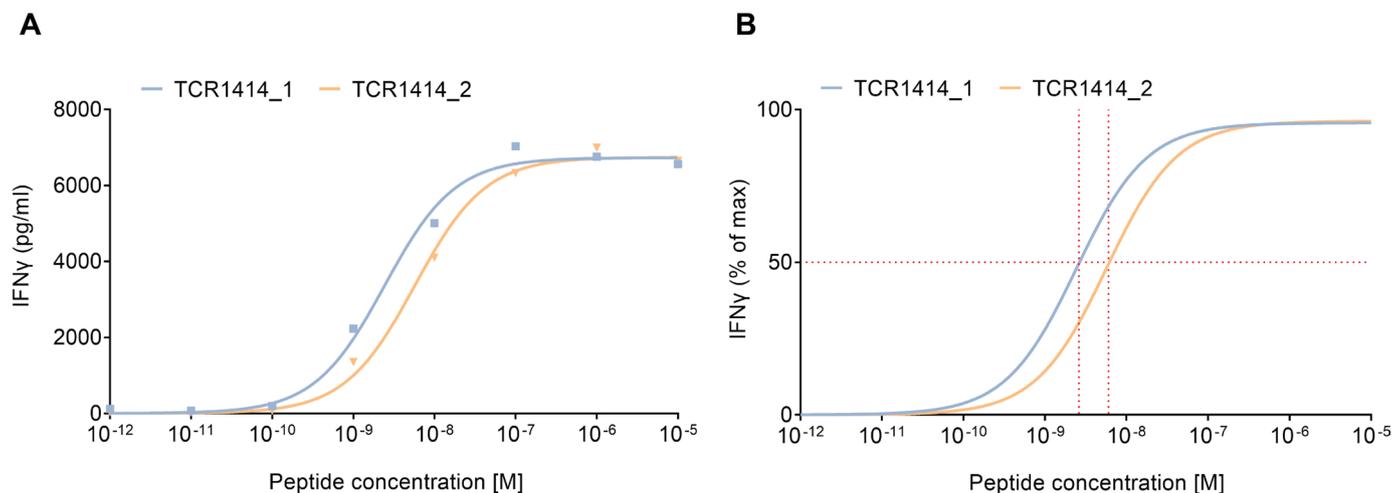


Figure 4 TCR1414_1 and TCR1414_2 sensitivity to TGFβR2(-1) peptide. (A) ELISA results reflecting responsiveness of CD4⁺ T cells transduced with TCR1414_1 (blue line) or TCR1414_2 (yellow line) to varying doses of the TGFβR2(-1) peptide (VALMSAMTTSSSQKN). TCR-transduced CD4⁺ T cells were co-cultured with BSM cells pulsed with decreasing peptide amounts. IFNγ was measured in the supernatant after 16 hours. (B) Normalized data to the maximum IFNγ release. Both investigated TCRs show recognition up to 10⁻⁹ M TGFβR2(-1) peptide with EC50 values around 2,65 nM and 6 nM for TCR1414_1 and TCR1414_2 as indicated by the red dotted line. The results are representative of two independent experiments with two different human PBLs donors.

34% of the Caucasian population regarding the HLA-DRB1 locus. No alloreactivity was detected (figure 7A). BSM and BM14 LCLs naturally expressing HLA-DR4 were additionally loaded with TGFβR2(-1) peptide and used as positive control.

TGFβR2(-1)-specific TCRs show no self-peptide cross-reactivity

Peptide cross-reactivity is characterized by shared amino acid (AA) motifs recognized by the TCR.⁴³ To identify which AA of the TGFβR2(-1) peptide take part in the interaction with the TGFβR2(-1)-reactive TCRs, we sequentially exchanged each AA position in the TGFβR2(-1) peptide to alanine. Peptides were added at concentration 10⁻⁷ M or 10⁻⁹ M to the co-culture of K562-DR4 cells with TCR1414_1 or TCR1414_2-transduced CD4⁺ T cells. At peptide concentration 10⁻⁹ M, the recognition motif for TCR1414_1 was x-x-L-M-x-x-M-T-T-S-x-x-x-x and for TCR1414_2 x-x-L-M-x-x-M-T-T-x-x-x-x-x (figure 7B). Within the human proteome no identical recognition motif was found for TCR1414_1, while the recognition motif of TCR1414_2 showed one peptide (ILLMfgMTTtaiypw) derived from taste receptor type 2 member 10 but with low affinity at IC50 460 nM and, therefore, was not taken into further investigation. At a concentration 10⁻⁷ M peptide, the recognition motifs were x-x-L-M-x-x-M-x-T-x-x-x-x-x and x-x-L-M-x-x-M-T-x-x-x-x-x for TCR1414_1 and TCR1414_2, respectively. Both TCRs required leucine at position 3, methionine at position 4 and 7 as well as threonine at position 9 for TCR1414_1 and threonine at position 8 for TCR1414_2. Within the human proteome, we detected nine peptides that shared the recognition motif of TCR1414_1 and 6 for TCR1414_2 with predicted binding affinity <300 nM (online supplemental table 2). Next, we investigated if

any of those peptides are cross-reactive, meaning if TCRs can bind them and activate the T cell to response. No cross-reactivity was observed for any of 15 tested peptides (figure 7C).

DISCUSSION

We isolated and characterized TGFβR2(-1)-specific TCRs including a comprehensive analysis of their functional activity and safety profile. We confirmed that the TGFβR2(-1) frameshift peptide is immunogenic in ABabDR4 mice with a humanized T cell recognition system and is processed and presented by HLA-DR4 molecules. Importantly, TGFβR2(-1)-specific TCRs transduced onto human CD4⁺ T cell recognized HLA-DR4-transduced CRC cell lines HCT116 and SW48 with natural TGFβR2(-1) expression levels. SW48 cells were better recognized, correlated with higher TGFβR2(-1) expression when compared with HCT116. TCR1414_1 appeared to better recognize the CRC cell lines HCT116-DR4 and SW48-DR4 as lower amount of peptide compared with TCR1414_2 was needed to trigger response (TCR1414_1: EC50~2,65 nM and TCR1414_2: EC50~6 nM). TCR1414_1 was the most abundant clone in line with our assumption that on repeated immunization in sufficient (4 weeks) interval, the most effective T cell clone is reactivated from the memory pool, thus the ABabDR4 mice serve as an optimal selection system. TCRs against human neoantigens can similarly be isolated from humans and mice, since both should not be tolerant to them. There is no reason to assume that one of the two species delivers higher-quality TCRs. In humans, TCRs can be isolated from patients with cancer, which in most cases are directed against patient-individual neoantigens.¹

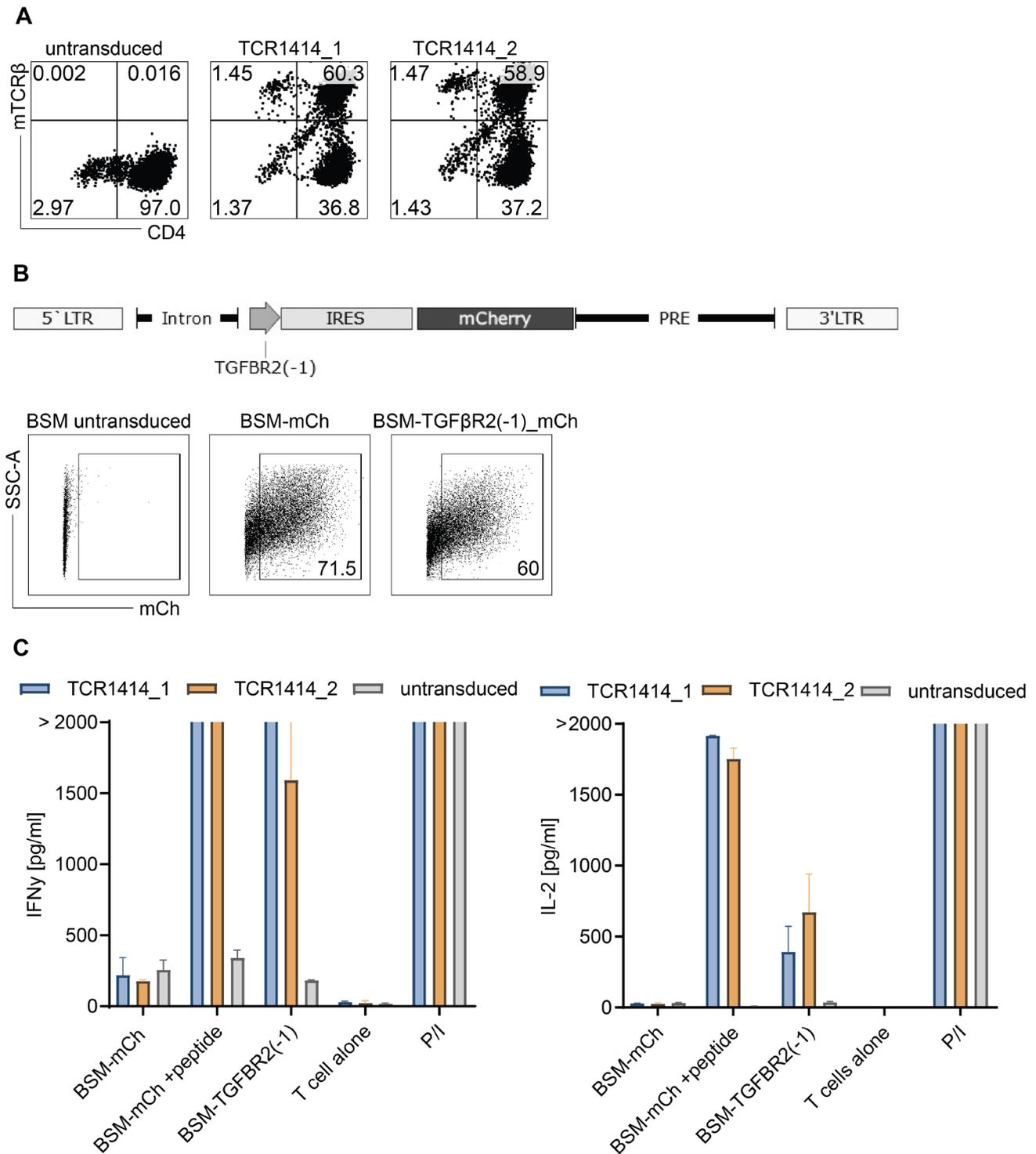


Figure 5 Recognition of naturally processed TGF β R2(-1) neoantigen by TCR1414_1 and TCR1414_2. (A) Expression of codon optimized TGF β R2(-1)-reactive TCR1414_1 and TCR1414_2 in human CD4⁺ T cells with given transduction efficiency in percentages. After CD8⁺ T cells depletion, TCRs were retrovirally transduced into human PBL-derived CD4⁺ T cells and stained with antibody for mouse TCR β constant region (mTCR β) and human CD4. Cells were gated on CD3⁺ lymphocytes. The results are representative of three different human PBLs donors. (B) Target cells transduction profile with TGF β R2(-1) minigene. BSM-TGF β R2(-1) cells were made by retroviral transduction of BSM cells with TGF β R2(-1) minigene encoding 47 amino acids containing the HLA-DRB1*04:01-restricted epitope and coupled to mCherry as reporter (top). BSM-mCh cells were used as negative control and transduced with same vector without the minigene. Numbers in gates show transduction frequencies in percentages. (C) Recognition of naturally processed TGF β R2(-1)-specific HLA-DR4-restricted epitope by TCR1414_1 and TCR1414_2. TCR-transduced CD4⁺ T cells were co-cultured with BSM-TGF β R2(-1) cell line (HLA-DR4⁺) for 16 hours. Data reflect IFN γ and IL-2 production measured by ELISA in the supernatant. TGF β R2(-1) peptide at 10⁻⁶ M (+peptide) or PMA/Ionomycin (P/I) were added as indicated. Results are representative of three (for IFN γ ELISA) and two (for IL-2 ELISA) independent experiments with different PBLs donors. PBLs, peripheral blood leukocytes.

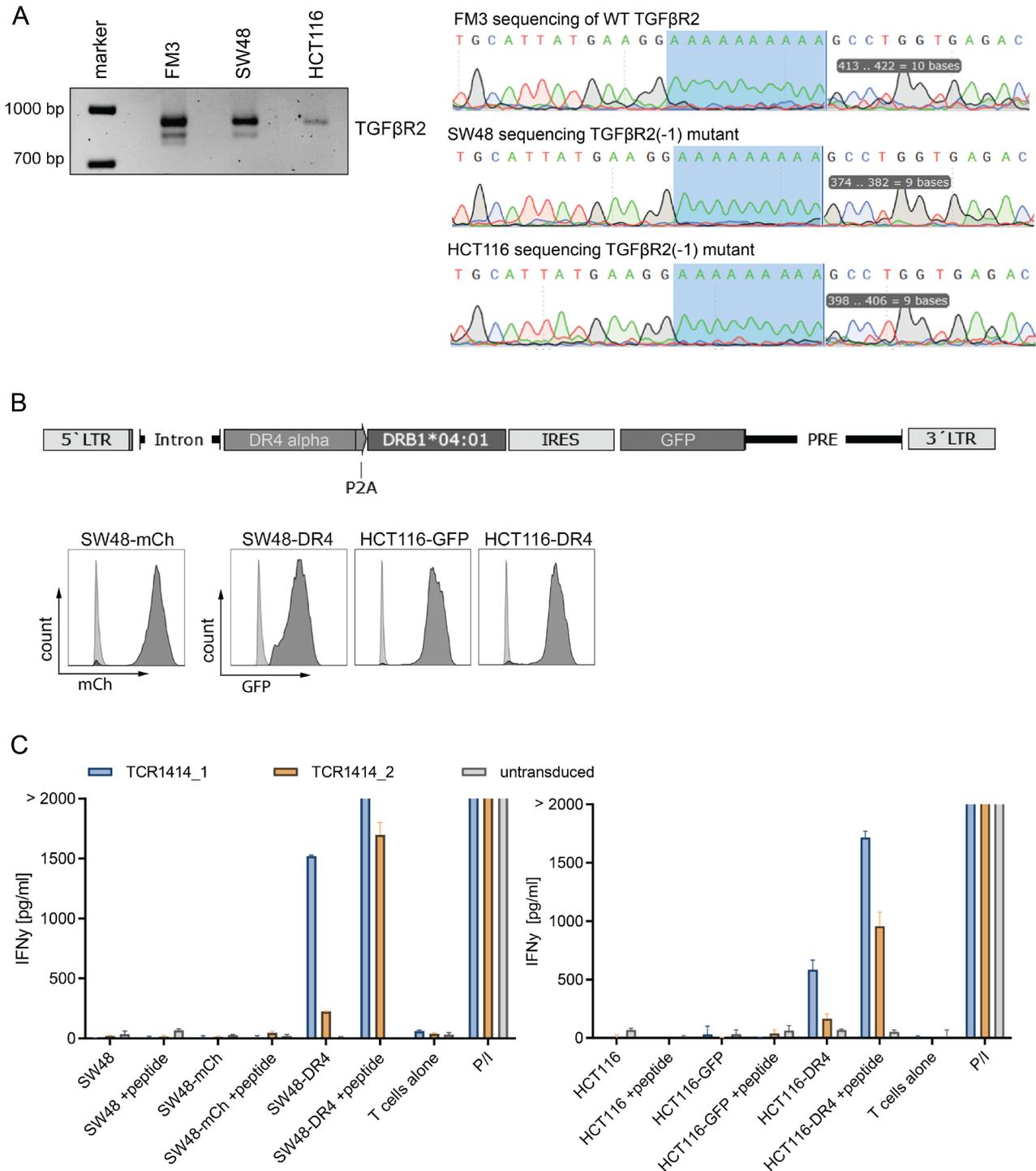


Figure 6 Recognition of naturally expressed TGFβR2(-1) neoantigen in colorectal cancer (CRC) cell lines by TCR1414_1 and TCR1414_2. (A) TGFβR2(-1) mutation detection in CRC cell lines SW48 and HCT116. Electrophoresis of amplified PCR products from cDNA shows the TGFβR2 region of interest containing the frameshift mutation. Primers for PCR were designed to cover the two splicing variants of the TGFβR2 gene. Sanger sequencing results show one adenine deletion (9A) in the 10-adenine microsatellite of TGFβR2 gene in both CRC cell lines. As control, the melanoma cell line FM3 containing the wild type (WT) TGFβR2 DNA sequence was used. (B) CRC cell lines SW48 and HCT116 (both HLA-DR*04:01) were transduced with HLA-DRA/HLA-DRB1*04:01 construct (top), as indicated, or mock controls (mCh or GFP). Dark gray represents transduced cells and light gray, untransduced, parental cell lines (bottom). Expression of reporter genes GFP or mCh was measured by flow cytometry. (C) Recognition of naturally expressed TGFβR2(-1) neoantigen in SW48-DR4 and HCT116-DR4 cell lines by TCR1414_1 and TCR1414_2. TCR-transduced human CD4⁺ T cells were co-cultured with CRC cell lines SW48-DR4 and HCT116-DR4 that endogenously express TGFβR2(-1) frameshift mutation. As positive controls, PMA and Ionomycin (P/I) were used, whereas for negative control, T cells alone were used. After 24 hours of incubation, IFN γ was measured in the supernatant by ELISA. The results are representative of four independent experiments performed with three different PBLs donors. PBLs, peripheral blood leucocytes.

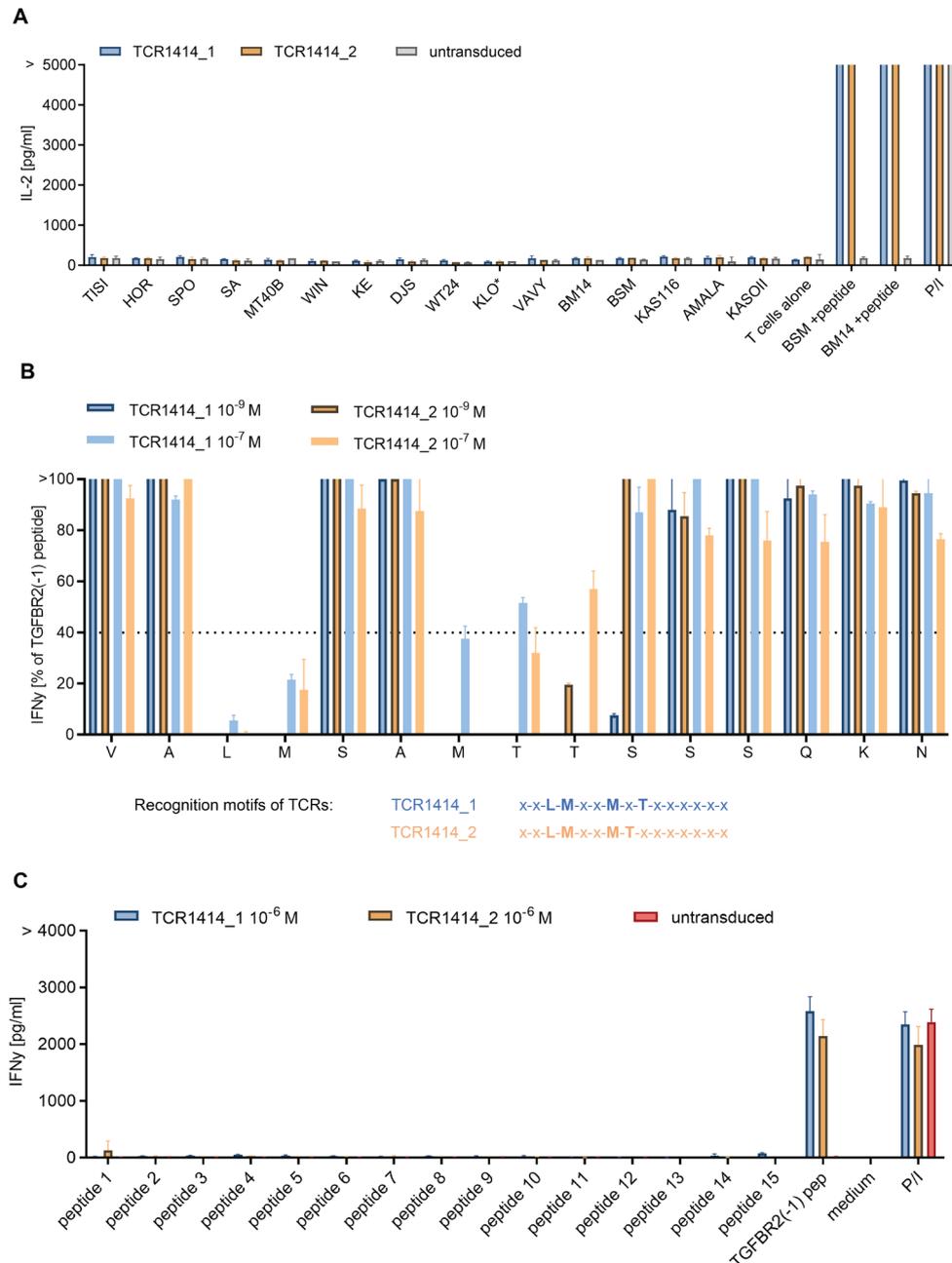


Figure 7 HLA alloreactivity and peptide cross-reactivity test of ABabDR4-derived TCRs. (A) TCR1414_1-transduced and TCR1414_2-transduced human CD4⁺ T cells were co-cultured with a panel of B-LCLs expressing different HLA allotypes, including both MHC I and II molecules (full HLAs profile of each LCL in online supplemental table 1). As positive control, PMA/Ionomycin (P/I) and BSM, BM14 (both HLA-DR*04:01⁺) loaded with 10^{-6} M TGF β R2(-1) peptide were used, whereas for negative control T cells alone were used. After 24 hours of incubation, IL-2 was measured in the supernatant by ELISA. The results are representative of three independent experiments performed with T cells from three different PBLs donors. For all experiments, all cell lines were used simultaneously except for *KLO cell line. (B) TGF β R2(-1) recognition motifs identification. Each amino acid of TGF β R2(-1) peptide was exchanged for alanine and such peptides were added to co-culture of K562-DR4 cells with TCR1414_1/2-transduced CD4⁺ T cells at 10^{-7} M or 10^{-9} M concentration. After 20 hours incubation, IFN γ in the supernatant was measured by ELISA. Data reflect percentage of IFN γ concentration relative to the response of unmodified TGF β R2(-1) peptide. Amino acids identified as recognition motifs are those which showed recognition of less than 40% when compared with unmodified TGF β R2(-1) peptide given by the dotted line. Amino acids mandatory for T cell recognition in TCR1414_1 are in position 3, 4, 7 and 9 (x-x-L-M-x-x-M-x-T-x-x-x-x-x-x) and for TCR1414_2 (x-x-L-M-x-x-M-T-x-x-x-x-x-x-x) position 3, 4, 7, 8, respectively. The results are representative of three independent experiments. (C) Cross-reactivity test of TCR1414_1 and TCR1414_2 with peptides containing similar recognition motifs (list of peptides in online supplemental table 2). TCR1414_1 and TCR1414_2-transduced human CD4⁺ T cells were co-cultured with peptides loaded onto K562-DR4 cells at 10^{-6} M concentration. As positive control, PMA/Ionomycin (P/I) and TGF β R2(-1) peptide were used, whereas for negative control T cells alone in medium were used. After 20 hours of incubation, IFN γ was measured in the supernatant by ELISA. The results are representative of two independent experiments using two different T cell donors. PMA, peripheral blood leucocytes.

Alternatively, TCRs against defined neoantigens can be isolated from autologous T cell-APC in vitro priming. Here, one cannot exclude that culture conditions skew the repertoire toward lower-avidity clones. In mice, T cells are activated through immunization in their natural environment. More importantly, by prime-boost immunization in sufficient long intervals, memory T cells are reactivated, which we believe are selected for optimal avidity and fitness. We note, however, that mouse-derived TCRs may have an increased risk of alloreactivity, since the mice expressed a single human MHC II gene and part of the selecting peptides in the thymus differ between humans and mice. To minimize the risk of off-target reactivity or HLA-alloreactivity, we performed robust assay systems, in which the TGF β 2(-1)-specific TCRs were negative.

While the majority of current immunotherapies focus on MHC I-restricted TCRs or non-MHC-dependent chimeric antigen receptor (CAR) technologies to engineer therapeutic CD8⁺ T cells, several studies have shown the importance of CD4⁺ T cells for tumor rejection. Early experiments showed that adoptively transferred CD4⁺ T cells eliminated MHC II-negative leukemia, likely by activating tumoricidal macrophages.^{22–23} Other models showed that CD4⁺ T cell-mediated antitumor immunity depends on IFN γ acting on stroma cells and that IFN γ and TNF, both often produced by effector CD4⁺ T cells, acted on and destroyed the tumor vasculature.^{44–45} CD4⁺ T cells can also directly lyse MHC II-positive melanoma cells.²⁶ Importantly, CD4⁺ T cells synergize with CD8⁺ T cells in eradication of large established tumors.^{27–28} Thus, varying mechanisms have been proposed, how CD4⁺ T cells contribute to tumor rejection.⁴⁶ The relevance of antitumor CD4⁺ T cells from experimental models is supported by several clinical case studies that demonstrated tumor regression following transfer of in vitro expanded tumor-infiltrating lymphocytes (TILs), which contained neoantigen-specific CD4⁺ T cells.^{24–47–48}

It is relevant to target MHC class II-restricted antigens, since MSI-CRC are instable and can downregulate or lose MHC I expression.^{49–51} Carcinomas rarely express MHC II and, therefore, cannot be recognized by CD4⁺ T cells. Efficacy by CD4⁺ T cells likely requires antigen uptake from dying cancer cells or vesicles released by the cancer cells, which are presented by MHC II molecules of tumor stroma cells like macrophages that are typically abundant in solid tumors. Mechanistically, the CD4⁺ T cells are expected to release IFN γ and TNF on recognition of the cross-presented antigen on MHC II, which leads to destruction of the tumor vasculature and leading to bystander killing and cancer regression.^{27–28–52} In this regard, it is important to note that TGF β 2(-1)-reactive CD4⁺ T cells were detected within the TIL of CRC tumors, indicating that TGF β 2(-1) is a targetable recurrent neoantigen. These CD4⁺ TIL were not restricted to HLA-DR4.¹⁵ Furthermore, TGF β 2(-1)-specific CD8⁺ T cells have been detected within the TIL of MSI-CRC.⁵³

The TGF β 2(-1) neoantigen occurs in 77% of MSI-CRC,^{16–17} 71% gastric and 17% endometrial cancers,⁵⁴

which makes it an attractive target for TCR gene therapy. On the other hand, MSI-CRC patients respond particularly well to immune checkpoint inhibitors (ICI), likely because of their high mutational burden.⁵⁵ Despite pronounced responses to ICI, half of the patients relapsed.⁵⁶ In such patients, especially if MHC I loss variants had been selected, MHC II-restricted TCRs could still be a treatment option. In conclusion, the DRB1*04:01-restricted TGF β 2(-1)-specific TCRs isolated from ABabDR4 mice are promising candidates for TCR gene therapy.

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Contributors NP conceived and designed the study, developed the methodology and performed all experiments, analyzed and interpreted the data, wrote and revised the manuscript. TB conceived, designed and supervised the whole project, analyzed and interpreted the data, wrote and revised the manuscript. TB is also the guarantor of the study. LP conceived and designed the study, contributed to development of methodology and analysis and revised the manuscript.

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Competing interests NP, LP, and TB are inventors of a patent (EP22155486) covering the TGF β 2(-1)-specific T cell receptors described in this study.

Patient consent for publication Not applicable.

Ethics approval Mouse experiments were approved by Landesamt für Gesundheit und Soziales Berlin (G-322/10).

Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as online supplemental information.

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