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Mutant p53-specific CD8TCR-therapy combined with a CD4TCR prevents relapse of cancer and outgrowth of micrometastases

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ABSTRACT

Relapse remains challenging in the treatment of metastatic cancers. More than 50% of human cancers harbor mutant p53 (mp53) as a cancer-specific target. We present the spontaneously metastasizing tumor model Ag104A to advance mp53-specific T cell receptor engineered T cell therapy (TCR-therapy). We identified in Ag104A an autochthonous $p53^{D256E}$ mutation as neoantigen recognized by a TCR isolated from $CD8^+$ T cells (CD8TCR). Cloning of the Ag104A cancer revealed mp53 expression in >99% of cancer cells. Targeting mp53 by CD8TCR-therapy was initially therapeutic, but tumors escaped as cancer cells with reduced or lack of antigen expression. Therefore, we determined whether escape could be prevented by combining the mp53-specific CD8TCR with a $CD4^+$ T cell-derived TCR (CD4TCR) recognizing a mutant antigen presented on the stroma of the cancer. No relapse occurred when the mp53-specific CD8TCR was combined with the stroma-recognizing CD4TCR. The combination therapy also prevented the development of macrometastases from cancer cells that had already spread to the lung at the time of TCR-therapy. Macrometastases were only observed after monotherapy. Thus, in a spontaneously metastatic model, tumor relapse and development of macrometastases can be prevented by combining a CD8TCR targeting an autochthonous p53-mutation with a mutation-specific CD4TCR recognizing tumor stroma.

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Introduction

Cancer is caused by mutations in the genome. Many of these mutations are cancer-specific somatic point mutations encoded by non-synonymous single nucleotide variants (nsSNVs). They cause single amino acid substitutions and are the basis of unique tumor-specific antigens,[1] now often referred to as neoantigens. Indeed, extensive studies in human cancers have shown that about 99% of these neoantigenic determinants are not shared between patients and are therefore appropriately being referred to as "unique".[2]

Cancer-specific mutations in the tumor suppressor gene *TP53* are almost universal in human and murine malignancies. Therefore, various approaches to p53-targeted therapies, including immunological ones, have been developed over the past several decades.[3] Changes in p53 are mostly the results of point mutations that occur in evolutionarily conserved codons in certain regions of the gene, also referred to as mutational hotspots.[4] These point mutations are highly variable between patients, and therapeutic reagents must be tailored to the particular neoantigenic determinant defined by the particular point mutation and the particular HLA presenting the mutant peptide found in the patient's cancer. By contrast, wildtype (wt) p53 sequences flanking a given p53 mutation (mp53) are shared with most patients and often highly overexpressed compared to the relatively low levels of the p53 protein in normal cells. Therefore, it was originally hoped that there was a therapeutic window and that epitopes encoded by these wtp53 sequences could serve as a "general tumor antigen".[5,6] Indeed, a wtp53 vaccine was able to induce $CD8^+$ T cells producing IFN-γ in response to multiple wtp53 peptides in most subjects with cancer.[7] Furthermore, T cell receptors (TCRs) of high-affinity to human wtp53 could be induced when HLA-A *02:01 transgenic mice that lacked tolerance to the human wtp53 sequences were used. T cells transduced with these TCRs killed very effectively a broad spectrum of human cancer cell lines and fresh isolated cancer cells.[8] However, it was subsequently found that these high-affinity TCRs also reacted to normal human cells raising doubts about wtp53

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sequences being a safe target for p53-specific TCR-therapy.[9] This led to a re-orientation of the field to targeting mutations in p53 since they are not found in normal cells and encode targets exclusively on malignant or premalignant cells.

A small percentage of cancer patients may share a given p53 mutation as well as the presenting HLA molecule with other patients.[10,11] Such so-called public, cancer-specific neoantigens, even though relatively rare, still can result in a substantial number of patients that could be treated.[12] For example, the most frequent p53 mutation R175H in human cancer with the presenting HLA molecule HLA-A *02:01 that is most frequent in people of European, Middle Eastern or North African ancestry, results in ~9,000 new cases each year in the United States alone.[13] Therefore, libraries of TCRs potentially useful for specific subsets of cancer patients are being developed in multiple locations. Adoptive transfer of autologous peripheral blood lymphocytes engineered with these allogeneic TCRs is being evaluated in the treatment of metastatic cancers that have failed other therapies. For instance, gene transfer of a $CD8^+$ T cell-derived TCR (CD8TCR) recognizing the HLA-A *02:01-restricted $p53^{R175H}$ mutation resulted in an objective response that lasted 6 months, after which the cancer escaped as an HLA-negative variant.[14]

Because of the attractiveness but also limitations of current mp53-specific TCR-therapy in patients,[15] an experimental model is needed for further improvement and investigation of mechanisms of tumor escape. Here, we present a syngeneic murine tumor model with a CD8TCR recognizing the autochthonous p53 mutation of a spontaneous cancer that naturally metastasizes to the lung. Well-established cancers exhibited a significant growth delay as CD8TCR T cells eliminated mp53-expressing cancer cells. However, all tumors eventually relapsed, and several mice succumbed of lung metastases. Tumor relapse correlated with presence of 0.3% of cancer cells within the Ag104A tumor cell population that carried only wtp53. Combining a tumor-stroma recognizing CD4TCR (derived from a $CD4^+$ T cell) with a mp53-specific CD8TCR not only eradicated the solid tumors but also prevented the outgrowth of metastatic cancer cells that had already spread to the lungs at the time TCR-therapy started. Thus, the combination of a tumor-stroma recognizing CD4TCR with a mp53-specific CD8TCR could overcome cancer relapse due to tumor heterogeneity.

Material and methods

Mice

This study adhered to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines for the reporting of experimental results. This study used male and female mice. Mice were 3–8 months old. The endpoint for *in vivo* experiments was reached when tumors reached a size of 2 cm^3 or when mice appeared hunched. Random littermates were selected and tagged to minimize confounding on the day of treatment. Envigo (Huntingdon, Cambridgeshire, United Kingdom) was the vendor from which C3H/HeN mice (RRID:MGI:2160972) were purchased from, while Douglas Hanahan (University of California, San Francisco, CA, USA) provided the C3H $Rag2^{-/-}$ (C3H.129S6-$Rag2^{tm1Fwa}$) mice. C3H $CD8^{-/-}$ (C3H.129S2-$Cd8a^{tm1Mak}$) and C3H $CD4^{-/-}$ (C3H.129S2-$Cd4a^{tm1Mak}$) mice were generated in house and described before.[16] All animals were maintained at the Max-Delbrück-Center for Molecular Medicine or at The University of Chicago under pathogen-free conditions in specific facilities. Splenocytes from C3H/HeN, C3H $CD8^{-/-}$ and C3H $CD4^{-/-}$ mice were used as T cell source for TCR-engineering.

Study approval

A specific pathogen-free barrier facility at the Max-Delbrück-Center for Molecular Medicine or at The University of Chicago was used to maintain mice. The Landesamt für Gesundheit und Soziales, Berlin, Germany and the Institutional Animal Care and Use Committee (IACUC) of The University of Chicago approved all mouse experiments according to institutional and national guidelines.

Cells

Ag104A is a spontaneous fibrosarcoma that was isolated at The University of Chicago.[17] The Ag104A-mL9-GFP cell line was generated in our laboratory by retroviral transduction as described.[18] Briefly, Plat-E cells were transfected by calcium phosphate precipitation with an pMP71 vector containing $(mL9\text{-}AAY)_3$-EGFP and viral supernatants were used to stably transduce 1×10^4 Ag104A cells in 24-well plates. GFP-expressing cells were sorted to acquire a pure population. Cell lines were shortly cultured and never repeatedly transferred *in vivo*. Ag104A, Ag104A-mL9-GFP and Plat-E packaging cells were maintained in Dulbecco's modified Eagles medium (Gibco, ThermoFisher, Waltham, MA, USA) supplemented with 10% heat-inactivated FCS and 100 U/mL penicillin/streptomycin. Culture medium for Plat-E cells was supplemented with 10 µg/mL blasticidin and 1 µg/mL puromycin (Sigma-Aldrich) for selection. RPMI 1640 GlutaMAX medium (Gibco, ThermoFisher) with 10% fetal calf serum (FCS, heat-inactivated, PAN Biotech), 1 mM sodium pyruvate, 100 U/mL penicillin/streptomycin, 50 µM 2-mercaptoethanol and 100 µM non-essential amino acids (all Gibco, ThermoFisher) was used for primary T cells and other cell lines. Master cell stocks were created shortly after thawing and expanding the original cell line. All cell lines were kept in culture for no more than one month.

T cell cultures, isolation of TCR genes and construction of transgene cassettes

Ex vivo T cell cultures from spleen of immunized mice and generation of T cell clones by limiting dilution have been described.[17,19] For identification of TCR-sequences, total RNA was extracted from the T cell line YL1 and the two distinct T cell clones A2 and A3 (RNeasy Micro Kit, Qiagen) and used for 5'-RACE PCR (SMARTer RACE cDNA Amplification kit, TaKaRa) with 3' – primers specific for the constant region of the mouse TCR α- and β-chain (mCa-RACE : 5'-cag gca gag ggt gct gtc ctg aga-3', mCb-RACE: 5'-gga gac ctt

ggg tgg agt cac att tct-3'). TCR sequences of M2/3[20] were determined as TRAV9D–3* 01-CAVSGGGSNYKLTF-TRAJ53* 01 and TRBV13–1* 02-CASSGRDRNAEQFF-TRBJ2 -1* 01, synthesized by GeneArt (Life Technologies, Carlsbad, CA, USA) and integrated in pMP71-PRE.[21,22]

TCR-transduction

TCR-transduction using $CD4^+$ and $CD8^+$ T cells has been described.[16,20] In brief, calcium phosphate precipitation was used for transfection of Plat-E cells with pMP71-M2/3 or pMP71-H6,[16] pMP71-OTI,[23] pMP71-P14,[21] pMP71-1D9,[24] pMP71-TCRI, pMP71-TCRIV[25] as described. 48 h after transfection, viral supernatants were harvested, filtered (0.45 µm pore size) and used for transduction. Splenocytes were harvested and red blood cells were lysed by adding TRIS ammonium chloride. T cells (2×10^6 cells/mL) were stimulated with 10 IU/mL recombinant human IL-2 (Proleukin, Novartis), 1 µg/mL anti-CD3 (clone 145-2C11, BD Biosciences) and 0,1 µg/mL anti-CD28 (clone 37.51, Biolegend). The next day, 24-well plates were coated for 2 h with RetroNectin (12,5 µg/mL, Takara). Blocking was done with a 2 % bovine serum albumin solution in PBS (30 min, 37°C). Plates were further coated with viral particles (centrifugation at 3,200 × g, 90 min, 4 °C) followed by spinoculation (800 × g, 90 min, 32 °C) with T cells (1.5×10^6 per well). For spinoculation, 8 µg/mL protamine sulfate, 10 IU/mL recombinant IL-2 and 4×10^5 Dynabeads^{TM} mouse T-activator CD3/CD28 (Invitrogen) were added per 24-well. A second viral supernatant, 10 IU/mL IL-2 and 4 µg/mL protamine sulfate was added the next day and T cells were spinoculated again at 800 × g for 90 min (32 °C). After 6 h, T cells were harvested and 1×10^6 cells/mL were seeded into culture flasks with 50 ng/mL recombinant IL-15 (Miltenyi Biotec, Bergisch Gladbach, Germany) for $CD8^+$ T cells and 40 IU/mL IL-2 for $CD4^+$ T cells. Adoptive transfer into tumor bearing mice was performed 72 h later. For *in vitro* analyses, TCR-transduced T cells were cultured for additional 10 days before used.

Isolation of antigen presenting cells from stroma of tumors

Ag104A-mL9-GFP tumors were used as source for the isolation of antigen presenting cells ($CD11b^+$) as described.[16] Tumors were grown in C3H $Rag2^{-/-}$ mice. To obtain single cell suspensions, 2 mg/mL Collagenase D and 100 U/mL DNAse I (both Roche, Indianapolis, IN, USA) were added to fragmented tumors. A horizontal shaker was used to incubate tumor fragments for 20 min at 37 °C. Afterwards, trypsin resuspended in Hanks' Balance Salt Solution (HBSS, MP Biomedicals LLC, Solon, OH, USA) was added to a concentration of 0.025% and incubated for additional 15 min, 37 °C. before filtered through a 40 µm cell strainer (ThermoFisher). Magnetic cell sorting (Miltenyi Biotec) was used to isolate $CD11b^+$ cells according to manufacturer's protocol. Purity was determined by flow cytometry before used for T cell stimulation.

Determination of antigen presentation

TCR-engineered T cells were cultured together with either cancer or stromal cells as described.[16] In brief, 5×10^4 T cells were cocultured with 1×10^5 target cells. Supernatants were removed and tested for IFN-γ by ELISA (Ready-SET-Go!, eBioscience or BD Biosciences), following the manufacturer's protocol. No stimulation and TCR-independent stimulation using 8 mg/mL αCD3- (University of Chicago, Frank W. Fitch Monoclonal Antibody Facility, Clone 145-2C11.1) and 2 mg/mL αCD28-specific (Clone 37.51, BioLegend) antibodies or ionomycin (1 mmol/L, Sigma-Aldrich) and phorbol 12-myristate 13-acetate (5 ng/mL, Sigma-Aldrich) were used.

Cytotoxicity assays

^{51}Cr-release: ^{51}Cr-release assay was performed as described.[17] In brief, 5×10^3 ^{51}Cr-labeled cancer cells were cultured for 4.5 h together with either the T cell line or the distinct T cell clones in 96-well V-bottom microtiter plates (Dynatech, Chantilly, VA). The percentage of specific lysis was calculated: % cytolysis = [(experimental release – spontaneous release)/(maximum release – spontaneous release)] x 100. *Flow cytometry-based*: 5×10^4 Ag104A-mL9-GFP cancer cells were cocultured with 2×10^5 TCR-transduced T cells (effector to target ratio 4:1) in a 96-well flat bottom plate (Greiner Bio-one, Austria). After 9 h, 16 h, 24 h and 48 h, samples were stained for CD3 to exclude T cells and viable (propidium iodide negative), GFP-positive cancer cells were analyzed by flow cytometry using a NovoCyte Quanteon (Agilent, Santa Clara, CA, USA). For quantification, 10 µL AccuCount Rainbow Fluorescent beads (Spherotech, Lake Forest, IL, USA) were added and recorded cell numbers were then normalized per 3,000 beads for data presentation.

Neoantigen determination

To determine the genetic origin of the Ag104A-specific CD8TCR M2/3, short DNA sequences of 75 bp in length for all potential neoantigens (n = 77, Table S1) were synthesized. The 75 bp DNA sequence encodes for 25 amino acids with the mutated amino acid in the center at position 13 and flanked by the corresponding wild type sequence. A maximum of ten minigene fragments were concatenated as tandem decamer separated by an alanine-alanine-tyrosine (AAY) spacer to improve proteasomal cleavage.[26] Each of these eight tandem minigenes (TMGs) was linked to GFP using a P2A sequence as linker element and integrated using NheI and XhoI restriction sides into the expression vector pcDNA3.1. Constructs were designed *in silico*, generated and codon optimized by GeneArt (Thermo Fisher Scientific, Waltham, MA, USA). The control TMGs (CG-1, CG-2, CG-3, CG-4) were constructed based on TMG7 with the positions indicated in Figure 2a replaced by 25 amino acids of ovalbumin, LCMV gp33, mp68, and peptides I and IV of SV40 large T encoding for the described H-$2K^b$- and H-$2D^b$-presented T cell epitopes in the center position.[25,27–29] The control TMG constructs (CGs) were directly linked to GFP. Linearized TMG or CG vectors (1 µg) were used to generate *in vitro* transcribed RNA (ivt-RNA) with

the mMESSAGE mMACHINE T7 transcription kit (Thermo Fisher). XbaI was used for linearization. Subsequent tailing was performed by polyadenylation (poly(A) tailing kit, Thermo Fisher) and ivt-RNA was extracted by phenol/chloroform purification. Generation of vectors encoding H-2K^k and H-2D^k. Splenocytes from C3H/HeN mice were harvested and total RNA was isolated. A random primer mix (Promega) was used to synthesize cDNA (SuperScript II, Invitrogen). PCR was performed on cDNA using primers encoding for NotI and EcoRI restriction sites for subsequent cloning of H-2K^k (Forward 5'-cag cgg ccg cca cca tgg cac cct gca tgc *t*-3', Reverse 5'-tcg aat tct cac gct aga gaa tga ggg tca-3') and H-2D^k (Forward 5'-cag cgg ccg cca cca tgg ggg cga tgg tac c-3', Reverse 5'-tcg aat tct cac gct tta caa tct ggg aga-3') into pMP71. Plate-E cells were transiently transfected by calcium phosphate precipitation with pMP71-H-2K^k or MP71- H-2D^k vectors and 48 h later virus supernatants were harvested for transduction. To generate surrogate antigen-presenting cancer cells, the lymphoma cell line EL4 which is of H-2^b background was used. 24-well plates were coated with RetroNectin (12,5 µg/mL). EL4 cells (2×10^5/mL) together with 1 mL virus supernatant and protamine sulfate (8 µg/mL) were added and spinoculated at 800 × g, for 90 min (32°C). The following day, EL4 cells were again transduced with a second round of viral supernatant together with 4 µg/mL protamine sulfate at 800 × g for 90 min (32°C). H-2K^k and H-2D^k positive EL4 cells were enriched by flow cytometry based cell sorting. The MicroPulser Electroporator instrument (Bio-Rad Laboratories) was used to electroporated 1×10^6 EL4 cells with 10 µg ivt-RNA. TMG expression was determined by GFP fluorescence using flow cytometry 24 h later.

Tumor injection and treatment

5×10^6 Ag104A or 1×10^5 Ag104A-mL9-GFP cancer cells were injected *s.c.* into C3H Rag2$^{-/-}$. T cells were transferred by *i.p.* or *i.v.* injections 14 or 25 days later. Splenocytes from C3H/HeN, C3H CD8$^{-/-}$ or C3H CD4$^{-/-}$ mice were used for TCR-engineering. Effectivity of TCR-engineering was determined on the day of treatment and used to adoptively transfer 1 – 2×10^6 M2/3-CD8TCR- or H6-CD4TCR-T cells per recipient for monotherapy. When M2/3-CD8TCR-T cells were combined with H6-CD4TCR-T cells, the number of TCR$^+$ T cells of each population injected *i.p.* per recipient was 1×10^6. Randomization of the different treatment groups was done on the day of adoptive T cell transfer. Tumor volume was measured along three axes two to three times a week and calculated by: (a*b*c) ÷ 2. Study endpoint was 100 days after adoptive T cell transfer, when tumor sizes reached more than 2 cm^3 or mice appeared hunched and weak.

Determination of lung metastasis

C3H Rag2$^{-/-}$ mice were *s.c.* injected with 1×10^5 Ag104A-mL9 -GFP cancer cells. 14 and 22 days later, the lung was removed, digested with DNAse and Collagenase D and a single cell suspension in DMEM containing 10% FBS and 2 mM L-glutamine was prepared (as described in isolation of stromal CD11b$^+$ cells). Cells were plated into 96-well flat bottom plates at different concentrations using 10-fold dilutions: 10,000 cells/well, 1,000 cells/well, 100 cells/well, 10 cells/well and 1 cell/well. After 10 days, plates were analyzed with goggles "miner's lamp" FHS/F-01 (BLS, Budapest, Hungary) equipped with the light source FHS/LS-1B and emission filter FHS/EF-4Y1 for GFP. Alternatively, lungs were inflated either with 1 mL 10% formalin solution (Sigma-Aldrich, St. Louis, MO, USA) alone or 1 mL 4% formalin containing 50 % India ink (Sanford Black Stamp Pad Ink, Bellwood, IL, USA)

Sequencing

Whole-exome and RNA sequencing: AllPrep DNA/RNA mini kit (Qiagen, Venlo, The Netherlands) was used to isolate genomic DNA and total RNA from Ag104A cancer cells. A germline control from the original Ag104 mouse of tumor origin was also available by isolating genomic DNA and total RNA from heart-and-lung fibroblasts (Ag104-HLF) from the mouse where the Ag104A cancer grew. Whole-exome DNA libraries were prepared from 2 to 3 µg of genomic DNA (SureSeletXT Mouse All Exon V1, Agilent Technologies, Santa Clara, CA). TruSeq Stranded Total RNA Library Prep kit (Illumina) was used to prepare RNA libraries from 1 µg of total RNA. Quantification and quality control of prepared libraries was done using the 2200 Tape Station (Agilent Technologies) prior to sequencing by 150 bp paired-end reads (NextSeq 500 Desktop Sequencer or HiSeq2500 Sequencer, both Illumina). *Variant calling*: Somatic variants (SNVs and indels) were called using a Fisher's exact test-based method with the following parameters (i) base quality ≥ 15, (ii) sequence depth ≥ 10, (iii) variant depth ≥4, (iv) variant frequency in tumor ≥ 10%, (v) variant frequency in normal <5%, and (vi) Fisher p value< 0.05.[19] SNVs and indels were annotated using ANNOVAR[30] based on RefGene. *Sanger sequencing*: Presence or absence of mp53 in the parental Ag104A cell line and its relapse variants was verified by sanger sequencing using p53-specific primers for amplification of genomic DNA. Forward 5' – gaa gac agg cag act ttt cg – 3'. Reverse 5' – aga ggc gct tgt gca ggt – 3'.

Barcoding Trp53-specific PCR and deep sequencing

The *Trp53*-barcoding approach was based on a previously described method.[31] Genomic DNA was isolated (Purelink™ Pro 96 Genomic DNA Purification kit (Thermo Fisher)) from bulk Ag104A cancer cells or clonal cultures of Ag104A cancer cells sorted and propagated in 96 wells. The first step amplified a region between intron 5 and 6 of the *Trp53* locus using Phase 1 primers from genomic DNA. Ph1-F 5'- gga tcc tgt gtc ttc ccc cag –3' and Ph1-R 5'- aaa gag cgt tgg gca tgt g –3'. Cycle conditions: Step 1, 95°C for 15 min; Step 2, 94°C for 30 s, 62°C for 1 min and 72°C 1 min with 25 cycles; Step 3, 72°C 10 min. Next 1 µl together with Phase 2 primers was used for a nested PCR amplifying exon 6 and intron 6. Ph2-F: 5'- cca ggg ttt tcc cag tca cga ccg gct ctg agt ata cca cca tcc A-3' and Ph2-R 5'- agc gga taa caa ttt cac aca gga tag ggg gcg gga ctc gtg gaa cag-3'. Cycler conditions: Step 1, 95°C for 15 min; Step 2. 94°C for 30 s, 64°C for 1 min, 72°C for 1 min with 25 cycles; Step 3. 72°C for 7 min. The Phase 2 primers contained a common 23-base sequence at

the 5′-end for further amplification. Lastly, 1 µl of the nested PCR was used for library preparation adding barcodes and Illumina P5 and P7 adaptors to enable next-generation-sequencing (NGS). Phase 3 primers shared common sequences with Ph2 primers (adaptors) and 5' (row-specific) and 3' (column-specific) barcodes containing unique 5-nucleotide sequences.[31] The PCR products were combined, run on a 1.2 % agarose gel and a band of approximately 360 bp was purified (Qiaquick gel extraction kit, Qiagen). NGS was performed using the Illumina MiSeq platform. Paired-end (MiSeq reagent Kit v2, 2 × 250 nucleotides) sequencing reads were merged using PEAR[32] and assigned to individual plates and wells following our scripts for single cell TCR sequencing (https://github.com/HansmannLab/TRECA). In each well, p53 reads were identified by alignment with Bowtie 2[33] and reads with exact matches for mutated (5'-CAT CAC ACT GGA AGA gTC CAG GTA GGA AGG-3', mutated nucleotide in lower case letter) and wt (5'-CAT CAC ACT GGA AGA CTC CAG GTA GGA AGG-3') sequences were counted.

Antibodies used for flow cytometry

Samples were stained with 0.2 to 1 µg of indicated anti-mouse antibodies in PBS for 20 min (4°C) and analyzed either on the FACSCantoII Symphony A1 (BD Biosciences), the MACSQuant (Miltenyi Biotec), or the NovoCyte Quanteon (Agilent, Santa Clara, CA, USA). TCR-engineered mouse T cells were analyzed using anti-mouse CD3ε (PE, FITC, APC, BV421^{TM}, clone 145-2C11), CD4^{+} [APC, clone GK1.5], CD8α (PE, FITC, APC, BV421^{TM}, clone 53–6.7). Antibodies directed against mouse TCRβ were used to determine expression of TCRs in engineered T cells. M2/3 (TCRvβ8.3, PE, clone 1B3.3, BD Biosciences) H6 and 1D9 (TCRvβ6, PE, clone RR4–7), OT-I (TCRvβ5, PE, clone: MR9–4), P14 (TCRvβ8.1, PE, clone: MR5.2), TCRI (TCRvβ7, PE, clone: TR310), and TCRIV (TCRvβ9, PE, clone: MR10–2). Surface MHC I expression was determined by using anti-mouse H-2K^{k} (APC, clone 36–7–5) and H-2D^{k} (PE, clone 15–5–5). Isolation of stromal cells was confirmed using CD11b^{+} (APC, clone M1/70). Unless otherwise indicated antibodies were purchased from BioLegend. FACSorting of desired cells was performed using FACSAria (BD Biosciences). Data were analyzed with FlowJo (BD Biosciences).

Data and statistical analysis

Statistical analysis was done using GraphPad Prism 9 (GraphPad, La Jolla, CA, USA) and data are displayed as mean ± standard deviation as indicated. Sample size is indicated and based on statistical significance. Method used to determine statistical significance is indicate. p values ≥ 0.05 were considered not significant (n.s.). p values are indicated as $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$. Biological repeats are defined as independent experiments, done on separate days with freshly generated TCR-transduced T cells.

Results

A cancer-specific CD8TCR for therapy of a spontaneous tumor

We used the Ag104A cancer cell line which was derived from a tumor that developed spontaneously in an aging immunocompetent normal C3H/HeN mouse.[17] A cancer-specific cytolytic T cell line (CTL line YL1) was previously established using Ag104A cancer cells expressing the costimulatory molecules B7–1 and CD48 for immunization of C3H/HeN mice (Figure 1a).[19] We generated two distinct T cell clone cultures, A2 and A3, from the CTL line YL1 and evaluated their cytolytic activity against the autochthonous Ag104A cancer cell in a 4 h Cr^{51}-release assay (Figure 1b). T cell clones A2 and A3 as well as the parental YL1 T cell line all lysed Ag104A cancer cells similarly well and did not react to 4102, a syngeneic UV-induced cancer cell control. TCR-sequencing revealed identical TCR α- and β-chains for the two separate T cell clone cultures A2 and A3 which were also the dominant chains in the parental YL1 line (Figure 1c). This Ag104A-specific TCR, hereafter referred to as M2/3-CD8TCR was cloned into a retroviral vector and used for TCR-engineering of T cells isolated from the spleen of C3H/HeN mice (Figure 1d). M2/3-engineered CD8^{+} T cells again specifically recognized Ag104A but not 4102 cancer cells determined by IFN-γ release (Figure 1e).

Each position on a tandem decamer minigene construct supports antigen processing

An established method for screening specificities of TCRs is using surrogate target cells that express short gene fragments, also named "minigenes".[34] These minigenes usually encode 25 amino acids of a potential immunogenic target linked to GFP. Routinely, ten different minigenes are concatenated in tandem generating tandem decamer minigenes (TMG). Correct proteasomal processing of each minigene is facilitated by adding an AAY cleavage site after every position.[16] However, these studies did not determine whether all ten positions in a given TMG are equally well processed and presented, leading to potential bias.[35] Therefore, we validated this for the TMG system and generated four control TMGs (CG-1, CG-2, CG-3, CG-4, Figure 2a). Each of the ten positions in the CGs encodes for one of five model antigens for which TCRs were available. TCR OT-1 recognizes an H-2K^{b}-restricted peptide derived from the antigen ovalbumin (OVA), TCR P14 from the antigen gp33 of the lymphocytic choriomeningitis virus (LCMV, H-2D^{b}), TCR 1D9 from mutant p68 (H-2K^{b}), and TCRI and TCRIV recognize peptide I (H-2D^{b}) and peptide IV (H-2K^{b}) of the SV40 large T antigen. The positions between the known minigenes encoded for unrelated sequences. *In vitro* transcribed RNA of the four CGs was electroporated into EL4 cells which were then used to stimulate T cells engineered with either of the TCRs OT-1, P14, 1D9, TCRI and TCRIV. Importantly, all four populations of electroporated EL4 cells are similarly well recognized by the different TCR-engineered T cells as measured by IFN-γ release (Figure 2b).

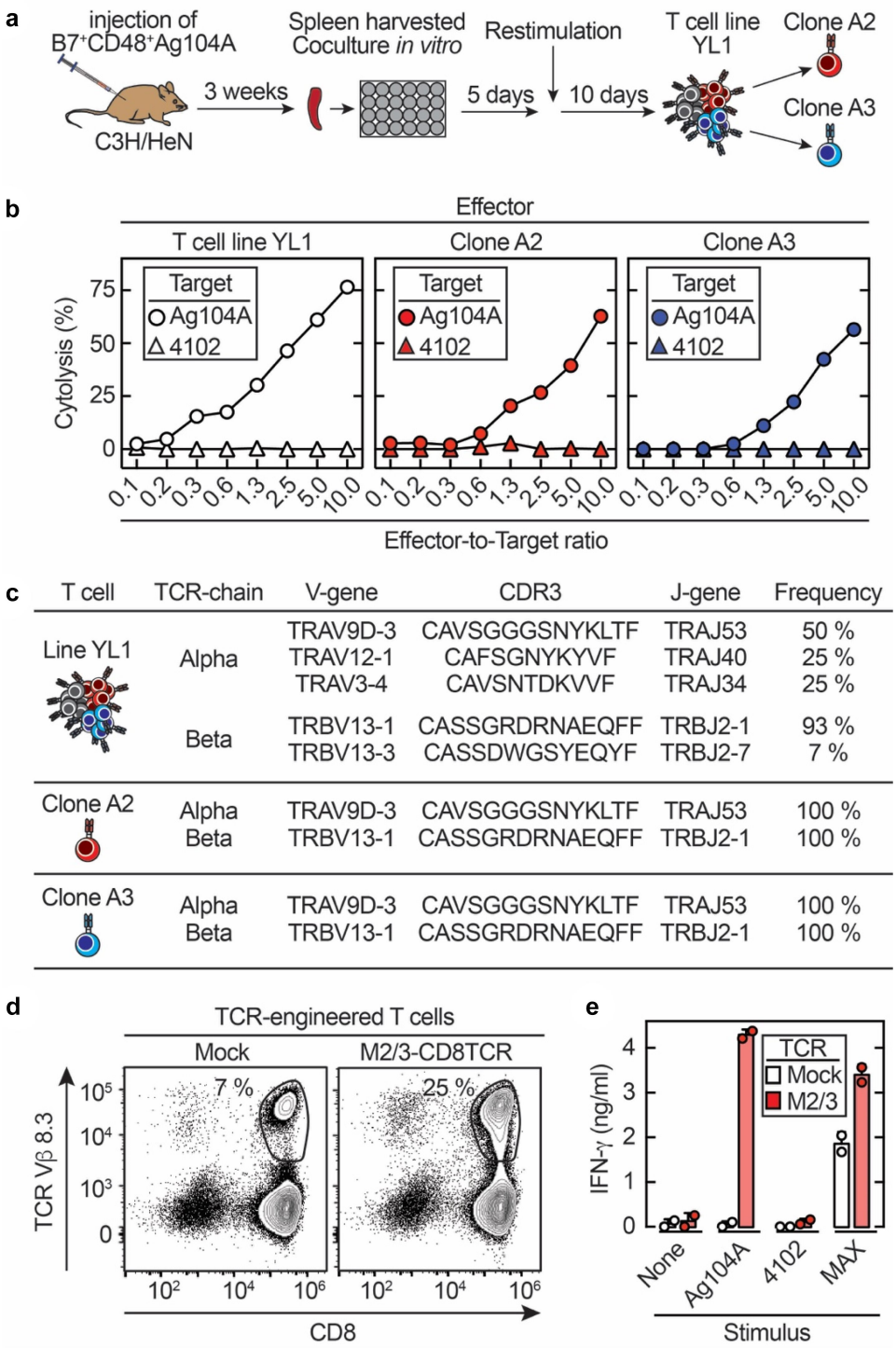

Figure 1. Selection of a CD8TCR from an Ag104A-specific CTL line. (a) overview of the generation of an Ag104A-specific CTL line done by Li et. al.[19] (b) specificity of the CTL line YL1 and its derived clones A1 and A2 was determined by a 4 h Cr^{51}-release assay. (c) the Ag104A-specific clones A1 and A2 express the most frequent TCR α- and β-chains of the CTL line YL1 as detected by 5′-RACE PCR. (d) T cells from the spleen of C3H/HeN mice were retrovirally transduced to express the M2/3-CD8TCR. (e) TCR-engineered T cells were cocultured for 24 h with indicated cancer cells and supernatants were analyzed for IFN-γ concentrations by ELISA. TCR-engineered T cells specifically recognized Ag104A cancer cells. Shown is one out of three independent biological repeats. Data are mean ± standard deviation from technical duplicates. Stimulation with αCD3/28 (MAX) was used as nonspecific T cell activation.

The M2/3-CD8TCR recognizes the D256E mutation in the tumor suppressor Trp53

To determine the recognized antigen of the M2/3-CD8TCR, we identified all non-synonymous single nucleotide variants (nsSNV) present in Ag104A and absent in the nonmalignant Ag104 heart-lung fibroblast (Ag104-HLF) autochthonous control by whole-exome sequencing. Out of 172 nsSNV mutations, 77 were expressed as identified by RNA-sequencing (Figure 2c, Table S1). We designed eight different TMGs covering all 77 mutations (Figure 2d). EL4 cells were modified to express the

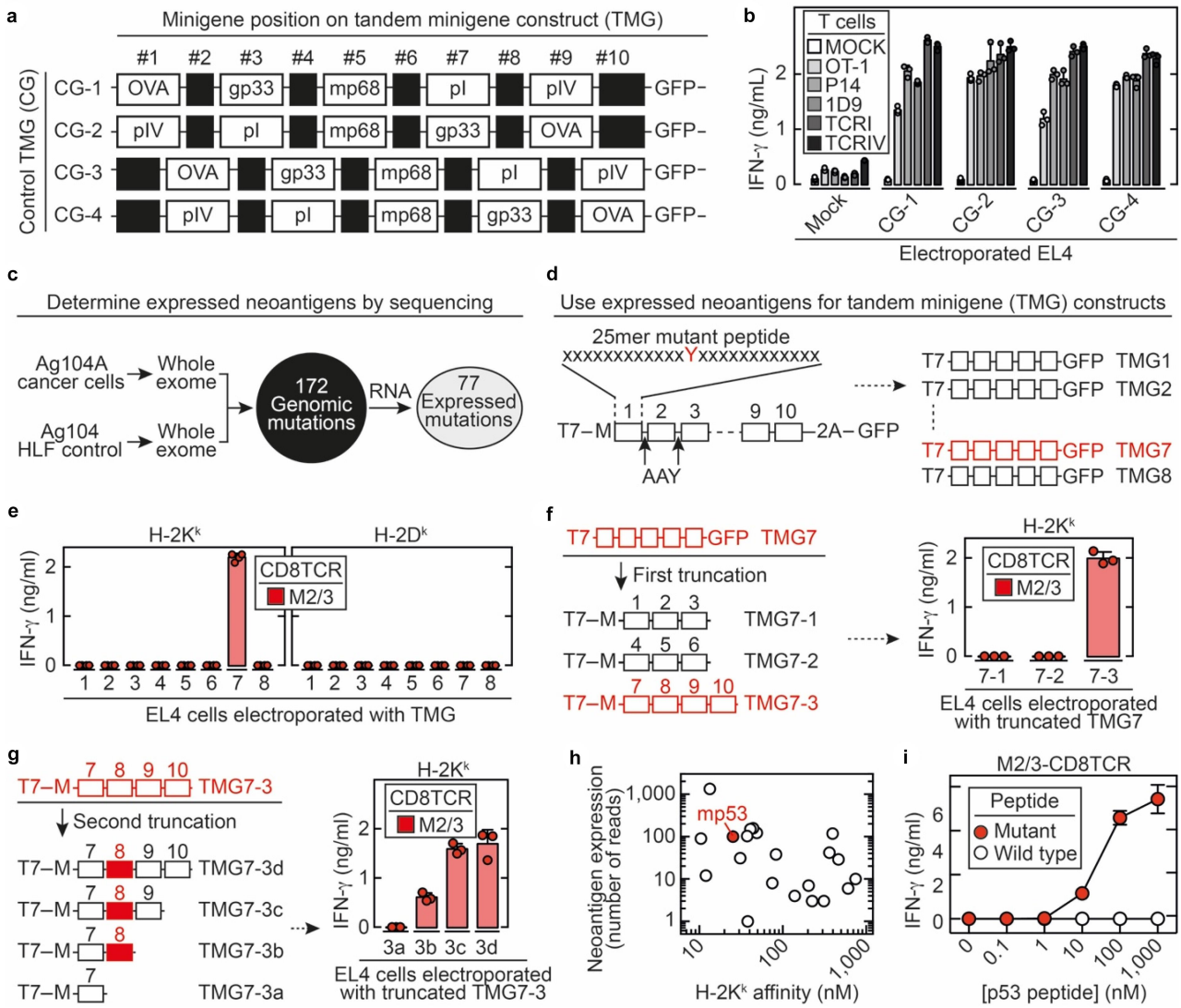

Figure 2. The CD8TCR M2/3 is specific for a D256E mutation in the tumor suppressor gene *Trp53*. (a–b) every position on a tandem minigene supports antigen processing for recognition by TCR-engineered T cells. (a) overview of minigenes encoding known antigens. Minigenes depicted as black box represent unrelated antigen sequences. Four different minigenes were designed to cover all ten positions in a decamer tandem minigene construct (CG-1, −2, −3 and − 4). (b) EL4 cells were electroporated with the different CG constructs and a minigene encoding unrelated sequences (mock). EL4 cells were then cultured for 24 h with TCR-engineered T cells. Supernatants were analyzed for IFN-γ concentrations by ELISA. Spleen of C57BL/6 mice were used as T cell source for TCR-engineering. Shown is one out of two independent biological repeats. Data are mean ± standard deviation from technical triplicates. (c) results of whole-exome-sequencing and RNA-sequencing were compared between Ag104A cancer cells and Ag104 heart-lung fibroblasts (HLF) as syngeneic normal control to determine expression of 77 Ag104A-specific mutations. (d) schematic representation of the tandem decamer minigene (TMG) design encoding for 25mer neoepitope sequences with the mutated amino acid at the center position, concatenated with an AAY proteasomal cleavage site and linked via 2A to GFP. Eight tandem minigenes were constructed to cover 77 mutations. (e – g) spleen of C3H/HeN mice were used as T cell source for TCR-engineering. EL4 cells were electroporated with different TMG constructs and cultured for 24 h with M2/3-engineered T cells. Supernatants were analyzed for IFN-γ concentrations by ELISA. Shown is always one out of two independent biological repeats. Data are mean ± standard deviation from technical (e) quadruplicates or (f and g) triplicates. (e) eight different TMG constructs were introduced into EL4 cells either positive for H-2K^k or H-2D^k MHC class I alleles. Only TMG7 expressed by H-2K^k positive EL4 cells was recognized by M2/3-engineered CD8$^+$ T cells. (f) TMG7 was divided into sub-TMGs 7–1, 7–2 and 7–3 containing only three or four minigenes. EL4 H-2K^k cells expressing TMG7–3 were recognized. (G) TMG7–3 was truncated to contain only one (7-3a), two (7-3b), three (7-3c) or four (7-3d) neoantigens. TMG7-3b, -3c and 3-d were all recognized indicating position eight of the original TMG7 as target for M2/3-engineered CD8$^+$ T cells. (h) NetMHC 4.0 predicted 21 out of 77 nsSNVs to bind to H-2K^k. The affinities of these 21 neoantigens were plotted against their total number of reads obtained by RNA-sequencing. (i) position eight of TMG7 contained the D256E mutation in *Trp53*. The 9mer sequence LEESSGNLL was predicted to be one of the highest H-2K^k binders. Splenocytes from C3H/HeN were cultured either with graded concentrations of mutant or wild type 9mer peptide and M2/3-engineered T cells. Spleen of C3H/HeN mice were used as T cell source. Supernatants were analyzed for IFN-γ concentrations by ELISA. Shown is one out of two independent biological repeats. Data are mean ± standard deviation from technical triplicates.

C3H MHC I haplotypes H-2K^k and H-2D^k (Fig. S1). Both cell lines were electroporated with eight different TMG constructs. The M2/3-engineered CD8$^+$ T cells released IFN-γ specifically after recognition of TMG7 when introduced into H-2K^k but not in H-2D^k positive EL4 cells (Figure 2e). TMG7 was divided into three smaller TMGs harboring either three or four different mutations instead of all ten and used again for stimulation of M2/3-T cells (Figure 2f). TMG7–3, which contained mutations of position seven, eight, nine and ten was still able to induce IFN-γ release. Further truncation of TMG7–3 generated four TMGs containing either only the mutation of position seven (TMG7-3a), mutations of position seven and eight

(TMG7-3b), mutations of position seven, eight and nine (TMG7-3c) or all four mutations (seven, eight, nine and ten, TMG7-3d). M2/3-T cells did not recognize TMG7-3a but all other constructs (TMG7-3b, -3c and 3-d) indicating mutation encoded by position eight of the original TMG7 to be the most likely target (Figure 2g). Position eight encoded a D256E mutation in the tumor suppressor gene *Trp53*. We used the open access computer algorithm NetMHC 4.0[36] to predict which 8mer or 9mer peptide derived from all 77 expressed nsSNVs will bind to H-2K^k (affinity <1,000 nM). NetMHC predicted 21 neoantigens with an affinity of below 1,000 nM (Table S2). The affinity of these 21 H-2K^k binders was compared to their total number of mRNA reads (Figure 2H). The p53 9mer peptide sequence LEESSGNLL was predicted to have one of the highest binding affinities to H-2K^k (25 nM) together with moderate gene expression (100 reads, Table S1 and S2) which we used to confirm specificity of the M2/3-CD8TCR. Peptide stimulation of M2/3-T cells verified recognition of the D256E mutation in p53. M2/3-T cells specifically reacted to the mutant LEESSGNLL and not the wild type LEDSSGNLL 9mer peptide presented by spleen cells from C3H/HeN mice (Figure 2i).

Tumor escape from mutant-p53 specific CD8TCR-therapy by antigen-negative variants

To understand how homogeneous the Ag104A cancer cell line carries the mutant-p53 neoantigen, we generated 128 clones from the Ag104A cancer cell line and evaluated their ability to stimulate M2/3-T cells (Figure 3a). All 128 clones were recognized by M2/3-T cells with the weakest clone inducing secretion of ~2.5 ng/mL IFN-γ and the strongest ~5.3 ng/mL IFN-γ. Thus, the D256E mutation in p53 is present in all analyzed clones and might therefore be a suitable target for mutation-specific TCR-therapy. We transduced the M2/3-CD8TCR into splenic T cells from C3H/HeN mice and adoptively transferred M2/3-T cells into C3H Rag$^{-/-}$ mice bearing established Ag104A tumors (Figure 3b). The M2/3-CD8TCR arrested tumor growth for about 20 days before tumors regularly relapsed (Figure 3c). Nonetheless, mice treated with M2/3-T cells still had a significant ($p = 0.00007$) survival advantage compared to mice treated with mock T cells (Figure 3d). We readapted eight relapse variants *in vitro* to investigate possible reasons of tumor escape. Interestingly, relapse variants still stained positive by flow cytometry for the MHC class I molecules H-2K^k and H-2D^k (Figure 3e). However, only one relapse variant (M2) was still recognized by M2/3-T cells. A second relapse variant (M1) induced strongly reduced IFN-γ release compared to parental Ag104A cancer cells (Figure 3f). Six additional relapse variants (M3 to M8) were no longer recognized by M2/3-T cells. Since relapse variants were still positive for MHC class I, we evaluated whether they were still able to process and present the mp53 neoantigen. Indeed, relapse variants were recognized by M2/3-T cells when electroporated with the TMG7 minigene (Fig. S2) indicating that antigen processing and presentation were still functioning. Nonetheless, loss of recognition by M2/3-T cells was heritable since relapse variants were not able to induce IFN-γ secretion even after 48 passages, ~ 4 months of *in vitro* culture (Fig. S3). We now determined whether the D256E mutation was still present in the *Trp53* gene. We isolated genomic DNA from relapse variants and amplified the p53 region that included the D256E mutation by standard PCR. Sanger-sequencing done with amplified genomic DNA from the original Ag104A cancer cell line detected two similar peaks at the position of the mutation (Fig. S4). One peak represented a C (blue) resulting in the GAC codon which encodes aspartic acid (D) in wild type p53. The second peak represents a G (black) resulting in the GAG codon which encodes glutamic acid (E) in mutant p53 (Fig. S4). The equal detection of both codons by Sanger-sequencing confirms results from RNA-sequencing (Table S1). However, when genomic DNA from the relapse variants M1, M2, M3 and M4 were analyzed, the mutant GAG codon was strongly reduced in the first two relapse variants (M1 and M2) and not detected in the two subsequent variants (M3 and M4) in which only the wildtype GAC codon remained (Figure 3g). Sanger-sequencing also detected a reduced peak of the mutant GAG codon in the relapse variants M1 and M2 which could still stimulate M2/3-T cells to secrete IFN-γ (Figure 3f). Thus, tumor escape might be due to selection of antigen-negative Ag104A variants. To investigate whether pre-existing mp53-negative cancer cells exist in the original Ag104A bulk population, we developed a barcoding, *Trp53*-specific PCR approach (Fig. S5) to simultaneously screen for the wild type and the D256E-encoding regions across a large number of Ag104A cancer cell clones.[31] Genomic DNA analysis of the Ag104A bulk cell line determined an equal ratio of wild type and mutant *Trp53* reads (Figure 3h) confirming results from RNA-sequencing (Table S1). However, when we performed deep sequencing of 1,828 Ag104A clones, we identified five clones (~0.3%) that showed only the *Trp53* wild type genotype (Figure 3i). This result indicates that, in our experimental model, approximately 15,000 of the 5,000,000 transplanted Ag104A bulk cells had only wild type p53.

Prevention of escape by combining the mp53-specific CD8TCR with a neoantigen-specific CD4TCR

We showed in a previous study that a combination of one CD8TCR with one CD4TCR can eradicate tumors and thereby prevent escape of antigen-negative variants.[16] In order to evaluate whether such a combinational TCR-therapy can also prevent tumor escape after mp53-specific CD8TCR-therapy in the spontaneous Ag104A tumor model, we transduced Ag104A cancer cells to express the MHC class II, I-E^k-restricted mutant neoantigen mL9.[1] The resulting Ag104A-mL9 cancer cell line is being killed by the M2/3-CD8TCR (Fig. S6) and develops tumors in which stromal CD11b$^+$ cells can be recognized by the mL9-specific CD4TCR H6 (Fig. S7).[37] Thus, Ag104A-mL9 cancer cells and CD11b$^+$ stromal cells from Ag104A-mL9 tumors can be targeted respectively by M2/3-CD8TCR and H6-CD4TCR engineered T cells. C3H Rag$^{-/-}$ mice bearing established Ag104A-mL9 tumors were treated either with the M2/3-CD8TCR, the H6-CD4TCR or a combination of both (Figure 4, left). Tumors treated with the M2/3-CD8TCR alone relapsed after 20 days of growth arrest while tumors treated

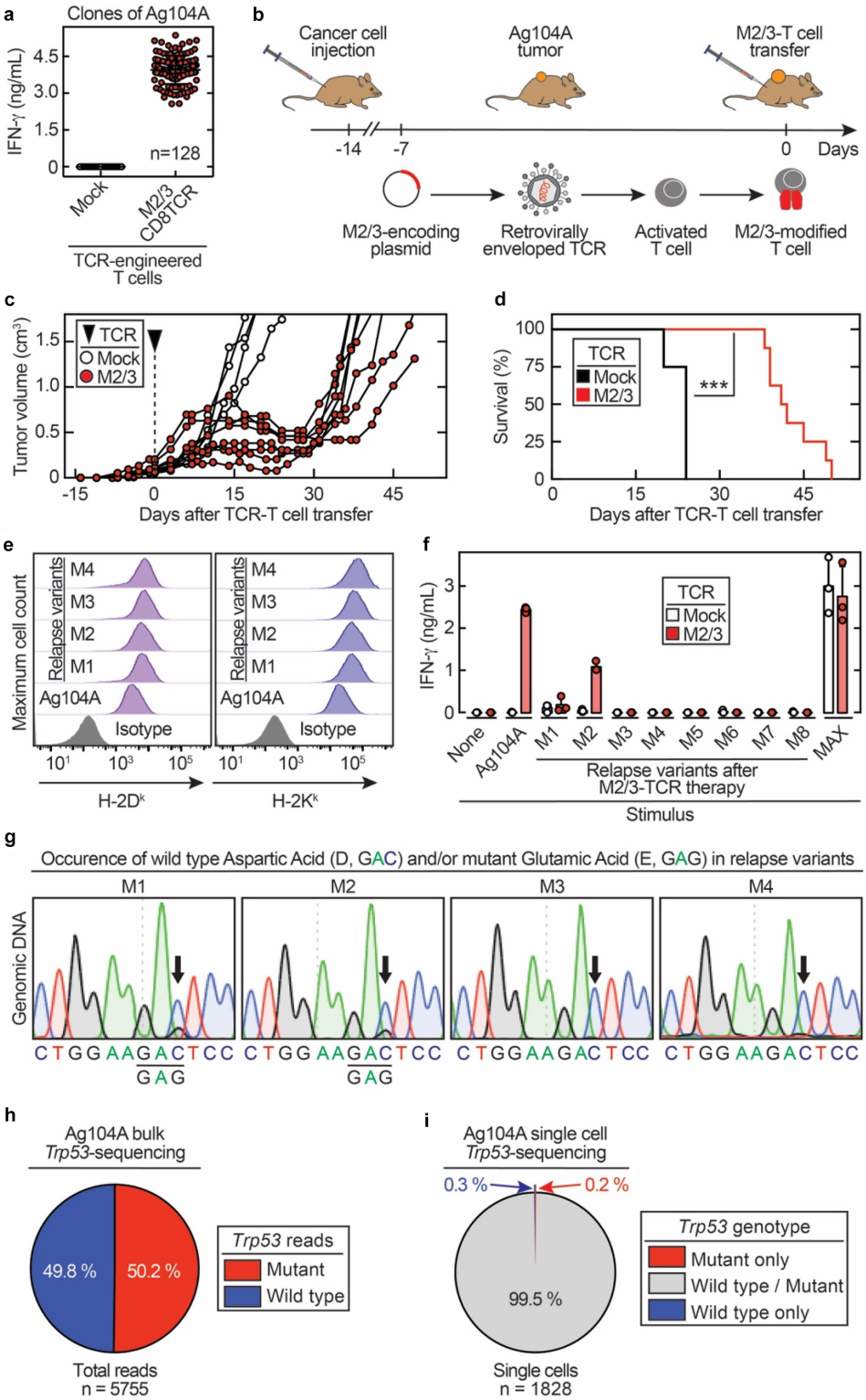

Figure 3. Escape of mutant-p53 specific TCR-therapy by antigen-loss variants. (a) cancer cell clones ($n = 128$) generated from the Ag104A cell line were cocultured for 24 h with M2/3-engineered T cells. Supernatants were analyzed for IFN-γ concentrations by ELISA. Spleen from C3H/HeN was used as source for T cells. Two independent biological repeats were done. One is shown. (b) overview of TCR-therapy. C3H Rag$^{-/-}$ mice were injected *s.c.* with Ag104A. 14 days later, mice were treated with M2/3-engineered T cells. Spleen from C3H/HeN mice were used as source for T cells. (c) treatment of Ag104A tumor-bearing mice is indicated by the red arrowhead at day 0 (total numbers of mice, $n = 8$). Average tumor size at day of treatment: 0.098 ± 0.048 cm^3. Mock-transduced CD8$^+$ T cells were used for treatment of control mice ($n = 4$). Data are summarized from three independent biological repeats. (d) mice treated with either M2/3- ($n = 8$) or mock-engineered ($n = 4$) T cells were compared in a Kaplan-Meier survival analysis (***$p < 0.001$). Log-rank test was used to determine significance. (e) relapse variants M1, M2, M3 and M4 from (c) were readapted *in vitro* and stained for expression of H-2K^k and H-2D^k. (f) relapse variants (M1 – M8) and the parental Ag104A cells were cultured for 24 h with M2/3-engineered CD8$^+$ T cells. Supernatants were analyzed for IFN-γ concentrations by ELISA. Spleen from C3H/HeN mice were used as source for T cells. T cells were cultured alone (none) as negative control. Ionomycin and PMA (MAX) was used as non-specific positive control for T cell stimulation. Shown is one out of three independent biological repeats. Data are mean ± standard deviation from technical triplicates. (g) PCR was used to amplify the genomic *Trp53* region harboring the D256E mutation and analyzed by Sanger sequencing. Electropherograms of relapse variants are shown. Arrows indicate the position where the C to G mutation is located. Nucleotide codon GAC, indicated in blue, encodes aspartic acid (D, wild type p53), while GAG, indicated in black, encodes glutamic acid (E, mutant p53). (H–I) *Trp53*-specific sequencing of either (H) the Ag104A bulk cell line showing an equal ratio of wild type and mutant *Trp53* reads or (I) Ag104A clones ($n = 1,828$). 99.5% of all clones ($n = 1,820$) harbor the heterogeneous *Trp53*$^{wt/mt}$ genotype while 0.2% harbor only *Trp53*^{mt} ($n = 3$) and 0.3 % harbor only *Trp53*^{wt} ($n = 5$).

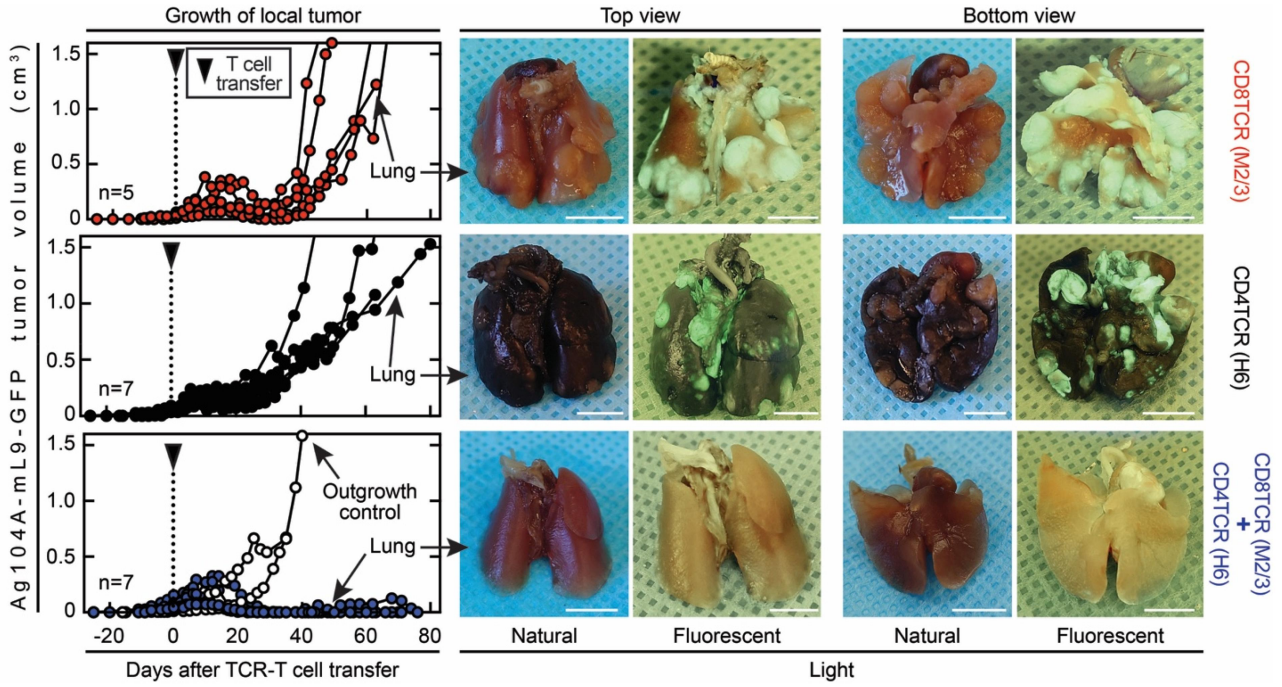

Figure 4. Prevention of relapse and outgrowth of macrometastases of cancer that had spread to the lung at time of combination TCR-therapy. (left) established Ag104A-mL9-GFP tumors were treated by TCR-therapy 25 days after *s.c.* injection in C3H Rag$^{-/-}$ mice as indicated by the arrow head. Average tumor size at day of treatment was 0.055 ± 0.031 cm^3 standard deviation. Mice were treated either with the M2/3-CD8TCR (top, $n = 5$), or the H6-CD4TCR (middle, $n = 7$) or with a combination of both (bottom, $n = 7$). Untreated outgrowth controls ($n = 2$) are indicated. Spleen from C3H CD8$^{-/-}$ or C3H CD4$^{-/-}$ mice were used as source for T cells. Data are summarized from three independent biological repeats. (right) shown are representative pictures of lungs from each treatment group under natural or fluorescent light. Under fluorescent light, GFP-expressing cancer cells become visible. Top and bottom view of the same lung is indicated. White sizing bar indicates a length of 500 µm. (top) lung from a mouse treated with the M2/3-CD8TCR. (middle) lung from a mouse treated with the H6-CD4TCR and inflated using India ink. (bottom) lung from a mouse treated with the combination of the M2/3-CD8TCR and the H6-CD4TCR.

with the H6-CD4TCR alone seemed to only have delayed outgrowth. In contrast, mice treated with a combination of the M2/3-CD8TCR and the H6-CD4TCR rejected tumors without signs of relapse.

Prevention of lung metastases from cancer cells that had spread to the lung at time of treatment by combinational TCR-therapy

Ag104A-mL9 cancer cells spread from the local tumor into the lung where cancer colony forming cells can be detected as early as 14 days after *s.c.* injection of Ag104A-mL9 cancer cells (Fig. S8). Lungs from C3H Rag$^{-/-}$ mice were removed, minced and plated. The plates were evaluated under fluorescent light 10 days later to detect cancer colony forming units. Since the combination of M2/3-CD8TCR with the H6-CD4TCR is able to eradicate the local *s.c.* tumor, we also evaluated lungs from either single TCR-treated mice or from mice treated with the combination (Table 1). The lungs of mice were removed, fixed overnight in 4 % formaldehyde with or without India ink and images were taken under natural or fluorescent light. Since Ag104A-mL9 cancer cells carry GFP, it was possible to detect fluorescent cells in the lungs forming the macroscopically visible metastases (Figure 4, right). The single treatments with either the M2/3-CD8TCR or the H6-CD4TCR 25 days after *s.c.* injection of cancer cells was not able to prevent formation of macroscopically detectable lung metastases. However, mice treated with the combination of one CD8TCR with one CD4TCR stayed free of macroscopic lung metastases (Table 1) as lungs from these mice (Figure 4, bottom right) appeared as lungs from tumor-free mice (Fig. S9).

Table 1. Combining one CD8TCR with one CD4TCR prevents the development of macrometastases.

Administered TCR-therapy	Type of TCR	Mice per group	Detected lung metastases[a]	p-Value[b]	Significance
Control[c]	Control[d]	6	4/4	1.0	n.s.
Mono-therapy	CD8TCR[e]	6	4/4	1.0	n.s.
	CD4TCR[f]	7	3/3	1.0	n.s.
Combination-therapy	CD8TCR[e] + CD4TCR[f]	7	0/4	0.003	**

[a]Only mice that were evaluated for lung metastases are included.
[b]Two tailed Fisher's exact test was used for calculation of p-values. Only mice that were examined for lung metastases were considered. The mono-therapy group was combined for comparison with the control and combination-therapy group.
[c]Control TCR-therapy includes untreated mice ($n = 3$) and mice receiving a TCR of unrelated specificity ($n = 3$).
[d]The CD8TCR anti-A4 specific for the syngeneic 6132A cancer cell line[16] was used as control TCR with unrelated specificity.
[e]The CD8TCR M2/3 specific for mp53 was used.
[f]The CD4TCR H6 specific for mL9 was used.

Discussion

Here we describe Ag104A as a tumor model for mutant p53-specific TCR-therapy. Monotherapy with a $CD8^+$ T cell-derived CD8TCR had significant therapeutic effects targeting mp53 in this spontaneous tumor at autochthonous, unmanipulated expression levels. The CD8TCR was highly lytic and specific *in vitro* and more than >99% of cancer cells expressed the antigen in the parental Ag104A cancer cell population. However, the therapeutic effects were only transient and tumors relapsed. Ag104A cancer cells that only expressed the wtp53 were found to be pre-existent at a small percentage (~0.3%) in the original Ag104A cancer cell population that we targeted. These antigen-negative cancer cells most likely replaced the mp53-expressing cancer cells that had been eliminated during the course of the mp53-specific TCR-therapy. Small subpopulations of cancer cells that lack the p53 mutation found in the bulk of the tumor have also been observed in human malignancies.[38]

Our present study shows the need for $CD4^+$ and $CD8^+$ T cell cooperation to prevent escape and to eradicate a well-established cancer that has already spontaneously spread into the lungs when therapy is initiated. In other tumor models, therapeutic effects of a CD4TCR in euthymic mice depended on the participation of endogenous $CD8^+$ T cells.[39] Conversely, when only one CD8TCR was used for adoptive transfer in euthymic mice, endogenous $CD4^+$ T cells were needed.[40] Therefore, to prove that in our model two TCRs were effective without the participation of other TCRs, it was essential that the recipient mice lacked endogenous T cells. However, our reductionist approach does not examine how preconditioning protocols may affect our TCR combination therapy.[41]

Escape of cancer variants after an objective but transient therapeutic effect has also been found in a patient treated with a mutant p53-specific CD8TCR.[14] Clinically and experimentally, heritable cancer variants that lacked sensitivity to the CD8TCR-engineered T cells caused the escape from therapy. Resistance of the cancer cells to the TCR-T cells whether caused by lack of the mutant target as in our model or lack of the presenting HLA/MHC molecule as in the patient, is the major common hurdle for therapeutic success. One underlying reason for escape is tumor heterogeneity which is a reality for most if not all autochthonous tumors in mouse and human. TCR diversity with multiple different specificities seems crucial to overcome the natural genetic instability of cancers and their antigenic heterogeneity to prevent escape.[42] It may also be possible to combine multiple CD8TCRs targeting independent neoantigens to overcome heterogeneity. However, more than two TCRs might be needed for this approach to be effective when targeting only MHC class I restricted autochthonous neoantigens.[16] Considering cost and time limitations during manufacturing, two TCRs, one CD8TCR and one CD4TCR, appears currently most realistic for a given patient to receive TCR-therapy with the highest chance of success.

Whether killing of an overwhelming majority of antigen-positive cancer cells will result in the death of few antigen-negative cells as "bystanders" was studied decades ago with contradictory results.[43–45] It is now clear that bystander killing of cancer variants occurs but depends on the tumor stroma presenting antigen released by the parental antigen-positive cancer cells.[24,46–49] If, however, cancer cells expressed the antigen at levels too low to be presented indirectly in the tumor microenvironment, then the tumor escaped as variant. It would be ideal if a single CD8TCR could eradicate a cancer containing variants. Experimentally, eradication of bystanders by a single type of CD8TCR can be achieved but only when cancer cells are engineered to express antigen at very high levels thereby achieving cross-presentation sufficient to sensitize tumor stroma for the destruction by T cells.[47] Such levels may not frequently be found in an unmanipulated tumor, and was probably neither found in the clinical study[14] nor in our model; both showed escape of cancer variants after mp53-specific CD8TCR monotherapy.

We showed previously that for targeting tumor-specific antigens expressed at autochthonous unmanipulated levels, at least one CD4TCR and one CD8TCR were required. Only combination TCR-therapy could eradicate cancers containing loss variants.[16] These $CD4^+$ and $CD8^+$ T cells cooperate at the effector phase by recognizing indirectly antigens in the stromal microenvironment[50,51] and thereby acquire the capability to eliminate cancer cells indirectly as bystanders.[51] Bystander killing of variants depended on the $CD4^+$ T cell- and the $CD8^+$ T cell-recognized antigen to be expressed on the same cancer cell. While these results were obtained using artificially high expressed antigens, our recent result finds that this T cell cooperation is also essential for unmanipulated tumors in which autochthonous $CD4^+$ and $CD8^+$ T cell-recognized antigens are both expressed by the same cancer cell.[16] $CD4^+$ T cells can eliminate MHC Class II-negative cancer cells,[52,53] but $CD4^+$ T cells seem not to cause any bystander killing,[53] unlike $CD8^+$ T cells.[47,48,54] Even though bystander killing may usually or always be accomplished by $CD8^+$ T cells, bystander killing depended on both, $CD4^+$ and $CD8^+$ T cell-recognized antigens being produced by the same cancer cell[51] and bystander activation may depend on forming a 4-cell cluster between cancer cells, stromal APCs, $CD4^+$ and $CD8^+$ T cells.[16] However, neither antigen needed to be recognized directly on the cancer cell by the T cells.[51] Thus, HLA/MHC-Class I haplotype loss from the cancer cell surface would still allow for bystander killing, if the cancer cell also expresses a cancer-specific $CD4^+$ T cell recognized antigen.

Our present study shows the need for $CD4^+$ and $CD8^+$ T cell cooperation to eradicate a well-established spontaneously metastatic cancer. While the mutant p53 target is at autochthonous unmanipulated levels, the $CD4^+$ T cell-recognized neoantigen mL9 was artificially introduced and future studies should establish that an autochthonous $CD4^+$ T cell recognized antigen on Ag104A can replace the engineered model antigen. Ag104A regularly induces a $CD4^+$ T cell-dependent antibody response to a cancer-specific mutant glycopeptide.[55] In addition, some of the 77 cancer-specific nsSNVs of Ag104A likely express additional $CD4^+$ T cell-recognized epitopes. Interestingly, the same p53 mutation can be recognized by $CD4^+$ as well as $CD8^+$ T cells, e.g., the $p53^{R175H}$ and $p53^{R248W}$ mutations frequently found in patients.[11,56,57] Thus, combination therapy as we propose

could soon be examined in metastatic cancer patients using allogeneic mutant p53-specific TCRs from libraries currently being established. Since a $CD4^+$ TCR selection may be very difficult based on *in vitro* assays alone,[37] our syngeneic pre-clinical animal model may help to learn which CD4TCR and CD8TCR could be combined to achieve therapeutic efficacy *in vivo*.

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All authors approved the final version of the manuscript.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

CRediT: **Vasiliki Anastasopoulou:** Data curation, Investigation, Methodology, Visualization, Writing – review & editing; **Hans Schreiber:** Conceptualization, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; **Ching-En Lee:** Data curation, Investigation, Writing – review & editing; **Kazuma Kiyotani:** Data curation, Investigation, Writing – review & editing; **Leo Hansmann:** Data curation, Methodology, Writing – review & editing; **Yusuke Nakamura:** Data curation, Project administration, Writing – review & editing; **Matthias Leisegang:** Conceptualization, Data curation, Funding acquisition, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing; **Steven P. Wolf:** Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Data availability

Whole-exome- and RNA-sequencing data of Ag104A and its normal heart-lung-fibroblast control Ag104A-HLF are publicly available at the Sequence Read Archive (SRA), accession number PRJNA1232138. All other data and materials that support the findings of this study are available from the corresponding author(s), [M.L. and S.P.W.], upon reasonable request.

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