Supplemental Material for

# Targeting human melanoma neoantigens by T cell receptor gene therapy

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**Methods**

*Isolation of TCR genes and construction of retroviral vector plasmids*

The patient-derived T cell clone 14/35 has been described (7) and was kindly provided by Thomas Wölfel (University Medical Center of the Johannes Gutenberg University Mainz, Mainz, Germany). TCR sequences of 14/35 (TRAV20-CAVQSGTSGSRLTF-TRAJ58, TRBV9-CASSVVAGFNEQFF-TRBJ2-1) and DMF5 (19) were determined and integrated in pMP71-PRE (28) as described (11). The high affinity tyrosinase-specific TCR T58 (11) was used as control (anti-TYR). Genes of human CDK4 harboring the mutations R24C (codon 24: TGT) or R24L (codon 24: CTT) were synthesized by GeneArt (Life Technologies, Carlsbad, CA, USA). Human genes encoding full length CDK4-R24C, CDK4-R24L or tyrosinase (11) were ligated into pMP71-i-GFP (29) via Eco72I restriction site as described (30) to generate the retroviral vector plasmids pMP71-R24C-i-GFP, pMP71-R24L-i-GFP and pMP71-TYR-i-GFP. Fusion constructs of trimer minigenes (ACDPHSGHFV-AAY (ACD), ALDPHSGHFV-AAY (ALD), AAGIGILTV-AAY (AAG), ELAGIGILTV-AAG (ELA) or YMDGTMSQV-AAY (YMD)) and GFP were designed as previously described (31), generated by GeneArt and integrated into pMP71-PRE (pMP71-ACD-GFP, pMP71-ALD-GFP, pMP71-AAG-GFP, pMP71-ELA-GFP, pMP71-YMD-GFP).

*Cell lines*

Plat-E packaging cells (32) and 293T (ATCC: CRL-11268, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagles medium supplemented with 10% heat-inactivated fetal calf serum (FCS, PAN Biotech, Aidenbach, Germany) and 100 IU/ml penicillin, streptomycin. The selection culture medium of Plat-E cells contained 10 µg/ml blasticidin and 1 µg/ml puromycin (Sigma-Aldrich, St. Louis, MO, USA). T2 cells (ATCC: CRL-1992) and the melanoma cell lines SKMEL-29.1 (SKMEL-29) (33), WM-902B (9) (ESTDAB-085, purchased from European

Searchable Tumor Cell Bank and Database (University Tübingen, Tübingen, Germany)), SKMEL-37 (34, 35) and 624MEL-38 (36) were cultured in cell medium (CM, RPMI 1640 supplemented with 10% FCS, 100 IU/ml penicillin-streptomycin, 1 mM sodium pyruvate, 1x non-essential amino acids, 50 µM 2-mercaptoethanol). WM-902B+A2 is a transfectant of WM-902B, generated by transduction with retroviral vectors encoding HLA-A2 and GFP as described (37). Unless otherwise indicated, reagents were purchased from Invitrogen (Life Technologies).

*Generation of retrovirus supernatant*

To produce amphotropic murine leukemia virus (MLV)-pseudotyped retroviruses, 293T cells were transiently transfected with the corresponding retrovirus construct and plasmids encoding Moloney MLV *gag/pol* genes (pcDNA3.1-MLVg/p, provided by Christopher Baum (Hannover Medical School, Hannover, Germany)) and the MLV-10A1 *env* gene (pALF-10A1 (38)). 293T cells were transfected with 18 µg DNA (6 µg of each plasmid) by calcium phosphate precipitation. Ecotropic retroviruses encoding the TCRs 14/35, DMF5 or T58 were obtained after transient transfection of Plat-E cells with only retroviral vector plasmids. 48 h after transfection, 3 ml of virus supernatant were harvested, filtrated and used for transduction.

*Mice*

HHD mice were provided by François A. Lemonnier (Institute Pasteur, Paris, France) and have been described (13). HHD and B6.129S7-*Rag1*$^{tm1Mom}$/J mice were crossed to obtain HHDxRag$^{-/-}$ mice (B6.Cg-Tg(HLA-A/H2-D/B2M)1Bpe *H2-D1*$^{tm1Bpe}$ *B2m*$^{tm1Unc}$ *Rag1*$^{tm1Mom}$/Luck). Offspring were genotyped as described (39) and samples of peripheral blood were analyzed for presence of mature lymphocytes (anti-mouse CD45R).

*MC703 cells*

MC703 fibrosarcoma cells were generated by subcutaneous injection of 50 µg 3'-methylcolanthrene (Sigma Aldrich) in 100 µl sesame oil into the left flank of a 12-week-old female HHD mouse. 135 days later at a size of 400 $mm^3$ the tumor was excised, cells were cultured in CM, cloned by limited dilution and a single clone was selected based on HHD expression. $5x10^4$ MC703 cells were transduced with viruses containing genes of tumor antigens twice by spinoculation (800 x g, 90 min, 32 °C) in 24-well plates using 1 ml virus supernatant. Bulk cultures were expanded and either enriched by preparative FACS using GFP as a marker (MC703-ACD, MC703-ALD, MC703-AAG, MC703-ELA, MC703-YMD) or clones were generated by limiting dilution cloning (MC703-R24C, MC703-R24L, MC703-TYR). Expression of antigen and HHD in MC703 cells was analyzed by flow cytometry and co-cultures with 14/35- , DMF5- and T58-engineered HHD T cells.

*TCR gene transfer into primary T cells*

Spleen cells were isolated from HHD mice and erythrocytes were lysed by ammonium chloride treatment. Cells were incubated in CM supplemented with 1 µg/ml anti-mouse CD3, 0.1 µg/ml anti-mouse CD28 antibodies (both BD Biosciences (BD), Franklin Lakes, NJ, USA) and 10 IU/ml human IL-2 (Proleukin S, Novartis, Basel, Switzerland) at a concentration of $2x10^6$/ml. On the following day, $1x10^6$ cells were transduced by spinoculation in 24-well non-tissue culture-treated plates pre-coated with 12.5 µg/ml RetroNectin (TaKaRa, Otsu, Japan) and virus particles (3200 x g, 90 min, 4 °C) in 1 ml CM supplemented with 10 IU/ml IL-2 and $4x10^5$ mouse T-Activator beads (Life Technologies). A second transduction was performed on the following day by spinoculation with 1 ml virus supernatant (+ 10 IU/ml IL-2). T cells were expanded in CM (+ 50 ng/ml IL-15 (Miltenyi Biotec, Bergisch Gladbach, Germany)) for 3 (ATT) or 10 days (co-cultures), respectively. Human PBMCs were isolated from healthy donors by ficoll gradient centrifugation. Initial stimulation was performed at a

concentration of $1x10^6$ PBMCs per well and 1 ml of CM (+ 100 IU/ml IL-2) for 72 h in non-tissue culture-treated 24-well plates, coated with 5 µg/ml anti-human CD3 and 1 µg/ml anti-human CD28 antibodies (BD). Human T cells were transduced by spinoculation at day 2 after isolation by adding 1 ml amphotropic virus supernatant supplemented with 4 µg/µl protamine sulfate (Sigma Aldrich) and IL-2 (100 IU/ml). On the next day, a second transduction was performed on RetroNectin-coated 6-well plates, which were pre-coated with 3 ml virus supernatant. Cells were expanded in CM (+ 100 IU/ml IL-2). After 10 days, the cells were rested for 2 days (10 IU/ml IL-2).

*Tumor challenge and adoptive T cell transfer*

$3\text{-}5x10^6$ tumor cells were subcutaneously injected in 100 µl PBS into the left flank of HHDxRag$^{-/-}$ mice (12-20 weeks old, female or male). Tumor growth was analyzed 2-3 times a week by determination of tumor volume by caliper measurements according to $\pi/6$ x (abc). On the day of T cell transfer, mice were ranked by tumor size and sequentially allocated to treatment groups to ensure equal average tumor sizes between groups. Mice were treated by adoptive transfer of TCR-engineered HHD T cells earliest 3-5 weeks after tumor cell injection when tumors were established. HHD T cells were analyzed for expression of CD8, 14/35 (TCRvβ1), DMF5 (A2/K$^b$:ELA) and T58 (TCRvβ23) by flow cytometry and intravenously injected in 100 µl PBS (adjusted to $1x10^6$ CD8$^+$TCR$^+$ HHD T cells per mouse) 3 days after transduction. Examiners were not blinded with respect to treatment groups. Mice were sacrificed when either tumors reached the maximum permitted size or if due to tumor burden the overall health-condition was poor. Animals were excluded from analysis, if they died due to reasons unrelated to tumor burden.

*T cell analysis*

$5x10^4$ TCR-engineered HHD T cells (14/35, DMF5, T58) or TCR-engineered human PBLs (14/35, T58) were co-cultured at an E:T ratio of 1:1 for 24 h with indicated target cells in 200 µl CM in 96-well

round bottom plates. Untransduced HHD T cells or human PBLs served as controls. CD8 and CD4 subpopulations of 14/35-transduced HHD T cells were separated using magnetically-labeled antibodies and MACS (Miltenyi Biotech, following manufacturer's protocol). For target cell titration, graded amounts of MC703-R24C, -R24L, -ACD and -ALD cells (ranging from $1.25x10^4$ - 98) were diluted in $5x10^4$ unmodified MC703 tumor cells and incubated with $5x10^4$ effector cells. For peptide titration, TCR-engineered human PBLs were incubated with T2 cells in presence of graded amounts of peptide (ACD (ACDPHSGHFV), ALD (ALDPHSGHFV), ARD (ARDPHSGHFV), Biosyntan, Berlin Germany). Unloaded T2 cells served as control. Supernatants of co-cultures were analyzed for IFN-γ or IL-2 content by enzyme-linked immunosorbent assay (ELISA, BD). For TCR-independent maximal stimulation of effector cells, 1 µM ionomycin (Merck, Darmstadt, Germany) and 5 ng/ml phorbol-12-myristate-13-acetate (PMA, Promega, Fitchburg, WI, USA) were used. Cytotoxic activity of $2x10^5$ 14/35-transduced human PBL or HHD T cells was analyzed by 16 h incubation with target cells (E:T ratio of 2:1) in 200 µl CM in presence of 1 µg CD107a-specific antibodies (anti-human: H4A3; anti-mouse: 1D4B, both Brilliant Violet 421, BioLegend, San Diego, CA, USA) and subsequent FACS analysis using MACSQuant (Miltenyi Biotec). To detect T cell numbers in peripheral blood, 50 µl peripheral blood were incubated with Fc block (BD) and indicated antibodies. Erythrocytes were lysed by ammonium chloride treatment, samples were washed in PBS and measured using FACSCanto II (BD). Total cells in each sample were measured to determine total cell counts per 50 µl blood. Numbers of $CD8^+$ and $CD8^-$ $CD3^+TCR\nu\beta^+$ lymphocytes were calculated per ml blood.

*Cytokine concentrations in peripheral blood*

Peripheral blood was collected from vena facialis, cell-free supernatants were obtained by centrifugation and stored at -80 °C until analyzed using cytometric bead array (CBA, Mouse Th1/Th2/Th17 Cytokine Kit, BD) following the manufacturer's instructions.

*Flow cytometry and antibodies*

Unless stated otherwise, cells or blood samples were incubated with 50 µl PBS containing 1 µg of indicated anti-mouse antibodies: CD3 (145-2C11, allophycocyanin (APC) or fluorescein isothiocyanate (FITC), BioLegend), CD8 (53-6.7, Brilliant Violet 421 or APC, BioLegend), CD45R (RA3-6B2, PE, BD). Anti-human TCRvβ-specific antibodies were used to analyze expression of TCRs 14/35 and T58: TCRvβ1 (14/35, BL37.2, PE, Beckman Coulter, Brea, CA, USA), TCRvβ23 (T58, AF23, PE, Beckman Coulter). Expression of TCR DMF5 on HHD T cells was analyzed using chimeric A2/Kb pentamers loaded with ELAGIGILTV peptide (PE, ProImmune, Oxford, UK). TCR-engineered human PBL were analyzed using anti-mouse TCRcβ (H57-597, APC, BD), anti-human CD8 (HIT8a, PE, BD) and anti-human CD3 (UCHT1, FITC, BD) antibodies. Anti-human HLA-A2 (BB7.2, Alexa Fluor 647, AbD Serotec, Bio-Rad, Hercules, CA, USA) and isotype control antibody (mouse IgG2b, Alexa Fluor 647, AbD Serotec) were used to detect expression of HHD or HLA-A2. Anti-mouse H-2K$^b$ (AF6-88.5, PE, BD) and isotype control antibody (mouse IgG2a, κ, PE, BD) were used to detect expression of H-2K$^b$. 1 µg Fc block was added to samples of peripheral blood 10 min prior addition of specific antibodies (anti-mouse CD16/CD32, 2.4G2, BD). Erythrocytes were lysed by ammonium chloride treatment (when necessary), samples were washed in PBS and acquired using MACSQuant or FACSCalibur (BD). 1 µM SYTOX Blue were used for live/dead cell discrimination. Data analysis was performed using FlowJo software (TreeStar, Ashland, OR, USA).

*Analysis of CDK4 mutations in human melanoma cell lines*

Total RNA was isolated from cultured tumor cells (SKMEL-29, WM-902B, SKMEL-37, 624MEL-38) and complementary DNA (cDNA) was synthesized using 1 µg total RNA as template (SuperScript II Reverse Transcriptase (Life Technologies), following manufacturer's protocol). Sequences of the CDK4 gene were amplified by PCR (5'-tgg tgt cgg tgc cta tgg ga-3' (sense), 5'-ggc caa agt cag cca gct

tga-3' (antisense)), purified by gel electrophoresis (Invisorb Fragment Cleanup, Stratec) and analyzed by sequencing (Eurofins Genomics).

*Western blot analysis of CDK4 in human melanoma cell lines and MC703 cells*

Western Blot analysis was performed as previously described (40). Briefly, 30 µg of tumor cell lysates (SKMEL-29, WM-902B, SKMEL37, 624MEL-38, MC703, MC703-R24C and MC703-R24L) were separated on a 12% SDS-polyacrylamid gel and proteins were transferred to a nitrocellulose membrane. The membrane was incubated in blocking and wash buffer and probed with polyclonal anti-human CDK4 (host: rabbit, LS-C290866/66675, 0.5 µg/ml, LifeSpan BioSciences, Seattle, WA, USA) and secondary HRP-coupled goat anti-rabbit antibodies (Santa Cruz Biotechnologies, Dallas, TX, USA). Chemiluminescence was captured on a X-ray film after adding Luminol substrate (both: Santa Cruz Biotechnologies). The membrane was stripped from antibodies and a second detection using an anti-β-actin antibody (AC-15, HRP-coupled, cross-reactive to human and mouse β-actin, Sigma Aldrich) was performed to control that equal amounts of protein were loaded.

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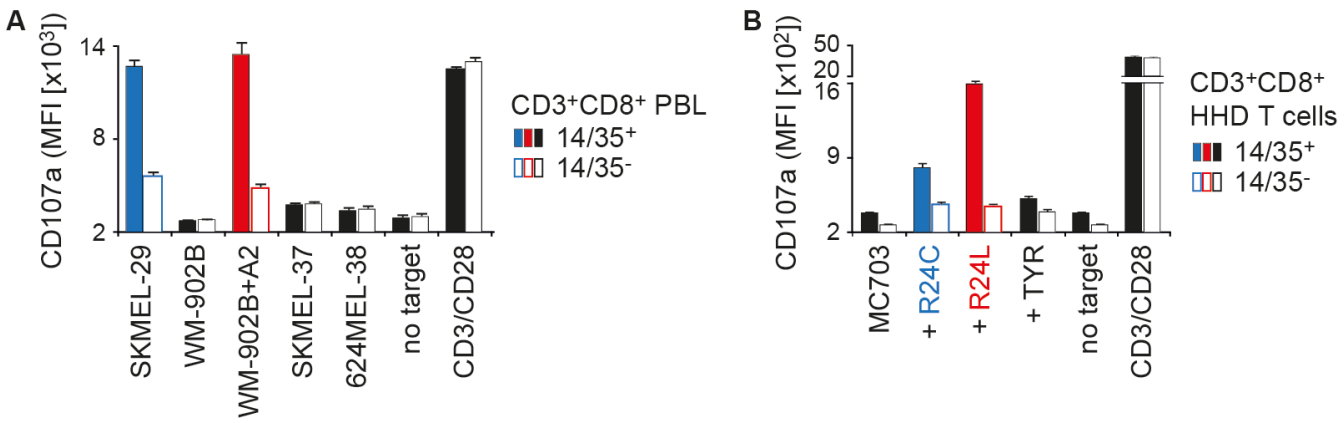
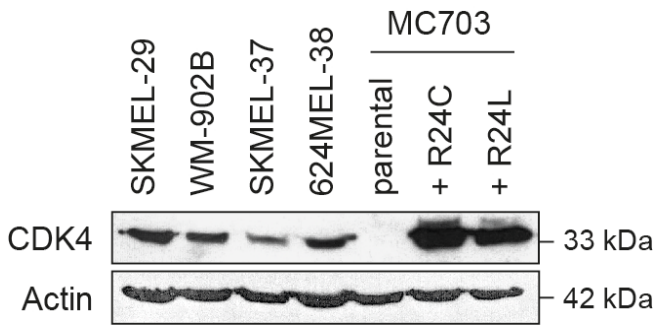


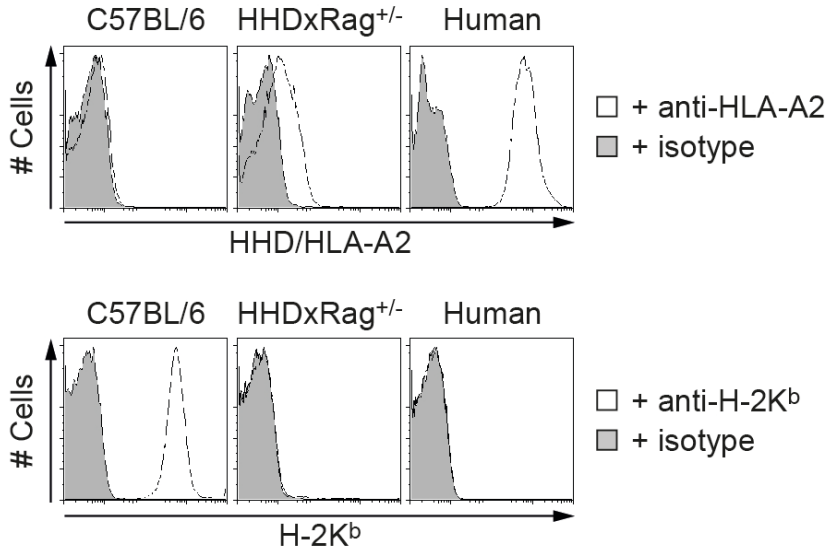

**Supplemental Figure 1** - Cytotoxicity of 14/35-transduced human PBL and mouse HHD T cells against R24C- and R24L-expressing cancer cells.

**(A)** 14/35-transduced human PBL or **(B)** mouse HHD T cells were incubated with indicated target cells in presence of CD107a-specific antibodies. Co-cultured cells were analyzed by flow cytometry and the mean fluorescence intensity (MFI) of bound CD107a antibodies is shown for either $14/35^+$ or $14/35^-$ $CD3^+CD8^+$ cells. Data are means of duplicates ± mean deviation. Two independent experiments were performed.

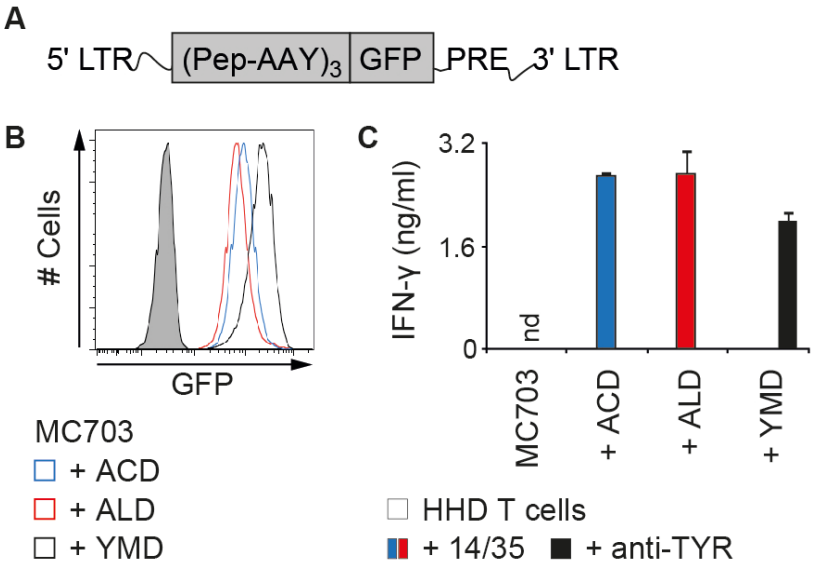



**Supplemental Figure 2** - Native CDK4 expression in human melanoma cell lines is lower than transgenic CDK4 expression in mouse MC703 cells.

Lysates (30 µg of protein) of indicated cancer cells were analyzed by Western blot using polyclonal human CDK4-specific antibodies. Transfer of equal amounts of protein was confirmed by detecting β-actin. Results are representative for three independent experiments.

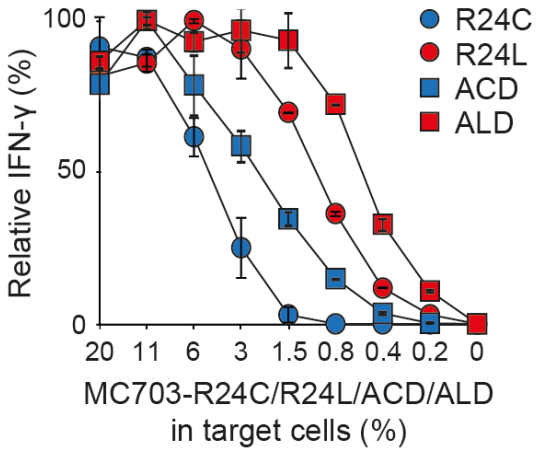



**Supplemental Figure 3** - MHC I expression on lymphocytes of HHD-transgenic mice is low. Peripheral blood of C57BL/6, HHDxRag$^{+/-}$ or humans was incubated with anti-human-HLA-A2 (top) or anti-mouse-H-2Kb antibodies (bottom). MHC expression on lymphocytes was analyzed by flow cytometry. One representative analysis of C57BL/6 (n=3), HHDxRag$^{+/-}$ (n=3) or humans (n=2) is shown.



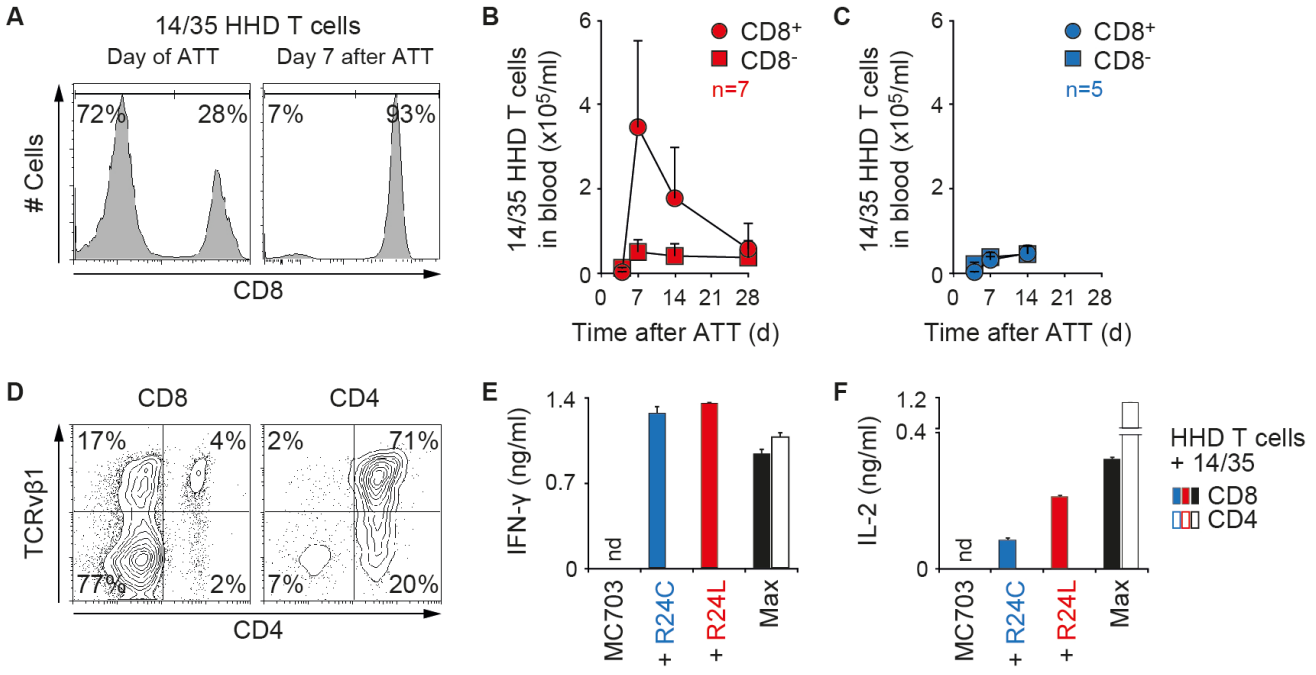

**Supplemental Figure 4** - MC703 tumor cells expressing high amounts of R24C and R24L peptide are similarly recognized by 14/35-transduced HHD T cells *in vitro*.

(**A**) MP71 vector encoding trimeric minigenes of peptide (Pep)-AAY fused to GFP (LTR: long terminal repeat, PRE: post-transcriptional regulatory element of woodchuck hepatitis virus). (**B**) Antigen (GFP) expression in MC703-ACD, -ALD and -YMD cells. (**C**) IFN-γ secretion of 14/35-engineered HHD T cells after co-culture with MC703-ACD, -ALD and -YMD tumor cells. HHD T cells either unmodified or expressing a tyrosinase-specific TCR (anti-TYR) were used as control. Means of duplicates ± mean deviation, representative for two independent experiments. nd: not detectable.




**Supplemental Figure 5** - 14/35-transduced HHD T cells are better stimulated by R24L than R24C at low target cell density and by using minigenes encoding multiple epitopes.

Titrated amounts of MC703 cells expressing full length genes of mutant CDK4 (R24C, R24L) or trimer minigenes encoding R24C and R24L epitopes (ACD, ALD) were mixed with $5x10^4$ unmodified MC703 cells. The percentage of antigen-expressing tumor cells in the target cell population is given on the x-axis (1.5% correspond to ~800 antigen-expressing MC703 cells). IFN-γ secretion of 14/35-engineered HHD T cells after co-culture with target cells was set in relation to the maximum amount of IFN-γ secreted. HHD T cells either unmodified or expressing a tyrosinase-specific TCR (anti-TYR) showed no IFN-γ secretion after co-culture with indicated target cell populations (not shown). Data are means of duplicates ± mean deviation, representative for two independent experiments.




**Supplemental Figure 6** - The anti-tumor response of 14/35-$T_E$ cells is CD8-restricted.

(**A**) CD8 expression of 14/35-transduced HHD T cells before and 7 days after adoptive transfer into a MC703-R24L tumor-bearing HHDxRag$^{-/-}$ mouse (shown in Figure 2B). (**B, C**) Total numbers of CD8$^+$ and CD8$^-$ 14/35-$T_E$ in blood during ATT of mice bearing MC703-R24L (B, n=7) or MC703-R24C tumors (C, n=5). Data correspond to analyses shown in Figure 2A, B, E and F. (**D**) Percentages of CD4- and TCRvβ1-expressing 14/35-transduced HHD T cells in cultures enriched for either CD8 or CD4 expression. (**E, F**) IFN-γ (E) and IL-2 (F) secretion of 14/35-engineered HHD T cells shown in panel D after co-culture with indicated target cells. T cells were incubated with PMA and ionomycin for TCR-independent stimulation (Max). Means of duplicates ± mean deviation, representative for three independent experiments. nd: not detectable.

**Supplemental Table 1** - Summary of animal experiments

| Designation | ATT | | Regression | Relapse |
|---|---|---|---|---|
| *MC703-* | *Time (d)* | *Tumor size ($mm^3$)* | *(d after ATT)* | *(d after ATT)* |
| R24C | 24 | 220 | - | - |
| R24C | 24 | 207 | - | - |
| R24C | 24 | 165 | - | - |
| R24C | 24 | 118 | - | - |
| R24C | 24 | 359 | - | - |
| **Mean** | **24** | **214** | **-** | **-** |
| R24L | 31 | 636 | 7 | 22 |
| R24L | 31 | 242 | 7 | 22 |
| R24L | 31 | 264 | 7 | 34 |
| R24L | 34 | 754 | 7 | 28 |
| R24L | 34 | 490 | 7 | 21 |
| R24L | 34 | 1106 | 7 | 18 |
| R24L | 34 | 749 | 7 | 21 |
| **Mean** | **33** | **606** | **7** | **24** |
| ACD | 27 | 126 | - | - |
| ACD | 27 | 242 | - | - |
| ACD | 27 | 220 | - | - |
| ACD | 27 | 424 | - | - |
| ACD | 27 | 242 | - | - |
| ACD | 27 | 586 | - | - |
| ACD | 28 | 377 | - | - |
| **Mean** | **27** | **317** | **-** | **-** |
| ALD | 27 | 436 | 7 | - |
| ALD | 27 | 282 | 7 | - |
| ALD | 27 | 157 | 7 | - |
| ALD | 27 | 330 | 7 | - |
| ALD | 27 | 282 | 7 | - |
| ALD | 28 | 402 | 7 | - |
| **Mean** | **27** | **315** | **7** | **-** |

| Designation | ATT | | Regression | Relapse |
|---|---|---|---|---|
| *MC703-* | *Time (d)* | *Tumor size ($mm^3$)* | *(d after ATT)* | *(d after ATT)* |
| AAG | 26 | 264 | - | - |
| AAG | 26 | 205 | - | - |
| AAG | 26 | 28 | - | - |
| AAG | 26 | 298 | - | - |
| AAG | 26 | 323 | - | - |
| **Mean** | **26** | **224** | **-** | **-** |
| ELA | 26 | 205 | 11 | - |
| ELA | 26 | 352 | 9 | - |
| ELA | 26 | 198 | 9 | - |
| ELA | 26 | 226 | 9 | - |
| **Mean** | **26** | **245** | **10** | **-** |