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Targeting human melanoma neoantigens by T cell receptor gene therapy

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In successful cancer immunotherapy, T cell responses appear to be directed toward neoantigens created by somatic mutations; however, direct evidence that neoantigen-specific T cells cause regression of established cancer is lacking. Here, we generated T cells expressing a mutation-specific transgenic T cell receptor (TCR) to target different immunogenic mutations in cyclin-dependent kinase 4 (CDK4) that naturally occur in human melanoma. Two mutant CDK4 isoforms (R24C, R24L) similarly stimulated T cell responses in vitro and were analyzed as therapeutic targets for TCR gene therapy. In a syngeneic HLA-A2–transgenic mouse model of large established tumors, we found that both mutations differed dramatically as targets for TCR-modified T cells in vivo. While T cells expanded efficiently and produced IFN-γ in response to R24L, R24C failed to induce an effective antitumor response. Such differences in neoantigen quality might explain why cancer immunotherapy induces tumor regression in some individuals, while others do not respond, despite similar mutational load. We confirmed the validity of the in vivo model by showing that the melan-A–specific (MART-1–specific) TCR DMF5 induces rejection of tumors expressing analog, but not native, MART-1 epitopes. The described model allows identification of those neoantigens in human cancer that serve as suitable T cell targets and may help to predict clinical efficacy.

Introduction

Melanoma regression, either through adoptive T cell therapy (ATT) or T cell checkpoint inhibitors blocking CTLA4 or PD-1/PD-1 ligand, correlated with increased frequencies of neoantigen-specific T cells, as deduced from cancer genome sequencing (1–3). However, the potential of neoantigen-specific T cells to induce tumor regression is largely unexplored (4). Neoepitopes may vary in their suitability as target (5), but identifying suitable epitopes remains difficult because in vitro analysis of T cells often cannot predict in vivo efficacy (6). Similarly, although T cells against neoepitopes are not subjected to central tolerance mechanisms, their fate in cancer-bearing individuals is unclear, as is the functional quality of T cell receptors (TCRs) obtained from individuals with cancer. Therefore, we investigated different human melanoma mutations as targets for TCR gene therapy in a syngeneic HLA-A2–transgenic cancer model in which specific immune recognition relies on human molecules (MHC, TCR, and tumor antigen), while cellular components (tumor cells, T cells and host) are of mouse origin. Using the model, we also compared a native melanoma epitope with its anchor-modified variant to determine whether experiments using peptide analogs are of predictive value for the design of clinical applications.

Results and Discussion

Three cancer-driving mutations have been described in the cyclin-dependent kinase 4 (*CDK4*) gene of human melanomas, substituting arginine at position 24, which is critical for binding to $p16^{INK4A}$ (R24C, R24H, and R24L) (7–9). Since these mutations modify an HLA-A2 anchor position, the CDK4 peptide becomes antigenic and is recognized by specific T cells (7, 10) if the mutation stabilizes the peptide:MHC complex. We isolated the TCR 14/35 from a T cell clone, which was established by culture with the autologous melanoma cell line SKMEL-29 bearing the R24C mutation (ref. 7 and Figure 1A). The 14/35-transduced human T cells (Figure 1B) recognized HLA-$A2^+$ SKMEL-29 (R24C) and WM-902B melanoma cells bearing the R24L mutation, as indicated by IFN-γ release and cytotoxic activity, by measuring CD107a expression (Figure 1, A and C, and Supplemental Figure 1A; supplemental material available online with this article; doi:10.1172/JCI83465DS1). In contrast, 14/35-transduced human T cells did not recognize SKMEL-37, containing the R24H mutation (Figure 1, A and C, and Supplemental Figure 1A), which probably does not support MHC binding and was therefore excluded from further analysis. The 624MEL-38 melanoma cells, containing wild-type CDK4, were also not recognized (Figure 1, A and C, and Supplemental Figure 1A). Western blot analysis confirmed expression of CDK4 in all human melanoma cell lines (Supplemental Figure 2). The antitumor response of 14/35-transduced T cells was similar to that of T cells modified with a tyrosinase-specific TCR that was derived from a nontolerant T cell repertoire (anti-TYR; ref. 11 and Figure 1, B and C). R24L, but not R24C or R24H, is predicted to increase the affinity of the mutated CDK4 peptide to HLA-A2 (Table 1). However, the binding prediction of the cysteine-containing peptide R24C is problematic, because algorithms do not account for unavoidable oxidation and cysteinylation during

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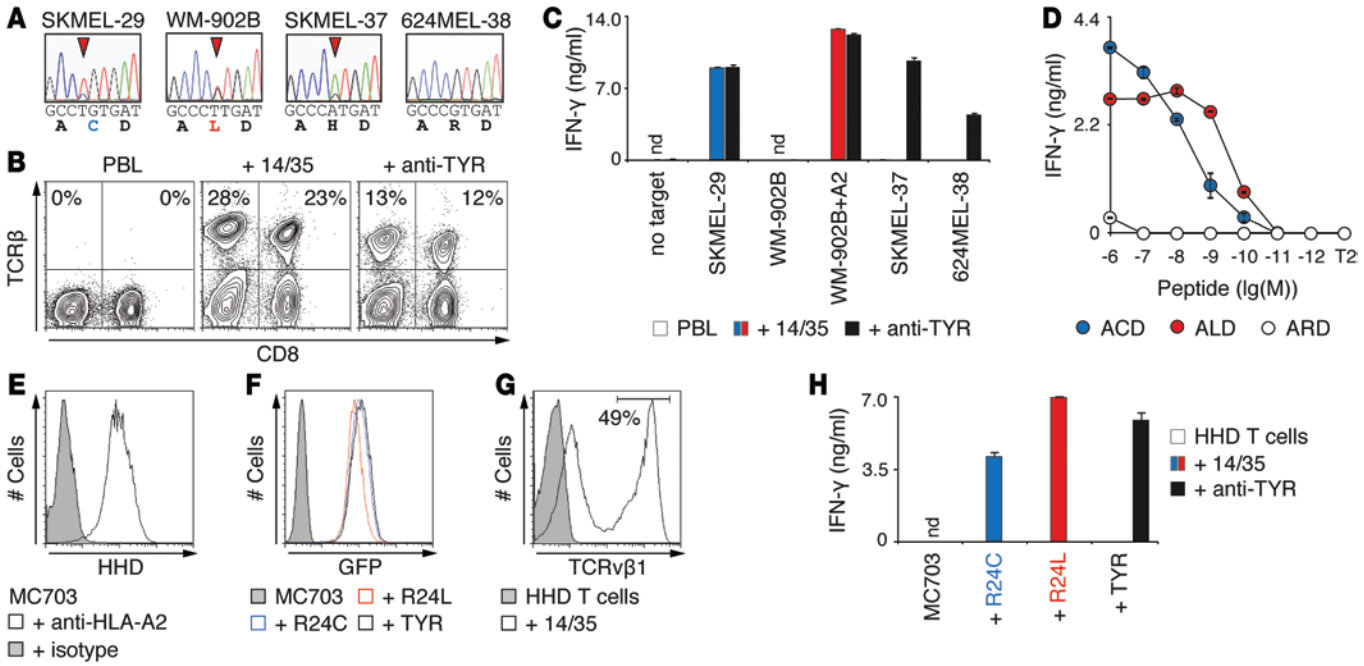

Figure 1. In vitro analysis suggests suitability of 2 different CDK4 epitopes as targets for TCR gene therapy. (**A**) Nucleotide and amino acid sequences of $CDK4_{23–25}$ in melanoma cell lines. Mutations in codon 24 are indicated. (**B**) Percentages of human $CD8^+$ and $CD8^-$ peripheral blood lymphocytes (PBLs) expressing 14/35 or a tyrosinase-specific TCR (anti-TYR). (**C** and **D**) IFN-γ secretion of human 14/35-expressing PBLs after coculture with (**C**) indicated melanoma cells or (**D**) T2 cells loaded with graded amounts of peptide (ACD, $CDK4_{23–32(24C)}$; ALD, $CDK4_{23–32(24L)}$; ARD, $CDK4_{23–32}$). WM-902B+A2 is an HLA-A2–transfected variant of $HLA\text{-}A2^-$ WM-902B melanoma cells. PBLs expressing a tyrosinase-specific TCR and/or unmodified PBLs were used as control. Data are means of duplicates ± mean deviation and representative of independent experiments ($n = 4$ [**C**]; $n = 2$ [**D**]) using PBLs of different donors. (**E**) HHD (HLA-A2) expression in MC703 cancer cells. (**F**) Antigen (GFP) expression in MC703-R24C and MC703-R24L tumor cells. (**G**) Percentage of 14/35-expressing HHD T cells (TCRvβ1) 10 days after retroviral transduction. (**H**) IFN-γ secretion of 14/35-expressing HHD T cells after coculture with MC703-R24C, MC703-R24L, and MC703-TYR tumor cells. HHD T cells either unmodified or expressing a tyrosinase-specific TCR were used as control. Data are means of duplicates ± mean deviation and representative of 3 independent experiments. ND, not detectable.

assays. Furthermore, it was suggested that the highly oxidizable sulfur residue in cysteine may contribute to MHC binding and could function as "pseudo"-anchor residue (5), an exception that is not considered when evaluating the R24C peptide in silico (12). Experimentally, 14/35-transduced human T cells recognized both the R24C and the R24L peptides, and differences were only detectable at low peptide concentrations (Figure 1D). This does not reflect the large differences obtained from MHC-binding predictions. To analyze the quality of both neoepitopes as therapeutic targets in a model of large established tumors, the mutant variants of the *CDK4* gene (linked by an internal ribosome entry site [IRES] sequence to GFP) were expressed in mouse MC703 tumor cells. The fibrosarcoma MC703 was generated in an HLA-A2–transgenic mouse (HHD, chimeric HLA-A2/H-2D^b) (13) and expressed HHD (Figure 1E). The tumor cells MC703-R24C and MC703-R24L expressed similar amounts of mutant CDK4 (Figure 1F) and were similarly recognized by HHD-derived T cells that were transduced with 14/35 (Figure 1, G and H, and Supplemental Figure 1B). Transgenic expression of CDK4 variants in MC703 tumor cells was somewhat higher if compared with the native expression in human melanoma cell lines (Supplemental Figure 2). In vitro analysis of both CDK4

Table 1. HLA-A2 binding prediction for CDK4 and MART-1 peptides

Designation	Abbreviation	Epitope sequence	NetMHC[A]	IEDB[A]	References
$CDK4_{23–32}$	ARD	ARDPHSGHFV	17871	15222	
$CDK4_{23–32(24C)}$	R24C/ACD	A**C**DPHSGHFV	10279	12008	7
$CDK4_{23–32(24H)}$	R24H	A**H**DPHSGHFV	18698	15083	8
$CDK4_{23–32(24L)}$	R24L/ALD	A**L**DPHSGHFV	55	184	9
$MART\text{-}1_{27–35}$	AAG	AAGIGILTV	2498	1429	16
$MART\text{-}1_{26–35(27L)}$	ELA	E**L**AGIGILTV	137	146	18

Computer algorithms for MHC I binding prediction are available as follows: NetMHC (http://www.cbs.dtu.dk/services/NetMHC/); IEDB Analysis Resource (http://tools.immuneepitope.org/mhci/). [A]Values indicate predicted IC_{50} (correct as of 12/07/15). Mutations or modifications are in bold.

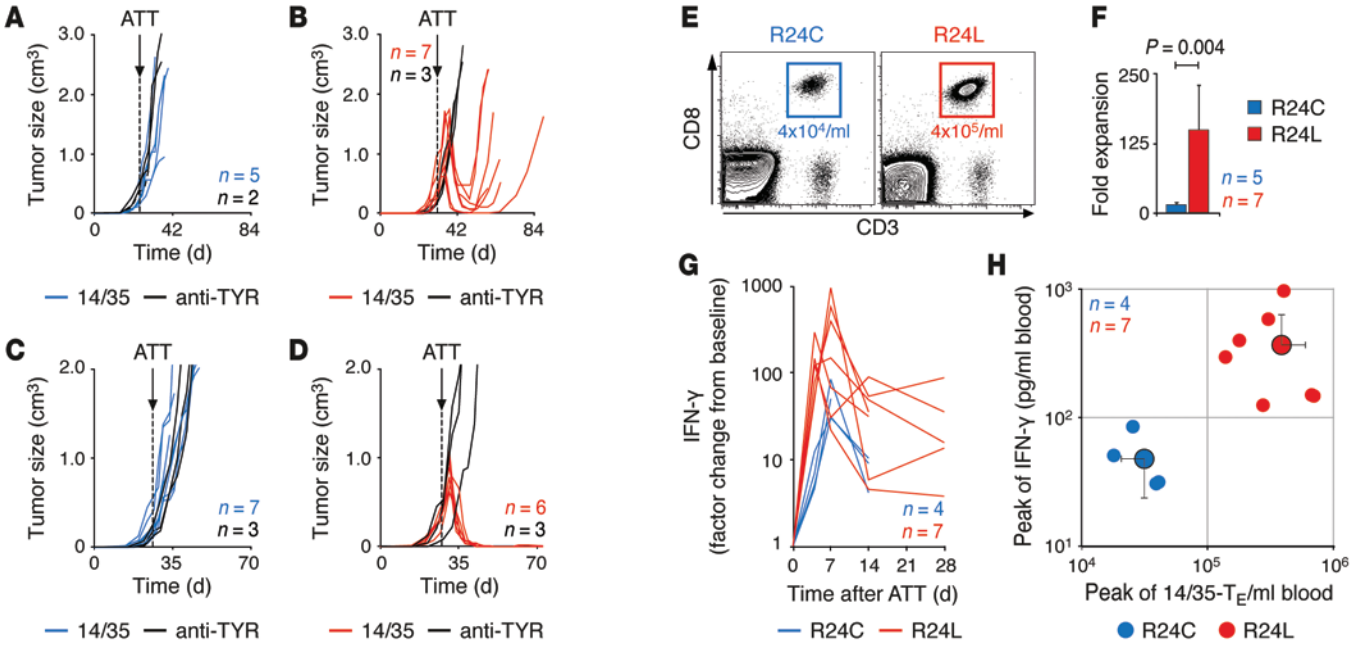

Figure 2. Neoepitope quality determines rejection of large HLA-A2⁺ tumors by TCR gene therapy. (**A**–**D**) HHD *Rag*$^{-/-}$ mice bearing established MC703-R24C (**A**, n = 5, average tumor size: 214 ± 90 mm³; mean values ± SD), MC703-R24L (**B**, n = 7, 606 ± 304 mm³), MC703-ACD (**C**, n = 7, 317 ± 155 mm³), or MC703-ALD (**D**, n = 6, 315 ± 100 mm³) tumors were treated with 14/35-T_E (adjusted to 1 × 10⁶ CD8⁺ 14/35-T_E). Tumor-bearing HHD *Rag*$^{-/-}$ mice that received T cells expressing a tyrosinase-specific TCR are shown as control. Tumor growth in these animals and untreated mice was comparable. (**E** and **F**) Number (**E**, day 7) and fold expansion (**F**, from day 4 to 7) of CD8⁺ 14/35-T_E after ATT of mice bearing MC703-R24C (n = 5) or MC703-R24L tumors (n = 7) shown in **A** and **B**. Data shown in **E** are representative for the analysis of 5 (R24C) and 7 samples (R24L). Independent 2-sample t test was used to compare data sets in **F**. (**G**) Blood samples were collected on days 4, 7, 14, and 28 after ATT of mice bearing MC703-R24C (n = 4) or -R24L tumors (n = 7), and serum was analyzed for IFN-γ content. Factor change from baseline was deduced from untreated mice (2 ± 1 pg/ml, mean values ± SD, n = 5). (**H**) Peak values in blood determined for the number of CD8⁺ 14/35-T_E and concentration of IFN-γ. Mean ± SD (R24C: n = 4, R24L: n = 7) is shown.

mutations, using human 14/35-transduced T cells and human melanoma cells that naturally express the CDK4 mutations or T cells and tumor cells from HHD mice, suggested R24C and R24L as relevant targets for specific T cells.

For in vivo analysis, we used HHD *Rag*$^{-/-}$ mice lacking mouse MHC I molecules, B cells, and T cells to focus on a single human MHC I molecule and to exclude endogenous T cell responses. HHD *Rag*$^{-/-}$ mice bearing large tumors (grown for 3 to 4 weeks, having an average diameter of 9–10 mm) were treated with 14/35-transduced effector T cells derived from HHD mice (14/35-T_E). Remarkably, 14/35-T_E did not even delay progression of MC703-R24C tumors (Figure 2A and Supplemental Table 1), while large MC703-R24L tumors regressed upon 14/35-T_E treatment (Figure 2B). However, probably because expression of HHD in transgenic mice is low (Supplemental Figure 3) and the antigen expression level is critical for successful ATT (14), tumors eventually relapsed. We repeated the experiments using MC703 cells that express minigenes encoding 3 copies of the R24C (MC703-ACD) or the R24L (MC703-ALD) epitope (Supplemental Figure 4, A and B) to compensate for low MHC expression. Both cancer cell lines were similarly recognized by 14/35-T_E in vitro (Supplemental Figure 4C). To determine the improvement in target cell recognition by increasing the amount of epitopes on the cancer cells, we titrated the number of antigen-expressing MC703 cells necessary to stimulate 14/35-transduced HHD T cells. Using minigenes improved recognition of MC703 cells, while the relative difference in recognition of both neoepitopes remained unchanged (Supplemental Figure 5). Under these limiting conditions, a better recognition of the R24L compared with the R24C mutation became clearly apparent. However, tumors expressing increased amounts of the R24C epitope still progressed after transfer of 14/35-T_E (Figure 2C and Supplemental Table 1). In contrast, all R24L epitope–expressing tumors were eradicated (Figure 2D), suggesting that R24L is a rejection epitope in human melanoma if sufficient peptide:MHC I complexes are present on the tumor cells.

To investigate differences in therapeutic outcome when targeting R24C or R24L, we followed 14/35-T_E after transfer into mice bearing MC703-R24C or MC703-R24L tumors. On day 7 after ATT, high numbers of CD8⁺ 14/35-T_E were detected when tumors expressed the R24L mutation (Figure 2E), while T cell expansion was significantly impaired when tumors expressed R24C (Figure 2, E and F). We asked whether 14/35-transduced CD4⁺ T cells contributed to the response to R24L. However, only CD8⁺ but not CD8⁻ 14/35-T_E expanded in vivo in response to R24L (Supplemental Figure 6A). Similarly, the total number of CD8⁺ but not CD8⁻ 14/35-T_E increased during the course of ATT (Supplemental Figure 6, B and C). Furthermore, only CD8⁺ but not CD4⁺ 14/35-transduced HHD T cells (Supplemental Figure 6D) secreted IFN-γ and IL-2 after incubation with MC703 target cells (Supplemental Figure 6, E and F), sug-

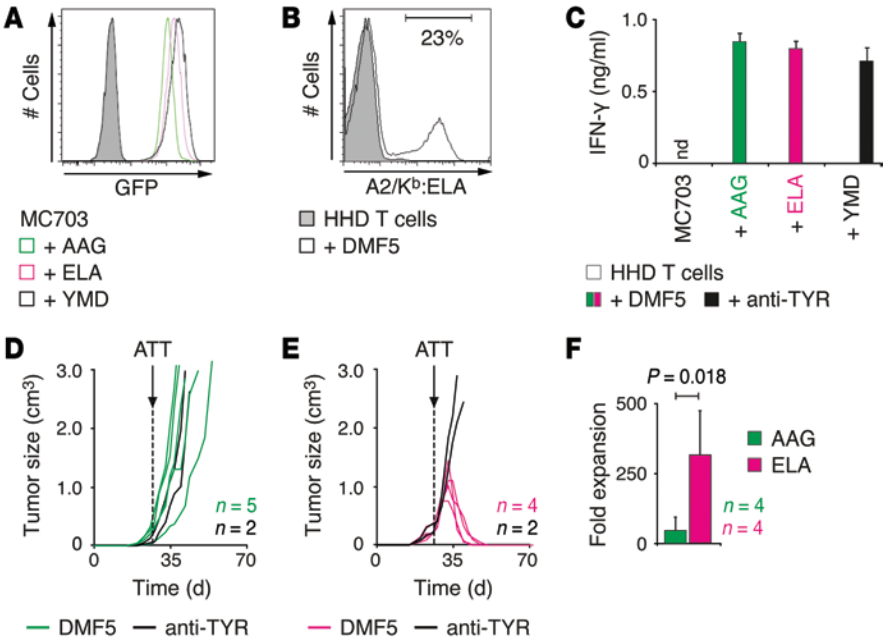

Figure 3. The anchor-modified but not the native MART-1 epitope elicits tumor rejection by TCR gene therapy. (**A**) Antigen (GFP) expression in MC703-AAG, MC703-ELA, and MC703-YMD tumor cells. (**B**) Percentage of DMF5-expressing (A2/K^b:ELA) HHD T cells 5 days after retroviral transduction. (**C**) IFN-γ secretion of DMF5-expressing HHD T cells after coculture with indicated cells. HHD T cells either unmodified or expressing a tyrosinase-specific TCR were used as control. Data are means of duplicates ± mean deviation and are representative of 3 independent experiments. (**D** and **E**) HHD *Rag*$^{-/-}$ mice bearing established MC703-AAG (**D**, $n = 5$, average tumor size: 224 ± 118 mm^3; mean values ± SD) or MC703-ELA tumors (**E**, $n = 4$, 245 ± 72 mm^3) were treated with DMF5-T$_E$ (adjusted to 1×10^6 CD8$^+$ DMF5-T$_E$). Tumor-bearing HHD *Rag*$^{-/-}$ mice that received T cells expressing a TYR-specific TCR are shown as control. (**F**) Fold expansion (from day 3 to 14) of CD8$^+$ DMF5-T$_E$ after ATT of mice bearing MC703-AAG ($n = 4$) or MC703-ELA tumors ($n = 4$). Data sets were compared using independent 2-sample *t* tests.

gesting that therapeutic effects can be ascribed to CD8$^+$ 14/35-T$_E$. Next, we analyzed serum levels of IFN-γ, a critical effector cytokine for tumor rejection (15), to obtain evidence for functional T cell activation in vivo. The concentration of IFN-γ in blood correlated with the number of CD8$^+$ 14/35-T$_E$ and was higher if tumors expressed the R24L mutation (Figure 2, G and H). This is in line with the observation that 14/35-transduced HHD T cells showed higher sensitivity for tumor cells expressing the R24L mutation (Supplemental Figure 5), which likely improved T cell activation/expansion in vivo and supported IFN-γ production in lymphopenic tumor-bearing mice.

To confirm the critical role of the target epitope in ATT, we employed native and anchor-modified melan-A (MART-1) epitopes. The MART-1 nonamer peptide AAGIGILTV (AAG) (16) is assumed to be relevant in HLA-A2$^+$ melanoma (17), whereas MART-1–specific T cells are often analyzed using the decameric peptide ELAGIGILTV (ELA) (18). ELA is modified from its original sequence in position 2 to improve MHC binding (Table 1). Biochemical analyses showed that the MART-1–specific TCR DMF5 engages both ligands similarly, with only minor differences in affinity (19). To evaluate the potential of DMF5-transduced T cells to eliminate AAG-expressing cancer, we generated MC703 cells expressing minigenes that encode either AAG (MC703-AAG) or ELA epitopes (MC703-ELA, Figure 3A). Both cancer cell lines were equally recognized by DMF5-transduced HHD T cells in vitro (Figure 3, B and C). However, the growth of MC703-AAG tumors was not influenced by ATT using DMF5-T$_E$ (Figure 3D and Supplemental Table 1), while MC703-ELA tumors were rejected (Figure 3E). In a manner comparable to the differences in the recognition of the 2 CDK4 neoepitopes by 14/35-T$_E$, CD8$^+$ DMF5-T$_E$ expanded in mice bearing ELA- but not AAG-expressing MC703 tumors (Figure 3F).

In mice and humans, effective T cell responses seem primarily directed against somatically mutated neoepitopes (20–23). The 2 CDK4 mutations in human melanoma represent an unprecedented case of the stochastic nature of creating immunogenic neoepitopes of dramatically different quality. It is likely that both neoepitopes were selected for their oncogenicity and were recognized on the autologous tumor, leading to expansion of a specific T cell response. In the case of CDK4^{R24C}, T cells were clonally expanded on the autologous melanoma cells (7), while in the case of CDK4^{R24L}, neoepitope-specific T cells were detected at low frequency within the tumor-infiltrating lymphocytes (10). Both CDK4 neoepitopes were naturally expressed by human melanoma cells and stimulated specific T cells in vitro. Also in common, T cells to both neoepitopes neither prevented melanoma nor selected escape variants in the respective patient, which is compatible with data from a sporadic cancer model (24). Paradoxically, the mutant CDK4-specific TCR, raised against the R24C mutation, was only effective against R24L. Given the generally high mutation load in human melanoma, we hypothesize that deviation of the T cell response to the R24C mutation might deter the T cell response to other recessive neoepitopes (25). Thus, our data may give one explanation for the differential response of patients to checkpoint inhibitors despite similar mutational load. We note, however, that the R24L mutation needed to be expressed in sufficient amounts to prevent tumor recurrence, as suggested (14). Data on targeting MART-1 confirmed the difficulty of predicting therapeutic efficacy based on in vitro analysis and the importance of high peptide:MHC affinity (6). The results are at variance with clinical trials in melanoma patients with the DMF5 TCR that showed some efficacy (26). However, in these trials, high-dose IL-2 was additionally applied, which alone can achieve responses in 16% of the patients (27). Alternatively, low MHC I expression in HHD-transgenic cells could have impeded TCR gene therapy. The current syngeneic model using large established tumors and only the T cell recognition system that is of human origin allows identification of (un)suitable epitopes, which, in turn, may help to improve clinical success rates.

Methods

Statistics. Independent 2-sample *t* test was used to evaluate statistical differences between R24C/R24L and AAG/ELA data sets. $P < 0.05$ was considered significant, $P < 0.01$ highly significant.

Study approval. All animal experiments were performed according to institutional and national guidelines, after approval by the responsible authority (Landesamt für Gesundheit und Soziales, Berlin). Blood collection from healthy human donors was done after prior informed consent. A complete, detailed description of all methods is provided in Supplemental Methods.

Author contributions

ML conducted experiments and acquired data. ML, TK, WU, and TB designed the research study, analyzed data, and wrote the manuscript.

Acknowledgments

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Supplemental Material for

Targeting human melanoma neoantigens by T cell receptor gene therapy

Matthias Leisegang, Thomas Kammertoens, Wolfgang Uckert, Thomas Blankenstein

Index of Supplemental Material

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 $CD8^-$ $CD3^+TCRv\beta^+$ 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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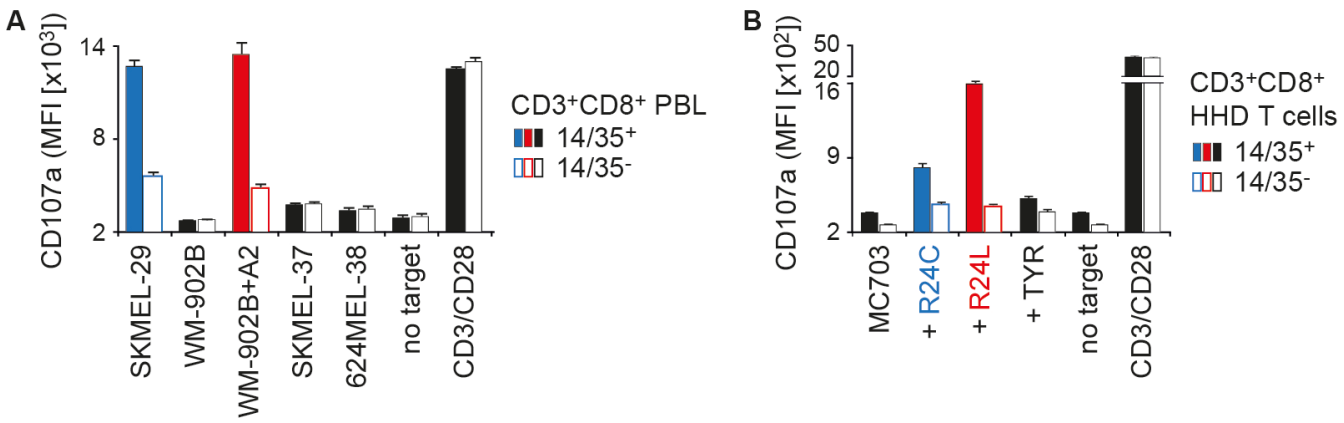
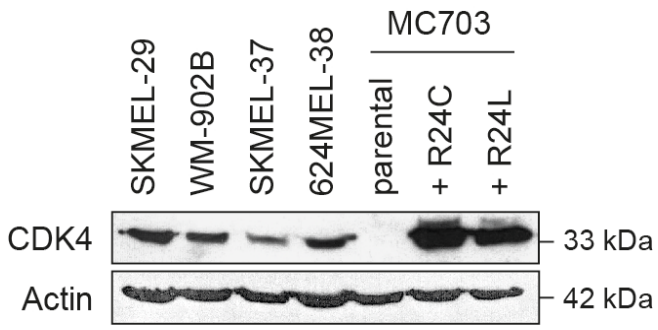


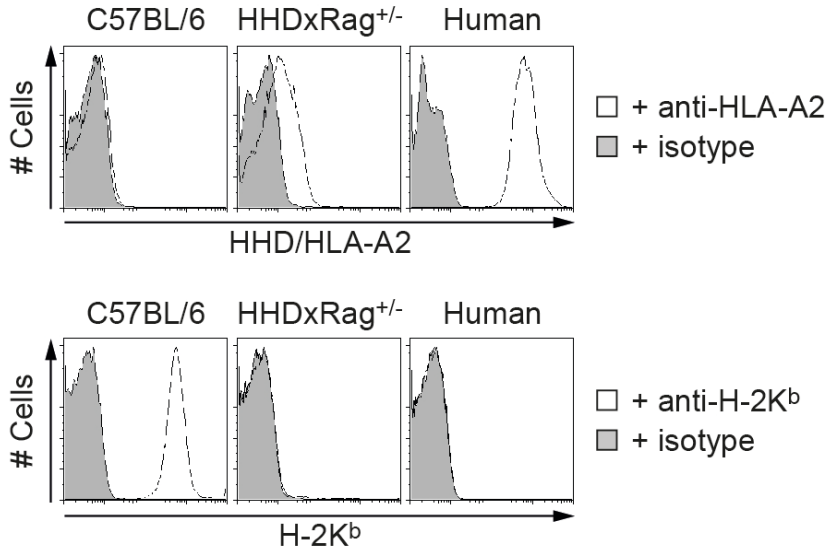
Supplemental Figure 1 - Cytotoxicity of 14/35-transduced human PBL and mouse HHD T cells against R24C- and R24L-expressing cancer cells.

(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.

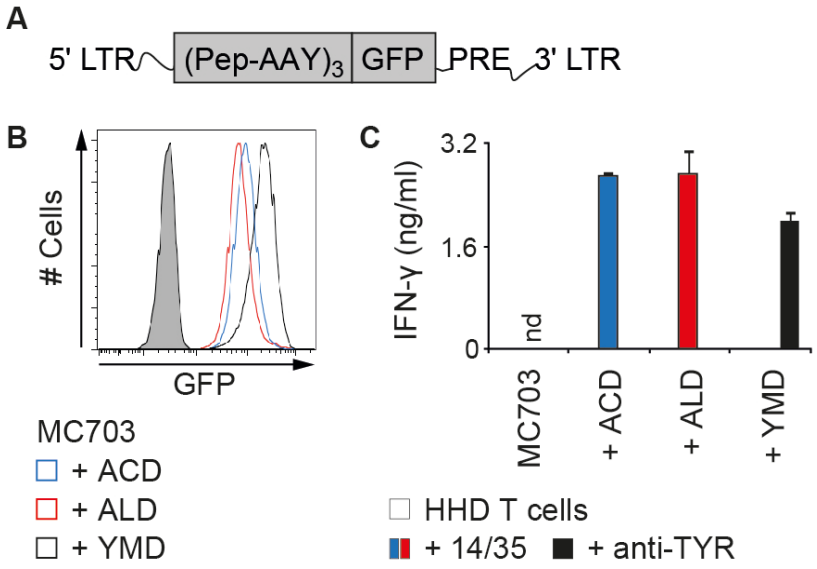


Supplemental Figure 2 - Native CDK4 expression in human melanoma cell lines is lower than transgenic CDK4 expression in mouse MC703 cells.

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.

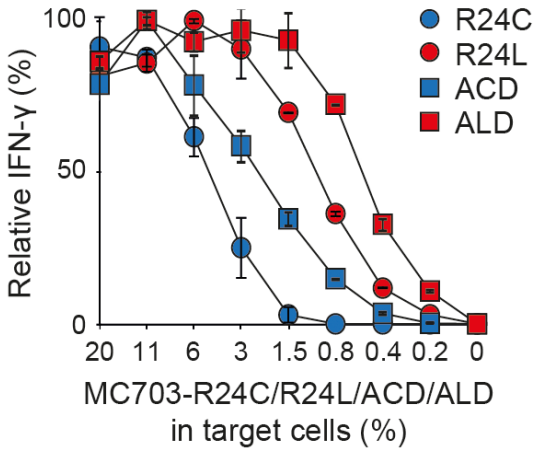


Supplemental Figure 3 - MHC I expression on lymphocytes of HHD-transgenic mice is low. Peripheral blood of C57BL/6, HHDxRag$^{+/-}$ or humans was incubated with anti-human-HLA-A2 (top) or anti-mouse-H-2Kb antibodies (bottom). MHC expression on lymphocytes was analyzed by flow cytometry. One representative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



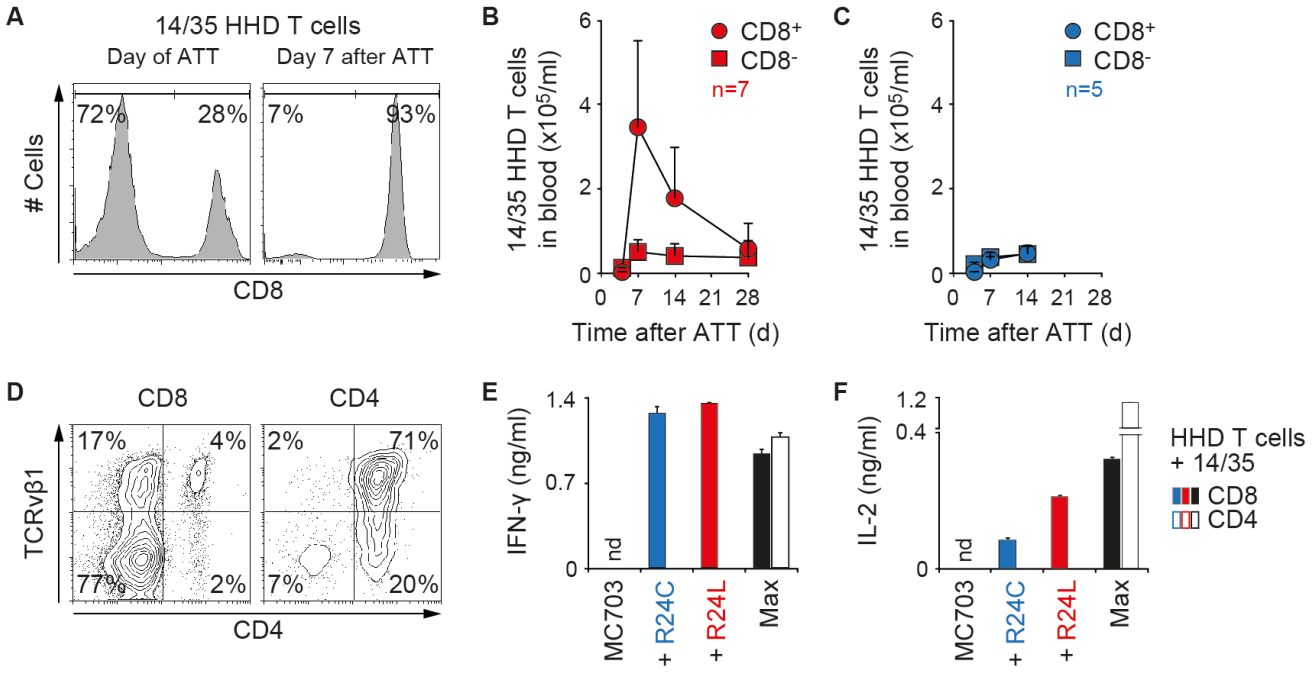
Supplemental Figure 4 - MC703 tumor cells expressing high amounts of R24C and R24L peptide are similarly recognized by 14/35-transduced HHD T cells *in vitro*.

(**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**	**-**