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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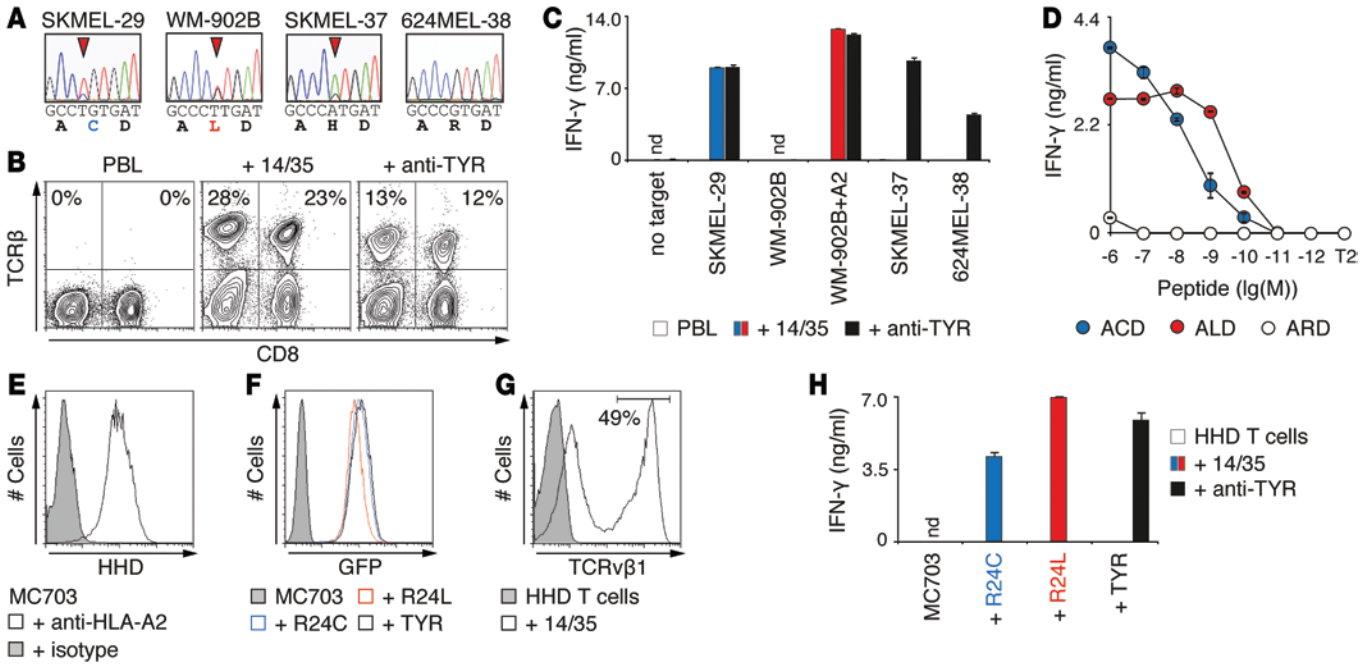

Figure 1. In vitro analysis suggests suitability of 2 different CDK4 epitopes as targets for TCR gene therapy. (**A**) Nucleotide and amino acid sequences of $CDK4_{23–25}$ in melanoma cell lines. Mutations in codon 24 are indicated. (**B**) Percentages of human $CD8^+$ and $CD8^-$ peripheral blood lymphocytes (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.

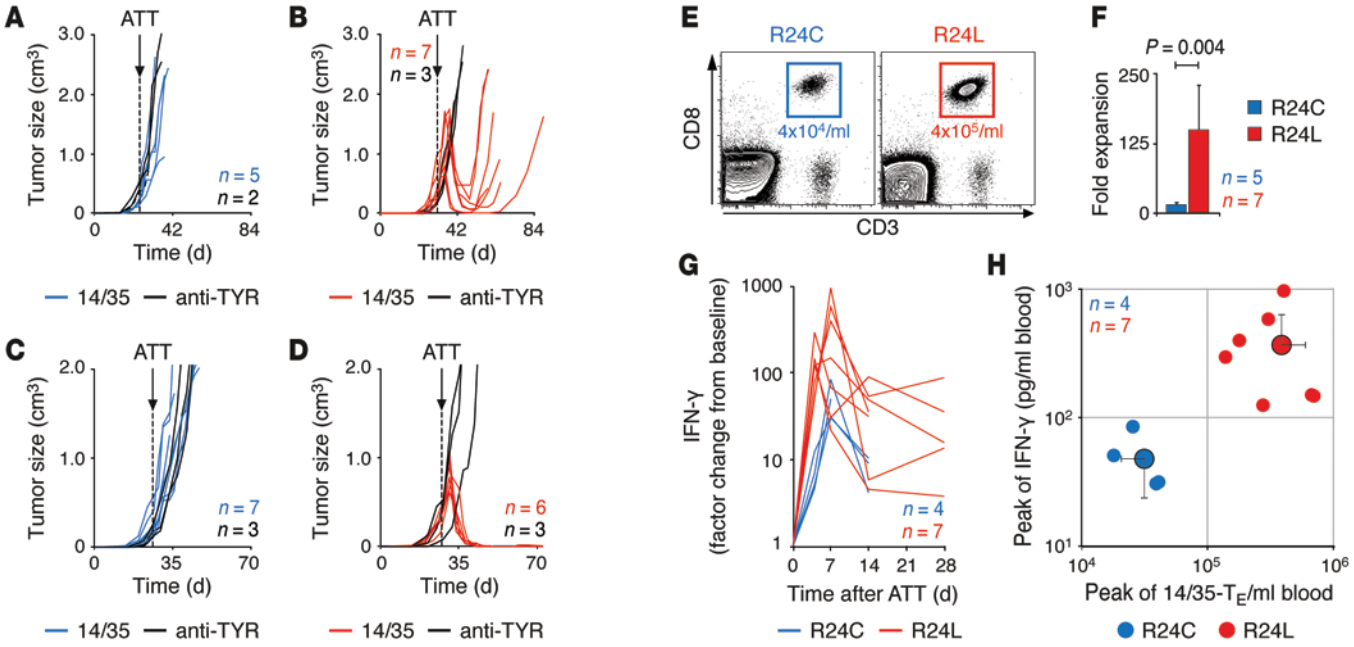

Figure 2. Neoepitope quality determines rejection of large HLA-A2⁺ tumors by TCR gene therapy. (**A**–**D**) HHD *Rag*$^{-/-}$ mice bearing established MC703-R24C (**A**, n = 5, average tumor size: 214 ± 90 mm³; mean values ± SD), MC703-R24L (**B**, n = 7, 606 ± 304 mm³), MC703-ACD (**C**, n = 7, 317 ± 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-

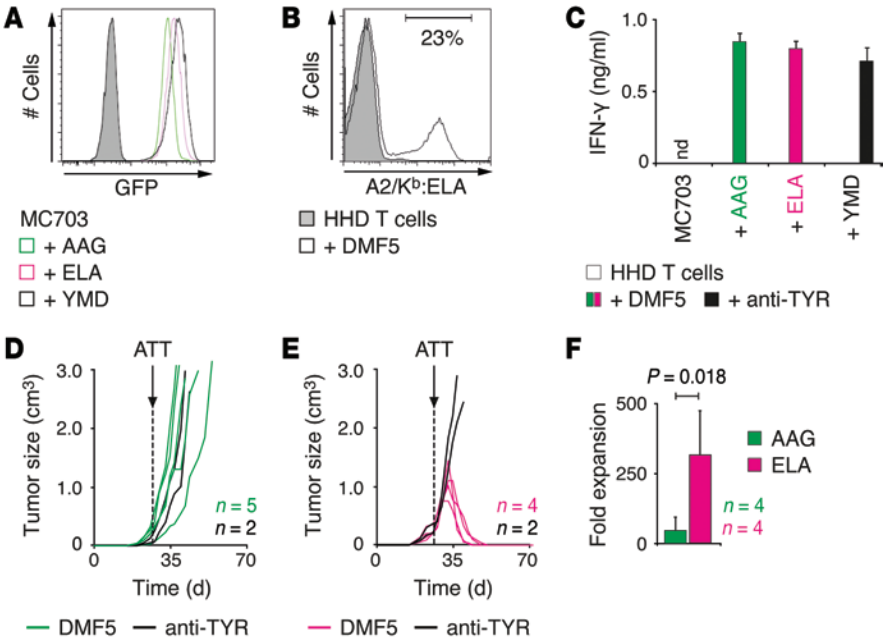

Figure 3. The anchor-modified but not the native MART-1 epitope elicits tumor rejection by TCR gene therapy. (**A**) Antigen (GFP) expression in MC703-AAG, MC703-ELA, and MC703-YMD tumor cells. (**B**) Percentage of DMF5-expressing (A2/K^b:ELA) HHD T cells 5 days after retroviral transduction. (**C**) 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.

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