

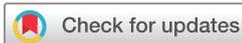
# H3.3K27M mutation is not a suitable target for immunotherapy in HLA-A2<sup>+</sup> patients with diffuse midline glioma

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## ABSTRACT

Diffuse midline glioma is the leading cause of solid cancer-related deaths in children with very limited treatment options. A majority of the tumors carry a point mutation in the histone 3 variant (H3.3) creating a potential HLA-A\*02:01 binding epitope (H3.3K27M<sub>26-35</sub>). Here, we isolated an H3.3K27M-specific T cell receptor (TCR) from transgenic mice expressing a diverse human TCR repertoire. Despite a high functional avidity of H3.3K27M-specific T cells, we were not able to achieve recognition of cells naturally expressing the H3.3K27M mutation, even when overexpressed as a transgene. Similar results were obtained with T cells expressing the published TCR 1H5 against the same epitope. CRISPR/Cas9 editing was used to exclude interference by endogenous TCRs in donor T cells. Overall, our data provide strong evidence that the H3.3K27M mutation is not a suitable target for cancer immunotherapy, most likely due to insufficient epitope processing and/or amount to be recognized by HLA-A\*02:01 restricted CD8<sup>+</sup> T cells.

## BACKGROUND

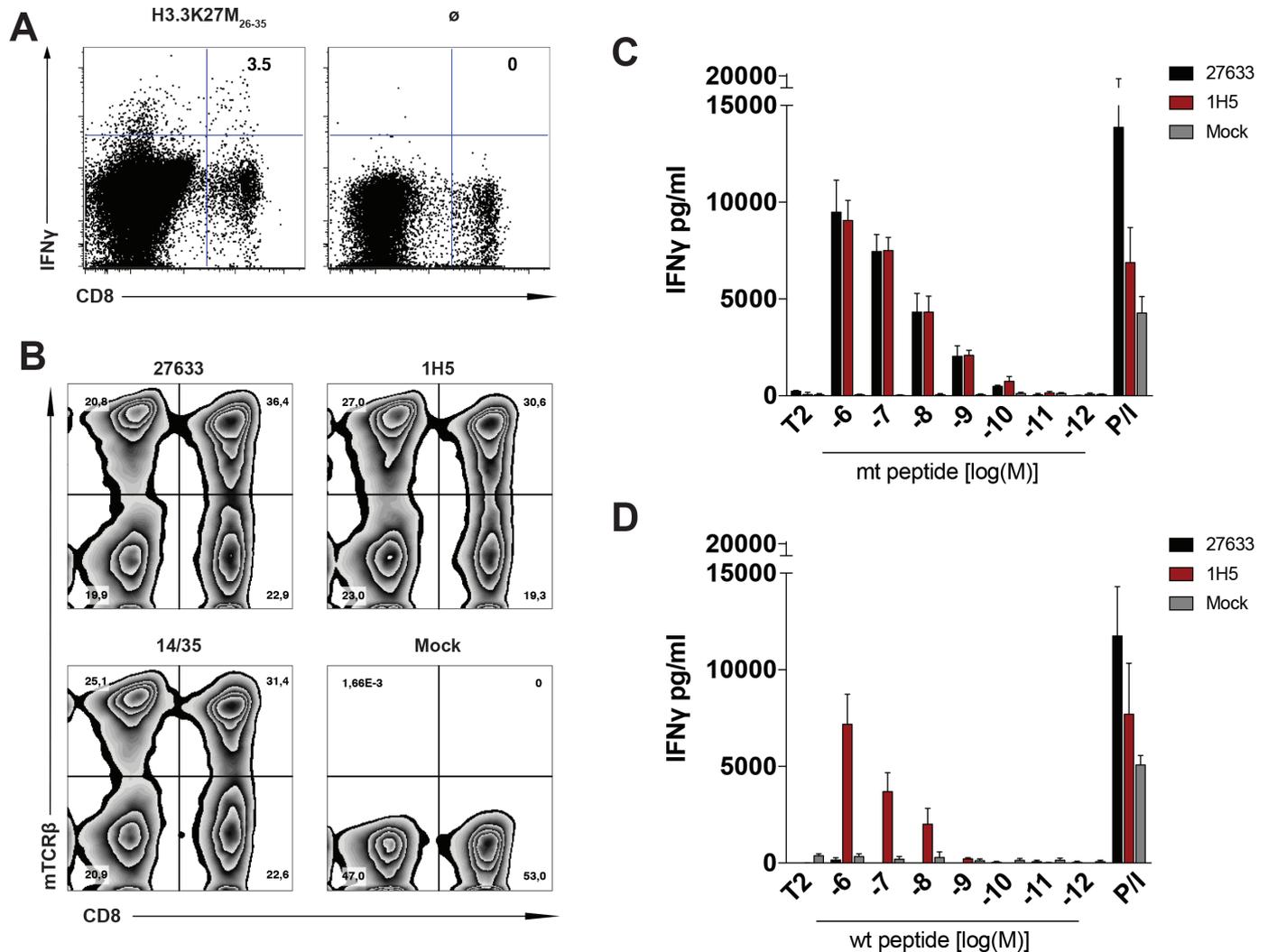
Diffuse midline glioma (DMG) is a devastating disease affecting primarily children and is the leading cause of solid cancer-related deaths in children, with a median survival of less than 1 year after diagnosis. Due to the proximity of the tumors to the brainstem, surgical removal is challenging and radiotherapy remains the only available treatment option.<sup>1</sup>

A point mutation in the histone H3 gene (*H3F3A*) causing a lysine to methionine amino acid exchange (K27M) leads to a reduced histone-3 trimethylation (H3K27me3) pattern and is found in the vast majority of DMG, including over 75% of diffuse intrinsic pontine glioma (DIPG) and 60% of non-brainstem midline tumors in the thalamus and spinal cord. The mutation is located at the N-terminus of the histone, a region which is highly conserved and crucial for the regulation and accessibility of chromatin.<sup>2</sup> It has been reported that H3.3K27M peptides inhibit the enzymatic activity of the histone methyltransferase Polycomb repressive

complex 2<sup>3</sup> and K27M mutated histones impede methylation at their cognate residues leading to aberrant epigenetic silencing. Tumors harboring the H3.3K27M mutation show a particularly aggressive progression.<sup>4</sup>

Cancer immunotherapy has developed into a promising approach for cancer therapies in the last decades. Adoptive T cell therapy (ATT) in particular, as in the case of using CAR T cells against the B cell antigen CD19, achieved remarkable regressions in leukemia and lymphoma patients.<sup>5</sup> Translation of this concept to solid tumors has proven to be difficult, a major hurdle being the identification of suitable tumor-associated auto-antigens as T cell targets that mediate effective tumor-killing without causing dose-limiting pathology in normal somatic tissues.<sup>6</sup> However, identification of truly cancer-specific antigens such as point-mutation-derived epitopes with good binding affinities to a frequent HLA molecule might prevent on-target toxicity and thus improve patient outcome.<sup>7</sup> The H3.3K27M mutation has been reported to create an epitope (H3.3K27M<sub>26-35</sub>, RMSAPSTGGV) that binds HLA-A\*02:01 and is recognized by a high-affinity T cell receptor (TCR) obtained from mutant peptide-stimulated HLA-A\*02:01-positive donor PBMCs.<sup>8</sup> In addition, this epitope is currently under investigation for peptide vaccination in an ongoing clinical trial for HLA-A\*02:01 and H3.3K27M positive patients with DMG.<sup>9</sup>

Here, we discuss the identification of an H3.3K27M mutation-specific TCR after peptide immunization of transgenic mice expressing a diverse human TCR repertoire and human HLA-A2 transgene.<sup>10</sup> We show that human PBMCs retrovirally transduced with the TCR possess high functional avidity specific to their target epitope, however, fail to recognize glioma cells naturally expressing or overexpressing the H3.3K27M mutation. We obtained similar results with the previously



**Figure 1** H3.3K27M peptide is recognized by a high avidity human TCR isolated from TCR transgenic mice. (A) Representation of a reactive CD8<sup>+</sup> cell population secreting IFN $\gamma$  on H3.3K27M<sub>26-35</sub> peptide (RMSAPSTGGV) restimulation of splenocytes isolated from immunized ABAbDII mice, measured by intracellular staining 7 days after the last immunization. Stimulation without peptide ( $\emptyset$ ) was used as a negative control. (B) Transgenic TCR expression levels in human PBMCs after retroviral transduction with H3.3K27M-specific TCRs 27633 and 1H5 (8), as well as CDK4-specific control TCR14/35 (11) and untransduced control, were measured by flow cytometry staining for the mouse TCR $\beta$  constant region. Percentage of positive transduced CD8<sup>+</sup> T cells are shown in the top right quadrant in (A, B), cells were gated on CD3<sup>+</sup>. (C, D) Levels of IFN $\gamma$  secretion of H3.3K27M-specific TCRs 27633 and 1H5-transduced PBMCs, cocultured with T2 cells loaded with decreasing concentrations of either H3.3K27M mutant (C) or H3.3 wild-type (WT) (D) peptide. A total of 10<sup>4</sup> CD8<sup>+</sup> transduced-TCR<sup>+</sup> cells were cocultured in a 1:1 E:T ratio with T2 cells. P/I represents maximum IFN $\gamma$  secretion from PBMCs stimulated with PMA/Ionomycin. The experiment was performed three times with similar results and graphs represent means of quadruplicate cultures  $\pm$ SD. TCR, T cell receptor; PBMC, peripheral blood mononuclear cells; E:T ratio, effector to target ratio.

published TCR 1H5.<sup>8</sup> Taken together, with this study we provide evidence that the H3.3K27M mutation is not a suitable target for HLA-A\*02:01 restricted, CD8<sup>+</sup> T cell-mediated immunotherapy.

## METHODS

The methods are described in online supplemental methods.

## RESULTS

The recurrent mutation in the histone 3 variant that results in a lysine to methionine amino acid exchange (K27M) creates an H3.3K27M<sub>26-35</sub> decamer peptide epitope (RMSAPSTGGV) which is predicted to bind HLA-A\*02:01 at substantial affinity (NetMHC v3.4: 285 nM) compared with the wild type counterpart (RKSAPSTGGV, NetMHC v3.4: 22651 nM). In order to isolate functional TCRs specific for the H3.3K27M mutation, we immunized ABAbDII mice, which express a diverse human TCR repertoire restricted to HLA-A\*02:01.<sup>10</sup> On H3.3K27M<sub>26-35</sub> peptide re-stimulation of splenocytes isolated from immunized mice, a reactive CD8<sup>+</sup> population secreting IFN $\gamma$  was

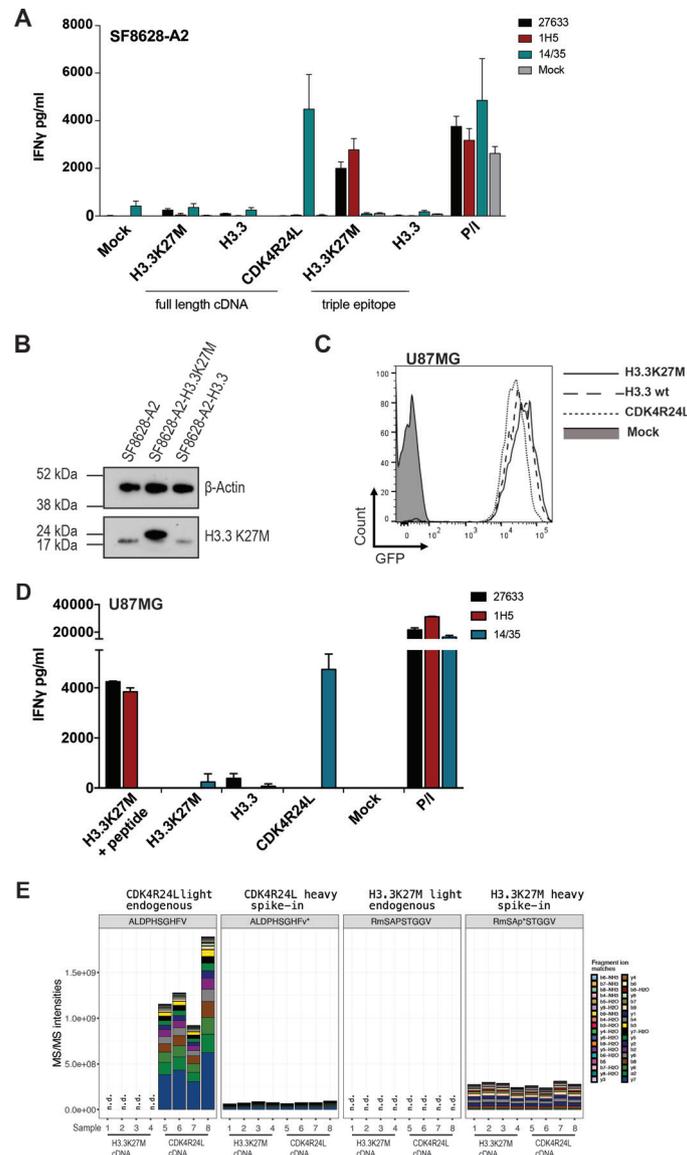
detected, CD8<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells were bulk sorted (figure 1A) and each dominant  $\alpha$  and  $\beta$  chain was identified in the reactive T cell population of mouse #2727633. The human constant regions were replaced by the respective murine ones and the identified TCR $\alpha\beta$  pair was synthesized as a bicistronic cassette separated by a P2A element. The TCR cassette was subsequently cloned into the MP71 vector, followed by retroviral transduction into primary human T cells of healthy donors. To better assess and compare the functionality of our mouse-derived human TCR (henceforth 27633), we also cloned and transduced a published human-derived TCR (henceforth 1H5),<sup>8</sup> into T cells of the same donor, as well as a well-characterized TCR (TCR 14/35) against an irrelevant target.<sup>11</sup> After a 10-day expansion of the PBMCs, we detected 36.4%, 30.6%, and 31.4% CD8<sup>+</sup> TCR-transduced T cells, respectively (figure 1B).

Functional avidity of both TCRs was analyzed by coculture with TAP deficient T2 cells, exogenously loaded with decreasing concentrations of the H3.3K27M<sub>26-35</sub> or the wild type H3.3<sub>26-35</sub> peptide. Both TCRs showed strong IFN $\gamma$  secretion down to a concentration of 10<sup>-10</sup> M H3.3K27M<sub>26-35</sub>, suggesting a high functional avidity of both TCRs against the mutant peptide (figure 1C). On binding to the wild type peptide, the human-derived TCR 1H5 induced a robust IFN $\gamma$  secretion down to 10<sup>-8</sup> M peptide concentration, while the mouse-derived TCR 27633 was specific for the mutant peptide (figure 1D).

To explore whether cells endogenously harboring the mutation are recognized by our TCR—an essential requirement for a potential clinical target, we retrovirally transduced the H3.3K27M<sup>+</sup> DIPG cell line SF8628 to express HLA-A\*02:01 (SF8628-A2) (online supplemental figure 1A). Unexpectedly, T cells transduced with either H3.3K27M-specific TCR failed to secrete IFN $\gamma$  during a 24-hour coculture with 1 $\times$ 10<sup>4</sup> tumor SF8628-A2 effector cells (E:T, 1:1) (figure 2A). To investigate whether this could be due to potential low (heterozygous) expression of the mutant allele in this cell line, we overexpressed either the H3.3K27M mutant or the wild type full-length cDNAs. Expression of the mutation was confirmed by western blot (figure 2B) and the level of overexpression of both mutant and wild-type cDNA was determined by qPCR (online supplemental figure 1B). However, even an 18-fold overexpression of the H3.3K27M cDNA in SF8628-A2 cells did not induce IFN $\gamma$  secretion by the TCR-transduced T cells (figure 2A). In contrast, when a minigene encoding three copies of the H3.3K27M<sub>26-35</sub> epitope (triple epitope), a robust IFN $\gamma$  response was observed, confirming HLA-A\*02:01-epitope binding and TCR specificity (figure 2A). To confirm that SF8628-A2 cells are in general capable of processing and presenting epitopes in an HLA-A\*02:01 dependent manner, we transduced T cells from the same healthy donor with TCR 14/35, which specifically recognizes the R24L mutation in CDK4.<sup>11</sup> As expected, overexpressed full-length CDK4R24L cDNA in the same SF8628-A2 target

cells was recognized by the respective TCR, inducing strong IFN $\gamma$  secretion (figure 2A). These results suggest that although both H3.3K27M-specific TCRs are of high functional avidity, they fail to lead to IFN $\gamma$  production against a glioma cell line, which is functional for antigen processing and presentation. In addition, we also investigated the recognition of a second DIPG cell line, SF7761, harboring the H3.3K27M mutation, which we also transduced to express HLA-A\*02:01 (SF7761-A2) (online supplemental figure 1A). Corroborating the above results, both TCRs were unable to recognize the naturally expressed mutant epitope, while the exogenously loaded mutant peptide elicited a robust immune response by both TCRs (online supplemental figure 1C). The heterozygous expression of the H3F3A alleles in the DIPG cell lines was confirmed by RT-PCR and Sanger Sequencing (online supplemental figure 1D). To further support this notion, we sought to use the well-established, processing and presentation competent HLA-A\*02:01<sup>+</sup> melanoma cell line Mel624. In order to assess recognition by our TCR, we transduced Mel624 cells to overexpress wild type or mutant H3.3 cDNAs, validated by western blot (online supplemental figure 2A) and qPCR analysis (online supplemental figure 2B). Here too, on coculture with H3.3K27M-specific TCR-transduced T cells, 27 633 and 1H5 TCR-transduced T cells failed to produce detectable amounts of IFN $\gamma$  (online supplemental figure 2C). To exclude that recognition through the transduced TCRs leads to expression of cytokines other than IFN $\gamma$ , we also tested for TNF $\alpha$  and IL-2 secretion, but we were unable to detect either of the two cytokines (online supplemental figure 2C). In contrast, overexpression of the CDK4R24L mutant cDNA in Mel624 cells led to recognition by the 14/35 control TCR and secretion of all three cytokines. These results show that the H3.3K27M TCR-transduced T cells fail to produce cytokines associated with T cell cytotoxicity on coculture with cells naturally expressing or overexpressing the H3.3K27M mutation.

To account for possible differences between DMG/DIPG and glioblastoma (GBM) cell lines,<sup>12</sup> we also overexpressed either H3.3K27M or H3.3, and as control, CDK4R24L full-length cDNAs in the GBM U87MG cell line used by Chheda *et al* (figure 2C). Again, only CDK4R24L expressing U87MG cells were recognized by the respective 14/35 TCR-transduced PBMCs, whereas neither mutant nor wild type H3.3-expressing U87MG cells induced IFN $\gamma$  release in 27 633 or 1H5 TCR-transduced PBMCs, unless the cells were exogenously loaded with peptide (figure 2D). To further analyze if the predicted H3.3K27M epitope is naturally presented in HLA-A\*02:01, we immunoprecipitated peptide/HLA-I complexes from the U87MG cells overexpressing either H3.3K27M or CDK4R24L and detected eluted peptides by liquid chromatography tandem mass spectrometry (LC-MS/MS). In contrast to spiked heavy synthetic H3.3K27M peptide, no endogenous mutant H3.3-derived peptides were detected in U87MG cells overexpressing the respective cDNA (figure 2E). As a control, mutant



**Figure 2** H3.3K27M TCR-transduced T cells fail to recognize cell lines naturally expressing or overexpressing the mutant H3.3 histone. (A) Levels of IFN $\gamma$  secretion of 27633- and 1H5-TCR transduced PBMCs, cocultured with the human SF8628 DIPG cell line. SF8628 cells were retrovirally transduced to express HLA-A\*02:01 (SF8628-A2) and additionally contained H3.3K27M full length cDNA (MP71\_H3.3K27M-CDS-P2A-GFP), H3.3 wild type full length cDNA (MP71\_H3.3-CDS-P2A-GFP), CDK4R24L full length cDNA (MP71-CDK4-R24L-P2A-GFP), H3.3K27M RMSAPSTGGV triple epitope (MP71\_H3.3K27M-triple-GFP), or H3.3 wild-type RKSAPSTGGV triple epitope (MP71\_H3.3-triple-GFP). A total of  $10^4$  CD8 $^+$  transduced cells were cocultured at a 1:1 E:T ratio. CDK4R24L specific TCR-transduced T cells (14/35) were used as positive control to assess processing and presenting capabilities of the cell line. P/I represents maximum IFN $\gamma$  secretion from PBMCs stimulated with PMA/Ionomycin. The experiment was performed three times with similar results and graphs represent means of triplicate cultures  $\pm$  SD. (B) H3.3K27M protein was detected by Western blot. Top panel shows  $\beta$ -actin (45 kDa), lower panel shows H3.3K27M (17 kDa) in parental or transduced SF8628-A2. Size discrepancy between naturally expressed and overexpressed mutant protein in SF8628-A2 cells is due to 18 amino acids at the C terminal end, encoded by the P2A element in the latter. (C) U87MG glioblastoma cell line was retrovirally transduced with MP71\_H3.3K27M-CDS-P2A-GFP, MP71\_H3.3-CDS-P2A-GFP or MP71-CDK4-R24L-P2A-GFP and sorted for GFP expression to overexpress either H3.3K27M, wild type H3.3 or CDK4R24L full length cDNA, respectively. (D) Levels of IFN $\gamma$  secretion of 27633 and 1H5 TCR-transduced PBMCs, cocultured with FACS-sorted transduced U87MG are shown, the CDK4R24L-specific TCR (14/35) served as positive control for functional antigen processing and presenting machinery in the U87MG cell line as well as IFN $\gamma$  detection by TCR-transduced T cells. (E) The mutated H3.3 peptide is not detectable by a LC-MS/MS-based immunopeptidomic screen in U87MG glioblastoma cell line overexpressing H3.3K27M cDNA. Samples 1–4 recombinantly express H3.3K27M, samples 5–8 express CDK4R24L. MS2 fragment intensities for the endogenous mutated CDK4 and H3.3 peptides eluted from HLA-A\*02:01 $^+$ /CDK4R24L $^+$  and HLA-A\*02:01 $^+$ /H3.3K27M $^+$  cells after W6/32 HLA pull-downs and their heavy synthetic counterparts are shown. V\* and P\* indicate a heavy amino acid with a mass shift of +6 on valine or proline, respectively; M: oxidized methionine; n.d.: not detected. An amount of 25 fmol for CDK4R24L or 5 fmol for H3.3K27M of the synthetic heavy peptides were injected along with each sample. Only charge state 2+ was used for the quantitation. LC-MS/MS, liquid chromatography tandem mass spectrometry.

CDK4-derived peptides were detected in U87MG cells overexpressing CDK4R24L cDNA. The sequence of the eluted CDK4R24L peptide was validated by matching its LC-MS/MS retention time and spectra with that of its synthetic heavy peptide counterpart (figure 2E, online supplemental figure 3).

To investigate the cytotoxic potential of the H3.3K27M TCR-transduced T cells, we used a live-cell imaging-based cytotoxicity assay with target cells expressing GFP. On coculture of these fluorescent target cells with the respective T cells, the number of target cells surviving was determined relative to the number of control cells over a time period of 72 hours. Consistent with our previous results, no cytotoxic effect of H3.3K27M TCR-transduced T cells was observed, neither when the mutation was expressed naturally, nor when we overexpressed the H3.3K27M cDNA. T cells transduced with either 27633 or 1H5 TCRs failed to lyse HLA-A\*02:01 melanoma cell line Mel21a overexpressing full-length H3.3K27M cDNA (Mel21a-H3.3K27M; figure 3A, top panel). In contrast, the control T cells expressing the CDK4R24L-specific TCR14/35 killed 50% of their target cells, Mel21a-CDK4R24L, within 11 hours (figure 3A, bottom panel). In addition, natural expression of the H3.3K27M mutation in the SF8628-CDK4R24L glioma cell line did not lead to lysis by the two H3.3K27M-specific T cells, while coculture with the control T cells showed substantial and prompt killing of 50% tumor cells within 9 hours (figure 3B, top panel). As depicted in figure 3B (bottom panel), cytotoxicity mediated by the H3.3K27M TCR-transduced T cells was only observed against target cells loaded with the mutant peptide (50% killing within 10 hours), confirming the cytotoxic potential of the H3.3K27M TCR-transduced T cells used in the assay.

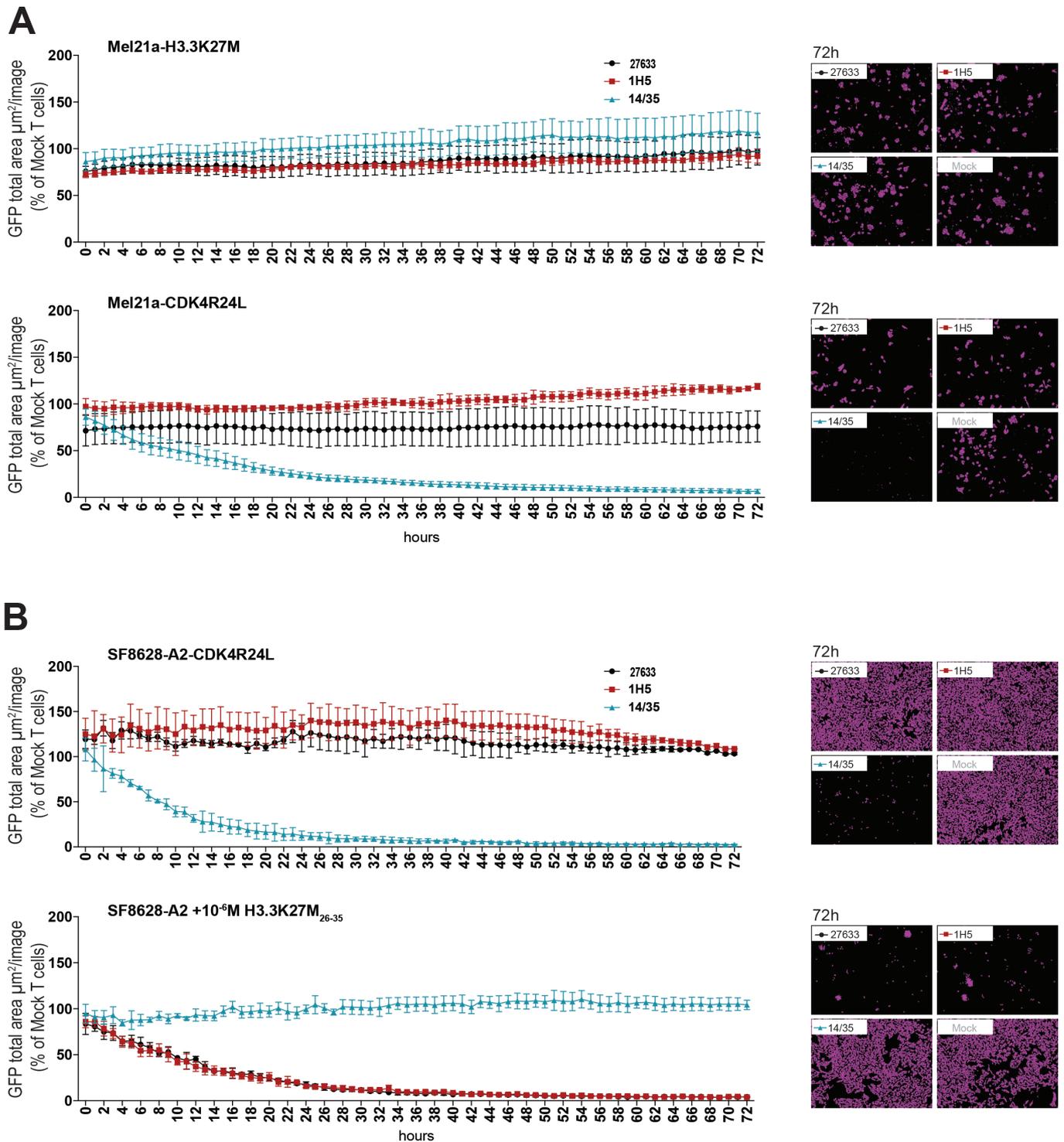
These experiments unequivocally show that the putative mutant H3.3K27M epitope is not recognized by two independently isolated TCRs, regardless of whether the mutant gene is naturally expressed or overexpressed. In addition, we analyzed eight ERAP1 polymorphisms in the genomic DNA of the cell lines Mel21a, Mel624, SF8628, and U87MG used in this T cell recognition analysis and identified different ERAP1 allotypes among the cell lines (online supplemental table 1). Thus, we exclude the possibility that lack of recognition is caused by differential epitope trimming or destruction due to specific ERAP1 allotypes present in all cell lines.<sup>13</sup>

To exclude that T cell recognition of H3.3K27M was hampered by the presence of the endogenous human TCR chains resulting in mixed dimers, we knocked out the endogenous TCR in the transduced human T cells by CRISPR/Cas9. To confirm the functionality of TRAC/TRBC-specific gRNAs, mock-transduced T cells were targeted with either RNP complex, which led to a knockout efficiency of 73.3% and 29.5%, respectively (online supplemental figure 4A). CRISPR targeting with both gRNAs simultaneously in mock-transduced PBMCs led to a marked decrease in the number of hTCR $\alpha$ / $\beta$ <sup>+</sup> cells, from 90.3% to 17.4% (online supplemental figure

4B). When 27633 and 1H5 TCR-transduced PBMCs were targeted, the hTCR $\alpha$ / $\beta$ <sup>+</sup>mTCR<sup>+</sup> double-positive populations, representing the population with potential mixed dimers, decreased to 5.8% from 11.6% and to 5.7% from 12.7%, respectively (online supplemental figure 4B). CRISPR/Cas9-edited TCR-modified T cells were cocultured with SF8628-A2 cells, either naturally expressing the H3.3K27M mutation, or overexpressing fulllength H3.3K27M cDNA, as well as with H3.3K27M<sub>26-35</sub> peptide-loaded T2 cells. As depicted in online supplemental figure 4C, no alteration in the capacity of T cells lacking the endogenous human TCR to recognize the expressed mutation was observed: The endogenous as well as the overexpressed mutation were still not recognized by the TCR-modified T cells, whereas H3.3K27M<sub>26-35</sub> peptide-pulsed T2 cells-induced IFN $\gamma$  secretion by TCR-modified T cells to previously observed levels. Again, 1H5 TCR-modified T cells also recognized wild type H3.3<sub>26-35</sub> peptide-pulsed T2 cells, as already shown in figure 1D. When analyzing binding of H3.3K27M-specific MHC I dextramer no significant difference in staining intensity was detected for CD8<sup>+</sup> T cells expressing H3.3K27M-specific TCRs. Thus CD8<sup>+</sup>Dextramer<sup>+</sup> cells modestly decreased on CRISPR/Cas9 knockout from 23.8% to 19.9% for 27633 TCR-transduced T cells and increased from 27.3% to 30% for 1H5 TCR-transduced T cells. In addition, no change in the mean fluorescence intensity was observed between both panels. Surprisingly, 22.6% (-Cas9 RNP) and 21.8% (+Cas9RNP) of CD4<sup>+</sup> T cells expressing the human-derived TCR 1H5 were also stained with the dextramer, suggesting that MHC-peptide binding might also be occurring in a CD8 independent manner<sup>14</sup> (online supplemental figure 4D). With these CRISPR/Cas9 modifications, we excluded that potential mispairing between endogenous and transduced human TCR chains might be affecting the recognition of the glioma target in any way, further strengthening the notion that the H3.3K27M<sub>26-35</sub> is not a suitable target for TCR gene therapy in HLA-A\*02:01<sup>+</sup> patients with DMG.

## DISCUSSION

Although we confirmed that the TCRs generated against the H3.3K27M mutation show high affinity to their cognate peptide *in vitro*, they do not recognize endogenously expressed mutation, which is a prerequisite for TCRs to possess a clinical potential. Overall, these findings are in contrast to those reported by Chheda *et al*, where 1H5 TCR-modified T cells were shown to mediate reactivity in a LDH cytotoxicity assay on coculture with DIPG cells naturally expressing the H3.3K27M mutation. Since we tested the 1H5 TCR side by side with a high-affinity H3.3K27M peptide-specific TCR, obtained from huTCR transgenic mice, in coculture experiments measuring IFN $\gamma$ , TNF $\alpha$ , and IL2 secretion as well as cytotoxicity, and found no signs of reactivity even to overexpressed H3.3K27M, the applicability of this adoptive T cell targeting approach in HLA-A\*02:01<sup>+</sup> DMG patients



**Figure 3** Lack of cytotoxicity against cells naturally expressing or overexpressing mutant H3.3 histone. (A)  $15 \times 10^3$  transduced CD8<sup>+</sup> cells were cocultured at a 5:1 E:T ratio with HLA-A\*02:01<sup>+</sup> Mel21a cells, retrovirally transduced to express either H3.3K27M full length cDNA (MP71\_H3.3K27M-CDS-P2A-GFP) (top panel) or CDK4R24 full length cDNA (MP71-CDK4-R24L-P2A-GFP) (bottom panel). Cytotoxicity was observed over 72 hours using the live cell imaging system IncuCyte Zoom (Essen bioscience). Representative images are shown in the right panels. Values are calculated by normalizing the average GFP total area ( $\mu\text{m}^2/\text{image}$ ) in the target cells cocultured with the respective TCR-transduced T cells to the average of that cocultured with mock transduced T cells. The experiment was performed three times with similar results and graphs represent means of triplicate cultures  $\pm$  SD. (B)  $15 \times 10^3$  CD8<sup>+</sup> transduced cells were cocultured at a 5:1 E:T ratio with SF8628-A2 cells, naturally expressing the H3.3K27M mutation and additionally either retrovirally transduced to express CDK4R24L full length cDNA (top panel) or loaded with  $10^{-6}$  M mutant H3.3K27M peptide (bottom panel). Cytotoxicity was observed over 72 hours using the live cell imaging system IncuCyte Zoom. Representative images are shown in the right panels. Values are calculated as in (A). The experiment was performed three times with similar results and graphs represent means of triplicate cultures  $\pm$  SD. GFP, green fluorescent protein.

is questionable. Detection of H3.3K27M-specific vaccine responses in a study with DMG patients by mass cytometry supports the notion that the H3.3K27M epitope may be presented by HLA-A\*02:01 to induce a T cell response, if the vaccine consists of the decamer mutant peptide.<sup>9</sup> However, it is unlikely that the CD8<sup>+</sup> T cells in this clinical trial were effective because we show here that the epitope is not presented in sufficient amounts to be recognized by T cells. In line with our findings, the H3.3K27M epitope vaccination treatment strategy (PNOC007) did not improve the overall outcome in H3.3K27M<sup>+</sup> patients with DMG.<sup>9</sup> We can rule out that factors such as different ERAP1 activity account for lack of recognition by using cell lines with different ERAP1 polymorphisms.<sup>13</sup> We excluded presence/absence of methionine oxidation in the H3.3K27M/HLA-A\*02:01 epitope<sup>15</sup> to account for the different results with recognition of cell lines by showing recognition of triple H3.3K27M<sub>25-35</sub> epitope speaking against a TCR specificity for the oxidized methionine only. Moreover, the discrepancy between our findings and previously published ones did not resolve, even when the same cell line (U87MG), authenticated by STR profiling, was used for antigen presentation. Here, a mass spectrometry-based immunopeptidomics screen further indicated that the predicted H3.3K27M epitope may lack sufficient T cell immunogenicity as it could not be detected in HLA-A\*02:01<sup>+</sup> U87MG cells overexpressing mutant H3.3 cDNA. Closing, we have to conclude that H3.3K27M is unlikely a suitable target for TCR gene therapy in HLA-A\*02:01<sup>+</sup> patients with DMG. We think it is important to share these negative data with the community, because clinical trials based on H3.3K27M could raise wrong hope at least in HLA-A\*02:01-positive patients and would lead to the wrong conclusion that an in principle ideal target cannot be made use of for immunotherapy. To open an immunotherapeutic perspective for DMG patients, further analysis of targeting H3.3K27M by either CD8<sup>+</sup> T cells restricted to HLAs other than HLA-A\*02:01 or CD4<sup>+</sup> T cells is therefore required. In general, immunogenicity of neoantigens predicted to bind the one or the other HLA need to be validated first by T cell assays or HLA ligandomics before these can be considered valuable targets for (peptide) vaccination or ATT.

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**Contributors** GW designed the study, LI, GP and OP conducted experiments. LI, GP, PM, TB and GW analyzed data and wrote the manuscript. TB and GW are joint last authors.

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

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## Supplemental Methods

**Peptide immunization of mice.** H3.3K27M-specific T cells were generated in ABAbDII mice expressing a diverse human TCR repertoire <sup>1</sup>. Mice were immunized by subcutaneous injection of 100 µg H3.3K27M (RMSAPSTGGV) peptide in a 1:1 solution of incomplete Freund's adjuvant and PBS containing 50 µg CpG. After priming, mice received the same immunization twice as boosts in a three weeks interval. To assess CD8<sup>+</sup> T cell responses, peripheral T cells were restimulated *in vitro* with either 10<sup>-6</sup> M peptide, PBS as a negative control, or 10<sup>6</sup> Dynabeads mouse T activator CD3/CD28 (Gibco) as a positive control. After 2h, Brefeldin A (BD) was added to the cultures and after overnight culturing, specific CD8<sup>+</sup> T cells were measured by intracellular IFN $\gamma$  staining (PE anti-mouse IFN $\gamma$  XMG1.2, Biolegend).

**Isolation and cloning of TCRs.** To isolate specific TCRs, immunized mice were sacrificed, splenocytes and lymphocytes from inguinal lymph nodes prepared and CD4<sup>+</sup> T cells depleted using microbeads (Miltenyi Biotec). 1x10<sup>6</sup> splenocytes were cultured in T cell media (TCM, RPMI (Gibco™) containing 10% FCS (Pan Biotech), 1 mM HEPES (Gibco™), 100 IU/ml PenStrep (Gibco™), 50 µM 2-Mercaptoethanol (Gibco™)) supplemented with 100 IU/ml IL-2 (Peprotech) for 10 days in the presence of 10<sup>-8</sup> M H3.3K27M peptide. Four hours prior to the *in vitro* assessment of IFN $\gamma$  secretion (mouse IFN $\gamma$  secretion assay, Miltenyi), cells were stimulated with a peptide concentration of 10<sup>-6</sup> M. To sort IFN $\gamma$ -secreting CD8<sup>+</sup> T cells, cells were stained with anti-mouse CD3-APC (145-2C11, Biolegend) and anti-mouse CD8-PerCP (53-6.7, Biolegend) at 4°C for 30 minutes before the cells were sorted (BD FACS Aria III) into RTL lysis buffer for RNA isolation with RNeasy Micro Kit (QIAGEN). SMARTer™ RACE cDNA Amplification Kit (Clontech Laboratories) was used to synthesize first-strand cDNA synthesis and 5'-RACE PCR. The TCR sequence was specifically amplified using 0.1 µM hTRAC (5'-cggccacttccaggaggaggattcggaaac-3') or hTRBC (5'-ccgtagaactggacttgacagcggaaagtgg-3') specific primer in a 1-2 µl reverse transcriptase reaction together with 1U Phusion® HotStart II polymerase (Thermo Scientific). The amplicons were analyzed on an agarose gel and specific bands were cut out and cloned using a Zero Blunt® TOPO® PCR cloning kit (Life Technologies). A T3 primer (5'-aattaacctactaaagg-3') was used to sequence plasmids from isolated individual clones (Eurofins Genomics). The dominant TCR- $\alpha/\beta$  chains were selected and paired as follows: 27633 TCR: TRAV13\*01 – CAVRGLTQGGSEKLVF - TRAJ57\*01, TRBV27\*01 – CASSWGGFPYEQYF - TRBJ2-7\*01). For 1H5 TCR we combined TRAV19\*01 – CALSEENDMRF – TRAJ43\*01, TRBV27\*01 – CASGWGGFPYEQYF - TRBJ2-7\*01) <sup>2</sup>. Corresponding TCR- $\alpha/\beta$  chains were linked using a P2A element and constant regions of both, the human-derived and mouse-derived TCRs were exchanged with mouse constant

regions. The codon optimized TCR cassettes were synthesized by GeneArt (Thermo Fisher Scientific) and cloned into a MP71 vector as NotI/EcoRI fragments.

**Plasmid constructs and cDNA synthesis.** All retroviral packaging plasmid vectors were based on plasmid MP71<sup>3</sup>. Plasmids MP71-A2 encoding HLA-A\*02:01, and MP71-CDK4-R24L-i-GFP encoding the full-length cDNA of CDK4 harboring the R24L mutation and co-expressing EGFP through an IRES element, were a kind gift from M. Leisegang. In order to construct MP71\_H3.3K27M-CDS-i-GFP and MP71\_H3.3-CDS-i-GFP, expressing H3F3A mutant and wild type full-length cDNA respectively, H3F3A coding sequences were cloned by RT-PCR from cDNA samples synthesized from RNA isolated from SF8628 cells. In brief, RNA was isolated from  $5 \times 10^5$  cells using the RNeasy Isolation Plus Mini Kit (QIAGEN). cDNA synthesis was performed with 1  $\mu$ g of total RNA as template and random hexamers, using the Advantage RT-for-PCR Kit (Takara Bio) according to manufacturer's protocol. H3F3A coding sequences were amplified by PCR using primers GP001\_H3F3A (5'-aaagcggccgcaccatggctcgtacaaagcag-3') and GP002\_H3F3A (5'-aaactcgagattcttaagcagcttccacgta-3') conferring NotI and XhoI restriction sites respectively. The IRES-EGFP element was cloned by PCR using primers GP003\_IRES (5'-aaactcgagacgtggcggctagtactcc-3') and GP004\_GFP (5'-aaagaattctgtacagctcgtccatgc-3') conferring XhoI and EcoRI restriction sites respectively. Fragments were digested with the respective restriction enzymes (NEB) and cloned in a two-fragment ligation (DNA ligation mix, Mighty Mix, Takara Bio) into NotI/EcoRI digested MP71. Clones were Sanger sequenced and both K27M and wild type clones were selected.

In order to construct MP71-CDK4R24L-P2A-GFP, MP71\_H3.3K27M-CDS-P2A-GFP and MP71\_H3.3-CDS-P2A-GFP, a P2A-EGFP fragment was PCR amplified from plasmid pcDNA3.1-Hygro(+)-M7PG (kind gift V. Anastasopoulou) using either primer H3.3-P2A\_F (5'-acgccgcatacgtggagaacgtgctggcagcggcgccaccaac-3'), or CDK4-P2A\_F (5'-acataaggatgaaggtaatccggagggcagcggcgccaccaac-3') combined with GFP-PRE\_Rev (5'-aatggcggtaagatgctcgaatttcattgtacagctcgtccatgc-3'). Produced amplicons bear homologies to the above plasmids and can serve to prime overlap extension PCR (OE-PCR), replacing the IRES-EGFP elements with P2A-EGFP. In brief, the respective OE-PCR amplicon was mixed in a 200 molar excess ratio to 10 ng of either one of MP71-CDK4-R24L-i-GFP, MP71\_H3.3K27M-CDS-i-GFP or MP71\_H3.3-CDS-i-GFP, in 25  $\mu$ l PCR reactions and were cycled, using 52°C annealing temperature and a 7-minute extension time at 72°C, for 21 cycles. Reactions were subsequently digested with DpnI and transformed into competent *E. coli*.

H3.3K27M and H3.3 triple-epitope minigenes, encoding three tandem copies of the respective decamer epitope followed by EGFP, separated by the preferred proteasome

cleavage site AAY, were synthesized (GeneArt Gene Synthesis, ThermoFischer Scientific) and were cloned into MP71 as NotI/EcoRI fragments (MP71\_[triple-H3.3K27M]-GFP and MP71\_[triple-H3.3]-GFP).

All PCRs described were performed using Q5, High fidelity 2x Master mix (NEB).

**Cells and cell culture.** The human diffuse intrinsic pontine glioma (DIPG) cell line SF8628 (RRID:CVCL\_IT46, SCC127 Sigma-Aldrich) was cultured in DMEM (Gibco™) supplemented with 10% FCS (Pan Biotech) and 100 IU/ml PenStrep (Gibco™). The human DIPG cell line SF7761 (RRID:CVCL\_IT45, SCC126 Sigma-Aldrich) was grown as neurospheres in suspension culture and cultured in ReNcell NSC Maintenance Medium (Cat. No. SCM005), containing 20 ng/mL EGF (Cat. No. GF001), 20 ng/mL FGF-2 (Cat. No. GF003) and 100 IU/ml PenStrep (Gibco™). The retroviral packaging cell line 293GP-GLV (producing amphotropic retroviral vectors) was cultured in DMEM supplemented with 10% FCS<sup>4</sup>. TAP deficient T2 cells (RRID:CVCL\_2211, ATCC: CRL-1992) were cultured in TCM. The melanoma cell lines Mel624 and Mel21a were cultured in RPMI (Gibco™) supplemented with 10% FCS (Pan Biotech) and 100 IU/ml PenStrep (Gibco™). U87MG cells were cultured in MEM $\alpha$  (Gibco™) supplemented with 10% FCS (Pan Biotech) and 100 IU/ml PenStrep (Gibco™). U87MG cells were authenticated by STR profiling (Cell Line Authentication Service of Eurofins Genomics, Ebersberg, Germany).

**Retroviral transduction.** TCR gene transfer was carried out as described before<sup>3,5</sup>. In brief, for retrovirus generation, 293GP-GLV cells were transfected with MP71 vector carrying the respective TCR cassettes using Lipofectamine 3000 (ThermoFisher Scientific). On the same day, PBMCs from healthy donors were seeded on plates coated with 5  $\mu$ g/ml anti-CD3 (OKT3, Invitrogen) and 1  $\mu$ g/ml anti-CD28 antibodies (CD28.2, Invitrogen) in TCM supplemented with 100 IU/ml IL-2 (Peprotech). 48 hours later, the virus supernatant was harvested, filtered and supplemented with 8  $\mu$ g/ml protamine sulfate (Sigma-Aldrich) and 100 IU/ml IL-2, before spinoculation with the activated T cells at 800g for 90 minutes at 32°C was performed. The next day, a second supernatant was harvested from the same 293GP-GLV cells, transferred to a RetroNectin (Takara Bio) coated plate and centrifuged at 3200g for 90 minutes at 4°C. The PBMCs were harvested, supplemented with 100 IU/ml IL-2 and 8  $\mu$ g/ml protamine sulfate and spinoculated with the virus-containing plates at 800g for 30 minutes at 32°C. After the second transduction, T cells were expanded for 10 days, before being transferred to low IL-2 (10 IU/ml). After 48 hours, transduced T cells were harvested, analyzed for TCR expression by flow cytometry and frozen for future experiments. To detect the transduction rate of the TCRs transduced into primary T cells, following antibodies were

used in a 1:100 dilution at 4°C for 30 min: anti-human CD3-PerCP (UCHT1, Biolegend), anti-human CD8-APC (HIT8a, Biolegend) and anti-mouse TCR  $\beta$  chain-PE (H57-597, Biolegend). Since both DIPG cell lines are HLA-A\*02:01 negative, SF8628-A2 and SF7761-A2 cells were generated by transfecting MP71-HLA-A\*02:01 into 293GP-GLV cells using Lipofectamine 3000 (ThermoFisher Scientific). 48 hours after transfection, 293GP-GLV virus-containing supernatant was harvested, supplemented with 8  $\mu$ g/ml protamine sulfate and transferred to the respective glioma cell line. Cells and virus supernatant were spinoculated at 800g for 90 minutes at 32°C. Medium was changed to the respective growth medium 6 hours later. The same transduction protocol was used to overexpress full-length cDNAs H3.3K27M, H3.3 wild type, CDK4R24L, wildtype and mutant triple epitopes in the SF8628-A2 DIPG cells and as control in HLA-A\*02:01 positive 624-Mel (RRID:CVCL\_8054) and SK-Mel-21 (Mel21a, RRID:CVCL\_3877) melanoma cells and U87MG ATCC (RRID:CVCL\_0022) glioblastoma cells<sup>6</sup>. To analyze successful transduction, flow cytometry was used to determine the fraction of GFP-positive cells. HLA-A\*02:01 transduction was confirmed by flow cytometry using an anti-human HLA-A2-APC (BB7.2, Biolegend) specific antibody and an APC mIgG2b, k isotype control (MPC-11, Biolegend).

**Co-culture experiments.** All co-culture experiments were conducted using  $1 \times 10^4$  transduced T cells with  $1 \times 10^4$  target cells for 24 hours in a 96 well plate. As a positive control for the T cell activation, 50 ng/ml PMA (Phorbol-12-myristat-13-acetat, (Calbiochem) and 1  $\mu$ g/ml Ionomycin (Calbiochem) were added to the transduced T cells. T2 cells were loaded with the H3.3K27M (RMSAPSTGGV) or the H3.3 wild type (RKSAPSTGGV) peptide (JPT Peptide Technologies GmbH) at the indicated concentrations. Secreted IFN $\gamma$ , TNF and IL-2 amounts in the supernatant were measured by ELISA (BD OptEIA; BD Biosciences).

**Western blot.** To determine the endogenous expression and overexpression of the H3.3K27M mutation in the SF8628 and Mel624 cells, we lysed  $2 \times 10^6$  tumor cells using a lysis buffer containing 2% SDS and 0,05 M TrisHCl for 10 minutes at 95°C. Subsequently, the protein concentration was measured using the Total Protein Kit, Micro-Lowry (Sigma). 25  $\mu$ g of protein in LDS sample buffer and DTT was loaded onto a NuPage 4-12% Bis-Tris 1,5 mm gel (Invitrogen) next to a Full Range Rainbow Protein ladder (Sigma-Aldrich) and ran at 200V, 250mA for 42 minutes. The separated proteins were transferred onto a PVCF membrane for 1h at 30V and 250mA. After blocking in 5% milk in TBST, the membrane was incubated in either H3.3K27M specific antibody (RM192, Rabbit mAb, Abcam) or a beta actin specific antibody (Rabbit mAb, Cell Signaling) diluted 1:1000 in 5% milk shaking at 4°C overnight. The membrane was washed three times for 15 minutes in TBST and stained with a secondary HRP coupled anti-rabbit IgG (Cell Signaling) diluted 1:2000 in 5% milk at room

temperature for one hour. After washing, the membrane was soaked in ELC substrates (ThermoFisher) and exposed for 10 minutes on a ChemiDoc XRS+ Gel Imaging System (Biorad).

**Quantitative analysis of cDNA overexpression.** cDNA synthesis from all samples was performed as described above. Samples were then diluted 1:5 and 5  $\mu$ l used as template in a 20  $\mu$ l qPCR reaction using SYBR Green PCR Master Mix (Thermo), with 500 nM primer concentration. Following primers were used for real-time PCR. H3.3: F:5'-taaagcaccaggaagcaac-3', R:5'-gtctcaaaaaggccaacca-3', GAPDH: F:5'-gtgaaggctggagtcacg-3', R:5'-tgaggtcaatgaaggggtc-3' Samples were run on the QuantStudio 3 Real-Time PCR system (ThermoFisher) and analyzed according to the comparative  $\Delta\Delta C_t$  method. Samples were sent for Sanger Sequencing (Eurofins Genomics) to confirm presence of wildtype and mutant H3.3 genes.

**ERAP1 allotype identification.** To identify the presence of ERAP1 polymorphisms, gDNA was extracted from cell lines using QIAamp DNA Mini Kit (Qiagen) and targeted ERAP1 loci amplified and sequenced using the following primers: E56K/R127P 5'-TCTGTGAATGCTGGGTGGAT-3' and 5'-CTCAGTTCCTTCCTTGGT-3'; G346D/M349V 5'-AAGGCCTGAGATGCAAGTCT-3' and 5'-CCATAGTGACCAGGTTCCCA-3'; K528R 5'-GTGTTATTGCCAGCCCCAAA-3' and 5'-AGGAGCATTACCCAGTGTCC-3'; D575N 5'-GCTGACACTCCCTACAAAGC-3' and 5'-GCAACTACATCTCTGGCCAT-3'; R725Q/Q730E 5'-GGTCCCTGTTCCCTGTACA-3' and 5'-GCATGGCTGTACCCGTTTAA-3'.

**CRISPR/Cas9 knockout of endogenous TCRs.** To knock out the endogenous TCR in transduced T cells, we used the CRISPR/Cas9 system with TRAC-gRNA: agagucucucagcugguaca<sup>7</sup> and gRNA2: uggcucaaacacagcgaccu<sup>8</sup> targeting the endogenous human TCR alpha and beta constant regions, respectively. Before RNP complexing, transduced cells were thawed and cultured for one hour in TCM and 10 IU/ml IL-2. Subsequently, cells were washed 2x in TCM and resuspended at  $5 \times 10^7$ /ml in OptiMEM. 150 pmol of Alt-R tracrRNA (IDT) were annealed to 150 pmol of the respective Alt-R crRNAs (IDT) according to manufacturer's instructions. The resulting TRAC-gRNA and gRNA2 were complexed with 50 pmol Alt-R S.p. HiFi Cas9 Nuclease V3 (IDT) and the RNP complexes were nucleofected into  $10^6$  T cells in 20  $\mu$ l OptiMEM, using pulse EO-115 on Lonza 4D-Nucleofector<sup>TM</sup> Core Unit in Opti-MEM (Gibco<sup>TM</sup>). The cells were cultured for 4 days in TCM containing 10 IU/ml IL-2 and the knockout efficiency of the endogenous TCR was measured by flow cytometry. To analyze the expression of endogenous and transduced TCR, the T

cells were first stained with 10  $\mu$ l of HLA-A\*02:01 H3.3K27M Dextramer-PE (Immudex) per 50  $\mu$ l PBS containing 50% FCS at room temperature for 30 minutes. After washing, the following antibodies were added in a 1:100 dilution at 4°C for 30 minutes in PBS containing 5% FCS: anti-human CD3-PerCP (UCHT1, Biolegend), anti-human CD8-BV421 (HIT8a, Biolegend), anti-human TCR  $\alpha/\beta$ -FITC (BW242/412, Miltenyi Biotec) and anti-mouse TCR  $\beta$  chain-APC (H57-597, Biolegend).

**Cytotoxicity assay.** The cytotoxic potential of transduced T cells was analyzed using the live cell imaging system IncuCyte Zoom (Essen Bioscience). Target cells were transduced with either MP71-CDK4R24L-P2A-GFP, or MP71\_H3.3K27M-CDS-P2A-GFP and  $3\text{-}5 \times 10^3$  GFP positive target cells were resuspended in TCM without phenol red and seeded into flat-bottom 96 well plates. The following day, for a 5:1 (effector : target) ratio  $1,4\text{-}2,5 \times 10^4$  transduced T cells were added to the respective wells in triplicates. GFP expression in target cells was determined every hour over a time period of 72 hours at 37°C and 5% CO<sub>2</sub>. For analysis, the average of GFP total area ( $\mu\text{m}^2/\text{image}$ ) in the target cells co-cultured with the respective TCR-transduced T cells was calculated and normalized to the average of GFP total area ( $\mu\text{m}^2/\text{image}$ ) of the same target cells co-cultured with mock-transduced T cells (% of mock T cells).

**Immunoprecipitation and LC-MS/MS analysis of HLA class I bound peptides.**  $5 \times 10^6$  U87MG cells overexpressing either full-length cDNAs H3.3K27M or CDK4R24L were injected into NSG mice (EPO GmbH, Berlin). At a size of 1.5-2 cm<sup>3</sup> tumors were isolated and 500 mg tumor pieces were snap frozen in liquid nitrogen. Samples were dry cryopulverized immediately using the cryoPREP (Covaris) device. Next, quadruplicate samples of each cell line were lysed using 2xCHAPS buffer containing 400mM TrisCl pH 8.0, 300mM NaCl, 2%CHAPS (Serva) and 2x protease inhibitor (ThermoFisher Scientific) in HPLC grade ultrapure water. To avoid unspecific binding to the beads, the samples were incubated at 4°C for 1 hour with 50 $\mu$ l/ml ProteinA-Sepharose beads (Merck). Next, samples were incubated with 60 $\mu$ g/ml HLA binding antibody (W6/32, Biolegend) at 4°C for 1 hour. For immune-affinity purification, 100 $\mu$ l/ml ProteinA-Sepharose beads were added to the precleared and stained samples overnight at 4°C. Samples were washed three times with HPLC grade ultrapure water and eluted with 10% acetic acid. Acidified samples were desalted on stage tips<sup>9</sup>. A mix of heavy synthetic versions of the mutated peptides (CDK4R24L: 25 fmol; H3-3K27M: 5 fmol; source JPT Peptide Technologies) was spiked into each sample and injected into liquid chromatography coupled to mass spectrometry on an orbitrap Exploris 480 mass spectrometer (Thermo Fisher Scientific). A 44 min gradient was applied using an EASY-nLC 1200 system (Thermo Fisher Scientific) with an in-house packed column (C18-AQ 1.9  $\mu$ m

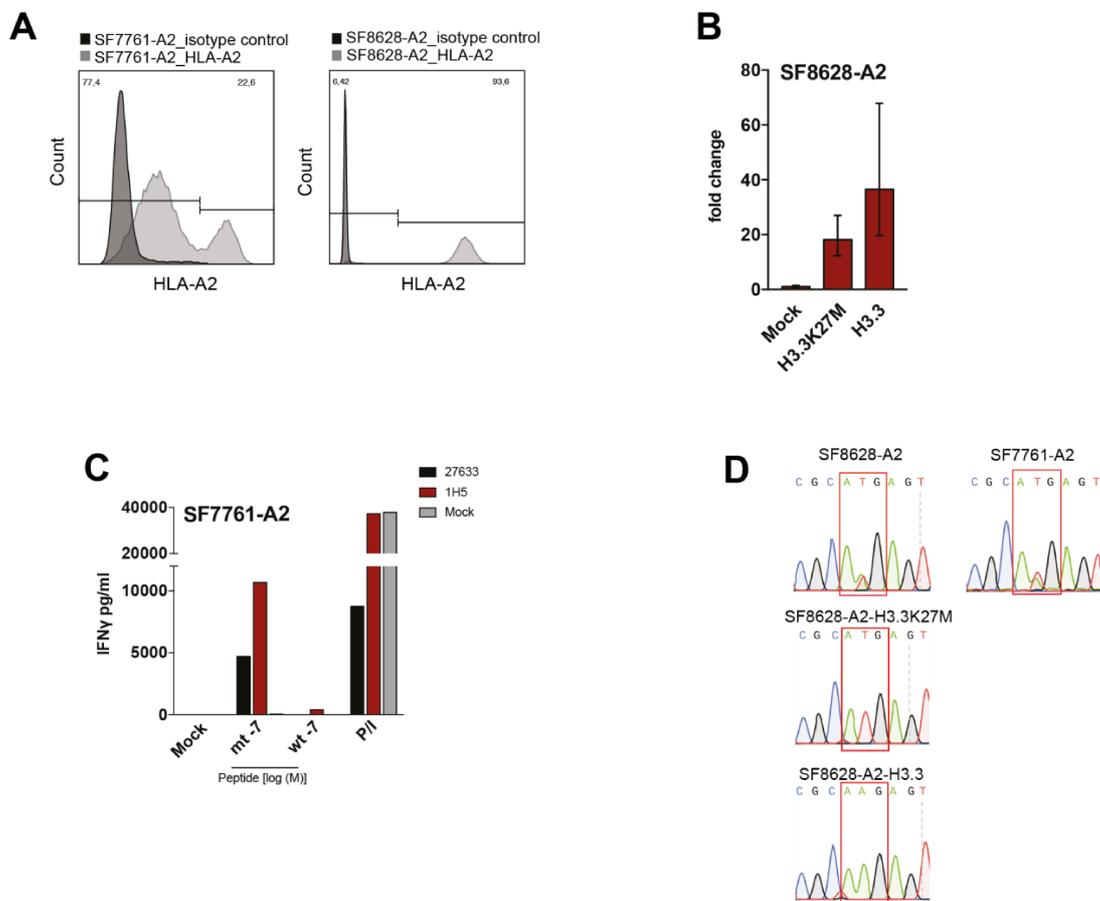
beads; Dr. Maisch Reprosil-Pur 120). MS1 resolution was set to 120'000, a MIPS peptide filter with relaxed restrictions was applied, the minimum intensity threshold was specified to 50'000, dynamic exclusion occurred for 20 s and charge states 1-5+ were allowed as precursors. A mixed mode of data-dependent MS2 scans and PRM was applied with an isolation width of 1.2 Th, an MS2 resolution of 15'000 and a maximum injection time of 100 ms. The cycle time was set to 3 s. For PRM scans, a resolution of 30'000 and a maximum injection time of 150 ms was chosen. The inclusion list contained the mutated H3.3K27M and CDK4R24L peptides in their heavy and light versions at charges 2 and 3 which were identified as most suitable in pre-experiments. For database search, MaxQuant version 2.0.3.0<sup>10</sup> was used. Peptide and site FDRs were specified to 1% while switching protein FDR off. The heavy amino acids of the mutated sequences were set as variable modifications (proline 6 and valine 6) along with oxidized methionine (H3.3K27M peptide) and acetylated protein N-termini. Digestion mode was set to unspecific with a maximum peptide length of 25 amino acids. Match-between runs was switched on. Andromeda search was done against a human Uniprot database (2021) plus the mutated CDK4R24L and H3.3K27M peptide sequences. Downstream analysis was done in R. Quantitation of the peptides of interest was done at MS2 level using the msms.txt table from the MaxQuant output. Fragment ions for the heavy and light versions of both sequences were used for quantitation. For the final quantitation, only charge 2 precursors of the mutated peptides were included for a more robust and consistent quantitation.

### Supplementary references

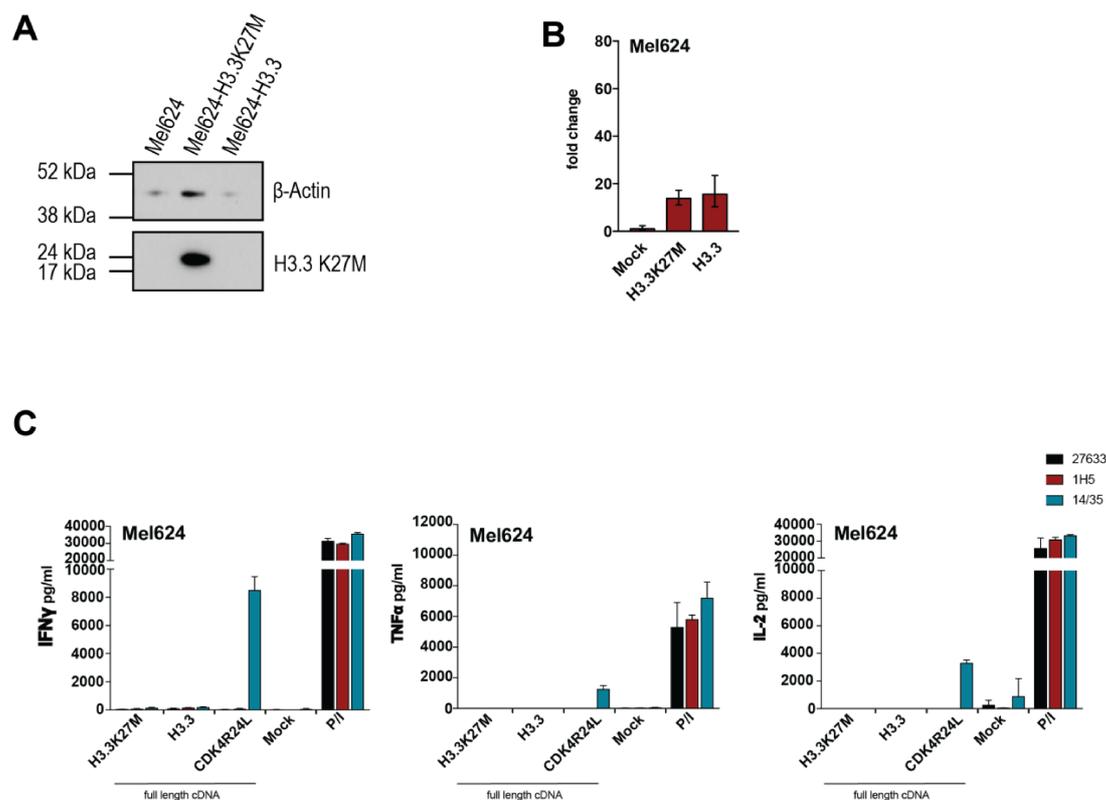
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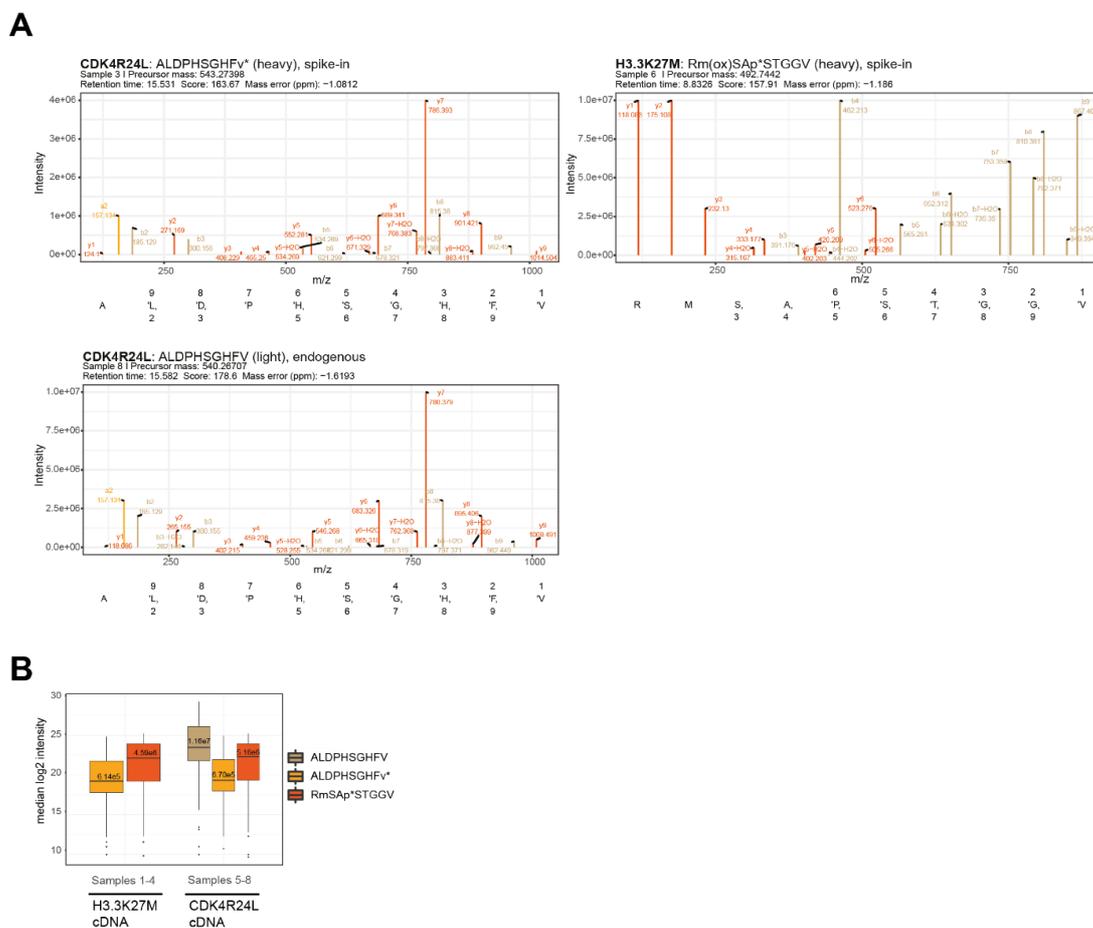
## Supplementary Figures



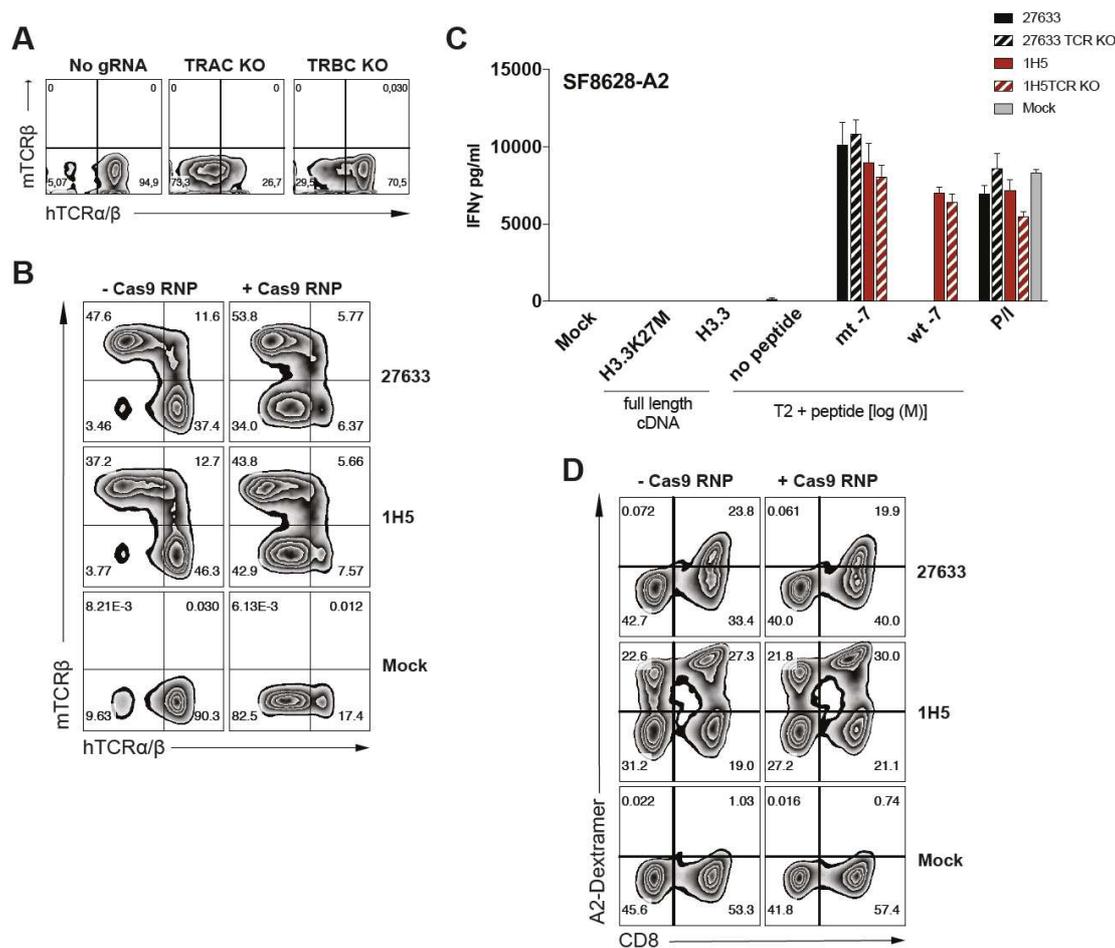
Supplementary Figure 1 **Mutant and wild type H3.3 overexpression profiles and failure of H3.3K27M TCR-transduced T cells to recognize DIPG cell line SF7761 naturally expressing mutant H3.3 histone.** (A) The glioma cells lines SF7761 and SF8628 were retrovirally transduced to express HLA-A\*02:01. Expression levels were determined by flow cytometry using a HLA-A\*02 specific antibody. (B) Relative quantitation of all H3.3 transcripts in SF8628\_A2 cells. Bars represent fold change of H3.3 expression, normalized to H3.3 expression in the parental cell line. Expression in parental cell line was arbitrarily set to 1. Error bars represent minimum and maximum relative level of gene expression. (C) Levels of IFN $\gamma$  secretion of 27633 and 1H5 TCR-transduced PBMCs, co-cultured with the human SF7761 glioma cell line, harboring a heterozygous copy of mutant H3.3 histone. The cells were retrovirally transduced to express HLA-A\*02:01 (SF7761-A2). A total of  $10^4$  CD8 $^+$  transduced T cells were co-cultured at an E:T ratio of 1:1. The experiment was performed twice and representative data are shown. (D) Presence of naturally expressed or overexpressed H3.3 wild type or H3.3K27M transcripts in SF8628-A2 (left panel) and SF7761-A2 (right panel) cells, was verified by RT-PCR and Sanger sequencing.



Supplementary Figure 2 **Lack of IFN $\gamma$ , TNF $\alpha$  and IL-2 production by H3.3K27M TCR-transduced T cells indicates failure to recognize Mel624 cells overexpressing the mutant H3.3K27M histone.** (A) H3.3K27M protein was detected by western blot. Top panel shows  $\beta$ -actin (45 kDa), lower panel shows H3.3K27M (17 kDa) in parental or transduced HLA-A\*02:01<sup>+</sup> Mel624 cells. (B) Relative quantitation of all H3.3 transcripts in Mel624 cells. Bars represent fold change of H3.3 expression, normalized to H3.3 expression in the parental cell line. Expression in parental cell line was arbitrarily set to 1. Error bars represent minimum and maximum relative level of gene expression. (C) Levels of IFN $\gamma$ , TNF $\alpha$  and IL-2 secretion of 27633 and 1H5 TCR-transduced PBMCs, co-cultured with the human Mel624 melanoma cell line retrovirally transduced to express either H3.3K27M full length cDNA, H3.3 wild type full length cDNA or CDK4R24L full length cDNA. A total of 10<sup>4</sup> CD8<sup>+</sup> transduced cells were co-cultured at a 1:1 E:T ratio. A CDK4R24L-specific TCR (14/35) served as positive control for functional antigen processing and presenting machinery in Mel624 cells. P/I represents maximum IFN $\gamma$  secretion from PBMCs stimulated with PMA/Ionomycin. Graphs represent means of triplicate cultures  $\pm$ SD.



Supplementary Figure 3 **The mutated CDK4 peptide, but not mutated H3.3 peptide is detectable in U87MG glioblastoma cells overexpressing the respective cDNAs.** Samples 1-4 recombinantly express H3.3K27M, samples 5-8 express CDK4R24L. **(A)** Representative annotated MS2 spectra generated from the MaxQuant output for the mutated CDK4 and H3.3 heavy synthetic peptides (top panel) and the endogenous CDK4R24L counterpart eluted from HLA-A\*02:01<sup>+</sup>/CDK4R24L<sup>+</sup> cells after W6/32 HLA pulldowns (bottom panel). Endogenous mutant H3.3 peptide could not be detected in any of the samples. Peaks represent b ions in ochre and y ions in red. **(B)** Boxplot of the MS2 intensities by group.



Supplementary Figure 4 **CRISPR/Cas9 knockout of endogenous human TCR does not lead to recognition of the H3.3K27M mutation.** (A) Expression of transduced TCR (mTCR $\beta$ , y-axis) and endogenous TCR (hTCR $\alpha/\beta$ , x-axis) after knockout of either endogenous TCR alpha or beta chain with CRISPR/Cas9 in mock-transduced T cells. S.p.HiFiCas9 V3 enzyme and gRNAs targeting human TRAC or TRBC loci were complexed and nucleofected into T cells. Endogenous (hTCR $\alpha/\beta$ ) and therapeutic (mTCR $\beta$ ) TCR expression was assessed by flow cytometry after 4 days in culture on 10 IU/ml IL-2, cells were gated on CD3<sup>+</sup>. (B) 27633-, 1H5- and mock-transduced T cells were nucleofected with sgRNAs and Cas9 protein (right panel) or without Cas9 (left panel) targeting the endogenous human TRAC and TRBC locus together. Endogenous (hTCR $\alpha/\beta$ ) and therapeutic (mTCR $\beta$ ) TCR expression was assessed by flow cytometry after 4 days in culture on 10 IU/ml IL-2, cells were gated on CD3<sup>+</sup>. Even though the proportion of hTCR $\alpha/\beta$ <sup>+</sup>mTCR<sup>+</sup> double positive cells in the therapeutic TCR-transduced PBMCs is quite low to begin with, probably due to stronger expression and preferential pairing of transduced TCR chains, the successful knockout is also reflected in the hTCR $\alpha/\beta$ <sup>+</sup>mTCR<sup>-</sup> populations. (C) Cells described in (B) were co-cultured with SF8628-A2, SF8628-A2-H3.3K27M, SF8628-A2-H3.3 wild type or with peptide-loaded T2 cells (10<sup>-7</sup> M mutant or wt peptide, respectively). Graphs represent mean IFN $\gamma$  secretion of triplicate cultures  $\pm$ SD. (D) Staining of the TCRs with H3.3K27M dextramer (y-axis) and CD8 (x-axis) after knockout of human TCR alpha and beta chains with CRISPR/Cas9. FACS staining was performed after 4 days in culture on 10IU/ml IL-2, cells were gated on CD3<sup>+</sup>.

Supplementary Table 1 **ERAP1 allotype identity in cell lines.**

	ERAP 1 polymorphisms								
Cell line	E56K	R127P	G346D	M349V	K528R	D575N	R725Q	Q730E	Allotype
<b>Mel21a</b>	E	R	<b>D</b>	M	<b>R</b>	D	R	<b>E</b>	*019
	E	R	<b>D</b>	M	<b>R</b>	D	R	<b>E</b>	*019
<b>Mel624</b>	E	R	G	M	K	D	R	<b>E</b>	*018
	E	R	G	M	K	D	R	<b>E</b>	*018
<b>SF8628</b>	E	R	G	M	K	D	R	Q	*002
	<b>K</b>	<b>P</b>	G	M	<b>R</b>	D	R	<b>E</b>	*014
<b>U87MG</b>	E	<b>P</b>	G	M	K	D	R	Q	*013
	E	<b>P</b>	G	M	<b>R</b>	D	R	<b>E</b>	*015

Bold type shows variants at the indicated amino acid position.