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In-depth characterization of vaccine-induced neoantigen-specific T cells in patients with IDH1-mutant glioma undergoing personalized peptide vaccination

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ABSTRACT

Isocitrate dehydrogenase (IDH) mutant glioma is a malignant primary brain tumor diagnosed in adults. In recent years, there has been significant progress in understanding the molecular pathogenesis and biology of these tumors. The first targeted IDH-inhibitor was approved by the US Food and Drug Administration in August 2024 for grade 2 gliomas, in light of results of a phase III trial which showed significant advantages in progression-free survival. However, biologic therapy is not curative, and subsequent treatment options offer only limited clinical benefit and often result in long-term toxicities. In addition, targeted treatment options for grade 3 and grade 4 IDH-mutant gliomas are still missing. In this study, we present n=52 patients with glioma (grade 2, 3 and 4) with confirmed IDH1 mutation (mutIDH1) in the newly diagnosed and recurrent setting who, in addition to standard-of-care, received a personalized neoantigen-targeting peptide vaccine. Each tumor was initially analyzed for somatic mutations by whole exome sequencing, and a peptide vaccine containing potential neoepitopes was designed, manufactured and vaccinated. Each vaccine consisted of peptides derived from numerous somatic mutations, including at least one peptide targeting the mutIDH1.

Vaccine immunogenicity was determined by intracellular cytokine staining and simultaneous measurement of four T-cell activation markers (Interferon-γ, Tumor Necrosis Factor, Interleukin-2, CD154) after 12-day in vitro expansion of pre and post vaccination peripheral blood mononuclear cells. Extracellular CD154 staining was used to sort mutIDH1-specific CD4+T cells.

Immunomonitoring revealed that the vaccines were immunogenic and induced mainly CD4 but also CD8 T cell responses. Vaccine-induced immune responses were robust and polyfunctional. Immunogenicity against mutIDH1 was high (89%). We implemented an assay which allowed us to isolate functional antigen-specific CD4+T cells in an HLA-independent manner. Subsequent T cell receptor (TCR) repertoire sequencing revealed that CD4+T cells reacting on mutIDH1 stimulation were polyclonal. Strikingly, we detected two mutIDH1-specific TCRβ candidate sequences in three different patients. These three patients had the same human leukocyte antigen (HLA) DQA-DQB alleles. The obtained TCRβ sequences could be tracked in autologous ex-vivo single-cell transcriptomic data. Our results provide a rationale for pursuing vaccination and T cell transfer strategies targeting IDH1. Furthermore, our findings indicate that

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ The eponymous isocitrate dehydrogenase (IDH) mutation represents an ideal therapeutic target for inhibitors and vaccination strategies. IDH1 mutation (mutIDH1)-derived peptides have shown to be highly immunogenic in clinical trials.

WHAT THIS STUDY ADDS

⇒ We comprehensively characterized the phenotype and functional cytokine profile of vaccine-induced T cell responses directed against mutIDH1. We are furthermore providing T cell receptor (TCR)β CDR3 sequences, including one full-length alpha and beta chain TCR sequence, of mutIDH1-reacting T cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ More publicly available data of paired alpha and beta chain sequences of neoantigen-recognizing TCRs will improve prediction algorithms for TCR-peptide-human leukocyte antigen (HLA) interactions. Such tools have great potential to improve antigen discovery and neoantigen vaccine design.

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personalized neoantigen-targeting vaccines might be considered for the treatment of IDH1-mutant gliomas.

INTRODUCTION

Gliomas are the most common primary malignant brain tumors in adults.[1] In the past, glioma subclasses had been solely determined based on histological subtype and grade.[2] In 2008, the discovery of mutations in isocitrate dehydrogenase (IDH) 1 in a subset of patients with glioblastoma multiforme initiated the systematic molecular classification of gliomas.[3] IDHs are essential metabolic enzymes involved in several metabolic processes, including the tricarboxylic acid (Krebs) cycle.[4] Significant progress in understanding the molecular pathogenesis and biology of these tumors finally led to the incorporation of the IDH-mutation status in the classification of diffusely infiltrating gliomas in the 2016 WHO report of central nervous system tumors.[5] The 2021 WHO classification includes two adult-type diffuse IDH-mutant gliomas, namely oligodendrogliomas and astrocytomas.[6] Among all IDH-mutant gliomas, most tumors have a heterozygous point mutation in *IDH1* leading to an arginine-to-histidine substitution at amino acid 132 (gain-of-function).[7] The resulting oncometabolite D-2HG of the mutated IDH1 (mutIDH1) induces a cold, immunosuppressive microenvironment that can antagonize the ability of T cells to mount an antitumor response.[8]

Standard of care therapy for IDH-mutant gliomas is maximal safe resection (if feasible) in order to reduce symptoms and collect tissue for molecular and histopathological analysis. Until recently, accepted practice included "watch and wait" following maximal resection. In tumors with significant residual or subsequent progression or in elderly patients, standard adjuvant treatments include a combination of radiation and chemotherapy.[9] The approval of vorasidenib by the US Food and Drug Administration, based on the significantly positive results of the phase III "Indigo" trial, has changed the treatment paradigm for grade 2 IDH-mutant glioma.[10] The biologically targeted agent can slow tumor growth and even lead to partial response, thereby potentially deferring the need for repeated surgery or chemo-radiation.

Although patients with grade 2 IDH-mutant gliomas have a significantly prolonged overall survival compared with patients with grade 3 and grade 4 IDH-wildtype gliomas (including glioblastoma), curative therapies are missing.[11] Furthermore, current treatment strategies offer only limited clinical improvements for the patient and may result in long-term toxicities including cognitive decline, indicating that there is an urgent need for advanced therapeutic options. At the same time, the eponymous mutIDH1 represents an optimal therapeutic target for therapies beyond the usage of IDH inhibitors like vorasidenib.[12] Certainly, IDH1 mutations also give rise to potential immunotherapy targets as tumor-specific neoantigen.[13] Several vaccines targeting mutIDH1 have been studied (NCT02454634, NCT02193347, NCT02771301). One of these trials demonstrated promising results using a peptide vaccine specific for the IDH1 mutation. 33 patients with newly diagnosed IDH1-mutant astrocytoma (grades 3 and 4) were vaccinated with an IDH1-R132H 20-mer peptide. Vaccine-induced T cell responses against mutant IDH1 were observed in 26 of 30 patients.[14]

In this study, we present immunogenicity data of a personalized neoantigen-derived multipeptide vaccine approach among patients with IDH1-mutant glioma administered in conjunction with established therapies. Each peptide vaccine was uniquely tailored to each individual's tumor and consisted of peptides derived from numerous tumor-specific mutations and contained at least one peptide targeting the mutIDH1 present within each tumor. The aim of this study was to characterize the phenotype and function of vaccine-induced T cell responses directed against mutIDH1. Here, we report on the vaccine-induced immune responses achieved among these patients.

MATERIAL AND METHODS

Study design

The study cohort includes patients with WHO grade 2–4 glioma with confirmed mutIDH1 who underwent guideline therapies in either the newly diagnosed or recurrent setting and additionally received a personalized peptide vaccine in the scope of an individual complementary healing attempt at the MVZ für Diagnostik, Prävention, Onkologie und Gastroenterologie Tübingen GmbH. All patients provided informed consent for personalized therapy.

Immune monitoring performed before and during vaccination was used to identify vaccine-induced neoantigen-specific T cells. Only patients who received at least seven vaccinations and had immune monitoring data available were included in the study. All patients provided additional informed consent for retrospective analysis. The patients did not receive any compensation.

Neoepitope prediction and peptide selection

Tumor material from the most recent surgical intervention was used for whole exome and transcriptome analysis and subsequent peptide vaccine design whenever possible. Neoepitope prediction and peptide selection were done as previously described.[15] Briefly, somatic variants were phased, and the resulting coding sequences were translated into the local amino acid sequence context to generate epitope candidates. The patient's Human leukocyte antigen (HLA) type was identified using the OptiType algorithm, and HLA binding affinity for potential neoepitopes was predicted.[16]

Potential HLA class I neoepitopes (peptide length between 8 and 12 amino acids) with high predicted binding affinity (using NetMHCpan, NetMHC, and SYFPEITHI), high variant allele frequency and confirmed expression of the mutant allele were selected. When possible, predicted neoepitopes for all patient HLA

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class I molecules were selected. Furthermore, peptides that potentially bind to multiple HLA class I molecules of the patient were preferred. In addition, possible HLA class II epitopes with high variant allele frequency and high expression were selected (peptide length preferably 17 amino acids). Expression of tumor mutations was confirmed in the patient's tumor transcriptome data or, if such data was lacking, expression of respective proteins in human gliomas was manually checked in the Human Protein Atlas database (https://www.proteinatlas.org/) and integrated into the peptide selection process.

We aimed to vaccinate 20 peptides per patient, consisting preferably of n=10 HLA class I and n=10 HLA class II peptides. However, for some patients fewer peptides could be selected due to reduced presence of mutations, predicted HLA binders, or synthesizable peptides.

Vaccination

Peptides were synthesized by solid-phase peptide synthesis and purified to at least 95% purity (synthesized by Intavis Peptide Services GmbH, Tübingen, Germany). Lyophilized peptides (HCl salt) were dissolved in water (Aqua ad iniectabilia; BBraun, Melsungen, Germany) + 33% dimethylsulfoxide (Miltenyi, Bergisch Gladbach, Germany). Peptides were mixed and sterile-filtered through a PTFE-membrane filter (Millex-LG sterile filter; Millipore) and bottled in glass vials (Thermo Scientific). The resulting peptide vaccine cocktails were controlled for identity and purity of contained peptides as well as sterility and absence of endotoxins followed by quality control/quality assurance release. Vaccine vials were stored at −80°C.

Per vaccination, 0.5 mL multipeptide solution (0.8 mg/mL per peptide) was injected intracutaneously in the left or right lower abdomen followed by subcutaneous injection of 83 µg sargramostim and/or superficial application of imiquimod (50 mg/g creme) in the same area. Patients were vaccinated four times in the first week followed by monthly boosters.

Detection of vaccine-induced T cell responses

To monitor vaccine-induced, neoantigen-specific T cells, 80 mL whole blood was routinely drawn before the first vaccination and before the seventh vaccination (3 months after the first vaccination). Detection of neoantigen-specific T cells was performed as previously described.[17] This assay primarily detects functional, non-anergic memory T cells in an HLA-independent manner.[18] Briefly, peripheral blood mononuclear cells (PBMCs) were isolated from whole blood using density gradient centrifugation and were cryopreserved in MACS Freezing Solution (Miltenyi Biotec; Bergisch-Gladbach) until further usage. After thawing, PBMCs were stimulated with peptides (1 µg/mL for HLA class I peptides and 5 µg/mL for HLA class II peptides) either separately or in pools of two to five peptides. Pools were formed in case cell numbers were too low to allow stimulation with all peptides individually, but HLA class I and II peptides were never combined in pools (see online supplemental methods). Cells were cultivated in the presence of Interleukin (IL) -2 (10 U/mL; Miltenyi Biotec, Bergisch-Gladbach, Germany) and IL-7 (10 ng/mL; Miltenyi Biotec). After 12 days of cultivation, expanded cells were or were not (mock-stimulated negative control; NC) restimulated with corresponding peptides at the same concentration and additionally incubated for 14 hours in the presence of Golgi inhibitors (1 µl/mL; Golgi Plug, BD Biosciences, Franklin Lakes, New Jersey, USA).

The readout was Flow Cytometric Analysis after Intracellular Cytokine Staining. After cultivation, cells were washed and stained extracellularly and intracellularly using fluorochrome-conjugated antibodies titrated to their optimal concentrations (see online supplemental material). Finally, cells were measured on a Novocyte 3005R cytometer (Agilent, Santa Clara, California, USA).

Data were analyzed using FlowJo V.10.5.3 (FlowJo, Ashland, Oregon, USA). A detailed gating strategy can be found in the supplement (see online supplemental figure 1). Briefly, CD4+and CD8+ T cells were gated within viable CD3+lymphocytes and analyzed separately for each functional marker (CD154, Interferon (IFN)-γ, Tumor necrosis factor (TNF), and IL-2). Peptide-specific responses were evaluated using the stimulation index (SI). The SI is the calculated ratio of polyfunctional activated CD4+or CD8+ T cells (positive for at least two markers of CD154, IFN-γ, TNF-α, and/or IL-2) in the peptide-stimulated sample to the mock-stimulated sample (NC). Cells stimulated with an antibody-based nonspecific stimulus (10 µl/mL; human CytoStim, Miltenyi Biotec) served as positive control. Neoantigen-specific T cells were defined as being present for SI≥2.

Fluorescence-activated cell sorting of mutIDH1-specific T cells

Cryopreserved PBMCs from patients with confirmed presence of mutIDH1-specific CD4+T cells were thawed and stimulated with mutIDH1-derived HLA class II peptides (5 µg/mL). Cells were cultivated in the presence of IL-2 (10 U/mL; Miltenyi Biotec, Bergisch-Gladbach, Germany) and IL-7 (10 ng/mL; Miltenyi Biotec). After 12 days of cultivation, expanded cells were either restimulated with the corresponding peptides at the same concentration and additionally incubated for 14 hours in the presence of 1 µg/mL anti-CD40 antibody (pure functional grade; Miltenyi Biotec) or left without restimulation.

After cultivation, restimulated cells were washed and stained extracellularly using fluorochrome-conjugated antibodies titrated to their optimal concentrations (see online supplemental methods). Finally, functional, neoantigen-specific T cells were sorted on a MACSQuant Tyto Cell Sorter (Miltenyi Biotec) using the following gating strategy: CD154+CD4+ T cells were gated within viable CD3+lymphocytes. These cells were collected in the positive collection chamber (positive fraction). All other cells were cumulated in the negative collection chamber (negative fraction) (for details, see online supplemental

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figure 2). Sorted and expanded but not restimulated cells (expanded fraction) were pelleted, supernatant was removed, and the pellet was stored at −20° until further usage.

DNA isolation

DNA from cell pellets was isolated using the QIAamp DNA Micro Kit (Qiagen) according to the instruction manual.

T cell receptor sequencing

T cell receptor (TCR) sequencing libraries were prepared from 100 to 250 ng DNA of each fraction using the AmpliSeq for Illumina TCR beta-SR Panel Kit (Illumina) according to the instructions given by the manufacturer. Resulting libraries were quality controlled using Qubit (Thermo Fisher Scientific) and Fragment Analyzer (Agilent).

All libraries were sequenced on the Illumina NovaSeq 6000 system with a read length of 2×100 bp. Demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.20). Adapters were trimmed with Skewer (V.0.2.2).[19] Quality trimming of the reads was not performed. The FASTQ files were downsampled to 2 million read pairs before TCR analysis. Overlapping paired reads were merged into single reads before reconstruction of the CDR3β regions using NGmerge.[20] Only read pairs that could be successfully merged into a single read were kept for further analysis. Reconstruction of TCR sequences was performed using RTCR.[21] Nonfunctional clones (alternative reading frames, premature stop codons) were discarded. All TCR clones were annotated with the corresponding read count and frequency (proportion of reads that belong to each clone).

We first excluded all TCR clones that were not present in all fractions. TCR clones that were more frequent in the negative fraction compared with the positive fraction (or expanded fraction), were also removed. We finally selected the top five clones from the positive fraction (or expanded fraction).

Single-cell sequencing and analysis

For single-cell sequencing, cryopreserved PBMC from one patient were thawed, washed and resuspended in phosphate-buffered saline (pH 7.4) with 0.1% bovine serum albumin following recommended steps from the 10x guideline to form a single-cell solution (CG000447 Handbook Cell Thawing Protocols Single-cell Assays Rev B). Cell viability and cell numbers were assessed using acridine orange/propidium iodide staining in combination with an automated cell counter (Cellaca MX, Cenibra GmbH, Germany). The single-cell solution was loaded onto four separate wells of a Chromium GEM-X Single-cell 5' chip (10x Genomics), each with a target cell recovery of 20,000 cells (targeting 80,000 cells in total). Library preparation was performed using the GEM-X Universal 5' Gene Expression v3 kit (10x Genomics) according to manufacturer's protocol. TCR V(D)J segments were amplified from cDNA using the Chromium Single-cell Human TCR Amplification kit (10x Genomics). The resulting libraries were sequenced on a NovaSeq 6000 using the cycle numbers recommended by 10x Genomics. Demultiplexing of sequencing data, barcode processing, cell and V(D)J sequence calling were performed with Cell Ranger V.9.0.0 (10x Genomics). For downstream single-cell and TCR analysis, combined data was processed with the R packages Seurat V.5.2.1 and scRepertoire V.2.2.1.[22 23] Here, cell clusters were calculated via principal component analysis and unsupervised clustering. Cell type annotation was performed with the local Seurat built-in application Azimuth and the reference dataset published by Hao *et al*.[24] For visualization of annotated cell clusters, de novo Uniform manifold approximation and projection (UMAP) plots were calculated. Regarding TCR analysis, scRepertoire processing V(D)J sequencing data and implementing them into the single-cell object was performed as described in the vignettes of Borcherding *et al* (https://www.borch.dev/uploads/screpertoire/). After the integration of single-cell and TCR data, the amino acid sequences of the most abundant TCR clones, which were identified via mutIDH1-specific immunogenicity assay, were used to highlight the specific clones within the single-cell data.

RESULTS

Patient characteristics

52 patients with IDH-mutant glioma received a personalized neoantigen-derived peptide vaccine between May 2016 and July 2024. Detailed patient characteristics are outlined in table 1 and in online supplemental table 1). Median time from first diagnosis to first vaccination was 15.6 months (range: 5.8–150.1). 24 patients (46%) did not progress before the first vaccination (primary), and 28 patients (54%) were treated after progression (recurrent).

Out of the 52 patients, 14 patients (27%) had WHO grade 2 glioma, 17 patients (33%) had grade 3 glioma, and 20 patients (38%) had grade 4 glioma before receiving the first vaccination. WHO grade was not available for one patient.

45 of 52 patients displayed an IDH1-R132H mutation (87%), 3 patients an R132S mutation (6%), 2 patients an R132G mutation (4%), and 2 patients an R132C mutation (4%).

At the time of first vaccination, 46 patients (88%) had received standard of care treatment with radiation therapy and temozolomide (TMZ) chemotherapy. Two patients had received TMZ only. Four patients did not have treatment data available. Furthermore, 16 patients were treated with glucocorticoids including dexamethasone for which timing and dosing were not comprehensively recorded.

At data cut-off (August 1, 2024), 44 of 52 patients were alive (85%), median observation time from initial diagnosis to date of last follow-up or death was 36 months

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Table 1 Patient characteristics

ID	Glioma subtype	Grade	IDH1 variant	Received TMZ (1=yes; 0=no)	Received radiation (1=yes; 0=no)	Time from first diagnosis to first vaccination (months)	Observation time from first diagnosis to date of last follow-up/death (months)	Status at cut-off date
1	astro	2	p.R132S	1	1	45	144	Alive
2	astro	2	p.R132H	1	0	147	150	Dead
3	astro	4	p.R132H	1	1	6	69	Alive
4	astro	2	p.R132H	1	1	116	170	Alive
5	astro	4	p.R132H	1	1	18	65	Alive
6	astro	3	p.R132G	1	1	66	78	Dead
7	astro	4	p.R132H	1	1	13	22	Dead
8	astro	3	p.R132C	1	1	15	47	Alive
9	oligo	2	p.R132H	NA	NA	150	185	Alive
10	astro	3	p.R132H	NA	NA	44	77	Alive
11	astro	4	p.R132H	1	1	8	24	Dead
12	astro	3	p.R132H	1	1	19	51	Alive
13	astro	3	p.R132H	1	1	7	39	Alive
14	astro	4	p.R132H	1	1	7	36	Alive
15	astro	3	p.R132H	1	1	59	71	Dead
16	astro	2	p.R132G	NA	NA	56	77	Alive
17	astro	3	p.R132H	1	1	9	36	Alive
18	astro	3	p.R132C	1	1	14	35	Alive
19	oligo	3	p.R132H	1	1	34	58	Alive
20	astro	4	p.R132H	1	1	11	36	Alive
21	astro	4	p.R132H	1	1	16	42	Alive
22	astro	4	p.R132H	1	1	13	31	Dead
23	astro	2	p.R132H	1	1	13	34	Alive
24	astro	2	p.R132H	1	1	104	119	Alive
25	astro	3	p.R132S	1	1	13	27	Alive
26	astro	3	p.R132H	1	1	19	27	Alive
27	astro	4	p.R132H	1	1	12	31	Alive
28	astro	3	p.R132H	1	1	22	41	Alive
29	astro	4	p.R132H	1	1	11	28	Alive
30	astro	4	p.R132H	1	1	10	25	Alive
31	astro	3	p.R132H	1	1	11	27	Alive
32	astro	3	p.R132H	1	1	16	29	Alive
33	astro	4	p.R132H	1	1	10	24	Alive
34	astro	4	p.R132H	1	1	24	39	Alive
35	oligo	2	p.R132H	1	1	17	32	Alive
36	astro	2	p.R132H	1	0	12	24	Alive
37	astro	2	p.R132H	1	1	41	57	Alive
38	astro	4	p.R132H	1	1	13	27	Alive
39	astro	2	p.R132H	1	1	120	125	Dead
40	astro	NA	p.R132H	1	NA	127	139	Alive
41	astro	4	p.R132H	1	1	16	27	Dead

Continued

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Table 1 Continued

ID	Glioma subtype	Grade	IDH1 variant	Received TMZ (1=yes; 0=no)	Received radiation (1=yes; 0=no)	Time from first diagnosis to first vaccination (months)	Observation time from first diagnosis to date of last follow-up/death (months)	Status at cut-off date
42	astro	4	p.R132H	1	1	15	27	Alive
43	astro	2	p.R132H	1	1	31	38	Alive
44	astro	4	p.R132H	1	1	11	19	Alive
45	astro	3	p.R132S	1	1	13	24	Alive
46	astro	3	p.R132H	1	1	16	20	Alive
47	astro	2	p.R132H	1	1	87	99	Alive
48	astro	2	p.R132H	1	1	38	44	Alive
49	astro	4	p.R132H	1	1	12	19	Alive
50	astro	4	p.R132H	1	1	11	18	Alive
51	astro	3	p.R132H	1	1	29	36	Alive
52	astro	4	p.R132H	1	1	9	15	Alive

astro, astrocytoma; IDH1, isocitrate dehydrogenase 1; NA, not available; oligo, oligodendroglioma; TMZ, temozolomide.

(range 14–185 months, mean 53 months) (online supplemental table 1).

Vaccine

The median number of peptides that were vaccinated per patients was 20 (range 6–21); including a median of 9 short peptides predicted to bind HLA class I (range 3–11) and a median of 9 long peptides presumed to bind HLA class II molecules (range 1–12). At the cut-off date, patients had received between 7 and 42 vaccinations (median 11).

Vaccines of all n=52 patients included one long peptide targeting the mutIDH1. The five different long HLA class II mutIDH1 peptides were: VKPIIIGSHAYGDQY (R132S; vaccinated in one patient); WVKPIIIGCHAYGDQYR (R132C; vaccinated in two patients); WVKPIIIGGHAYGDQYR (R132G; vaccinated in two patients); WVKPIIIGSHAYGDQYR (R132S; vaccinated in two patients), WVKPIIIGHHAYGDQYR (R132H; vaccinated in 45 patients) (table 2 and online supplemental table 1).

The vaccines of n=29 patients contained at least one additional short mutIDH1-derived peptide predicted to bind at least one of the corresponding patients' HLA class I allele. The 10 different short HLA class I mutIDH1-derived peptides were: GSHAYGDQY (R132S; vaccinated in one patient); HHAYGDQYR (R132H; vaccinated in three patients); IIGHHAYGDQYR (R132H; vaccinated in one patient); IIIGHHAY (R132H; vaccinated in three patients); KPIIIGCHA (R132C; vaccinated in one patient); KPIIIGCHAY (R132C; vaccinated in one patient); KPIIGGHA (R132G; vaccinated in two patients); KPIIIGHHA (R132H; vaccinated in seven patients); KPIIIGHHAY (R132H; vaccinated in six patients); PIIIGHHAY (R132H; vaccinated in four patients) (table 2 and online supplemental table 1).

General immunogenicity

Among 20 vaccinated peptides per patient (median), each patient developed at least CD4+T cell responses against 5.8 peptides (median; range: 0–11), and CD8+T cell responses against three peptides (median; range 0–8). One patient did not develop any immune responses against the vaccinated peptides (patient 44). For a detailed list of all induced immune responses in all patients, see figure 1 and online supplemental table 1). Based on the above-mentioned median amount of vaccine-induced immune responses, we dichotomized patients into immunological responder (iR) and non-responder (iNR). Notably, we found that iR seemed to have a significantly higher overall survival than iNR although median survival time was not yet reached (HR 0.09 (0.01–0.74); p=0.0057; online supplemental figure 3 and table 1).

Immunogenicity against mutIDH1-derived peptides

Among n=52 patients that received the mutIDH1 HLA class II peptide, n=47 patients had single peptide immune monitoring data available. Two patients showed a CD8+T cell response (4%), 25 patients showed a CD4+T cell response (53%), and 15 patients showed a combined CD4+and CD8+ T cell response (32%). Five patients did not show vaccine-induced immune responses against their mutIDH1 HLA class II peptide (11%) (figure 2).

Among n=29 patients who received at least one HLA class I mutIDH1 peptide, n=18 patients had single peptide immune monitoring data available for this peptide.

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Table 2 List of all vaccinated, class I and class II mutIDH1 peptides

AA sequence	HLA	p.R132C	p.R132G	p.R132H	p.R132S
GSHAYGDQY	A*01:01, B*57:01				1 (0)
HHAYGDQYR	A*68:01			3 (0)	
IIGHHAYGDQYR	A*29:02			1 (0)	
IIIGHHAY	A*29:02, A*26:01			3 (0)	
KPIIIGCHA	B*55:01	1 (0)			
KPIIIGCHAY	B*35:01	1 (0)			
KPIIIGGHA	B*07:02		2 (0)		
KPIIIGHHA	B*07:02			7 (0)	
KPIIIGHHAY	A*29:02			6 (0)	
PIIIGHHAY	A*25:01, A*29:02, B*15:01			4 (0)	
VKPIIIGSHAYGDQY*	class II				1 (0)
WVKPIIIGCHAYGDQYR	class II	2 (2)			
WVKPIIIGGHAYGDQYR	class II		2 (0)		
WVKPIIIGHHAYGDQYR	class II			45 (10)	
WVKPIIIGSHAYGDQYR	class II				2 (0)

(x)=number of patients with TCR sequencing data available.
*This peptide was designed in 2016 using the first version of our prediction pipeline, resulting in 15 amino acid long putative HLA class II binders.
AA, amino acid; HLA, Human leukocyte antigen; mutIDH1, mutated isocitrate dehydrogenase 1; TCR, T cell receptor.

Five patients showed a CD8+T cell response (28%), and two patients showed a combined CD4+and CD8+ T cell response (11%). 11 patients did not show vaccine-induced immune responses against HLA class I mutIDH1 peptides (62%) (figure 2). Two of those patients (patient 22 and 42) did not show vaccine-induced immune responses against either HLA class I or HLA class II mutIDH1 peptides.

Multicytokine profile of mutIDH1-specific T cells

The use of multicolor flow cytometry allowed us to investigate the release/expression of four functional markers at a single-cell level within neoantigen-specific T cells. Boolean analysis of four parameters on the two T cell populations, that is, CD8+and CD4+ T cells, resulted in 16 different functional populations, each with a distinct cytokine/activation marker profile. Quadruple-negative cells, that is, cells that do not express cytokine/activation markers were not considered for further analysis. For CD4 responses against the HLA class II mutIDH1 peptide, the most frequent marker combination (median) was CD154+IFN-γ− IL-2− TNF+ (2.95% of all CD4+T cells) followed by CD154− IFN-γ− IL-2− TNF+ (1.44%) and CD154+IFN-γ−IL-2+TNF+ (1.40%). Hierarchical clustering of square root transformed values resulted in a cluster of double-positive CD154+IFN-γ− IL-2− TNF+and triple-positive CD154+IFN-γ−IL-2+TNF+ T cell populations (figure 3).

Hierarchical clustering of CD4 immune responses against mutIDH1 resulted in two distinct patient groups of approximately the same size (cluster 1=multicytokine low, upper half; cluster 2=multicytokine high, lower half) (figure 3). We compared glioma grade, mutIDH1, ATRX mutation, TP53 mutation, recurrence before start of vaccine treatment, receipt of an HLA class I peptide targeting the same mutIDH1, and general responsiveness against the whole vaccine as well as overall survival in both clusters but could not find statistically significant differences between both groups (figure 3 and online supplemental figure 4).

For CD8 responses against the HLA class II mutIDH1 peptide, the most frequent cytokine combination (median) was CD154− IFN-γ− IL-2− TNF+ (0.23% of all CD8+T cells) followed by CD154− IFN-γ+ IL-2− TNF+ (0.08%) (see online supplemental figure 5).

T cell receptor sequencing of mutIDH1-specific T cells

In order to investigate clonality of mutIDH1-specific T cells, we next sequenced the CDR3 region of the TCR beta chain. We focused on CD4+mutIDH1-specific T cells because they were more frequent. Multicytokine profiling of mutIDH1-specific CD4+T cells confirmed the dominant expression of CD154 in vaccine-induced T cells. We therefore used this marker for sorting of functional neoantigen-specific T-cells in an HLA-independent manner.

For n=12 patients with confirmed presence of mutIDH1-specific CD4+T cells, an additional PBMC sample was available for sorting (including 10 patients with R132H mutation and 2 patients with R132C mutation).

As expected for antigen-specific CD4+T cells, we identified multiple mutIDH1-recognizing TCRβ clones per patient. Table 3 summarizes the maximum top five identified TCRs expected to recognize mutIDH1. Interestingly,

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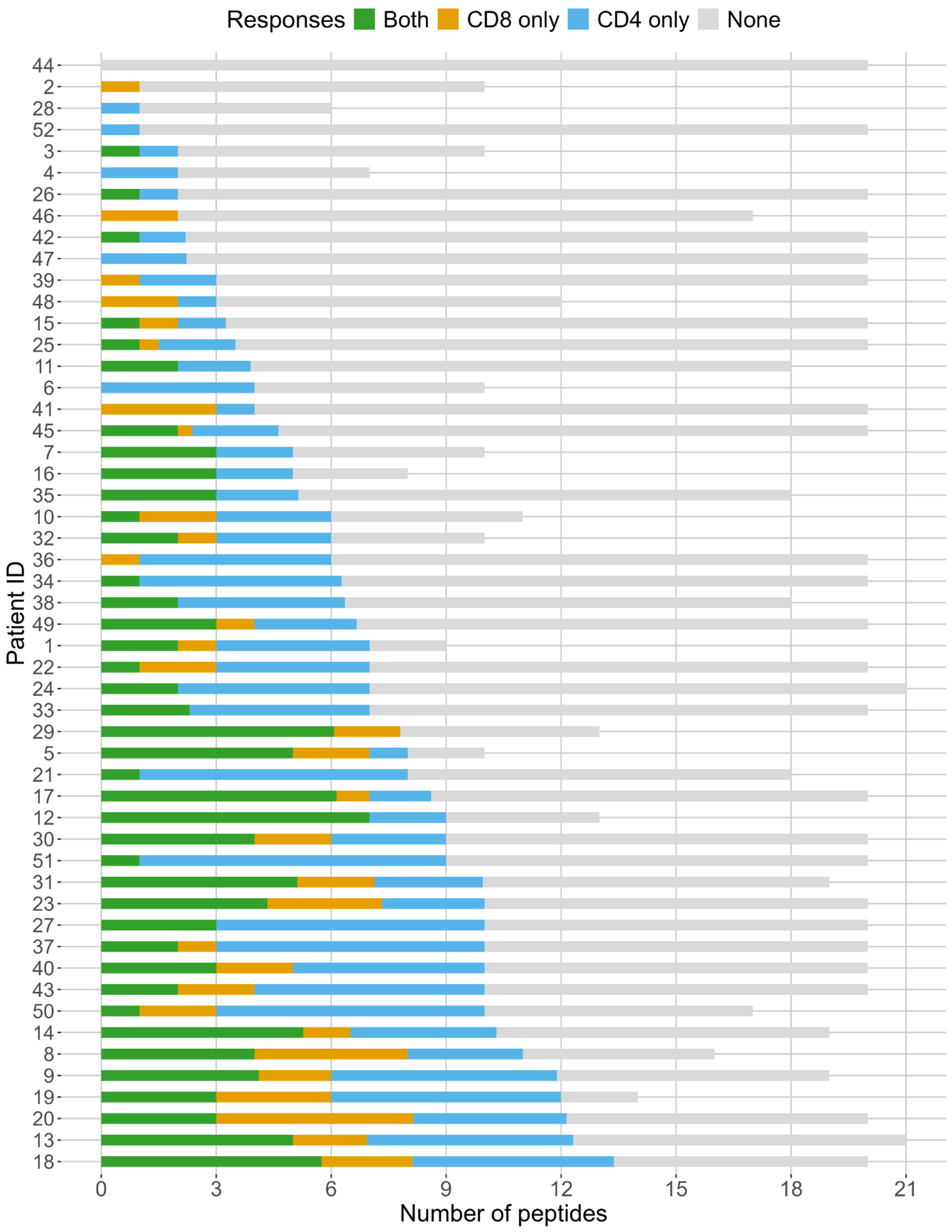

Figure 1 General immunogenicity data of the cohort. List of all study participants. Displayed are the number of total vaccinated peptides and the phenotype of the vaccine-induced neoantigen-specific T cells. Solely CD4 response (blue), solely CD8 response (orange), combined CD4 and CD8 response ("Both"; green), or no response (gray).

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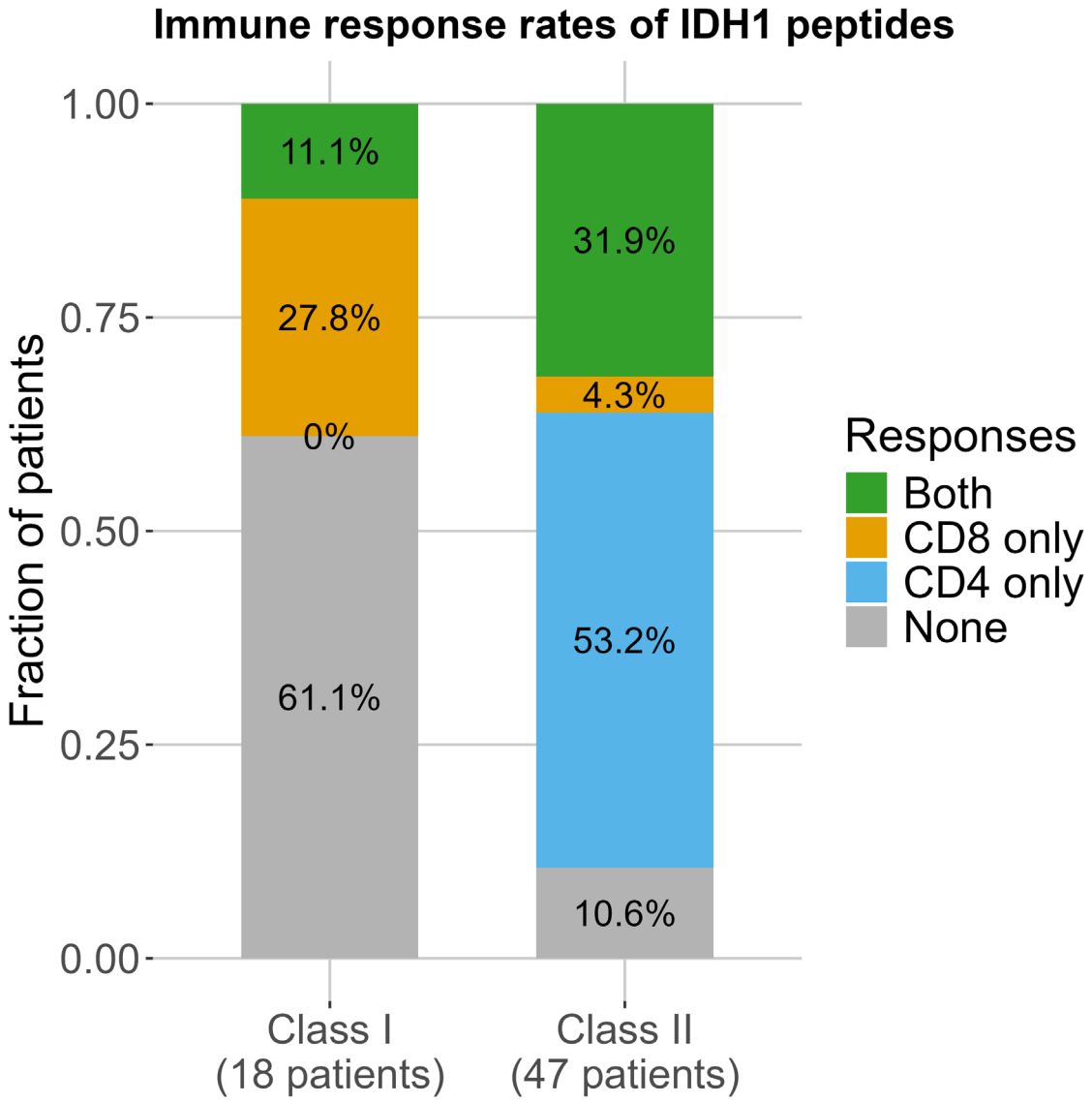

Figure 2 Immunogenicity of vaccinated mutIDH1 neoantigens. Percentage of patients that developed a solely CD4 response (blue), solely CD8 response (orange), a combined CD4 and CD8 response ("Both"; green) or no response (grey) against the HLA class II (right) or the HLA class I (left) mutIDH1-derived peptides. IDH1, isocitrate dehydrogenase 1; mutIDH1, mutated IDH1; HLA, Human leukocyte antigen.

two TCRβ sequences (CASTKNTEAFF, CASSLGPQAQYF) were identified in the expanded fractions of two different patients (patient 5 and patient 12). Both patients' tumors showed an R132H mutation, were vaccinated with the same mutIDH1-derived peptide and both patients were HLA-DQA1*01, DQA1*03 and DQB1*03, DQB1*06 (online supplemental table 2). Notably, the CDR3 sequence CASTKNTEAFF was also detected in the third patient (patient 14), but only in peripheral blood, not in the positive fraction (online supplemental figure 6). This patient had the same HLA DQA-DQB alleles. Only two other patients (patients 11 and 13) had these HLA DQA-DQB alleles. Both were negative for CASTKNTEAFF and CASSLGPQAQYF motive.

MutIDH1-specific TCR sequences can be tracked in single-cell sequencing data

The above-mentioned approach is resulting in sequences for the CDR3 region of the TCR beta chain only. Therefore, we used the identified sequences as a molecular barcode and tracked them ex vivo in single-cell transcriptomic data. PBMCs from patient ID14 (obtained after 14 vaccinations) were thawed and prepared for single-cell sequencing. Within 79,662 used cells, we identified 43 167 T cells (VDJ cells); here 37,243 different TCR clones. The shared sequence CASTKNTEAFF could not be detected. However, we were able to detect the most frequent sequence from the sorted positive fraction of this patient (CSARGAQETQYF; see table 3 and online supplemental figure 6). This was the 105th most frequent clone within all identified T cells in this sample (n=7 cells;

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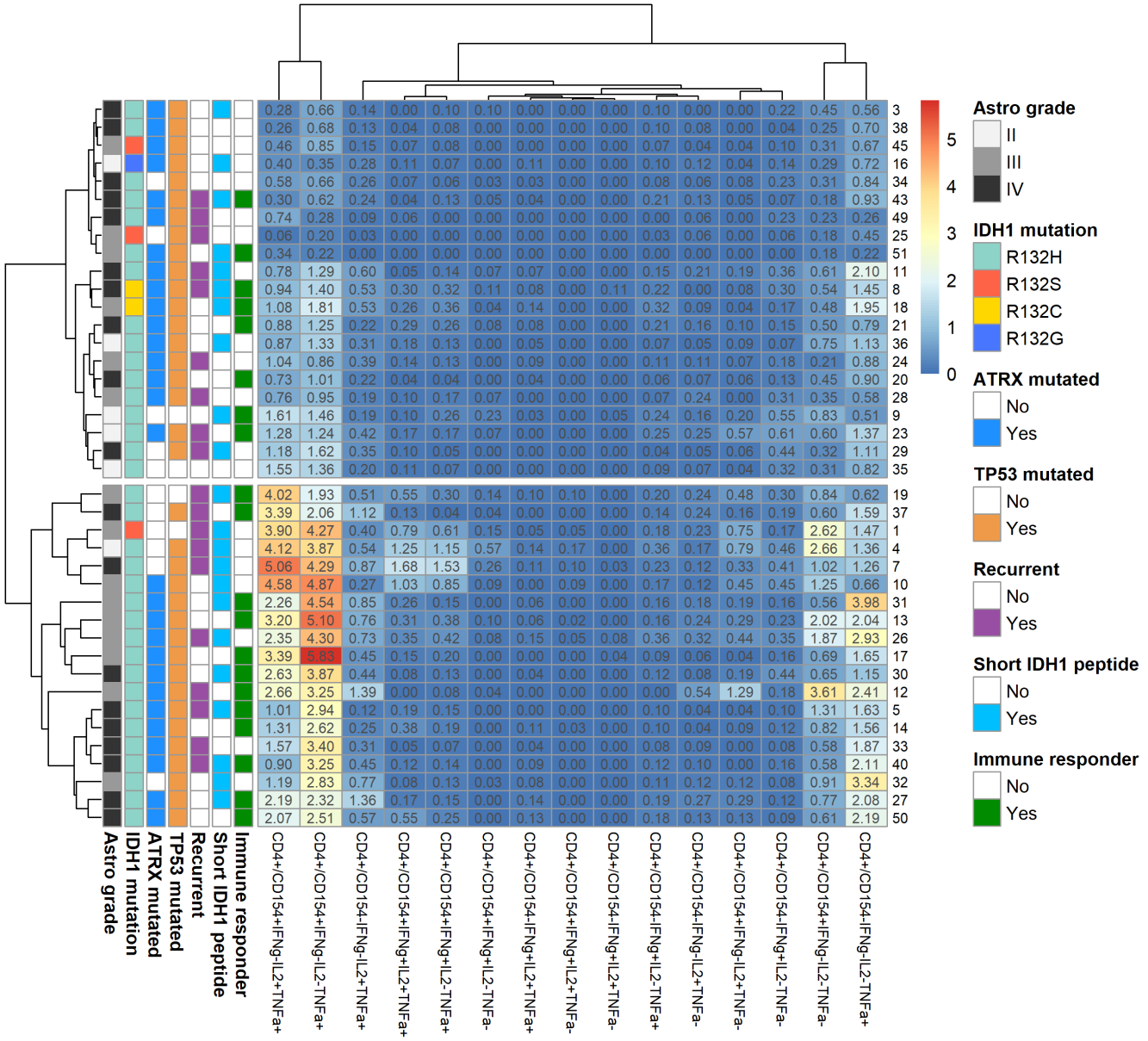

Figure 3 Multicytokine profile of CD4 T cell responses against vaccinated mutIDH HLA class II peptides Heat map showing the frequencies of each distinct multicytokine profile. Hierarchical clustering of patients was performed according to indicated frequencies of CD154, IFN-γ, IL-2, and TNF expressing CD4+T cells as determined by flow cytometry analysis and after square root transformation (color gradient from dark blue=0 to dark red=5 of the parent cell subset). Clinical parameters depicted on the left (glioma grade; presence of IDH1 mutation, ATRX mutation, or TP53 mutation; recurrence before start; receipt of an HLA class I peptide targeting the same IDH1 mutation, immune responder) were not included in the clustering. ATRX, alpha thalassemia/mental retardation syndrome X-linked gene; astro, astrocytoma; HLA, Human leukocyte antigen; IDH1, isocitrate dehydrogenase 1; IFN-γ, Interferon-γ; IL-2, Interleukin-2; mutIDH1, mutated IDH1; TNF, Tumor necrosis factor; TP53, Tumor protein p53.

frequency 0.016%). The usage of 5' single-cell transcriptomic data allowed us to reveal the full-length alpha and beta chain of this TCR (see online supplemental table 3). Furthermore, cell type annotation with a well-described reference dataset[24] confirmed that T cells expressing the TCRβ CSARGAQETQYF, were mainly CD4+T Helper cells having an effector-memory phenotype (figure 4). We refrained from further differential gene expression analysis due to the low number of specific clones. Here, analyses of sorted or expanded cells could help to analyze the transcriptomic profile of the clones of interest.

DISCUSSION

IDH-mutant gliomas are a common diffuse primary brain tumor and ultimately progress and cause morbidity and mortality to a typically younger group of patients. Despite significant improvements in understanding the molecular

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Table 3 mutIDH1 TCRβ sequences

Patient	Rank	CDR3.aa	Proportion*	mutIDH1
5	1	CASNGLAGPGETQYF	0.0026	R132H
5	2	CSARVWQLNTEAFF	0.0014	R132H
5	3	CATWDRPYEQYF	0.0014	R132H
5	4	CASTKNTEAFF†	0.0014	R132H
5	5	CASSLGPQAQYF‡	0.0014	R132H
9	1	CSAQSDRWGGYTF	0.2876	R132H
9	2	CSVFEQANYGYTF	0.0242	R132H
9	3	CASSFGTGKETQYF	0.0065	R132H
9	4	CAIKGGYNSPLHF	0.0052	R132H
9	5	CASRSRPGNTEAFF	0.0041	R132H
10	1	CASSLGQGGNEQFF	0.3973	R132H
10	2	CSAEAGRSTSPLHF	0.0218	R132H
10	3	CASSLASGLGYEQYF	0.0100	R132H
10	4	CASSYSTASYEQYF	0.0065	R132H
10	5	CASSIRGGLETQYF	0.0060	R132H
11	1	CASSGAPGSYEQYF	0.4078	R132H
11	2	CASRTLLGGSGNTIYF	0.0325	R132H
12	1	CSARGLVGSSHEQYF	0.1106	R132H
12	2	CASSLAVGQETQYF	0.0855	R132H
12	3	CASSLGPQAQYF‡	0.0307	R132H
12	4	CAISGTGRTDTQYF	0.0111	R132H
12	5	CASTKNTEAFF†	0.0077	R132H
13	1	CASSLESNTEAFF	0.1094	R132H
13	2	CSARDPSFDTQYF	0.0477	R132H
13	3	CASSLGPQGVTDTQYF	0.0400	R132H
13	4	CASSGGAETQYF	0.0325	R132H
13	5	CATSDIDRHNTEAFF	0.0286	R132H
14	1	CSARGAQETQYF§	0.5451	R132H
14	2	CSAPATLYGYTF	0.2252	R132H
14	3	CSARDPVGTEAFF	0.0778	R132H
14	4	CSAAGQEETQYF	0.0636	R132H
17	1	CASKTGNTGELFF	0.0028	R132H
17	2	CASSEQHGNTEAFF	0.0019	R132H
17	3	CASSPQGGNTQYF	0.0012	R132H
17	4	CASSTQGGMETQYF	0.0009	R132H
17	5	CASSAQHQNTEAFF	0.0008	R132H
26	1	CASRRTSEGVETQYF	0.0879	R132H
26	2	CATSRASGGQETQYF	0.0565	R132H
26	3	CSAPPGQPPYEQYF	0.0392	R132H
26	4	CASSWGLDTEAFF	0.0319	R132H
26	5	CSANRLETQYF	0.0237	R132H
29	1	CANSQWASGEQETQYF	0.2596	R132H
29	2	CAISESTDYGYTF	0.0178	R132H
29	3	CASGLARPTDTQYF	0.0118	R132H
29	4	CASSMTLGGYTF	0.0098	R132H

Continued

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Table 3 Continued

Patient	Rank	CDR3.aa	Proportion*	mutIDH1
29	5	CSASGRGGYEQYF	0.0083	R132H
8	1	CASSASTDTQYF	0.0776	R132C
8	2	CASSSGGGQADTQYF	0.0261	R132C
8	3	CSARDRDYGYTF	0.0237	R132C
8	4	CASTQQGANYGYTF	0.0114	R132C
8	5	CASRGWTGTEAFF	0.0113	R132C
18	1	CASSLEVGEQYF	0.0803	R132C
18	2	CATSRSLDGYSPLHF	0.0792	R132C
18	3	CASSEGGEETQYF	0.0322	R132C
18	4	CSARDGRQDTQYF	0.0075	R132C
18	5	CSARGPGTGTNEQFF	0.0046	R132C

*Proportion of clones within positive fraction.
†Identically reoccurring CDR3 sequences.
‡Identically reoccurring CDR3 sequences.
§CDR3 sequence that was detected in ex-vivo single cell transcriptome data.
mutIDH1, mutated isocitrate dehydrogenase 1; TCR, T cell receptor.

pathogenesis and biology of gliomas, they remain incurable. Because the mutated IDH represents an optimal therapeutic target for alternative therapies, efforts aiming at mutant IDH using vaccine strategies are currently an intensively investigated research area. Here, we present responses of patients with IDH1-mutant glioma who received personalized neoantigen-derived peptide vaccination in the setting of an individual healing attempt. Each peptide vaccine contained peptides targeting individual somatic mutations, but also at least one peptide targeting the IDH1 mutation.

Although IDH-mutant gliomas generally have a medium/low tumor mutational burden, we were able to identify a reasonable amount of potential neoantigens suitable for vaccination in each patient.

The vaccine was well tolerated. Minor temporal local skin reactions at vaccination sites such as redness, itching, and swelling resolved without interventions. This is consistent with previously reported results using peptide-based vaccines combined with i. a. sargramostim in larger trials.[25 26]

We observed that the personalized multipeptide vaccine was generally able to elicit robust CD4+and CD8+ T cell responses in a high proportion of patients. We previously observed similar results in a large cohort of patients with glioblastoma,[15] but also in a patient with urothelial carcinoma,[27] a patient with metastatic castration-sensitive prostate cancer,[17] a patient with cerebral metastasized ovarian carcinoma,[28] as well as four patients with breast cancer.[29] Furthermore, Hilf *et al* reported similar results in a large cohort of patients with glioblastoma receiving consecutively an off-the-shelf and personalized multipeptide vaccine.[30]

Single peptide immune monitoring data was available for 47 out of the 52 patients that received at least one HLA class II mutIDH1-derived peptide within their multipeptide vaccine cocktail. We detected vaccine-induced mutIDH1-specific T cells in 89% of patients, confirming the high immunogenicity of mutIDH1-derived peptides.[14 31] We observed that most vaccine-induced anti-mutIDH1 T cell responses were CD4-driven, and multicytokine profiling revealed that mutIDH1-specific T cells were polyfunctional. This is in line with previously reported results.[14 28] Associations between distinct multicytokine profiles and clinical parameters including overall survival were not observed, probably due to the small size of the cohort. Notably, these multicytokine profiles are measured after 12 days of in-vitro expansion and might not reflect the in-vivo conditions.

Since 85% of patients were still alive at data cut-off and after a median observation time of 36 months from initial diagnosis, the follow-up time may still be too short to detect statistically significant differences in survival between patient subgroups and especially between those with or without mutIDH1-specific polyfunctional T cell responses. However, we have observed a trend towards a prolonged overall survival in patients who developed T cell responses against multiple neoantigens (immunological responders) compared with those patients with no/few induced T cell responses. This is in consensus with previous reports, where induction of T cell responses against multiple targets was associated with prolonged overall and progression-free survival.[15 25 32] Given the intratumoral heterogeneity of gliomas in general,[33 34] immunoresistance to vaccines targeting only a single neoantigen (such as mutIDH1) may be encountered.

We have implemented an assay which allowed us to isolate functional antigen-specific CD4+T cells in an HLA-independent manner. TCR sequencing of the CDR3 region of the beta chain revealed that mutIDH1-specific

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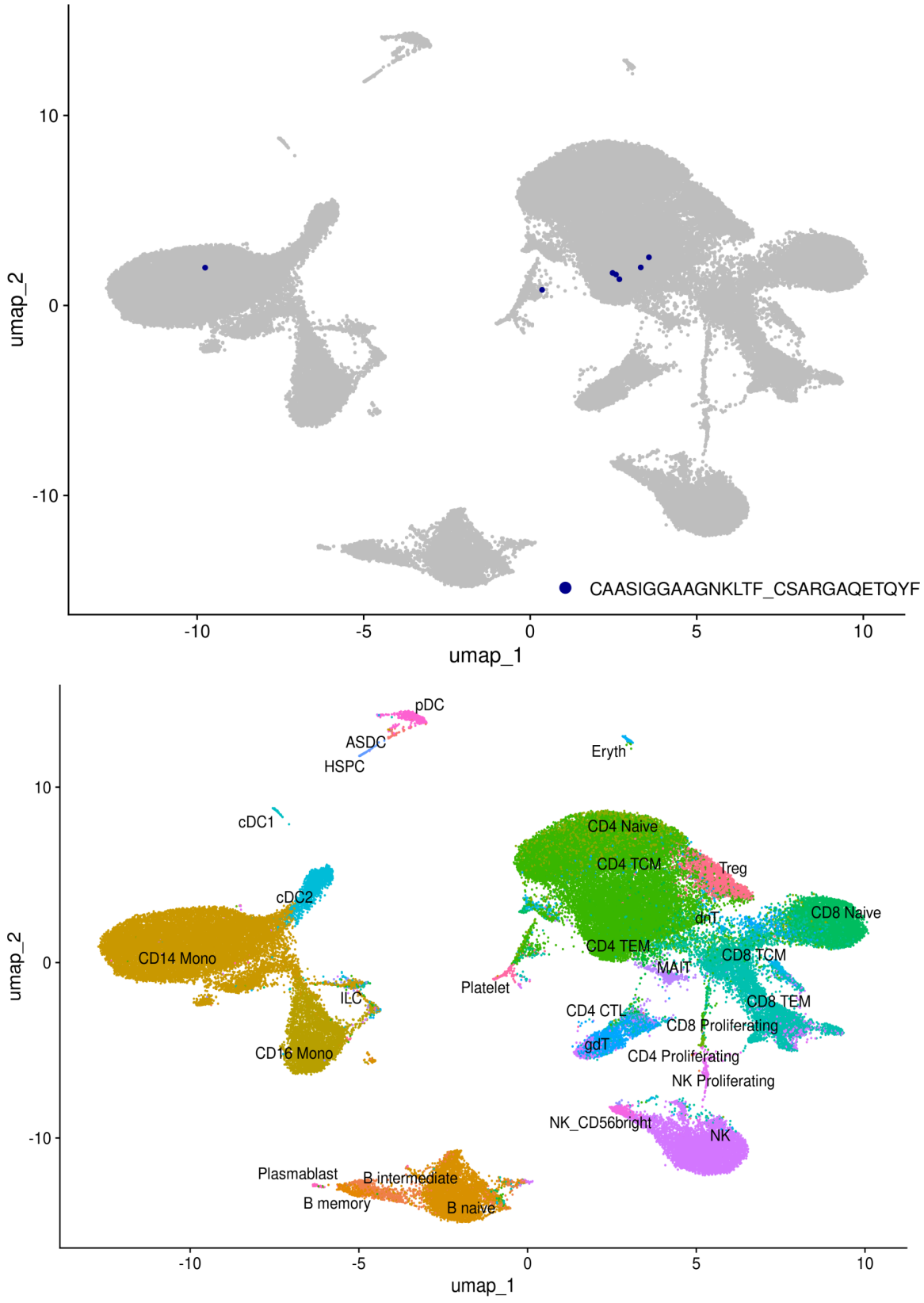

Figure 4 TCRβ sequences tracked in single-cell transcriptomic data UMAP plot showing 79,662 cells from a PBMC sample of patient ID14 after vaccination. Cells were clustered and annotated using the R package Seurat as seen in the lower UMAP plot. Paired V(D)J information was added to the single-cell data with scRepertoire. The amino acid sequence of the TCR beta chain that was identified via mutIDH1-specific immunogenicity assay was used to highlight the corresponding TCR clone in the single-cell data and is shown highlighted in blue in the upper UMAP plot in contrast to the other cells (gray). mutIDH1, mutated isocitrate dehydrogenase 1; PBMC, peripheral blood mononuclear cell; TCR, T cell receptor; UMAP, Uniform manifold approximation and projection.

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CD4+T cells were polyclonal. As suggested by Danilova *et al*, neoantigen-specific CDR3 sequences can be used as molecular barcodes to detect and track these clonotypes in blood and infiltrated tumor tissue, both before and during/after immunotherapy.[35] Single-cell transcriptomic data for one patient indeed confirmed that the CDR3 region of the TCR beta chain can be used to detect TCR-expressing T cells ex vivo and hence reveal the full-length beta and alpha chain of the TCR as well as their immune phenotype. Future studies can use the paired alpha and beta chain sequences of cancer-specific TCRs to improve prediction algorithms for TCR-peptide-HLA interactions.

Strikingly, we have identified two mutIDH1-specific TCRβ CDR3 sequences occurring identically in two different patients. One of these CDR3 sequences was detected in a third patient. All three patients had vaccine-induced CD4+T cells against the same peptide and expressed identical HLA DQA-DQB alleles. Once recognition of mutIDH1-derived peptide by these clonotypes can be experimentally confirmed, more matched beta and alpha chain TCR sequences could be collected. Consequently, such data might hold promise to accelerate the development of TCR-based diagnostic and therapeutic tools (eg, mutIDH1-targeting TCR-transgenic T cells). This approach might help improve treatment strategies not only for IDH1-mutant gliomas but for IDH-mutant tumors in general.[36]

Our study has several important limitations. First, the small sample size. Second, patients received different numbers of peptides and vaccinations at different times after initial diagnosis. Third, we cannot specifically estimate long-term clinical effects as the observation period is not adequately mature. In addition, detailed information on prior, concurrent and subsequent therapies relative to vaccination was not completely collected making it difficult to isolate the impact of the vaccine on outcome. Although we observed differences regarding overall survival between iR and iNR, the follow-up time is probably too short to assess longer-term survival. Lastly but most importantly, we did not ensure experimentally that the identified TCRβ CDR3 sequences are indeed exclusively mediating T cell recognition of IDH1-R132H-derived peptides in the context of HLA class II molecules.

Here, we provide further evidence that it is technically feasible to produce a fully personalized neoantigen-derived peptide vaccine. We were able to induce T cell responses against multiple target neoantigens without any additional treatment, such as immune-checkpoint inhibition.

There are several additional research questions that emerged, such as the role of HLA class I and II neoantigen presentation, the optimal ratio/amount of HLA class I versus II restricted/binding peptides, and the role of mutIDH1-specific CD8+T cells. Given the recent approval of the IDH-inhibitor vorasidenib, it is tempting to speculate regarding synergistic effects of targeted biologic therapy with a mutIDH1 targeting neoepitope peptide vaccine. Further investigation in the framework of a randomized, controlled clinical trial is warranted.

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