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Transcriptional and functional analyses of neoantigen-specific CD4 T cells during a profound response to anti-PD-L1 in metastatic Merkel cell carcinoma

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ABSTRACT

Background Merkel cell carcinoma (MCC) often responds to PD-1 pathway blockade, regardless of tumor-viral status (~80% of cases driven by the Merkel cell polyomavirus (MCPyV)). Prior studies have characterized tumor-specific T cell responses to MCPyV, which have typically been CD8, but little is known about the T cell response to UV-induced neoantigens.

Methods A patient in her mid-50s with virus-negative (VN) MCC developed large liver metastases after a brief initial response to chemotherapy. She received anti-PD-L1 (avelumab) and had a partial response within 4 weeks. Whole exome sequencing (WES) was performed to determine potential neoantigen peptides. Characterization of peripheral blood neoantigen T cell responses was evaluated via interferon-gamma (IFNγ) ELISpot, flow cytometry and single-cell RNA sequencing. Tumor-resident T cells were characterized by multiplexed immunohistochemistry.

Results WES identified 1027 tumor-specific somatic mutations, similar to the published average of 1121 for VN-MCCs. Peptide prediction with a binding cut-off of ≤100 nM resulted in 77 peptides that were synthesized for T cell assays. Although peptides were predicted based on class I HLAs, we identified circulating CD4 T cells targeting 5 of 77 neoantigens. In contrast, no neoantigen-specific CD8 T cell responses were detected. Neoantigen-specific CD4 T cells were undetectable in blood before anti-PD-L1 therapy but became readily detectible shortly after starting therapy. T cells produced robust IFNγ when stimulated by neoantigen (mutant) peptides but not by the normal (wild-type) peptides. Single cell RNAseq showed neoantigen-reactive T cells expressed the Th1-associated transcription factor (T-bet) and associated cytokines. These CD4 T cells did not significantly exhibit cytotoxicity or non-Th1 markers. Within the pretreatment tumor, resident CD4 T cells were also Th1-skewed and expressed T-bet.

Conclusions We identified and characterized tumor-specific Th1-skewed CD4 T cells targeting multiple neoantigens in a patient who experienced a profound and durable partial response to anti-PD-L1 therapy. To our knowledge, this is the first report of neoantigen-specific T cell responses in MCC. Although CD4 and CD8 T cells recognizing viral tumor antigens are often detectible in virus-positive MCC, only CD4 T cells recognizing neoantigens were detected in this patient. These findings suggest that CD4 T cells can play an important role in the response to anti-PD-(L)1 therapy.

INTRODUCTION

Merkel cell carcinoma (MCC) is a rare and aggressive neuroendocrine skin cancer with an increasing incidence estimated to exceed 3200 cases per year in the USA by 2025.[1] Before the availability of immune checkpoint inhibitors, diagnosis with metastatic MCC had a grim outlook with a 20% relative survival rate at 3 years.[2] In contrast, the 3-year overall survival rate for first-line anti-PD-1 (pembrolizumab) in the Keynote-017 trial was 59%.[3] Accordingly, MCC tumors are thought to be immune sensitive as indicated by a >10 fold increase in incidence among immune suppressed patients[4 5] as well as a markedly lower risk of recurrence when tumors have brisk intratumoral CD8 infiltration.[6]

Roughly 80% of MCCs are driven by the Merkel cell polyomavirus (MCPyV), in which the viral DNA is clonally integrated into the host genome, and T-antigen oncoproteins are persistently expressed by tumor cells.[7] The remaining ~20% of cases are thought to be truly virus-negative MCC (VN-MCC) and are characterized by high UV-mutational burdens resulting in numerous potential neoantigens. Indeed, there are a median of 1121 protein-coding somatic single nucleotide variants

(SSNVs) per exome compared with a median of 13 SSNVs per exome in MCC tumors harboring MCPyV.[8–10] The MCPyV proteins that drive tumor cell growth have been shown to be immunogenic as evidenced by robust T and B cell responses specifically observed in MCC patients.[11 12] However, there is a paucity of data describing the immune response to individual tumor neoantigens in VN-MCCs. The different etiologies for virus-positive (VP) and VN-MCCs, as well as the immune sensitive nature of MCC, makes this an ideal cancer to study the important role of anti-tumor T cells, which can be applied to other solid tumors more broadly.

Programmed death-ligand 1 (PD-L1), a major ligand for programmed cell death-1 (PD-1), is frequently expressed in MCC patient tumor specimens on immune cells and in some cases on tumor cells.[13 14] MCC has a strikingly high response rate to agents targeting the PD-1 pathway (56–62%), making it one of the most PD-(L)1 responsive solid tumors studied to date.[3 15 16] The high response rate to PD-1 pathway blockade is consistent across multiple studies for both VP-MCC and VN-MCC. The immune response has been extensively studied for VP-MCC. It is clear that MCPyV oncoproteins are frequently recognized by CD8[12 17 18] and CD4 T cells,[17 19] the number and avidity of which correspond to clinical outcomes.[20] In contrast, little is known about the nature of the immune response in VN-MCC patients. Based on analogy to other cancers,[21–24] the abundant UV-induced neoantigens in VN-MCC are presumed to be relevant immune targets, but the contributions of CD8 vs CD4 T cells and nature of the response have not been characterized to the best of our knowledge.

Herein, we describe a patient with an impressive and rapid partial response (RECIST V.1.1)[25] to anti-PD-L1 (avelumab) that was deep and durable (87% reduction in tumor burden after the first 2 years of therapy and remained disease free 5 years after discontinuing treatment) despite a large burden of chemotherapy-refractory disease. Our results reveal robust CD4 T cell responses to patient-specific tumor neoantigens even though neoepitope peptides were selected using class I HLA binding prediction algorithms to identify CD8 T cell responses. Indeed, CD4 T cells were identified that selectively recognized five different neoantigens from this patient. These neoantigen-specific T cells actively secreted interferon gamma (IFNγ) and upregulated cellular activation markers when stimulated with neoantigens compared with wild-type normal self-antigens. Furthermore, detailed transcriptional analysis showed a T-helper (Th) 1 skewed T cell phenotype.

METHODS

Patient consent and specimens

Informed consent was received. HLA typing was performed via PCR at Bloodworks Northwest (Seattle, Washington, USA). Peripheral blood mononuclear cells were collected in heparinized tubes, ficolled and cryopreserved at the Fred Hutchinson Cancer Center (FHCC) Specimen Processing/Research Cell Bank (Seattle, Washington, USA). Formalin-fixed paraffin-embedded (FFPE) archival biopsy material was used for immunohistochemistry and DNA isolation. Tumor whole exome sequencing (WES) was performed on DNA from the tumor biopsy peripheral blood mononuclear cells (PBMCs), and somatic mutation and neoantigen calls were performed as previously described.[8] Neoantigen-specific peptides for immunoassays were selected based on HLA class I predicted binding affinity using NetMHCv3.4[26] (https://services.healthtech.dtu.dk/?NetMHC-3.4), with a cut-off of ≤100 nM (n=77 peptides). Neoepitopes were excluded from analysis if they were within genes that are not expressed in MCC.[6]

IFNγ enzyme-linked immunospot (ELISpot) assay

Cryopreserved PBMC were thawed and rested overnight in R10 (RPMI, 10% fetal bovine serum, 100 nmol/L L-glutamine, and 100 U/mL penicillin-streptomycin) at 37°C. Concurrently, 96-well MultiScreen-IP filter plates (Millipore) were coated with anti-IFNγ capture antibody (1-D1K, Mabtech) at a concentration of 10 µg/mL in and incubated overnight at 4°C. The next day, 1×10^5 PBMC/well were plated in R10 with either neoantigen peptide pools (containing 8–9 peptides each at 5 µg/mL), DMSO (negative control) or Staphylococcal enterotoxin B (SEB) as a positive control, in triplicate and incubated at 37°C overnight (online supplemental figure 1B). After 16 hours of culture, plates were developed.[27] Briefly, antibody coated 96-well plates were washed before incubation with detection antibody (7-B6-1; Mabtech), followed by Avidin-peroxidase (Vectastain ABC kit; Vector Labs), and AEC substrate (AEC kit; Vector Labs). Developed plates were scanned with a C.T.L. ELISpot reader and counted (ImmunoSpot 5.0 Software, C.T.L.). Wells with 2× above DMSO or with at least 10 spot forming units (SFU) were considered positive. Peptide pool hits were mapped to individual peptides in follow-up ELISpot assays. ELISpot experiments were performed as previously described with the addition of an anti-HLA class I antibody (clone W6/32, 10 µg/mL; Thermo Fisher) or anti-HLA-DR antibody (clone G46-6, 10 µg/mL; BD Biosciences) 30 min before peptides were added.

Generation and testing of T cell lines

On day 1, cryopreserved PBMC were thawed and allowed to rest overnight at 37°C in T cell media (TCM) (RPMI, 10% human serum, 10 mM HEPES, 50 µM β-mercaptoethanol, 100 nmol/L L-glutamine, 100 U/mL penicillin-streptomycin and 2 ng/mL IL-7). On day 2, the media was refreshed and volume adjusted so that PBMCs were at a concentration of 2×10^6 cells/mL. Each well of a 24-well plate had one neoantigen peptide or control peptide added at a concentration of 1 µg/mL. On day 3, 10 U/mL of recombinant human IL-2 was added. TCM and cytokines were refreshed every 2–3 days thereafter. On day 13, cells were washed and allowed to rest overnight without

cytokines. For functional readouts, 2.5×10^4 cultured cells were combined with 5×10^5 patient-derived LCLs (EBV-transformed lymphoblastoid cell line) in each well of a 96-well round bottomed plate. The antigen source was serial dilutions of the neoantigen peptide used to generate the cell lines or wild-type counterparts. Plates were incubated overnight at 37°C. Supernatants from cocultured cells were collected and assessed for IFNγ secretion via Ready-SET-Go human IFNγ ELISA per manufacturer's protocol (eBioscience).

Single cell sorting of antigen-specific T cells

Cryopreserved PBMC were thawed and cultured at 2×10^6 cells/mL in a 24-well plate with anti-CD40 at 500 ng/mL and each of the five neoantigen peptides as well as a control HIV NEF (RYPLTFGWCF) peptide at 1 µg/mL overnight at 37°C. After 16 hours, cells were stained for with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen), followed by lineage markers CD14 (clone M5E2, Biolegend), CD19 (clone HIB19, Biolegend), CD4 (clone SK3, eBioscience), CD8 (clone 3B5, Invitrogen) and activation-induced markers CD69 (clone L78, BD Biosciences), CD137 (clone 4-1BB, Biolegend) and CD154 (clone TRAP1, BD Bioscience). Single neoantigen-reactive CD4 cells were sorted into RNAlater (Thermo Fisher) based on coexpression of CD69, CD137 and CD154 on a BD FACSAria II (BD Biosciences).

Single-cell RNA sequencing and analysis

RNA from sorted cells was purified using RNA Solid Phase Reversible Immobilization beads (Beckman), and cDNAs were generated with Smart-Seq2 (Illumina). Purified DNA was quantified with a Qubit dsDNA High Sensitivity Assay Kit (Invitrogen) before library preparation (Nextera, Illumina). Paired-end, next-generation sequencing was performed. Kallisto (V.0.44.0) was used for pseudoalignment of sequencing reads to transcripts. All further single cell RNA sequencing (scRNAseq) analyses were performed in R (V.3.6.0) primarily using packages Seurat (V.3.2.1) and scater (V.1.22.0) for normalization and plotting. Cells with less than 2000 genes or more than 5% mitochondrial genes were removed as low quality. Gene counts were log-normalized and regressed using mitochondrial gene expression via the Seurat function SCTransform. Code is available on request.

Multiplex immunohistochemistry

FFPE tissues were stained on a Leica BOND Rx autostainer using the Akoya Opal Multiplex IHC assay (Akoya Biosciences, Menlo Park, California, USA) with the following changes: additional high stringency washes were performed after the secondary antibody and Opal fluor applications using high-salt Tris-buffered saline + 0.1% Tween-20 (0.05M Tris, 0.3M NaCl, and 0.1% Tween-20, pH 7.2–7.6). Tris-casein + 0.1% Tween was used as the blocking buffer (0.05M Tris, 0.15M NaCl, 0.25% Casein, 0.1% Tween 20, pH 7.6±0.1). All primary antibodies were incubated for 1 hour at room temperature. OPAL Polymer HRP Mouse plus Rabbit (Akoya Biosciences) was used for all secondary applications. Slides were mounted with ProLong Gold and cured for 24 hours at room temperature in the dark before image acquisition at 20× magnification on the Akoya Vectra 3.0 Automated Imaging System. Images were spectrally unmixed using Akoya Phenoptics inForm software and analyzed using HALO software (Indica Labs, Corrales, New Mexico, USA). Cell nuclei=DAPI, CTLA-4=clone BSB-88 OPAL 540, T-bet=-clone EP263 OPAL 570, CD4=clone EP204 OPAL 620, FoxP3=clone 236A OPAL 690, CD8=clone 144B OPAL 520, CD3=clone SP7 OPAL 690 and PD-L1=clone E1L3N OPAL 620.

RESULTS

Case presentation: clinical response to anti-PD-L1 therapy

A patient in her mid-50s with a previous history of breast cancer presented with a skin lesion on her chest, which was biopsied and diagnosed as VN-MCC based on positive staining for CK-20 and negative staining for MCPyV Large-T antigen (CM2B4 clone). Staging PET-CT scan revealed multiple metastatic tumors in the liver. Pathological evaluation a liver biopsy indicated metastatic MCC. The patient was treated with four cycles of cytotoxic chemotherapy (cisplatin and etoposide). After a brief partial response to chemotherapy that lasted less than 2 months, the patient progressed while still on treatment resulting in four liver masses, the largest two of which had diameters of 11 and 7.5 cm (figure 1A).

She received anti-PD-(L)1 (avelumab) as a second-line treatment and experienced a rapid decrease of tumor size within 4 weeks from treatment initiation (figure 1A). All four measurable lesions had significant shrinkage over the course of 29 months of anti-PD-L1 therapy. She did not experience any significant side effect other than grade 1 fatigue and remains in remission with a deep partial response (87% reduction in tumor burden) more than 7 years after beginning and 5 years after discontinuing anti-PD-L1 therapy. High-throughput T cell receptor sequencing (see online supplemental figure 3) of the tumor and on-treatment blood specimens showed an expansion of tumor-resident T cell clones in the blood suggesting an antitumor response that tracked with clinical response (figure 1A).

To understand this T cell response, we performed WES to identify tumor neoantigen mutations that could potentially be targeted by T cells. Neoantigen peptides were selected for synthesis based on prediction of their binding strength (see methods and figure 1B) to the following patient-specific HLA class I types: HLA-A*24:02, HLA-A*68:02, HLA-B*14:02, HLA-B*51:09, HLA-C*01:02 and HLA-C*08:02.

T cells recognize neoantigens and expand during profound clinical response

Following WES and mutant peptide identification, 77 potential neoantigen peptides were selected for synthesis

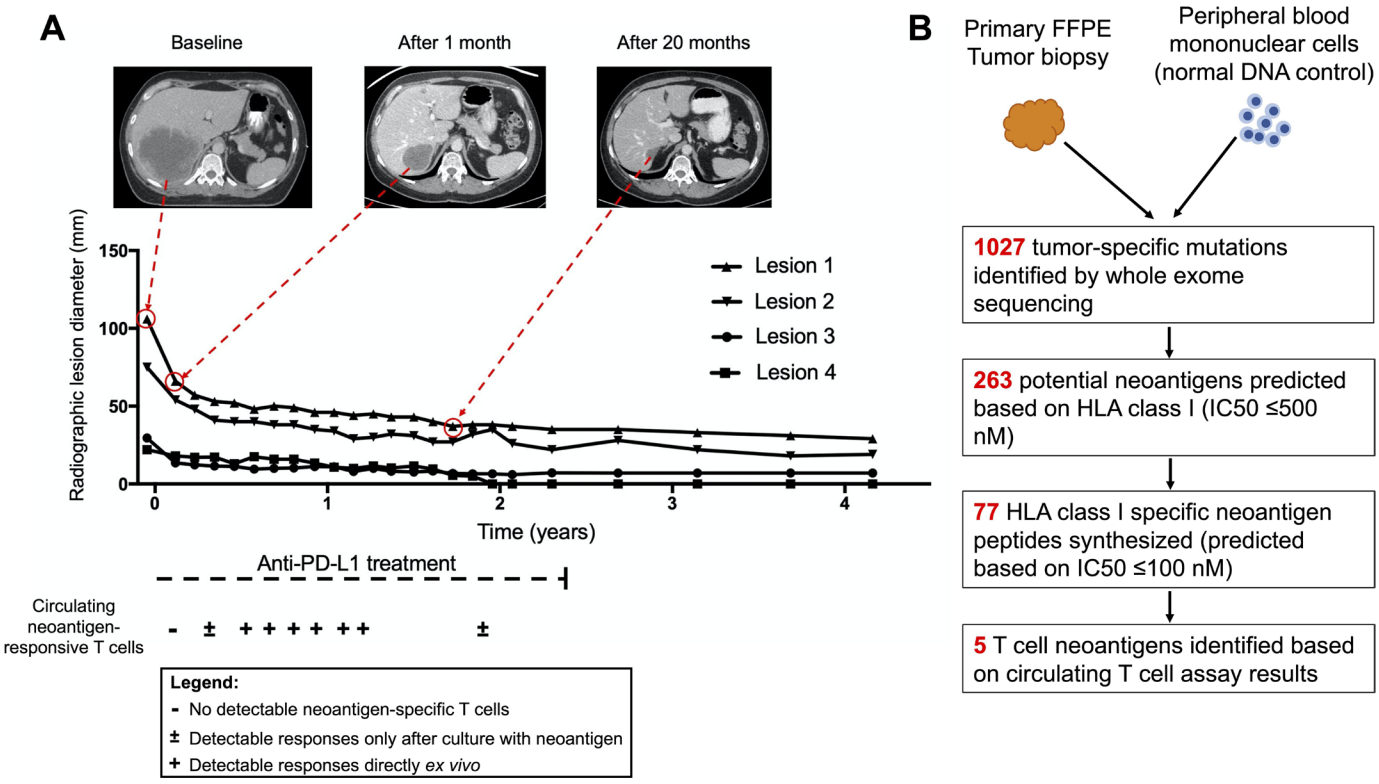

Figure 1 Clinical response to anti-PD-L1 therapy. (A) Timeline describing treatment, clinical response and T cell responses. Patient received four doses of cisplatin/etoposide and discontinued due to disease progression. Shortly thereafter, patient started anti-PD-L1 therapy (avelumab) and experienced a rapid partial response. Computed tomography (CT) scan of a tumor (lesion 1) in the liver before initiation of anti-PD-L1 therapy, after two doses of anti-PD-L and after 20 months of anti-PD-L1. Detection of neoantigen-specific T cells is denoted below the x axis: a '-' sign for no detectable neoantigen-specific T cells, a '±' sign for neoantigen-specific T cells detected after culture with target peptides, or a '+' for neoantigen-specific T cells detected directly ex vivo. (B) Tumor specific non-synonymous somatic mutations were identified via WES and compared against peripheral blood DNA for reference. WES, whole exome sequencing.

based on whether their predicted binding affinity for any cognate HLA class I allele was less than or equal to 100 nM (online supplemental figure 1A and table 1). Neoantigen peptides were pooled (online supplemental figure 1B), and patients PBMC from 6 months post-treatment initiation were assessed for response to peptides in an IFNγ ELISpot. Eleven neoantigen peptide pools elicited IFNγ secretion from T cells (online supplemental figure 1C), and then individual reactive peptides were then identified. Due to limited patient PBMC, only peptides from the strongest IFNγ pool hits were tested in follow-up IFNγ ELISpot assays.

Of the 18 individual peptides tested, five neoantigen peptides were considered positive based on responses above the baseline threshold (greater than 10 SFU/5×10^5 PBMC cells; online supplemental figure 1D). The following genes, and specific amino acid changes, elicited a positive IFNγ ELISpot result: *CASR* (P39S), *DMXL2* (P154S), *RNF38* (P287L), *TANGO6* (R172S), and *ZNF280C* (R301M). To the best of our knowledge, none of the mutations are considered driver mutations.

To determine if IFNγ secretion was specific for mutant neoantigen peptides and not a response against self-antigens, we performed an IFNγ ELISpot with side-by-side neoantigen and self (wild-type) peptides (figure 2A). Four of the five self-peptides tested did not elicit a T cell response; however, the self-peptide originating from the *RNF38* gene stimulated~45% of the IFNγ response that was induced by the mutant/neoantigen version of that peptide. To determine the sensitivity of neoantigen responses, we performed a dose–response curve in T cell lines specific for two of the mutant peptides, *TANGO6* and *ZNF280C*. Results of the dose–response experiments show specificity for the mutated form of the peptides as indicated by response to the neoantigen peptides versus wild type as low as 1 µg/mL (figure 2C,D).

After identifying neoantigen T cell epitopes, we used serially collected PBMC to determine kinetics. We were unable to detect T cell responses in the blood until ~4 months after anti-PD-L1 treatment initiation, consistent with either expansion or migration of epitope-specific T cells during immunotherapy. Furthermore, we were unable find epitope-specific T cell responses directly *ex vivo* after tumors markedly shrank (76% reduction at~22 months post treatment initiation).

Neoantigen-specific T cells are HLA class II restricted CD4+, CD8- T cells

To determine if IFNγ-positive T cells responding to neoantigen peptides were restricted by HLA class I or HLA class

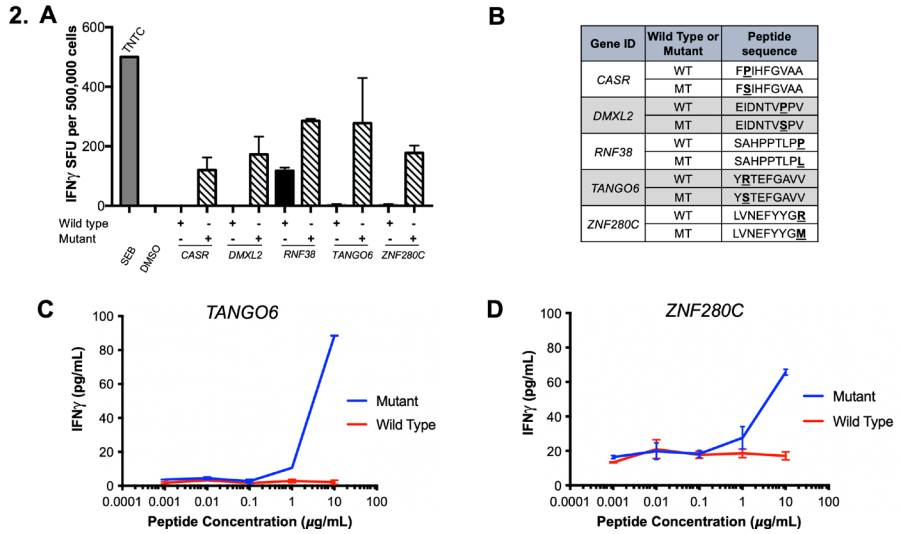

Figure 2 Circulating T cells recognize tumor-specific neoantigens. (A) Top: PBMC was tested for neoantigen T cell specificity by comparing IFNγ reactivity of mutant and wild-type peptides (5 µg/mL). Detection at twice the DMSO control level or >10 SFU per 5×10^5 PBMC were considered positive. Experiments were performed in triplicate. The mean is plotted, and SD is indicated by error bars. (B) Table of mutant peptides with reactivity in direct *ex vivo* IFNγ ELISpot and wild type peptide homologs. (C and D) T-cell line dose response curves for neoantigen peptides from the *TANGO6* (C) and *ZNF280C* (D) genes. Experiments performed in duplicate or triplicate. The mean is plotted and error bars represent SD from the mean. ELISpot, enzyme-linked immunospot; IFNγ, interferon gamma; SFU, spot forming units; TNTC, too numerous to count.

II, we performed an IFNγ ELISpot with the addition of blocking antibodies specific for either HLA class I A, B, and C, or the HLA class II loci. Addition of HLA class II specific antibodies to ELISpot wells blocked production of IFNγ spots (figure 3A) indicating that T cell responsiveness was HLA-DR restricted.

To confirm these findings, we measured the upregulation of three activation-induced T cell surface markers: CD69,[28] CD137,[29] and CD154[30] on CD4 T cells and two activation-induced cell markers, CD69 and CD137, on CD8 T cells after stimulation with neoantigen peptides. The specimen used was PBMC from ~7 months after

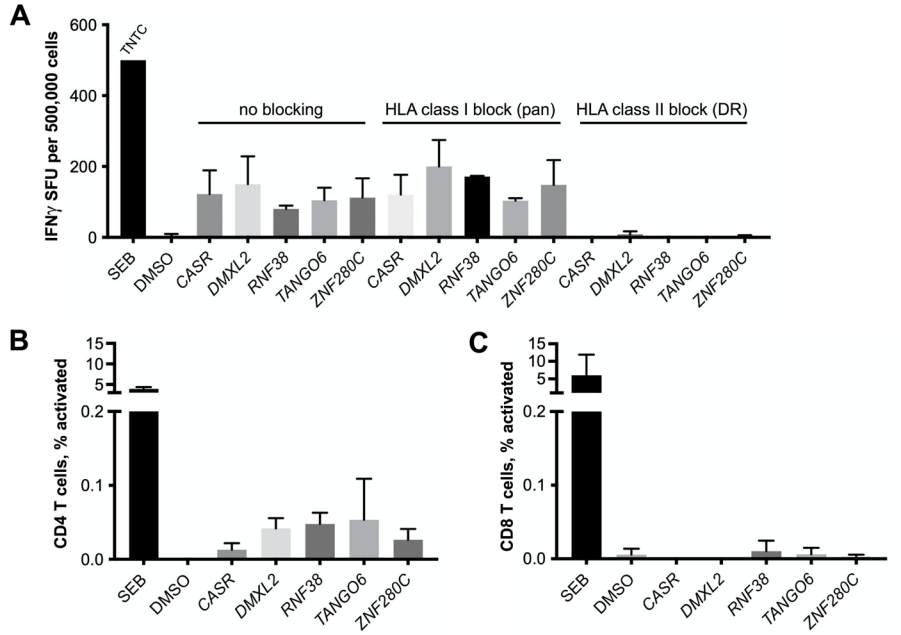

Figure 3 Neoantigen-specific T cells are HLA class II restricted and CD4 T cells upregulate activation markers on peptide stimulation. (A) IFNγ ELISpot incorporating anti-HLA class I antibody clone W6/32 or anti-HLA class II DR clone G46-6. Experiments were performed with two to six replicates. Positive responses were indicated by twice the DMSO control level or >10 SFU per 5×10^5 PBMC. (B and C) Per cent of CD4 T cells (B) that upregulate all three activation markers (CD69, CD137 and CD154) or CD8 T cells (C) that upregulate two activation markers (CD69 and CD137) after stimulation with each neoantigen peptide. Results are an average of two flow cytometry experiments; error bars indicate the SD. ELISpot, enzyme-linked immunospot.

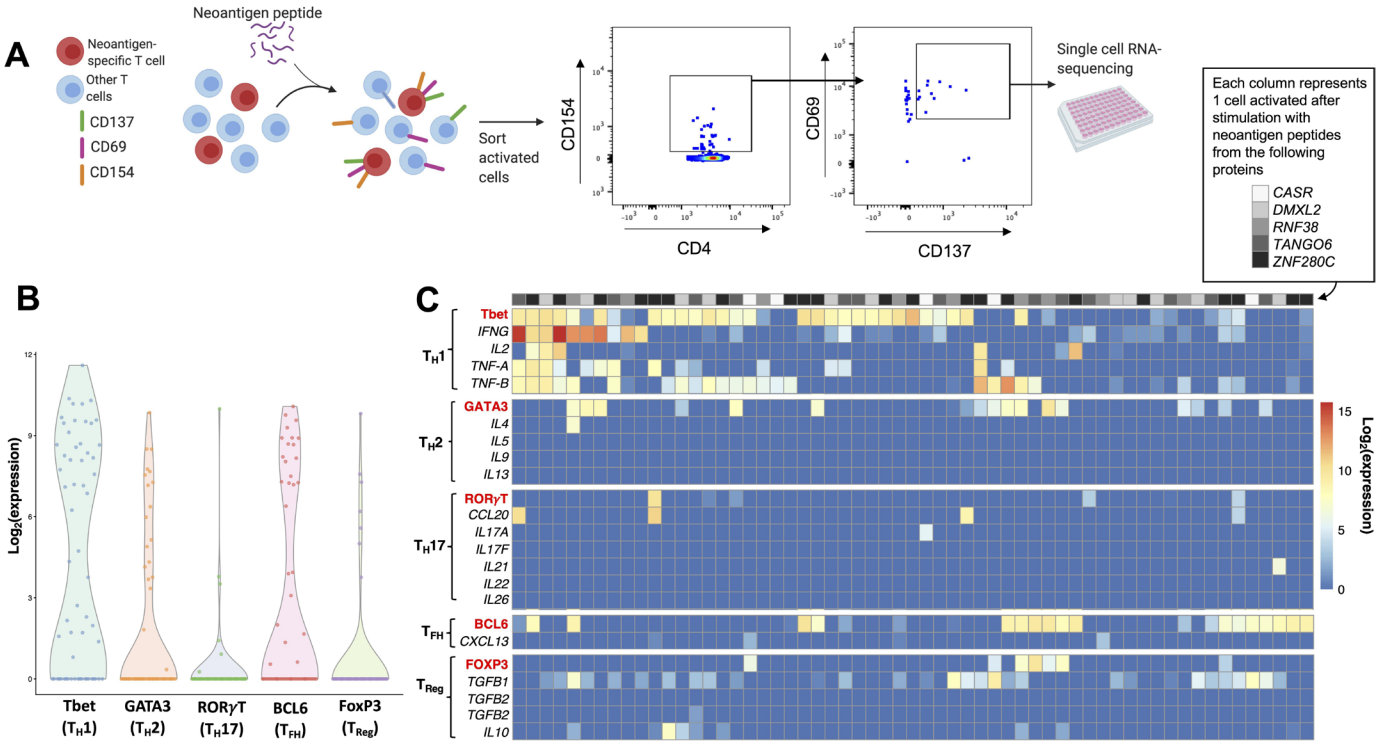

Figure 4 Neoantigen-specific CD4 T cells have a Th1-skewed transcriptional phenotype. (A) Overview of experimental method. Peripheral blood mononuclear cells were stimulated for 16 hours with neoantigen peptides and CD4 T cells were single-cell sorted into a 96 well plate based on upregulation of three activation markers (CD69, CD137 and CD154, representative dot plots shown). (B) Sorted CD4 T cell T-bet RNA levels were significantly elevated when compared with other CD4 T cell master regulator transcription factors (p values as compared with T-bet were: p<0.001 for GATA3, RORγT and FoxP3, and p<0.01 for BCL6). (C) RNA expression of effector cytokines indicates a Th1 skewed phenotype.

initiation of anti-PD-L1 therapy at a time the patient had experienced a partial response. Measurement by flow cytometry showed that all five of the neoantigens stimulated upregulation of the three activation markers on CD4+ but not CD8+ T cells (figure 3B,C).

IFNγ detection via flow cytometry can be difficult due to low secretion and infrequent numbers of T cells, as is common with antigen-specific CD4 T cells. To overcome this limitation, PBMCs were cultured with individual neoantigen peptides to first expand the T cell populations. Intracellular production of IFNγ was then assessed after restimulation with the corresponding neoantigen peptide for each culture condition. CD4 T cells, and not CD8 T cells, produced IFNγ in response to re-stimulation with neoantigen peptides, confirming our direct *ex vivo* findings (online supplemental figure 2A,B).

Single-cell transcriptional profiling of neoantigen-reactive CD4 T cells

To determine the transcriptional phenotype of neoantigen-specific T cells, we sorted CD4 T cells that upregulated activation markers CD69, CD137 and CD154 *ex vivo* in response to stimulation with neoantigen peptides (figure 4A). Single cell RNAseq was then used to characterize T cell phenotypes. Expression of CD4 T cell subset master transcription factor mRNAs showed a plurality of cells expressing the Th1-associated transcription factor (T-bet), suggesting that a Th1 phenotype is the most common phenotype that the neoantigen-specific CD4 T cells develop. This is further supported by the expression of cytokine mRNAs that showed a predominance of the Th1-weighted cytokines IFNγ, tumor necrosis factor alpha (TNFα) and IL-2 (figure 4B,C).

Tumor microenvironment exhibits a T cell excluded phenotype involving T-bet expressing CD4 T cells

To determine the T cell characteristics into and around the pre-therapy tumor, we used multiplex immunohistochemistry. While the tumor did not have appreciable infiltrating immune cells, the peritumoral and stromal areas had abundant CD3+, CD8+ and PD-L1+ cells indicating an 'excluded' T cell phenotype (figure 5A,B).

We also used a separate phenotyping panel to characterize CD4 T cells at the tumor edge. CD4+/T-bet+ cells were abundant while CD4+/FoxP3+ cells were not, indicating a predominantly inflammatory (Th1) response (figure 5A bottom and C).

DISCUSSION

The use of anti-PD-(L)1 agents in MCC has changed patient management and dramatically improved long-term outcomes for a substantial subset of patients with advanced disease. Indeed, the first-line response rate for MCC (~56–62%) is essentially the highest among solid tumors.[31–35] Across multiple trials, the response rate

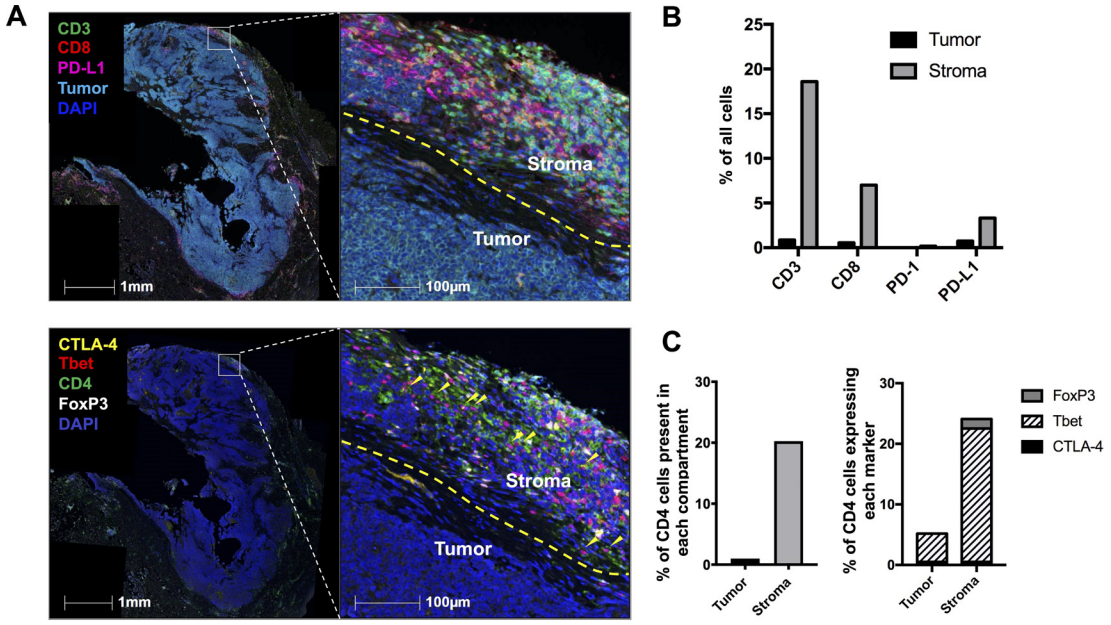

Figure 5 Primary tumor exhibits a T cell-excluded phenotype. (A) Top: representative image of multicolor staining for adaptive immune markers. Light blue=tumor (CD56), green=CD3, red=CD8, magenta=PD-L1, dark blue=cell nuclei. Bottom: representative image of multicolor staining for CD4 phenotyping in tumor. Dark blue=cell nuclei, green=CD4, yellow=CTLA-4, red=T bet, and white=FoxP3. Yellow arrowheads indicate examples of CD4+T-bet+ cells. (B) Staining was quantified with HALO software. Per cent of cells expressing CD3, CD8, PD-1, and PD-L1 within the tumor and on the peritumoral edge (stroma). (C) Left: per cent CD4 cells in the tumor or stroma; right: proportion of CD4 cells expressing each of the phenotypic markers listed.

has been similarly high for both VN-MCC and VP-MCC patients.[3 16 36]

VP-MCC has frequently been used to understand the immunological correlates of response and tumor antigen-specific T cells, due to the invariant MCPyV oncoprotein antigens expressed by these virally driven tumors. In contrast, relevant tumor-specific T cell responses have not been characterized in VN-MCCs to the best of our knowledge. Accordingly, we sought to determine whether neoantigen-specific T cells were associated with a deep and durable, partial response to anti-PD-L1 therapy in a patient with VN-MCC.

A screen of 77 putative neoantigen peptides lead to identification of five T cell responses that preferentially recognized the mutated version of a peptide (neoepitope) as compared with its wild type counterpart (figure 2A). Although the initial goal was to characterize CD8 T cell responses by selecting neoantigen peptides predicted to bind to class-I HLA, all neoantigen-reactive T cells identified were CD4+ and recognized neoepitopes in the context of class-II HLA (figure 2). Similar results have been reported for therapeutic vaccination wherein neoantigen peptides optimized for presentation on class-I HLA elicited in a surprisingly robust expansion of epitope-specific CD4 as well as CD8 T cells.[21 22 37] The fact that only CD4 T cells were identified using potential neoantigen peptides in the present study contrasts with prior findings in VP-MCC for which the majority of T cell responses identified have been CD8+.[17 19 20 38]

To understand the role these cancer-specific CD4 T cells were playing in this patient's clinical response to anti-PD-L1 therapy, we sought to quantify and phenotype these cells over the course of treatment. When studying their quantity, we observed that these CD4 T cells increased in the weeks following initiation of anti-PD-L1 (figure 1A). However, several months after the patient experienced a partial response, CD4 T cell responses became undetectable. This potentially could be due to the reduction in tumor neoantigens and subsequent contraction of the neoantigen-specific T cell population. This finding is consistent with previously published studies of MCPyV-specific T cell activity that reproducibly decreased in MCC patients shortly after surgical removal of their tumors.[38] We also sought to functionally characterize these neoantigen-specific T cells because CD4 T cells are known to have diverse potential roles within the tumor microenvironment. While one proposed mechanism for antitumor activity of CD8 T cells is direct killing of tumor cells, it is becoming more evident that cancer-specific CD4 T cells are a critical aspect of the antitumor response. Indeed, in experimental mouse models in which CD4 T cells were depleted, mice could no longer control tumors, suggesting that the CD4 T cell response is required for antitumor immunity.[39 40]

There are multiple potential 'helper' roles for antitumor CD4 T cells. In MCC, perhaps the most significant role may be secretion of IFNγ to upregulate HLA class I molecules, in turn making tumor cells visible to cytotoxic CD8 T cells.[41] This is particularly relevant in MCC because 85% of MCC tumors downregulate HLA class I molecules, and this downregulation is reversible with stimulation by interferons.[42 43] CD4 T cells can also have

direct cytotoxic effects on tumors through production of granzyme and perforin[44 45] or immunosuppressive function in the case of regulatory T cells.[46] Importantly, CD4 T cells are crucial for the formation of tertiary lymphoid structures in the tumor microenvironment.[47–49] These structures of coordinated B and CD4 T cells are associated with improved survival across several cancer types including MCC[50] and likely lead to improved antitumor immunity via increased support for cancer-specific CD8 T cells.[51]

To determine which of many roles neoantigen-specific CD4 T cells were playing in this patient's clinical response, we phenotyped these cells via scRNAseq (figures 3B and 4A). We observed a Th1 pattern (figure 4C), consistent with the observation in figure 2 that neoantigen-reactive T cells release IFNγ in response to their cognate neoantigen peptide. Furthermore, these cells express T-bet, the canonical transcription factor that enforces a Th1 phenotype and supports CD8 T cell function (figure 4B). To confirm that intratumoral CD4 T cells had a Th1 phenotype in the tumor microenvironment, multiplexed immunohistochemistry was performed. This showed an 'immune excluded' tumor with numerous T cells on the tumor periphery and very few within the tumor itself (figure 5A). The most frequently expressed master transcription factor in CD4+ cells in both tumor and stroma was T-bet, further suggesting these cells are Th1 skewed (figure 5C). These data show that neoantigen-specific CD4 T cells were present in this patient, increased over the course of anti-PD-L1 therapy and had a Th1 phenotype that could improve antitumor immunity via promotion of an inflamed tumor microenvironment.

It is noteworthy that neoantigen-specific CD8 T cells were not detected despite this patient's dramatic clinical response. There are several possibilities that could explain this perplexing finding. Even though data for *in silico* predictions of relevant peptides is robust, it is possible that not all immunogenic neoantigen peptides were appropriately identified. Additionally, high-avidity CD8 T cells could have been present but later deleted.[52] T cells with higher avidity TCRs for their cognate peptide-HLA molecules may be particularly prone to exhaustion and elimination, and higher peptide-HLA affinities are associated with higher TCR avidity.[52] Pertinent to this, only neoantigens with predicted peptide-HLA affinities of <100 nM were used in this study. Alternatively, it is possible that CD4 T cells were directly cytotoxic as appears to be the case in bladder cancer and melanoma, both of which are known to express HLA class II in some cases.[44 45] However, we do not believe this is the case for two main reasons: (1) HLA class II is typically not expressed on MCC tumor cells[43] and thus there would be no target ligand available for CD4 T cell recognition and possible cytotoxic activity (online supplemental figure 4) and (2) scRNAseq data revealed no significant expression of cytotoxic perforin or granzymes in the neoantigen-specific CD4 T cells (data not shown), further suggesting that it would be unlikely for these cells to be directly responsible for this patient's tumor regression.

Another potential explanation for why neoantigen-specific CD8 T cells were not detected is that the sensitive assays required to identify these rare cells depend on inducible cytokine secretion or activation marker expression. IFNγ inducibility is lost late in the process of CD8 T cell exhaustion, after suppression of other key cytokines such as IL-2 and TNFα. If neoantigen-specific CD8 T cells were too dysfunctional to release IFNγ or upregulate activation markers, then they would not be detectable via these functional assays. We have previously observed this contrast for MCPyV-specific CD8 T cells in blood, for which tetramer-positive populations are detectable in some patients who have absent IFNγ ELISpot responses *ex vivo*.[17] Additionally, the frequency of neoantigen-specific CD8 T cells in the blood may have been below the threshold of IFNγ ELISpot assays (less than 1 in 50,000). It is possible that neoantigen-specific CD4 T cells could also have greatly outnumbered their CD8 counterparts in the blood but could have been present in the tumor at sufficient frequency to mediate regression seen in this patient (figure 5). Regardless, this study suggests that future clinical trials of immunotherapies, including possible therapeutic vaccination for MCC,[53] should include both CD4 and CD8 T cell biomarker analyses to further elucidate the role of both T cell subsets.

While many studies have characterized cancer-specific CD8 T cells,[52 54 55] the focus has recently shifted to investigating the role of CD4 T cells in immunotherapy largely in part to emerging technologies. In this study, we report that neoantigens can elicit a CD4 T cell immune response in MCC and that tumor-specific T cells expand in the periphery following immunotherapy in a patient with a profound durable response. Future and ongoing clinical trials aiming to boost the CD4 immune response via adoptive T cell transfer (NCT03747484) or therapeutic vaccination[53 56] could further strengthen this response and improve antitumor immunity. Immunotherapy as well as personalized vaccination strategies, which often result in robust tumor-specific CD4 T cells, should be considered for MCC patients including those with chemotherapy-refractory disease. Reinvigorating and generating new CD4 and CD8 T cell responses are key to eliminating tumors and providing patients with long-term clinical benefit.

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Competing interests PN reports personal fees from Pfizer, Inc, Bristol Myers Squibb, EMD Serono, Rain Therapeutics, Almirall, and Instill Bio. In addition, PN has pending patents for 'Merkel cell polyomavirus T antigen-specific TCRs and uses thereof' and 'Novel epitopes as T cell targets in Merkel Cell Carcinoma (MCC)'. CC has a pending patent 'Merkel cell polyomavirus T antigen-specific TCRs and uses thereof'. VM reports a patent (#EP3090066A2), Determinants of cancer response to immunotherapy. NR reports grants from REPARE Therapuetics, Repertoire Immune Medicines, and from Bristol Myers Squibb. RA is an employee of and reports stocks in Pfizer. JSC reports stock from Sensei Bio. RG reports personal fees from Takeda, Ozette Technologies, and Modulus Therapeutics. RHP is an employee of and reports stock in Sensei Bio. TC reports stock from Gritstone Bio, grants from Pfizer and InterVenn, personal fees from Illumina, and royalties from PGDx. Additionally, TC has a patent 'Use of TMB to identify immunotherapy responders licensed to PGDx'. DK reports grants from Sensei Bio and a pending patent related to 'high-affinity T-cell receptors that target the Merkel polyomavirus'.

Patient consent for publication Not applicable.

Ethics approval This study was conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonisation Guidelines for Good Clinical Practice. This study was approved by the Fred Hutchinson Cancer Center (FHCC) Institutional Review Board (IRB Protocol #6585). Patient provided written informed consent and the protocol was approved by the Institutional Review Board at the Fred Hutchinson Cancer Center.

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