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Mutation-derived Neoantigen-specific T-cell Responses in Multiple Myeloma

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ABSTRACT

Purpose: Somatic mutations in cancer cells can give rise to novel protein sequences that can be presented by antigen-presenting cells as neoantigens to the host immune system. Tumor neoantigens represent excellent targets for immunotherapy, due to their specific expression in cancer tissue. Despite the widespread use of immunomodulatory drugs and immunotherapies that recharge T and NK cells, there has been no direct evidence that neoantigen-specific T-cell responses are elicited in multiple myeloma.

Experimental Design: Using next-generation sequencing data we describe the landscape of neo-antigens in 184 patients with multiple myeloma and successfully validate neoantigen-specific T cells in patients with multiple myeloma and support the feasibility of neoantigen-based therapeutic vaccines for use in cancers with intermediate mutational loads such as multiple myeloma.

Results: In this study, we demonstrate an increase in neoantigen load in relapsed patients with multiple myeloma as compared with newly diagnosed patients with multiple myeloma. Moreover, we identify shared neoantigens across multiple patients in three multiple myeloma oncogenic driver genes (*KRAS, NRAS*, and *IRF4*). Next, we validate neoantigen T-cell response and clonal expansion in correlation with clinical response in relapsed patients with multiple myeloma. This is the first study to experimentally validate the immunogenicity of predicted neoantigens from next-generation sequencing in relapsed patients with multiple myeloma.

Conclusions: Our findings demonstrate that somatic mutations in multiple myeloma can be immunogenic and induce neoantigen-specific T-cell activation that is associated with antitumor activity *in vitro* and clinical response *in vivo*. Our results provide the foundation for using neoantigen targeting strategies such as peptide vaccines in future trials for patients with multiple myeloma.

Introduction

Multiple myeloma is a malignancy of plasma cells affecting >30,000 individuals per year (1, 2). Multiple myeloma is clinically and pathologically heterogeneous, making treatment of relapsed patients especially challenging, despite advances in therapeutics over the last decade. Peptides corresponding to somatic mutations in tumor cells are recognized as "non-self" neoantigens by the adaptive immune system (3). An increase in T cells recognizing cancer-specific neoantigens following immune checkpoint inhibition has been described in several tumors (4–8). Immunomodulatory drugs such as thalidomide, lenalidomide, and pomalidomide have been ascribed to have immunostimulatory effects in multiple myeloma (9, 10). Immunological checkpoint blockade therapies targeting cytotoxic T-lymphocyte antigen-4 (CTLA-4), programmed cell death-1 (PD-1), and programmed death ligand-1 (PD-L1) produce responses in only a subset of patients with multiple myeloma, with potentially increased toxicity in combination with immunomodulatory drugs (11–13). As biomarkers of response to immunotherapeutics have not been clearly defined in multiple myeloma, determining which patients would derive clinical benefit from immunotherapy is a compelling clinical question. To address this, we sought to analyze and experimentally validate immunogenic neoantigens from next-generation DNA and RNA-sequencing (RNA-seq) data from newly diagnosed as well as relapsed patients with multiple myeloma. We used algorithms that predict peptide binding to HLA class I and validated our *in silico* prediction *in vitro* using primary multiple myeloma samples in coculture systems. Results from this study support the feasibility of neoantigen targeting immunotherapy for tumors with intermediate mutational load such as multiple myeloma.

Materials and Methods

Patient selection

The study was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. The study protocol was reviewed and approved by the Institutional Review Board (IRB#11-1669) at the Icahn School of Medicine at Mt. Sinai, NY. Ninety-two patients with relapsed/refractory multiple myeloma were included in the study after written informed consent had

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Tumor-specific mutations are excellent targets for cancer immunotherapy as they may be recognized as neoantigens by mature T cells. Tumors with more mutations have higher likelihood of neoepitopes, which can be recognized by tumor-infiltrating T cells. As a result, cancers with high mutation rates are more responsive to checkpoint blockade therapies. Immune recognition of neoantigens in cancers with relatively low to moderate mutational load as in multiple myeloma, is therefore considered less likely, thus limiting the potential application of mutanome-targeted immunotherapy. However, recent studies in several cancers with low mutational load have contradicted this hypothesis. Here our results confirm that neoantigens identified in multiple myeloma are immunogenic and can elicit T-cell–specific responses. Our findings are particularly relevant for relapsed multiple myeloma, because these patients are immunocompromised from disease infiltration of marrow and immunosuppression from multiple lines of therapy. In addition, we also highlight that the mutation burden and neoantigen burden increases in relapsed patients with multiple myeloma compared with newly diagnosed patients. Further, shared neoantigens are predicted in oncogenic driver genes in newly diagnosed and relapsed patients with multiple myeloma. Our data suggest that expansion of neoantigen specific T-cell responses may serve as direct pharmacodynamics biomarkers of immunotherapeutic interventions in myeloma and shared neoantigens could be targeted for "off-the-shelf" approaches.

been obtained. DNA and RNA from 92 relapsed patients with multiple myeloma were extracted from sorted $CD138^+$ cells from bone marrow aspirates performed at Mt. Sinai (New York, NY). At the time of sample collection, all patients had relapsed following at least five lines of therapy including autologous stem cell transplantation (ASCT). Patient data were collected retrospectively from clinical records. RNA-seq and WES data from 92 newly diagnosed patients with multiple myeloma enrolled in the CoMMpass study was provided by Multiple Myeloma Research Foundation (MMRF).

Detection of somatic mutations, HLA typing, and epitope prediction by next-generation sequencing

DNA and RNA from 92 relapsed patients with multiple myeloma were extracted from sorted $CD138^+$ cells from bone marrow aspirates performed at Mt. Sinai. At the time of sample collection, all patients had relapsed following at least five lines of therapy including ASCT. The exome capture for DNA sequencing was carried out using the Agilent human whole-exome SureSelect assay. RNA-seq libraries were prepared using Illumina mRNA-seq protocol. All libraries were sequenced on an Illumina HiSeq2500 to generate 100 nucleotide reads. Raw fastq files from 92 newly diagnosed patients with multiple myeloma were downloaded from IA7 release of MMRF CoMMpass study. Whole-exome sequence (WES) data were mapped to human reference genome by Burrows-Wheeler Aligner software (14) and somatic missense variants were detected using MuTect (15). Variants were called if there were more than five variant reads, a minimum of 10% variant allele frequency (VAF), and less than 1% VAF in the normal DNA. We restricted our neoantigen prediction to missense mutations as they account for majority of somatic mutations identified and excluded other types of rare mutations such as frame shifts, NeoORFs/indels. RNA-seq libraries were prepared using Illumina mRNA-seq protocol. RNA reads were aligned to human reference genome (hg19) and assembled into transcripts using Bowtie–TopHat–Cufflinks (16). Expression was evaluated by determining the fragment per kilobase per million reads (FPKM) values from the RNA-seq analysis. Four-digit HLA class I (HLA-A, HLA-B, and HLA-C) alleles of each patient were determined from RNA-seq using Seq2HLA (17). The identified mutations led to candidate antigenic peptides that were filtered by tumor expression level (FPKM >2) using RNA-seq data. The Immune Epitope Database analysis resource tool NetMHCpan (18) was used to predict MHC class I binding of 8- to 11-mer mutant peptides to the patients' HLA-A, HLA-B, and HLA-C alleles. Candidate peptides with an IC_{50} value less than 500 nmol/L were considered strong binders. Peptides were custom synthesized at JPT with high purity of >90%.

Analysis of T-cell responses by intracellular cytokine staining

PBMC (fresh or thawed) was stimulated with specific and nonspecific peptides on day 1 and cultured for 14 to 21 days along with IL2 (R&D Systems, 202-IL-010) and IL7 (R&D Systems, 207-IL-005). On day 14 or 21, cells were pulsed with 1 μg/mL specific peptide or control peptides from JPT Peptide Technologies (CEFT-positive control pool of 27 peptides selected from defined HLA class I and II restricted T-cell epitope from Cytomegalovirus, Epstein–Barr virus, Influenza virus, or Clostridium tetani, MOG-negative control pool of 29 peptides derived from a peptide scan through myelin-oligodendrocyte glycoprotein (MOG) of Homo sapiens, phorbol 12-myristate 13-acetate (PMA; Sigma Aldrich, P1585) and Ionomycin (Sigma Aldrich, I3909) peptide for 6 hours at 37°C, and then washed two to three times prior to the start of the staining. Cells undergoing intracellular staining were treated with monensin (Golgi Stop; BD Biosciences, 554724) and brefeldin A (Golgi Plug; BD Biosciences, 555029) to block cytokine secretion. Labeling of dead cells, fixation, and permeabilization were performed as described previously (19). Cells were surface stained with anti-CD4 (BD Biosciences, 555346), anti-CD8 (BD Biosciences, 341051) for 30 minutes at 4°C, or, following permeabilization, with anti-CD3 (BD Biosciences, 562280), anti-IL2 (BD Biosciences, 559334), PE Rat IgG2a κ isotype control (BD Biosciences, 559317), anti-TNFα (BD Biosciences, 557647), PE-Cy7 mouse IgG1 κ isotype control (BD Biosciences, 557646), anti-IFNγ (Biolegend, 502520) and Alexa Fluor 700 mouse IgG1 κ isotype control (Biolegend, 400143) for 30 minutes at room temperature. Cells were finally resuspended in 250 μL 1% paraformaldehyde and filtered prior to acquisition on a flow cytometer (BD Biosciences). Compensation was performed using tubes of CompBeads (BD Biosciences, 552844) individually stained with each fluorophore and compensation matrices were calculated with FACSdiva. Data were analyzed using FlowJo software version 10 (TreeStar). T-cell reactivity for every neo-epitopes was validated by ≥2 independent experiments.

Generation of MHC tetramers and flow cytometry analysis

As described previously (20), biotinylated HLA monomer HLA-A*03:01 was loaded with UV-cleavable epitopes produced by the Bhardwaj Laboratory. Potential HLA-A3-binding (predicted IC_{50} < 500 nmol/L by netMHC) neoantigen peptides from a patient with HLA-A3 were synthesized (JPT Peptide Technologies),

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exchanged, and multimerized. Briefly, HLA loaded with UV-sensitive peptide monomers were subjected to UV light in the presence of 10 μmol/L individual candidate synthetic peptide for 1 hour on ice. Then, the monomers were tetramerized in the presence of fluorescent (PE or APC) streptavidin (Thermo Fisher Scientific, S886 and S868) and kept at 4°C for further use. Prior to tetramer analysis, T cells were incubated in PBS containing 5% FCS and Dasatinib (50 nmol/L; Selleck Chemicals, S1021) for 30 minutes at 37°C to enhance tetramer binding. Between 2×10^4 and 5×10^4 cells were stained with 1 ng/μL MHC tetramer in PBS containing 5% FCS for 45 minutes at room temperature. Following two washes in PBS, immunofluorescence was analyzed as the relative log fluorescence of live cells gated on the on populations of cells with relatively low levels of forward and side scatter. Immunofluorescence of the gated lymphocyte population was measured using a BDFortessa flow cytometer. Fluorophore-labeled antihuman CD8 (clone SK1), CD4 (clone L200) antibodies were purchased from BD Biosciences.

Sorting and T-cell expansion

As described previously (20), following 1 hour incubation with fluorophore-conjugated Abs or tetramers, T cells were isolated using a FACSAria cell sorter (BD Biosciences) and collected in sterile PBS containing 50% FCS. T cells were expanded to large numbers using a rapid-expansion protocol with IL2, anti-CD3 antibody, and irradiated feeder cells.

T-cell receptor-β repertoire sequencing and clonality

DNA was extracted from peripheral blood mononuclear cells, then T-cell receptor-β (TCR-β) CDR3 regions were amplified and sequenced using ImmunoSEQ (Adaptive Biotechnologies) from 500 ng of DNA template. Clonality values were obtained through the ImmunoSeq Analyzer software. Independently, we evaluated differential abundance of TCRs between samples using two methods. First, TCRs for which with the total counts in the two samples that do not exceed 10 are filtered out. In method 1, counts were normalized to effective library size (total counts in the sample adjusted using RLE normalization as implemented in edgeR package; ref. 21). Variance was estimated using negative binomial model with over dispersion parameter phi = 0.011 (estimated from replicate samples). Normalized counts are log transformed and Z test performed. In method 2, Fisher exact test comparing counts for a given TCR in the two samples and counts for nonproductive TCRs in the same samples was performed. Computed P values were adjusted using Benjamini–Hochberg (FDR) method (22). All computations were done using R.

Cytotoxic assay by fluorescent dye labeling

Cytotoxic assay was performed using by fluorescent dye labeling using CFSE, CTV (both from Molecular Probes, Invitrogen) kept in DMSO (Sigma, D2650) and stored at 20°C. T cells pulsed with target peptides or control peptides were labeled with either CFSE (Thermo Fisher Scientific, C34554) or CTV (Thermo Fisher Scientific, C34557) for proliferation tracking. For dye labeling of target cells, a final concentration of 500 nmol/L of each dye were used in 1 to 2 mL aliquots of cells with immediate vortexing to ensure rapid homogenous staining of cells. Target cells and effector cells were incubated in 96-well plate at 1:100 ratio for 10 minutes at 37°C and 5% CO_2. After labeling, cells were washed two times with RPMI1640 supplemented with 10% FCS. The labeled cells were then analyzed sing a BDFortessa flow cytometer. The following formula was used to calculate % specific killing:

$$\%\text{Specific killing} = (1 - \text{experimental ratio}/\text{control ratio}) \times 100.$$

Results

Relapsed patients with multiple myeloma have greater mutation burden and higher frequency of neoantigens as compared with newly diagnosed patients with multiple myeloma

We utilized a neoantigen discovery pipeline in newly diagnosed and relapsed multiple myeloma tumor samples from MMRF CoMMpass and Mount Sinai, respectively, to predict *in silico* patient-specific tumor mutations. Demographic information of the MMRF CoMMpass and Mount Sinai patients is provided in Supplementary Tables S1 and S2. A detailed schema outlining the pipeline and the methodologies followed in this study is shown in **Fig. 1A**. We investigated the distribution of the number of predicted peptides with high HLA binding affinity ($IC_{50} < 500$ nmol/L) across 92 newly diagnosed multiple myeloma and 92 relapsed patients with multiple myeloma with available HLA typing information. Consistent with the previously published study (23), we observed frequencies of up to several hundred nonsynonymous somatic mutations per patient in relapsed populations as well as in newly diagnosed patients, resulting in an average of 150 potential neoantigenic peptides (range 10–600) in individual patients. **Figure 1B** and **D** shows the number of nonsynonymous somatic mutations per megabase (Mb) and the predicted neoantigens with high binding affinity ($IC_{50} < 500$ nmol/L) in patients with multiple myeloma. Relapsed patients with multiple myeloma have greater mutation burden [median number of coding somatic mutations per megabase (MB) = 67] as well as predicted neoantigens (median number of neoantigens = 142) as compared with newly diagnosed patients (median number of coding somatic mutations per MB = 16, median number of neoantigens = 62; **Fig. 1C** and **D**). Our results confirm that potential neoantigens are detectable in both relapsed and newly diagnosed populations.

Neoantigens are found in recurrently mutated genes in both newly diagnosed and relapsed multiple myeloma

We first set out to identify most commonly recurrent somatic mutations in our 184 patients with multiple myeloma. We identified top 10 recurrent somatic mutations in both 92 relapsed and 92 newly diagnosed patients with multiple myeloma as shown in **Fig. 2A** and **B** and Supplementary Tables S3 and S4. $NRAS^{Q61R}$ was the top recurrent mutation in our relapsed patients with multiple myeloma, whereas $CDC27^{L85F}$ was the top recurrent mutation in our newly diagnosed patients with multiple myeloma. We next asked whether the frequency of recurrent somatic mutations relates to the frequency of immunogenic mutations (neoantigens) and what mutations have the most immunogenic potential. To address this, we first identified top immunogenic mutations (neoantigens) in relapsed and newly diagnosed patients with multiple myeloma. The top 10 observed immunogenic mutations in relapsed and newly diagnosed patients with multiple myeloma are shown in **Fig. 2C** and **D**. For the majority of the identified neoantigens, we observed that mutations in the same gene are not shared between patients and are highly patient-specific. For example, immunogenic mutation in *PKD1* gene was observed in 14 relapsed patients but none of them shared the same variant effect. We observed a total of 18,636 somatic mutations and a total of 6,330 immunogenic

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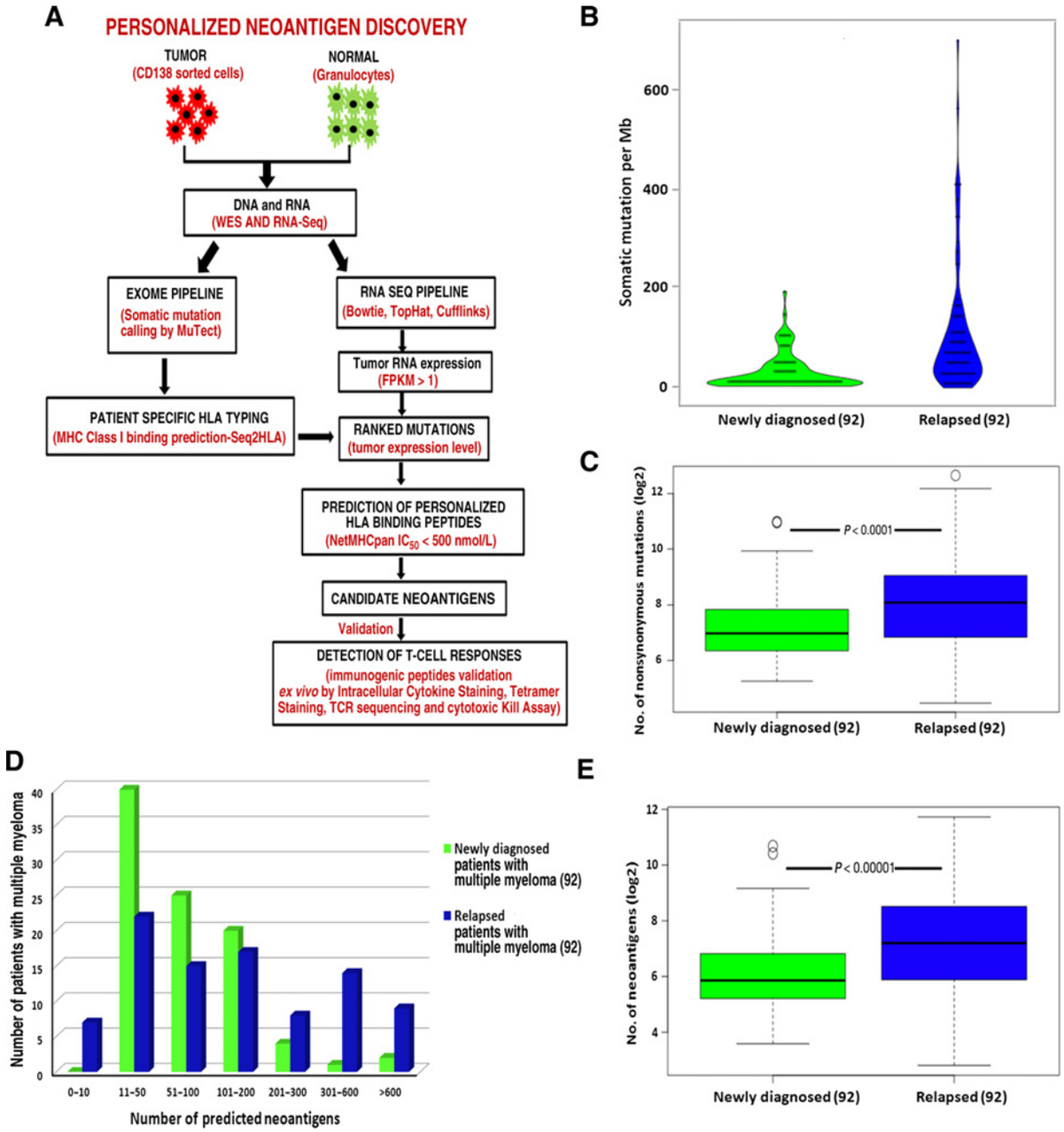

Figure 1.

High frequency of neoantigens observed in relapsed patients with myeloma as compared with newly diagnosed patients with multiple myeloma. **A**, Neoantigen discovery pipeline used in this study. **B,** Distribution of mutational burden [i.e., number of somatic mutations per megabase (Mb) detected in newly diagnosed and relapsed patients with multiple myeloma from WES data]. **C,** High mutational load in relapsed patients with multiple myeloma as compared with newly diagnosed myeloma patients ($P < 0.0001$, Wilcoxon rank sum test). **D,** Frequency of predicted neoantigens in newly diagnosed and relapsed patients with multiple myeloma. **E,** High frequency of neoantigen load in relapsed patients with multiple myeloma as compared with newly diagnosed patients with myeloma ($P < 0.0001$, Wilcoxon rank sum test).

mutations in 92 relapsed patients with multiple myeloma. Similarly, we observed a total of 17,788 somatic mutations and a total of 5,194 immunogenic mutations in 92 newly diagnosed patients with multiple myeloma. We compared our frequency of immunogenic mutations with that of the frequency of somatic mutations and found that 81 immunogenic mutations in relapsed and 60 immunogenic mutations in newly diagnosed patients (Supplementary Tables S5 and S6) were distinct from our somatic mutations in multiple myeloma. Among the top 10 recurrent somatic mutations (**Fig. 2A** and **B**), we found *NRAS* gene in relapsed patients and *KRAS* gene in newly diagnosed patients to be highly immunogenic as they overlapped with the top 10 immunogenic mutations (**Fig. 2C** and **D**). Notably, only two potential

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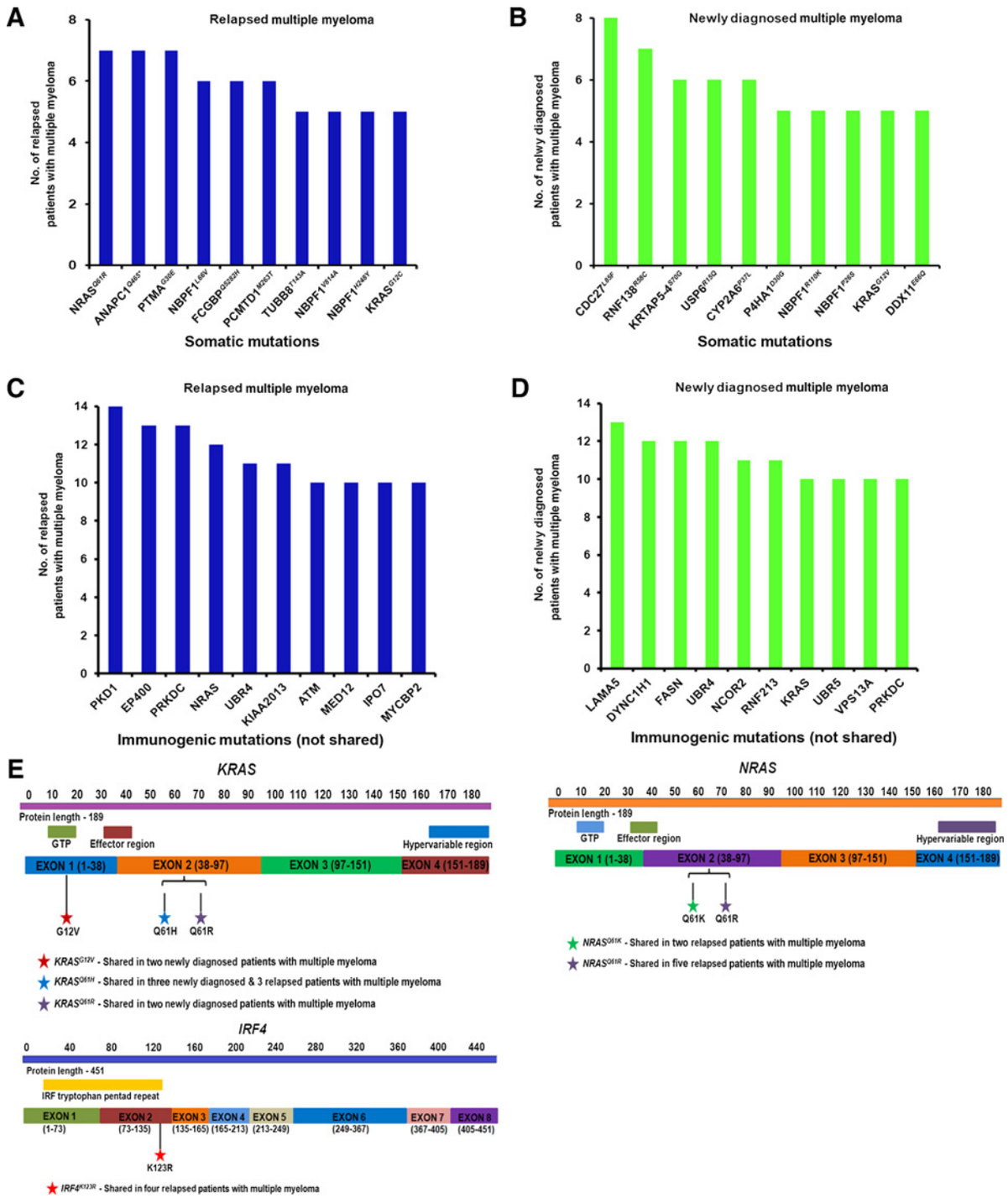

Figure 2.

Neoantigens are observed in recurrently mutated multiple myeloma genes. **A,** The top 10 recurrent somatic mutations observed in 92 relapsed patients with multiple myeloma. *NRAS*^{Q61R} was the top recurrent mutation in our relapsed patients with multiple myeloma. **B,** The top 10 recurrent somatic mutations observed in 92 newly diagnosed patients with multiple myeloma. *CDC27*^{L85F} was the top recurrent mutation in our newly diagnosed patients with multiple myeloma. **C,** The top 10 most frequently observed immunogenic mutations in relapsed patients with multiple myeloma. Mutated genes that could yield potentially immunogenic neoantigens in relapsed patients with multiple myeloma were *PKD1, EP400, PRKDC, NRAS, UBR4, KIAA2013, ATM, MED12, IPO7*, and *MYCPB2*. For the majority of the identified neoantigens, we observed that mutations in the same gene are not shared between relapsed patients with multiple myeloma and are highly patient-specific **D,** The top 10 most frequently observed immunogenic mutations in newly diagnosed patients with multiple myeloma. In newly diagnosed patients with multiple myeloma *LAMA5, DYNC1H1, FASN, UBR4, NCOR2, RNF213, KRAS, UBR5, VPS13A*, and *PRKDC* were identified. (*Continued on the following page.*)

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neoantigens, *PRKDC* and *UBR4* overlapped between both the relapsed and newly diagnosed multiple myeloma populations. Functionally, protein kinase, DNA-activated, catalytic polypeptide (PRKDC) has a major role in nonhomologous end joining (NHEJ) DNA repair in cancer cells (24). Ubiquitin N-recognin domain-containing E3 ligase 4 (UBR4) is an E3 ubiquitin ligase that functions as an adaptor for other E3 ubiquitin ligases and essential for proteasomal, autophagosomal, and lysosomal degradation of several cytoplasmatic and membrane proteins (25).

Next, we examined two previously published studies on mutational profiles in multiple myeloma for overlap with our immunogenic neoantigens. The mutational landscape of multiple myeloma was published by the Multiple Myeloma Research Consortium (MMRC) with the most commonly recurrent mutations of 11 genes seen in 203 multiple myeloma patient samples (26). Recently, 63 oncogenic driver genes (27) were identified by the Myeloma Genome Project (MGP) in 1,273 newly diagnosed patients with multiple myeloma. We observed that neoantigens from our dataset overlapped the known recurrent (90%, 10/11 genes in MMRC) and oncogenic driver mutations (79%, 50/63 genes in MGP) in both relapsed and newly diagnosed patients with multiple myeloma (Supplementary Table S7).

We next sought to identify whether oncogenic driver mutations shared across patients could also be immunogenic (shared neoantigens). Our analysis revealed shared neoantigens in *NRAS, KRAS, IRF4* genes in relapsed patients and *KRAS* gene in newly diagnosed patients. Interestingly, we observed that 5 relapsed patients shared $NRAS^{Q61R}$ mutations, 4 relapsed patients shared $IRF4^{K123R}$ mutations, 3 relapsed patients shared $KRAS^{Q61H}$ mutations, and 2 relapsed patients shared $NRAS^{Q61K}$ mutations (**Fig. 2E**). Similarly, we observed that 3 newly diagnosed patients shared the $KRAS^{Q61H}$ mutations, 2 newly diagnosed patients shared $KRAS^{G12V}$ neoantigenic mutations, and 2 newly diagnosed patients shared $KRAS^{Q61R}$ neoantigenic mutations (**Fig. 2E**). Our results suggest that there may be multiple immunogenic neoepitopes derived from recurrently mutated genes that could be effective targets for immunotherapy in myeloma.

We next asked whether the number of immunogenic mutations (neoantigens) differ between multiple myeloma molecular subgroups/cytogenetic abnormalities in our 184 patients with multiple myeloma. Each patient in this study was assigned to one of the 10 classes defined by MMNet, our previously published network model of multiple myeloma (28) based on the MMRF CoMMpass cohort, using a support vector machine classifier trained on the 450 patients in MMNet. We observed the enrichment of neoantigens to various multiple myeloma molecular subgroups/cytogenetic abnormalities in 57 of 92 relapsed multiple myeloma (62%) and 55 of 92 newly diagnosed patients (60%; Supplementary Tables S8 and S9). However, we did not find any statistically significant correlation with multiple myeloma molecular subgroups/cytogenetic abnormalities with respect to high or low immunogenic mutation burden.

PRKDC neoantigen-specific T-cell expansion is associated with complete response to combination checkpoint inhibitor-based therapy in a refractory patient with multiple myeloma

Although mutation and neoantigen load have been previously correlated with prolonged survival in newly diagnosed multiple myeloma (29) setting, the relationship between predicted neoantigens and their functional immunogenicity has not been investigated in the relapsed setting. To this end, we validated the neoantigenic T-cell responses in three relapsed patients with multiple myeloma in this study using a combination of coculture and TCR-sequencing techniques and correlated with clinical response. Supplementary Tables S10 and S11 show the total number of nonsynonymous somatic mutations, HLA class-I haplotypes and number of predicted and validated neoepitopes in these patients. A 63-year-old patient with multiple myeloma with relapsed myeloma presented after four lines of chemotherapy including bortezomib, lenalidomide, cyclophosphamide, dexamethasone, carfilzomib, and pomalidomide. The patient had high-risk cytogenetics including t(4;14) translocation, deletion of *TP53*, and complex karyotype (>5 abnormalities), and a high recurrence score by gene expression profiling (MyPRS score >67; **Fig. 3A**; Supplementary Table S12). Given the progressive nature of the disease and lack of CAR-T or other active investigational options at that time for the patient, we attempted to salvage the patient using an immunotransplant approach. Multiple studies have demonstrated that effector CD8 T cells undergo robust homeostatic proliferation/activation in the lymphopenic environment and that PD-1 blockade during the expansion phase potentiates tumor-reactive T-cell responses and tumor eradication (30–32). We treated the patient using dual checkpoint inhibition with anti-CTLA-4 (ipilimumab) and anti-PD-1 (nivolumab) treatment to expand tumor-specific T cells, followed by mini-ASCT with high-dose alkylators Melphalan-100 mg/m^2 and BCNU 100 mg/m^2 to induce "immunogenic" cell death, followed by a second dose of ipilimumab and nivolumab (**Fig. 3A**). Therapy was then continued with nivolumab and lenalidomide resulting in a stringent complete remission (sCR). To evaluate neoantigen-specific T-cell activation, we conducted RNA and exome sequencing (WES) on CD138$^+$ selected tumor cells from a bone marrow aspirate obtained prior to dual checkpoint blockade and immunotransplant to identify immunogenic neoantigens. The number of nonsynonymous mutations and the predicted immunogenic neoantigens are shown in **Fig. 3B** and Supplementary Fig. S1. PBMC were isolated from pre- and post-immunotransplant blood to test T-cell responses. The top 20 mutated peptides (Supplementary Tables S13 and S14) based on high HLA-binding affinity and RNA expression were custom synthesized and used for *ex vivo* validation by intracellular cytokine staining assay (ICS).

We detected expansion of neoantigen specific T-cell activation in the post anti-CTLA-4 + anti-PD-1-treated PBMCs (**Fig. 3C**; Supplementary Figs. S2A–S2C). Significantly, this patient demonstrated the highest T-cell activity against the neoantigen $PRKDC^{Q823E}$ restricted to HLA-A03:01. We observed significant IFNγ, TNFα, and IL2 production (increase from 0.19% to 5.19%, 0.38% to 3.01%,

(*Continued.*) For the majority of the identified neoantigens, we observed that mutations in the same gene are not shared between newly diagnosed patients with multiple myeloma and are highly patient-specific **E,** Shared neoantigens identified in patients with multiple myeloma were found in recurrently mutated genes *KRAS, NRAS*, and *IRF4*. Shared neoantigens in *KRAS, NRAS*, and *IRF4* and their corresponding mutants in observed in both relapsed and newly diagnosed patients with multiple myeloma are shown in the plot. We observed that five relapsed patients shared $NRAS^{Q61R}$ mutations (strong binding, $IC_{50} < 150$ nmol/L), four relapsed patients shared $IRF4^{K123R}$ mutations (very strong binding, $IC_{50} < 50$ nmol/L), three relapsed patients shared $KRAS^{Q61H}$ mutations (strong binding, $IC_{50} < 150$ nmol/L), and two relapsed patients shared $NRAS^{Q61K}$ mutations (strong binding, $IC_{50} < 150$ nmol/L) (**Fig. 2c**). Similarly, we observed that three newly diagnosed patients shared the $KRAS^{Q61H}$ mutations (strong binding, $IC_{50} < 150$ nmol/L), two newly diagnosed patients shared $KRAS^{G12V}$ neoantigenic mutations (very strong binding, $IC_{50} < 50$ nmol/L), and two newly diagnosed patients shared $KRAS^{Q61R}$ neoantigenic mutations (intermediate binding, IC_{50} of 150–250 nmol/L).

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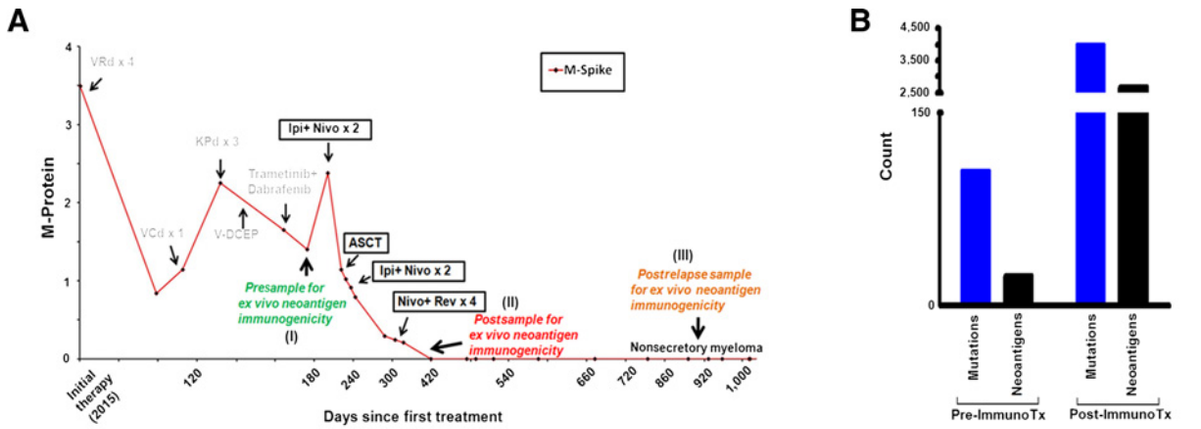

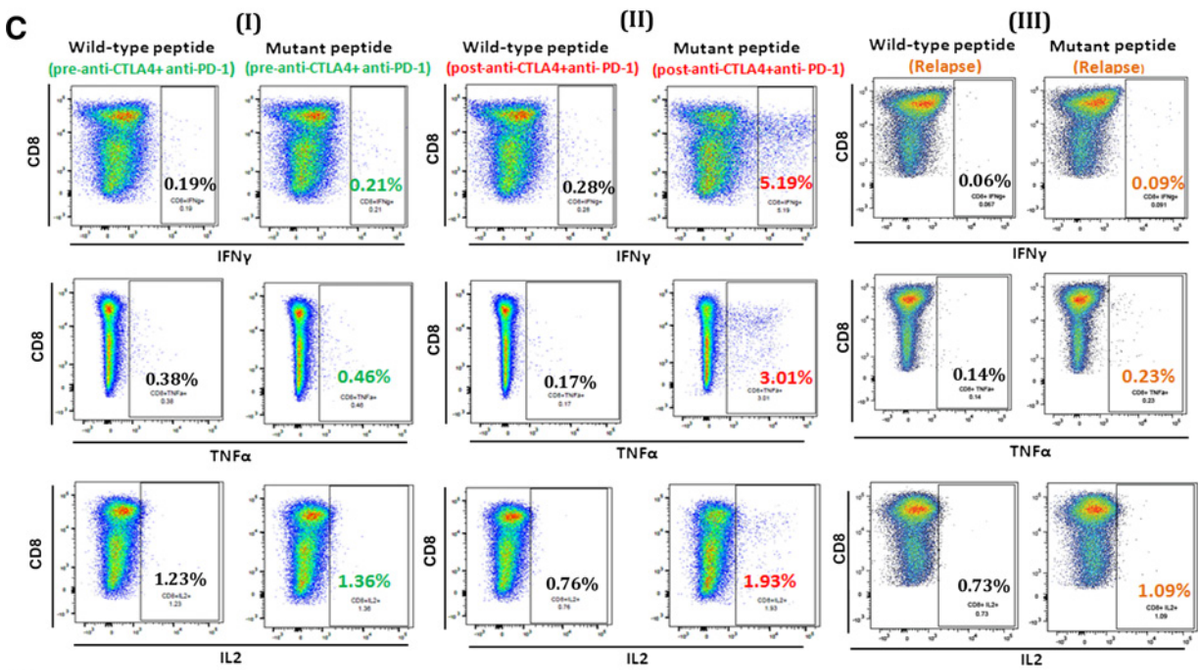

D

	Pre ImmunoTx	Response after ImmunoTx	Relapse
PRKDC RNA expression (FPKM)	7.2	5.4	3.5
Clinical response	No	Yes	No
T-cell response to Neoantigen	No	Yes	No

Figure 3.

Checkpoint-based inhibitor therapy elicits *PRKDC*-neoantigen specific T-cell response in a primary refractory patient with multiple myeloma. **A,** Timeline of clinical response of patient with multiple myeloma to dual checkpoint inhibitor (anti-CTLA4 + anti-PD-1) therapy. **B,** The number of nonsynonymous mutations and the predicted immunogenic neoantigens. **C,** $CD8^+$ T-cell response to neoantigen peptide (PRKDC) measured by IFNγ, TNFα, and IL2 pre- and post-dual checkpoint inhibition (anti-CTLA4 + anti-PD-1). **D,** The clinical response and T-cell response to neoantigen of this patient prior to and after immunotransplant and checkpoint inhibition.

and 1.23% to 2.05% as compared with wild-type peptide) by tumor derived $CD8^+$ T cells in response to stimulation. No T-cell response was evident in the pre-anti-CTLA-4 + PD-1-treated cells. However, following discontinuation of treatment with the checkpoint blockade therapies the patient relapsed after 15 months, at which point the peripheral blood demonstrated a decrease in the $PRKDC^{Q823E}$-specific T-cell responses, now at a level similar to the wild-type peptide (**Fig. 3C**; Supplementary Figs. S2A–S2C). It must be noted that the sequencing data captured from the bone marrow biopsy post relapse of this patient confirmed the presence of the $PRKDC^{Q823E}$ neoantigen but at a lower expression level (FPKM = 3.5) compared with the early time point (FPKM = 7.2). The clinical

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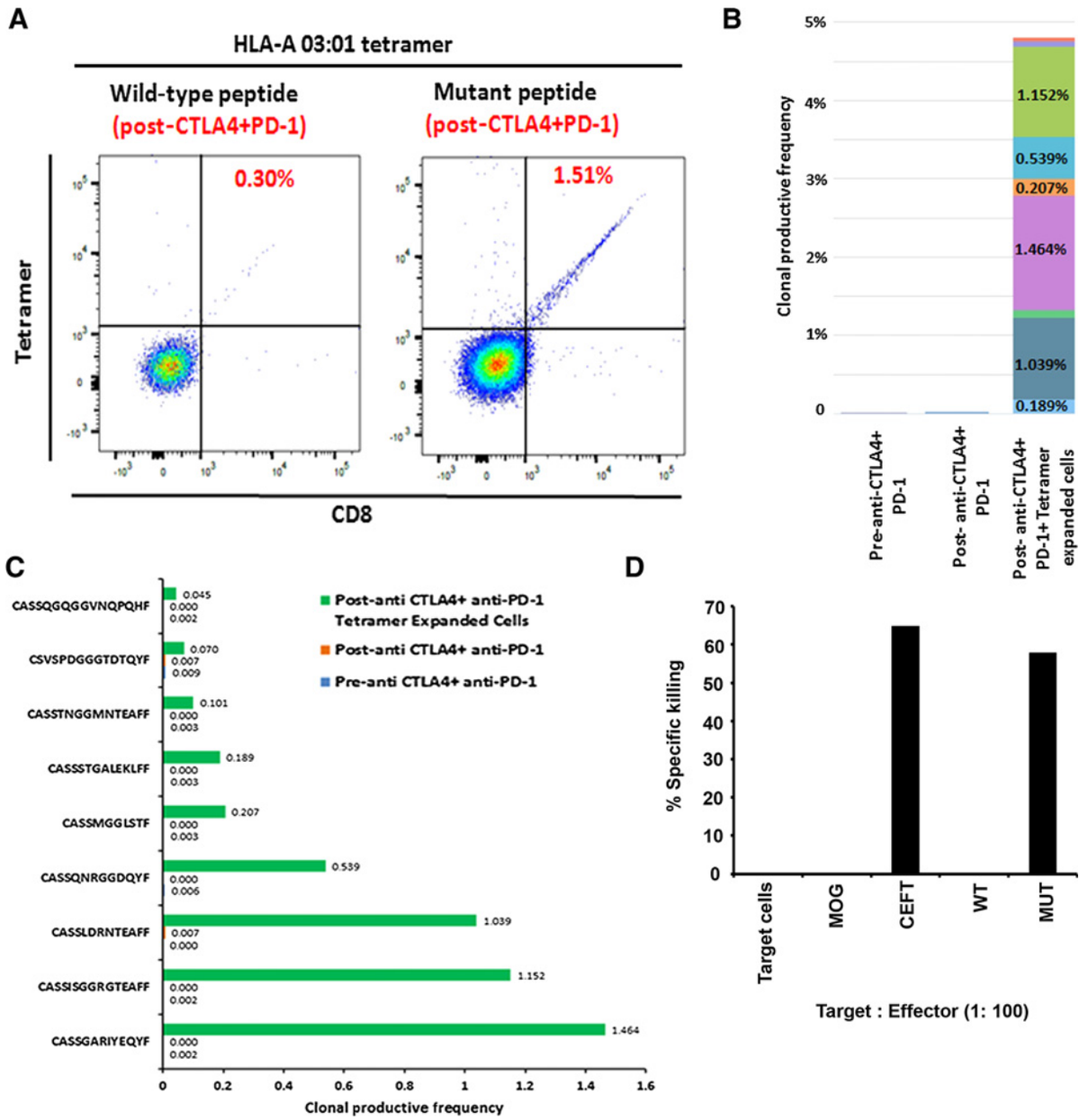

Figure 4.

Assessment of CD8$^+$ T-cell responses to *PRKDC* neoantigen using MHC class I tetramer, TCR sequencing, and cytotoxic T-cell killing. **A,** Validation of HLA-A*03:01 tetramer with neoantigen peptide PRKDC from the patient with multiple myeloma. Peripheral lymphocytes obtained after checkpoint blockade treatment was incubated with pMHC tetramer (HLA-A03:01). There was a significant increase in tetramer positive CD8$^+$ T cells with the mutant peptide (1.51%) as compared with the wild-type peptide (0.3%). **B,** TCR-sequencing reveals increase in oligoclonal expansion after dual checkpoint inhibition. Tetramer-binding T cells were subjected for sequencing of TCR-β chains. We observed peripheral T-cell expansion of the top 10 most dominant intratumoral clones, with the most dominant clones reaching a 1.464% and 1.152% increase in abundance in the *ex vivo* expanded cells after immune checkpoint therapy than at the time of pretreatment (cells without *ex vivo* expansion). **C,** The specific TCR-β clones are shown in the bar plot. The patient had a high proportion of preexisting dominant clones after the administration of checkpoint therapy compared with the low proportion of such preexisting dominant clones before ASCT and checkpoint therapy. **D,** Neoantigen specific CD8$^+$ T-cell cytotoxicity. Antigen-specific effector T cells were expanded with peptides [Mutant (Mut), Wild Type (WT), CEFT (+ ctrl), and MOG (−ve ctrl)] for 10 days following which patients target (T) cells were incubated with expanded effector (E) T cells with T:E ratio of 1:100. Shown is the antigen-specific lysis in %, which is the specific lysis specific to appropriate peptides.

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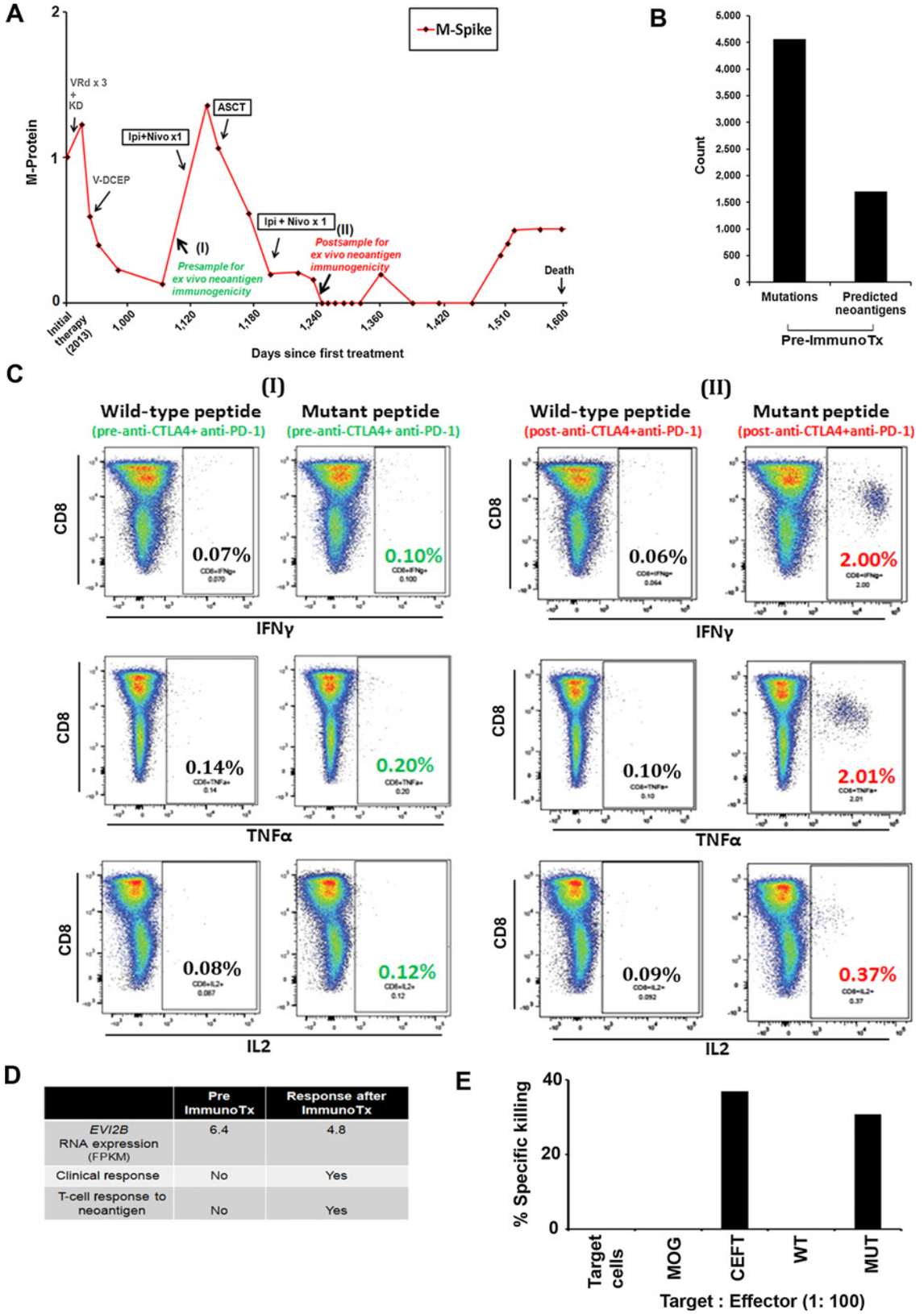

	Pre ImmunoTx	Response after ImmunoTx
EVI2B RNA expression (FPKM)	6.4	4.8
Clinical response	No	Yes
T-cell response to neoantigen	No	Yes

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response and T-cell response to neoantigen of this patient prior and after immunotransplant and checkpoint inhibition is tabulated in **Fig. 3D**. Our results are the first demonstration and validation of a mutation-derived neoantigen-specific T-cell recognition and activation associated with clinical response in multiple myeloma.

TCR-sequencing confirms increases in *PRKDC*-specific tetramer-positive CD8 T cells that are cytotoxic for antigen-bearing target cells

To confirm the specificity of the $PRKDC^{Q823E}$ neoepitope-specific T cells from the endogenous T-cell repertoire, we used peptide-MHC-I (pMHC) complex tetramers to detect high-frequency populations of neoepitope-specific T cells following antigen-induced clonal expansion. Peripheral lymphocytes obtained after checkpoint blockade treatment were incubated *in vitro* with pMHC tetramer (HLA-A03:01) that had been labeled with fluorophores (see Materials and Methods), thus allowing separation of antigen-specific T cells. **Figure 4A** depicts the flow cytometric analysis of both *PRKDC* mutant and wild-type neoepitope-specific $CD8^+$ T cells in the patient's mononuclear cells following pMHC-I (HLA-A03:01) tetramer-based enrichment. We observed a significant increase in tetramer positive $CD8^+$ T cells with the mutant peptide (1.51%) as compared with the wild-type peptide (0.3%; **Fig. 4A**). Further, we sorted and expanded these mutated antigen-specific tetramer-positive T cells by *in vitro* stimulation with feeder cells. Ten days later, T cells were assessed for tetramer binding and again the levels of tetramer-specific T cells detected were significant with the mutated peptide tetramer, which bound to 3.3% of the $CD8^+$ T cells from the patient (Supplementary Fig. S3).

We hypothesized that neoantigens could lead to an increase in clonal T-cell populations after stimulation with the peptides *ex vivo*, and that this expansion could be detected in the patient's blood. Towards this end, the tetramer-binding T cells were subjected to sequencing of TCR-β chains. We also profiled this patient's T cells by TCR repertoire sequencing of peripheral blood cells collected before and after the dual checkpoint blockade therapy (cells without *ex vivo* expansion) to identify specific TCR clones that could be induced or expanded following treatment. In addition, approximately 500 sorted tetramer-positive T cells were cultured in the presence of irradiated feeder cells, which led to 500-fold expansion after 14 to 21 days. The resultant 250,000 tetramer-positive T cells were also subject to TCR-sequencing along with the blood cells collected before and after double check point therapy and analyzed for clonal frequency. We observed peripheral T-cell expansions of the top 10 most dominant intratumoral clones, with the most dominant clones reaching a 1.464% and 1.152 % increase in abundance in the *ex vivo* expanded cells after immune checkpoint therapy than at the time of pretreatment (cells without *ex vivo* expansion; **Fig. 4B** and **C**). Shannon diversity indices of the productive clones in the neoepitope-specific tetramer-positive T cells were smaller (2.7) than those of the pre- (8.8) and post-checkpoint blockade (7.3) treatments. This decrease in the T-cell population diversity is expected because the clones selectively bind to tetramer. Also, reactive clones are expected to selectively expand when they undergo stimulation with the peptide, which is done before tetramer binding. Our findings indicate that T cells specific for mutated tumor antigens can be isolated and expanded from the peripheral blood of patients with myeloma.

Next, to determine if antigen-specific effector T cells were cytotoxic to HLA-matched cells, we expanded PBMCs from the patient with peptides mutant (MUT), wild type (WT), CEFT (positive control pool of 27 peptides selected from defined HLA class I and II restricted T-cell epitope from cytomegalovirus, Epstein–Barr virus, influenza virus, or *Clostridium tetani*) and MOG (negative control pool of 29 peptides derived from a peptide scan through MOG of *Homo sapiens*) for 10 days following which patients autologous PBMCs were used as target (T) cells and incubated with expanded effector (E) T cells with T:E ratio of 1:100. Because autologous myeloma cells do not survive in culture for a 10-day assay outside the functional bone marrow, we used the patients PBMCs as HLA-matched target cells for the assay. Shown in **Fig. 4D** is the specific lysis of target cells following exposure of antigen-presenting cells to cognate peptides. *PRKDC* effector T cells exerted significantly higher cytotoxic activity against mutant peptide against target cells than wild-type peptide. Our results demonstrate that $PRKDC^{Q823E}$ neoantigen-specific $CD8^+$ T cells can be expanded with checkpoint inhibitor therapy and that they can recognize autologous cells presenting the specific mutant peptide.

Checkpoint blockade enhances *EVI2B* neoantigen-specific T-cell responses in a relapsed patient with multiple myeloma

A 79-year-old patient with multiple myeloma relapsed after induction therapy with bortezomib, lenalidomide, dexamethasone (VRd) for three cycles followed by one cycle of carfilzomib, daratumumab (KD), and dexamethasone, cyclophosphamide, etoposide, and cisplatin in combination with bortezomib (V-DCEP). Similar to patient 1 reported above, we treated this patient using the immunotransplant approach with the patient entering complete remission. We used combination checkpoint inhibitor (anti-CTLA4 + anti-PD1) treatment followed by mini-ASCT and a second dose of combination checkpoint inhibitors (**Fig. 5A**; Supplementary Table S15). RNA-seq and WES data from the bone marrow biopsy of this patient received prior to checkpoint inhibitor therapy were analyzed to predict and synthesize immunogenic neoantigens. The number of nonsynonymous mutations and the predicted immunogenic neoantigens are shown in **Fig. 5B**. PBMC were isolated from pre and post-immunotransplant blood. When we interrogated the top 20 predicted neoantigens (Supplementary Tables S16 and S17), we found increased levels of neoantigen specific T-cell activation in the post anti-CTLA4 + PD-1-treated PBMCs (**Fig. 5C**; Supplementary Figs. S4A and S4B). Similar to patient 1, this patient also demonstrated the highest T-cell activity against the neoantigen $EVI2B^{G327A}$ restricted to HLA-A68:02. Functionally, *Ecotropic Viral Integration site 2B* (EVI2B), also known as CD361 is a common

Figure 5.

Checkpoint blockade therapy enhances *EVI2B*-neoantigen specific T-cell response in a relapsed patient with multiple myeloma. **A,** Timeline of clinical response of patient with multiple myeloma to dual checkpoint inhibitor (anti-CTLA4 + anti-PD-1) therapy. **B,** The number of nonsynonymous mutations and the predicted immunogenic neoantigens. **C,** $CD8^+$ T-cell response to neoantigen peptide (EVI2B) measured by IFNγ, TNFα, and IL2 pre- and post-dual checkpoint inhibition (anti-CTLA4 + anti-PD-1). **D,** The clinical response and T-cell response to neoantigen of this patient prior to and after immunotransplant and checkpoint inhibition. **E,** Neoantigen-specific $CD8^+$ T-cell cytotoxicity. Antigen-specific effector T cells were expanded with peptides [Mutant (Mut), Wild Type (WT), CEFT (+ ctrl) and MOG (-ve ctrl)] for 10 days following which patients target (T) cells were incubated with expanded effector (E) T cells with T:E ratio of 1:100. Shown is the antigen specific lysis in %, which is the specific lysis specific to appropriate peptides.

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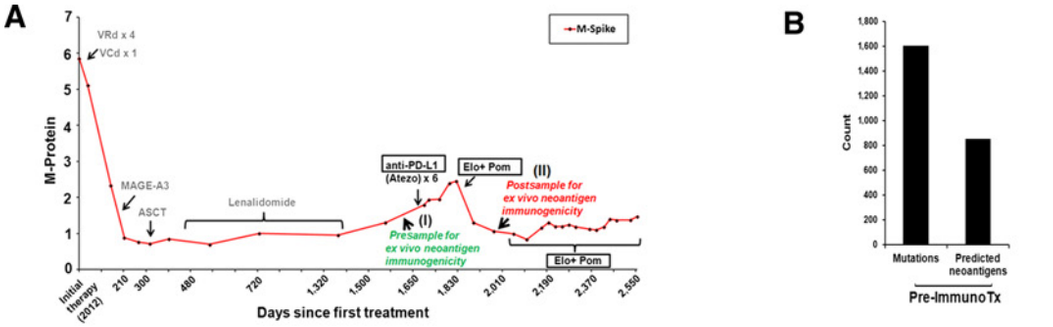
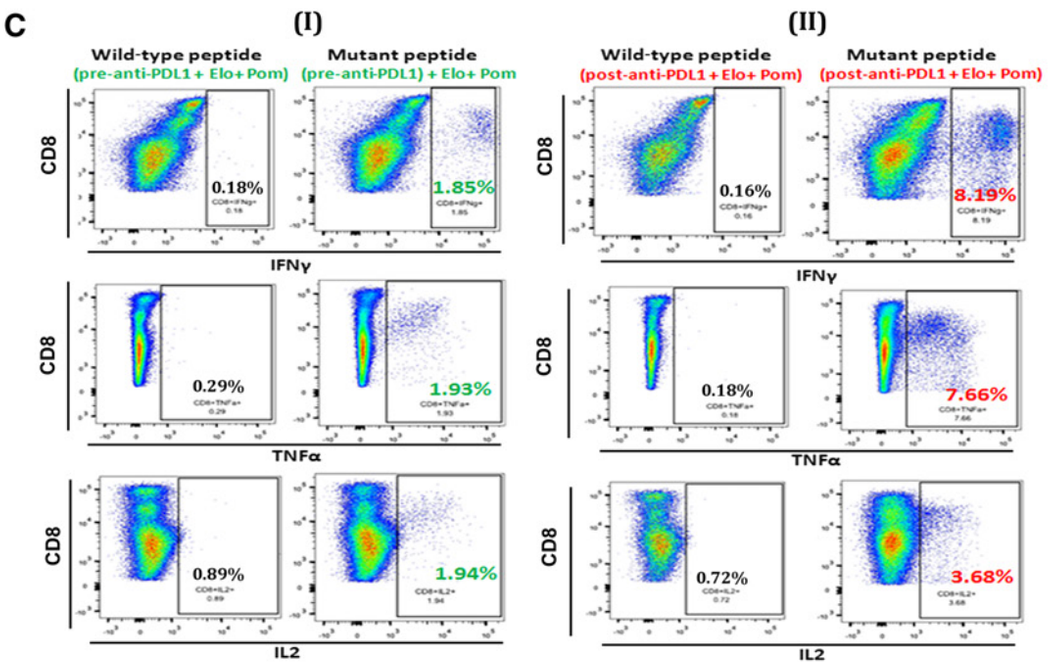
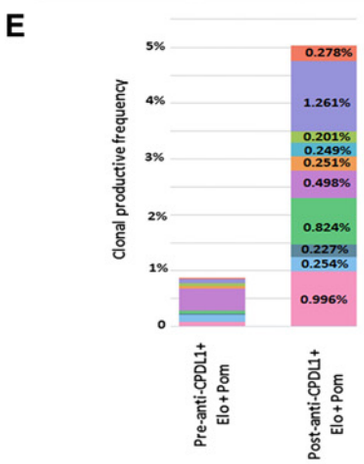
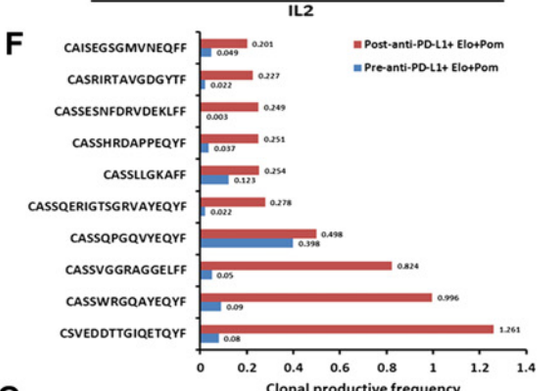


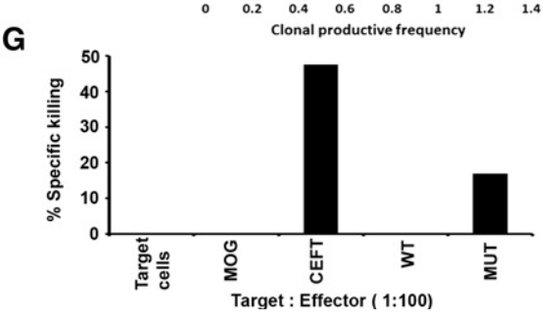


D

	Pre ImmunoTx	Response after ImmunoTx
S100A9 RNA expression (FPKM)	6.1	2.1
Clinical response	No	Yes
T-cell response to neoantigen	No	Yes







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viral integration site in retrovirally-induced murine leukemia with a physiological role in myeloid differentiation and functionality of hematopoietic progenitors (33). We observed significant IFNγ, TNFα, and IL2 production (increase from 0.09% to 2%, 0.14% to 2.01%, and 0.08% to 0.37% as compared with wild-type peptide) by tumor derived $CD8^+$ T cells in response to peptides. No T-cell response was evident in the pre-anti-CTLA4 + PD-1-treated cells. The clinical response and T-cell response to neoantigen of this patient prior to and after immunotransplant and checkpoint inhibition is shown in **Fig. 5D**.

Next, we evaluated the peptide-dependent cytotoxic activity in which the patients target (T) cells were incubated with expanded effector (E) T cells at a T:E ratio of 1:100. This analysis of effector T cells revealed cytotoxic activity exclusively against *EVI2B* mutant peptide and not against wild-type peptide (**Fig. 5E**). Our data from the above two relapsed patients with multiple myeloma treated with checkpoint blockade therapies confirm the immunogenicity of *in silico* predicted neoantigens.

Checkpoint blockade in combination with pomalidomide and elotuzumab treatment enhances *S100A9* neoantigen-specific T-cell response in a relapsed patient with multiple myeloma

Our initial observations from the above two cases were recapitulated in a patient treated with immunomodulatory therapies (pomalidomide and elotuzumab). This patient diagnosed with multiple myeloma was administered induction therapy with bortezomib, lenalidomide, dexamethasone (VRd) for four cycles and bortezomib, cyclophosphamide, dexamethasone (VCd) followed by ASCT and lenalidomide maintenance. Subsequently, the patient received anti-PD-L1 (Atezolizumab) upon relapse for 6 months on a clinical trial and on progression was administered elotuzumab (elo) and pomalidomide (pom; **Fig. 6A**; Supplementary Table S18).

Bone marrow aspirate was performed for $CD138^+$ selection followed by WES and RNA-seq prior to checkpoint blockade and immunotransplant to identify immunogenic neoantigens. The number of nonsynonymous mutations and the predicted immunogenic neoantigens are shown in **Fig. 6B**. PBMC were isolated from pre-anti-PD-L1+ elo+pom and post-anti-PD-L1+ elo+pom blood. The top 20 mutated peptides based on affinity and RNA expression were used for *ex vivo* validation for T-cell responses using patient's PBMCs (Supplementary Tables S19 and S20). Significantly, this patient demonstrated the highest T-cell activity against the top scoring neoantigen, that is T18N mutant of the *S100A9* gene, against the patients HLA-B44:03 (**Fig. 6C**; Supplementary Figs. S5A and S5B). We observed significant IFNγ, TNFα, and IL2 production (increase from 0.18% to 8.19%, 0.29% to 7.66%, and 0.89% to 3.68% as compared with wild-type peptide) by tumor-derived $CD8^+$ T cells in response to peptide tested with the post PD-L1 PBMCs. The pre-PD-L1 PBMCs also elicited $CD8^+$ T-cell responses, albeit at much lower levels (increase from 0.18% to 1.94%, 0.29% to 1.93%, and 0.89% to 1.94% as compared with wild-type peptide). Functionally, S100A9 a calcium binding protein A9 plays a prominent role in the regulation of immune response with the promotion of cytokines and chemokines and is upregulated in 1q21 amplified patients (34). The clinical response and T-cell response to neoantigen prior and post to checkpoint inhibition and elotuzumab plus pomalidomide treatments is shown in **Fig. 6D**.

We also profiled this patient's T cells for TCR repertoire sequences in peripheral blood cells (without *ex vivo* expansion) collected before and after atezolizumab and elotuzumab plus pomalidomide therapy. We observed peripheral T-cell expansion of the top 10 most dominant intratumoral clones, with the most dominant clones reaching a 1.261 % (15-fold) and 0.996 % (11-fold) increase in abundance in the blood post elo plus pom therapy than at the time of pre-atezolizumab (**Fig. 6E** and **F**). Next, antigen-specific effector T cells were expanded with peptides mutant (Mut), wild-type (WT), CEFT (+ve ctrl), and MOG (-ve ctrl) for 10 days following which patients target (T) cells were incubated with expanded effector (E) T cells. Similar to other 2 patients, there was a significant killing of target cells by the *S100A9* neoantigen-specific CD8+ effector T cells from the mutant peptide in the patient cells (**Fig. 6G**). In conclusion, our evaluation of 3 patients demonstrate that neoantigen-specific immunogenic T-cell responses can be detected and expanded in relapsed patients with multiple myeloma undergoing immunotherapy with combinations of immune checkpoint inhibitors, mAbs, and immunomodulatory drugs.

Discussion

Our results demonstrate that the mutation and neoantigen burdens increase in relapsed patients with multiple myeloma compared with newly diagnosed patients with multiple myeloma. In some respects, these findings align with the solid tumors, where somatic mutational and neoantigen burden have been shown to correlate with long-term benefit from checkpoint blockade therapies (4, 5). This reasoning predicts that immune recognition of neoantigens in tumors with relatively low mutational load would be unlikely, thus limiting the potential application of neoantigen-targeted immunotherapy. However, recent studies in several cancers with low mutational load have contradicted this hypothesis (35, 36). Luksza and colleagues (37) presented a neoantigen fitness model, in which the ability of neoantigens to activate T-cell recognition and the quality of the T-cell response are more important than neoantigen quantity in determining immune responses during tumor evolution. We found that some antigenic mutations, such as those in *PRKDC* and *UBR4*, are present in both newly diagnosed and relapsed multiple myeloma. We posit that neoantigens in relapsed myeloma may serve as targets for immunotherapy.

Figure 6.

Checkpoint blockade therapy in combination with pomalidomide and elotuzumab treatment elicits *S100A9* neoantigen-specific T-cell response in a relapsed patient with multiple myeloma. **A,** Timeline of clinical response of patient with multiple myeloma to checkpoint inhibitor anti-PD-L1 + elotuzumab and pomalidomide therapies. **B,** The number of non-synonymous mutations and the predicted immunogenic neoantigens. **C,** $CD8^+$ T-cell response to neoantigen peptide (S100A9) measured by IFNγ, TNFα, and IL2 pre- and post-dual treatment (anti-PD-L1 + elotuzumab and pomalidomide). **D,** The clinical response and T-cell response to neoantigen of this patient prior to and after checkpoint inhibition and elotuzumab plus pomalidomide treatments. **E,** TCR-sequencing reveals increase in oligoclonal expansion after anti-PD-L1 + elotuzumab and pomalidomide treatments. We observed peripheral T-cell expansion of a subset of the top 10 most dominant intratumoral clones, with the most dominant clones reaching a 1.261% and 0.996 % increase in abundance in the blood posttreatment (without *ex vivo* expansion). **F,** The specific TCR-β clones are shown in the bar plot. The patient had a high proportion of preexisting dominant clones after the administration of immunomodulatory therapies compared with the low proportion of such preexisting dominant clones before treatment. **G,** Neoantigen-specific $CD8^+$ T-cell cytotoxicity. Antigen-specific effector T cells were expanded with peptides [mutant (Mut), wild type (WT), CEFT (+ ctrl) and MOG (-ve ctrl)] for 10 days following which patients target (T) cells were incubated with expanded effector (E) T cells with T:E ratio of 1:100. Shown is the antigen specific lysis in %, which is the specific lysis specific to appropriate peptides.

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Our results using primary cells from relapsed patients with myeloma demonstrate that cellular immune responses against autologous neoantigens can be primed or expanded in the setting of combination immunotherapies. These combinations leveraged different agents to induce immunogenic cell death in tumor cells, inhibit central and/or peripheral tolerance, and mobilize adaptive immune components to positively affect critical nodes of the "cancer-immunity" cycle (38). Checkpoint inhibitors such as anti-CTLA-4, anti-PD-1, and anti-PD-L1 antagonize principle mechanisms of central and peripheral tolerance to promote T-cell activation and effector function (39). Pomalidomide and lenalidomide are commonly used in multiple myeloma therapy and target cereblon, an ubiquitously expressed E3 ligase protein (40). They have a broad range of immunomodulatory functions, including maturation of dendritic cells (41), promotion of antigen presentation (42), activation of B and T cells (43), and enhancement of NK-cell cytotoxicity and antibody-dependent cell-mediated cytotoxicity against myeloma cells (44). Elotuzumab, an immunostimulatory mAb targeting signaling lymphocytic activation molecule F7 (SLAMF7), showed activity in combination with lenalidomide in patients with relapsed or refractory multiple myeloma (45). This mAb can cross-link SLAMF7 on NK cells and potentiate their antitumor activity, which could contribute to immunogenic cell death and tumor antigen presentation to T cells (46). We showed that selected combination immunotherapeutic strategies are efficacious and associated with adaptive immune responses against multiple myeloma-associated neoantigen, even in heavily treated patients with impaired immunity, and that neoantigen-specific T cells are a direct biomarker of these activities. The challenge is to understand the biology that dictates successful combination immune therapy, as increased mortality risk was detected in phase III studies combining pembrolizumab (anti-PD-L1) with lenalidomide or pomalidomide in two halted multiple myeloma studies (NCT02576977, NCT02579863). Further clinical investigation testing safer alternatives should be supported by scrupulous safety monitoring and correlative studies to illuminate proper patient selection and optimal disease settings for these therapies.

Our data reveal a pathway in which neoantigens may be harnessed to salvage relapsed patients with multiple myeloma. Shared tumor-associated antigens, such as MelanA/MART-1 and gp100, are typically over expressed in tumor cells, but also exist in normal cells (47, 48). Our analysis revealed for the first time shared neoantigens are detectable in *NRAS, KRAS*, and *IRF4* genes in relapsed patients and in *KRAS* in newly diagnosed patients supporting the possibility of neoantigen-based vaccines in patients with multiple myeloma with these mutations. Although most mutation-derived neoantigens are highly specific to individual patients, we expect that targeting these shared neoantigens could be immediately applicable to a subset of patients with relapsed multiple myeloma. Recently, it was shown in metastatic colorectal cancer that $KRAS^{G12D}$ mutation can be successfully targeted by specific tumor-infiltrating lymphocytes (49). Therefore, our findings provide a rationale for a similar "off-the shelf" approach that could be implemented with the validation of these shared neoantigenic mutants in patients with multiple myeloma. Currently, several neoantigen vaccine trials are being implemented with promising early results (50). Towards this purpose, our colleagues are pursuing a clinical trial (PGV-001, NCT02721043) investigating the safety and immunogenicity of a personalized, multipeptide, neoantigen vaccine for the treatment of cancers including multiple myeloma (51).

Despite the fact that functional neoantigen-specific CD8 T-cell responses are generated *ex vivo*, the durability of T-cell responses clinically in patients may depend on the treatment and schedule of interventions. In this study, we demonstrated CD8+ T-cell responses at 200 days for patient 1, 240 days for patient 2 after dual checkpoint inhibition, and at 300 days for patient 3 after anti-PD-L1 treatment. In addition, longer follow up from our PGV-001 study, which uses a strategy of 10 vaccinations over 180 days with POLY-ICLC adjuvant will inform directly about the durability of responses. Preliminary data from the PGV-001 study demonstrates *ex vivo* responses to neoantigen peptides were undetectable at week 0 but were clearly evident at week 27 after vaccination (51). We believe these could potentially be sustained with booster strategies such as additional vaccines; immunomodulatory drugs or checkpoint inhibitors. Perspectively, we are testing various combinations of these strategies *in vitro* and intend to publish the results from PGV-001 personalized genomic vaccine study in the future.

Despite the rigid analyses, our study is not free from limitations. We restricted our neoantigen prediction to missense mutations as they account for majority of somatic mutations identified. Our strategy may have excluded other potentially immunogenic changes such as frame shifts, NeoORFs/indels, and gene fusions. Although results from cytotoxicity assays using primary myeloma cells as targets would provide stronger and more direct evidence of peptide neoantigen presentation, in this study we were limited in the number of primary cells that can be obtained in a bone marrow aspirate while the patients are in remission. An immunopeptidomic approach using peptide elution from HLA complexes and LC/MS would provide direct evidence of antigen presentation; however, this requires millions of cells and is unfortunately not feasible due to the limited number of primary cells obtained from the patients treated. To prove antigen processing, we could hypothetically take an antigen negative cell line and transfect it with a peptide sequence that could be processed and test antigen presentation with (HLA matched) allogeneic T-cell activation, unfortunately this would still be indirect evidence of antigen presentation. In addition, there is concern for high background from allogeneic T cells. Another potential experiment is to perform a down-titration of antigen reactive T cells to reach levels similar to native endogenously presented immunogenic peptides such as NY-ESO-1. However, this too would require additional primary cells which we cannot obtain as the patients are in remission.

Overall, the data presented here support that mutational burden is associated with increased neoantigen frequency in relapsed/refractory patients with multiple myeloma, and these neoantigens can elicit cytotoxic $CD8^+$ T-cell activity in the setting of combination immunotherapy.

Disclosure of Potential Conflicts of Interest

S. Parekh is a paid consultant for Foundation Medicine; has received research funding from Celgene and Karyopharm. N. Bhardwaj is an advisory board member for Neon Therapeutics, Curevac, Roche, Boehringer Ingelheim and Parker Institute for Cancer Immunotherapy; has received research funding from Regeneron, Oncovir, Genentech, Celldex and Novocure. J. Brody has received research funding from Merck, Genentech, Acerta, Janssen, Seattle Genetics, Bristol-Myers Squibb, Celgene and Kite. A. Chari is a paid consultant for Amgen, Bristol Myers Squibb, Celgene, Antengene, Takeda, Janssen, Karyopharm; has received research funding from Amgen, Array Biopharma, Celgene, Glaxo Smith Klein, Janssen, Takeda, Novartis, Oncoceutics, Pharmacyclics, Seattle Genetics; is an advisory board member for Amgen, Celgene, Millenium/Takeda, Janssen, Karyopharm, Sanofi, Seattle Genetics. H.J. Cho is a paid consultant for the MMRF; has received research funding from Takeda, Celgene and Genentech. S. Gnjatic is a paid consultant for Neon Therapeutics; has received research funding from Agenus. B. Greenbaum is a paid consultant for PMV Pharmaceuticals and Rome Therapeutics; has received research funding from Bristol-Meyers Squibb; has

received honoraria for speaking engagements from Merck, Bristol–Meyers Squibb and Chugai Pharmaceuticals; Icahn School of medicine has a patent related to neoantigens (WO 2016/131048 Al) on which B. Greenbaum is an inventor. S. Jagannath is a paid consultant for AbbVie, Celgene, Bristol-Meyers Squibb, Karyopharm, Janssen and Merck. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D. Perumal, N. Imai, J.P. Finnigan, H.J. Cho, J.D. Brody, S. Gnjatic, N. Bhardwaj, S. Parekh
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D. Perumal, N. Imai, A. Laganà, J.P. Finnigan, D. Melnekoff, A. Solovyov, H.J. Cho, J.T. Dudley, J.D. Brody, B. Greenbaum, N. Bhardwaj, S. Gnjatic, N. Bhardwaj, S. Parekh
Writing, review, and/or revision of the manuscript: D. Perumal, D. Madduri, A. Chari, H.J. Cho, J.D. Brody, S. Jagannath, S. Gnjatic, N. Bhardwaj, S. Parekh
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Greenbaum
Study supervision: D. Perumal, S. Gnjatic, S. Parekh
Other (processed patient samples): V.V. Leshchenko

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