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Authors

Perumal, Deepak
Imai, Naoko
Laganà, Alessandro
et al.

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Mutation-Derived Neoantigen Specific T cell Responses in Multiple Myeloma

Deepak Perumal[1,2], **Naoko Imai**[1,2], **Alessandro Laganà**[3,4], **John P. Finnigan**[1,2,5], **David T Melnekoff**[3,4], **Violetta Leshchenko**[1,2], **Alexander Solovyov**[5,6,7], **Deepu Madduri**[1,2], **Ajai Chari**[1,2], **Hearn Jay Cho**[1,2], **Joel T Dudley**[3,4], **Joshua S Brody**[1,2,5], **Sundar Jagannath**[1,2], **Benjamin Greenbaum**[5,6,7], **Sacha Gnjatic**[2,5], **Nina Bhardwaj**[1,2,5], **Samir Parekh**[1,2,8]

[1]Department of Hematology and Medical Oncology, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

[2]Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[3]Department of Genetics and Genomic Sciences, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

[4]Institute for Next Generation Healthcare, Icahn School of Medicine at Mount Sinai, New York, NY, USA.

[5]Precision Immunology Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

[6]Department of Oncological Sciences, Icahn School of Medicine at Mount Sinai, New York, New York, USA.

[7]Center for Computational Immunology, Tisch Cancer Institute, Icahn School of Medicine at Mount Sinai, New York, NY, USA

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 (MM).

[8]Corresponding author: samir.parekh@mssm.edu.

AUTHORS' CONTRIBUTIONS

Conception and design: D.Perumal, S.Parekh

Development of methodology: D.Perumal, N. Imai, J.S. Brody, S. Gnjatic, N. Bhardwaj, S. Parekh

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): D.Perumal, J.P. Finnigan, D. Madduri, H.J.Cho, J. Dudley, A. Chari, S.Jagannath, S. Parekh

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): D.Perumal, A. Lagana, D.T. Melnekoff, A. Solovyov, B. Greenbaum.

Writing, review, and/or revision of the manuscript: D.Perumal, H.J.Cho, J.S.Brody, S. Gnjatic, N. Bhardwaj, S. Parekh

Study supervision: D.Perumal, S. Parekh.

Other (processed patient samples): V. Leshchenko

DATA AND MATERIALS AVAILABILITY:

Raw sequencing data from this study is available at NCBI SRA (accession number: PRJNA577591) (http://www.ncbi.nlm.nih.gov/bioproject/577591).

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

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

Conclusions—Our findings demonstrate that somatic mutations in MM 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 MM patients.

INTRODUCTION

Multiple Myeloma (MM) is a malignancy of plasma cells affecting >30,000 individuals per year (1,2). MM 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 neo-antigens 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 MM (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 MM, with potentially increased toxicity in combination with immunomodulatory drugs (11–13). As biomarkers of response to immunotherapeutics have not been clearly defined in MM, 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 data from newly diagnosed as well as relapsed MM patients. We used algorithms that predict peptide binding to HLA class I and validated our *in silico* prediction *in vitro* using primary MM samples in co-culture systems. Results from this study support the feasibility of neoantigen targeting immunotherapy for tumors with intermediate mutational load such as MM.

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 Mount Sinai, NY. Ninety two patients with relapsed/refractory multiple myeloma were included in the study after

written informed consent had been obtained. DNA and RNA from 92 relapsed MM patients 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 Autologous Stem Cell Transplantation (ASCT). Patient data were collected retrospectively from clinical records. RNA-seq and WES data from 92 newly diagnosed MM patients 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 MM patients 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 Autologous Stem Cell Transplantation. 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 MM patients were downloaded from IA7 release of MMRF CoMMpass study. Whole Exome Sequence (WES) data was mapped to human reference genome by Burrows-Wheeler Aligner software (BWA) (14) and somatic missense variants were detected using MuTect (15).Variants were called if there were more than 5 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 human leukocyte antigen (HLA) class I (HLA-A, HLA-B, and HLA-C) alleles of each patient were determined from RNA sequencing using Seq2HLA (17). The identified mutations led to candidate antigenic peptides that were filtered by tumor expression level (FPKM >2) using RNA sequence data. The Immune Epitope Database (IEDB) 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 IC50 value less than 500 nM were considered strong binders. Peptides were custom synthesized at JPT, Germany with high purity of >90%.

Analysis of T cell responses by Intracellular cytokine staining (ICS)

PBMC (fresh or thawed) was stimulated with specific and non-specific peptides on day 1 and cultured for 14–21 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, Germany (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, PMA (Phorbol 12-myristate 13-acetate (Sigma Aldrich, P1585) and Ionomycin (Sigma Aldrich, I3909) peptide for 6 hours at 37°C, and then washed 2–3 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 previously described (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-IL-2 (BD Biosciences 559334), PE Rat IgG2a κ isotype control (BD Biosciences 559317), anti-TNFα (BD Biosciences 557647), PE-Cy™7 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 250μL 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, Ashland, OR). T-cell reactivity for every neo-epitopes was validated by ≥ 2 independent experiments.

Generation of MHC tetramers and flow cytometry analysis

As previously described (20), biotinylated HLA monomer HLA-A*03:01 was loaded with UV-cleavable epitopes produced by the Bhardwaj lab at Mount Sinai, NY, USA. Potential HLA-A3-binding (predicted IC_{50} < 500 nM by netMHC) neoantigen peptides from a patient with HLA-A3 were synthesized (JPT Peptide Technologies, Germany), exchanged, and multimerized. Briefly, HLA loaded with UV-sensitive peptide monomers were subjected to UV light in the presence of 10μM individual candidate synthetic peptide for 1 hour on ice. Then, the monomers were tetramerized in the presence of fluorescent (PE or APC) streptavidin (ThermoFisher Scientific, S886 & 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 nM, 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 2 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 anti-human CD8 (clone SK1), CD4 (clone L200) antibodies were purchased from BD Biosciences.

Sorting and T cell expansion

As previously described (20), following 1 hour incubation with fluorophore-conjugated Abs or tetramers, T cells were isolated using a FACSAria cell sorter (BD) and collected in sterile PBS containing 50% FCS. T cells were expanded to large numbers using a rapid-expansion protocol with IL-2, anti-CD3 antibody and irradiated feeder cells.

TCR-β repertoire sequencing and Clonality

DNA was extracted from peripheral blood mononuclear cells, then TCR-β CDR3 regions were amplified and sequenced using ImmunoSEQ (Adaptive Biotechnologies, Seattle, Washington, USA) 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 (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 non-productive 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 −20C. T cells pulsed with target peptides or control peptides were labeled with either CFSE (ThermoFisher Scientific, C34554) or CTV (ThermoFisher Scientific, C34557) for proliferation tracking. For dye labeling of target cells, a final concentration of 500 nM of each dye were used in 1–2 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 min at 37°C and 5% CO2. After labeling, cells were washed two times with RPMI 1640 supplemented with 10% FCS. The labeled cells were then analyzed sing a BDFortessa flow cytometer. The following formula was used to calculate % specific killing:

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

RESULTS

Relapsed MM patients have greater mutation burden and higher frequency of neoantigens as compared to newly diagnosed MM patients

We utilized a neoantigen discovery pipeline in newly diagnosed and relapsed MM 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 nM) across 92 newly diagnosed MM and 92 relapsed MM patients with available HLA typing information. Consistent with the previously published study (23), we observed frequencies of up to several hundred non-synonymous 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. Fig. 1B

and 1D shows the number of non-synonymous somatic mutations per megabase (Mb) and the predicted neoantigens with high binding affinity (IC_{50}<500nM) in MM patients. Relapsed MM patients 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 to newly diagnosed patients (median number of coding somatic mutations per MB= 16, median number of neoantigens =62) (Fig. 1C **and** 1E). 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 MM

We first set out to identify most commonly recurrent somatic mutations in our 184 MM patients. We identified top 10 recurrent somatic mutations in both 92 relapsed and 92 newly diagnosed MM patients as shown in Fig. 2A **and** 2B; Supplementary Table S3 and S4. *NRAS*^{Q61R} was the top recurrent mutation in our relapsed MM patients where as *CDC27*^{L85F} was the top recurrent mutation in our newly diagnosed MM patients. 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 MM patients. The top 10 observed immunogenic mutations in relapsed and newly diagnosed MM patients are shown in Fig. 2C **and** 2D. 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 18636 somatic mutations and a total of 6330 immunogenic mutations in 92 relapsed MM patients. Similarly, we observed a total of 17788 somatic mutations and a total of 5194 immunogenic mutations in 92 newly diagnosed MM patients. 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 Table S5 and S6) were distinct from our somatic mutations in MM. Among the top 10 recurrent somatic mutations (Fig. 2A **and** 2B) 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** 2D). Notably, only two potential neoantigens, *PRKDC* and *UBR4* overlapped between both the relapsed and newly diagnosed MM populations. Functionally, PRKDC (Protein Kinase, DNA-activated, Catalytic polypeptide), has a major role in non-homologous end joining (NHEJ) DNA repair in cancer cells (24). UBR4 (ubiquitin N-recognin domain-containing E3 ligase 4) 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 MM for overlap with our immunogenic neoantigens. The mutational landscape of MM was published by the MM Research Consortium (MMRC) with the most commonly recurrent mutations of

11 genes seen in 203 MM patient samples (26). Recently, 63 oncogenic driver genes (27) were identified by the Myeloma Genome Project (MGP) in 1,273 newly diagnosed MM patients. We observed that neoantigens from our dataset overlapped the known recurrent (90%, 10 out of 11 genes in MMRC) and oncogenic driver mutations (79%, 50 out of 63 genes in MGP) in both relapsed and newly diagnosed MM patients (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 MM molecular subgroups/cytogenetic abnormalities in our 184 MM patients. Each patient in this study was assigned to one of the 10 classes defined by MMNet, our previously published network model of MM (28) based on the MMRF CoMMpass cohort, using a support vector machine (SVM) classifier trained on the 450 patients in MMNet. We observed the enrichment of neoantigens to various MM molecular subgroups/cytogenetic abnormalities in 57 out of 92 relapsed MM (62%) and 55 out of 92 newly diagnosed MM patients (60%) (Supplementary Table S8 and S9). However, we did not find any statistically significant correlation with MM 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 MM patient

While mutation and neoantigen load have been previously correlated with prolonged survival in newly diagnosed MM (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 MM patients in this study using a combination of co-culture and TCR sequencing techniques and correlated with clinical response. Supplementary Table S10 and S11 show the total number of non-synonymous somatic mutations, HLA class-I haplotypes and number of predicted and validated neo-epitopes in these patients. A 63 year old MM patient 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 and 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-100mg/m^2 and BCNU 100mg/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 non-synonymous 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 Table 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 and Supplementary Fig. S2A–C). Significantly, this patient demonstrated the highest T cell activity against the neoantigen *PRKDC*^{Q823E} restricted to HLA-A03:01. We observed significant IFN-γ, TNF-α and IL-2 production (increase from 0.19% to 5.19%, 0.38% to 3.01%, and 1.23% to 2.05% as compared to 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 and Supplementary Fig. S2A–C). 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 to the early time point (FPKM=7.2). The clinical response and T cell response to neoantigen of this patient prior and post to 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 post checkpoint blockade treatment were

incubated *in vitro* with pMHC tetramer (HLA-A03:01) that had been labeled with fluorophores (see methods), thus allowing separation of antigen-specific T cells. Fig. 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 to 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 post 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 T-cell receptor (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. Additionally, approximately 500 sorted tetramer-positive T cells were cultured in the presence of irradiated feeder cells, which led to 500-fold expansion after 14–21 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 post 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 since 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 myeloma patients.

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 Myelin-oligodendrocyte glycoprotein (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. Since autologous myeloma cells do not survive in culture for a ten 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 MM patient

A 79 year old MM patient relapsed after induction therapy with bortezomib, lenalidomide, dexamethasone (VRd) for 3 cycles followed by 1 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 **and** 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 non-synonymous 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 Table S16 and S17), we found increased levels of neoantigen specific T cell activation in the post anti-CTLA4 + PD-1 treated PBMCs (Fig. 5C and Supplementary Fig. S4A and B). Similar to patient 1, this patient also demonstrated the highest T cell activity against the neoantigen *EVI2B* ^{G327A} restricted to HLA-A68:02. Functionally, EVI2B (*Ecotropic Viral Integration site 2B)*, also known as CD361 is a common viral integration site in retrovirally-induced murine leukemia with a physiological role in myeloid differentiation and functionality of hematopoietic progenitors (33). We observed significant IFN-γ, TNF-α and IL-2 production (increase from 0.09% to 2%, 0.14% to 2.01%, and 0.08% to 0.37% as compared to 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 post 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 MM patients 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 MM patient

Our initial observations from the above two cases were recapitulated in a patient treated with immunomodulatory therapies (pomalidomide and elotuzumab). This patient diagnosed with MM was administered induction therapy with bortezomib, lenalidomide, dexamethasone (VRd) for 4 cycles and bortezomib, cyclophosphamide, dexamethasone (VCd) followed by

ASCT and lenalidomide maintenance. Subsequently the patient received anti-PD-L1 (Atezolizumab) upon relapse for six months on a clinical trial and on progression was administered elotuzumab (elo) and pomalidomide (pom) (Fig. 6A and Supplementary Tables S18).

Bone marrow aspirate was performed for CD138+ selection followed by WES and RNA sequencing prior to checkpoint blockade and immunotransplant to identify immunogenic neoantigens. The number of non-synonymous 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 Table S19 and S20). Significantly, this patient demonstrated the highest T cell activity against the *top* scoring neoantigen, i.e. T18N mutant of the *S100A9* gene, against the patients HLA-B44:03 (Fig. 6C and Supplementary Fig.S5A and B). We observed significant IFN-γ, TNF-α and IL-2 production (increase from 0.18% to 8.19%, 0.29% to 7.66%, and 0.89% to 3.68% as compared to 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 to 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 two 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 MM patients undergoing immunotherapy with combinations of immune checkpoint inhibitors, monoclonal antibodies and immunomodulatory drugs.

DISCUSSION

Our results demonstrate that the mutation and neoantigen burdens increase in relapsed MM patients compared to newly diagnosed MM patients. 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 et al (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 MM. We posit that neoantigens in relapsed myeloma may serve as targets for immunotherapy.

Our results using primary cells from relapsed myeloma patients 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-cytotoxic T-lymphocyte antigen 4 (anti-CTLA-4) ,anti- programmed death-1 (anti-PD-1) and anti-programmed cell death ligand 1 (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 MM therapy and target cereblon (CRBN), 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 (ADCC) against myeloma cells (44). Elotuzumab, an immunostimulatory monoclonal antibody targeting signaling lymphocytic activation molecule F7 (SLAMF7), showed activity in combination with lenalidomide in patients with relapsed or refractory multiple myeloma (45). This monoclonal antibody can cross-link SLAMF7 on NK cells and potentiate their anti-tumor 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 MM-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 3 studies combining pembrolizumab (anti-PD-L1) with lenalidomide or pomalidomide in two halted multiple myeloma studies (,). 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 MM patients. 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 MM patients 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 MM. Recently, it was shown in metastatic colorectal cancer that $KRAS^{G12D}$ mutation can be successfully targeted by specific tumor-infiltrating lymphocytes (TILs) (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 MM patients. 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,) investigating the safety and immunogenicity of a personalized, multipeptide, neoantigen vaccine for the treatment of cancers including MM (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. Additionally, 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 post vaccination (51). We believe these could potentially be sustained with booster strategies such as additional vaccines; immunomodulatory drugs (IMIDs) 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-mass spectrometry 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. Additionally 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 MM patients, and these neoantigens can elicit cytotoxic CD8+ T cell activity in the setting of combination immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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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 Multiple Myeloma Research Foundation; 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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Translational Relevance

Tumor-specific mutations are excellent targets for cancer immunotherapy as they may be recognized as neo-antigens 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 neo-antigens in cancers with relatively low to moderate mutational load as in MM, 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 MM are immunogenic and can elicit T cell specific responses. Our findings are particularly relevant for relapsed MM, since 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 neo-antigen burden increases in relapsed MM patients compared to newly diagnosed patients. Further, shared neoantigens are predicted in oncogenic driver genes in newly diagnosed and relapsed MM patients. 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.

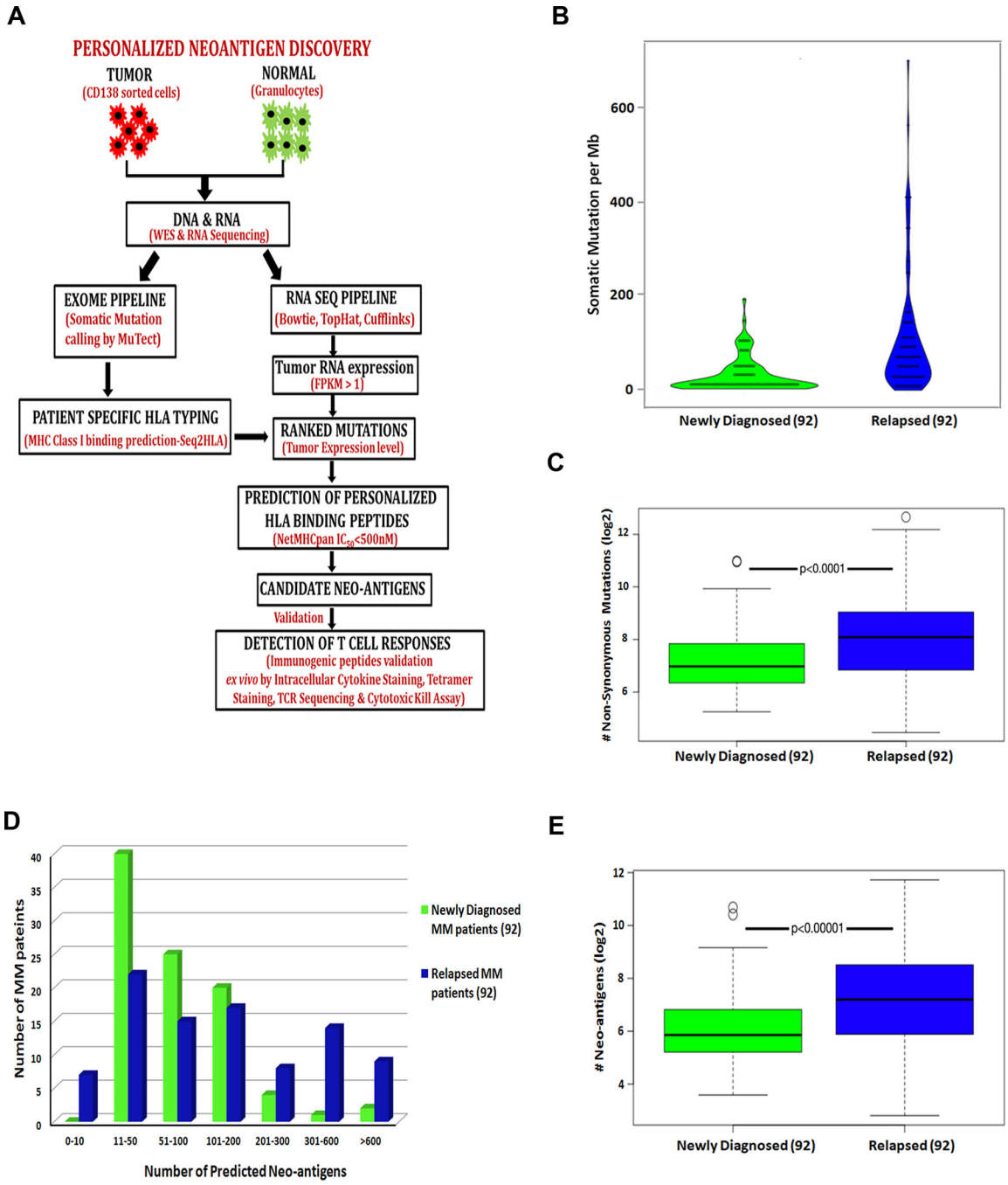


Figure 1. High frequency of neoantigens observed in relapsed myeloma patients as compared to newly diagnosed MM patients

A, Neoantigen discovery pipeline used in this study. **B,** Distribution of mutational burden (ie, number of somatic mutations per megabase [Mb] detected in newly diagnosed and relapsed MM patients from whole-exome sequencing (WES) data. **C,** High mutational load in relapsed MM patients as compared to newly diagnosed myeloma patients ($p < 0.0001$, Wilcoxon Rank Sum Test). **D,** Frequency of predicted neoantigens in newly diagnosed and

relapsed MM patients. **E,** High frequency of neoantigen load in relapsed MM patients as compared to newly diagnosed myeloma patients ($p < 0.0001$, Wilcoxon Rank Sum Test).

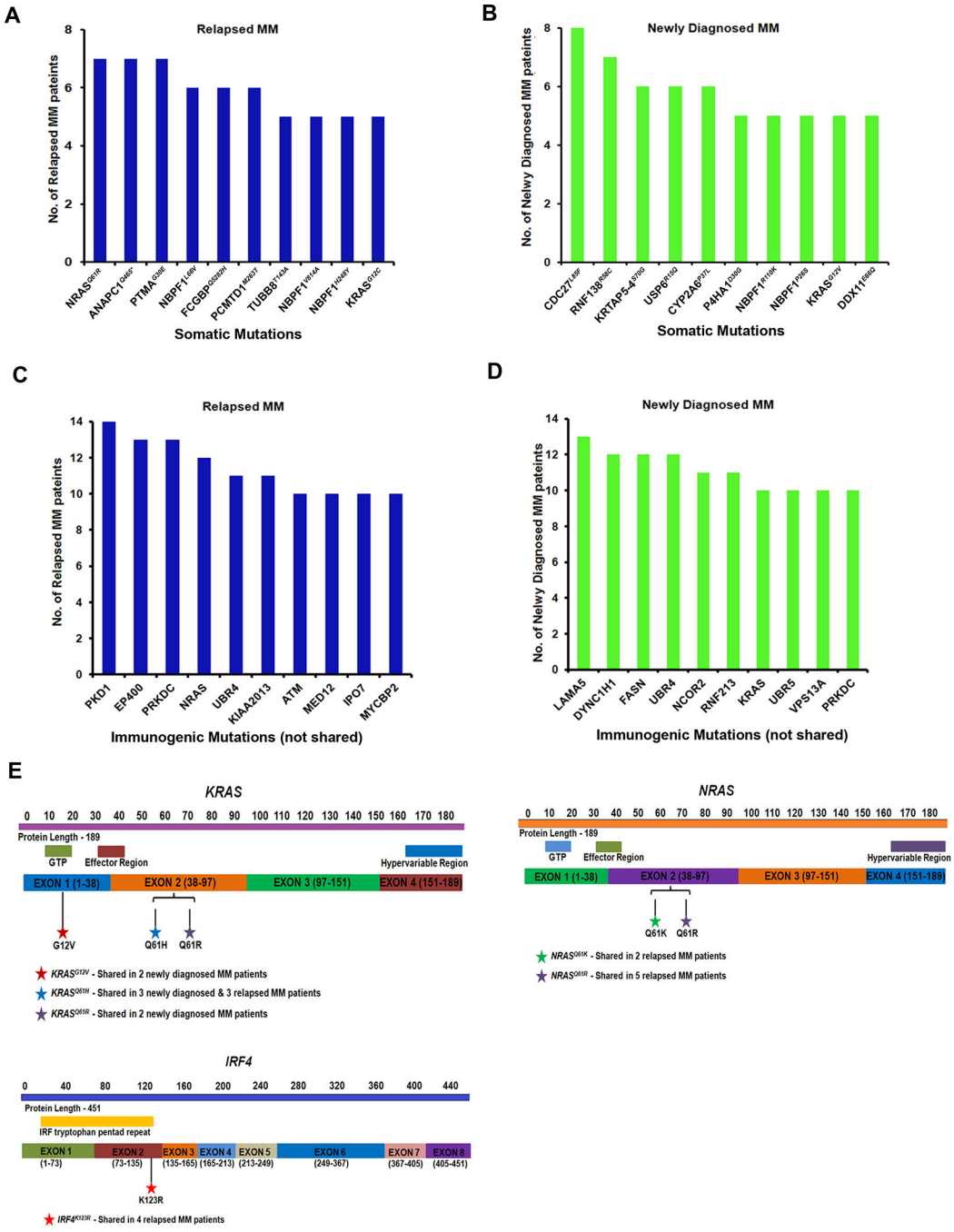
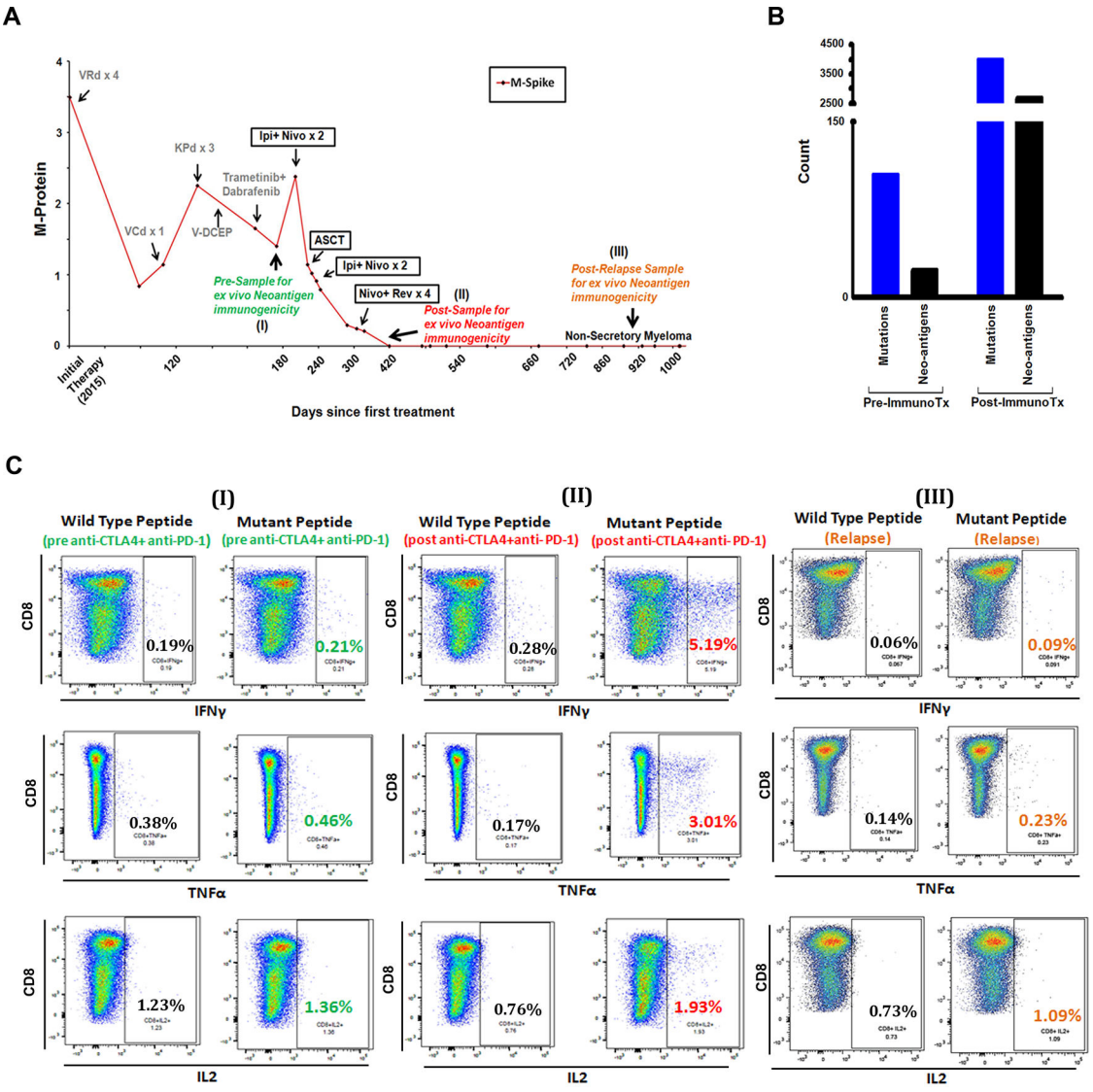


Figure 2. Neoantigens are observed in recurrently mutated MM genes

A, The top 10 recurrent somatic mutations observed in 92 relapsed MM patients. *NRAS*^{Q61R} was the top recurrent mutation in our relapsed MM patients, **B,** The top 10 recurrent somatic mutations observed in 92 newly diagnosed MM patients. *CDC27*^{L85F} was the top recurrent mutation in our newly diagnosed MM patients. **C,** The top 10 most frequently observed immunogenic mutations in relapsed MM patients. Mutated genes that could yield potentially immunogenic neoantigens in relapsed MM patients were *PKD1*, *EP400*, *PRKDC*, *NRAS*, *UBR4, KIAA2013, ATM, MED12, IPO7* and *MYCPB2*. For the majority of the identified

neo-antigens, we observed that mutations in the same gene are not shared between relapsed MM patients and are highly patient-specific **D,** The top 10 most frequently observed immunogenic mutations in newly diagnosed MM patients. In newly diagnosed MM patients *LAMA5*, *DYNC1H1*, *FASN*, *UBR4*, *NCOR2*, *RNF213*, *KRAS*, *UBR5*, *VPS13A* and *PRKDC* were identified. For the majority of the identified neo-antigens, we observed that mutations in the same gene are not shared between newly diagnosed MM patients and are highly patient-specific **E,** Shared neoantigens identified in MM patients 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 MM patients are shown in the plot. We observed that 5 relapsed patients shared $NRAS^{Q61R}$ mutations (Strong binding, IC_{50} < 150 nM), 4 relapsed patients shared $IRF4^{K123R}$ mutations (Very strong binding, IC_{50} < 50 nM), 3 relapsed patients shared $KRAS^{Q61H}$ mutations (Strong binding, IC_{50} < 150 nM) and 2 relapsed patients shared $NRAS^{Q61K}$ mutations (Strong binding, IC_{50} < 150 nM) (Fig. 2c). Similarly, we observed that 3 newly diagnosed patients shared the $KRAS^{Q61H}$ mutations (Strong binding, IC_{50} < 150 nM), 2 newly diagnosed patients shared $KRAS^{G12V}$ neoantigenic mutations (Very strong binding, IC_{50} < 50 nM) and 2 newly diagnosed patients shared $KRAS^{Q61R}$ neoantigenic mutations (Intermediate binding, IC_{50} of 150–250 nM).



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 Neo-antigen	No	Yes	No

Figure 3. Checkpoint based inhibitor therapy elicits *PRKDC*-neoantigen specific T cell response in a primary refractory MM patient

A, Timeline of clinical response of MM patient to dual checkpoint inhibitor (anti-CTLA4 +anti-PD-1) therapy. **B,** The number of non-synonymous mutations and the predicted immunogenic neoantigens. **C,** CD8+ T cell response to neoantigen peptide (PRKDC) measured by IFNg, TNFa and IL2 pre & post dual checkpoint inhibition (anti-CTLA4 + anti-PD-1). **D,** The clinical response and T cell response to neoantigen of this patient prior and post to immunotransplant and checkpoint inhibition.

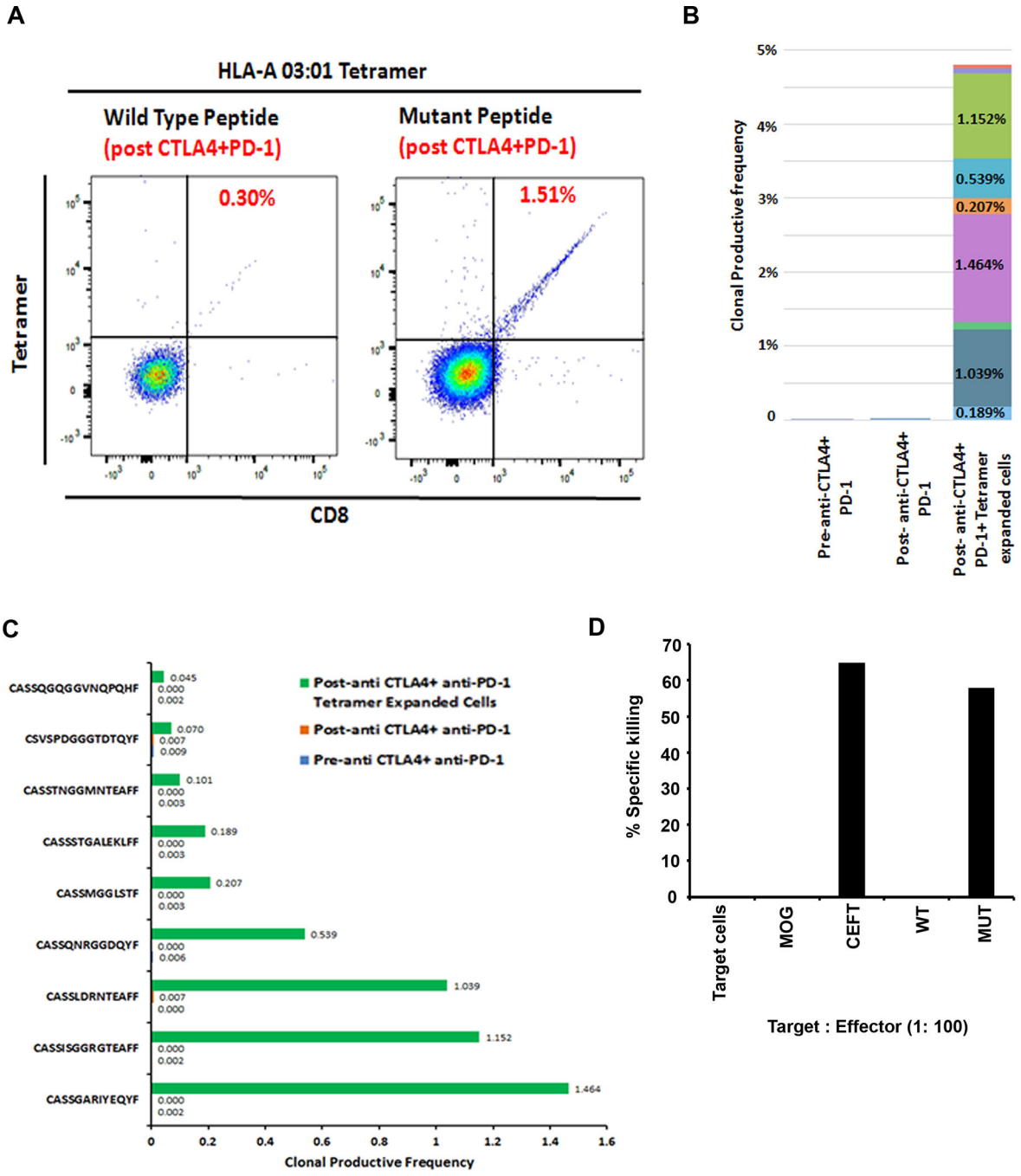
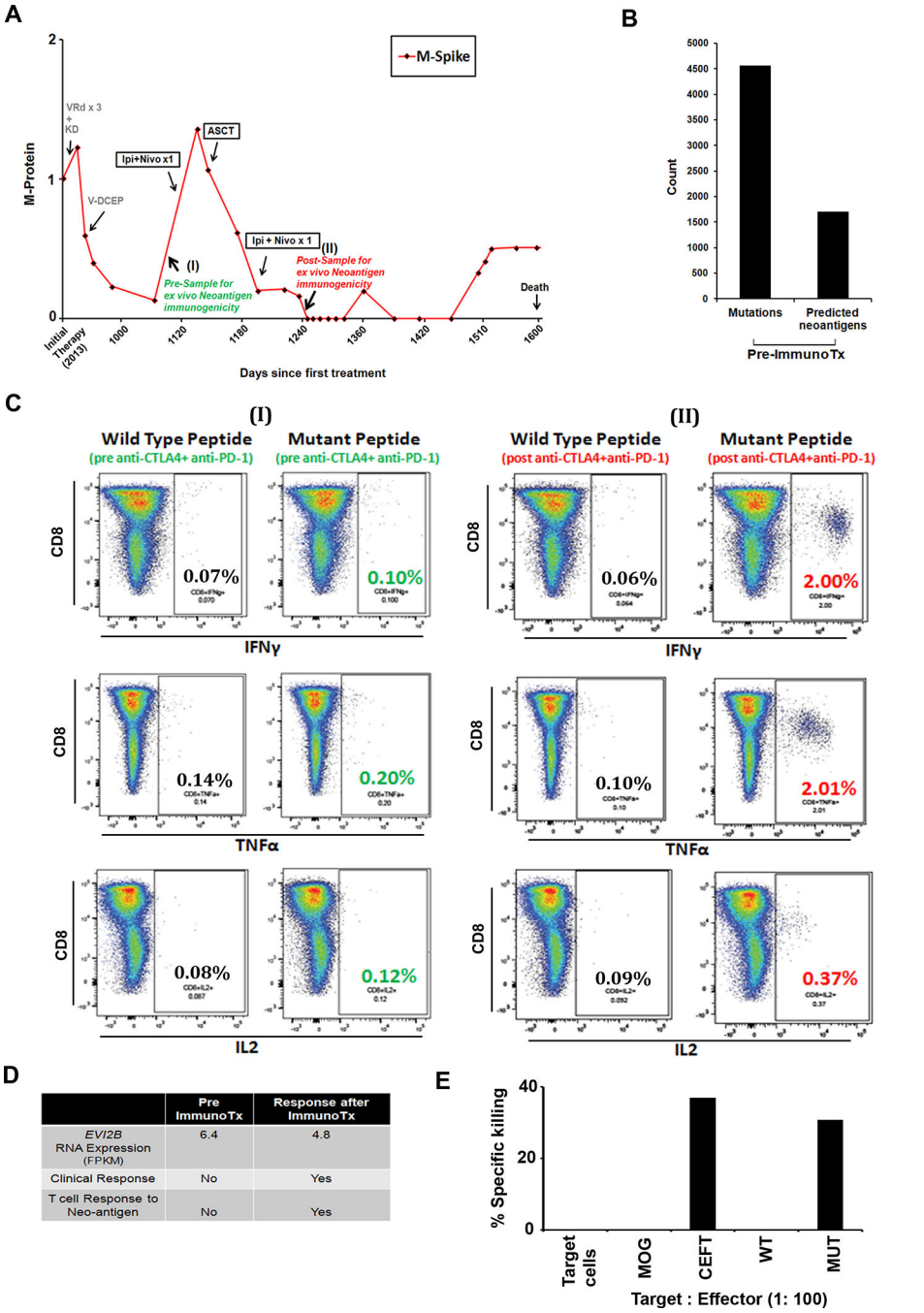


Figure 4. Assessment of CD8$^+$ T Cell Responses to *PRKDC* neoantigen using MHC Class I Tetramer, TCR sequencing and Cytotoxic T cell killing

A, Validation of HLA-A*03:01 tetramer with neoantigen peptide PRKDC from the MM patient. Peripheral lymphocytes obtained post 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 to the wild type peptide (0.3%). **B,** TCR-Seq reveals increase in oligoclonal expansion after dual checkpoint inhibition. Tetramer-binding T cells were subjected for sequencing of T-cell receptor (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 post 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 pre-existing dominant clones after the administration of checkpoint therapy compared to the low proportion of such pre-existing 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.



	Pre ImmunoTx	Response after ImmunoTx
EVI2B RNA Expression (FPKM)	6.4	4.8
Clinical Response	No	Yes
T cell Response to Neo-antigen	No	Yes

Figure 5. Checkpoint blockade therapy enhances *EVI2B*-neoantigen specific T cell response in a relapsed MM patient

A, Timeline of clinical response of MM patient to dual checkpoint inhibitor (anti-CTLA4 +anti-PD-1) therapy. **B,** The number of non-synonymous mutations and the predicted immunogenic neoantigens. **C,** CD8+ T cell response to neoantigen peptide (EVI2B) measured by IFNg, TNFa and IL2 pre & post dual checkpoint inhibition (anti-CTLA4 + anti-PD-1). **D,** The clinical response and T cell response to neoantigen of this patient prior and post to 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.

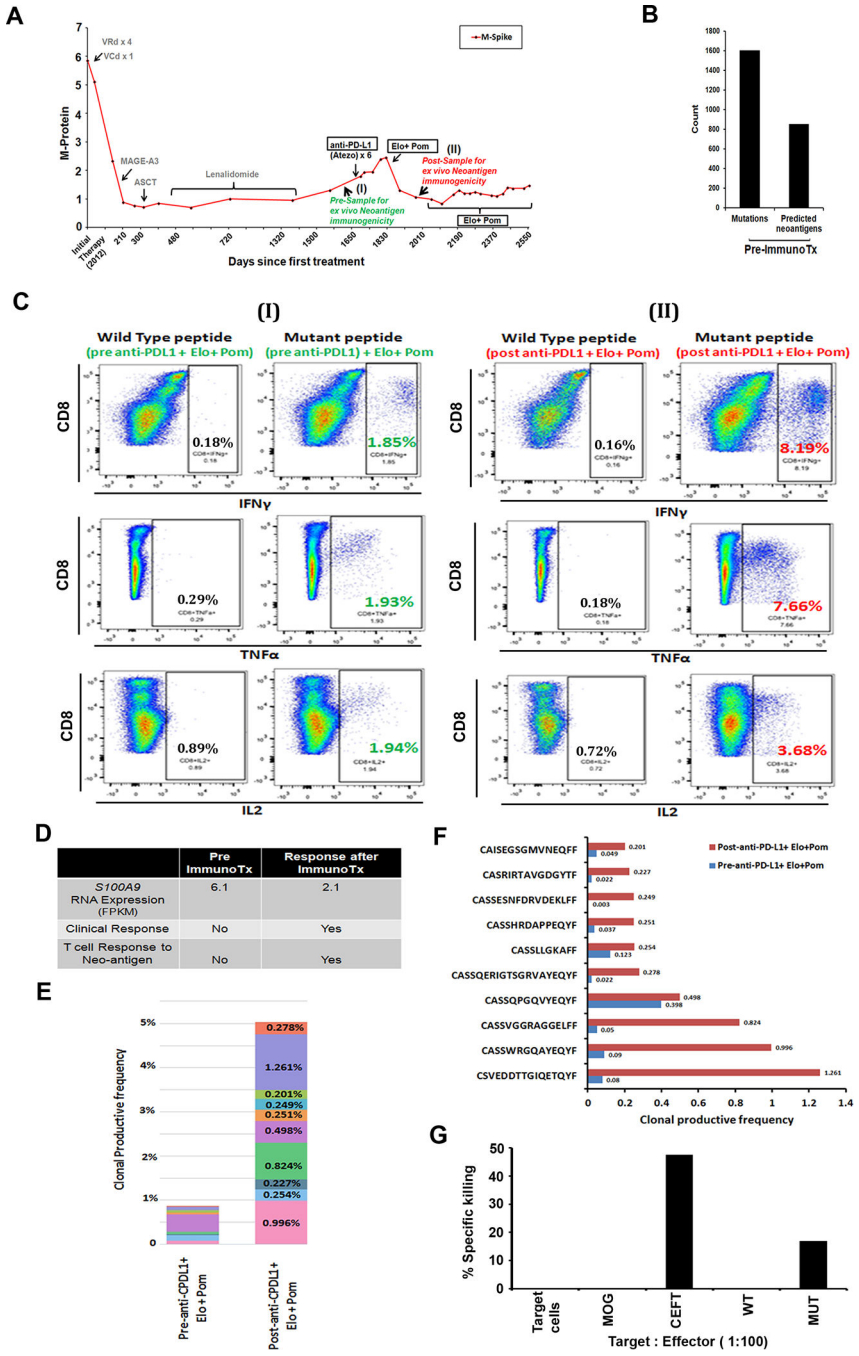


Figure 6. Checkpoint blockade therapy in combination with Pomalidomide and elotuzumab treatment elicits *S100A9*-neoantigen specific T cell response in a relapsed MM patient

A, Timeline of clinical response of MM patient 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 IFNg, TNFa and IL2 pre & post dual treatment (anti-PD-L1 + elotuzumab and pomalidomide). **D,** The clinical response and T cell response to neoantigen of this patient prior and post to checkpoint inhibition and elotuzumab plus pomalidomide

treatments. **E,** TCR-Seq 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 post treatment (without *ex vivo* expansion). **F,** The specific TCR-β clones are shown in the bar plot. The patient had a high proportion of pre-existing dominant clones after the administration of immunomodulatory therapies compared to the low proportion of such pre-existing 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.