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Translational Research

In vitro induction of neoantigen-specific T cells in myelodysplastic syndrome, a disease with low mutational burden

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ABSTRACT

Therapies that utilize immune checkpoint inhibition work by leveraging mutation-derived neoantigens and have shown greater clinical efficacy in tumors with higher mutational burden. Whether tumors with a low mutational burden are susceptible to neoantigen-targeted therapy has not been fully addressed. To examine the feasibility of neoantigen-specific adoptive T-cell therapy, the authors studied the T-cell response against somatic variants in five patients with myelodysplastic syndrome (MDS), a malignancy with a very low tumor mutational burden. DNA and RNA from tumor (CD34⁺) and normal (CD3⁺) cells isolated from the patients' blood were sequenced to predict patient-specific MDS neopeptides. Neopeptides representing the somatic variants were used to induce and expand autologous T cells *ex vivo*, and these were systematically tested in killing assays to determine the proportion of neopeptides yielding neoantigen-specific T cells. The authors identified a total of 32 somatic variants (four to eight per patient) and found that 21 (66%) induced a peptide-specific T-cell response and 19 (59%) induced a T-cell response capable of killing autologous tumor cells. Of the 32 somatic variants, 11 (34%) induced a CD4⁺ response and 11 (34%) induced a CD8⁺ response that killed the tumor. These results indicate that *in vitro* induction of neoantigen-specific T cells is feasible for tumors with very low mutational burden and that this approach warrants investigation as a therapeutic option for such patients.

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Introduction

Neoantigen-specific T cells have been credited for the recent therapeutic success of immune checkpoint inhibitors in treating various malignancies, supporting the use of neoantigens as targets for cancer immunotherapies [1–4]. Tumor-infiltrating lymphocytes contain T cells reactive against cancer neoantigens, and adoptive transfer of neoantigen-reactive tumor-infiltrating lymphocytes effectively reduces tumor burden in specific contexts [5,6]. Results obtained with checkpoint inhibitors indicate that approximately 1% of predicted HLA-binding neopeptides are immunogenic, consistent with data that checkpoint blockade is more effective in cancers with high mutational burden [7].

These results contrast with data obtained in viral systems where 30–40% of HLA class I binding peptides are immunogenic and recognize virally infected cells [8], highlighting the need for further investigation of T-cell responses against cancer neoantigens.

Recently, results obtained by vaccinating melanoma patients with an array of class I HLA-binding neopeptides showed that 18% of the peptides induced a CD8⁺T-cell response; however, the CD8⁺ effector cytotoxic T lymphocytes were not capable of killing the tumor cells [9]. To address the discrepancy between data on the immunogenicity of viral antigens versus cancer mutation-dependent neopeptides, the authors studied patients with higher-risk myelodysplastic syndrome (MDS), a malignancy of hematopoietic stem cells that results from the accumulation of a few mutations per patient, therefore allowing for a systematic analysis of each mutation's immunogenic potential. Moreover, information obtained from this analysis can inform therapeutic approaches for MDS patients for whom there are very few therapeutic options.

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Here the authors assessed the neoantigen burden in peripheral blood CD34⁺ stem cells from five patients with higher-risk MDS and tested the ability of their T cells to generate autologous neoantigen-specific responses *in vitro*. Remarkably, despite a very low tumor mutational burden (TMB) (four to eight non-synonymous mutations per cancer genome), robust neopeptide-specific CD4⁺ and CD8⁺T-cell responses were obtained from all five patients, and more than one neoantigen-specific T-cell population per patient mediated autologous cancer cell lysis. Therefore, the authors' *in vitro* results indicate the feasibility of generating neoantigen-reactive T cells in such patients and support the further investigation of adoptive T-cell transfer as a possible therapy for tumors with a low TMB.

Methods

Patient samples

Five patients with MDS per French-American-British criteria (Table 1) [10] were recruited from the Moores Cancer Center at the University of California, San Diego in La Jolla, CA (NCT-03072498), and samples were collected under a clinical protocol approved by the University of California San Diego Institutional Review Board and in accordance with the Declaration of Helsinki. A non-mobilized leukapheresis for mononuclear cell collection was performed using a COBE Spectra or Optia system. CD34⁺, CD14⁺, CD8⁺ and CD4⁺ cells were separated with a CliniMACS (Miltenyi Biotec, Auburn, CA, USA) per the manufacturer's instructions, resulting in >85% purity.

Media and buffers

Cell freezing medium consisted of 90% heat-inactivated fetal bovine serum (FBS) (Seradigm, Providence, UT, USA) and 10% dimethyl sulfoxide (Sigma-Aldrich, St Louis, MO, USA). For cell culture and assays, the authors used R10 medium [(RPMI 1640; HyClone, Pittsburgh, PA, USA), 50 ng/mL gentamicin (Sigma-Aldrich), 10% heat-inactivated FBS (Seradigm) supplemented with 2% GlutaMAX (Life Technologies, Carlsbad, CA, USA), 1% nonessential amino acids and 1% sodium pyruvate (HyClone)], complete GMP SF5 medium [(GMP DC serum-free medium; CellGenix, Portsmouth, NH, USA), 50 ng/mL gentamicin and 5% human serum (Valley Biomedical, Winchester, VA, USA)].

DNA and RNA extraction and sequencing

The overall methodology is outlined in Figure 1. The authors identified the somatic variants in the MDS stem and progenitor cells by sequencing the DNA (exome) and RNA [11] of purified CD34⁺ cells, cultured MDS cell lines (MCLs) and purified CD3⁺ control T-cell blasts from each patient [12,13]. Nucleic acid extractions were performed using the AllPrep kit (Qiagen, Germantown, MD, USA) for purifying DNA and RNA simultaneously from the same cells per the manufacturer's recommended procedures. DNA and RNA samples were sequenced at the Institute for Genomic Medicine Center at the University of California, San Diego on Illumina (San Diego, CA, USA) platforms (100 bp paired-end). The SureSelect XT human clinical research exome V2 (Agilent Technologies, Santa Clara, CA, USA) kit was used to prepare DNA and RNA libraries.

GATK best practices (Broad Institute, Cambridge, MA, USA) were used to analyze sequences and identify short variants (single nucleotide variants or small insertions and deletions) and produce a joint call set in a variant call format (VCF) file. The sequence reads obtained by the whole-exome sequencing were aligned to the reference human genome build hg19 using the Burrows-Wheeler aligner (Illumina). Following alignment, a BAM file was generated with Samtools and Picard (Broad Institute). The BAM file was processed using GATK 3.4–3.6 and set to remove variants with a call quality less than 2, resulting in a VCF file. For RNA sequencing, the Burrows-Wheeler

aligner step was replaced with STAR aligner 2.4 (Illumina) and Sambamba 0.5 [14]. GATK 3.4 best practices using the RNA sequencing setting (Broad Institute) were used for all subsequent steps. The raw variant list obtained in this pipeline was filtered on the Phred scale using the Fisher test to detect strand bias (FisherStrand >30.0) and variant confidence/quality by depth value greater than 2, generating the final VCF file (Broad Institute). VCF files were uploaded in Ingenuity Variant Analysis software (Qiagen, Redwood City, CA, USA), and the authors selected somatic variants that were present in the DNA and RNA of CD34⁺ cells, with a variant allele fraction of at least 0.05 (5%), and that were not present in the DNA and RNA of autologous CD3⁺ control cells. Only variants that led to a change in the protein sequence (e.g., missense and frameshift) were selected. All variants were confirmed by manual review of aligned reads. MDS stem cell-specific recurrent variants (i.e., driver mutations) were defined using the list of 263 genes provided by Duncavage *et al.* [15]; all others were considered passenger mutations.

Peptides

The authors obtained synthesized peptides (purity >99%) from Synthetic Biomolecules (San Diego, CA, USA) and PepTivator CEF mix (cytomegalovirus, Epstein-Barr and flu virus peptides) from Miltenyi Biotec (Auburn, CA, USA). To test the immunogenicity of the 32 somatic variants, the authors prepared peptide pools consisting of the peptide containing the mutation flanked by 12–14 amino acids (25–29 mer). For mutations with frameshifts of fewer than 15 amino acids, the mutated peptide sequence was flanked by 12 amino acids at the amino terminus. For frameshifts over 30 amino acids, a stretch of 25 amino acids of the mutated protein was selected. The authors also selected shorter k-mers for each long peptide based on predicted HLA-A and HLA-B binding with a percentile rank <2% or half maximal inhibitory concentration (IC50) <1000 nM and class II k-mers based on percentile rank <5% or IC50 <2000 nM using the prediction tool SMM-align hosted on the Immune Epitope Database (www.iedb.org) [16]. The authors selected 40 k-mers among the highest binders for class I. Of these, 22 had predicted binding to HLA-B molecules and 18 to HLA-A. A total of 28 of the 32 (88%) somatic variants contained at least one peptide predicted to bind to each patient's HLA-A or HLA-B (see supplementary Tables 1, 2). The authors also selected 19 k-mers with predicted binding to HLA-DRB1. To verify these results, the authors repeated the analysis with the recently developed binding prediction tool NetMHCpan 4 [17]. The authors found that 29 of the 32 variants (91%) contained at least one 8–12 mer (percentile rank <2) with predicted high affinity to an HLA-A, HLA-B or HLA-C allele in the respective patient.

Generation of MCLs

To generate a short-term MDS stem cell line, CD34⁺ cells or lymphodepleted peripheral blood mononuclear cells (2×10^6) were centrifuged and resuspended in 2 mL R10 culture medium containing 50 ng/mL stem cell factor, 1000 U/mL granulocyte-macrophage colony-stimulating factor and 50 ng/mL IL-3 (PeproTech, Rocky Hill, NJ, USA). This cytokine cocktail was added to cell culture every 3–4 days until cryopreservation. The authors generated short-term cell lines from four of the five patients tested. Samples were sent to the University of California, San Diego Institute for Genomic Medicine for sequencing, and all mutations associated with a T-cell response were maintained in the MCLs.

Generation of dendritic cells derived from monocytes

Monocyte-derived dendritic cells (moDCs) were generated as previously described [18,19] starting with CD14⁺ magnetically purified cells.

Table 1
Patient characteristics and somatic variants.

Patient	MDS-002	MDS-003	MDS-004	MDS-005	MDS-009
MDS/CMML classification	MDS-EB-1	MDS-EB-2	MDS-EB-2	CMML-1	CMML-1
Age	76	78	63	84	70
Sex	M	M	M	M	M
WBCx10⁹/L	1.9	0.8	1.5	10.6	9
Platelets, x10⁹/L	32	71	6	43	350
Hgb, g/L	87	86	68	88	107
ANC, x10⁹/L	1	0.1	5	6.2	3.2
% Blasts, BM	6	10	5–10	2–3 (IHC)	4
Cytogenetics	normal	normal	complex	normal	normal
IPSS-R risk group/score	High/5.5	High/5	Very high/9	N/A	N/A
Prior disease therapy	no	yes	no	no	yes
DNA somatic variants % Variant Allelic Fraction (VAF)	ASXL1* 34%	ARRDC3 26%	CACNA1H 47%	ASXL1 26%	MAGI2* 62%
	ATP6V0A1 36%	ASXL1 29%	ETV2 51%	ATP6V1B2 13%	C1orf27 45%
	EXOSC2 29%	BTBD7 27%	MIB2 54%	RUNX1 21%	HERC2 31%
	L3HYPDH 32%	DDX41 30%	PHLPP1 20%	SMARCB1 17%	SRSF2 40%
	WDR6 38%	CES2 27%	PKD1 26%		NOP56 41%
	ZNF131 38%	LRRC58 29%	TP53 96%		PHRF1 43%
		SPRTN 22%	WDR91 29%		RUNX1 50%
		SSH1 30%			SMC3 18%
					ZXDC 39%

Variants in red represent reported driver mutations [15].

MDS-002: ASXL1

MDS-003: ASXL1, DDX41

MDS-004: TP53

MDS-005: ASXL1, RUNX1

MDS-009: MAGI2, SRSF2, RUNX1, SMC3

ANC, absolute neutrophil count; Hgb, hemoglobin; IPSS-R, Revised International Prognostic Scoring System; N/A, not applicable; n/a, not available; WBC, white blood cell count.

*Variant not expressed.

Induction of neopeptide-specific T cells

To stimulate neopeptide-specific T cells, the authors pulsed autologous moDCs for 2–4 h at 37°C with 1–5 μ M of each peptide pool representing a somatic variant. Pulsed moDCs ($0.5\text{--}1 \times 10^5$) were then used to stimulate the MDS patients' autologous purified CD4⁺ T

cells alone (1×10^6) and purified CD8⁺ T cells (1×10^6) plus irradiated CD4⁺ T cells. To each condition, the authors added 10 ng/mL IL-7 and 800 U/mL IL-4 (PeproTech). After 9–15 days, T cells were stimulated a second time with autologous peptide-pulsed dendritic cells. Cultures were fed as needed with IL-2 or IL-15. Killing was tested after 7–14 days using T-cell killing (TCK) assays.

Figure 1. Overall manufacturing process. Patient T cells, MNCs and CD34⁺ cells were enriched using a leukapheresis product. Purified DNA and RNA from CD34⁺ cells and CD3⁺ cells were sequenced to identify variants exclusive to CD34⁺ cells with VAF >0.05 (5%). Candidate neopeptides were selected from all peptide-representing variants tested by assessment of *in silico* prediction of HLA binding affinity. Autologous moDCs were pulsed with the synthesized peptides and used to stimulate autologous CD4⁺ and CD8⁺ T cells. The induced T cells were tested for neopeptide specificity in cytolytic assays with autologous CD19⁺ cells with/without neopeptides and for tumor killing with autologous MDS stem cells and allogeneic CD34⁺ cells. To test the immunogenicity of the 32 somatic variants, the authors prepared peptide pools consisting of the entire peptide containing the mutation flanked by 12–14 amino acids (25–29 mer). The authors also selected shorter k-mers for each long peptide based on the predicted HLA-A and HLA-B binding with a percentile rank <2% or IC50 <1000 nM and class II k-mers based on percentile rank <5% or IC50 <2000 nM using the prediction tools hosted on the Immune Epitope Database. MNCs, mononuclear cells; VAF, variant allele fraction.

TCK assay

A sample of neopeptide-specific T cells (responders) was collected 6–7 days after restimulation and tested for cytolytic activity using a flow cytometry-based killing assay. Target cells for initial immunogenicity testing were autologous Epstein-Barr virus-transformed B-cell (EBV-B) lymphoblasts with or without the peptide pool used to generate the T cells. All neopeptide-stimulated T cells that elicited a significant specific response to neopeptide-pulsed EBV-B lymphoblasts and at least one negative control (peptide pool that did not elicit a T-cell response in the same experiment) were examined for tumor cell killing. Targets for tumor cell killing were the patients' tumor cells (CD34⁺ cells or MCLs) or, as controls, allogeneic acute myeloid leukemia CD34⁺ cells. Targets were labeled with CellTrace Violet or CellTrace carboxyfluorescein succinimidyl ester fluorescent dye (Life Technologies, Carlsbad, CA, USA), combined with responders at an E:T ratio of 20:1 and incubated at 37°C. After overnight incubation, the cells were harvested, and 7-aminoactinomycin D plus mitochondrial dye was added to discriminate between live and dead cells. The cells were analyzed on an iQue Screener PLUS (IntelliCyt, Albuquerque, NM, USA) flow cytometer, and the percentage killing of target cells was calculated as $[(\text{live cells in control} - \text{live cells in test samples}) / \text{live cells in control}] \times 100 = \text{cytotoxicity (\%)}.$ All experiments were repeated a minimum of three times. Positive responses were defined as those that yielded a minimum of 10% specific lysis that was at least 2-fold greater than the background, with a significance of at least $P < 0.05$, calculated using the GraphPad Prism multiple *t*-tests analytical tool.

Clonogenicity assay

A sample of neopeptide-specific T cells was collected 6–7 days after restimulation and plated in R10 medium at various E:T ratios with autologous tumor cells (CD34⁺ cells or MCLs) and incubated overnight at 37°C. The authors used the non-tumor-specific immunizing peptide pool PepTivator CEF mix (Miltenyi Biotec), which had 95% peptide-specific lysis in the TCK assay, as a negative control for

tumor cell killing. The next day (16–24 h), cells were collected, washed and resuspended in Iscove's Modified Dulbecco's Medium (STEMCELL Technologies, Vancouver, Canada) with 2% FBS and transferred at a 1:10 v/v in MethoCult medium (STEMCELL Technologies). Cell mixtures were transferred to six- or 12-well plates. After 12–16 days of incubation at 37°C, the authors quantified blast colonies using an inverted microscope as previously described [20].

Results

Identification of tumor-specific non-synonymous mutations

The goal of this study was to determine the proportion of tumor-specific somatic variants that are immunogenic to autologous T cells *ex vivo*. To this end, the authors selected the somatic variants expressed by purified CD34⁺ cells and not expressed by CD3⁺ T-cell blasts of five MDS patients. A total of 32 variants were identified with these characteristics. Table 1 shows the patients' characteristics and their expressed somatic variants plus two driver mutations that were not expressed. Of the 32 expressed variants, three were >30aa frame-shifts. The authors found four to eight somatic variants per patient, and all patients had at least one known recurrent MDS mutation (range, 1–4). These data are consistent with the results of a study by da Silva-Coelho *et al.* [21], who reported eight to 27 mutations per patient and zero to six known recurrent mutations. In the current study, the patients' recurrent mutations and phenotype were also consistent with those reportedly found for higher-risk MDS patients [13,22–25]. Specifically, mutations in the *ASXL1* gene were found in three patients (patients MDS-003 and MDS-005 had a G646fs*12 mutation in the DNA and RNA, and patient MDS-002 had an E574fs*15 mutation present only in the DNA), a *RUNX1* mutation was found in two patients and *TP53* and *SRSF2* mutations were found in one patient each. The remaining mutated genes were unique and not shared among the tested patients. Mutations with an allelic fraction above 50% may have been the result of copy number variations or copy neutral loss of heterozygosity within the parent clone.

Figure 2. CD8⁺ neopeptide immunogenicity induced using neopeptide pools representing a single mutation. (A) CD8⁺ T cells induced using neopeptide pools showed cytotoxicity against autologous EBV-B lymphoblasts ± peptide pool as well as against (B) autologous MDS stem cells (CD34⁺ cells or MCLs) or HLA-unrelated CD34⁺ leukemia cells. Results represent mean ± SD and are representative of at least three experiments. Statistical significance is indicated by red bars. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. SD, standard deviation.

T-cell response to neopeptides

Although predicting HLA binding is a useful step for reducing the number of potentially immunogenic peptides required for testing, whether neopeptide-specific T cells are present within the T-cell repertoire of the patient cannot be predicted and needs experimental validation [26]. The authors tested purified, stimulated CD8⁺ cells (with irradiated CD4⁺ cells) to evaluate the response of the CD8⁺ T cells independently of responding CD4⁺ T cells. Among the 32 total variants tested for immunogenicity, 14 (44%) induced a CD8⁺ T-cell response (Figure 2A). Notably, of the four variants that did not have a predicted HLA class I binder (ATP6V0A1, DDX41, PKD1 and ZNF131), none induced a neopeptide-specific CD8⁺ T-cell response.

Next, the authors analyzed CD4⁺ T-cell responses to the same peptide pools. A total of 17 (53%) of the 32 variants induced a specific response in CD4⁺ T cells (Figure 3A). In summary, of the 32 somatic variants, 21 induced a peptide response, and of those, 10 induced a

CD8⁺ and CD4⁺ T-cell response, whereas four stimulated CD8⁺ only and seven stimulated CD4⁺ only. Retrospective analysis indicated that 16 of the 17 somatic variants contained neopeptides with a predicted HLA binding affinity to a DRB allele of less than 1000 nM (using NetMHCIIpan 3.2). It is possible that the one peptide for which this was not the case (MIB2) was restricted by HLA-DP or HLA-DQ, but this was not tested.

Lysis of autologous MDS stem cells

Not all predicted HLA-peptide binders induce an immune response that leads to killing of cells expressing the endogenously processed antigen [8,27,28]. Therefore, the authors next tested CD8⁺ or CD4⁺ neopeptide-specific T cells and at least one negative control for tumor cell killing in TCK assays against autologous and allogeneic CD34⁺ stem cells or MCLs. Of the 31 neopeptide-specific T-cell populations specific for 21 of the somatic variants, 22 were also tumor-specific (Figures 2B, 3B). Nine of the 31 (29%) neopeptide-

Figure 3. CD4⁺ neopeptide immunogenicity induced using neopeptide pools representing a single mutation. (A) CD4⁺ T cells induced using neopeptide pools showed cytotoxicity against autologous EBV-B lymphoblasts ± peptide pool as well as against (B) autologous MDS stem cells (CD34⁺ cells or MCLs) or HLA-unrelated CD34⁺ leukemia cells. Results represent mean ± SD and are representative of at least three experiments. Statistical significance is indicated by red bars. *P < 0.05, **P < 0.01, ***P < 0.001 and ****P < 0.0001. SD, standard deviation.

specific T-cell populations did not lyse the tumor cells. The very low, non-specific killing of HLA non-matched CD34⁺ cells supports the specificity of lysis. Similarly, the lack of autologous MDS stem cell killing by T cells that did not recognize peptide-coated EBV-B lymphoblasts supports the neopeptide specificity of autologous tumor cell killing.

To confirm the validity of the autologous tumor cytotoxicity detected in the TCK assay, the authors assessed MCL killing in clonogenic assays. The authors incubated neoantigen- and tumor-specific T cells with MCLs and measured residual MCL growth using the non-tumor-specific immunizing peptide pool PepTivator CEF mix (Miltenyi Biotec), which generated 95% peptide-specific lysis of target cells in the TCK assay as a negative control for tumor cell lysis (Figure 4). The results were in accordance with those obtained by the TCK assay.

In summary, among the 32 total somatic variants tested for immunogenicity, 21 (66%) were immunogenic and generated a total of 31 T-cell responses, of which 17 were CD4⁺ and 14 were CD8⁺. A total of 10 variants induced both CD4⁺ and CD8⁺ T-cell responses. A peptide-specific T-cell response occurred in four of the eight expressed known recurrent mutations [29]. The recurrent mutations that did elicit a T-cell response were seen in *TP53* for patient MDS-004, *AXSL1* and *RUNX1* for patient MDS-005 and

RUNX1 for patient MDS-009, whereas a T-cell response was not present in *DDX41* and *ASXL1* for patient MDS-003 and *SMC3* and *SRSF2* for patient MDS-009. Of the 31 neopeptide-specific T-cell populations, 22 (59%), 11 of 14 CD8⁺ and 11 of 17 CD4⁺ T cells, were capable of autologous tumor cell lysis (Figures 2B, 3B), whereas nine (41%) were not. This highlights the need for *in vitro* anti-tumor validation of candidate neopeptides prior to their use *in vivo* [26].

Correlation between tumor cell lysis and RNA read depth

The expression level of somatic variants on tumor cells might affect the level of tumor cell lysis and is therefore a possible criterion for selecting mutations for the generation of tumor-specific T cells. To evaluate the influence of expression level on tumor-specific lysis, the authors plotted the percent tumor cell lysis from the TCK assays against the RNA read depth of the somatic variants (Figure 5). Interestingly, there was essentially no correlation; for example, the authors found that mutations with the lowest and highest read depth (*CACNA1H* and *NOP56* genes) induced the same level of CD8⁺ T-cell-mediated tumor lysis (~40%) (Figure 5A), and somatic variants with the same read depth did not induce the same level of CD4⁺ T-cell-mediated tumor lysis (*ZXDC* and *C1orf27* genes) (Figure 5B).

Figure 4. Clonogenicity assay confirms TCK results. Purified CD4+ and CD8+ cells from patient MDS-004 were induced and restimulated with autologous DCs and the indicated peptide pools or CEF peptide mix as a negative control for tumor cell killing. Cell cultures that were positive against autologous CD34+ cells or MCLs during immunogenicity testing were plated in a clonogenicity assay with autologous MCLs. Conditions are no T-cell control (MCL only), MCL and tumor-specific CD4+ or CD8+ T cells directed against the indicated mutation or CEF. DCs, dendritic cells.

Figure 5. Protein expression level does not correlate with level of lysis. Percent tumor cell lysis (cytotoxicity) induced by (A) CD8+ and (B) CD4+ T cells compared with RNA read depth for each mutation. Peptides that did not induce a T-cell response were not analyzed for ability to induce tumor cell lysis and therefore were not included. Spearman's r 0.1564 and -0.0726 for CD8 and CD4, respectively.

Discussion

The authors present a systematic analysis of the immunogenicity of neopeptides representing 32 somatic variants derived from cancer stem and progenitor cell mutations in five patients with MDS, which, like acute myeloid leukemia, is a malignancy with low mutational burden [7]. The primary finding from the results is that, for each of the five patients, the authors identified more than one somatic variant that effectively immunized autologous T cells *ex vivo* to kill autologous tumor cells while sparing cells not bearing the mutations. This result diverges from the widely held belief that malignancies with low mutational burdens are not amenable to neoantigen-based T-cell immunotherapy because of low TMB [30].

The identification of neoantigen-specific T cells is commonly based on the use of bioinformatic algorithms that predict HLA binding. Among the 14 variant neopeptides that induced a CD8⁺T-cell response, Immune Epitope Database software predicted 12 (HLA-A and HLA-B) with IC₅₀ <500 nM and two with IC₅₀ <1200 nM. These results support the use of major histocompatibility complex class I predictive binding algorithms [31] to identify neopeptides with potential immunogenicity and to reduce the number of individual peptides that require initial immunogenicity screening. This is an important practical consideration since an individual point mutation can give rise to 120 potential HLA-A or HLA-B binders of 9 mer length, not including HLA-C or the less common 8- or 12-mer peptides. Taking these considerations into account, along with evidence from viral systems that about 1% of peptides bind to a given HLA allele with IC₅₀ <500 nM [8], the authors expect to find one or more peptides binding with IC₅₀ <500 nM for a given somatic variant, which is consistent with the experimental data presented here.

In the present study, the authors show that 28 of 32 (88%) somatic variants generated neopeptides predicted to bind to HLA-A or HLA-B, where 14 of 28 (50%) induced a CD8⁺ response and 11 of 14 (79%) effectively induced CD8⁺ T cells to kill autologous tumor cells. Although many variants induced a CD4⁺ and CD8⁺T-cell response, among the 31 total neopeptide-specific T-cell responses obtained, nine (29%) were unable to kill the tumor.

The authors suspect that failure of some neopeptide-specific T cells to lyse tumor cells was perhaps due to destruction of the neopeptide during endogenous processing and transport to the endoplasmic reticulum, where binding to nascent class I molecules may occur [27]. This phenomenon has also been shown for class II neopeptides resulting from the processing of endogenously synthesized antigens. For instance, up to 50% of peptides eluted from class II molecules were found to derive from proteins associated with the nuclear and cytoplasmic compartments [32]. Differences in protein or peptide processing might explain why peptide-specific T cells fail to kill autologous tumor cells [33,34]. Since an increase in the amount of HLA-peptide complex leads to an increase in T-cell activation [35], it seems reasonable that proteins expressed at higher levels might lead to better recognition by T cells. However, the authors' results show no such correlation, which might be explained by different T-cell receptors having different affinities for the peptide-HLA complex. However, it would be necessary to compare a larger number of neoantigen-specific T cells obtained from the same individual to verify if there is a relationship between T-cell receptor affinity and target expression level of peptides with similar HLA-binding affinities. This finding would have significant implications in the selection of T-cell therapeutic targets. In summary, the authors' findings indicate that for tumors with low mutational burden, tumor cell lysis should be assessed systematically to validate neoantigen-specific T cells for adoptive T-cell therapy.

Prior studies have found a low amount of cytotoxic T-cell responses in patients with cancer—on the order of 1.2% of potential neopeptides [36]. The authors found that 34% of the tested variants induced a CD8⁺T-cell response recognizing autologous tumor. This

high rate of T-cell response likely results from the expansion of both memory and naive T-cell precursors since the authors' *in vitro* protocol was designed to activate naive T cells [18]. Similarly, in high-mutational-burden melanoma, Carreno *et al.* [37] found that dendritic cells pulsed with HLA-A2-binding neopeptides (<500 nM) resulted in nine of 21 neopeptides inducing a T-cell response, with seven (33%) recognizing the tumor cells. Stronen *et al.* [38] studied the T-cell neoantigen-specific repertoire of healthy HLA-A02:01 subjects that were presumably naive to the tumor antigens, finding that 10 of the 45 variants (22%) tested *in vitro* for immunogenicity induced a CD8⁺T-cell response that recognized endogenously processed antigen [38].

Conclusions

The authors demonstrate the induction of neoantigen-specific CD4⁺ and CD8⁺ T cells and recognition of processed antigens in autologous tumor cells in a high proportion of cases in patients with higher-risk MDS. Collectively, the authors' results demonstrate that a low mutational burden should not be viewed as an obstacle to developing adoptive T-cell therapies targeting tumor-specific somatic mutations [39–41] and that T-cell responses require validation by demonstrating effective tumor cell killing [26,42].

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Declaration of Competing Interest

AV and FF have an equity interest in PersImmune, Inc.

Author Contributions

Conception and design of the study: AV. Acquisition of data: VF, AT, HF, CM, TNT, RB, EDB, DT and AB. Analysis and interpretation of data: VF, AT, HF, LF, TC, FF, ZKY, BP, AS, TL and AV. Drafting or revising the manuscript: VF, TL and AV. All authors have approved the final article.

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Supplementary materials

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