

Immunogenicity evaluation of mutant peptides identified from lung cancer patients

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Abstract

Tumor genome sequencing can identify genetic mutations that, when translated, lead to mutant peptides. Such mutant peptides, when shown to be immunogenic, may be designated as neoantigens. We identified three mutant peptides (PLVAP, MBOAT7, and Neurofascin) from lung cancer patients and characterized their antigenicity in this study. T cells and B cells were isolated from peripheral blood mononuclear cells (PBMC) and tumor-infiltrating lymphocytes (TILs); dendritic cells (DCs) generated from PBMC were pulsed with the mutant peptides or their corresponding wild type (WT) peptides, in order to assess peptide anti-tumor immunogenicity. DCs presenting mutant peptides conferred TILs, PBMC CD3⁺ T cells as well as CD19⁺ B cells to directly kill tumor cells, and enhanced proinflammatory cytokine (IFN- γ , TNF) secretion and antibody (IgG, IgM) production significantly more than DCs presenting WT peptides. Of note, we observed mutant epitope-induced B cell and antibody responses that signify the role of B cells in immune response to malignancy, which was largely overshadowed by our focus on T cells. In addition, we found that identified epitopes from one patient are immunogenic in 5 of the 6 patients tested in autologous settings, suggesting the existence of these mutant epitopes in multiple lung cancer patients as shared antigens. Together, the results encourage the generation of antitumor T cells and B cells *via in vitro* activation with the identified antigenic mutant epitopes for subsequent T cell and/or B cell-based immunotherapy in lung cancer patients.

Introduction

Lung cancer is the leading cause for approximately 1.8 million deaths and 2.5 million new cases per year worldwide[1]. Despite progress made in surgery, immunotherapy and targeted therapy, lung cancer remains a poor prognosis cancer[2]. Lung cancer is a heterogeneous malignancy characterized by a high mutation profile.

As these cancers develop, they acquire mutations affecting the response to immune checkpoint inhibitor[3]. McGranahan et al. highlighted the significance of clonal neoantigen burden in lung cancer, and described a correlation of overall survival and enhanced effectiveness of PD-1 and CTLA-4 blockade therapies with a higher clonal neoantigen presence in tumors[4]. Łuksza et al. proposed a fitness model for tumors focusing on neoantigen interactions with the immune system[5]. These studies indicate that neoantigens, resulting from somatic mutations in cancers, can significantly influence the efficacy of immune therapies. However, several factors attenuate the anti-tumor immune responses in lung cancer. These include a limited patient T cell repertoire that is responsive to only a small subset of neoantigens.

Whole exome and RNA sequencing of fresh tumor tissues, transcriptomics and mass spectrometry-based proteogenomics, along with bioinformatics analysis informs the design and manufacture of mutant peptides. However, only those mutant peptides that are strongly immunogenic could be designated as neoantigens. Identifying therapeutically relevant neoantigens remains challenging, with positive responses in only 6% of candidates tested, emphasizing the significance to filter predicted neoantigens based on their immunogenicity.

To induce tumor specific T cells as well as B cells for immune therapies, we have previously developed tumor antigen-targeted dendritic cell (DC) vaccines. DCs loaded with the lysate of cancer stem cell (CSC)-enriched populations demonstrated significant protective antitumor immunity. In parallel, immune monitoring assays showed that sera from CSC-DC vaccinated hosts contained high levels of IgG which bound to CSCs, resulting in CSC lysis. These investigations established that CSC-DC could prime B cell/antibodies as well as T cells with anti-CSC immunity[6]. Our follow-up study further offered pre-clinical evidence that DC-based vaccine strategies are most efficacious if deployed in the adjuvant setting after surgical excision of established tumors[7]. We also developed integrin $\beta 4$ (ITGB4) protein-pulsed DC (ITGB4-DC) for vaccination with potential clinical benefit. ITGB4-DC induced endogenous T cell cytotoxicity and immune plasma against tumor which expressed ITGB4 [8]. Overall, these studies offered preclinical proof of concept for the use of tumor antigen-DC vaccines in cancer immunotherapy to induce both cellular and humoral anti-tumor immunity.

In this study, we conducted a comprehensive immunogenicity evaluation of mutant peptides identified *de novo* from lung cancer patients and hypothesize that immunogenic mutant peptides loaded to DCs can stimulate not only T cell but also B cell immune responses, thus conferring anti-tumor reactivity of these T cells and B cells in the peripheral blood as well as in the tumor-infiltrating lymphocytes (TILs) for future immunotherapy.

Materials and Methods

Patient sample collection

Resected tumors and peripheral blood samples were collected from six patients who underwent curative intent resection for lung cancer larger than 2 cm at a single institution (Table 1). The study was approved by the Institutional Research Board (HUM00174116). The freshly collected samples were sent to the lab immediately after the collection for the following processing. Patient tumor tissue samples were processed for TIL isolation. PBMC were isolated from whole blood, followed by T cell and B cell isolation (Fig. 1).

Mutant peptides identification and synthesis

The tumor tissue from patient 1 was used for whole exome sequencing (WES) and bulk RNA sequencing(RNA-seq). Most potential candidate peptides were selected based on the surface markers Ly9 (CD229), PLVAP, MBOAT7 and non-surface molecules TLN1, ABLIM3, DAXX, DST and neurofascin. The selected peptides were synthesized at Genemed Synthesis, Inc. (San Antonio, TX).

Tumor and normal tissue processing

To generate tumor cells as target cells in subsequent function analysis, part of the tumor tissue was finely minced and resuspended in Collagenase (Stemcell Technologies, US) within RPMI media for digestion. Normal tissue samples were processed similarly to generate cell suspensions as controls.

Serum, CD3, CD19, and CD3CD19 PBMC collection

Upon receipt of the blood sample, serum samples were first isolated by centrifugation. This was followed by the isolation of PBMCs using Ficoll density gradient centrifugation. The freshly collected PBMCs were rapidly sorted with magnetic cell isolation technology (Miltenyi Biotec, US), leading to the specific collection of CD3⁺ T cells, CD19⁺ B cells, and CD3⁻CD19⁻ monocytes.

DC Generation

The CD3⁻CD19⁻ PBMCs (monocytes) were cultured in RPMI media supplemented with 10% human serum and 1% Antibiotic-Antimycotic. To initiate DC generation, 50 ng/mL IL-4 and 100 ng/mL GM-CSF were added.

Table 1

Subjects' characteristics. LUAD: lung adenocarcinoma; CRC: colorectal cancer; LUSC: lung squamous cell carcinoma.

Subjects	Gender	Age at Surgery	Diagnosis	WES	WES Analysis	RNA-seq	RNA-seq analysis	Mutant Peptides	PBMC isolated from blood	TIL isolated
1	Female	70	LUAD	+	+	+	+	40	Yes	Yes
2	Male	59	Metastatic CRC	+	+	+	+	3	Yes	Yes
3	Male	71	LUAD	+	+	+	+	0	Yes	Yes
4	Female	71	LUSC	+	+	+	+	1	Yes	Yes
5	Female	68	LUAD	+	+	+	+	6	Yes	Yes
6	Female	65	LUAD	-	-	-	-	—	Yes	Yes

Every other day, for six days, half of the media was replaced with fresh media containing IL-4 and GM-CSF. On the seventh day, half of the media was substituted with fresh media that included 10 ng/mL IL-1 β , 10 ng/mL IL-6, and 10 ng/mL TNF α in addition to IL-4 and GM-CSF. On the ninth day, selected peptides were added to the DC culture at a concentration of 100 μ g/mL and incubated for one day.

Generation of TILs

Part of the tumor sample was utilized to generate TILs. Tumors were minced (3 to 4 mm³) and put into a 24-well plate. High concentration IL-2 (6000 IU/mL) was used for the generation of TILs in RPMI supplemented with 10% human serum (Valley Biomedical Products & Services, Inc., Winchester, VA) and 1% Antibiotic-Antimycotic (Gibco, Thermo Fisher Scientific Inc., US) in a total volume of 2 mL per well. Half of the media was periodically removed and replenished with fresh media containing IL-2. The TIL generation process lasted for 12 days. Subsequently, the TILs were collected *via* filtration and cryopreserved for further use.

TIL and CD3 PBMC T cell activation

For T cell activation, each plate was coated with 5 μ g/mL anti-human CD3 and 5 μ g/mL anti-human CD28 one day before cells plating. Then the wells were washed, and TILs or CD3⁺ PBMC T cells were added and cultured in RPMI supplemented with 10% human serum and 1% Antibiotic-Antimycotic, along with 1000 IU/mL IL-2. Such T cell activation and expansion was carried out for two days prior to their co-culture with DCs.

CD19 PBMC B cell activation

To activate B cells, CD19⁺ PBMCs were cultured in RPMI supplemented with 10% human serum and 1% Antibiotic-Antimycotic, along with 5 μ g/mL anti-human CD40, 5 μ g/mL Lipopolysaccharide (LPS), and 300 IU/mL IL-2. B cell activation and expansion was carried out for two days prior to their co-culture with DCs.

Co-culture of DCs with T cells and B cells

On the tenth day of DC generation, DCs were harvested, counted, and loaded with the mutant peptide, or corresponding wide-type (WT) peptide as control. Subsequently, these DCs were co-cultured with activated and expanded TILs, CD3⁺ PBMC T cells, and CD19⁺ PBMC B cells, respectively (Fig. 1).

Cytotoxicity Assays

One day after the co-culture of DCs with T cells or B cells, the supernatants from the co-culture were collected for subsequent ELISA analysis. TILs, CD3⁺ PBMC T cells, and CD19⁺ PBMC B cells were collected and counted. These immune cells were then co-cultured with either tumor cells derived from the patients or A549 cells, respectively, to examine their killing effects (cytotoxicity). Cytotoxic T lymphocyte (CTL)-mediated cancer cell killing was assessed using the lactate dehydrogenase (LDH) release assay kit (CytoTox 96 Non-Radioactive Cytotoxicity Assay; Promega, Madison, WI), following the manufacturer's instruction in round bottom 96-well culture plates (Fig. 1). In the experimental wells, effector cells (lymphocytes) and target cells (cancer cells) at various ratios were incubated at 37°C for 4 hours. Subsequent to the incubation, LDH levels were measured. The degree of cytotoxicity was calculated as per the instructions provided in the assay manual:

$$Cytotoxicity (\%) = \frac{Exp_{LDH} - Eff_{spon} - Tar_{spon}}{Tar_{max} - Tar_{spon}} \times 100$$

A549 cell line was utilized in some experiments. To sensitize cancer cells to cytotoxic immune cells, we treated these cells with 2 μ mol/L Pemetrexed (Sigma-Aldrich, US) [9], and incubated for two days in DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin.

Measurement of cytokine and antibody production

Cytokines produced by activated/expended TILs and CD3⁺ PBMC T cells in the supernatants of cell culture were detected using commercially available ELISA kits (Fig. 1). Specific kits for human interferon (IFN- γ), tumor necrosis factor (TNF), IL-6 and IL-10 (all from BD Biosciences, US) were used as instructed by the manufacturer's instructions. Antibody production of total IgG and IgM from CD19⁺ PBMC B cells was detected also using the ELISA kits (Invitrogen, Thermo Fisher Scientific Inc., US) (Fig. 1).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software Inc., San Diego, CA). Data were evaluated by unpaired Student t test. The statistical significance was set at $p < 0.05$.

Results

Mutant peptides identified from lung cancer patients

We collected tumor tissues and peripheral blood (Fig. 1) from six lung cancer patients (Table 1). Among them, four patients were diagnosed with lung adenocarcinoma (LUAD, patients 1, 3, 5, 6); one with lung squamous cell carcinoma (LUSC, patient 4), and one with metastatic colorectal cancer (metastatic CRC, patient 2). Patients' age ranged from 59 to 71 years old with 2 males and 4 females.

Mutant peptide sequences were identified from four patients (Table 1). In Patient 1, the identification was based on the analysis that 19.6% of non-synonymous coding mutations displayed a predicted affinity of less than 500 nM, categorizing them as potential neoantigen sequences (Fig. 2). In 4/6 patients from the cohort, we identified at least one mutated peptide sequence from each patient (arranged from 1–40) (Table 1). Among them, PLVAP[10], MBOAT7[11], and Neurofascin [12] from Patient 1 were used for this study, based on their expression as cell surface markers and previous studies indicating their potential roles in tumor progression. Accordingly, we synthesized three pairs of peptides. Each pair consisted of a mutant peptide (Mu peptides) and its respective wild type (WT) counterpart (Table 2). PLVAP and MBOAT7 mutant peptides bear a single amino acid mutation from Val(V) to Leu(L), while Neurofascin mutant peptide was from Arg(R) to Leu(L) (Table 2).

Table 2
Three pairs of mutant peptides identified vs. WT peptides from subject 1 as described in Materials and Methods. Peptides were synthesized from Genemed Synthesis, Inc.

Gene	Peptide Sequence
PLVAP-WT	VLFMVYGNVHVSTESNLQATE
PLVAP-Mutation	VLFMVYGNVHVLSTESNLQATE
MBOAT7-WT	RAYDYMCMGFVLLSLADTLRY
MBOAT7-Mutation	RAYDYMCMGFLLSLADTLRY
Neurofascin-WT	NSTAISLQWNRVYSDTVQGQL
Neurofascin-Mutation	NSTAISLQWNLVYSDTVQGQL

TILs, CD3⁺ PBMC T cells, CD19⁺ PBMC B cells stimulated by mutant peptide-presenting DCs showed effective killing of cancer cells

In our initial experiments, DCs presenting the mutant peptide PLVAP or Neurofascin significantly enhanced the ability of TILs and PBMC CD3⁺ T cells to kill both tumor cells and tumor cell lines, compared to DCs presenting the WT peptide. Specifically, Patient 1 DCs presenting the mutant PLVAP peptide stimulated autologous (Patient 1) TILs ($p = 0.002$) and PBMC CD3⁺ T cells ($p = 0.046$) to kill autologous (Patient 1) tumor cells significantly higher than DCs presenting the WT PLVAP peptide (Fig. 3A). Interestingly, we noticed that Patient 4 DCs presenting the mutant PLVAP peptide stimulated autologous (Patient 4) TILs ($p =$

0.004) and PBMC CD3⁺ T cells ($p = 0.013$) to kill autologous (Patient 4) tumor cells also significantly higher than DCs presenting the WT PLVAP peptide (Fig. 3B). Similarly, we found that Patient 6 DCs presenting the mutant neurofascin peptide stimulated autologous (Patient 6) TILs to kill PEM treated autologous (Patient 6) tumor cells significantly ($p = 0.048$) higher than DCs presenting the WT neurofascin peptide (Fig. 3C), with negligible difference in cytotoxicity against A549 cell cultures. Previous reports showed that pemetrexed (PEM) enhances the sensitivity of lung cancer cells to immune cells[9]. However, T cells activated by mutant MBOAT7 peptide identified from Patient 1 demonstrated significant killing of tumor cell line A549 (Fig. 3D), revealing mutant peptide-induced killing effects could cross tumor cells and cell line. Although the cytotoxicity against A549 without PEM treatment was limited, mutant MBOAT7-activated T cells enhanced cytotoxicity in PEM-treated A549 cells significantly ($p = 0.010$), confirming that chemotherapy may condition tumors for mutant peptide-induced killing. More importantly, Patient 2 DCs presenting the mutant peptide MBOAT7 stimulated autologous (Patient 2) PBMC CD3⁺ T cells to kill PEM-treated A549 cells significantly ($p < 0.001$) higher than DCs presenting the WT MBOAT7 peptide (Fig. 3D).

In these experiments, these T cells showed no killing effects on normal tissues (Fig. 3D), demonstrating tumor specificity. We confirmed the specific killing of tumor cells, as opposed to normal tissue cells by autologous (Patient 2) PBMC CD19⁺ B cells as well as PBMC CD3⁺ T cells and TILs stimulated with autologous (Patient 2) DCs presenting the mutant PLVAP peptide. As shown in Fig. 3E, we observed that the herein activated B cells directly killed tumor cells as observed with activated T cells and TILs, which represented a novel phenomenon as we previously reported[13–15]. As observed with mutant MBOAT7, when activated with PLVAP, T cells, B cells and TILs killed autologous (Patient 2) tumor cells extensively, with minimal effect in normal tissue cells.

Together, these killing studies confirm that the mutant peptides identified from Patient 1 are absent in normal tissue, and strongly suggest that mutant peptides PLVAP and Neurofascin may exist in multiple lung cancer patients, e.g. Patients 1, 2, 4, 6, representing 4 out of 6 or ~ 67% of the patients examined in this study.

Mutant peptide-DCs induced proinflammatory cytokine secretion

Proinflammatory cytokines play fundamental roles in anti-tumor immunity in addition to tumor cell killing. Therefore, we examined the potential of mutant peptide (s) to induce proinflammatory cytokine production.

In these experiments, we found that Patient 1 DCs presenting mutant PLVAP peptide stimulated autologous (Patient 1) PBMC CD3⁺ T cells, resulting in significant IFN γ production (Fig. 4A). In addition, Patient 2 DCs presenting mutant MBOAT7 peptide stimulated autologous (Patient 2) PBMC CD3⁺ T cells to produce high levels of IFN- γ (Fig. 4A). Similarly, Patient 3 DCs presenting mutant MBOAT7 peptide stimulated autologous (Patient 3) PBMC CD3⁺ T cells to produce IFN- γ (Fig. 4A) as well. These experiments reveal mutant peptide-DC induces proinflammatory cytokine IFN γ secretion, and again demonstrate common expression of mutant peptides PLVAP and MBOAT7 in different lung cancer patients.

We further tested the tumor-specificity of above observed IFN- γ production. In comparison, IFN γ production of TILs from Patient 2 was significantly ($p = 0.0442$) higher when stimulated with autologous (Patient 2) DCs presenting mutant PLVAP than when stimulated with DCs presenting WT PLVAP (Fig. 4B). Similarly, PBMC T cells from Patient 2, when activated with autologous (Patient 2) DCs presenting mutant MBOAT7 peptide, produced significantly ($p = 0.0384$) more IFN- γ than when stimulated with DCs presenting WT MBOAT7 (Fig. 4B), confirming tumor-specific production of IFN γ .

To confirm this proinflammatory response we measured the generation of cytokine TNF. When Patient 1 DCs presenting the mutant PLVAP peptide to autologous (Patient 1) PBMC CD3⁺ T cells, these T cells produced significantly ($p = 0.01$) higher level of TNF compared with DCs presenting WT PLVAP as demonstrated in Fig. 4C. Notably, we found that Patient 6 DCs presenting the mutant peptide Neurofascin to autologous (Patient 6) PBMC CD3⁺ T cells resulted in remarkably higher amount of TNF production compared with DCs presenting the WT Neurofascin peptide when co-cultured with autologous (Patient 6) tumor cells (Fig. 4D). We got very similar results ($p = 0.04$) when utilizing autologous (Patient 6) TILs (Fig. 4E). In both Fig. 4D and 4E, we used normal tissue cells as control as in the above cytotoxicity experiments. Both Fig. 4D and 4E reveal that only mutant peptide Neurofascin vs. WT conferred significant TNF production, and this induced proinflammatory cytokine production was specifically

against cancer cells. In addition, as observed in our cytotoxicity assays, these mutant peptide-DC induced proinflammatory cytokine secretion experiments confirm that the mutant peptides identified from Patient1, e.g., PLVAP, MBOAT7 and Neurofascin may exist in multiple lung cancer patients, e.g. Patients 1, 2, 3, 6.

Mutant peptide-DC stimulation is associated with downregulated immuno-suppressive cytokine secretion

Interleukin-6 (IL-6), a pleiotropic cytokine, exerts potent anti-inflammatory effects by modulating the differentiation of regulatory T cells (Tregs) and suppressing the excessive activation of pro-inflammatory immune cells in pathological conditions[16]. In additional experiments, we found that DCs presenting mutant peptides conferred autologous PBMC CD3⁺ T cells to secrete significantly less IL-6. Specifically, DCs presenting mutant peptides PLVAP, MBOAT7 significantly reduced the IL-6 secretion by the respective autologous PBMC CD3⁺ T cells of Patient1 ($p = 0.03$, Fig. 5A), Patient 2 ($p = 0.02$, Fig. 5B), and Patient 4 ($p = 0.002$, Fig. 5C) compared with DCs presenting the corresponding WT peptides. In contrast, we observed that generation of IL-10 did not differ in similar experiments comparing mutant and WT peptide PLVAP-DCs (Fig. 5D).

Collectively, these data indicate that in addition to CTL activity, DCs presenting the mutant peptides polarized the autologous PBMC CD3⁺ T cells towards a Type 1 profile. This polarization was characterized by increased secretion of IFN- γ and TNF, but reduced secretion in IL-6, compared to the responses to DCs presenting the WT peptides. This polarization is in favor of the anti-tumor immunity, thus confirming the immunogenicity of the mutant peptides tested in this study.

Mutant peptide-DCs induced antibody production

Humoral response represents another arm of the host anti-cancer immunity. However, over the years, the role of B cells in the host immune response to malignancy has been largely overshadowed by focus on T cell responses. To further evaluate the immunogenicity of the mutant peptides regarding their potential to modulate the humoral responses, we conducted separate experiments examining their impact on B cell activation and antibody production.

DCs generated from Patient 1 presenting the mutant peptide PLVAP significantly stimulated autologous (Patient 1) PBMC CD19⁺ B cells to secrete higher levels of IgG and IgM ($p = 0.045$) compared to DCs presenting the WT PLVAP (Fig. 6A). DCs generated from Patient 3 presenting the mutant peptide MBOAT7 to autologous (Patient 3) PBMC CD19⁺ B cells resulted in high levels secretion of IgG and IgM (Fig. 6B). Together, these results suggest that mutant peptides PLVAP and MBOAT7 are immunogenic to B cells as well as to T cells, and they may exist in other lung cancer patients than Patient 1.

Discussion

Dendritic cells (DCs) are critical antigen-presenting cells (APC) bridging innate and adaptive immunity. They are essential for activating and directing T cells against tumor cells [17]. Neoantigens identified from tumor-specific mutations have been shown to serve as novel targets for cancer immunotherapy [18–20]. Several clinical trials are exploring the feasibility of neoantigen-based vaccines for solid tumors, including lung cancer[21, 22]. A single-arm trial involving neoantigen-DC cell-activated neoantigen specific T cell therapy (ClinicalTrials.gov NCT03205930) assessed the safety and efficacy in a total of 13 patients with relapsed non-small cell lung cancer (NSCLC)[23].

To test our hypothesis that in lung cancer, patient-derived mutant peptides loaded to DCs can stimulate T cell and B cell immunity, thus conferring the anti-tumor responses of these T cells and B cells for future immunotherapy, we identified mutant peptides from resected tumor tissues and isolated T cells from both peripheral blood and TILs of these patients. We also isolated B cells from the peripheral blood. DCs were generated from patient blood samples. These DCs were then pulsed with the mutant peptide epitopes or relevant WT peptides as control. We stimulated the isolated T cells and B cells with the peptide-DCs. Both T and B cells were tested for their killing efficacy against tumor, cytokine production, and antibody secretion, respectively, ensuring a comprehensive assessment of their anti-tumor immunogenicity. The mutant peptide(s) presented by DCs induced significant killing of tumor cells as well as a proinflammatory immune response, marked by increased levels of IFN- γ and TNF.

Simultaneously, there was a notable decrease in the immune suppressive response, as evidenced by reduced IL-6 levels. This shift in cytokine production towards a Type 1 profile is crucial in immune responses against cancer in addition to tumor cell killing by T cells. Of note, the mutant peptide-DC conferred B cell direct killing on tumor cells and antibody production, which represents a novel finding in this study.

We identified mutant peptides in this study with a frequency consistent with previous reports in NSCLC[24]. This alignment with previous findings reinforces the validity of our approach in identifying and testing these mutant peptides as potential neoantigens in lung cancer. The presentation of these mutant peptides by DCs elicited significant cytotoxicity, cytokine secretion and antibody production vs. the corresponding WT peptides, demonstrating their immunogenicity. Importantly, our data suggest that the identified mutant peptides may exist in multiple lung cancer patients, indicating possible shared antigen expression across different individuals of lung cancer patients.

In 6 out of 7 NSCLC-patients analyzed, Lennerz et al[25] proved that for one of these patients, engineered TCR-T cells with 4 candidate tumor specific TCRs showed reactivity against the patient's tumor. Using these TCR-T cells to screen for candidate neoantigens, 3 TCRs recognized recurrent driver-mutation KRAS Q61H-peptide ILDTAGHEEY. The finding of homologous immunogenicity of independent KRAS Q61H-positive cancers may suggest ILDTAGHEEY as a shared antigen epitope for the patients analyzed in their study. In our study, 5 out of 6 lung cancer patients were analyzed. We found that in Patients 1, 2, 3, 4, 6), PLVAP, MBOAT7, and Neurofascin mutant peptides identified from Patient 1 reacted against autologous tumors when presented by their autologous DCs to each patient's own T cells and B cells respectively. Of note, we cannot conclude that our identified mutant peptides are neoantigens, as patient HLA matching was not completed due to limited tissue availability. The data presented herein suggest that the identified mutant peptide epitopes may exist in multiple lung cancer patients, supporting the concept of shared antigen across different lung cancer patients. Further studies are needed to characterize their "cross-presentation" by various type of HLAs. This may help with future characterization of these mutant peptides as potential lung cancer neoantigens[26] in future study. Successful neoantigen prediction lays the foundation for the development of "off-the-shelf" neoantigen vaccines in future cancer therapy.

The results presented in this study may encourage the exploration of novel T cell immunotherapy of lung cancer patients. Using identified immunogenic mutant peptide(s) to *in vitro* stimulate TILs or PBMCs acquired from lung cancer patients may result in the generation of lung cancer-specific T cells that can mediate anti-lung cancer efficiency, including tumor regression, when infused back to lung cancer patients. We have significant experience in developing T cell immunotherapy both in clinical trials and in pre-clinical studies[27–36]. In addition, prior studies have demonstrated that a subset of TILs or PBMCs are tumor specific and cytotoxic, which play major roles in anti-tumor immunity[26, 37]. Stimulating the TILs and PBMCs with immunogenic mutant antigen(s) identified from lung cancer patients would significantly augment their anti-tumor responses in these patients.

Moreover, the mutant peptides identified in this study triggered not only T cell-mediated killing and anti-tumor cytokine production against lung cancers, but also induced B cell-mediated immune responses. This was evident by B cell killing on tumor cells and IgG, IgM production in response to lung cancer cells in this study. However, the lung cancer-specificity of these IgG and IgM was not examined due to the lack of samples. In extended studies, the lung cancer-specificity can be tested by binding assays of these IgG and IgM to lung cancer cells. Specific binding would lead to the lung cancer cell lysis in the presence of complement (CDC) or with the addition of NK cells, macrophages, or neutrophils (ADCC). In addition, we previously reported anti-tumor effector B cell which are capable of direct killing the tumor cells in the absence of complement and ADCC, just like the CTL T cells[14, 37]. Specifically, we find that *in vitro* activated B cells could mediate significant tumor regression in cancer adoptive immunotherapy[13]. The direct killing effects of these B cells involve the Fas/FasL[15] as well as the CXCR4/CXCL12[37] signaling pathways. The results of this current study further support our efforts to stimulate the TILs and PBMCs with the mutant antigen (s) to significantly augment B cell anti-tumor responses in lung cancer patients.

We also note that PBMC CD3 + T cell cytotoxicity is enhanced significantly by pembrolizumab. These findings suggest that chemotherapy e.g. PEM treatment, may play a role in conditioning tumors for mutant peptide-based immunotherapy. Novel synergies with chemotherapy followed by immunogenic mutant peptide-based T cell, B cell and vaccination therapy may open a new avenue to enhance the treatment of lung cancer.

Li et.al.[38] predicted neoantigen-derived CTL epitopes as well as B cell epitopes and constructed a multiple epitope vaccine (MEV) by rationally conjugating selected CTL and B cell epitopes for lung adenocarcinoma. Very recently, Zhang et. al.[39] conducted a single-arm phase Ib trial (NCT04193098) using mutant peptide-pulsed DC-stimulated multiple target cytotoxic T lymphocytes (MCTL) in 21 patients with advanced NSCLC. Our previous study also found that using T cells together with B cells in adoptive immunotherapy is significantly more potent than using each cell type alone to mediate tumor regression[13]. We reported targeting ALDH^{high} tumor cells using two antigenic peptides derived from ALDH. ALDH 1A1(LLYKLADLI) or 1A3 (LLHQLADLV) peptide-DC vaccines primed CTLs that specifically killed ALDH^{high} tumor cells, with ALDH 1A1 + 1A3 dual peptides-DC vaccine mediating an additive CTL effect compared to single peptide-DC vaccines[40]. Together, these studies support using the identified mutant peptides in combination to simultaneously activate both T cells and B cells for future cancer immunotherapy.

Individualized vaccine is considerably prolonged. In this study, mutant peptides identified from one lung cancer patient demonstrated broad immunogenicity for multiple lung cancer patients, making it possible to apply the mutant peptide(s) identified from one lung cancer patient to generate vaccine for the use of other lung cancer patients. We have significant experience developing peptide vaccines [6–8] [40]. Recently, we successfully generated peptide-nanodisc (ND) vaccines[41, 42] which efficiently targeted cancer stem cells. Further studies are warranted to develop lung cancer mutant peptide(s)-based ND vaccine.

Declarations

Competing Interests:

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval:

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Institutional Research Board from University of Michigan Hospital (HUM00174116).

Consent to participate:

Informed consent was obtained from all individual participants included in the study.

Consent to publish:

The authors affirm that human research participants provided informed consent for research data publication.

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Author Contribution

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by all authors. Jianyi Yang, Ying Xin and Jiahua Liu wrote the manuscript. All authors commented on previous and current versions of the manuscript.

Data Availability:

The datasets generated and analyzed during the current study are available from the first authors and corresponding authors on reasonable request.

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Figures

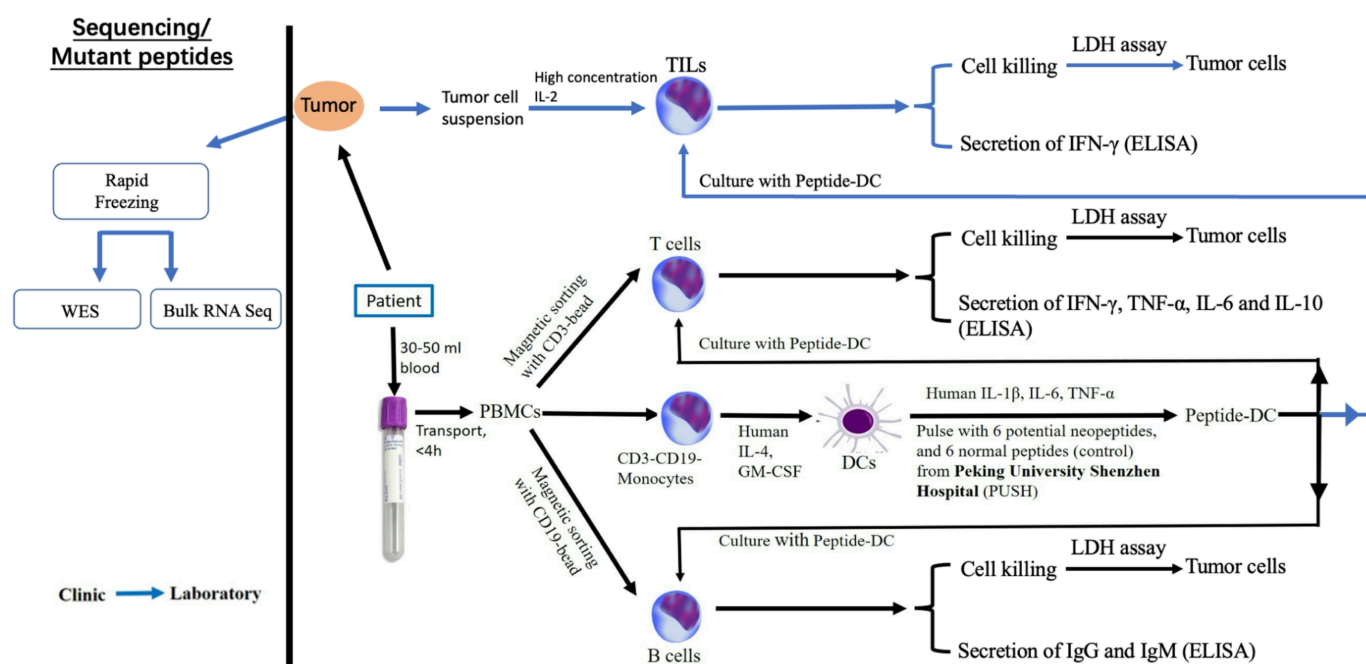


Figure 1

Experiment protocol to evaluate the immunogenicity of mutant peptides

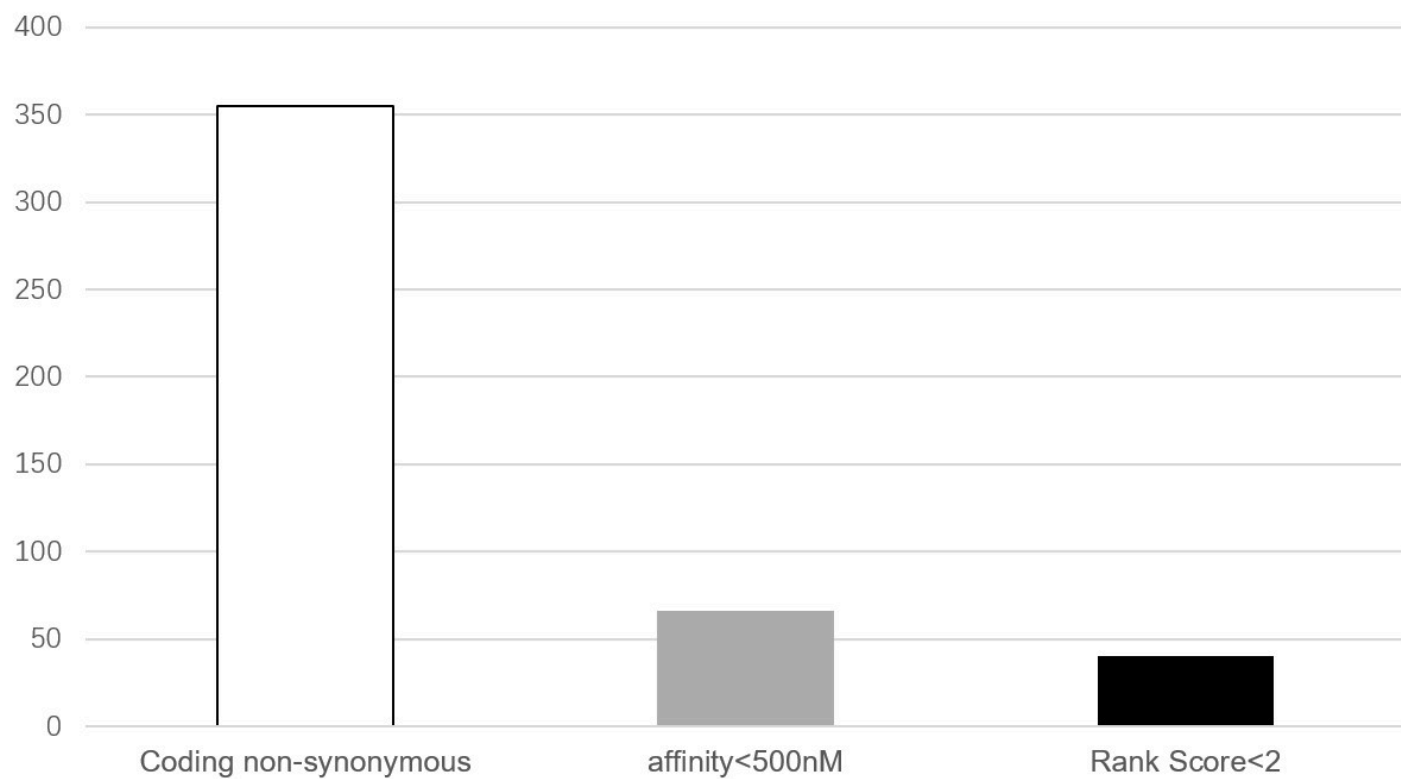


Figure 2

19.6% of non-synonymous coding mutations have a predicted affinity <500nM.

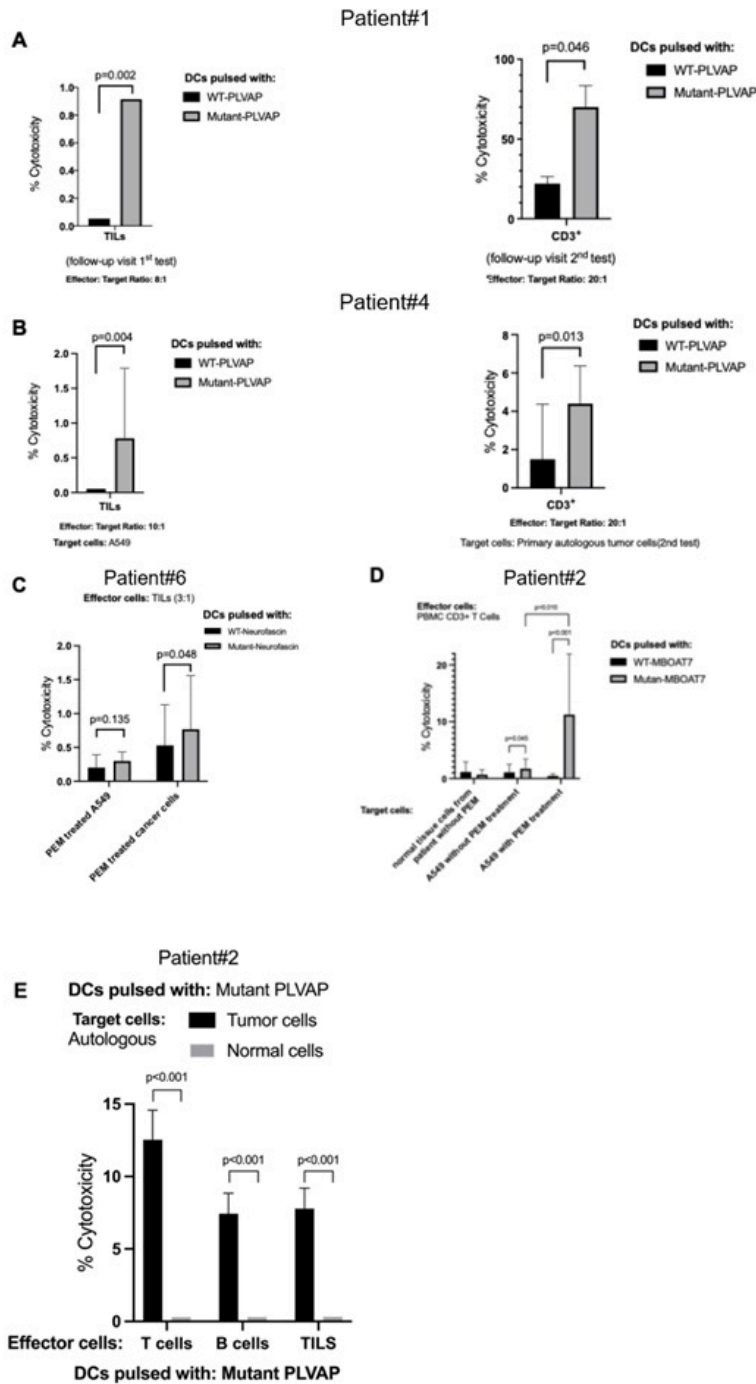


Figure 3

Stimulation with mutant peptide-pulsed DCs conferred TILs, CD3⁺ PBMC T cells and CD19⁺ PBMC B cells with potent cancer cell-killing ability. (A, B) DCs presented mutant peptide PLVAP and stimulated autologous TILs and PBMC CD3⁺ T cells to kill tumor cells significantly more than DCs presenting WT peptide (LDH Assay, as described in Materials and Methods). (C) DCs presented mutant peptide Neurofascin and stimulated autologous TILs to kill tumor cell line A549 and autologous tumor cells. (D, E) Specific killing of tumor cells vs. normal tissue cells by autologous effector cells stimulated with mutant peptide-DC, and (E) Direct killing of tumor cells by mutant peptide-DC stimulated B cells as well as T cells and TILs.

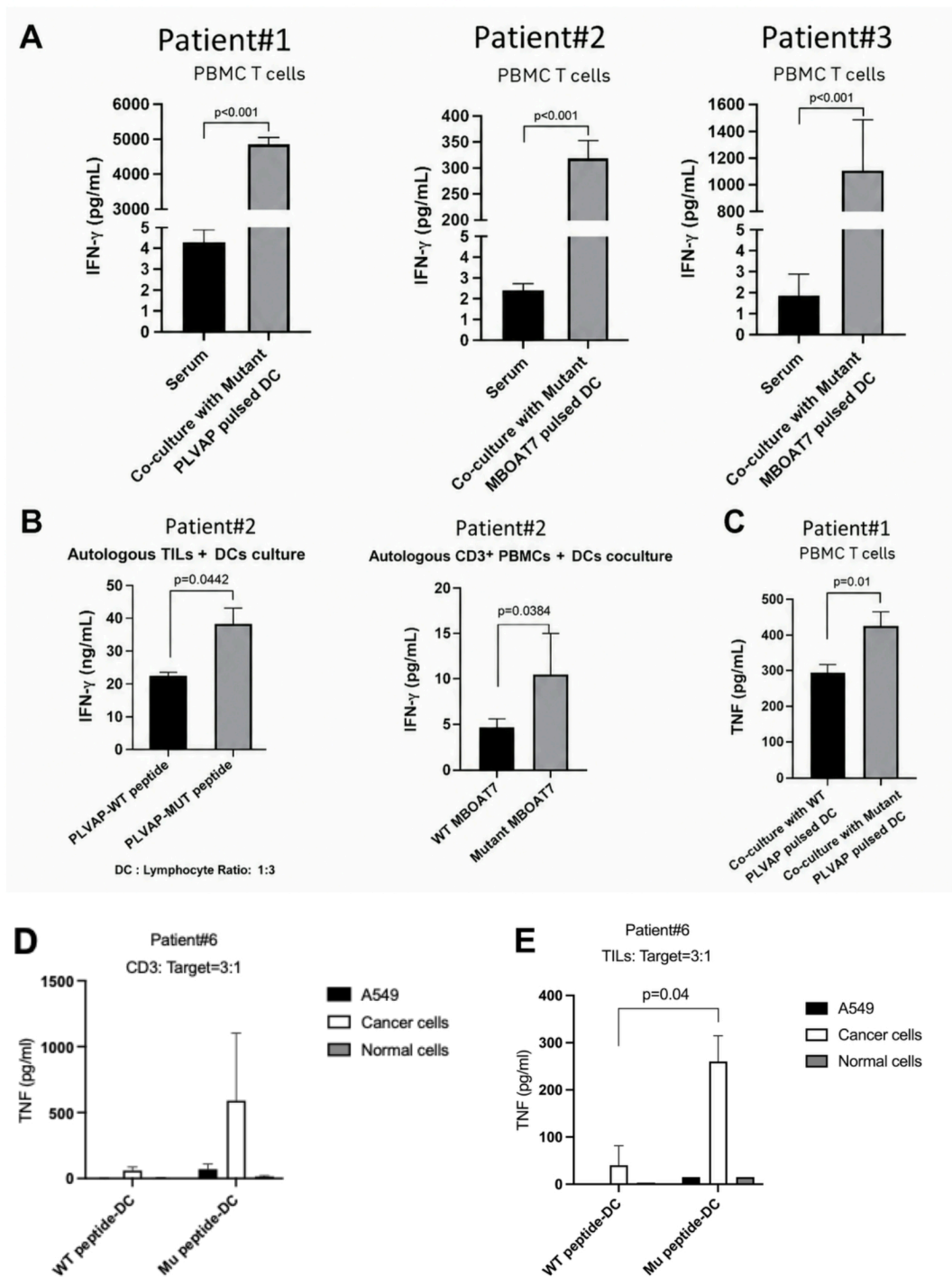


Figure 4

Mutant peptide-pulsed DCs elicited a robust proinflammatory cytokine response of autologous CD3⁺ PBMC T cells and TILs. **(A)** DCs presented mutant peptides to autologous PBMC T cells to secrete IFN- γ (ELISA Assays, as described in Materials and Methods). **(B)** DCs presented mutant peptides and stimulated autologous TILs and PBMC T cells to secrete IFN- γ . **(C)** DCs presented mutant peptide PLVAP to autologous PBMC CD3⁺ T cells to secrete significantly more TNF than DCs presenting WT peptide. **(D, E)** DCs presented mutant peptide Neurofascin to autologous CD3⁺ PBMC T cells and TILs to secrete significantly more TNF than DCs presenting WT peptide when co-cultured with autologous tumor cells, but not to the normal tissue cells.

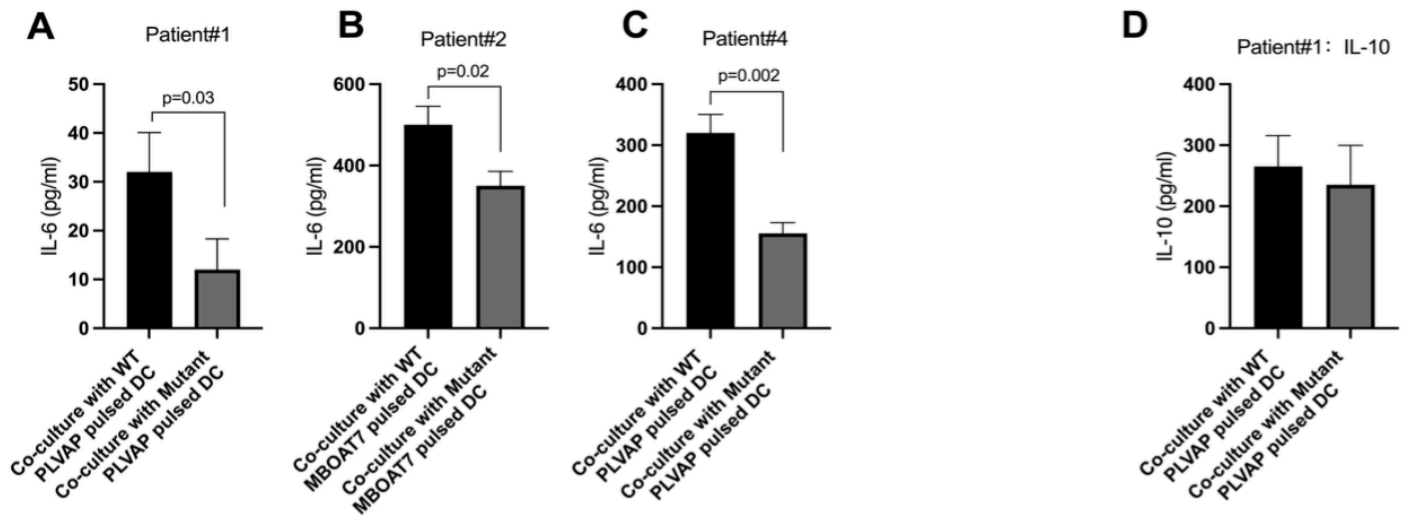


Figure 5

Mutant peptide-presenting DCs significantly decreased the levels of immune suppressive cytokine IL-6, but showed no significant impact on IL-10. **(A)** DCs presented mutant peptide PLVAP to autologous PBMC CD3⁺ T cells of Patient 1, resulting in significantly less IL-6 secretion (ELISA Assays, as described in Materials and Methods). **(B)** DCs presented mutant peptide MBOAT7 to autologous PBMC CD3⁺ T cells of Patient 2, resulting in significantly less IL-6 secretion. **(C)** DCs presented mutant peptide PLVAP to autologous PBMC CD3⁺ T cells of Patient 4, resulting in significantly less IL-6 secretion. **(D)** DCs presenting mutant peptide PLVAP to autologous PBMC CD3⁺ T cells of Patient 1 did not alternate the IL-10 secretion compared with DCs presenting WT PLVAP.

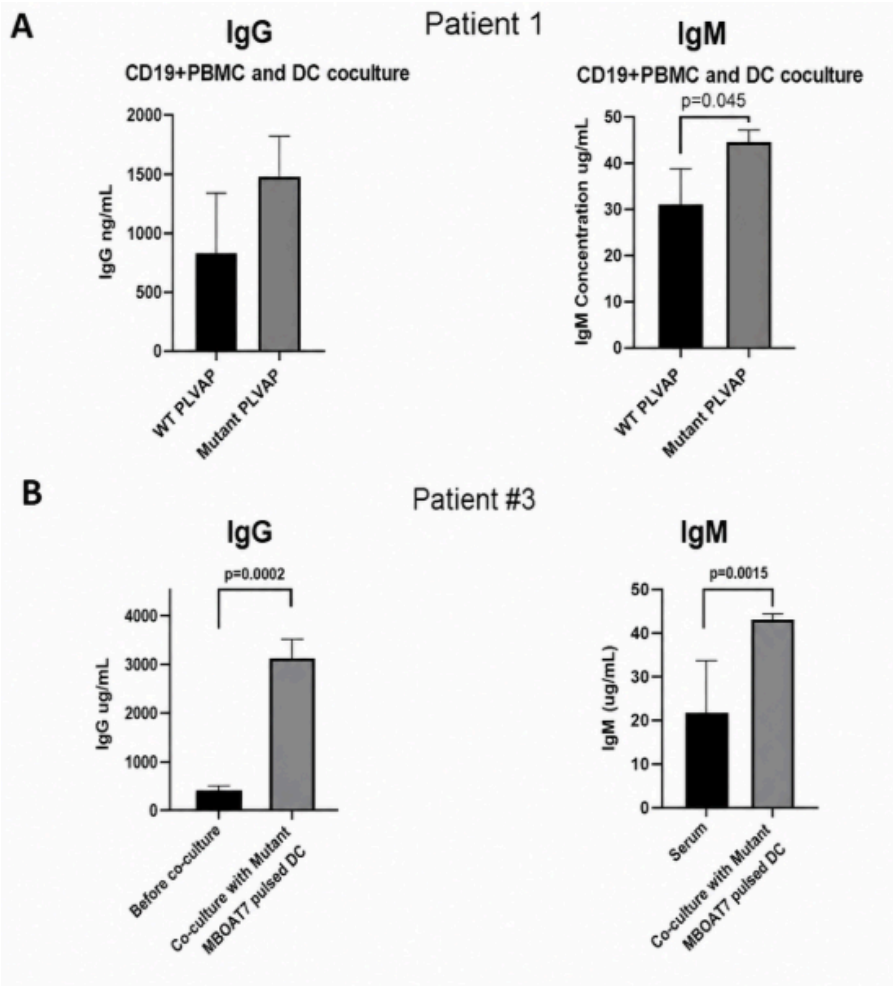


Figure 6

Mutant peptide-pulsed DCs remarkably promoted antibody production by CD19⁺B cells. **(A)** DCs presented mutant peptide PLVAP and stimulated autologous PBMC CD19⁺ B cells to secrete more IgG and IgM than DCs presenting WT PLVAP (ELISA Assays, as described in Materials and Methods). **(B)** DCs presented mutant peptide MBOAT7 to autologous PBMC CD19⁺ B cells to secrete IgG and IgM at high levels.