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ARTICLE

Clinical Study

Phase I trial of a heat-conditioned tumor lysate vaccine (TRIMELVax) in anti-PD-1 refractory melanoma: safety and immunological aspects (NCT06556004)

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BACKGROUND: TRIMELVax is a cancer vaccine prototype derived from heat-conditioned melanoma cell lysates combined with a natural adjuvant. Preclinical studies demonstrated robust antitumor immune responses and tumor regression. We conducted a Phase I clinical trial to evaluate the safety, tolerability, immunogenicity, and preliminary efficacy of TRIMELVax in patients with unresectable stage IV melanoma who had progressed after first-line anti-PD-1 therapy.

METHODS: Eligible patients had stage IV melanoma with documented progression or unacceptable toxicity following anti-PD-1 treatment. TRIMELVax was administered subcutaneously every four weeks for a total of four doses. The primary endpoints of the study were safety and feasibility, which were assessed according to CTCAE v5.0. Secondary endpoints included efficacy as assessed by RECIST 1.1, overall survival (OS), progression-free survival (PFS), and immunogenicity, evaluated by peripheral blood immune cell responses and delayed-type hypersensitivity (DTH) reactions.

RESULTS: Seventeen patients received ≥ 1 dose; 13 completed all four doses. Treatment-related adverse events occurred in 9 patients, with a grade 1 or 2 severity. Two patients experienced manageable grade 3 events. No grade 4–5 toxicities were observed. No complete responses were observed in this cohort. Notably, a partial response was observed in 1 patient, stable disease in 6 patients, yielding a 41% disease control rate; 10 patients progressed. Median OS was 14 months, and median PFS was 5.2 months. DTH positivity was observed in six of the nine patients tested, correlating with the induction of memory T cells in peripheral blood after treatment. Immunomonitoring revealed increased CXCR3 expression in $CD8^+$ T cells and decreased CD39 expression in both $CD4^+$ and $CD8^+$ subsets in patients with disease control. One case with lung metastasis regression exhibited a significant expansion of $TCF1^+PD\text{-}1^+$ $CD4^+$ and $CD8^+$ T cell populations, and an enriched $perforin^+granzyme\ B^+$ $CD8^+$ T cell compartment, consistent with vaccine-associated immune activation.

CONCLUSIONS: TRIMELVax demonstrated acceptable safety and early signs of clinical activity in anti-PD-1 refractory melanoma patients. These findings support its immunogenic potential and warrant further evaluation in larger, controlled studies. CLINICALTRIAL.GOV: NCT06556004.

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BACKGROUND

Immunotherapy has transformed the treatment landscape for advanced melanoma, with immune checkpoint inhibitors (ICIs) targeting CTLA-4 and PD-1 showing unprecedented survival benefits [1, 2]. Despite these advances, many patients do not respond or develop resistance, particularly those with poorly

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inflamed or "cold" tumors characterized by limited T-cell infiltration, as widely reported in the literature [3–5]. Moreover, these groundbreaking therapies have led to the observation and reporting of various types of adverse events, commonly referred to as immune-related adverse events (irAEs) [6–8]. This highlights an urgent need for novel and complementary strategies to convert non-responsive tumors into immune-inflamed states, enhancing durable clinical benefits with a lower rate of adverse events. Therapeutic cancer vaccines represent a promising approach to achieve this goal [9–11]. By delivering relevant tumor-associated antigens (TAAs) along with danger-associated signals, vaccines can prime and expand tumor-specific T-cell responses, potentially restoring sensitivity to ICIs. Whole tumor cell (WTC) vaccines are a desirable alternative because they provide a naturally diverse repertoire of antigens, including neoantigens and shared TAAs, while also incorporating endogenous stress signals that facilitate dendritic cell (DC) activation and antigen cross-presentation [12]. Despite encouraging preclinical evidence, clinical experience with cancer vaccines has remained limited by suboptimal immunogenicity, a lack of standardization in adjuvant strategies, and heterogeneous clinical outcomes [12, 13].

Our research group has contributed to the development of a new generation of WTC-based immunotherapies over the past two decades [14–17]. DC vaccines loaded with heat-shock–conditioned melanoma cell lysates (TAPCells) elicited delayed-type hypersensitivity (DTH) responses, correlating with improved survival in clinical trials [14–17]. We recently advanced this concept with TRIMELVax, a therapeutic vaccine composed of heat-shocked allogeneic melanoma cell lysates combined with the potent natural adjuvant *Concholepas concholepas* hemocyanin (CCH) [18]. CCH is a high-molecular-weight glycoprotein with strong immunostimulatory capacity, previously shown to induce potent Th1-polarized immune responses, promote antigen cross-presentation, and act as an effective vaccine adjuvant [17, 19, 20]. Preclinical studies in mice demonstrated that TRIMELVax inhibited tumor growth and increased mouse survival, inducing $CD8^+$ T cell-mediated immune responses against melanoma-associated antigens [18]. The vaccine increased the frequency of intratumor cDC1s and augmented infiltration of $CD3^+$, $CD4^+$, and $CD8^+$ T cells. Moreover, TRIMELVax promoted an increased proportion of PD-1^{low} $CD8^+$ T cells in tumors, a phenotype associated with prototypic effector cells required for tumor growth control and for preventing dysfunctional T-cell accumulation [18]. Although TRIMELVax itself has not previously been tested in humans, the TRIMEL lysate component was extensively evaluated in phase I/II clinical trials as the antigenic payload for DC vaccines (TAPCells) developed by our group. In these studies, the lysate concentration and vaccination schedule were empirically optimized based on immunogenicity, DTH induction, and safety outcomes in melanoma patients, providing a clinically validated framework for the current trial [14–17].

The development of optimized formulations such as TRIMELVax, specially designed to overcome early WTC vaccine limitations, provides a renewed opportunity to revisit and position cancer vaccines as viable therapeutic options in patients with advanced, treatment-refractory melanoma. Here, we report the results of a first-in-human phase I clinical trial evaluating the safety, tolerability, and preliminary efficacy of TRIMELVax in patients with unresectable stage IV melanoma refractory to anti-PD-1 therapy.

METHODS

Study design and patient recruitment

This was a phase I, open-label clinical trial evaluating the safety, clinical response, and immunogenicity of TRIMELVax in patients with unresectable stage IV melanoma who had experienced progression or, in one case, unacceptable toxicity (patient #10) following first-line anti-PD-1 immunotherapy. Eligible patients were aged 18 years or older, had histologically confirmed melanoma, an Eastern Cooperative Oncology Group (ECOG) performance status of 0–1, and had adequate organ function. Exclusion criteria included active autoimmune disease and concurrent immunosuppressive therapy. Protocol details are available at clinicaltrials.gov NCT06556004.

Ethical approval

This study was approved by the Institutional Review Board at the Faculty of Medicine, Universidad de Chile (Protocol ID: 066-2020) and the Regional Ethics Committee of Santiago, Chile (CEC SSM Oriente). All participants provided written informed consent before any study procedures, in accordance with the Declaration of Helsinki.

Vaccine composition

TRIMELVax is a therapeutic cancer vaccine composed of heat-shock-conditioned allogeneic melanoma cell lysates, named TRIMEL, combined with a natural adjuvant, hemocyanin from *Concholepas concholepas* (CCH). CCH (Biosonda, Chile) was purified under sterile, endotoxin-free conditions. The endotoxin content was assessed using a PyroGene Recombinant Factor C Endotoxin Detection Assay kit (Lonza Group, Walkersville, MD, USA). The melanoma component is derived from three established human melanoma cell lines (MEL1, MEL2, and MEL3). Database Name for MEL1, MEL2, and MEL3: International Depositary Authority of Canada (IDAC) Accession Numbers: 260916-01, 260916-02, and 260916-03, respectively. The HLA class I alleles represented in the TRIMEL cell lines are as follows: MEL1: HLA-A02:01, HLA-A05; HLA-B35, HLA-B40; MEL2: HLA-A02:01; MEL3: HLA-A29, HLA-A68; HLA-B44, HLA-B*51. Cells were routinely tested and confirmed to be free of mycoplasma contamination. The cell lines were subjected to heat stress before lysis [16, 18]. This procedure promotes translocation of calreticulin across the plasma membrane and the release of high-mobility group box-1 (HMGB1) and ATP, thereby enhancing the lysate's immunogenicity via danger-associated molecular patterns (DAMPs) [16]. The resulting lysates provide a broad repertoire of TAAs, including proteins MART-1, gp100, tyrosinase, NY-ESO-1, MC1R, MAGE, and GAGE [16].

Immunization protocol and vaccine dose

Each TRIMELVax dose consisted of 500 µL of conditioned tumor cell lysate equivalent to 10×10^6 melanoma cells/mL mixed with 100 µL CCH (1 mg/mL) under sterile conditions. Patients received TRIMELVax by subcutaneous administration every four weeks for a total of four doses (days 0, 28, 56, and 84). Vaccinations were administered at predefined cutaneous sites under aseptic conditions. Patients were monitored for at least 30 min after each injection for the occurrence of immediate local or systemic adverse events. The dose and vaccination schedule of TRIMELVax were determined based on prior clinical and preclinical experience. TRIMEL lysate had previously been used as the antigenic source in DC-based vaccines (TAPCells) evaluated in phase I/II clinical trials in melanoma patients, in which lysate concentration and vaccination schedule were empirically optimized based on safety, DTH induction, and immunogenicity outcomes [14–17]. A four-dose vaccination schedule administered over approximately 3 months was established as clinically feasible and immunogenic and served as the basis for the vaccination periodicity used in the present study. TRIMEL lysate concentration per dose was established in preclinical murine studies based on the demonstration of a robust antitumor immune response and tumor growth inhibition, without evidence of toxicity, whereas lower concentrations were ineffective [18].

Toxicity

The primary objective was to assess the therapy's safety, as evaluated by medical controls under the supervision of a clinical oncologist. Adverse events were measured according to Common Terminology Criteria for Adverse Events (CTCAE) 5.0 [21].

Peripheral blood mononuclear cell (PBMC) isolation

Peripheral blood samples were collected into EDTA-coated vacutainers at baseline and before each subsequent vaccination to monitor systemic immune responses. An aliquot of 500 µL of whole blood was stabilized with 25 µL of TransFix® (Cytomark) and stored at 4 °C in the dark until staining. The remaining blood was processed on the same day to isolate PBMCs. For PBMC isolation, whole blood was diluted 1:1 in RPMI-1640 medium (Gibco) and layered onto SepMate tubes (Stemcell Technologies)

preloaded with 15 mL of Lymphoprep. Samples were centrifuged at 1200 × g for 20 min at room temperature without a break. The PBMC layer was collected, washed with RPMI, and treated with ACK lysis buffer (Thermo Fisher) to remove erythrocytes. The cells were then resuspended in RPMI 1640 medium. Approximately 3×10^6 PBMCs were stabilized in 5% TransFix and stored at 4 °C in the dark for 5–7 days before staining.

Immune response monitoring

Flow cytometry was performed on either 1×10^6 PBMCs or 100 µL of stabilized whole blood per patient, depending on the staining panel. PBMCs were stained in 96-well V-bottom plates, while whole blood was stained in 5 mL polypropylene tubes. Fc receptor blocking (BioLegend) was performed before surface staining with fluorescent-conjugated monoclonal antibodies. Cells were fixed and permeabilized for intracellular staining (e.g., granzyme B) using IC Fixation and Perm Buffers (BioLegend) according to the manufacturer's protocol. After surface staining, whole blood samples were lysed with EasyLyse™ Erythrocyte-Lysing Reagent (Agilent). The following antibodies were obtained from BioLegend and used at optimized dilutions (1:100 or 1:200): CD4 FITC (OKT4); CD138 PE (DL-101); CD56 PerCP/Cy5.5 (HCD56); CD123 APC (6H6); CD14 Alexa Fluor 700 (63D3); CD8 APC/Cy7 (SK1); CD38 BV421 (HIT2); CD11c B510 (3.9); CD16 BV605 (3G8); CD19 BV650 (HIB19); PD-1 BV711 (EH12.2H7); CCR7 PE (G043H7); CCR4 PerCP/Cy5.5 (L291H4); CD27 APC (M-T271); CXCR3 Alexa fluor 700 (G025H7); CD127 APC/Cy7 (A019D5); Granzyme B BV421 (QA18A28); CCR6 BV510 (G034E3); CXCR5 BV605 (J252D4); PD-1 BV711 (EH12.2H7); CD11b FITC (ICRF44); PD-L1 PE (29E.2A3); CD19 PE/Dazzle 594 (HIB19); CD86 BV650 (IT2.2); CD64 BV711 (10.1); CD24 PE/Dazzle 594 (ML5); IgM APC/Cy7 (MHM-88); CD38 BV421 (HIT2); IgG BV510 (M1310G05). Additional antibodies used on a 1:200 dilution were: CD3 PE/Dazzle 594 (UCHT1); CD45 PE/Cy7 (HI30); HLA-DR BV785 (L243); CD45RA BV650 (HI100); IgD FITC (IA6-2). For spectral flow cytometry, PBMC were incubated with a mixture of Human TruStain FcX (BioLegend) and the viability dye Zombie Near Infrared (BioLegend). Chemokine receptor antibodies were labeled in staining buffer (50% FACS Buffer (PBS with 2% FBS) and 50% Brilliant stain buffer (BD Bioscience)) for 20 min at 37 °C, protected from light. Cell surface labeling was performed in a staining buffer for 30 min at 4 °C in the dark. Cells were fixed and permeabilized using the eBioscience FOXP3 Transcription Factor Staining Buffer Set (Invitrogen) according to the manufacturer's protocol. The following antibodies were used for spectral flow cytometry at the indicated dilutions: from BioLegend, CD8 Spark PLUS UV 395 (RPA-T8) 1:1600, CCR7 BV650 (G043H7) 1:200, CD45RO BV711 (UCHL1) 1:100, GZMB Alexa Fluor 700 (QA16A02) 1:200, CD69 APC-Cy7 (FN50) 1:200, PD-1 PE-Cy7 (EH12.2H7) 1:400; from BD Bioscience, CD3 BUV496 (UCHT1) 1:600, CD4 BUV563 (SK3) 1:1600, CD101 BV480 (V7.1) 1:600, GNLY Alexa Fluor 488 (RB1) 1:300, FOXP3 PE-CF594 (236 A/E7) 1:400, CD25 PE-Cy5 (M-A251) 1:200; from Invitrogen, CD62L BUV615 (DREG56) 1:400, Perforin eFluor 450 (dG9) 1:100, CD127 Alexa Fluor 532 (eBioRDR5) 1:200, CD45 PerCP (2D1) 1:800; and from Cell Signaling, TCF1 PE (C63D9) 1:400. Data acquisition was performed on a BD LSRFortessa X-20 flow cytometer using the FACSDiva software and a Cytek Aurora spectral flow cytometer using the SpectroFlo software. Data were analyzed using FlowJoTM v10.10 software (BD Bioscience).

Delayed-type hypersensitivity (DTH) reaction associated with vaccine-induced immune recall

DTH tests using TRIMEL lysate were performed 30 days after the last immunization to evaluate the in vivo immune response, as described in López et al. [15]. Skin tests were performed by intradermal injection of 150 µL TRIMEL (2 mg/mL), or 150 µL of saline solution at different sites. A positive reaction was defined as skin induration of 5 mm or greater 48 hours after injection.

Clinical response and survival analysis

Objective response was evaluated clinically and radiologically using Positron Emission Tomography (PET) and/or Computed Tomography (CT) scans performed at baseline, after completion of the four vaccine administrations, and every 12 weeks thereafter until disease progression. Tumor response was assessed according to the Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 [22]. Progression-free survival (PFS) was defined as the interval from the date of the first TRIMELVax administration to the first documentation of disease progression by imaging or physical examination, or to death from any cause, whichever occurred first. Overall survival (OS) was measured from the date of the first vaccine dose to the date of death from any cause or last follow-up. The disease control rate (DCR) was calculated as the proportion of evaluable patients who achieved complete response (CR), partial response (PR), or stable disease (SD) as their best overall response, divided by the total number of evaluable patients, according to RECIST 1.1 criteria.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 10 software (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between the two groups were performed using the non-parametric Mann-Whitney U test. The Kruskal-Wallis test was followed by Dunn's multiple comparison post hoc tests for multiple group comparisons. Data were presented as individual values with mean ± standard error of the mean (SEM), unless otherwise specified. Survival analysis was made using the Kaplan–Meier method and the log-rank test. A p-value < 0.05 was considered statistically significant.

RESULTS

Patient characteristics and treatments

Between January 2022 and November 2023, 20 patients with unresectable stage IV melanoma signed informed consent and were enrolled in this phase I trial. Eligibility was based on prior disease progression, and in one case (Patient #10), unacceptable toxicity following anti-PD-1 therapy (Supplementary Table 1). All enrolled patients had received previous systemic treatment, including at least one anti-PD1 immunotherapy agent. Seven patients had received a second agent in combination with anti-PD1 therapy (Table 1). The most used first-line treatment was nivolumab, followed by pembrolizumab and cemiplimab. The duration of first-line therapy ranged from 3 to 24 months (Supplementary Table 1).

Of the 20 registered patients, two did not initiate the protocol due to rapid clinical deterioration, and one was excluded after the first dose of the vaccine because of a diagnosis of anti-PD1 immune-mediated pneumonitis, ruling out what had been interpreted as pulmonary progression. Following vaccination, the patient reported grade 1 dyspnea; therefore, a new CT was performed. The lung lesions had changed location on the CT scan, so he was re-evaluated. Finally, anti-PD1 immune-mediated pneumonitis was diagnosed, ruling out pulmonary progression. For this reason, the patient was excluded from the study as a screening failure. Thus, 17 patients received at least one dose of TRIMELVax starting May 16, 2022. The final dose was administered on March 19, 2024. Thirteen patients completed the full four-dose protocol. Reasons for early discontinuation included the patient's death or rapid disease progression ($n = 4$). Baseline demographic and clinical characteristics are summarized in Table 1 and Supplementary Table 1.

Safety and adverse effects

All patients were clinically evaluated before and after each administration and followed until protocol discontinuation (due to disease progression or death). Overall, the therapy was well tolerated, with an acceptable toxicity profile and no serious adverse events observed. Nine patients experienced treatment-related adverse events (AEs). Two patients (#4 and #14) experienced grade 3 AEs that required a delay in subsequent dose administration. One patient developed palmoplantar keratoderma (PPK), characterized by persistent thickening of the palms and soles, while another patient had elevated gamma-glutamyltransferase (GGT) levels, a biomarker of hepatobiliary damage. Both AEs were managed medically, and the patients completed the protocol. Patient #4 restarted 8 weeks after the event, after topical treatment by dermatology, and patient #14 restarted 2 weeks later after normalizing her GGT values without medical intervention. No grade 3 AEs required hospitalization or led to long-term functional impairment. Seven patients presented grade 1 or 2 adverse events, including headache, fatigue, local

pain, and flu-like symptoms. A summary of all reported AEs is provided in Table 2.

Clinical response

No CR was observed in this cohort; however, one patient achieved PR as their best RECIST response. Among the 17 patients who received treatment, seven patients, including the patient with PR (#8, #9, #12, #14, #16, #17, and #20), achieved stable disease (SD), yielding a disease control rate (DCR) of 41% (Fig. 1a and Table 3). The remaining ten patients experienced progressive disease (PD). With a median follow-up of 18.5 months, the median overall survival (OS) in the cohort was 14 months (Fig. 1b), and the median progression-free survival (PFS) was 5.2 months (Fig. 1c).

Following disease progression, nine patients received additional systemic therapies, including: three patients treated with BRAF/MEK inhibitors (patients #10, #12, and #20), one patient was treated with an anti-KIT therapy (#4), and seven who received subsequent anti-PD1 immunotherapy (patients #4, #8, #9, #10, #14, #16, and #17). Notably, three of these patients, who had previously progressed on anti-PD1 therapy, achieved long-term stable disease upon re-exposure to anti-PD1 treatment following TRIMELVax therapy (patients #8, #9, and #17).

Case analyses highlighted patient #8, who had previously progressed on anti-PD1 (cemiplimab) and IL-2 therapy for three months, and subsequently achieved a partial response to TRIMELVax, with complete resolution of measurable pulmonary nodules (Fig. 1d). One year after starting the TRIMELVax protocol, patient #8 experienced disease progression and was re-treated with anti-PD1 therapy, achieving stable disease that was maintained through the last follow-up (Supplementary Table 1). Patient #9, who had progressed on anti-PD1 therapy (nivolumab for ten months), showed a significant reduction of a lesion in the left knee after TRIMELVax therapy, although not enough to be classified as a partial response, achieving a stable disease (Fig. 1e). Seven months after starting the protocol, patient #9 experienced progression and was re-exposed to nivolumab, again achieving stable disease at the most recent follow-up. Finally, patient #17, who had previously progressed after 13.5 months of anti-PD1 therapy (nivolumab), achieved stable disease following TRIMELVax treatment. Nine months after starting the protocol, patient #17 experienced disease progression. This patient was re-treated with nivolumab and palliative radiotherapy, achieving stable disease (Supplementary Table 1).

Immune response

Immunological responses were assessed one month after completion of the vaccination schedule by measuring DTH reactions to the TRIMEL lysate in patients who completed all four immunizations. DTH testing was used as an in vivo indicator of vaccine-induced immune recall to antigens contained within the

Table 1. Baseline patient characteristics ($n = 17$).

Characteristic	*n* (%) or Median [Range]
Age, years (mean, range)	65 (36–81)
Sex	
– Male	9 (53)
ECOG PS	
– 0–1	15 (88)
LDH levels	
– ≤ ULN	9 (53)
– ≥ ULN	6 (35)
– Unknown	2 (12)
M stage (AJCC-8)	
– M1a	4 (23)
– M1b	3 (18)
– M1c	9 (53)
– M1d	1 (6)
Number of lesion sites	
– 1	1 (6)
– 2	5 (29)
– ≥ 3	11 (65)
Subtype	
– Acral	6 (35)
– Mucosal	2 (12)
– Cutaneous, non-acral	9 (53)
Liver metastases present	4 (23)
BRAF status	
– Mutant	4 (23)
– Wild type	7 (42)
– Unknown	6 (35)
Previous systemic therapy	
– Nivolumab only	8 (47)
– Nivolumab + others*	3 (18)
– Pembrolizumab only	2 (12)
– Pembrolizumab + others**	2 (12)
– Cemiplimab + IL-2	2 (12)

*Others = surgery/chemotherapy/radiotherapy; surgery; dabrafenib/trametinib. **Others = surgery/radiotherapy. *AJCC-8* eighth edition of the American Joint Committee on Cancer, *ECOG PS* Eastern Cooperative Oncology Group performance status, *ULN* upper limit of normal, *Unk* unknown.

Table 2. Adverse effects associated with TRIMELVax treatment.

Event	CTCAE grade (number of patients)			
	Grade 1	**Grade 2**	**Grade 3**	**Grade 4**
Palmar-plantar erythrodysesthesia syndrome (1)	0	0	1	0
Headache	1	0	0	0
Fatigue	1	0	0	0
GGT increased level	1	0	1	0
Injection site reaction	2	1	0	0
Flu-like symptoms	0	1	0	0
Total	**5**	**2**	**2**	**0**

CTCAE Common Terminology Criteria for Adverse Events, *GGT* gamma-glutamyl transferase;
(1) hyperkeratosis with pain.

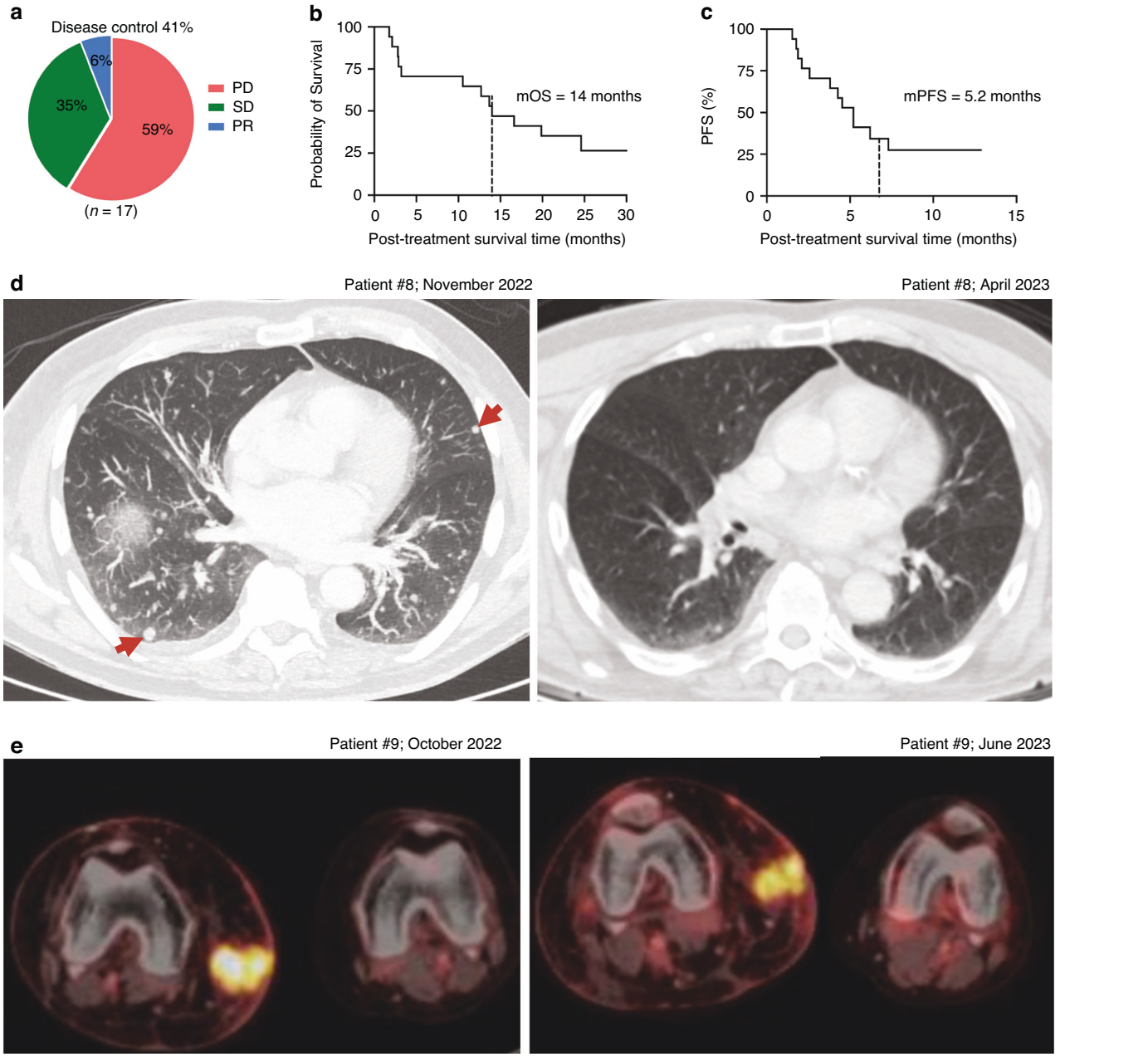

Fig. 1 Clinical response. a Pie chart with percent disease control (SD + PR) and PD according to RECIST 1.1 by investigator review of all patients ($n = 17$). **b** Kaplan–Meier curve of OS in all 17 treated patients. **c** Kaplan–Meier curve of PFS in all 17 treated patients. **d** Metastatic lung nodules regressions (red arrows) based on computed tomography (CT) scan imaging at 5 months after administration of the four doses of TRIMELVax (right) compared with baseline (left) for patient #8. **e** Reduction of a target lesion in the left knee of patient #9. An 18F-fluorodeoxyglucose (FDG) PET/CT comparison of the basal and post-treatment (four doses of TRIMELVax) images shows that the lesion decreased in size and intensity, as indicated by the standardized uptake value (SUV).

TRIMELVax formulation, as previously reported for other tumor lysate-based vaccination strategies [13–15]. Patients exhibiting an induration greater than 5 mm in diameter were classified as DTH-positive (DTH$^+$).

DTH assessments were performed in 9 of the 17 treated patients, of whom six were classified as DTH$^+$. Four of the 13 patients who completed the full vaccination schedule were not evaluated because they failed to attend the scheduled DTH testing visit. Among evaluable patients, three individuals with stable disease (patients #9, #14, and #17) and the patient who achieved a partial response (patient #8) exhibited a positive DTH reaction to the TRIMEL lysate (Fig. 2a and Table 3).

Immunophenotypic analysis of peripheral blood mononuclear cells revealed dynamic post-vaccination changes in T-cell populations relative to baseline. In four DTH$^+$ patients analyzed, TRIMELVax administration promoted a shift in both CD4$^+$ and CD8$^+$ compartments from naïve/stem-cell memory ($T^N + T^{SCM}$) phenotypes toward memory subsets, showing post-treatment increases in central (T^{CM}) and/or effector memory (T^{EM}) populations and relative decreases in $T^N + T^{SCM}$ and effector (T^{EF}) subsets (Fig. 2b).

Following vaccination, an overall increase in CXCR3 expression was observed in CD8$^+$ T cells at the cohort level, although inter-individual variability was noted (Fig. 2c). These changes are consistent with modulation of chemokine receptor expression

Table 3. Clinical and immunological effects of TRIMELVax vaccination.

Patient	TRIMEL doses	RECIST	CTCAE	DTH
6	1	PD	(−)	nt
18	4 complete	PD	(−)	nt
2	2	PD	(−)	nt
5	2	PD	(−)	nt
13	3	PD	G2	nt
3	4 complete	PD	(−)	nt
7	4 complete	PD	(−)	(+)
1	4 complete	PD	G2	(+)
10	4 complete	PD	G1	(+)
4	4 complete	PD	G3	(−)
17	**4 complete**	**SD**	**(−)**	**(+)**
20	**4 complete**	**SD**	**(−)**	**nt**
12	**4 complete**	**SD**	**G2**	**nt**
16	**4 complete**	**SD**	**(−)**	**(−)**
14	**4 complete**	**SD**	**G3**	**(−)**
9	**4 complete**	**SD**	**G1**	**(+)**
8	**4 complete**	**PR**	**(−)**	**(+)**

AE Adverse Events, *DTH* Delayed type hypersensitivity, *PD* Progressive disease.
SD Stable disease, *PR* Partial response.

associated with T-cell trafficking, but do not allow conclusions regarding migration into tumor tissues in the absence of paired tumor biopsies. Conversely, CD39 expression decreased in both $CD4^+$ and $CD8^+$ subsets (Fig. 2c, d). These shifts were more pronounced in patients with stable disease than in those who progressed.

The responder patient with partial regression of lung metastases (patient #8) showed a marked expansion of perforin$^+$ granzyme B^+ $CD8^+$ T cells (from 5.7% to 12.2%), consistent with enhanced cytotoxic potential (Fig. 2e). Similar increases were observed in patient #9 (from 30.3% to 48%) and patient #10 (from 5.7% to 12.5%) (Supplementary Fig. 2A). Patient #8 also displayed a substantial expansion of TCF1$^+$ PD-1$^+$ $CD8^+$ (from 2.5% to 56.8%) and TCF1$^+$ PD-1$^+$ $CD4^+$ (from 3.2% to 39.4%) T-cell populations (Fig. 2e), a phenotype associated with progenitor-like cells that sustain durable antitumor immunity. A moderate increase in TCF1$^+$ PD-1$^+$ $CD8^+$ cells was also detected in patient #10 (from 13.4% to 24.4%) (Supplementary Fig. 2B). Finally, a reduction in $CD8^+$ CX3CR1$^+$ granzyme B^+ T cells was observed in patients #9 and #10, but not in patient #8. Collectively, these immune phenotype shifts may serve as potential biomarkers for future studies. However, such modulation was not evident in all patients, as illustrated by patient #17, who exhibited no significant immunological changes after vaccination compared with baseline (Supplementary Fig. 2C).

DISCUSSION

This phase I trial demonstrates that TRIMELVax, a heat-conditioned whole-tumor lysate vaccine combined with the natural adjuvant CCH, is feasible and well-tolerated in patients with advanced melanoma who have progressed on anti-PD-1 therapy. The safety profile was acceptable, with most adverse events grade 1 or 2 and only 2 grade 3 toxicities, both manageable without requiring hospitalization or sequelae. These findings compare favorably with the spectrum of irAEs reported with ICIs, which can include severe or life-threatening manifestations.

Although the trial was not designed as a primary efficacy endpoint, a partial response with regression of lung metastases was reported. Notably, stable disease was observed in 7 of the treated patients. Several patients who progressed following TRIMELVax subsequently achieved stable disease upon re-exposure to anti-PD-1 therapy. This suggests that vaccination affected the immune contexture and restored sensitivity to checkpoint blockade. Similar synergistic effects between cancer vaccines and ICIs have been reported in other early-phase studies, further supporting the potential of combinatorial strategies [23, 24]. Consistently, a previous preclinical study demonstrated that TRIMELVax, but not anti-PD1 treatment, induced enhanced tumor infiltration of effector $CD3^+$ $CD8^+$ T cells with a reduced exhausted phenotype [18]. However, when evaluating the response to the experimental treatment, we cannot fully rule out prior pseudo-progression. For this reason, blood-based markers related to immune activation were explored as a complement to clinical findings.

The induction of DTH reactions in a subset of patients who completed the vaccination schedule indicates that TRIMELVax can elicit in vivo vaccine-induced immune recall responses to antigens present in tumor lysate. While DTH positivity does not constitute direct evidence of tumor-specific immunity, particularly in the context of an allogeneic lysate, it has been consistently associated with clinical benefit in prior studies using TRIMEL-based TAPCells and other tumor lysate-based vaccination strategies, where DTH responses correlated with improved survival and durable disease control [15, 16].

In parallel, the post-vaccination modulation of peripheral T-cell compartments, characterized by a shift from naïve and stem-cell memory phenotypes toward central and effector memory subsets, is consistent with the induction of memory-type T-cell differentiation. Although the functional specificity and intratumoral activity of these populations cannot be directly assessed in the absence of paired tumor biopsies, these systemic immunological changes support the notion that TRIMELVax promotes immune priming and memory formation that may contribute to sustained immune surveillance.

Following vaccination, an overall increase in CXCR3 expression was observed in $CD8^+$ T cells at the cohort level, although inter-individual variability was noted. These changes are consistent with modulation of chemokine receptor expression associated with T-cell trafficking, but do not allow conclusions regarding migration into tumor tissues in the absence of paired tumor biopsies. Moreover, the expansion of TCF1$^+$PD-1$^+$ progenitor-like populations in responders is consistent with emerging evidence that this subset is critical for sustaining long-term effector responses under PD-1 blockade [25, 26]. The observation of an enriched perforin$^+$ granzyme B^+ $CD8^+$ T-cell compartment in a subset of patients, including one with metastatic regression, further supports the capacity of TRIMELVax to modulate cytotoxic effector programs, although tumor specificity cannot be directly inferred from peripheral analyses alone.

In the context of cancer immunotherapy, the concept of *immune fitness* has been used to describe the capacity of the adaptive immune system to sustain effective antitumor responses, characterized by the presence of functional, non-terminally exhausted T-cell populations capable of proliferation, differentiation, and effector function upon antigen encounter [27]. Features commonly associated with immune fitness include preservation of memory T-cell subsets, reduced expression of exhaustion-associated markers, and the ability to generate cytotoxic responses upon appropriate stimulation. In the present study, post-vaccination changes observed in peripheral blood, such as reduced expression of exhaustion-associated markers and shifts toward memory T-cell phenotypes, may be consistent with modulation of systemic immune fitness. However, these findings do not constitute direct evidence of functional immune fitness or tumor-specific T-cell activity and should be regarded as

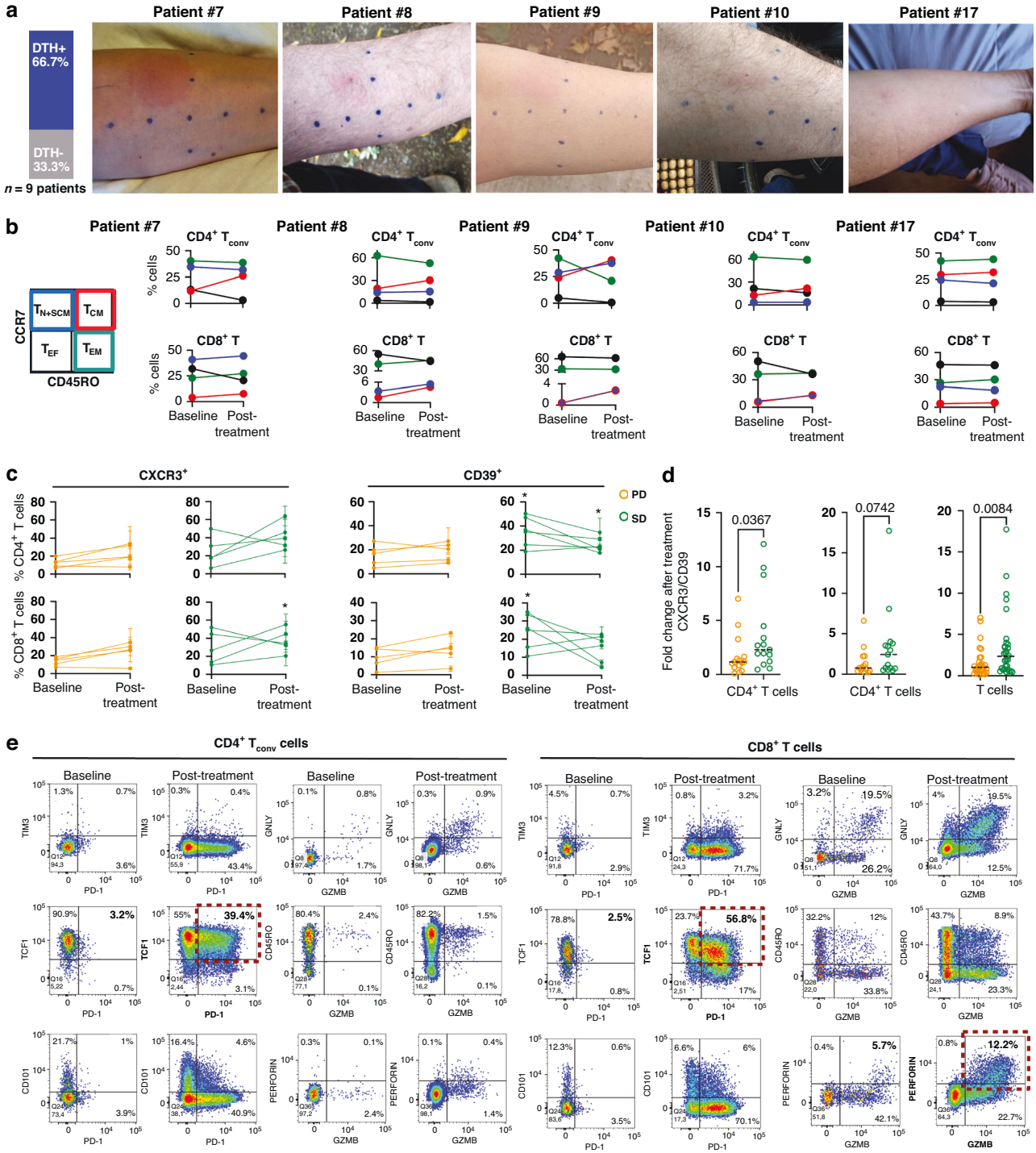

Fig. 2 Immunological parameters associated with clinically stable disease. a TRIMELVax vaccine triggers a lysate-specific DTH response in treated patients. One month after the last dose of TRIMELVax, vaccinated patients were intradermally challenged with 150 µL of the TRIMEL lysate. The DTH reaction was evaluated after 48 h. Representative photographs of DTH$^+$ reactions (patients #7, #8, #9, #10, and #17) are shown. **b** Memory populations, based on CCR7 and CD45RO expression levels, were evaluated in conventional CD4$^+$ and CD8$^+$ T cells, at baseline and after the last TRIMELVax vaccine dose (post-treatment) in peripheral blood samples of DTH$^+$ patients #8, #9, #10, and #17. T_{N+SCM}: naive+ stem cell memory T cells (blue lines); T_{CM}: central memory T cells (red lines); T_{EF}: effector T cells (black lines); T_{EM}: effector memory T cells (green lines). **c** TRIMELVax vaccination induces changes in CXCR3 and CD39 expressions in peripheral blood T cells associated with clinical responses of patients. The percentage of CXCR3$^+$ or CD39$^+$ cells among CD4$^+$ and CD8$^+$ T cells at baseline and after each TRIMELVax vaccine dose (post-treatment; average of the four doses) is shown for patients with progressive disease (PD, orange) or stable disease (SD, green). * $p < 0.05$ SD vs. PD. **d** Fold change of the ratio CXCR3/CD39 on CD4$^+$, CD8$^+$, and total T cells after treatment. **e** TRIMELVax vaccine induces a change in T cell activation markers in populations of a patient with partial response (#8).

exploratory, given the absence of paired tumor biopsies or antigen-specific functional assays.

The immunological and clinical responses observed in this trial are particularly relevant given the refractory nature of the treated population. Despite substantial advances in first-line treatment outcomes, such as those reported in CheckMate 067, with three-year survival rates of 58% for nivolumab plus ipilimumab and 52% for nivolumab alone, a significant proportion of patients experience disease progression [28]. In the second-line setting, salvage strategies combining anti-PD-1 therapy with other agents, including lenvatinib [29] or ipilimumab [30, 31], have shown limited durability, with most patients progressing within six months. These findings underscore the ongoing need for novel, well-tolerated approaches that can be integrated with existing immunotherapies.

Emerging evidence indicates that therapeutic cancer vaccines can expand and activate tumor-reactive T-cell repertoires and, when combined with immune checkpoint inhibitors, may help overcome tumor-induced immunosuppression and promote a more inflamed, responsive microenvironment [32–34]. Recent studies demonstrate that next-generation vaccines, whether RNA-based, peptide-based, or whole-tumor lysate formulations, can synergize with PD-1 inhibition to overcome adaptive resistance mechanisms and induce durable responses in melanoma and other solid tumors [24, 35, 36]. In this context, the ability of TRIMELVax to elicit memory-type and cytotoxic T-cell populations, together with clinical stabilization in anti–PD-1–refractory patients, supports the rationale for combining tumor lysate vaccines with checkpoint inhibitors to potentiate therapeutic immunity.

Although the observation that several patients achieved disease stabilization upon re-exposure to anti–PD-1 therapy following vaccination raises the possibility that TRIMELVax modulated systemic immune responsiveness, alternative explanations must be considered. In particular, nonspecific immune stimulation provided by the vaccine adjuvant or other inflammatory components of the formulation may have contributed to immune activation that was subsequently amplified by checkpoint blockade. In this regard, recent evidence has shown that unrelated immunological stimuli, such as mRNA COVID-19 vaccination, can be associated with enhanced responses to immune checkpoint inhibitors in patients with lung cancer and melanoma [37]. Moreover, clinical responses following immune checkpoint inhibitor rechallenge have been reported in melanoma patients even in the absence of additional immunomodulatory interventions [38, 39]. Consequently, while our findings are compatible with the hypothesis that TRIMELVax influenced immune contexture and responsiveness to checkpoint blockade, they do not establish causality. These observations underscore the need for prospective studies specifically designed to disentangle vaccine-specific effects from nonspecific immune activation and checkpoint rechallenge phenomena.

Previous clinical experience with WTC vaccines has been limited by modest immunogenicity and heterogeneous outcomes. Our results build on earlier TAPCells trials, in which TRIMEL lysates elicited DTH responses correlated with prolonged survival [15, 16]. Importantly, unlike TAPCells, TRIMELVax does not require patient-derived cells, enabling a standardized, universal formulation suitable for large-scale clinical application. The present study confirms the feasibility of this approach and extends prior findings by providing mechanistic insights into systemic T-cell modulation.

Based on these results and the developmental trajectory of other oncology vaccines, TRIMELVax may represent a promising adjunct to checkpoint inhibitor therapy in both curative and palliative settings. Its favorable safety profile and immunogenic potential warrant further evaluation in a Phase II clinical trial.

This study has limitations, including its small sample size and potential confounding effects of subsequent therapies on survival outcomes. Nonetheless, the demonstration of safety, disease control in a refractory population, and associated immunological changes support continued clinical development. Future studies should evaluate TRIMELVax in combination with PD-1 blockade in earlier disease settings or as part of maintenance strategies, where immune priming may translate into greater clinical benefit. Moreover, the inclusion of detailed analyses of T-cell receptor (TCR) clonality and specificity, particularly in relation to vaccine-induced responses and their modulation by subsequent immune checkpoint inhibitor administration, would provide valuable mechanistic insights.

In conclusion, TRIMELVax exhibited an acceptable toxicity profile and immunogenic potential, with preliminary signals of clinical activity in patients with anti-PD-1–refractory melanoma. These findings warrant validation in larger, controlled studies and support continued exploration of whole-tumor cell vaccines as complementary agents to immune checkpoint inhibition.

DATA AVAILABILITY

All published data and material are available upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization and research design (RE, BM, FSO, MNL), Clinical data acquisition (RE, AC, FT, CM, CF, AR, NR, PC, KB, MNL), data analysis and interpretation (RE, BM, FT, MAG, AT, CP, IF, FEG, AA, ALL, SH, MNL, MIB, FSO), writing the manuscript (RE, AT, FSO), supervision and final manuscript approval (MNL, FSO).

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COMPETING INTERESTS

The authors declare no conflict of interest. The funders had no role in the study's design, data collection, analysis, or interpretation, nor in the writing of the manuscript or the decision to publish the results.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study was conducted in accordance with the Declaration of Helsinki and approved by the Ethical Committee for Human Research of the University of Chile, Faculty of Medicine (363/290102), and the Ethical Committee for Human Research of the University of Chile's Clinical Hospital (227/280803). Informed consent was obtained from all subjects involved in the study.

CONSENT FOR PUBLICATION

All authors have reviewed and approved the final version of the manuscript and consent to its submission and potential publication.

ADDITIONAL INFORMATION

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