

RESEARCH ARTICLE



Langerhans-type dendritic cells electroporated with TRP-2 mRNA stimulate cellular immunity against melanoma: Results of a phase I vaccine trial

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ABSTRACT

Purpose: We conducted a phase I vaccine trial to determine safety, toxicity, and immunogenicity of autologous Langerhans-type dendritic cells (LCs), electroporated with murine tyrosinase-related peptide-2 (mTRP2) mRNA in patients with resected AJCC stage IIB, IIC, III, or IV (MIA) melanoma. **Experimental Design:** Nine patients received a priming immunization plus four boosters at three week intervals. Vaccines comprised 10×10^6 mRNA-electroporated LCs, based on absolute number of CD83⁺CD86^{bright}HLA-DR^{bright}CD14^{neg} LCs by flow cytometry. Initial vaccines used freshly generated LCs, whereas booster vaccines used viably thawed cells from the cryopreserved initial product. Post-vaccination assessments included evaluation of delayed-type hypersensitivity (DTH) reactions after booster vaccines and immune response assays at one and three months after the final vaccine. **Results:** All patients developed mild DTH reactions at injection sites after booster vaccines, but there were no toxicities exceeding grade 1 (CTCAE, v4.0). At one and three months post-vaccination, antigen-specific CD4 and CD8 T cells increased secretion of proinflammatory cytokines (IFN- γ , IL-2, and TNF- α), above pre-vaccine levels, and also upregulated the cytotoxicity marker CD107a. Next-generation deep sequencing of the TCR-V- β CDR3 documented fold-increases in clonality of 2.11 (range 0.85–3.22) for CD4 and 2.94 (range 0.98–9.57) for CD8 T cells at one month post-vaccines. Subset analyses showed overall lower fold-increases in clonality in three patients who relapsed (CD4: 1.83, CD8: 1.54) versus non-relapsed patients (CD4: 2.31, CD8: 3.99). **Conclusions:** TRP2 mRNA-electroporated LC vaccines are safe and immunogenic. Responses are antigen-specific in terms of cytokine secretion, cytolytic degranulation, and increased TCR clonality, which correlates with clinical outcomes.

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Introduction

The advent of immune checkpoint inhibitors targeting anti-cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell-death protein 1 (PD-1) has revolutionized the treatment landscape for advanced melanoma, with a subset of patients achieving long-term survival.^{1,2} A significant number of patients do not benefit, however, due to the low frequency or absence of tumor antigen-specific cytotoxic T lymphocytes (CTLs) at baseline.^{3–6} Priming patients with a vaccine to induce or augment the pool of CTLs against a tumor is one strategy to complement or synergize with checkpoint blockade and/or other therapeutic modalities to improve clinical responses.

Dendritic cells (DCs) are key regulators of immunity, and translational studies have demonstrated the safety and feasibility of DC-based immunization in patients with cancer.⁷ These trials have achieved mixed success with regard to immune and objective clinical responses,⁷ which may well depend on the type of

DC used. Most DC cancer vaccines have used monocyte-derived DCs (moDCs), but Langerhans-type DCs (LCs) derived from CD34⁺ hematopoietic progenitor cells (HPCs) are superior at stimulating antigen-specific CTLs against tumor antigens *in vitro*.^{8–11} LCs, but not moDCs, can also stimulate CTLs *de novo* against a self-differentiation tumor antigen like Wilms tumor 1 (WT1) *in vitro* by an IL15 α /IL15-dependent mechanism.¹⁰ Clinical trial data have also shown greater efficacy of LC-containing vaccines,¹² as well as greater tetramer reactivity stimulated by LCs when compared with moDCs.¹³

Electroporation of DCs with mRNA encoding tumor-associated antigens effectively induces T cell responses *in vitro* and *in vivo*.^{14,15} Antigen loading by electroporation is more efficient than peptide pulsing¹⁴ and avoids the risk of genome integration seen with retroviral transgenes.^{16,17} Electroporation with full-length mRNA also facilitates DC presentation of a more diverse array of multiple peptides for presentation, obviating

the need to predetermine class I and II MHC restrictions. This in turn supports a more robust immune response than achieved by single class I MHC-peptide pulsing.

Tumor-infiltrating lymphocytes from patients with melanoma recognize tyrosinase-related peptide 2 (TRP2), a melanosomal differentiation antigen.¹⁸ In addition, vaccination with highly homologous xenogeneic antigen can augment immune responses against differentiation antigens, like TRP2^{19,20}.

We performed a phase I vaccine study to determine the safety and toxicity of autologous LCs electroporated with murine TRP2 mRNA in patients with resected American Joint Committee on Cancer (AJCC) stage IIB, IIC, III, or IV (M1a) melanoma. Post-vaccination studies included evaluation of delayed type hypersensitivity (DTH) reactions and immune response assessments. Clinical outcomes were also monitored after completion of vaccines.

Results

LCs electroporated with TRP2 mRNA are safe and well-tolerated

Figure 1 outlines the treatment schedule. Tables 1a and 1b summarize the characteristics of the ten patients enrolled in this study, which comprised an equal number of male and female participants, with a median age of 56 (range, 34 to 69). None of the patients received adjuvant therapy, with a median interval of 3.3 months (range 1.9 to 11.9) from surgical resection to start of vaccination. Nine patients completed all five scheduled vaccines. One patient received three vaccines before being withdrawn from the study due to disease recurrence. All patients developed some degree of erythema, induration, and mild pruritus at the injection sites after booster vaccines (Figure S1). No patient developed toxicities greater than grade 1, possibly or probably related to these vaccines.

Post-vaccination T-cell subset phenotype analysis

Unmanipulated PBMCs were analyzed by flow cytometry to assess for changes in lymphocyte composition at one and three months after completion of vaccines. There were no significant fold-changes from pre-vaccine baseline in the levels of naïve (CCR7⁺CD45RO^{neg}), central memory (CCR7⁺CD45RO⁺), effector (CCR7^{neg}CD45RO^{neg}), or effector memory (CCR7^{neg}CD45RO⁺) CD8 T cells (Figure 2A). CD8 T cells maintained relatively static inhibitory receptor expression at one and three months after vaccination (Figure 2B). The post-

vaccination trends of CD4 T-cell subsets and inhibitory receptor expression were similar to those of CD8 T cells (data not shown). Inducible co-stimulator (ICOS) expression by CD4 and CD8 T cells increased 2.51- and 1.99-fold, respectively, at one month, indicating T-cell activation (Figure 2C). Vaccines did not significantly alter regulatory T cell (CD3⁺CD4⁺CD25^{bright}CD127^{neg}) to CD3⁺CD8⁺CD25⁺ effector T cell ratios (Figure 2D).

LC vaccines induce CD4 and CD8 T-cell cytokine secretion and expression of activation epitopes

To amplify antigen-specific reactivity from the pool of responder T cells, PBMCs obtained at one and three months post-vaccination underwent two rounds of restimulation *in vitro* with freshly generated LCs electroporated with TRP2 mRNA. Compared with pre-vaccine levels, CD4 T-cell secretion of IFN- γ , IL-2, and TNF- α increased 6.15-, 2.27-, and 2.25-fold, respectively, at one-month post-vaccination, and remained 3-, 2.92-, and 2.3-fold elevated, respectively, at three-months post-vaccination (Figure 3A). CD4 T cells also upregulated CD107a 1.84- and 1.7-fold, as a marker of degranulation, and Mip-1- β 3.47- and 2.67-fold, as a marker of activation, at the one and three-month time points, respectively (Figure 3A). Similar to CD4 T cell responses, antigen-specific CD8 T-cell secretion of IFN- γ , IL-2, and TNF- α increased 1.71-, 2.05-, and 1.37-fold, respectively, at one-month post-vaccination, and further increased 2.28-, 3-, and 1.61-fold, respectively, at three-months post-vaccination (Figure 3B). CD8 T cells upregulated CD107a 1.55- and 1.6-fold, and Mip-1- β 1.72- and 1.24-fold, at the one and three-month time points, respectively (Figure 3B). On an individual basis, cytokine secretion and activation epitope markers in both the CD4 and CD8 T-cell compartments increased in all patients, including all five patients (#5–9) with sufficient cells for replicate analyses showing positive T-cell responses, defined as greater than 2 standard deviation increases over pre-vaccine levels (Figure 3C–E). A limited assessment of tetramer-specific reactivity against two different TRP2 residues showed no change (data not shown). Available cell yields precluded evaluation of antigen-specific CTL activity in killing assays.

CD4 and CD8 T-cell clonality increases after vaccination with TRP2 mRNA-electroporated LCs

Clonality, as defined in Methods, is a composite measure of the abundance (frequency) and diversity (uniqueness) of the TCR-

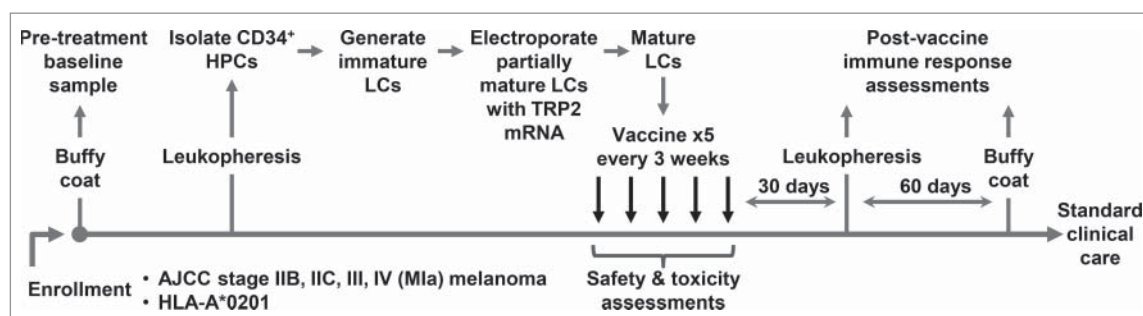


Figure 1. Study schema.

Table 1a. Patient demographics and disease characteristics.

Patient	Age	Gender	Stage	KPS	Disease status at study entry	LDH at study entry	Disease status at EOS (3 months after last vaccine)	Interval from surgical resection to first vaccine (months)	RFS from surgical resection (months)	OS from surgical resection (months)	F/u from last vaccine (months)
1	54	M	IIIA	100	NED	177	NED	2.9	67.3	67.3	61.6
2	65	F	IIB	100	NED	186	NED	3.2	55.7	55.7	49.8
3	62	M	IV (Mlc)	100	NED	161	Relapsed ^A	5.1	8.3	55.8	47.9
4	69	M	IIIB	100	NED	137	Relapsed ^B	3.3	7.4	51.6	45.4
5	58	F	IIB	100	NED	212	NED	3.1	50.5	50.5	44.6
6	47	M	IIIC	100	NED	158	NED	11.9	57.7	57.7	43.7
7	41	M	IIIB	100	NED	152	NED	4.9	44.6	44.6	36.9
8	46	F	IIC	100	NED	172	Relapsed ^C	1.9	5.2	23.4	18.7
9	59	F	IIIA	100	NED	187	NED	3.6	37.6	37.6	31.3
10	34	F	IIIB	100	NED	103	Relapsed ^D	2.7	4	45.6	41.6
Median:	56	M 50%	—	100	—	167	NED: 6	3.3	41.1	51.1	44.2
Range:	(34–69)	F 50%	—	—	—	(103–212)	Relapsed: 4	(1.9–11.9)	(4–67.3)	(23.4–67.3)	(18.7–61.6)

Table 1b. Relapsed patients (A–D from Table 1a).

Patient	Time of relapse	Site(s) of relapse	Treatment	Treatment duration	Response	RFS from start of treatment* (months)	RFS from end of treatment* (months)
A	1 mo after last vaccine 8.3 mo from resection	Lung, spleen	Ipilimumab + nivolumab	14 months	CR	44	30
B	1 mo after last vaccine 7.4 mo from resection	Liver, spleen	Ipilimumab + nivolumab	4 months	CR	43	39.3
C	1 mo after last vaccine 5.2 mo from resection	Bone	Multiple including ipilimumab & pembrolizumab	16 months total	POD	NA	NA
D	On study after 3rd vaccine 4 mo from resection	Skin, CNS	Lirilumab + nivolumab	One dose	CR	40.2	40.2

CR = complete response; EOS = end of study; F/u = follow-up; NA = not applicable; NED = no evidence of disease; POD = progression of disease; RFS = recurrence-free survival

*cutoff date 07/01/2017, approximately 31 months from the end of the study

V- β repertoire.²¹ CD4 and CD8 T cells isolated from PBMCs collected at one month after vaccination underwent next-generation deep sequencing of the TCR-V- β CDR3. Pre- to post-vaccination clonality increased in all cases, except one CD4 and one CD8 pair (different subjects); and clonality was significantly higher in the CD8 ($P = 0.0097$) but not in the CD4 compartment ($P = 0.1084$) (Figure 4A). Clonality increased 2.11-fold (range 0.85–3.22) for CD4 T cells and 2.94-fold (range 0.98–9.57) for CD8 T cells (Figure 4B). Subset analysis showed a trend in overall lower fold-increases in clonality in patients who relapsed (CD4: 1.83, CD8: 1.54; $n = 3$) versus non-relapsed patients (CD4: 2.31, CD8: 3.99; $n = 4$) (Figure 4C). CD8 T cells showed higher clonal frequencies than CD4 T cells one month after LC vaccines (Figure 4D). The mean frequencies in the top clones of post-vaccine samples were higher in both CD4 and CD8 T cells but did not reach statistical significance due to the small sample size (Figure 4D).

Clinical outcomes

Patient outcomes are summarized in Tables 1a/1b and Figure 5. At a median follow-up of 18.7 to 61.6 months, six of the nine patients who completed the study remain free of disease recurrence, with a median of 51.1 months (range, 34.6 to 67.3) from initial surgical resection. The other three patients who completed the study (patients 3, 4, and 8) relapsed at approximately one month post-vaccines, which was a median of 7.4 months (range, 5.2 to 8.3) from initial surgical resection. An additional

participant (patient 10) received three vaccines before withdrawing from the study due to cutaneous and CNS metastases. All relapsed patients (patients 3, 4, 8, and 10) subsequently received checkpoint inhibitor-based therapy. Patient 8 died of progressive disease, while the others (patients 3, 4, and 10) are in complete remission, with a median recurrence-free survival of 39.3 months (range, 30 to 40.2) from the completion of last therapy.

Discussion

This phase I clinical trial demonstrates the safety and immunogenicity of mRNA-electroporated LC vaccines. Aside from mild DTH reactions at injection sites after booster vaccines, there were no toxicities. Post-vaccination immune responses detected enhanced CD4 and CD8 T-cell activation and proinflammatory cytokine secretion, as well as increased T-cell clonality that correlated with clinical outcomes.

The vaccine formulation in this study combined the superior potency of LCs^{8–11} with electroporation of full-length tumor antigen mRNA^{14,15,22} to stimulate a more diverse T-cell response. In addition to promoting CD4 and CD8 T-cell activation by traditional phenotypic measures, mRNA-electroporated LCs induced greater CD4 and CD8 T-cell clonality in patients who remained free of disease. Greater T-cell diversity after vaccination correlates with clinical benefit,^{23,24} indicating an advantage of oligoclonal expansion of several T-cell clones over a more restricted clonal profile. In addition, low baseline levels

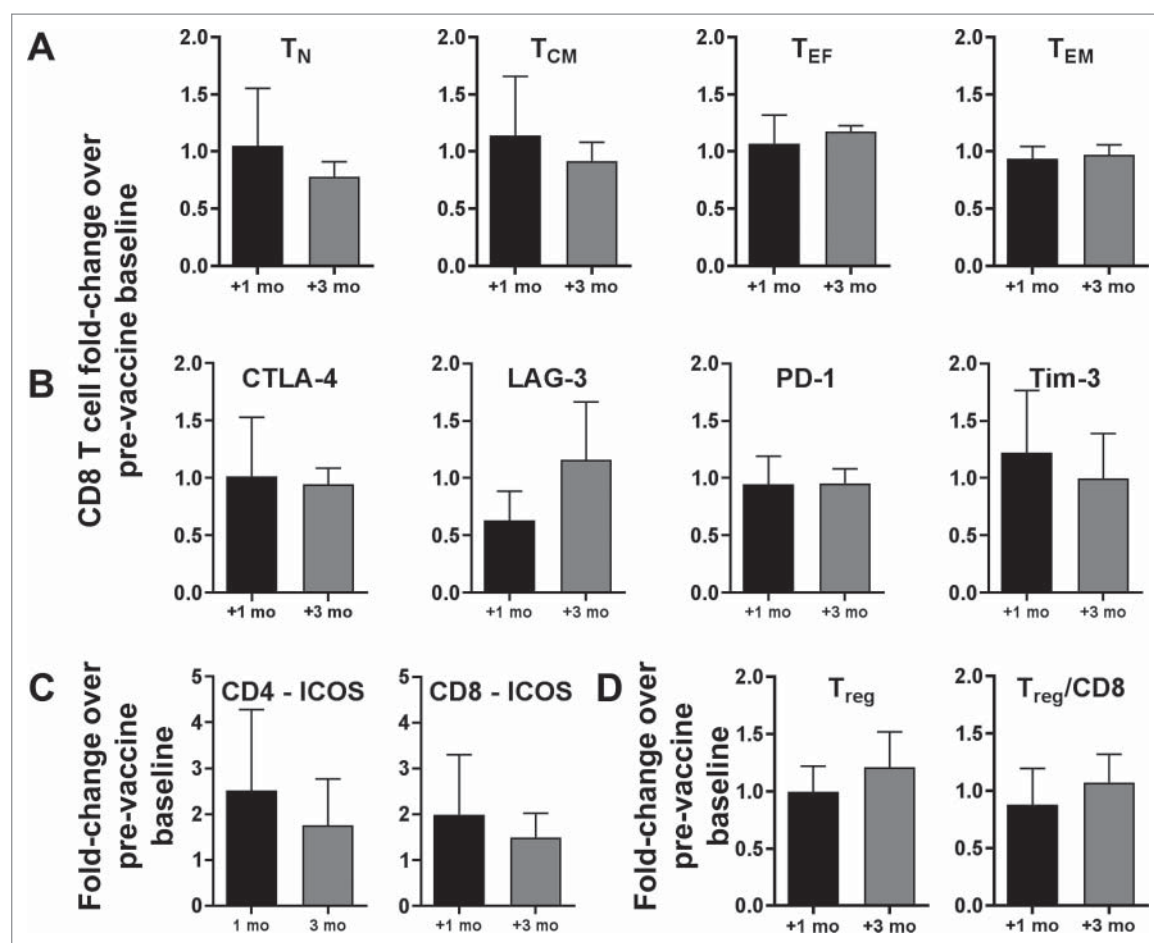


Figure 2. CD8 T cell subset analysis and Treg content after LC vaccines. PBMCs were analyzed by flow cytometry to assess fold-changes in lymphocyte composition at one and three months post-vaccination, compared with pre-vaccine baseline. (A) CD8 T-cell subsets: naïve (T_N; CCR7⁺CD45RO^{neg}), central memory (T_{CM}; CCR7⁺CD45RO⁺), effector (T_{EF}; CCR7^{neg}CD45RO^{neg}), and effector memory (T_{EM}; CCR7^{neg}CD45RO⁺). (B) Expression of inhibitory receptors (CTLA-4, LAG-3, PD-1, and TIM-3) by CD8 T cells. (C) ICOS expression by CD4 and CD8 T cells. (D) Regulatory T cell (T_{reg}; CD3⁺CD4⁺CD25^{bright}CD127^{neg}) to CD3⁺CD8⁺CD25⁺ effector T cell ratios. For all panels (A-D), pooled data (mean \pm SD; $P = NS$) from seven patients are shown.

of TCR diversity associate with inferior outcomes after treatment with ipilimumab,²⁵ and maintenance of high-frequency TCR clones after CTLA-4 blockade correlates with superior outcomes.²⁶ In this study, LC vaccines also induced CD4 and CD8 T-cell expression of ICOS, which predicts response to ipilimumab therapy.^{27,28} These findings, as well as emerging pre-clinical²⁹⁻³¹ and clinical³²⁻³⁴ data, support the sequential administration of vaccines followed by immune checkpoint blockade to augment T-cell responses and optimize antitumor immunity.

T-cell tetramer reactivity against two well-defined TRP2 epitopes restricted by HLA-A*0201 did not increase after vaccination. This was not unexpected in the setting of antigen presentation by LCs after electroporation with mRNA encoding full-length antigen, where expansion of antigen-specific CTLs recognizing other immunologically relevant TRP2 epitopes is supported by the TCR clonality results. This highlights another challenge in monitoring immune therapy, however, which is the need for more robust assessment tools that increase sensitivity for detecting subtle but significant changes after an intervention.

Four patients in this trial relapsed, three after completing vaccines and one while on study. All received checkpoint

inhibitor-based therapy, and three are alive with no evidence of disease, including one patient who had CNS metastases. In a previous study comparing melanoma peptide-pulsed moDCs and LCs in patients with AJCC stage III/IV melanoma,¹³ clinical responses, although not the primary endpoint of the trial, were observed solely in the LC recipients. For the current study, pre/post-treatment samples from relapsed patients were not available for analyses. Thus, whether the LC vaccines altered local or systemic T-cell subset composition in patients treated with checkpoint inhibitors for relapsed disease is an important unknown that merits future investigation.

Investigators recently discovered that a neoantigen-based DC vaccine increases the breadth and diversity of antigen-specific T-cell responses in melanoma.³⁵ Another study has found induction of DC-mediated antitumor immunity after tumor mRNA delivery to DCs *in vivo* with nanoparticles.³⁶ These studies and our results with LCs underscore the potential of DC-based vaccines to stimulate immune responses against cancer. For LCs, we acknowledge that resource limitations at most centers to collect and process CD34⁺ HPCs, as well as the labor, time, and cost of manufacturing LCs *in vitro*, restrict their exportability for broad clinical application. Strategies aimed at activating LCs directly *in vivo* to circumvent the

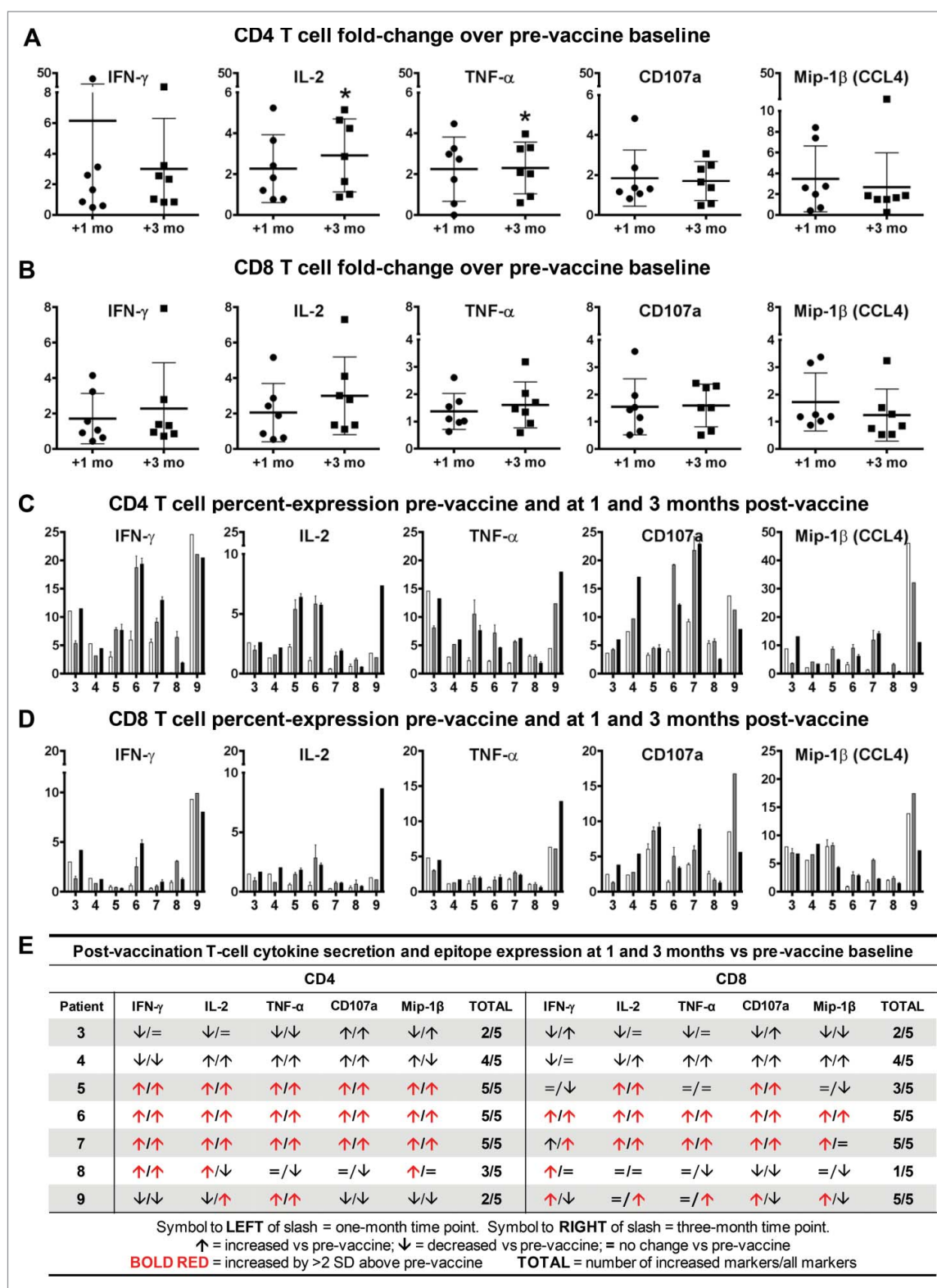


Figure 3. LC vaccines stimulate CD4 and CD8 T-cell cytokine secretion and activation epitope expression in patients with melanoma. Responses against TRP2 mRNA-electroporated LCs were measured at approximately one and three months after completion of vaccines, and compared with pre-vaccine levels. (A) CD4 and (B) CD8 T-cell pro-inflammatory cytokine (IFN- γ , IL-2, and TNF- α) secretion and activation epitope (CD107a and Mip-1 β) expression, fold-change. For panels A and B, pooled data (triplicate means \pm SEM; * $P < .05$) from seven patients are shown. (C) CD4 and (D) CD8 T-cell pro-inflammatory cytokine secretion and activation epitope expression, percent-expression for individual patients pre-vaccine (white bar) and at one (gray bar) and three (black bar) months after completion of vaccines. For panels C and D, Y-axis indicates percent-expression for each marker, and X-axis indicates patient number. For patients #3 and 4, single data points are shown. For patients #5–9, pooled data (triplicate means \pm SD) are shown. (E) Composite cytokine secretion and activation epitope expression profile of individual patients.

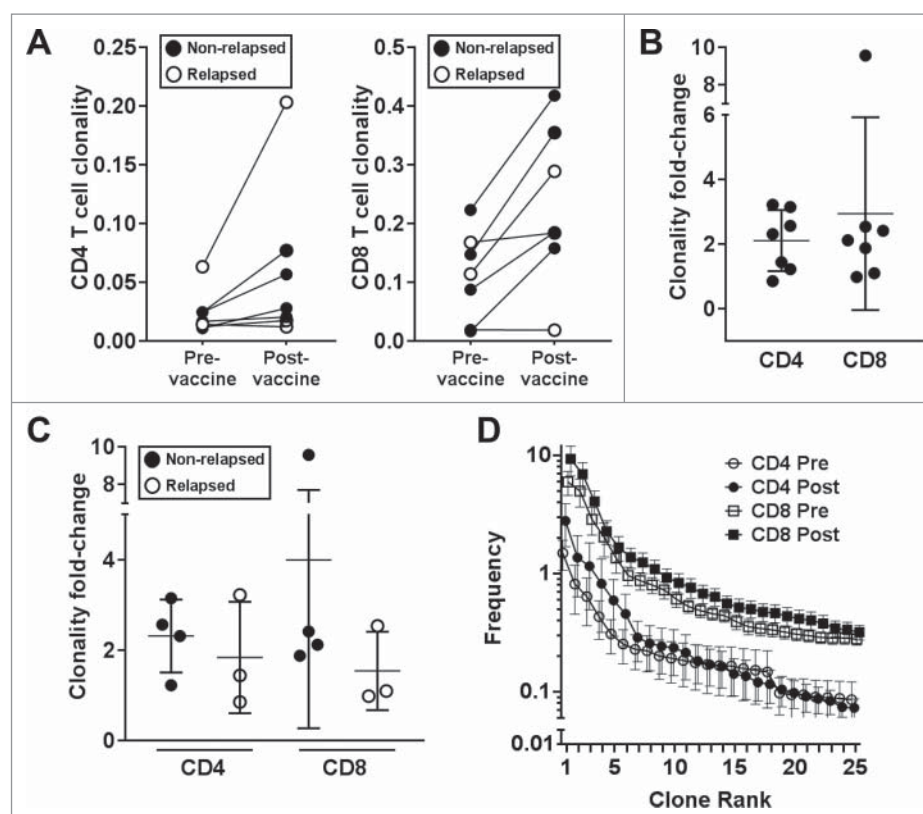


Figure 4. T-cell clonality increases after vaccination with TRP2 mRNA-electroporated LCs. Next-generation deep sequencing of the TCR-V β CDR3 was performed on CD4 and CD8 T cells isolated from PBMCs obtained from seven patients at one month post-vaccination and compared for changes from pre-vaccine levels. (A) CD4 (left panel; $P = 0.1084$) and CD8 (right panel; $P = 0.0097$) T-cell clonal frequency for individual patients. (B) CD4 and CD8 T-cell clonality fold-change. (C) CD4 and CD8 T cell clonality fold-change comparing patients with no evidence of disease (non-relapsed; $n = 4$) with patients who relapsed ($n = 3$) after completing vaccines. (D) Mean frequency of each of the top 25 CD4 and CD8 T-cell clones before and after vaccination. Pooled data (mean \pm SEM; $P = \text{NS}$).

production process *ex vivo* or other approaches to modify more simply derived DC subsets to mimic the superior antigen presentation and accessory functions of LCs for CTL stimulation are underway.

This pilot study highlights the feasibility of immunizing patients with mRNA-electroporated LCs to induce measurable cellular immune responses. Future progress in vaccine development for melanoma and other malignancies will require the identification of rational combinations and sequencing of immune-directed agents to improve clinical outcomes.

Materials and methods

Patients and clinical trial design

Patients expressing HLA-A*0201 with resected AJCC stage IIB, IIC, III, or IV (M1a) melanoma were enrolled in a phase I clinical trial (NCT01456104) to test safety and toxicity and to assess immune responses stimulated by TRP-2 mRNA-electroporated LCs. The use of influenza matrix peptide as a positive control was the only reason necessitating HLA-A*0201-expressing patients. The trial was conducted at Memorial Sloan Kettering Cancer Center (MSKCC), approved by the Institutional Review and Privacy Board of Memorial Hospital/MSKCC and the Food and Drug Administration, and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines.

Patients received a total of five vaccines, comprising a priming immunization followed by four boosters at 3-week intervals (Figure 1). Target enrollment was nine patients, with the trial designed to identify a promising agent for further study from a large pool of candidates, while minimizing the total number of patients studied.³⁷ Of note, a total of ten patients accrued to the trial, including an extra patient enrolled to replace a patient who relapsed before completing vaccines. For this trial, the vaccine was considered promising if more than four out of the nine patients had an immunologic response. For cytokine secretion and activation epitope expression, a positive T-cell response was defined as greater than 2 standard deviations over pre-vaccine replicates. The decision rule aimed to identify an agent that produced an immunologic response rate greater than 50%. The error rates of missing a promising agent or selecting a non-promising agent were set to be at most 10%. The decision rule and error rates were devised using a historical database of vaccine trials performed at MSKCC.³⁷ For clonality, a positive T-cell response required an increased above baseline without respect to degree of increase, as there are no validated metrics to provide cutoffs for determining significance.

Vaccine production and administration

Generation of human LCs

G-CSF-elicited CD34⁺ HPCs collected from patients by leukopheresis were used to generate LCs with media, media

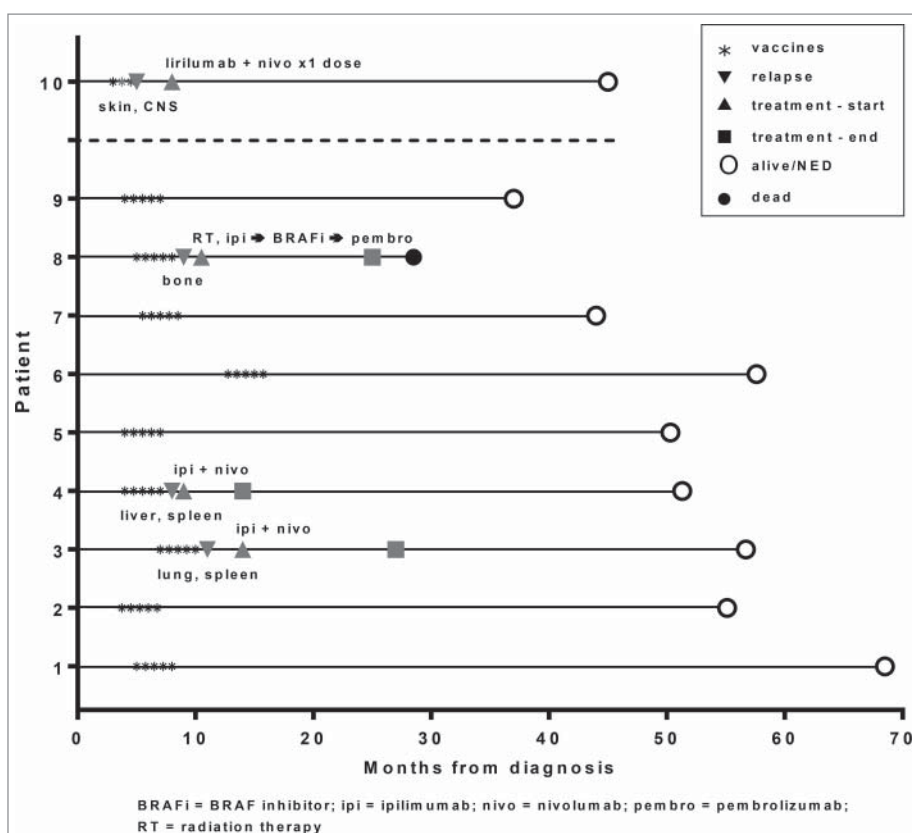


Figure 5. Clinical outcomes. The clinical status timeline of the nine patients who completed treatment on study, from diagnosis to vaccinations, relapse and treatment (if applicable), and follow-up. Also shown is a tenth patient who received 3/5 planned vaccines but relapsed and was removed from the study. Site of relapse/metastasis is indicated under the down-pointing triangle. Treatment is indicated over the up-pointing triangle.

supplements, and cytokines, exactly as published.⁸ In brief, CD34⁺ HPCs were cultured in serum-free X-VIVO 15, supplemented with GM-CSF, TGF- β , and TNF- α , to which c-kit ligand and FLT-3 ligand were added for the initial 5–6 days of a 10–12-day culture. LCs underwent terminal maturation with TNF- α , IL-1 β , IL-6, and prostaglandin E₂. All cultures used autologous plasma or serum, avoiding the introduction of any allogeneic or xenogeneic plasma or serum (e.g., fetal calf serum).

Several epitopes distinguish LCs from other conventional DC subtypes. LCs are consistently negative for CD11b, express e-cadherin before exposure to terminal maturation stimuli, and express CD207/Langerin primarily by less mature forms and with apparent asynchrony during differentiation in culture.⁸ LCs also express the chemokine receptor, CCR3, independent of maturation status,³⁸ and upregulate the chemokine receptor, CCR7, with maturation (Figure S2).

Production of *in vitro*-transcribed mRNA

A pING plasmid expression vector containing the gene for murine TRP2 under the control of a T7 promoter was used to synthesize TRP2 mRNA. The murine TRP2 cDNA was cloned in the laboratory of Dr. Alan Houghton (MSKCC). The plasmid was propagated in Max Efficiency DH5- α competent cells (Invitrogen) and purified using a Plasmid Maxi Kit (QIAGEN). For TRP2 mRNA transcription, the plasmid was linearized with Hind III (New England Biolabs) before mRNA transcription *in vitro* with T7 RNA polymerase (mMessage mMachine

T7 kit; Ambion). Production of full-length capped mRNA was confirmed by agarose gel electrophoresis, and mRNA concentration was determined by spectrophotometry.

Electroporation of LCs

Partially-matured LCs were electroporated with TRP2 mRNA on day 12–13.²² LCs were harvested, washed twice, and resuspended in OptiMEM (Gibco, Invitrogen) at 15×10^6 cells/ml. 200 μ L of cell suspension were then mixed with 40 μ g mRNA and electroporated in a 2 mm gap cuvette at 700 V for two pulses, using a BTX ECM 830 square-wave electroporator (BTX Harvard). After electroporation, cells were immediately transferred to culture and terminally matured with inflammatory cytokines for 24–48 hours.⁸

Control antigen-loading of LCs

Synthetic influenza matrix peptide (fluMP₅₈₋₆₆ GILGFVFTL; Multiple Peptide Systems) served as a positive control for HLA-A*0201-restricted responses. Peptide (1 μ M) was added to terminally maturing LCs in cultures overnight at 37°C.¹³

Vaccine administration

Cells were washed and met release criteria (Table S1A) before proceeding with vaccination. Each vaccine consisted of 10×10^6 mRNA-electroporated LCs/vaccine, based on the absolute number of CD83⁺CD86^{bright}HLA-DR^{bright}CD14^{neg} LCs by flow cytometry (Table S1B). The initial vaccines used freshly generated LCs, whereas booster vaccines used viably thawed cells

from the initial product, which was cryopreserved using controlled-rate freezing followed by storage in liquid nitrogen. Vaccines were administered as ten x 0.1 ml, deep intradermal injections, five of which were given at each of two sites adjacent to axillary or inguinofemoral nodes. Areas of prior lymph node resections were excluded.

Clinical assessments

Patients were monitored with serial vital sign measurements once every 15 minutes for one hour after vaccine administration. Surrounding erythema and induration at vaccine injection sites were measured for DTH reactions 48 hours after each booster vaccine. Toxicities were graded according to Common Terminology Criteria for Adverse Events (CTCAE), version 4.0. For disease status monitoring, patients were followed for melanoma recurrence according to National Comprehensive Cancer Network (NCCN) Guidelines for Melanoma.

Post-vaccination immune response assessments

Response assessments used peripheral blood mononuclear cells (PBMCs) collected at pre-vaccine baseline and then approximately one and three months after the fifth and final vaccine. Two of the nine patients (#1 and #2) who received all five vaccines were inevaluable for immune response assessments because of inadequate cell yields.

Flow cytometric analysis

PBMCs were incubated with fluorochrome-conjugated antibodies and analyzed on an LSRFortessa flow cytometer (Becton Dickinson). FITC-, PE-, PE-Texas Red-, ECD-, APC-, PE-Cy5-, PE-Cy7-, PerCP-Cy5.5-, Pacific Blue-, and AF700-conjugated mouse anti-human mAbs included anti-CD3, anti-CD4, anti-CD8, anti-CD14, anti-CD25, anti-CD45RA, anti-CD45RO, anti-CD80, anti-CD86, anti-CTLA-4, anti-HLA-DR, anti-IL-2, anti-CD83, anti-Mip-1- β (Beckman Coulter), anti-CD127, anti-IFN- γ , anti-LAG-3, anti-PD-1, anti-TNF- α (eBioscience), anti-CCR7, and anti-TIM-3 (R&D Systems). LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies) was used to exclude dead cells. Gates were set for collection and analysis of at least 50,000 live events. Data were analyzed with FlowJo software (TreeStar).

T-cell subset analysis and intracellular cytokine secretion

PBMCs were restimulated with TRP2 mRNA-electroporated autologous LCs generated from the same CD34⁺ HPCs used to make patient vaccines. Responder PBMCs were resuspended in 10% autologous serum in complete RPMI-1640 and plated at a 1:10 ratio of LCs:PBMCs in a 96-round bottomed well plate (Costar). Cultures were supplemented with recombinant human IL-2 (10 IU/ml; Chiron, Emeryville, CA) and IL-15 (10 ng/ml; R&D Systems). After 6–7 days, fresh LCs were added for a second round of stimulation in the same culture conditions, followed by harvesting of cells for staining and analysis by flow cytometry. T-cell subsets and intracellular cytokine levels were compared for fold-changes over pre-vaccine baseline. Two HLA-A*0201-restricted TRP2 tetramers, one PE-labeled for TRP2 residues 180–188 and one APC-labeled for TRP2 residues 360–368 (TC Matrix), were used for tetramer analyses.

T-cell receptor variable beta chain sequencing

PBMCs from the one-month post-vaccine time point were separated into CD4 and CD8 T-cell fractions using MS columns (Miltenyi Biotec), then cryopreserved and shipped to Adaptive Biotechnologies (Seattle, WA) for immunosequencing of the CDR3 of human TCR-V- β chains using the ImmunoSEQ™ Assay. Extracted genomic DNA was amplified in a bias-controlled multiplex PCR, followed by high-throughput sequencing. Sequences were collapsed and filtered to identify and quantitate the absolute abundance of each unique TCR-V- β CDR3 for further analysis.^{39–41}

Statistics

For immune response assessments, replicate means from ≥ 3 independent experiments were averaged and SEM calculated as the measure of variability. Otherwise, SD was calculated for the replicate mean of a single representative experiment of at least three. Unpaired or paired t tests assessed differences in expression levels. Statistical significance required a *P* value less than 0.05. All statistical analyses were calculated using Prism 6 software (GraphPad).

Statistical Analyses of TCR- β sequencing results

Clonality was defined as 1- Peilou's evenness²¹ and was calculated on productive rearrangements by:

$$1 + \frac{\sum_i^N p_i \log_2(p_i)}{\log_2(N)}$$

where p_i is the proportional abundance of rearrangement i and N is the total number of rearrangements. Clonality values range from 0 to 1 and describe the shape of the frequency distribution. Clonality values approaching 0 indicate a very even distribution of frequencies, whereas values approaching 1 indicate an increasingly asymmetric distribution in which a few clones are present at high frequencies. Statistical analyses were performed in R version 3.2.

Conflict of interest

The authors declare no conflicts of interest.

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