



Divergent clinical outcomes in a phase 2B trial of the TLPLDC vaccine in preventing melanoma recurrence and the impact of dendritic cell collection methodology: a randomized clinical trial

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Abstract

Background A randomized, double-blind, placebo-controlled phase 2b trial of the tumor lysate, particle-loaded, dendritic cell (TLPLDC) vaccine was conducted in patients with resected stage III/IV melanoma. Dendritic cells (DCs) were harvested with and without granulocyte-colony stimulating factor (G-CSF). This analysis investigates differences in clinical outcomes and RNA gene expression between DC harvest methods.

Methods The TLPLDC vaccine is created by loading autologous tumor lysate into yeast cell wall particles (YCWPs) and exposing them to phagocytosis by DCs. For DC harvest, patients had a direct blood draw or were pretreated with G-CSF before blood draw. Patients were randomized 2:1 to receive TLPLDC or placebo. Differences in disease-free survival (DFS) and overall survival (OS) were evaluated. RNA-seq analysis was performed on the total RNA of TLPLDC + G and TLPLDC vaccines to compare gene expression between groups.

Results 144 patients were randomized: 103 TLPLDC (47 TLPLDC/56 TLPLDC + G) and 41 placebo (19 placebo/22 placebo + G). Median follow-up was 27.0 months. Both 36-month DFS (55.8% vs. 24.4% vs. 30.0%, $p=0.010$) and OS (94.2% vs. 69.8% vs. 70.9%, $p=0.024$) were improved in TLPLDC compared to TLPLDC + G or placebo, respectively. When compared to TLPLDC + G vaccine, RNA-seq from TLPLDC vaccine showed upregulation of genes associated with DC maturation and downregulation of genes associated with DC suppression or immaturity.

Conclusions Patients receiving TLPLDC vaccine without G-CSF had improved OS and DFS. Outcomes remained similar between patients receiving TLPLDC + G and placebo. Direct DC harvest without G-CSF had higher expression of genes linked to DC maturation, likely improving clinical efficacy.

Keywords Cancer vaccine · Melanoma · Dendritic cell · Immunotherapy · Personalized medicine

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Abbreviations

TLPLDC Tumor lysate, particle-loaded, dendritic cell
 YCWPs Yeast cell wall particles

Introduction

Immunotherapy, particularly with the introduction of checkpoint inhibitor (CPI) therapy, has drastically changed the management of advanced stage melanoma [1–3]. Additional immunotherapeutic options, such as cancer vaccines, may also have a role in the management of melanoma. Cancer vaccines, in particular, may have a benefit when administered in combination with CPIs [4–6]. Multiple vaccination approaches exist, including dendritic cell (DC)-based vaccines, with promising results [7].

DC-based vaccines capitalize on the active cellular immune response using the most potent antigen-presenting cell (APC), the DC, to prime both cytotoxic T-cell (CTL) and T-helper cell responses specific to tumor antigens. The only FDA-approved dendritic cell-based cancer vaccine, sipuleucel-T (Provenge), targets metastatic castration-resistant prostate cancer by priming DCs *ex vivo* with the tumor-associated antigen prostatic-acid phosphatase (PAP) linked to granulocyte–macrophage colony-stimulating factor (GM-CSF) [8, 9].

The tumor lysate, particle-loaded, dendritic cell (TLPLDC) vaccine uses the same potent DC delivery mechanism but loads DCs *ex vivo* with yeast cell wall particles (YCWPs) containing autologous tumor lysate from an individual's tumor. This utilizes the highly immunogenic properties of YCWPs to create a personalized vaccine targeting each patient's unique tumor antigens. The TLPLDC vaccine strategy was first studied in a phase 1/2a trial, which demonstrated safety and potential for efficacy in a variety of solid tumors [10]. This led to the current phase 2b trial of TLPLDC used to prevent recurrence in patients with resected stage III/IV melanoma. The primary analysis of this trial showed no difference in the primary endpoint of 24-month disease-free survival (DFS) by intention-to-treat (ITT) analysis at median follow-up of 19.1 months (38.5 vs 27.0%, $p=0.974$). However, in a pre-specified per-treatment (PT) analysis of patients completing the primary vaccine series (PVS) without early recurrence in the first six months, the TLPLDC vaccine group had significantly increased 24-month DFS (62.9 vs 34.8%, $p=0.041$) [11].

A key aspect of creating each patient's individualized TLPLDC vaccine creation is the harvest of DCs. In the current trial, this was accomplished by two methods: a 120 cc blood draw for harvest or a lower volume blood draw (50–70 cc) 24–48 h after administering G-CSF. In previous trials, G-CSF may be used to augment DC collection for production of DC-based vaccines [3, 12, 13]. The protocol

allowed for investigators or patients to choose between these two methods. Here, we report the final outcomes from the phase 2b trial comparing placebo to the TLPLDC vaccine, with 36-month disease-free and overall survival. In addition, as prespecified in the trial statistical analysis plan, we compare outcomes of patients vaccinated using the two different DC harvest methods.

Methods

Patient Selection and Randomization

The protocol was approved by the Western Institutional Review Board. Patients with stage IIIA-C or stage IV resectable melanoma were identified prior to definitive surgery and screened for inclusion criteria including anticipation for being clinically disease-free after surgery and other standard of care (SoC) therapies, ability to provide approximately 1 cm³ (or 1 mg minimum) of tumor for vaccine production, and ECOG 0–1 performance. Due to a change in standard of care therapy for advanced melanoma, the protocol was amended partway through the trial to allow enrollment of patients on CPI therapy, and to allow treatment with vaccine or placebo concurrently with CPI therapy; patients were required to tolerate CPI therapy alone for 3 months prior to starting study inoculations. Patients were excluded if they had evidence of residual disease after surgery or SoC therapies, immune deficiency such as HIV, active HBV or HCV, or were taking steroids or immunosuppressants. Patients were screened and approached by a research nurse and/or study coordinator. Patients were initially consented prior to definitive surgery during the process of tissue collection by a research nurse coordinator, study coordinator, and/or principal investigator. Patients were consented again after completion of SoC therapy, prior to beginning the vaccination series. Patients, treating physicians, study personnel, and clinical and medical monitors were blinded to treatment groups.

Beginning in February 2015 patients were randomized 2:1 to vaccine or control, with computer-generated randomization tables and a site-balancing algorithm. Initial sample size was calculated to require at least 120 patients with an estimated 2-year recurrence rate of 60% and treatment effect corresponding to a hazard ratio of 0.50, in order to reach a power of 80% to detect a statistical difference between treatment arms with a two-sided alpha of 0.05. Sample size was increased to 140 when a blinded assessment of the first 75 patients found an early recurrence rate of 12% prior to completion of the primary vaccine series (within the first six months). Once patients recurred in either treatment arm, they were offered open-label TLPLDC vaccination as previously described, but were followed for overall survival [14].

Vaccine Production

Vaccine production has been described in greater detail elsewhere [10, 14]. Briefly, tissue collection occurred at time of surgical resection. After completion of SoC therapy, peripheral blood was collected for dendritic cell (DC) generation. Based on patient and provider preference, patients either had 120 ml of peripheral blood drawn, or they received a single injection of 300 µg of subcutaneous G-CSF for pre-DC mobilization with a 50–70 ml peripheral blood draw 24–48 h later. Peripheral blood was sent to a central facility for extraction of peripheral blood monocytes and derivation of immature DCs after exposure to IL-4 and GM-CSF.

Tumor lysate (TL) was created through freeze/thaw cycling of tumor tissue and loaded into yeast cell wall particles (YCWP) derived from *S. cerevisiae*. The YCWPs were loaded with TL, stock CpG oligonucleotides, and tetanus helper peptide (amino acids 948–968, sequence FNNFT-VSFWLRVPKVSASHLE). TL-loaded YCWPs were then exposed to immature DCs at day two of incubation for phagocytosis, in the presence of cytokines TNF- α , IL-1 β , IL-6, and PGE2. Successful production of the TLPLDC vaccine was confirmed by tests for sterility, DC counts, and DC viability. The process of vaccine production was the same regardless of the blood draw method (with or without G-CSF). For control patients, empty YCWPs were phagocytosed by autologous DCs generated in the same fashion, and the placebo vaccines were similarly tested for sterility and viability.

The vaccine contained $1\text{--}1.5 \times 10^6$ TLPLDC per dose, while the placebo contained the same number of empty

YWCP-loaded DCs. Each dose was provided in individual vials with 250 µl of freezing media. A batch of six doses per patient, to cover the full series, was created and sent to the site regardless of treatment arm. Each dose was thawed and diluted with 500 µl of sterile saline for intradermal injection. These doses were administered at months 0, 1, 2, 6, 12, and 18 months. The inoculations were given in the same lymph node draining area for each respective patient. The primary vaccine series (PVS) was defined as the first three doses. The 6-month, 12-month, and 18-month vaccines were considered boosters.

Analysis Plan

Survival analysis was performed using Kaplan–Meier (K-M) analysis. The primary endpoint of this study was 24-month disease-free survival (DFS), which was previously reported [11]. The secondary endpoints included 36-month DFS and overall survival (OS), as well as safety. Time to recurrence was defined as date of randomization to time of confirmed recurrence. The pre-specified analysis plan included a three-arm subgroup analysis of DFS and OS, analyzing patients receiving G-CSF for vaccine production (TLPLDC + G), those who did not (TLPLDC), and placebo. We additionally performed an exploratory four-arm analysis of DFS and OS comparing TLPLDC, TLPLDC + G, placebo, and placebo with G-CSF (placebo + G). Other exploratory outcomes included investigation of immunologic response to vaccination and correlation of vaccine immune response to clinical outcome.

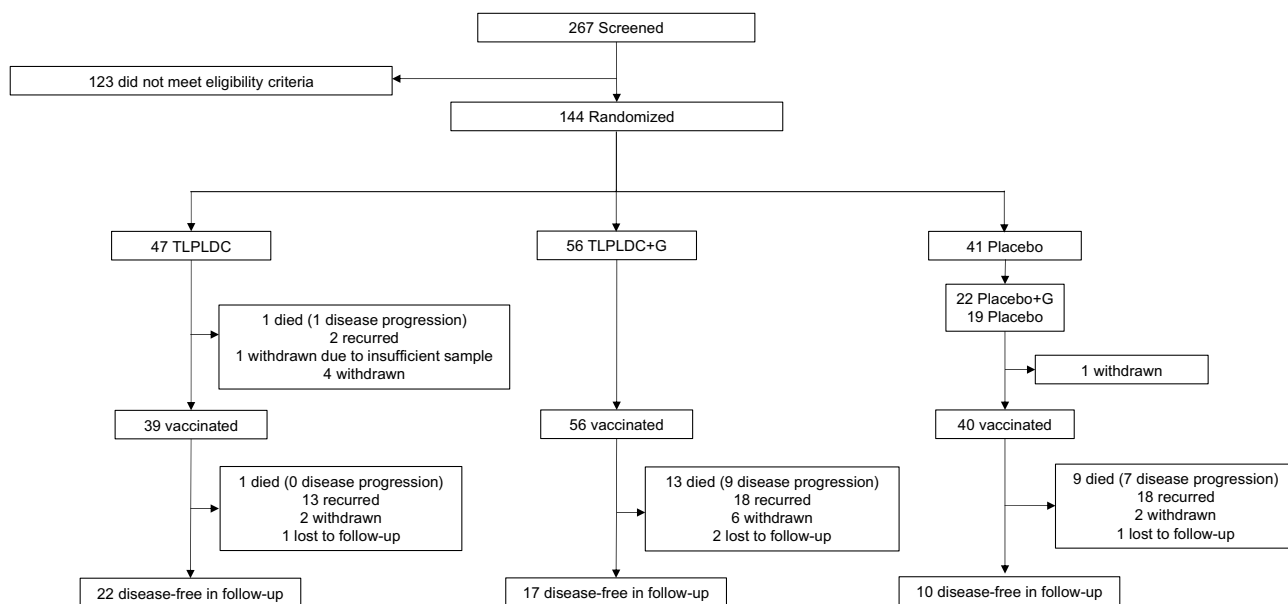


Fig. 1 CONSORT diagram

Table 1 Demographic and pathologic data with comparison between Vaccine vs. Placebo, and in the 3-arm analysis (TLPLDC vs TLPLDC + G vs placebo)

Category	Intention to treat analysis			Three-arm analysis			
	Vaccine (%)	Placebo (%)	<i>p</i> -value	TLPLDC (%)	TLPLDC + G (%)	Placebo (%)	<i>p</i> -value
Age							
Median	65.5	58.7	0.037	69.5	61.7	58.7	0.042
IQR	55.6 - 73.0	49.5-67.8		57.2-75.2	55.5-70.1	49.5-67.8	
Race							
Asian	0 (0)	1 (2.4)	0.320	0 (0)	0 (0)	1 (2.4)	0.583
Black	0 (0)	1 (2.4)		0 (0)	0 (0)	1 (2.4)	
Hispanic	3 (2.9)	1 (2.4)		1 (2.1)	2 (3.5)	1 (2.4)	
Native American	1 (1.0)	0 (0)		0 (0)	1 (1.8)	0 (0)	
Unknown	1 (1.0)	0 (0)		0 (0)	1 (1.8)	0 (0)	
White	98 (95.1)	38 (92.7)		46 (97.9)	52 (92.9)	38 (92.7)	
Ulceration							
Present	17 (16.5)	12 (29.3)	0.316	7 (14.9)	10 (17.9)	12 (29.3)	0.316
Absent	22 (21.4)	7 (17.1)		11 (23.4)	11 (19.6)	7 (17.1)	
Not available	64 (62.1)	22 (53.7)		29 (61.7)	35 (62.5)	22 (53.6)	
TILS							
Brisk	3 (2.9)	2 (4.9)	0.462	1 (2.1)	2 (3.6)	2 (4.9)	0.247
Mild	2 (1.9)	0 (0)		0 (0)	2 (3.6)	0 (0)	
Non-Brisk	14 (13.6)	10 (24.4)		9 (19.1)	5 (8.9)	10 (24.4)	
Absent	8 (7.8)	1 (2.4)		5 (10.6)	3 (5.4)	1 (2.4)	
Not available	76 (73.8)	28 (68.3)		32 (68.1)	44 (78.6)	28 (68.3)	
Biopsy Margins							
Positive	26 (25.2)	13 (31.7)	0.756	16 (34.0)	10 (17.9)	13 (31.7)	0.330
Negative	46 (44.7)	19 (46.3)		18 (38.3)	28 (50.0)	19 (46.3)	
Not available	31 (30.1)	9 (22.0)		13 (27.7)	18 (32.1)	9 (22.0)	
BRAF Mutation							
Yes	18 (17.5)	6 (14.6)	0.648	10 (21.3)	8 (14.3)	6 (14.6)	0.768
No	31 (30.1)	10 (24.5)		13 (27.7)	18 (32.1)	10 (24.4)	
Not available	54 (52.4)	25 (61.0)		24 (51.1)	30 (53.6)	25 (61.0)	

Statistics were calculated with SPSS (version 22, IBM Corp, Released 2013, Armonk, NY). Demographic data were analyzed using student's t-test if quantitative and Chi-square if categorical. Comparison of DFS and OS between groups was performed with log-rank analysis with Mantel-Cox log rank test.

Phenotypic Characterization of the TLPLDC Vaccine

RNA-sequencing analysis on the total RNA of patients' prepared TLPLDC and TLPLDC + G vaccines was performed by ROSALIND® (San Diego, CA; <https://rosalind.onramp.bio/>) using the TruSeq Stranded mRNA Library Prep Kit High Throughput. Equal samples of randomly selected TLPLDC and TLPLDC + G vaccine doses were analyzed. Data were analyzed with a HyperScale architecture developed by ROSALIND®, reads were trimmed using cutadapt, and quality scores were assessed using FastQC [15,

16]. After aligning reads to the *Homo sapiens* genome build *hg19*, individual sample reads were quantified using HTseq and normalized with relative log expression using DESeq2 R library [17–19]. Quality control was performed using RSeQC [20]. DESeq2 was used to calculate fold changes and *p*-values, and perform covariate correction [19]. Relative RNA expression (RRE) was compared between TLPLDC and TLPLDC + G. These results were correlated with clinical outcomes.

Results

A total of 267 patients were screened and 144 patients were enrolled and randomized. A total of 103 patients were in the vaccine group (47 received TLPLDC, 56 received TLPLDC + G), and 41 in the control group (19 received placebo, 22 received placebo + G) (Fig. 1). Patients in the

Table 2 Staging data with comparison between Vaccine vs. Placebo, and in the 3-arm analysis (TLPLDC vs TLPLDC + G vs placebo)

Category	Intention to treat analysis			Three-arm analysis			
	Vaccine (%)	Placebo (%)	<i>p</i> -value	TLPLDC(%)	TLPLDC + G(%)	Placebo (%)	<i>p</i> -value
AJCC Stage							
III	82 (79.6)	32 (78.0)	0.835	42 (89.4)	40 (71.4)	32 (78.0)	0.081
IV	21 (20.4)	9 (22.0)		5 (10.6)	16 (28.6)	9 (22.0)	
T stage							
T0	2 (1.9)	0 (0)	0.354	0 (0)	2 (3.6)	0 (0)	0.587
Tis	1 (1.0)	0 (0)		0 (0)	1 (1.8)	0 (0)	
T1	7 (6.8)	7 (17.1)		4 (8.5)	3 (5.4)	7 (17.1)	
T2	21 (20.4)	4 (9.8)		10 (21.3)	11 (19.6)	4 (9.7)	
T3	23 (22.3)	8 (19.5)		12 (25.5)	11 (19.6)	8 (19.5)	
T4	24 (23.3)	7 (17.1)		10 (21.3)	14 (25.0)	7 (17.1)	
TX	11 (10.7)	7 (17.1)		5 (10.6)	6 (10.7)	7 (17.1)	
Unavailable	14 (13.6)	8 (19.5)		6 (12.8)	8 (14.3)	8 (19.5)	
N stage							
N1	18 (17.5)	14 (34.1)	0.168	12 (25.5)	6 (10.7)	14 (34.1)	0.296
N2	26 (25.2)	9 (22.0)		11 (23.4)	15 (26.8)	9 (22.0)	
N3	32 (31.1)	7 (17.1)		13 (27.7)	19 (33.9)	7 (17.1)	
Unavailable	27 (26.2)	11 (26.8)		11 (23.4)	16 (28.6)	11 (26.8)	
M stage							
M0	71 (68.9)	26 (63.4)	0.667	40 (85.1)	31 (55.4)	26 (63.4)	0.111
M1a	5 (4.9)	4 (9.8)		1 (2.1)	4 (7.1)	4 (9.8)	
M1b	6 (5.8)	1 (2.4)		1 (2.1)	5 (8.9)	1 (2.4)	
M1c	8 (7.8)	3 (7.3)		2 (4.3)	6 (10.7)	3 (7.3)	
Unavailable	13 (12.6)	7 (17.1)		3 (6.4)	10 (17.9)	7 (17.1)	

vaccine group were significantly older than those in the placebo group (median age 65.5 vs. 58.7, $p=0.037$), as well as in the three-arm analysis of TLPLDC vs. TLPLDC + G vs. placebo (median age 69.5 vs. 61.7 vs. 58.7, $p=0.042$). There were no other significant clinicopathologic or treatment differences between the vaccine and placebo groups or between the three treatment arms when comparing TLPLDC vs. TLPLDC + G vs. placebo (Tables 1, 2, 3). While individual M stage was significantly different between TLPLDC vs. TLPLDC + G vs. placebo vs. placebo + G on 4-arm analysis ($p=0.014$), no differences in overall AJCC stage III vs. IV ($p=0.120$) or other factors were identified between groups.

As previously described, the vaccine was well-tolerated, and production was successful in all patients with a median delivery time of 18 days. The vaccine resulted in significantly improved estimated 24-month DFS by PT analysis as reported by our group [11].

Clinical Results

Secondary endpoints included 36-month DFS and OS. At a median follow-up of 27.0 months, by ITT analysis, there was no difference in 36-month estimated survival between the overall vaccine and placebo groups. 36-month estimated

DFS was 38.1% for the vaccine group and 30.0% for placebo (HR 0.95 $p=0.840$). The 36-month estimated OS was 80.1% for the vaccine group and 70.9% for placebo (HR 0.61, $p=0.218$; Fig. 2A/B).

By pre-specified three-arm analysis comparing TLPLDC vs. TLPLDC + G vs. placebo, patients receiving TLPLDC had significantly improved OS and DFS, while survival between TLPLDC + G and placebo remained similar. Comparing TLPLDC vs. TLPLDC + G vs. placebo by ITT analysis, respectively, 36-month estimated DFS was 55.8% vs. 24.4% vs. 30.0% ($p=0.010$). Similarly, 36-month estimated OS was 94.2% vs. 69.8% vs. 70.9% ($p=0.024$; Fig. 2C/D).

A four-arm analysis was additionally performed comparing TLPLDC vs. TLPLDC + G vs. placebo vs. placebo + G, demonstrating similar results. The 36-month estimated DFS was 55.8% vs. 24.4% vs. 30.7% vs. 28.6% ($p=0.025$), while 36-month estimated OS was 94.2% vs. 69.8% vs. 76.6% vs. 64.8% ($p=0.042$) (Suppl. Figure 1).

Phenotypic Characterization of the TLPLDC Vaccine Results

When compared to TLPLDC + G vaccine, RNA-seq from the TLPLDC vaccine showed upregulation of genes associated

Table 3 Prior treatment data with comparison between Vaccine vs. Placebo in the ITT and PT cohorts, and in the 3-arm analysis (TLPLDC vs TLPLDC + G vs placebo)

Category	Intention to treat analysis			Three-arm analysis			
	Vaccine (%)	Placebo (%)	<i>p</i> -value	TLPLDC (%)	TLPLDC + G (%)	Placebo (%)	<i>p</i> -value
Wide local excision							
Yes	100 (97.1)	40 (97.6)	0.876	46 (97.9)	54 (96.4)	40 (97.6)	0.895
No	3 (2.9)	1 (2.4)		1 (2.1)	2 (3.6)	1 (2.4)	
Lymph node surgery							
SLNB	17 (16.5)	6 (14.6)	0.077	7 (14.9)	10 (17.9)	6 (14.6)	0.267
LND	39 (37.9)	9 (22.0)		17 (36.2)	22 (39.3)	9 (22.0)	
SLNB and LND	20 (19.4)	16 (39.0)		11 (23.4)	9 (16.1)	16 (39.0)	
None	27 (26.2)	10 (24.4)		12 (25.5)	15 (26.8)	10 (24.4)	
Immunotherapy							
Yes	41 (39.8)	15 (36.6)	0.721	19 (40.4)	22 (39.3)	15 (36.6)	0.931
No	62 (60.2)	26 (63.4)		28 (59.6)	34 (60.7)	26 (63.4)	
Checkpoint inhibitor							
Yes	34 (33.0)	9 (22.0)	0.191	16 (34.0)	18 (32.1)	9 (22.0)	0.416
No	69 (67.0)	32 (78.0)		31 (66.0)	38 (67.9)	32 (78.0)	
Chemotherapy							
Yes	7 (6.8)	1 (2.4)	0.303	2 (4.3)	5 (8.9)	1 (2.4)	0.346
No	96 (93.2)	40 (97.6)		45 (95.7)	51 (91.1)	40 (97.6)	
Radiation therapy							
Yes	26 (25.2)	11 (26.8)	0.844	14 (29.8)	12 (21.4)	11 (26.8)	0.615
No	77 (74.8)	30 (73.2)		33 (70.2)	44 (78.2)	30 (73.2)	

with DC maturation and antigen presentation, including HLA-DMB (RRE: 3.60), IFIT1 (3.38), CD27 (3.37), IFI44L (3.24), MX1 (2.96), HLA-DQA1 (2.67), HLA-DRA (2.40), CD49D (2.34) and CD74 (2.09). Slight upregulation in CD83 (RRE: 1.14), CD86 (1.05), CD40 (1.05), CD40 ligand (1.43), and PD1 (1.02) were additionally noted.

Downregulated genes were associated with DC suppression or immaturity including CD14 (RRE: 9.50), SERPINA1 (7.8), CD163 (7.00), TLR2 (6.65), CCR1 (5.11), IL10 (4.19), IL6 (3.95), and CD93 (3.84). Figure 3 displays up- and downregulated genes with associated fold changes.

Discussion

In this randomized, double-blind, placebo-controlled phase 2b trial, the 36-month DFS and OS did not differ between the TLPLDC vaccine vs. placebo by ITT analysis. In a pre-specified three-arm analysis and exploratory four-arm analysis; however, patients receiving the vaccine created without G-CSF stimulation at the time of DC harvest had significantly improved DFS and OS compared to the TLPLDC + G and placebo groups. In these patients, TLPLDC vaccine demonstrated up-regulated genes associated with antigen presentation and DC maturation, and down-regulated genes

associated with DC immaturity compared to TLPLDC + G vaccine.

While the overall results of this trial were negative, we discovered a profound impact of G-CSF stimulation prior to DC harvest on clinical outcomes, with implications for dendritic cell vaccine production previously unrecognized. The effect of DC maturity on immune response has been demonstrated in some preclinical work. Jonuleit, et al. conducted an in vitro study comparing two DC populations, CD1a + immature DCs cultured in GM-CSF and IL-4, and terminally differentiated, or mature, CD83 + DCs stimulated with select cytokines. When stimulated with melanoma peptides MelanA/MART-1, the mature DCs showed rapid activation and expansion of CD8 + T cells with strong antigen-specific cytotoxicity, while no CD8 + expansion was seen with the immature DCs [21]. This effect was subsequently studied in vivo with stage IV melanoma patients, comparing the peptide-pulsed mature DCs versus immature DCs as described above. The mature DC-based vaccines induced recall antigen-specific CD4 + T-cell responses in 87.5% of patients, compared to only 37.5% of patients receiving DC-based vaccines using immature DCs [22]. The importance of DC maturity has also been described in DC-based vaccines for prostate cancer [23].

While this early data provided some information regarding the effect of DC maturity on immune response, little was

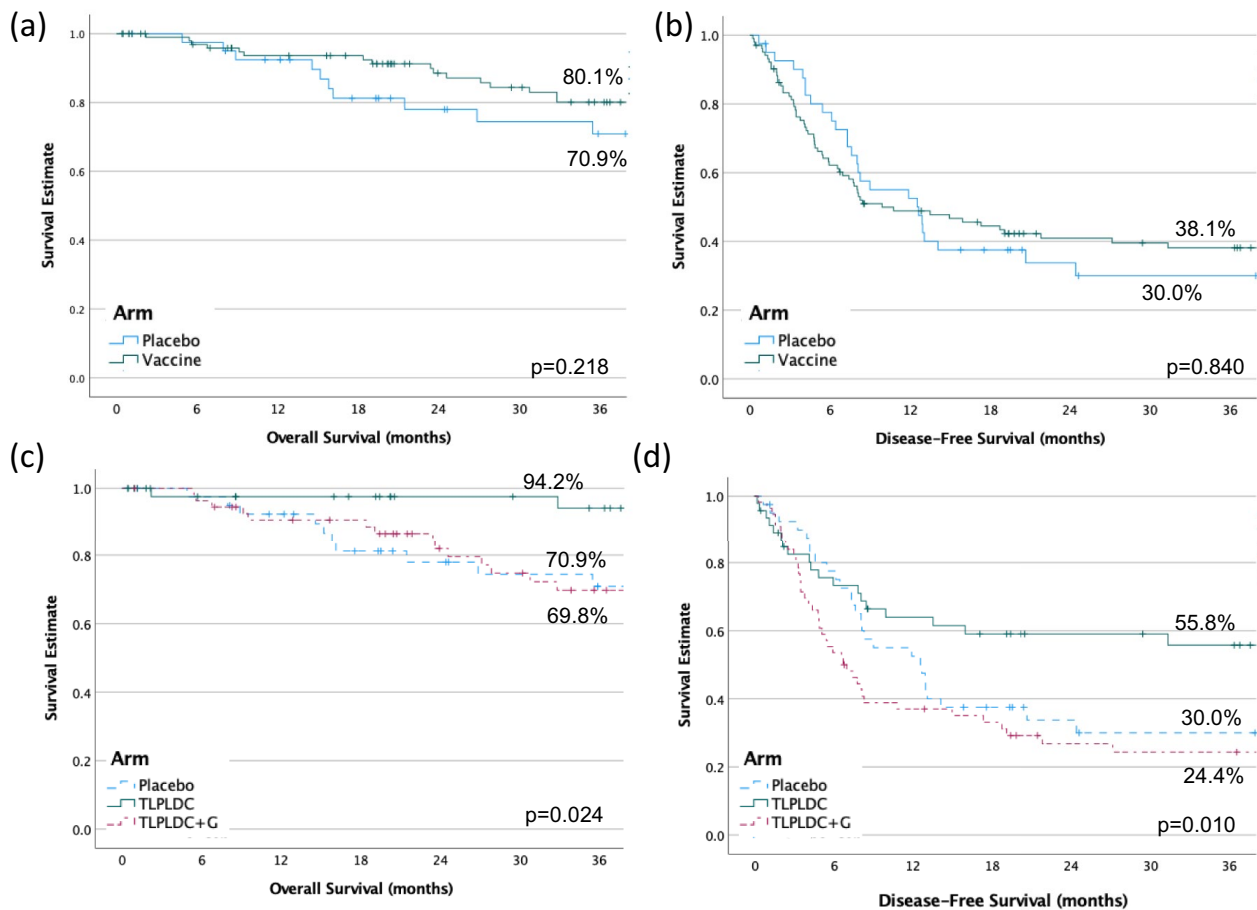


Fig. 2 36-month overall survival **A** and disease-free survival **B** comparing vaccine to placebo, and overall survival **C** and disease-free survival **D** comparing TLPLDC, TLPLDC + G, and placebo

known about the specific effects of stimulation with G-CSF in vivo on dendritic cell maturity. G-CSF was included in our clinical trial protocol to increase a patient's white blood cell count in order to reduce the overall blood volume required for isolation of sufficient monocytes for maturation into DCs. However, G-CSF functions by increasing the number of immature cells in circulation [24]. The vaccine production strategy utilizes a 72-h rapid maturation process, which only permits sufficient time for DC differentiation from mature, but not immature, monocytes as demonstrated by RNA-seq analysis we conducted to investigate differences in observed outcomes [10, 25]. Our current work suggests that the combination of G-CSF stimulation with our protocol timing leads to harvest of relatively immature DCs, which does not promote a tumor-specific immunologic response and, therefore, results in a less effective vaccine [26].

While DC maturity is clearly a significant contributor to clinical efficacy, there may be additional factors that are also

important. The TLPLDC + G had relatively higher expression of IL-10, CD14 and TLR2, suggesting a skewed response towards a Th1 phenotype. Specifically, steady-state IL-10 producing DCs may induce tolerance instead of a stimulatory immune response and induce T cell anergy and Tregs [26]. This phenomenon of immature DCs producing tolerance, or even exacerbation of disease, has been demonstrated in pre-clinical models [27, 28]. To avoid inducing tolerance, it is therefore important to ensure that DCs used for vaccines are mature [26]. Given the effects of G-CSF stimulation that we have documented on DC maturity, cells collected 24–48 h after pre-treatment with G-CSF may require additional time for maturation than previously known. Protocols utilizing G-CSF and similar production timing to ours should be cautioned that this combination may result in an ineffective vaccine. These results represent the most robust clinical data demonstrating the significant difference in efficacy based on the maturation

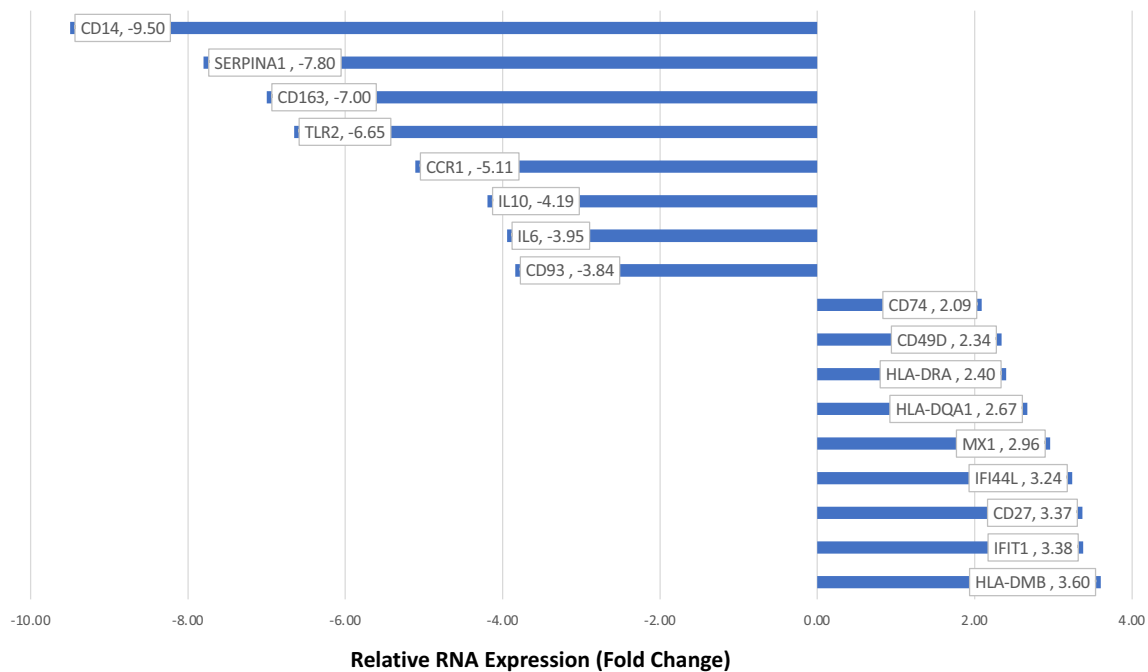


Fig. 3 Relative RNA expression of genes in the TLPLDC vs. TLPLDC + G vaccine, with associated fold changes

of DCs used to create dendritic cell-based cancer vaccines and provide important lessons for future protocols.

This study had several limitations. The study protocol had to be amended midway through the investigation to accommodate for changes to standard of care treatments, namely CPI therapy. Additionally, the use of additional immunotherapy was not randomized, but rather given at the treatment team's discretion. Exploratory outcomes included study of the immunologic response to vaccination which we have discussed above; however, given the nature of this investigation and the limited number of patients who underwent RNA sequencing, definitive conclusions cannot be made. Protein expression confirmation and detailed immunologic assessment from bio-banked specimens correlating to outcomes and DC function will be the subject of future investigations.

Conclusions

In pre-specified three-arm analysis, the TLPLDC vaccine appears to be clinically effective when produced without G-CSF pre-treatment for DC harvest. This difference between vaccine formulation efficacy is attributable to utilization of DCs with a mature phenotype, and merits further investigation. Confirmatory testing and detailed immunologic assessment correlating outcomes and DC function is forthcoming. A phase III trial is planned, which will standardize vaccine production without G-CSF and compare CPI therapy alone to CPI plus vaccine combination therapy. This

trial will not only provide a comparison of the vaccine to modern SoC therapy for melanoma, but also evaluate for synergy between the two treatments.

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Declarations

Conflict of interest The protocol was approved by the Western Institutional Review Board and all patients provided informed consent for participation. Dr. Faries is an advisor for Bristol-Myers Squibb, Sanofi, Array Bioscience and Pulse Bioscience. Dr. Wagner is an employee of Orbis Health Solutions. Dr. Peoples is employed by Orbis Health Solutions and Cancer Insight; is a consultant for Rapamycin Holdings, Heat Biologics, Abexxa Biologics, and Pelican Therapeutics; and has received funding from the above as well as Sellas Life Sciences and Genentech. Dr. Jakub served on a Novartis Melanoma Surgical Oncology Advisory Board. Dr. Clifton is employed by Parthenon Therapeutics. The view(s) expressed herein are those of the author(s) and do not reflect the official policy or position of Brooke Army Medical Center,

the US Army Medical Department, the Department of the Army, Department of the Air Force, Department of Defense, or the US Government. The voluntary, fully informed consent of the subjects used in this research was obtained as required by 32 CFR 219 and DODI 3216.02_AFI40-402.

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