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Durable and dynamic hTERT immune responses following vaccination with the long-peptide cancer vaccine UV1: long-term follow-up of three phase I clinical trials

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ABSTRACT

Background Therapeutic cancer vaccines represent a promising approach to improve clinical outcomes with immune checkpoint inhibition. UV1 is a second generation telomerase-targeting therapeutic cancer vaccine being investigated across multiple indications. Although telomerase is a near-universal tumor target, different treatment combinations applied across indications may affect the induced immune response. Three phase I/IIa clinical trials covering malignant melanoma, non-small cell lung cancer, and prostate cancer have been completed, with patients in follow-up for up to 8 years.

Methods 52 patients were enrolled across the three trials. UV1 was given as monotherapy in the lung cancer trial and concurrent with combined androgen blockade in the prostate cancer trial. In the melanoma study, patients initiated ipilimumab treatment 1 week after the first vaccine dose. Patients were followed for UV1-specific immune responses at frequent intervals during vaccination, and every 6 months for up to 8 years in a follow-up period. Phenotypic and functional characterizations were performed on patient-derived vaccine-specific T cell responses.

Results In total, 78.4% of treated patients mounted a measurable vaccine-induced T cell response in blood. The immune responses in the malignant melanoma trial, where UV1 was combined with ipilimumab, occurred more rapidly and frequently than in the lung and prostate cancer trials. In several patients, immune responses peaked years after their last vaccination. An in-depth characterization of the immune responses revealed polyfunctional CD4+ T cells producing interferon-γ and tumor necrosis factor-α on interaction with their antigen.

Conclusion Long-term immunomonitoring of patients showed highly dynamic and persistent telomerase peptide-specific immune responses lasting up to 7.5 years after the initial vaccination, suggesting a plausible functional role of these T cells in long-term survivors. The superior immune response kinetics observed in the melanoma study substantiate the rationale for future combinatorial treatment strategies with UV1 vaccination and checkpoint inhibition for rapid and frequent induction of anti-telomerase immune responses in patients with cancer.

Key messages

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Improving T cell responses against relevant tumor antigens is considered an important goal to advance immunotherapy efficacy. Although therapeutic cancer vaccines have shown T cell immune response induction in patients with cancer, the persistency and long-term dynamics of the immune responses are less established.

WHAT THIS STUDY ADDS

⇒ The data reported herein demonstrate that telomerase peptide vaccination induces long-lasting memory T cell responses exhibiting considerable fluctuations. The data also further contributes to the scientific rationale for combining therapeutic cancer vaccination with checkpoint inhibition.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE AND/OR POLICY

⇒ The association between telomerase-targeting CD4+ T cell responses and long-term survival substantiates telomerase as a relevant antigen for immunotherapy. Randomized clinical trials are warranted to validate the therapeutic potential of eliciting a CD4+ anti-hTERT T cell response by peptide vaccination.

BACKGROUND

In the current era of immunotherapy, therapeutic cancer vaccines (TCVs) have earned interest for their potential to stimulate a patient's immune system against tumors. Numerous studies link a lack of response to checkpoint inhibition (CPI) to an insufficient T cell effector response owing to insufficient T cell priming, effector cell generation, or

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memory formation.[1] TCVs do, therefore, hold promise as a potential next step to improve clinical outcomes through their combination with CPIs, providing an invigorated T cell response against relevant tumor antigens while simultaneously blocking T cell suppressor mechanisms. Despite past failures,[2 3] TCVs targeting both shared tumor-associated antigens and mutated neoepitopes have earned renewed enthusiasm, demonstrating promising clinical activity when combined with checkpoint inhibitors.[4–6]

Telomerase is activated in 85%–90% of all cancers and is thus a potential near pan-tumor antigen for immunotherapy.[7 8] Telomerase activation provides unlimited cell proliferation and increases metastatic potential, serving crucial functions for the tumor.[9–11] Its high activity level is a negative prognostic factor across several malignancies.[12–14] Conversely, spontaneous immune responses (IRs) against telomerase reverse transcriptase (hTERT) confer positive prognostic value in non-small cell lung cancer, renal cell carcinoma, and anal squamous cell carcinoma, and are associated with increased response to CPI in melanoma.[15–17] The tumor telomerase reliance and consequent continuous activation provide relevancy for an anti-hTERT IR along the cancer disease continuum. TCVs designed to cover the active site of hTERT are potentially broadly applicable and may serve as an 'off-the-shelf' approach to treat cancer.[18] Theoretically, there should be limited opportunities for resistance mutations to develop, as molecular alterations in the hTERT T cell epitopes would likely negatively affect telomerase activity leading to hampered tumor growth. Characterization of IRs induced by first-generation hTERT vaccines led to the identification of a now clinically validated immunogenic region derived from the active site of hTERT.[19 20] IRs against this region were robust CD4+ T helper 1 (Th1) responses associated with long-term survival. Three highly immunogenic peptides covering the identified region were selected to develop a second-generation telomerase-targeting vaccine, UV1.

Utilizing synthetic long peptides that require intracellular processing facilitates antigen presentation on human leukocyte antigen (HLA) class II, and HLA class I by cross-presentation, leading to induction of CD4+ and CD8+ T cell responses, respectively.[21] Cytotoxic CD8+ T cells have historically been the focus for describing immune-mediated antitumor capacities. Recently, however, the importance of the CD4+ component of the adaptive immune system is becoming more established. Released tumor antigens can be engulfed either in situ or in the draining lymph node by tissue and lymph node-resident antigen-presenting cells (APCs), respectively, and be presented on HLA class II to CD4+ T cells.[22] Activated CD4+ T cells serve as orchestrators of an IR by direct and indirect mechanisms.[23] First, CD40L on the CD4+ T cells binds CD40 on the dendritic cells (DCs) initiating heightened antigen presentation and expression of cytokines and the co-stimulatory molecules CD80 and CD86 by the DCs. These co-stimulatory molecules provide signal 2 for the CD8+ T cells, which in conjunction with the cytokines promote differentiation, effector function, and survival. Second, CD4 +T cells also secrete inflammatory cytokines, such as interleukin (IL)-2 and interferon (IFN)-γ, directly supporting the CD8+ T cells. This inflammatory response promoted by the CD4+ T cells may thus reshape the tumor microenvironment and result in epitope spreading.[24] Cancer cells can also express HLA class II on IFN-γ stimulation,[25] providing a more direct target for CD4+ T cell-mediated cytotoxicity in immunogenic tumors.[26 27] A recent publication from Dillard *et al* further supports this concept, demonstrating antitumor efficacy of an HLA class II-restricted hTERT-specific T cell receptor in an animal model.[28] In vitro studies have also shown recognition of a melanoma cell line by a CD4+ UV1-peptide specific T cell clone,[19] supporting target antigen detection at endogenous levels.

UV1 has been investigated in three completed phase I/IIa clinical trials covering malignant melanoma (MM),[29] non-small cell lung cancer (NSCLC),[30] and prostate cancer (PC).[31] In total, 52 patients have been treated in these studies. The long follow-up time and longitudinal immunomonitoring allow for in-depth characterization of the IR dynamics observed across these indications and treatment combinations. Herein, we provide a comprehensive overview of the IRs induced by UV1 vaccination and demonstrate its correlation with clinical outcomes.

METHODS

Patients and study design

Three phase I/IIa clinical trials evaluating UV1 have been completed, enrolling 52 patients with MM (NCT02275416),[29] NSCLC (NCT01789099),[30] or PC (NCT01784913).[31] All trials were conducted at Oslo University Hospital, Oslo, Norway, and patients were treated between 2013 and 2015. All trials enrolled patients with advanced disease; stage IV melanoma (n=12), locally advanced or metastatic NSCLC with stable disease after chemotherapy alone or combined with radiotherapy (n=18), and newly diagnosed PC with non-visceral metastases (n=22). All studies were open-label, single-armed, single-center studies, with the primary objective to assess the safety and tolerability of UV1 and the secondary objective of evaluating IRs to the UV1 peptides.

Patients who had left the studies due to progression in May 2017 and those progressing thereafter were asked to participate in an IR and survival follow-up study with the aim of monitoring UV1 vaccine responses in long-term surviving patients. The follow-up study encompassed 6-month intervals for assessment of survival only or immunomonitoring and survival (up to 8 years). All but 6 patients alive enrolled (n=25), and 12 patients agreed to be followed for survival and peripheral blood mononuclear cell (PBMC) sampling. The remaining patients agreed to be followed for survival only (online supplemental figure 2 and table 2). The original clinical trials and the follow-up study were approved by the competent

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regulatory authority and ethical committee, and patients provided written informed consent before enrollment.

Treatments

UV1 (Ultimovacs ASA, Oslo, Norway) consists of three peptides, one 30-mer (p719-20) and two 15-mers (p725 and p728), and is produced as a sterile aqueous solution, lyophilized, and stored at −20°C before reconstitution in water for injection. Three doses of UV1 (100 µg, 300 µg, and 700 µg) were administered in the NSCLC and PC trials, whereas only the 300 µg dose was administered in the MM trial. UV1 vaccinations were administered intradermally in the lower abdomen. The vaccine adjuvant granulocyte-macrophage colony-stimulating factor (sargramostim 75 µg) (lyophilized Leukine, Sanofi Aventis, Bridgewater, New Jersey, USA), was administered intradermally at the same injection site 10–15 min prior to UV1.

In the NSCLC trial, patients received UV1 monotherapy. In the PC trial, patients received upfront and concomitantly combined androgen blockade (goserelin 10.8 mg subcutaneously every third month and bicalutamide 50 mg per day). In the MM trial, patients received up to four infusions of ipilimumab (3 mg/kg) starting 1 week after the first UV1 vaccinations (see online supplemental figure 1 for vaccination and biological sampling schedule).

Immunological assessments

PBMCs were prepared from whole blood samples (50 mL in acid citrate dextrose tubes) collected at scheduled intervals prior, during, and after UV1 vaccinations (up to 5 years for the MM and NSCLC studies and 8 years for the PC study) (online supplemental figure 1). The samples from the three studies were processed similarly and at the same laboratory facility. PBMCs were isolated and frozen as previously described.[31] Thawed PBMCs were pre-stimulated once in vitro with UV1 vaccine peptides for 10–12 days before the T cell assays below, as previously described.[31]

Proliferation assay

The T cell proliferation assay (^{3}H-thymidine incorporation) was used to assess vaccine-specific IR, as previously described.[31] Briefly, after 10–12 days pre-stimulation, PBMCs were re-stimulated with 10 µM of the UV1 vaccine peptides (peptide 725, hTERT 691–705 (RTFVLRVRAQDPPPE); peptide 719–20, hTERT 660–689 (ALFSVLNYERARRPGLLGASVLGLDDIHRA); peptide 728, hTERT 651–665 (AERLTSRVKALFSVL) (Bachem AG, Switzerland) and irradiated, autologous PBMCs as APCs, and tested in triplicates for proliferation by ^{3}H-thymidine incorporation. A stimulation index (SI) (ratio of mean counts in wells with T cells stimulated with or without vaccine peptides) above or equal to three for any of the three peptides or a mix was considered positive. Doubling of the SI was required in patients with a spontaneous IR against UV1. Patients with a positive SI during the treatment period were considered a vaccine immune responder. The treatment period was defined as the time from the first vaccine dose to 16 weeks after the last dose. Staphylococcus aureus enterotoxin C3 (SEC3) was used as a positive control to determine immunocompetence. Samples were considered evaluable for IR if SEC3 SI was ≥3

Generation of vaccine-specific T cell clones

Samples containing T cells highly reactive to vaccine peptide stimulation were further utilized to isolate vaccine-specific T cells. T cells were cloned by limiting dilution seeding in Terasaki plates, essentially as previously described.[19] Briefly, T cell lines from immune responders were co-cultured in CellGro DC medium (Cellgenix, Freiburg, Germany) supplemented with 5% human serum (TCS BioSciences, Botolph Claydon, UK) with irradiated allogeneic PBMCs (30 Gy) used as feeder cells. IL-2 and IL-7 were added at 20 U/mL and 2200 U/mL, respectively. Phytohemagglutinin (PHA) was added at 2 µg/mL for polyclonal stimulation. After polyclonal stimulation and when sufficient cell numbers were reached, the clones were tested in proliferation assays against the individual peptides using autologous Epstein-Barr Virus-transformed B lymphoblastoid cell lines (EBV-LCL) as APCs. Unique clonotypes of T cell clone samples were verified by complementarity-determining region three sequencing performed by the laboratory of Dr Mascha Binder, Martin-Luther-University Halle-Wittenberg, Germany (online supplemental table 1).

HLA restriction of vaccine-specific T cell clones (TCCs) was determined by HLA Class II blocking antibodies used in the proliferation assay. Antibodies blocking HLA-DR, HLA-DQ, and HLA-DP were used (produced in the immune monitoring group of Department of Cellular Therapy, Oslo University Hospital, Norway, as described in.[32] HLA class II restriction analysis of TCCs was refined using a panel of EBV-LCLs as APCs, homozygous for patient HLA alleles.[33] The EBV-LCLs were irradiated (100 Gy), loaded with UV1 peptides, and co-incubated with TCCs at a 1:1 ratio in proliferation assays (3H-thymidine incorporation) as described for testing vaccine-specific IRs.

Phenotypic characterization of vaccine-specific T cells

After T cell expansion, the vaccine-specific T cells were phenotypically characterized by staining for surface markers. Briefly, cells were suspended in round-bottom test tubes (5 mL, Falcon, Corning Life Sciences, Corning, USA) in staining buffer (Dulbecco's phosphate-buffered saline (Lonza BioWhittaker) with 2% fetal calf serum (Thermo Fisher Scientific)). Aggregated γ-globulin (10 mg/mL) (Sigma-Aldrich Norway AS) was added before staining to block Fc receptors. The cells were stained for viability (LIVE/DEAD Fixable Aqua Dead Cell Stain Kit, Invitrogen) and surface markers as follows (antibody clones and manufacturer in parenthesis); CD3 (UCHT1, Invitrogen), CD8 (RPA-T8, eBioscience), CD4 (OKT4,

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BioLegend), CD45RO (UCHL1, BioLegend), CD197 (3D12, eBioscience), ICOS (ISA-3, Invitrogen), PD-1 (MIH4, eBioscience), LAG3 (11C3C65, eBioscience), TIM3 (F38-2E2, eBioscience), TIGIT (MBSA43, eBiosciences), and incubated in the dark on room temperature for 20 min. The acquisition was performed on FACSCanto IVD 10 color configuration (Becton Dickinson, New Jersey, USA) and analyzed using FlowJo V.10.8 (Becton Dickinson, New Jersey, USA)

For intracellular staining of cytokines, vaccine-specific TCCs were first stimulated with vaccine peptides. Autologous EBV-LCLs were used as APCs and loaded with vaccine peptides at 10 µM for 12 hours. The TCCs were co-cultured with vaccine-peptide-loaded EBV-LCL cells at a 1:2 ratio in a 96-well plate for 9 hours. CD107a (H4A3, BD BioSciences) and Golgi plug and stop (BD BioSciences) were added to the culture. TCCs stimulated with phorbol myristate acetate and ionomycin (50 ng/mL and 1 µM, respectively) were used as a positive control. TCCs stimulated with a non-cognate vaccine peptide were used as a negative control. The cells were harvested and stained with CD4 (OKT4, BioLegend) and, after fixation and permeabilization (FIX & PERM Cell Permeabilization Kit, Invitrogen), stained for IFN-γ (4S.B3, Invitrogen), and tumor necrosis factor-α (TNF-α) (Mab11, BD BioSciences). Acquisition was performed on FACSCanto IVD 10 color configuration (Becton Dickinson, New Jersey, USA) and analyzed using FlowJo V.10.8 software (Becton Dickinson, New Jersey, USA)

Multiplex cytokine analysis

The TCCs were co-cultured with vaccine-peptide-loaded EBV-LCL cells at a 1:2 ratio in a 96-well plate for 24 hours, using the same positive and negative controls as above. A multiplex cytokine assay (Bio-Plex Pro Human Cytokine Th1/T helper 2 (Th2) Assay, Bio-Rad Laboratories, Hercules, USA) was performed on the culture supernatant. Th1 and Th2 cytokine concentrations were assessed following the manufacturer's protocol on the Bio-Plex 200 instrument (Bio-Rad Laboratories, Hercules, USA). Supernatants were analyzed in triplicates. Concentrations for the negative control were subtracted from concentrations in supernatants from peptide-stimulated TCCs. Z-scores were calculated by subtracting the mean of all cytokine concentrations from the respective cytokine concentration and dividing by the SD.

HLA genotyping

HLA genotyping was performed on PBMCs from patients with available samples (n=50). The HLA typing was performed by ProImmune (Oxford, UK) utilizing PCR sequence-specific oligonucleotides. Major allele groups were resolved to four digits (eg, HLA-A*23:01). For patient 802, HLA genotyping resolved to two digits for class I and four digits for class II were performed by Oslo University Hospital, Rikshospitalet, Oslo, Norway.

Statistics

The study was designed to be analyzed descriptively, and no formal statistical plan was pre-defined. Sample sizes (n) represent the number of analyzed patients. P values presented in table 1 were calculated using Fisher's exact test (two-sided) for categorical variables (values listed with percentages) with two categories and the χ^2 test for values with three categories. P values for variables shown with mean and SD were calculated using unpaired Student's t-test (two-tailed). In figure 1, the p values were calculated using one-way analysis of variance and Tukey's multiple comparisons test. Statistical significance was set at $p<0.05$. Center values in figure 1C,D represent the median. The statistical analyses were performed in GraphPad Prism V.9.2.0. (GraphPad Software)

The survival analyses were performed using the Kaplan-Meier method with 95% CIs for median overall survival (OS) calculated with Greenwood's formula. The log-rank test was performed to determine the statistical difference between positive and negative immune response populations (figure 4A) and between the populations responding to none, one, or two or more UV1 peptides (figure 4B). Only IRs appearing within the treatment period, defined as up to 16 weeks after the last vaccination (online supplemental table 2), were included in the survival analyses.

RESULTS

Patients, treatment, and clinical outcomes

Between 2013 and 2015, a total of 52 patients were enrolled across the three studies. The median age was 65 (range 44–78), and 37 (71.2%) patients were men (male dominance due to PC study). Most patients were Eastern Cooperative Oncology Group performance status 0 (88.5%). The median time from initial diagnosis until trial enrollment was 6.2 months (range 1.1–207.1). All patients in the MM and PC studies had stage IV disease. In the NSCLC study, 6, 5, and 7 patients had stage III, III/IV, and IV disease, respectively. Patients were treated with a mean of 5.5 (range, 3–9), 12.4 (range, 9–18), and 13.5 (range, 7–18) UV1 vaccine doses in the MM, NSCLC, and PC studies, respectively. Patients have been followed for survival for a median of 55.7 months (range, 3.5–79.5), 28.2 months (range, 4.7–87.3), and 61.8 months (range, 11.7–96.3) for the MM, NSCLC, and PC studies, respectively. In the pooled analysis, patients received a mean of 11.2 (range, 3–18) doses of UV1 and were followed for a median of 46.0 months (range, 3.5–96.3).

UV1 vaccination was generally considered safe and well tolerated across the three trials.[29–31] With up to 79.5 months of follow-up, the median OS was not reached in the MM study. For the NSCLC and PC studies, the median OS was 28.2 and 61.8 months, respectively (table 2).

Patient characteristics and IR development

PBMCs were collected for longitudinal IR assessment across the three trials. One patient in the MM trial was not evaluable for IR analysis due to a lack of post-treatment

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Table 1 Patient characteristics and associations with immune response

		MM	NSCLC	PC	Overall	IR negative	IR positive	
		n=11	n=18	n=22	n=51	n=11	n=40	P value
Age (years)	Mean	58.2	65.1	66.6	64.3	65.6	63.9	0.55
	SD	9.4	7.7	7.5	8.5	7.5	8.8	
Gender (%)	Male	6 (54.5)	8 (44.4)	22 (100)	36 (70.6)	6 (54.5)	30 (75)	0.26
	Female	5 (45.5)	10 (55.6)	0 (0)	15 (29.4)	5 (45.5)	10 (25)	
Ethnicity (%)	Caucasian	11 (100)	18 (100)	21 (95.5)	50 (98.0)	11 (100)	39 (97.5)	1.0
	Asian	0 (0)	0 (0)	1 (4.5)	1 (2.0)	0 (0)	1 (2.5)	
ECOG (%)	0	11 (100)	14 (77.8)	21 (95.5)	46 (90.2)	10 (90.9)	36 (90)	1.0
	1	0 (0)	4 (22.2)	1 (4.5)	5 (9.8)	1 (9.1)	4 (10)	
Prior systemic therapy (%)	Yes	4 (36.4)	18 (100)	22 (100)	44 (86.3)	10 (90.9)	34 (85)	1.0
	No	7 (63.6)	0 (0)	0 (0)	7 (13.7)	1 (9.1)	6 (15)	
Stage (%)	III	0 (0)	6 (33.3)	0 (0)	6 (11.8)	2 (18.2)	4 (10)	0.39
	III/IV	0 (0)	5 (27.8)	0 (0)	5 (9.8)	2 (18.2)	3 (7.5)	
	IV	11 (100)	7 (38.9)	22 (100)	40 (78.4)	7 (63.6)	33 (82.5)	
Months since diagnosis	Mean	43.9	22.0	3.7	18.8	28.1	16.2	0.30
	SD	58.3	23.8	1.7	33.4	32.1	33.7	
LDH* (%)	>ULN	6 (54.5)	6 (33.3)	2 (9.1)	14 (27.5)	3 (27.3)	11 (27.5)	1.0
	≤ULN	5 (45.5)	12 (66.7)	20 (90.9)	37 (72.5)	8 (72.7)	29 (72.5)	
Lymphocytes* (%)	<LLN	1 (9.1)	6 (33.3)	1 (4.5)	8 (15.7)	2 (18.2)	6 (15)	1.0
	≥LLN	10 (90.9)	12 (66.7)	21 (95.5)	43 (84.3)	9 (81.8)	34 (85)	
Number of doses received	Mean	5.7	12.4	13.4	11.4	10.8	11.5	0.60
	SD	2.5	2.7	2.5	3.9	3.6	4.1	
Dose level (%)	100 µg	0 (0)	6 (33.3)	7 (31.8)	13 (25.5)	4 (36.4)	9 (22.5)	0.33
	300 µg	11 (100)	6 (33.3)	7 (31.8)	24 (47.1)	3 (27.3)	21 (52.5)	
	700 µg	0 (0)	6 (33.3)	8 (36.4)	14 (27.5)	4 (36.4)	10 (25.0)	

Demographics of immune response (IR) evaluable patients in each trial, and pooled IR negative and positive populations. The p values represent demographic differences between IR positive and negative populations.
*Levels of LDH and lymphocytes at baseline.
ECOG, eastern cooperative oncology group; LDH, lactate dehydrogenase; MM, malignant melanoma ; NSCLC, non-small cell lung cancer ; PC, prostate cancer.

PBMC samples. Patients with samples having a SI≥3 in the proliferation assay were considered immune responders. In evaluable patients (n=51), the IR rate was 90.9%, 66.6%, and 81.8% for the MM, NSCLC, and PC trials, respectively (figure 1A), providing a pooled IR rate of 78.4%. Spontaneous anti-UV1 IRs were observed in six patients at baseline, all of whom developed an increase in SI after vaccination (figure 1B). We found no associations between patient baseline characteristics and the development of vaccine-induced IRs (table 1)

UV1 induced IRs occurred earlier and more frequently when combined with ipilimumab

In the treatment period, the median highest SI observed in individual patients was 11.5 (range 2.3–60.0) in the MM study, 10.6 (range 1.1–64.8) in the NSCLC study, and 9.8 (range 1.8–38.1) in the PC study, and 10.7 (range, 1.1–64.8) overall (figure 1B.). The median time to a measurable IR was 4.0, 5.0, and 10.0 weeks for the MM, NSCLC, and PC trials, respectively, and 6.5 weeks (range, 1–40) for all studies combined (figure 1C). The median number of vaccinations received until the first detectable IR was 5 (range 3–6) in the MM study, 6 (range 5–13) in the NSCLC study, and 8 (range 3–13) in the PC study, and 6 (range 3–13) for all studies combined (figure 1D) (online supplemental figure 1).

UV1 vaccination induces persisting IRs

After the treatment period, patients were followed for long-term immunomonitoring every 6 months for up to 8 years. Robust IR peaks occurred in many patients several years after the last vaccination, often exceeding SIs detected during the treatment period, particularly in the PC study (figure 2A–C). Of note, several patients who mounted IRs against select peptides within UV1 during the treatment period displayed immune responses towards more peptides during the late peaks (patients N03 and N11 in the MM study, and patients 805, 816,

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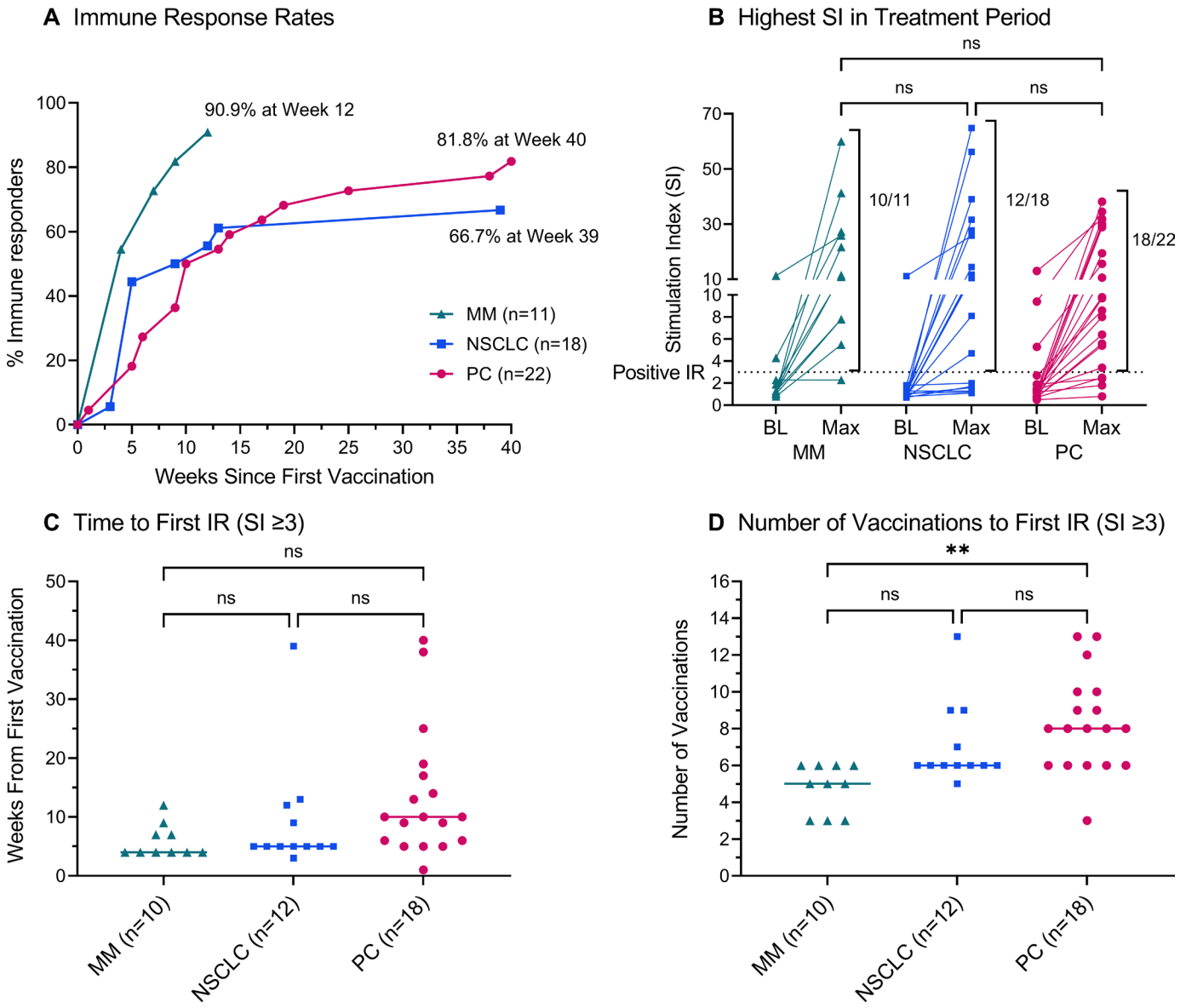

Figure 1 Dynamics of vaccine-induced immune responses across three phase I/IIa trials. (A) Comparative overview of immune response kinetics and immune response rates achieved in each trial. (B) Highest stimulation index (SI) score achieved within the treatment period by immune response evaluable patients. The differences across the studies are non-significant (one-way analysis of variance (ANOVA), p value 0.71). (C) The time from first vaccination to first detectable immune response across the three studies. The differences are not statistically significant (one-way ANOVA, p value 0.12). (D) The number of UV1 vaccinations administered before the first immune response was detectable in the proliferation assay. Immune responses in the MM study appeared after fewer vaccinations than in the PC and NSCLC studies (one-way ANOVA, MM vs PC, Tukey's adjusted p value 0.001; MM vs NSCLC, Tukey's adjusted p value 0.061; NSCLC vs PC, Tukey's adjusted p value 0.34). BL, baseline; IR, immune response; MM, malignant melanomal; NSCLC, non-small cell lung cancer; ns, non-significant; PC, prostate cancer.

and 821 in the PC study) (online supplemental figure 4). Patient 809 in the PC study, with no positive IRs during the treatment period, demonstrated an SI of 12.0 at week 234 after the first vaccination (69 weeks after the last vaccine dose). Interestingly, of the six patients with spontaneous IRs (N07, N11, 911, 802, 806, and 822), four were among the long-term survivors with late peaks in their immune responses (N07, N11, 806, and 822).

Although these studies were not designed to evaluate the effect of subsequent therapy on vaccine-induced IRs, we investigated whether there were any temporal associations between late peaks and receipt of successive CPI. Seven patients in the MM study (N01, N03, N04, N07, N08, N11, and N14) and one patient in the NSCLC study (902) received subsequent CPI (figure 2A) (online supplemental table 4). Unfortunately, samples post CPI initiation from patients N08, N14, and 902 were not available. Patient N03 received anti-programmed cell death protein-1 (PD-1) therapy between weeks 26 and 64 after the first vaccination, and at the nearest time point of IR assessment after CPI initiation, the patient had an SI of 36.3 (week 132) (up from 4.3 at week 23). Patient N07

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Table 2 Study population, treatment, and clinical outcomes

Trial	Population	Phase	Treatment	Follow-up time (up to)	mOS	IR
MM	Stage IV melanoma (n=12)	I/IIa	UV1 +Ipilimumab	79.5 mo	NR	90.9%
NSCLC	Locally advanced or metastatic NSCLC with SD after radiation therapy and/or chemotherapy (n=18)	I/IIa	UV1	87.3 mo	28.2 mo	66.7%
PC	Newly diagnosed PC with non-visceral metastases and eligible for CAB (n=22)	I/IIa	UV1 +CAB	96.3 mo	61.8 mo	81.8%

CAB, combined androgen blockade; IR, immune response rate; MM, malignant melanoma ; mo, months; mOS, median overall survival; NR, not reached; NSCLC, non-small cell lung cancer ; PC, prostate cancer ; SD, stable disease.

received anti-PD-1 between weeks 77 and 180 and showed a steady increase in SI in the following assessments at week 112 (SI 1.5), week 138 (SI 5.1), week 164 (SI 7.6), week 190 (SI 29.2), and week 216 (SI 70.3). Patient N11 received anti-PD-1 at week 155 after the first vaccination and experienced an IR boost at week 182 (SI of 12.3, up from 1.3 at week 154), although the duration of anti-PD-1 treatment is not known for this patient. The remaining patients (N01 and N04) did not experience SI peaks following CPI therapy.

UV1 vaccination induces polyfunctional CD4+ Th1 cells

In patients with an initial strong IR, vaccine-specific TCCs were generated by limiting dilution (online supplemental table 1). Based on the long-term IRs detected in patients, we assumed that vaccine-specific T cells had a memory phenotype with lasting proliferative potential. This was further corroborated by the in vitro characterization of the TCCs, showing CD4+ T cells with downregulation of CCR7 and expression of CD45RO, consistent with an effector memory T cell phenotype[34] (figure 3A). Furthermore, the TCCs expressed the activation marker ICOS (figure 3B). Few TCCs expressed the checkpoint molecules PD-1 and LAG3, whereas the majority expressed TIM3 and TIGIT (figure 3B). To further characterize the TCCs' functionality, we loaded autologous APCs (EBV-LCLs) with the UV1 peptides and co-cultured them with the TCCs to assess their response to antigen recognition. Vaccine-specific T cells produced both IFN-γ and TNF-α and were positive for the degranulation marker CD107a on stimulation with vaccine peptide-loaded APCs (figure 3C). Culture supernatant from vaccine peptide-stimulated TCCs was harvested and assessed for Th1 and Th2 cytokine concentrations, showing high production of Th1 cytokines (TNF-α, IFN-γ, granulocyte-macrophage colony-stimulating factor, and IL-2) (figure 3D). Th2 cytokines (IL-4, IL-5, and IL-13) were also produced, although to a lesser extent. TCCs isolated from patients with MM and NSCLC exhibited a stronger Th1 polarization than the TCCs isolated from patients with PC, primarily due to lower IFN-γ and higher IL-5 production by TCCs from patients with PC (online supplemental table 5). Characterization of two TCCs from patient 822 targeting different vaccine peptides revealed dichotomous T cell profiles. TCC #10 directed at the 15-mer peptide p725 exhibited robust Th1 cytokine production, whereas TCC #12 directed at the 30-mer peptide p719-20 had a more neutral cytokine profile.

The HLA class II restriction of TCCs was characterized in patients 802 and 822, showing TCCs restricted for epitopes presented on HLA-DQB1*03:01, HLA-DQB1*02:01, HLA-DRB1*12:01, HLA-DRB1*07:01, and -DPB1*03:01 molecules (online supplemental table 1). The range of HLA class II molecules able to bind and present UV1 epitopes is further supported by the diversity of HLA genotypes in all immune responders (online supplemental table 3).

UV1-specific IRs associated with long-term survival

Patients with a UV1-specific immune response elicited during the treatment period had improved OS compared with non-immune responders (median OS (mOS), 54.8 months vs 23.4 months, log-rank p value 0.05) (figure 4A). At time points with sufficient PBMCs, an IR against each of the three peptides and a mix of all were analyzed (online supplemental table 2). Patients responding to two or more peptides demonstrated improved OS as compared with patients responding to one or no peptides (mOS, IR positive ≥2 peptides 77.0 months, IR positive one peptide 44.8 months, IR negative 23.4 months, log-rank p value 0.09) (figure 4B). A similar trend of prolonged survival in immune responders versus non-immune responders was also observed in each study separately, mOS not reached versus 7.4 months in the MM study (log-rank p value 0.05), 38.4 months versus 21.3 months in the NSCLC trial (log-rank p value 0.67), and 76.0 months versus 30.0 in the PC study (log-rank p value 0.20), respectively (online supplemental figure 3).

DISCUSSION

The antigen selected for the UV1 vaccine represents an essential tumor-promoting factor, providing the immune system with a target that persists throughout the tumorigenesis. Furthermore, the peptides in UV1 were selected from the active site of hTERT to limit the potential for tumor resistance mutations to develop in the targeted epitopes. Indeed, non-synonymous mutations in the

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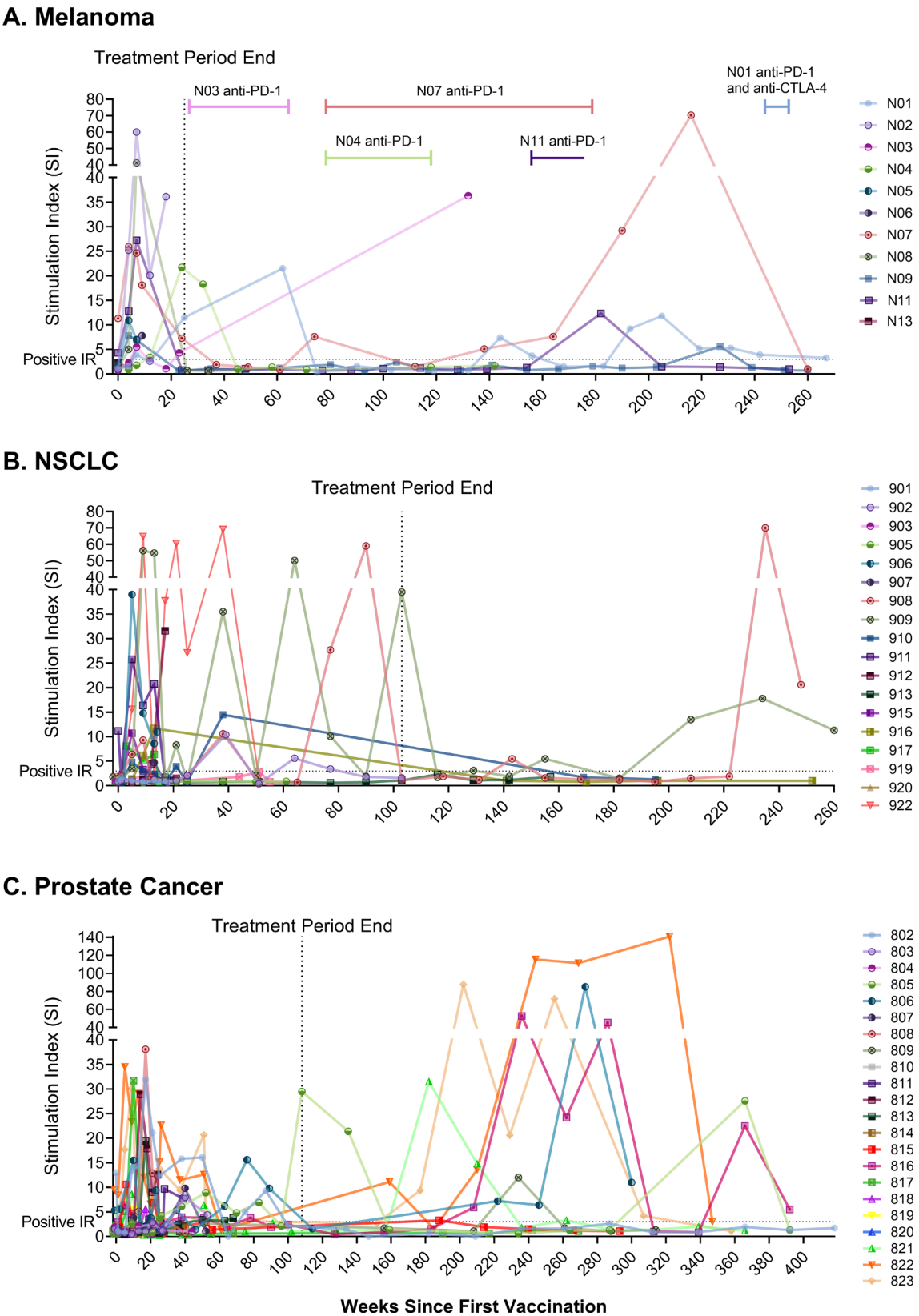

Figure 2 With up to 8 years of immune response follow-up, vaccinated patients demonstrate persisting immune responses. Many patients experienced natural or treatment-related immune response boosting occurring several years after receiving the last vaccine dose. (A) Melanoma (lines indicate the duration of new CPI therapy), (B) NSCLC, and (C) prostate cancer. The treatment period was assessed for individual patients and defined as 16 weeks after the last vaccine dose. 'Treatment Period End' represents the end of the treatment period for the last patient. Values represent the highest SI toward any UV1 peptide or a mix at the time point of assessment. The end of anti-PD-1 treatment for patient N11 is not known. IR, immune response; NSCLC, non-small cell lung cancer; PD-1, programmed cell death protein-1; CTLA-4, cytotoxic T lymphocyte antigen-4.

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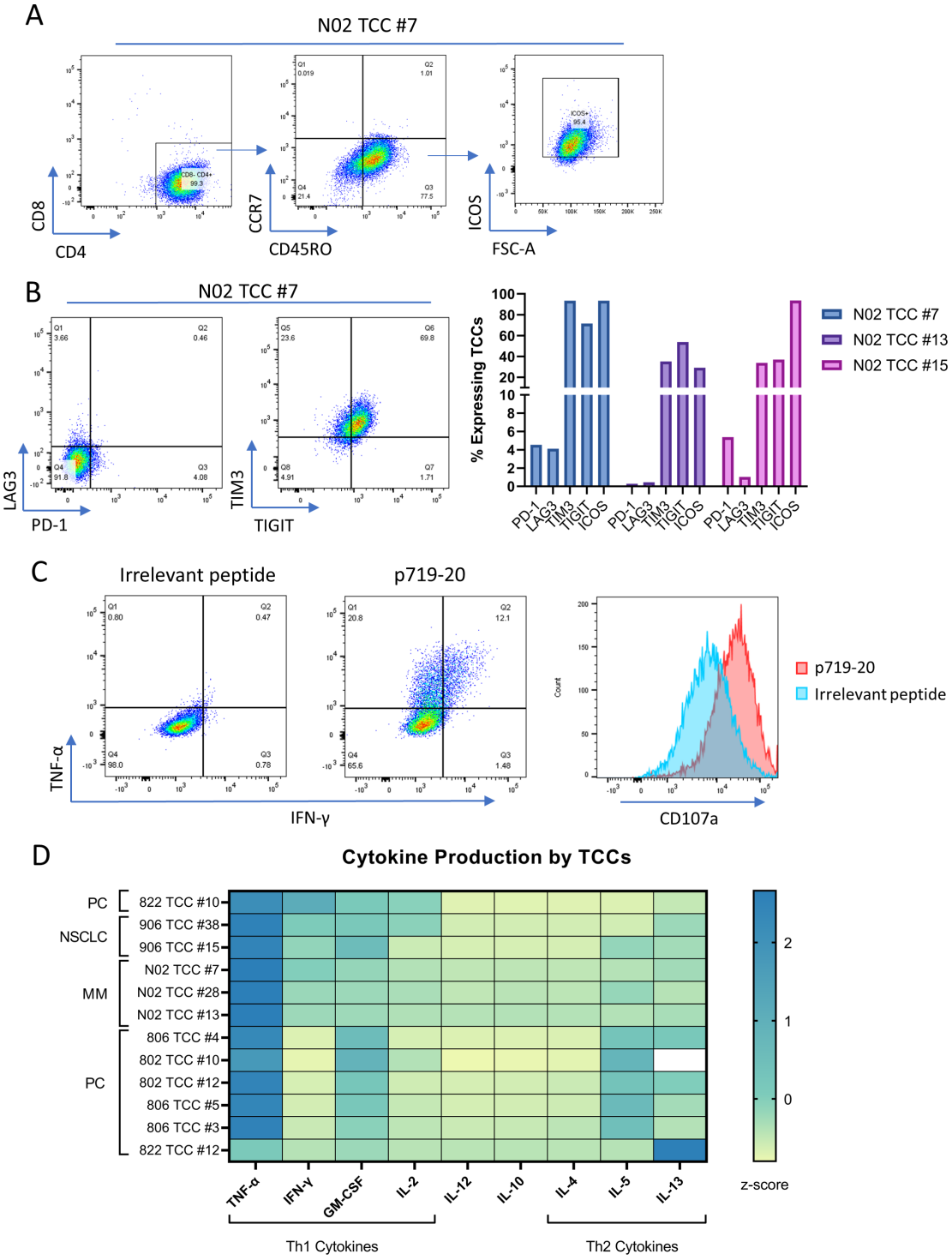

Figure 3 Vaccine-induced T cell responses are CD4+ Th1 T cells. (A) Representative vaccine-specific T cell clones (patient N02, TCC #7) characterized by extracellular staining of surface markers CCR7, CD45RO, and ICOS. Cells were gated on singlets, viability, and lymphocytes. (B) Staining of TCCs for surface expression of the checkpoints PD-1, LAG3, TIM3, and TIGIT, and the co-stimulatory receptor ICOS. Cells were gated on singlets, viability, lymphocytes, and CD4 positivity. (C) Intracellular staining of effector cytokines and the degranulation marker CD107a on vaccine-specific T cell clones (patient N02, TCC #7) after 9 hours of antigen-specific stimulation (p719–20). Cells were gated on singlets, lymphocytes, and CD4 positivity. (D) 24-hour stimulation of T cells with antigen-specific vaccine peptides elicit Th1 cytokine production. Background concentrations (TCC stimulated with irrelevant peptide) have been subtracted. The rows are sorted on the highest mean concentration of Th1 cytokines. For cytokines with concentrations above the standard curve, the highest measurable concentration is used (see online supplemental table 5 for numerical values). IL-13 cytokine concentration for 802 TCC #10 was not evaluable. IFN, interferon; IL, interleukin; MM, malignant melanoma; NSCLC, non-small cell lung cancer; PC, prostate cancer; PD-1 programmed cell death protein 1; TCC, T cell clones; Th1, T helper 1; TNF, tumor necrosis factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

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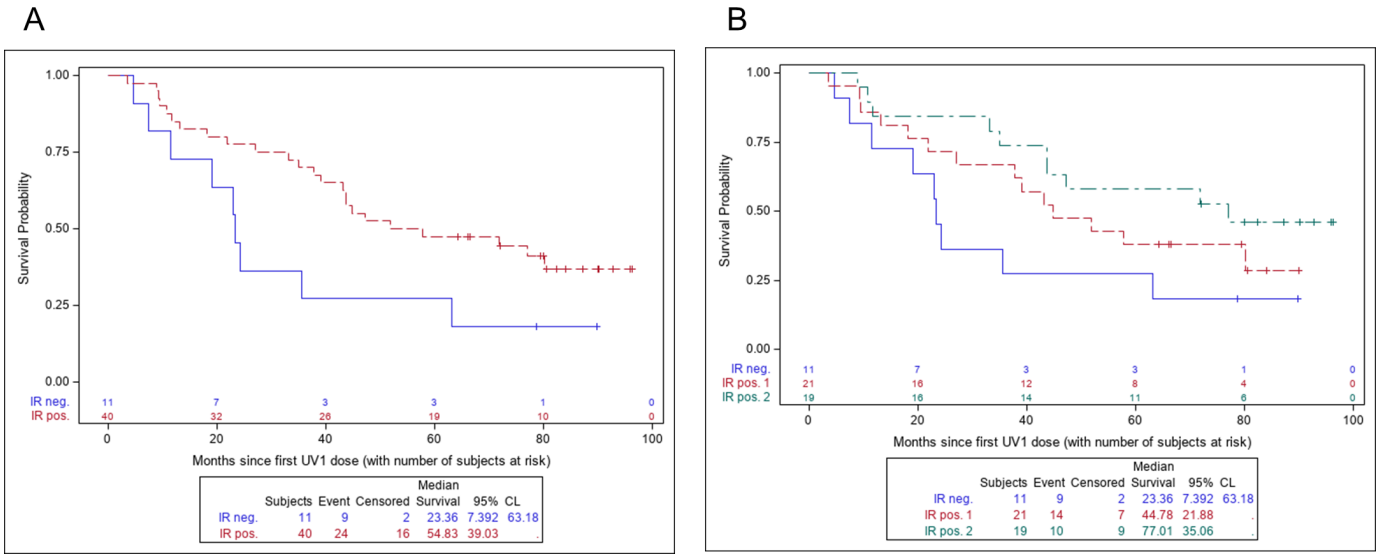

Figure 4 UV1 induced immune responses associated with improved overall survival. (A) With up to 8 years of follow-up time, immune response positive patients (red curve) demonstrate median overall survival of 54.8 months, as compared with 23.4 months in the immune response negative population (blue curve). (B) Breadth of the immune response associates with improved survival. Patients developing an immune response to two or more peptides (green curve) have prolonged survival as compared with patients responding to only one peptide (red curve) or none (blue curve) (77.0 vs 44.8 vs 23.4 months, respectively). IR, immune response.

reverse transcriptase domain of hTERT have been shown to cause defective enzymatic activity.[35–37] Theoretically, epitopes from less essential sequences in hTERT may well mutate away from an IR without compromising hTERT activity. Although hTERT is a frequently mutated gene across most cancers,[38] nearly all of these mutations map to the promoter (88% of hTERT mutations) (online supplemental figure 5), working to increase hTERT expression.[39] Three highly immunogenic synthetic long peptides were admixed in a vaccine peptide pool to allow individual selection of epitopes matching patient-specific HLA molecules, providing a broad population coverage. The data reported herein support that UV1 mounts strong and persistent IRs across an HLA-unselected population, further substantiated by the HLA restriction diversity demonstrated by vaccine-specific TCCs.

In the MM study, we hypothesized that the combination of UV1 and the anti-CTLA-4 monoclonal antibody ipilimumab would lead to synergy in the expansion of vaccine-specific T cells. The CTLA-4 immune checkpoint is upregulated on primed T cells and competitively inhibits the binding of CD28 on the T cells with B7 ligand on the APCs.[40] Thus, blocking CTLA-4 provides enhanced co-stimulation of primed T cells and could lead to more robust T cell responses after vaccination. We did indeed observe a more rapid and frequent induction of IRs in the MM study, as compared with both the NSCLC and PC studies, with 6/11 patients exhibiting IRs at the first time point of assessment in week 4 and additional four patients mounting IRs during the subsequent 8 weeks. The differences were most pronounced between the MM and PC studies. The sampling schedule was slightly different in the MM study as compared with the NSCLC and PC studies, which may impact when the first IR was detected (first and second sampling in week 4 and 7 in the MM study, and week 2 and 6 in the NSCLC and PC studies). Irrespective of the sampling schedule, the high IR rate (91%) further supports the postulated synergistic effect of vaccination and CPI.

A plausible explanation for the higher number of vaccine doses required to elicit IRs in the PC study could be the simultaneous use of combined androgen blockade (CAB). In the PC study, patients received CAB (goserelin and bicalutamide) upfront and concomitantly to vaccination. Although one study reported increased CD8+ T cell infiltration after androgen deprivation therapy,[41] murine studies have demonstrated an immunosuppressive effect of CAB on tumor-reactive T cells, disrupting the effect of concurrent immunotherapy.[42] Specifically, androgen receptor antagonists were shown to suppress priming and diminish the IFN-γ production by T cells. These observations correlate well with the higher number of vaccine doses required to induce IRs and the lower levels of IFN-γ produced by vaccine-specific T cells derived from patients with PC than patients with MM and NSCLC. However, the few patients with available TCCs significantly limit the interpretations of cross-study differences in cytokine responses by vaccine-induced T cells. Regardless of the potential adverse effect of CAB, a long-lasting and high rate of IRs was observed in the PC study (82%). Therapeutic cancer vaccination before starting hormonal therapy has been shown to salvage immune responses, thereby supporting an alternative sequential administration regime for future studies.[43]

The longevity of the induced IR and the persisting proliferative capacity of the T cells provide reasons for optimism for UV1's potential to deliver long-term clinical impact for the patients. The observed late peaks in the IR

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are remarkable and often surpass the SIs detected during the treatment period. Such late peaks reflect complex dynamics of the anti-hTERT peptide response, elicited by tumor antigen release or subsequent therapy rather than the vaccine itself. The broadening of the IR occurring during these peaks may result from intramolecular epitope spreading, as has been previously observed with hTERT peptide vaccination.[19] These observations may have been unnoticed in other cancer vaccine studies due to shorter follow-up periods. We investigated whether subsequent CPI had a temporal correlation with these late peaks, which was apparent in some patients with melanoma. Several studies have revealed that CPIs expand antitumor T cells that are detectable in peripheral circulation before entering the tumor bed,[44 45] providing a plausible explanation for this sudden surge of anti-hTERT T cells in these patients. Nardin and colleagues observed a correlation between improved clinical outcomes of CPIs and the presence of spontaneous anti-hTERT IRs in patients with melanoma.[16] The improved clinical responses could be due to a reinvigoration of the spontaneously primed anti-hTERT IR by CPIs. Whereas they reported spontaneous IRs to an hTERT peptide pool (a pool that does not include the UV1 region) in 52% of patients with stage IV melanoma (n=31), only two (17%) patients were spontaneous immune responders to the highly immunogenic UV1 region in our melanoma study. After UV1 vaccination, however, 91% of patients developed an IR, which could also support clinical responses to subsequent CPI, although a dedicated study would be required to test this hypothesis.

Based on the described mechanism by which CD4+ T cells contribute to antitumor immunity, their effector potential partly relies on their ability to secrete inflammatory cytokines (TNF and IFN-γ).[46] We, therefore, assessed the TCCs phenotype and cytokine response to target antigen recognition in vitro. The vaccine-specific T cells were polyfunctional CD4+ Th1 cells secreting TNF-α, IFN-γ, and IL-2 on antigen stimulation. The TCCs from patient N02 in the MM trial also expressed the degranulation marker CD107a supporting a hypothesis for cytotoxic potential against HLA class II expressing melanomas.[19 47] Although vaccine-specific T cells produced Th1 cytokines, a secretion of Th2 cytokines was also observed (IL-4, IL-5, and IL-13), disproving the classical dichotomy of Th1/Th2 CD4+ T cell differentiation. The high amount of IL-13 produced by hTERT peptide-specific T cells has been demonstrated in previous studies from our laboratory.[48] Importantly, the immunosuppressive T(reg) cytokine IL-10 was consistently low. While vaccine-specific TCCs derived from patients with PC exhibited less IFN-γ production and an increase in IL-5 levels, possibly due to the concomitant androgen blockade, IRs against hTERT were still associated with improved OS in this study (online supplemental figure 3E,F). Thus, the exact antitumor potential of T cells induced through vaccination may not entirely rely on their Th1/Th2 differentiation, as both phenotypes have described antitumor effects.[49 50]

The expression of checkpoint molecules by the TCCs is likely affected by repeated rounds of in vitro antigenic and polyclonal (PHA) stimulation, and the expression levels are therefore likely not directly translatable to the in vivo setting. We aimed to assess whether the TCCs were exhausted, and despite expressing TIM3 and TIGIT, the TCCs exhibited preserved functionality with continued proliferative and cytokine responses. Though vaccine-specific cells isolated through limiting dilution were CD4+, we have previously demonstrated CD8+ IRs in the NSCLC study, although to a relatively lower degree.[30] The lack of isolating hTERT-specific CD8+ T cells from blood may result from a difference in tissue distribution among vaccine-induced CD4+ and CD8+ T cells. However, the exact kinetics of T cell subtype emigration after vaccination is not comprehended. The detection of mainly CD4+ T cell responses in blood with synthetic long peptide and RNA cancer vaccines is also observed by others.[4 51–53]

We observed an association between vaccine-induced IRs and prolonged survival across three distinct cancer populations. The IR rates were 91%, 67%, and 82% in the MM, NSCLC, and PC studies, respectively, and the mOS was not reached in the MM study, 28.2 months in the NSCLC trial and 61.8 months in the PC trial. Hence, there were relatively more immune responders belonging to the studies with the longest survival times, skewing the data towards prolonged OS in the immune responder group. However, a similar trend was observed in each study individually, supporting the prognostic significance of the induced IRs (online supplemental figure 3). While we observed a survival time association with vaccine-induced anti-hTERT peptide IRs, others have established the prognostic relevance of spontaneous anti-hTERT immune responses across several cancers.[15–17] The now accumulating data strengthen the evidence of hTERT as a relevant antigen for immunotherapy.

FUTURE PERSPECTIVES

The results reported herein support further clinical evaluation of UV1, especially in combination with CPIs. Inhibiting regulatory mechanisms imposed on T cells by immune checkpoints will likely lead to more rapid and frequent induction of vaccine-specific T cells and support their antitumor effector functions. To determine the clinical efficacy of UV1 in combination with CPI, randomized controlled trials with UV1 combined with pembrolizumab in head and neck cancer, durvalumab in ovarian cancer, and two studies with ipilimumab and nivolumab in melanoma and mesothelioma, respectively, are currently being conducted (EudraCT number 2020-005910-17, NCT04742075, NCT04382664, NCT04300244).

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Competing interests EBE, SMM, SA, and GG are employees of Ultimovacs ASA or Ultimovacs AB. GG, SMM, and EMI are shareholders in Ultimovacs ASA. GG and EMI are inventors of the UV1 patent. SMM is the founder and shareholder of Immuneed AB, Vivologica AB, and Strike Pharma AB, none of which have had any role in the work herein. Other authors declare no competing interests.

Patient consent for publication Not applicable.

Ethics approval This study involves human participants and was approved by Regional Committees for Medical and Health Research Ethics (REC) (reference number: REK 2014/421, REK 2012/1114, REK 2012/1126, and REK 2017/702). Participants gave informed consent to participate in the study before taking part.

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