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Dose escalation study of a personalized peptide-based neoantigen vaccine (EVX-01) in patients with metastatic melanoma

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ABSTRACT

Background Neoantigens can serve as targets for T cell-mediated antitumor immunity via personalized neopeptide vaccines. Interim data from our clinical study NCT03715985 showed that the personalized peptide-based neoantigen vaccine EVX-01, formulated in the liposomal adjuvant, CAF09b, was safe and able to elicit EVX-01-specific T cell responses in patients with metastatic melanoma. Here, we present results from the dose-escalation part of the study, evaluating the feasibility, safety, efficacy, and immunogenicity of EVX-01 in addition to anti-PD-1 therapy.

Methods Patients with metastatic melanoma on anti-PD-1 therapy were treated in three cohorts with increasing vaccine dosages (twofold and fourfold). Tumor-derived neoantigens were selected by the AI platform PIONEER and used in personalized therapeutic cancer peptide vaccines EVX-01. Vaccines were administered at 2-week intervals for a total of three intraperitoneal and three intramuscular injections. The study's primary endpoint was safety and tolerability. Additional endpoints were immunological responses, survival, and objective response rates.

Results Compared with the base dose level previously reported, no new vaccine-related serious adverse events were observed during dose escalation of EVX-01 in combination with an anti-PD-1 agent given according to local guidelines. Two patients at the third dose level (fourfold dose) developed grade 3 toxicity, most likely related to pembrolizumab. Overall, 8 out of the 12 patients had objective clinical responses (6 partial response (PR) and 2 CR), with all 4 patients at the highest dose level having a CR (1 CR, 3 PR). EVX-01 induced peptide-specific CD4+ and/or CD8+T cell responses in all treated patients, with CD4+T cells as the dominating responses. The magnitude of immune responses measured by IFN-γ ELISpot assay correlated with individual peptide doses. A significant correlation between the PIONEER quality score and induced T cell immunogenicity was detected, while better CRs correlated with both the number of immunogenic EVX-01 peptides and the PIONEER quality score.

Conclusion Immunization with EVX-01-CAF09b in addition to anti-PD-1 therapy was shown to be safe and well tolerated and elicit vaccine neoantigen-specific CD4+and CD8+ T cell responses at all dose levels. In addition, objective tumor responses were observed in 67% of patients. The results encourage further assessment of the antitumor efficacy of EVX-01 in combination with anti-PD-1 therapy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Tumor mutational burden has previously been correlated to a better clinical outcome to immune checkpoint inhibitor (ICI) treatment. Several clinical trials are, therefore, aiming to boost a cancer immune response through personalized neoantigen vaccines. However, immunogenicity of different potential neoantigens can vary significantly and can therefore have a major impact on the antitumor response.

WHAT THIS STUDY ADDS

⇒ We demonstrate through a dose escalation that the neopeptide vaccine EVX-01-CAF09b is safe at high-dose levels (2000 µg total peptide) in combination with ICI therapy, where the majority of patients showed a clinical response.

⇒ The PIONEER predicted neoantigens included in the vaccine induced both CD4 and CD8 T cell responses.

⇒ Clinical responses correlated with PIONEER prediction score, which indicates PIONEER as a promising neoantigen prediction tool.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Neopeptide-based vaccines are safe to use in high-dose levels in combination with ICI therapy. This could potentially allow administration of higher number of different neopeptide for a broader coverage in future trials.

INTRODUCTION

Immune checkpoint inhibitors (ICIs) have resulted in major treatment advantages for solid cancers.[1] In particular, ICIs that target the PD-1/PD-L1 axis are now the standard of care for several cancers, including advanced melanoma.[2] Still, many patients do not benefit from ICI therapy.[3] The tumor mutational burden (TMB) is suggested to be a predictive biomarker for treatment outcome of ICI immunotherapy,[4 5] however, it is hypothesized to be of greater importance whether tumor mutations give rise to immunogenic MHC-presented neoantigens.[6] Therefore, combining ICI with a personalized neoantigen cancer vaccine to elicit tumor-specific T cell responses could be an attractive therapeutic avenue to augment the effect of ICI treatments.

Several clinical trials are currently evaluating personalized cancer vaccines against neoantigens using either peptides, mRNA, or DNA to induce neoantigen-specific immune response in cancer patients.[7] Results from these trials show that these treatments can induce profound neoantigen-specific immune responses in humans and have a clinical relevant effect. Noteworthy, it has been shown in resectable melanoma patients that neoantigen vaccination in combination with anti-PD-1 lowers the risk of recurrence compared with anti-PD-1 treatment alone.[8] Hence, neoantigens are promising targets for cancer treatment and are further subject of investigations.

One class of neoantigen vaccines uses synthetic long peptides to induce patients' neoantigen-specific T-cell responses. Peptides directly prime the immune system and have the advantages of easy and fast production. Clinical trials have shown that peptide vaccines targeting neoantigens can effectively induce T-cell responses,[9] predominantly reporting induction of tumor-specific CD4+T cells, while induction of neoantigen-specific CD8+T cells is observed less consistently.[10 11] In these trials, different strategies for neoantigen; selection (vaccine design), delivery (vaccine modality), dose, and formulation have been applied, profoundly affecting T cell activation and function and thus clinical effect.[12–14] Hence, concluding on those important parameters for peptide-based neoantigen vaccines is challenging based on previous studies.

Peptide dose has been investigated in other non-neoepitope-based cancer vaccine trials, where dose-dependent immune responses and clinical responses (CR) were observed.[14] Similar mechanisms might be true for neoantigen peptide vaccines. While multiple different bioinformatic pipelines have been developed for neoantigen identification[15] only a minority has been applied to design vaccines in clinical trials. Collaborative efforts, such as the TESLA consortium, have furthermore demonstrated that these pipelines, in general, do not identify the same neoantigens as important for treatment.[16] Hence, knowledge about the impact of selection algorithms on neoantigens, relevance, immunogenicity, and effect is limited. Therefore, further studies are needed to understand the potential of personalized neoantigen peptide vaccines.

We have previously reported interim phase I data demonstrating feasibility of the EVX-01 neoantigen targeting and CAF09b adjuvanted peptide vaccine.[9] Here, we report the complete findings of the study, including the impact of the neoantigen peptide dose and the PIONEER neoantigen quality score on T-cell activation as well as CR in patients. Patients with metastatic melanoma were vaccinated with the neopeptide vaccine EVX-01, at three different dose levels while keeping the CAF09b adjuvant/peptide ratio constant. To enhance CD8+T cell immune responses, the vaccine was administrated by three IP followed by three intramuscular (IM) injections (reference med IP og CD8). The patients received anti-PD-1 therapy during vaccine production and the dosing with EVX-01 vaccine. The CD4+ and CD8+ T cell immune responses toward vaccine neopeptides were measured and we investigated the relationship between neoantigen immune responses, vaccine doses, clinical outcomes, and PIONEER quality score for selecting neoantigens.

PATIENTS, MATERIALS, AND METHODS

Patients

Included patients (≥18 years of age) had biopsy-verified advanced unresectable melanoma. Patients were planned to either begin first-line treatment with an anti-PD-1 checkpoint inhibitor (cohort A) or had already been treated with an anti-PD-1 agent for at least 4 months with stable disease (SD) and qualified for continued treatment with anti-PD-1 (cohort B). Additional inclusion criteria were as follows: ≥1 measurable lesion as per investigator-assessed Response Evaluation Criteria in Solid Tumors (RECIST V.1.1); an Eastern Cooperative Oncology Group (ECOG) performance status of 0 or 1; adequate organ function; and tumor tissue available for whole-exome sequencing (WES). Main exclusion criteria included severe autoimmune disease and previous severe immune-related adverse events (AEs) and no other significant comorbidity.

Trial design

To assess the safety, immunogenicity, and feasibility of manufacturing the personalized neoepitope-based cancer vaccine; EVX-01-CAF09b, we conducted a phase I first-in-human clinical trial (EudraCT No. 2018-002892-16 and ClinicalTrials.gov NCT03715985) at the National Center for Cancer Immune Therapy (CCIT-DK) and the Department of Oncology, Copenhagen University Hospital, Herlev, Denmark. [17 18]

Patients were treated with an anti-PD-1 agent according to local guidelines (pembrolizumab 2 mg/kg, every 3 weeks or nivolumab 6 mg/kg, every 4 weeks), and the personalized cancer vaccine EVX-01-CAF09b was added to the treatment schedule when manufacturing was completed (figure 1A). Patients received EVX-01 treatment every 2 weeks for six EVX-01 vaccinations in total. The first three vaccinations were administered

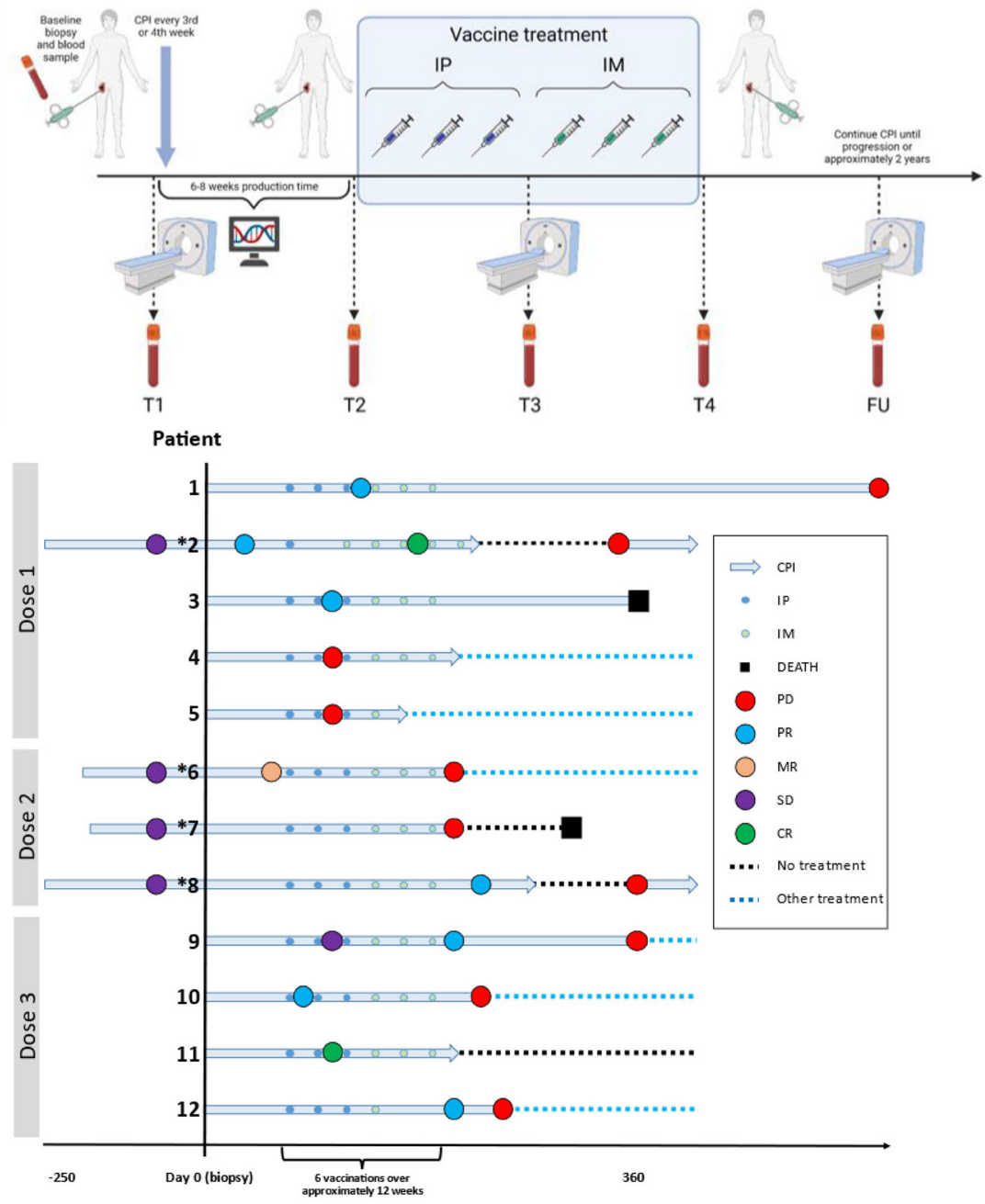

Figure 1 Clinical setup and response. (A) Clinical setup; biopsy, PET/CT scan, and blood samples were collected at baseline (T1). Treatment with aPD1 was either initiated around the first biopsy (group A) or had already been initiated for at least 4 months before the biopsy (group B: *). EVX-01 vaccination was administered approximately at weeks 6–8 and every second week for six vaccinations in total (3 IM+3 IP). Tumor biopsies were performed (if possible) at T2 and T4. In addition, radiographic imaging was done every 12 weeks. Blood samples were collected from T1 to T4, and thereafter every 12 weeks. Figure created with BioRender.com. (B) Overview of patients included in trial: checkpoint inhibitor (CPI) initiation, baseline biopsy (day 0), vaccine treatment, and follow-up information of the twelve patients at three different dose levels. Small blue and green dots indicate either IP vaccinations or IM vaccinations, respectively. The depiction of disease condition and patient status are indicated in various colors. CR, complete response (green); IM, intramuscular; IP, intraperitoneal; MR, mixed response (salmon); PD, progressive disease (red); PR, partial response (blue); SD, stable disease (purple); PET, positron emission tomography.

intraperitoneal (IP), and the last three vaccinations were administered intramuscular.

The trial was amended to include three-dose-level groups. At least two vaccine doses were administered to patients 1 and 2 before additional patients could be vaccinated. Dose level 1 (lowest dose) included five patients (500 µg total peptide, target 50 µg/peptide), three patients were included at dose level 2 (1000 µg total peptide, target 100 µg/peptide), and four patients at dose level 3 (2000 µg total peptide, target 200 µg/peptide). The initial individual peptide dose was chosen based on formulation studies and experience from another CAF09b adjuvanted cancer

vaccine starting clinical testing at CCIT-DK[19] Only if no vaccine-related grade 3–4 AEs occurred in relation to the first two vaccinations at each dose level, the next patient could receive the first vaccination.

The study's primary endpoints were safety and tolerability of the treatment at each dose level, based on the observation of AEs according to the NCI Common Terminology Criteria for Adverse Events (CTCAE V.4.0). The secondary endpoints were the feasibility of manufacturing a personalized neoantigen-based vaccine within 6–8 weeks of inclusion via the PIONEER AI prediction platform and evaluating the immune response before, during and after vaccination with the personalized neoantigen vaccine (EVX-01-CAF09b). The tertiary endpoints included efficacy, which was evaluated by best overall response (BOR), progression-free survival (PFS), and overall survival (OS).

Assessments in the study included physical examination, ECOG performance status, vital signs (pretreatment and post-treatment), and laboratory analyses to warrant the safety of the participants. Imaging (CT scan or PET-CT) was done at baseline, and every 3 months, followed by imaging every 12 weeks to evaluate the clinical efficacy of the trial treatment. Tumors were evaluated following the RECIST V.1.1 criteria. Tumor biopsies were obtained at baseline (obligatory), shortly before the initial vaccination, and just after the final vaccination (voluntary).

Design of personalized neoantigen vaccines

Personalized neoantigen vaccines were designed by the PIONEER platform. WES data from healthy and tumor tissue, mRNA sequencing data from tumor tissue, and human leucocyte antigen (HLA) typing data from healthy tissue were used to predict the neoantigens and design the neopeptides. The PIONEER system assigns a combined neoantigen quality score to each neoantigen/neopeptide by combining (1) the potential to be presented by MHC, (2) expression levels, and (3) the clonality. The manufacturing of the personalized neoantigen vaccine is described in detail in Mørk *et al*.[9] In some patients, it was decided to administer individual neopeptides in double dose, that is, some individual neoantigens in dose level 1 were given at 100 µg/dose instead of target 50 µg/dose, 200 µg/dose instead of target 100 µg/dose at dose level 2 and 400 µg/dose instead of target 200 µg/dose at dose level 3. This was accomplished by administrating a lower number of neopeptides while keeping the total peptide dose level constant between patients at the same dose level. Patient 1, patient 3, patient 5, patient 6 and patient 10 had 4, 1, 1, 4 and 2, peptides administrated, respectively, in double dose as the target of manufacturing 10 soluble peptides could not be met. For patient 7, patient 8 and patient 9, it was decided to administer 5, 5, 4; peptides in double dose, respectively, to test higher individual peptide doses earlier in the trial. Each EVX-01 vaccine comprises 5–10 synthetically manufactured peptides. The initial individual peptide dose was chosen based on the experience from a clinical trial performed at DK-CCIT (EudraCT No.: 2015-003719-39).[19]

CAF 09b and final vaccine formulation at all dose levels

EVX-01 vaccine peptides were solubilized in 100% dimethyl sulfoxide (DMSO) (NPV-dp001) ad-mixed with the CAF09b adjuvant. CAF09b is a cationic liposomal vaccine adjuvant produced by Statens Serum Institut and is based on dimethyldioctadecyl ammonium (DDA) combined with monomycoloyl glycerol (MMG) and poly I:C. Formulation of each EVX-01 vaccine with CAF09b was performed at CCIT within 2 hours before administration as follows; a total of 1.08 mL sterile filtered Tris reconstitution buffer was added to 0.12 mL sterile filtered NPV-dp001 and thoroughly mixed. Following this step, 1 mL of the peptide solution was added to a 2R vial containing 1.0 mL CAF09b (2500 µg DDA/mL, 500 µg MMG/mL, and 125 µg poly I:C/mL). Subsequently, the final vaccine product could be drawn into a syringe. At dose level 1, patients received 500 µg of total peptide and 0.25 mL CAF09b, administered as 1×0.5 mL (IM) or 0.5 mL (IP). At dose level 2, patients received 1000 µg of total peptide and 0.5 mL CAF09b, administered as 2×0.5 mL (IM) or 1.0 mL (IP). At dose level 3, patients received 2000 µg of total peptide and 1.0 mL CAF09b, administered as 2×1.0 mL (IM) or 2.0 mL (IP).

Peripheral blood mononuclear cells isolation and prestimulation

Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood at different time points (see figure 1A).

Before screening for peptide recognition, PBMCs were prestimulated with pools of patient-specific EVX-01 peptides. PBMCs were thawed and cultivated in X-vivo media (X-vivo 15, Lonza # + 5% human AB serum) supplemented with 10 ng/mL IL-15 and 50 ng/mL IL-21 (Preprotech) as culture media. At day 1, pooled vaccine peptides (final concentration 20 µg/mL) were added to the cell cultures in base media. From day 2, the culture media was supplemented with 40 IU/mL IL-2 (Preprotech). The cells were cultivated for 10–14 days and rested for 1–3 days in X-vivo media before cryopreservation and subsequent analysis.

Skin-test infiltrating lymphocytes

A voluntary delayed-type hypersensitivity (DTH) skin test was done approximately 2 weeks after the sixth vaccinations (figure 2A). Patients received two intradermal injections of the EVX-01 peptides and one injection with trisaminomethan buffer and DMSO as a negative control on the back. After 48 hours, 5 mm punch biopsies were obtained from each injection site for skin-test infiltrating lymphocytes (SKILs) culture. The tissue biopsies were divided into 1–3 mm^3 fragments and cultured with medium (90% RPMI-1640 plus GlutaMAX and 25 mM HEPES), 10% heat-inactivated AB Human serum (HS; Sigma-Aldrich), 100 U/mL penicillin, 100 µg/mL streptomycin (Pen Strep, Thermo Fisher Scientific), 1.25 µg/mL Amphotericin B (Fungizone, Bristol-Myers Squibb) and 100 IU/mL rhIL-2 (Proleukin, Novartis). Plates

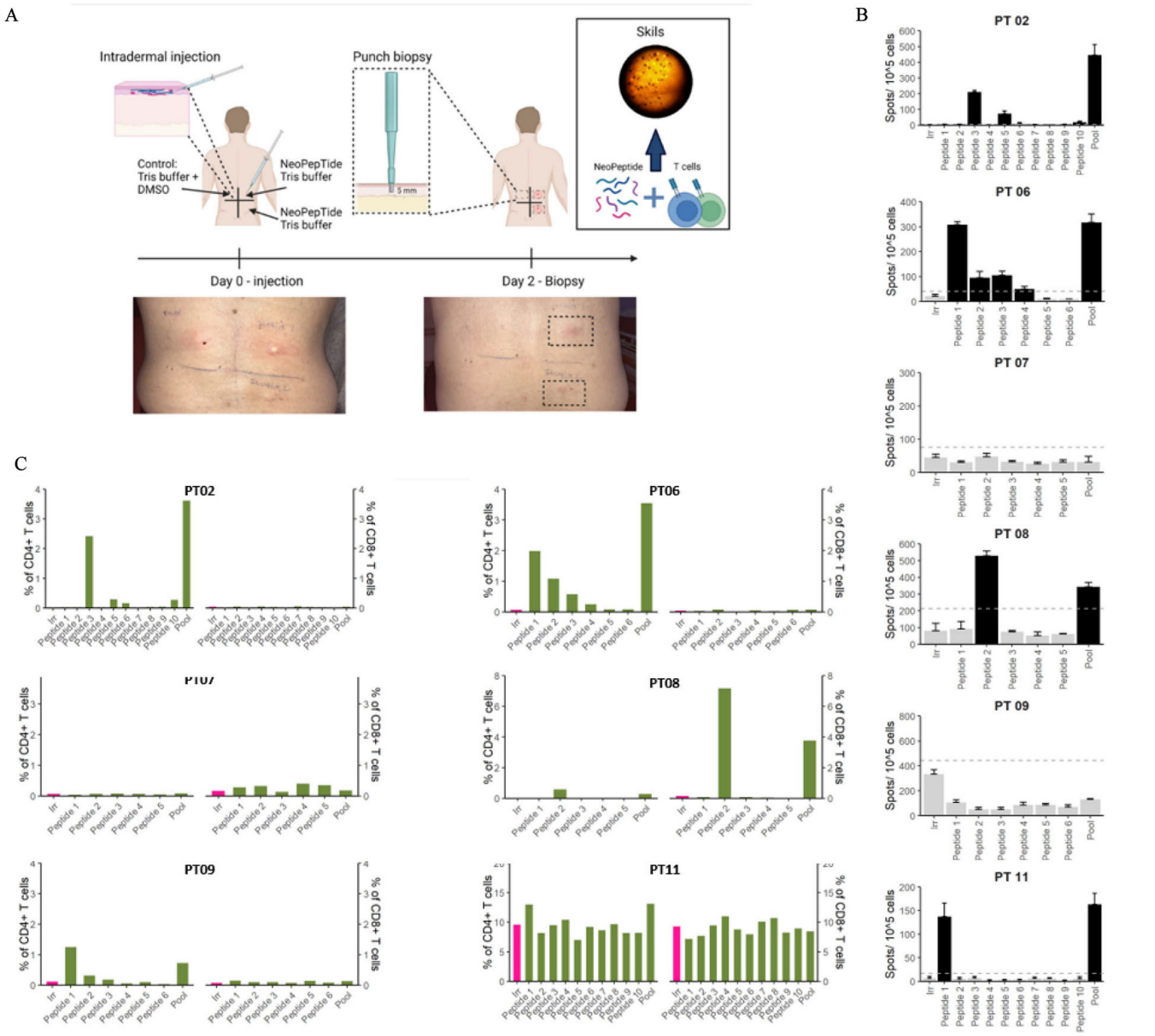

Figure 2 EVX-01 reactive SKIL derived T cell. (A) DTH skin test was done approximately 2 weeks after the last vaccination. Two intradermal injections of the EVX-01 peptides and one control (DSMO) injection were administered on day 0 (approximately 2 weeks from the last vaccination). After 48 hours, 5 mm punch biopsies were taken from the three injections site. The tissue was then transported to the laboratory for SKILs culture. Minimally expanded SKILs were expanded from tissue fragments for 3–6 weeks. Created with BioRender.com. EVX-01-specific T cell responses in SKILs (skin-test infiltrating lymphocytes) in patients 2 (dose level 1), 6, 7, 8 (dose level 2), and 9, 11 (dose level 3). (B) Elispot responses were examined in SKILs at T4 (after six vaccinations) from six patients. SKILs were co-cultured with EVX-01 peptide pool and individual EVX-01 peptides. Black bars represent significant responses, gray bars are not significant. The dotted line indicated the threshold value for a significant response; background (irrelevant peptide) plus 3×SD of the background and at least 10 spots over background response (C) T cells specific toward EVX-01 peptides were identified in SKILs, which were restimulated with EVX-01 peptide pool (green), single-vaccine peptides (green) or irrelevant peptide (pink) for 8 hours. The T cell reactivity was defined as the percentage of live CD8+ or CD4+ T cells staining positive for at least two of four markers (TNF-α, IFNγ, CD107a, CD137).

were incubated at 37°C, and 1/2 of the medium was replaced on day 5 and, hereafter, three times weekly. After 3–6, pooled SKILs were cryopreserved or further expanded with Rapid Expansion Protocol (REP).[20]

Tumor-infiltrating lymphocytes and tumor cell lines

As previously described,[21] fresh tumor tissue was collected and transported to the laboratory for tumor-infiltrating lymphocytes (TILs) culture. In short, to obtain minimally expanded TILs, the tumor biopsies were divided into 1–3

mm^3 fragments and placed in separate wells of a 24 well-culture plate with medium (90% RPMI-1640 plus GlutaMAX and 25 mM HEPES), 10% HS, 100 U/mL penicillin, and 100 µg/mL streptomycin, 1.25 µg/mL Amphotericin B and 6000 IU/mL rhIL-2, for 3–6 weeks. Minimally expanded TILs were then pooled and either cryopreserved or further expanded according to a standard 14-day REP.[21] Autologous tumor cell lines (TCLs) were established via serial passage of adherent cells from the same tumor biopsies.[20] The TCLs were routinely tested negative for mycoplasma (AppliChem; Darmstadt, Germany), and the number of passages between collection and use in the described experiments was <10.

T cell activation assay by IFN-γ ELISPOT

Using the IFN-γ ELISPOT assay, we screened for peptide recognition by T cells in both PBMCs and SKILs as described in the interim report.[9]

T cell activation assay by intracellular cytokine staining analyses

PBMCs (peptide-specific activation)

Rested PBMCs were cultured in a 96-well plate ($2–3\times10^6$ cells/well) and restimulated with the vaccine peptide pool (20 µg/mL) or irrelevant peptide (1 µg/mL) in X-vivo media and incubated at 37°C. After 2 hours, Golgi mix containing GolgiPlug, GolgiStop (BD biosciences), and CD107a (BD biosciences) were added and incubated for 6 hours. The cells were washed and stained with Live/Dead Fixable Dead Cell Stain Near-IR (Thermo Fisher) and Surface antibodies: CD3, CD4, and CD8 (BD biosciences) and fixed and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (Invitrogen). Next, the cells were intracellularly stained for TNF-α (Biolegend), IFN-γ, and CD137 (BD biosciences). Cells were analyzed using the LSRFortessa (BD biosciences), and flow cytometry data were analyzed by FlowJo V.10 (Becton Dickinson). Reactivity of the T cells was defined as a percentage of live CD8+or CD4+ T cells staining positive for at least two of four reactivity markers (TNF-α, IFN-γ, CD137, and CD107a). Irrelevant peptides with effector cells were used as control. Patients were defined as having a vaccine-specific response when the frequencies of reactive T cells stimulated with EVX-01 neopeptide pool exceeded the frequency of reactive T cells stimulated with irrelevant peptide.

SKILs and TILS (peptide-specific activation)

Prior to the initiation of the assays, SKILs and TILs were thawed and rested overnight. EVX-01-specific T cell activation was evaluated with an 8-hour co-culture at 37°C of effector cells (TILs or SKILs) and peptides in the presence of autologous monocytes. The SKIL to monocyte ratio was 10:1. The single peptides and peptide pool were added with a final concentration of 0.5 µg/mL alongside a positive control (PMA+Ionomycin) and a negative control (irrelevant peptide). After 2 hours of co-culture, anti-human CD107a antibody, brefeldin A (dilution of 1:1000, GolgiPlug), and monensin (dilution of 1:1000, GolgiStop) were added. After 8 hours of incubation, the cells were washed two times with DPBS (Sigma-Aldrich/Merck KGaA) and stained with live/dead reagents, as well as antibodies used for surface markers. Afterward, the cells were washed, fixed and permeabilized overnight using the FoxP3/Transcription Factor Staining Buffer Set (eBiosciences, Thermo Fisher Scientific). Following, the cells were stained with antibodies binding to intracellular targets. Cells were then analyzed on a NovoCyte Quanteon Flow Cytometer with FlowJo V.10 (Becton Dickinson). The T cell reactivity was defined as the percentage of live CD8+ or CD4+ T cells staining positive for at least two of four reactivity markers (TNF-α, IFN-γ, CD107a, CD137) minus the background (unstimulated control). Further details can be found in the interim manuscript presenting interim data.[9]

SKILs (tumor-specific activation)

Prior to the initiation of the assays, SKILs were thawed and rested overnight. SKILs were then tested for antitumor reactivity as previously described.[22] The definition of T cell reactivity was the percentage of live CD8+ or CD4+ T cells staining positive for at least two of four markers (TNF-α, IFNγ, CD107a, CD137) minus the background (unstimulated control).

Detection of peptide-specific CD8+ T cells by combinatorial fluorochrome encoding of peptide-MHC multimers

8–11 mer minimal peptides were predicted from every EVX-01 peptide (22–27 mer) by NetMHCpan V.4.1,[23] based on patient-specific HLA types. EVX-01-derived minimal peptides were selected per patient based on EL%rank <2. A selection process was performed to reduce the number of peptides; we selected the top 30–41 peptides with lowest EL%rank, grouped by the long EVX-01 peptides and the patient-specific HLA alleles (online supplemental figure 6). A few EVX-01 peptides did not include HLA class I binders (EL%rank <2), which is why no minimal peptides were included for these peptides. A panel of fluorochrome-labeled peptide-MHC (pMHC) tetramers was assembled for each patient, with each pMHC having a unique identifiable fluorochrome combination, as previously described.[24 25] EVX-01 prestimulated PBMCs for all collected time points were stained with the patient-specific pMHC tetramer panel, analyzed on an LSR fortessa (BD Bioences) and gated in FlowJo V.10.

Detection of peptide-specific CD8+ T cells using DNA barcode-labeled pMHC I multimers

Selected patients (patients 1, 2, 3, 4, 8, and 9—primarily patient with a CD8 response detected by intracellular cytokine staining (ICS)) were screened for the broad presence of neoantigen reactive CD8+T cells (NARTs) and virus-reactive CD8+T cells (VARTs). Patient-specific neopeptides were predicted with two different prediction pipelines, PIONEER and MuPeXi.[26] To construct patient-specific peptide libraries, the top 100 peptides from each prediction pipeline were selected (including potential overlapping peptides, resulting in <200 peptides per library). Additionally, EVX-01 vaccine minimal peptides described above and virus peptides were added to the patient-specific panel of peptides resulting in a total of 145–231 unique pMHC combinations per patient.

PBMCs, TILs, and SKILs were screened for CD8+T cell recognition using the patient-specific peptide panels, loaded into barcode labeled pMHC-multimer complexes.[27] In short, pMHCs and a short unique DNA barcode were both bound to a fluorochrome-labeled (PE: neo antigens, APC: viral antigens) dextran molecules, creating a DNA barcode-labeled pMHC I multimer, which is unique for each pMHC combination. The cells mentioned above were stained with a panel of these multimers in combination with a CD8 (BD Biosciences, RPA-T8) and CD3 (BD, clone SK7) antibody. PE and APC fluorochrome-labeled CD8+T cells were sorted on the FACSAria (BD Biosciences). DNA barcodes bound to the sorted cells were hereafter amplified by PCR, as were a reference DNA barcode baseline sample from the collected pMHC multimer panel that the cells were stained with. Amplified barcodes from sorted cells and baseline were hereafter sequenced by PrimBio. Sequence results were uploaded to Barracoda[27] for analysis, together with various information on primers, DNA barcodes, DNA barcode annotation for pMHC, and information on sample identification. Output files included log2 fold change of sorted barcodes compared with baseline barcodes and the related p value to determine significantly pMHC complexes among the sorted cells. Barcode-pMHC multimers were used to select pMHC, complexes possibly recognized by T cells in the pool (between 7 and 41 pMHC complexes) while this more restricted library of pMHC was included for analyses using combinatorial fluorochrome encoding of pMHCs as described above.

RESULTS

12 patients were enrolled from January 2019 to October 2021. Demographics and baseline disease characteristics of the patients are listed in table 1 and consort table overview of screened patients is listed in online supplemental table 2. As previously reported, five patients were enrolled at dose level 1. Of these, four patients were in cohort A (anti-PD-1 naïve) while one patient was in cohort B (SD on anti-PD-1).

To augment the effect of ICI treatment, we have developed a process for identifying and manufacturing a personalized cancer vaccine comprising cancer-specific neoantigens, EVX-01. Included neoantigens were identified based on genomic sequencing data from tumor tissue, genomic sequencing data from healthy tissue, and the patient's HLA type. We delivered the neoantigens as long peptides (neopeptides) with the liposomal adjuvant CAF09b to maximize the effect. In the current study, patients with metastatic melanoma were enrolled and treated with EVX-01-CAF09b in addition to anti-PD-1 treatment. The clinical protocol dictated six injections of EVX-01 CAF09b to each patient at 2 weeks intervals: three IP followed by three IM injections. During vaccine production lead time, patients received anti-PD-1 therapy, which continued during the vaccination period and beyond until progressive disease (PD), stable remission, or intolerable toxicity. Manufacturing time was between 6 and 8 weeks, however, as the first dose was to be administered on the same day as a dose of anti-PD-1, time from baseline biopsy until first vaccination ranged from 51 to 70 days (table 1). Three dose levels were tested; dose level 1 with 500 µg total peptide and 0.25 mL CAF09b in 5 patients (results previously reported in Mørk *et al*,[9] dose level 2 with 1000 µg total peptide and 0.5 mL CAF09b in hree patients, and dose level 3 with 2000 µg total peptide and 1.0 mL CAF09b in four patients.

EVX-01-CAF09b in combination with anti-PD-1 has no major safety concerns

All AEs observed at dose level 1 were grade 1, except for patient 2, who experienced grade two fatigue after the first vaccination.[9]

At dose level 2, three patients were enrolled, all in cohort B, who received all six vaccinations. One patient experienced grade 2 abdominal pain after IP injection. No severe vaccine-related AEs were observed at this dose level.

Table 1 Patient characteristics

Disease stage	Baseline LDH	PD-L1 receptor	BRAF mutation	Dosage	Days from biopsy until first vaccine
M1b	259	PD-L1>1% and <50%	Mutation	500 µg	56
M1c	147	PD-L1>1%	Mutation	500 µg	51
M1c	118	PD-L1>1% and < 2%	Negative	500 µg	53
M1a	184	PD-L1 5%	Mutation	500 µg	57
M1b	835	PD-L1<1%	Negative	500 µg	60
M1a	116	PD-L1<1%	Mutation	1000 µg	62
M1b	239	PD-L1>1%	Negative	1000 µg	56
M1a	180	PD-L1<1%	Mutation	1000 µg	60
M1b	160	PD-L1>50%	Positive	2000 µg	53
M1c	201	PD-L1>50%	Negative	2000 µg	70
M1a	210	PD-L1 negative	Positive	2000 µg	57
M1c	223	PD-L1>1%	Positive	2000 µg	56

Table 2 Adverse events at dose levels 2 and 3

	Dose level 2 (n=3)		Dose level 3 (n=4)	
	IP	IM	IP	IM
All adverse reactions within 0–14 days				
Any	12	3	13	12
Grade 2	1	0	2	2
Grade 3	0	0	0	3
Injection site adverse reactions within 0–14 days				
Pain grade 1	2	0	6	4
Systemic adverse reactions witin 0–14 days				
Abdominal pain grade 1	1	0	0	0
Abdominal pain grade 2	1	0	0	0
Chils grade 1	0	0	1	0
Cough grade 1	1	0	0	0
Fatigue grade 1	1	1	1	2
Fatigue grade 2	0	0	1	1
Fever grade 1	2	0	0	0
Influenza like symptoms grade 1	0	0	3	0
Headache grade 1	0	0	0	1
Joint pain grade 1	0	0	1	0
Joint pain grade 2	0	0	1	0
Nausea grade 1	3	1	0	0
Vomiting grade 1	2	1	1	0
Edema grade 2	0	0	0	1
Myositis grade 3	0	0	0	1
Adrenal insufficiency grade 3	0	0	0	1
Papilledema grade 1	0	0	1	0
Nefritis grade 3	0	0	0	1

IM, intramuscular; IP, intraperitoneal.

Four patients were enrolled at dose level 3, all in cohort A. Patient 11 developed grade 3 immune-related myositis (without any signs of myocarditis) and adrenal insufficiency after six vaccinations and six cycles of ICI. Patient 12 developed grade 3 immune-related nephritis after four vaccinations and five series of ICI. These side effects are well-documented toxicity risks in connection with ICI therapy and thus judged must likely related to this treatment. The myositis and nephritis were managed with steroid therapy and resolved within 4 weeks.

In summary, most AEs were grade 1, except for four events (abdominal pain, fatigue, edema, and joint pain), which were reported as grade 2, and two AEs (nephritis and myositis) were registered as grade 3, judged as related to ICI (table 2). Both patients with grade 3 AEs ceased treatment with ICI and EVX-01-CAF09b as defined per protocol. The most frequent reported AEs were pain at the injection site, fatigue, and nausea. Between the two vaccination routes, a tendency for more side effects was seen after IP injections compared with IM injections.

One patient from group 1 died unexpectedly 4 months after the sixth vaccination. It was determined as treatment-unrelated and cancer-unrelated reasons.

Majority of patients have CRs to the combined EVX-01 and anti-PD-1 treatment

Eight of the 12 patients treated with the combination of anti-PD-1 and EVX-01 vaccination achieved objective response: six patients with partial response (PR) and two patients with CR (figure 1B). One patient (patient 11) had a durable response while remaining patients progressed at some point during the trial.

At dose level 1, three patients had objective responses comprising one CR with a duration of response (DOR) of 10 months and two PR with DOR of 28 and 5 months.

At dose level 2, one patient had PR (60% regression in target lesions) with DOR of 9 months. This patient had been treated with 10 cycles of anti-PD-1 at the time of inclusion, with SD as the best response, and developed PR 7 months after the first vaccination. The patient developed temporary complete regression in target lesions (100 %) and a stable non-target lesion (online supplemental figure 1).

At dose level 3, all four patients obtained objective responses. Three patients obtained a PR with DOR of 8, 4, and 2 months. Patient 11 has ongoing CR for +16 months.

EVX-01 induces vaccine-specific T cell responses in all patients

EVX-01-specific T-cell responses were evaluated on peptide pool restimulation of in vitro stimulated (IVS) PBMCs using both IFN-γ ELISpot (ELISpot) and ICS analyses (figure 3A). To increase the resolution of the analysis, specific T-cell responses were evaluated by individual neoantigen-peptide stimulation using ELISpot. T-cell reactivity to the vaccine was evaluated at baseline (T1), before EVX-01-CAF09b vaccine initiation (T2), and after administration of EVX-01 vaccine (T3=after three IP vaccinations and T4=after three additional IM vaccinations). Some patients had up to five follow-up samples (FU1–FU5) for analysis of T-cell responses collected approximately at 12-week intervals.

Magnitude of T-cell responses to neoantigen peptides correlates with individual dose levels

The number of individual neoantigen responses detected by ELISpot before vaccination (T1 and T2), after vaccination (T3 and T4), and in follow-up samples (FU1–FU5) are delineated in table 3. PBMC samples from all 12 patients were analyzed by ELISpot assay, where EVX-01 prestimulated PBMCs were restimulated with individual EVX-01 peptides (n=91 neoantigen peptides in total). Overall, we observed T-cell responses toward 53 of the 91 analyzed EVX-01 peptides (58%). Among these, peptide-induced immune responses toward 45 EVX-01 peptides were exclusively detected after initiation of vaccination. Hence 85% (45/53) of the immunogenic neopeptides have induced a de novo responses. The three dose levels showed similar amounts of total neoantigen responses and de novo responses (table 3).

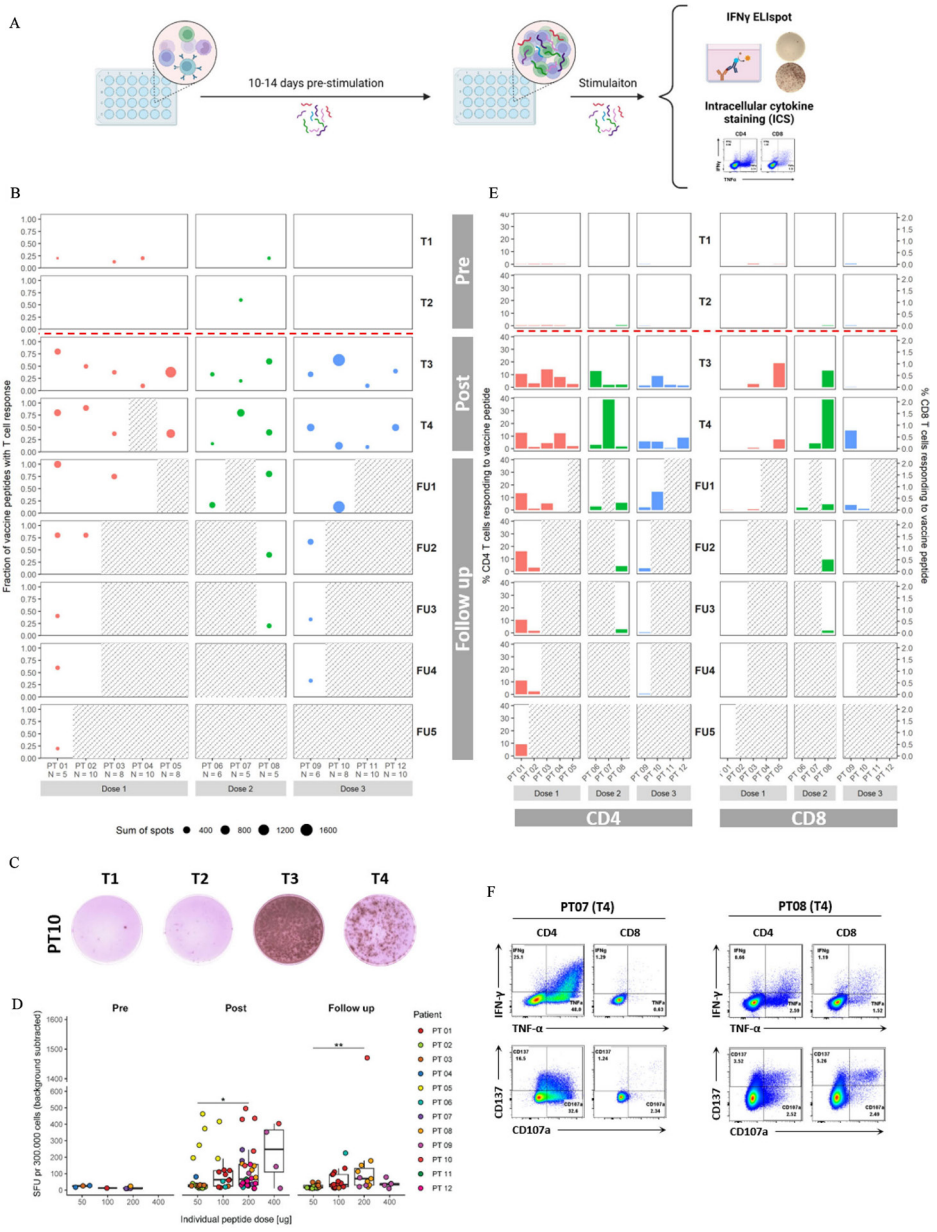

Figure 3 EVX-01-specific PBMC derived T cell responses analyzed by Elispot and ICS. (A) Experimental setup for detection of T cells responses. PBMCs were prestimulated with EVX-01 neopeptide pool for 10–14 days. Prestimulated PBMCs were hereafter stimulated with EVX-01 neopeptides in an ELISpot assay or intracellular cytokine staining assay. (B) An overview of patient IFN-γ responses detected by Elispot grouped by dose levels and time points. Red dots represent dose level 1, green dots dose level 2, and blue dots dose level 3. The height of the dots on the y-axis represents the fraction of single vaccine peptides with a T cell response out of total neopeptides per patient. A total number of single peptides (N) are stated under the patient number at the X-axis. The size of the dots represents the sum of the spot after subtraction of the background (Irrelevant peptide). This only includes spots from single peptide stimulation, which induces positive responses. Gray scattered boxes indicate time points which were not analyzed. (C) Representative ELIspot wells for patient 10 pre vaccination (T1 and T2), and post vaccination (T3 and T4). (D) The individual vaccine dose of the single EVX-01 peptides versus delta spots for peptides with T cell response (background stimulated with irrelevant peptide has been subtracted). Responses have been divided in pre vaccination (T1 and T2), postvaccination (T3 and T4) and follow-up (all FU time points). Each dot represents a single-peptide response detected with Elispot. Means were compared between peptide dose groups using Kruskal-Wallis test. Significant difference is indicated with asterisks (*p<0.05, **p<0.01). (E) T cell responses was tested after restimulation with pool vaccine peptides and stained intracellularly for IFN-γ, TNF-α, CD107a and CD137. The percentages of T cells which are positive for at least two of mentioned markers are shown in an overview of EVX-01-specific CD4+ and CD8+ T cells in all patient responses from all three dose levels and all time points. Red bars represent dose level 1, green bars dose level 2, and blue bars dose level 3. Bars show specific CD4+ and CD8+ T cells. Background response has been subtracted. Gray scattered boxes indicate time points which were not analyzed. (F) Flow cytometry dotplots for CD4+ and CD8+ T cells stained for IFN-γ, TNF-α, CD107a and CD137. The dotplots are shown for patient seven with the highest CD4+T cell response at T4, and patient eight with the highest CD8+T cell response at T4. ICS, intracellular cytokine staining; PBMC, peripheral blood mononuclear cell.

Table 3 Elispot responses overview

Dose level	Total peptides (n)	Total responses	De novo responses
All	91	53 (58%)	45 (49%|**85%**)
Dose 1	41	26 (63%)	22 (54%|**85%**)
Dose 2	16	11 (69%)	7 (44%|**64%**)
Dose 3	34	16 (47%)	16 (47%|**100%**)
% of total peptides			
% of total responses			

The fraction and magnitude of neoantigen-induced ELISpot responses are depicted in figure 3B. Overall, fewer and of lower magnitude pre-existing responses were detected before initiation of EVX-01 vaccination (T1 and T2). Post EVX-01 vaccination (T3 and T4), neoantigen responses were observed in all patients with a significant increase in response magnitude (sum of spot forming units) compared with baseline samples. Furthermore, ELISpot responses were detected in all follow-up samples showing that vaccine-induced T cells persisted in patients for up to 14 months from the last vaccination (last FU-sample time point). However, a decrease in both frequency and magnitude was observed over time (figure 3B,C, online supplemental figure 2).

To investigate the effect of EVX-01 peptide dose, the magnitude of T cell responses was plotted against the dose of the individual peptides (figure 3D, online supplemental figure 3). It could be observed that higher peptide dose correlates with T cell response magnitude and peptides administered in doses of 200 µg induced a significantly higher response (number spot forming units, SFU, background subtracted) than peptides delivered with a dose of 50 µg, maintained during follow-up.

EVX-01 induces both CD4+ or CD8+ T-cell responses

We evaluated whether the EVX-01-specific T-cell responses were CD4+ or CD8+ T cell-mediated. Therefore, EVX-01 prestimulated PBMCs were restimulated with patient-specific EVX-01 peptide pools and intracellularly stained for IFN-γ, TNF-α, CD137, and CD107a to detect T-cell reactivity against the peptide pool using flow cytometry. EVX-01-reactive CD4+T cells were observed in all 12 patients after vaccine administration at T3 and T4 (figure 3E,F). CD4+T cell responses persisted in follow-up samples, however, the frequency of reactive CD4+T cells decreased over time. CD8+T cell responses were observed in seven patients in total; five patients at T3 and T4 and additional two patients at FU1 (figure 3E,F). In general, minor reactivity was detected before vaccination (T1 and T2) for both CD4+ and CD8+ cells. There was no obvious difference in reactivity between the three dose levels. As opposed to ELISpot results, ICS results were only based on EVX-01-peptide pool stimulation and not individual EVX-01-peptide stimulation.

EVX-01-induced T cells can migrate into solid tissue

As antigen-specific homing of immune cells to the tumor tissue is important for tumor cell elimination, the homing capacity of EVX-01 induced T cells was analyzed by a DTH skin test in six patients (patients 2, 6, 7, 8, 9 and 11, figure 2A). We evaluated the presence of EVX-01-specific T cells in SKILs by IFN-γ ELISpot and ICS following peptide stimulation. We observed reactivity against individual EVX-01 peptides in the SKILs from four patients by ELISpot after EVX-01-CAF09b vaccination (figure 2B).

By use of ICS, we could confirm the presence of CD4+T cell responses in these 4 patients and a CD8+T cell response in the SKILs of one of these patients. Furthermore, a low frequency of EVX-01-specific response was detected in CD4+T cells in the SKILs of patient 9, not picked up with IFN-γ ELISpot (figure 2C, online supplemental figure 6). Responses to single peptides in ICS corresponded to responses detected in SKILs using IFN-γ ELIspot. The specific responses observed in SKILs had also been detected in PBMCs with Elispot, except for peptide 2 and 4 detected in SKILs from patient 6 (online supplemental figure 2). For patient 6, a TCL was established from a tumor biopsy collected before initiation of EVX-01-CAF09b vaccination (T2). In this patient, SKILs were evaluated for tumor reactivity, but no tumor recognition was observed (data are not shown). In summary, EVX-01 SKIL reactivity analyzed in Elispot and ICS assays is comparable to our results from EVX-01 prestimulated PBMCs.

Additionally, tumor biopsies were collected both before (T1 and T2) and after (T4) vaccination from three patients (patients 1, 6, and 7). TILs were successfully expanded from all tumor biopsies, but no reactivity was detected when these TILs were exposed to EVX-01 peptides(data are not shown). Of note, patients 6 and 7 did not respond to treatment and, in general, had low-quality neoepitopes (see paragraph below).

Vaccine-related neoantigen recognizing CD8+ T cells were detected in prestimulated and ex vivo PBMCs

EVX-01 prestimulated PBMCs were screened for CD8+T cells that recognize minimal epitopes within the EVX-01 vaccine peptides. CD8+T cells recognizing minimal peptides predicted from EVX-01 were defined as VaccNARTs (vaccine-embedded neoantigen-recognizing CD8+T cells). The presence of VaccNARTs was examined by staining with pMHC I tetramers. Any overall increase in VaccNARTs frequency after vaccination was not detected when summarizing the signal across patients (online supplemental table 1, figures 7 and 8). However, an increase in VaccNARTs was observed in selected patients after vaccination. In patient 6, a strong enhancement of low-level pre-existing and induction of de novo T-cell recognition was observed toward minimal peptides derived from vaccine peptide 2 and 6 (figure 4B). In patient 8, VaccNARTs were observed against a minimal peptide derived from vaccine peptide 3 (KLYASPSQFIK). This response was increased after ICI treatment T2 and again after IM vaccination T4 (figure 4B).

Furthermore, using barcoded-pMHC multimers, we evaluated CD8+T cell recognition toward 145–231 HLA-binding neoepitopes predicted across the full tumor mutagenome, that is, also beyond those embedded in the

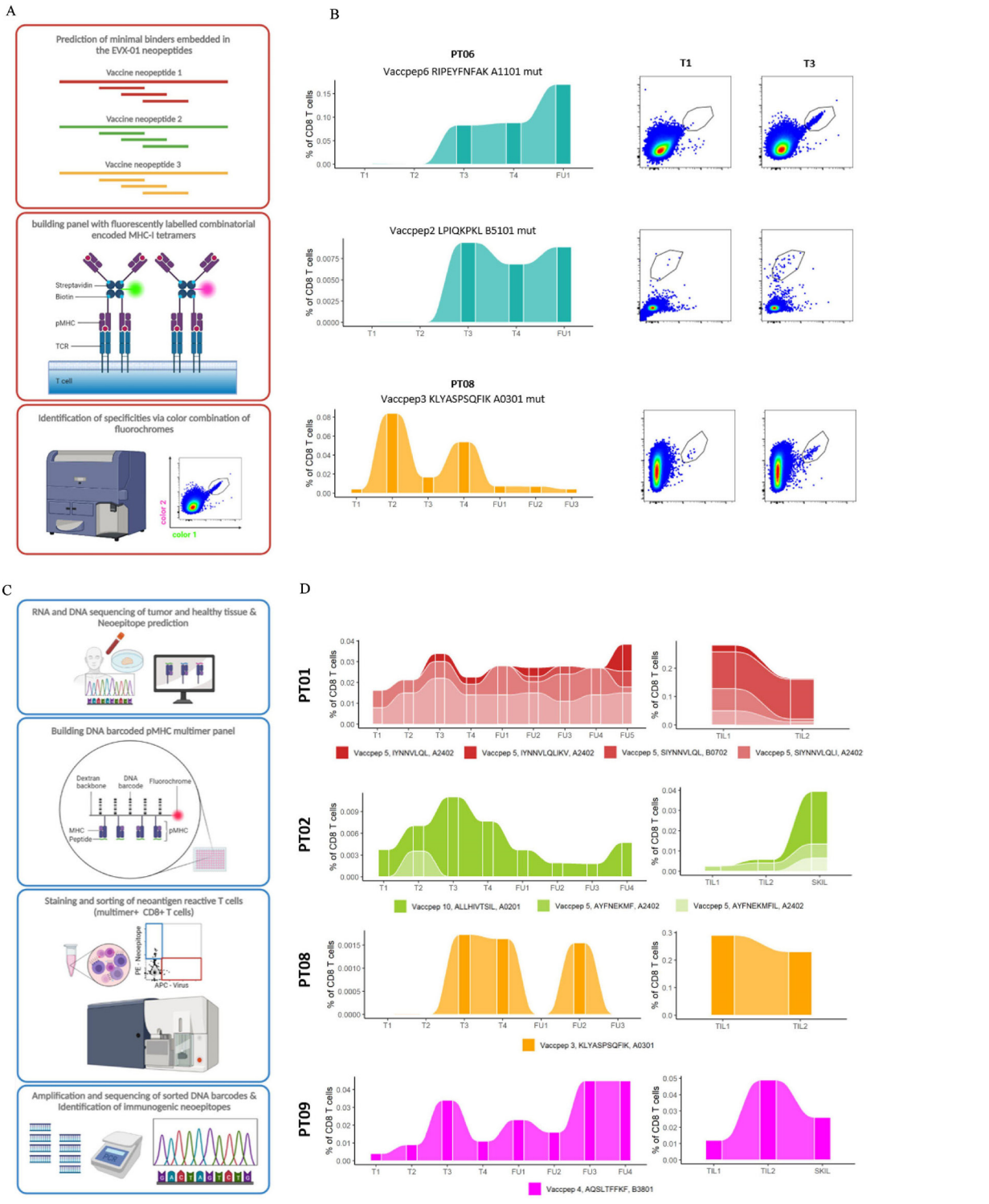

Figure 4 EVX-01 prestimulated PBMCs and ex vivo PBMC derived CD8+T cells screened for EVX-01 and other CD8+T cell neoepitopes. (A) A schematic overview of the experimental setup for detection of VaccNARTs in prestimulated PBMCs. Minimal peptides embedded in the EVX-01 neopeptides were predicted and loaded on tetramers conjugated to fluorochromes. Specific combinations of two fluorochromes were used for identification of the specificities of detected VaccNARTs. (B) The frequency of selected VaccNART with only one specificity, from patients 6, 8 and 10. EVX-01 peptide number (Vaccpep), short peptide sequence, HLA, and whether the short peptide includes the mutated region of the Vaccpep (mut) is shown above the graphs. dotplots for time point T1 (baseline) and T3 (after three IP vaccinations) are shown for each peptide. (C) A schematic overview of experimental setup used for detection of NARTs, VaccNARTs and VARTs in ex vivo PBMCs, expanded TILs and SKILs. CD8+T cells reactive toward predicted neoepitopes, including vaccine embedded neoepitopes, and virus epitopes were screened with DNA barcoded pMHC multimers. (D) The frequency VaccNARTs shown for selected patients, showing the single populations dynamics over time. EVX-01 peptide number (Vaccpep), short peptide sequence and HLA is shown next to the graphs. IP, intraperitoneal; PBMC, peripheral blood mononuclear cell.

EVX-01 vaccine peptides. We analyzed CD8+T cells that recognize such predicted neopeptides and categorized as either VaccNARTs (recognizing vaccine-embedded sequences) or NARTs (recognizing non-vaccine-embedded neoepitopes) directly ex vivo on PBMCs and expanded TILs and SKILs from six patient. We did not observe any overall increase in frequencies of NARTs after vaccination, hence no signs of epitope spreading occurring after vaccination. However, an increase in the number of NARTs was observed after ICI for patient 1 (online supplemental figures 9 and 10). When evaluating the VaccNARTs using this methodology, we find that frequency and number generally appeared to increase on ICI administration (T2) and then again after EVX-01 vaccination (T3) (figure 4C and online supplemental figures 9 and 10). New VaccNARTs detected in PBMCs after vaccination were also detected in TILs from tumor biopsies taken before vaccination, as seen for patient 1 and 8, and thus not a de novo response induced by the vaccine (figure 4C and online supplemental figure 9). This was supported by the observed recognition of an epitope for vaccine peptide 3 (KLYASPSQFIK) in patient eight prior to vaccinations (T2) in EVX-01 prestimulated PBMCs, as mentioned above (figure 4B). Finally, VaccNARTs were detected in SKILs from patients 2 and 9, where patient 2 had a larger frequency of VaccNARTs in SKILs compared with TILs (T1 and T2), indicating a boost in tissue homing VaccNARTs by the vaccine. In general, VaccNART and NARTs were only detected in low frequencies, although the frequency and the number of virus-responsive T cells (VARTs) were stable over time, demonstrating assay stability between time points (online supplemental figures 9 and 10).

CR correlates with neoantigen immunogenicity and PIONEER scores

Next, we investigated how the PIONEER quality score of administered EVX-01 neopeptides and the response to the vaccine correlated with clinical outcome. First, we compared the level of T cell responses with the BOR. We observed that patients who benefitted from treatment (PR or CR) responded to more EVX-01 neopeptides compared with patients not benefitting from treatment (SD or PD) (figure 5A,B). The difference in immune response between responders and non-responders seems to increase over time, with no difference observed before vaccination and the highest difference observed at follow-up. However, among the responders, CR did not have an overall better vaccine response compared with PR (figure 5A,B). Furthermore, we observed significantly more de novo T cell responses detected by IFN ELIspot in patients with a CR (figure 5C). The observed correlation between immunogenicity appeared to be connected to the broadness of response as we did not find a correlation between clinical outcome and the magnitude of IFN-γ ELIspot response or the magnitude of ICS-detected responses (online supplemental figure 11).

We then evaluated the impact of the PIONEER quality score assigned to each EVX-01 administered neoantigen and used to select neoepitopes for treatment of each patient. As shown in figure 5D, the PIONEER quality scores are significantly associated with immunogenic EVX-01 neopeptides. Although immune response toward cancer-specific epitopes is essential, this parameter alone does not dictate clinical efficacy. We, therefore, investigated if the PIONEER quality score of administered EVX-01 peptides could separate responders from non-responders, as depicted in figure 5E. In contrast to the magnitude of immune responses, the PIONEER quality scores are significantly higher for responders compared with non-responders. This might be attributed to the evaluation of neoepitope abundancy, expression, clonality, and tumor evasion embedded in the PIONEER quality score additionally to evaluating the immune response of neoepitopes.

Algorithms for identifying effective neoepitopes are a rapidly developing field.[15] Since initiating the EVX-01-CAF09b phase I clinical trial, we have progressed the prediction platform from version 2 to version 4. The development consists of improvements of submodules of PIONEER-2 as well as the addition of new models. The PIONEER-4 quality scores do more efficiently separate immunogenic from non-immunogenic peptides compared with the PIONEER-2 quality scores with significantly higher scores assigned to immunogenic neoantigens (figure 5F). Additionally, VaccNART detected EVX-01 peptides are all found within the highest prediction scores, except one. When comparing the PIONEER-4 quality scores for neopeptides administered to patients, these are, as the for the PIONEER-2 scores, highest for EVX-01 peptides in CR (CR/PR) (figure 5G). Compared with PIONEER-2 scores, the PIONEER-4 scores are lower in patients with SD and PD.

These interesting findings around the PIONEER quality score prompted us to investigate how the scores correlate with PFS. Interestingly, when separating the patient population based on high and low PIONEER-4 median quality score (balanced, n=6 in each group), a significantly longer PFS in patients with high scores was observed (figure 5H). To investigate if the longer PFS in the high-score group was driven by a higher mutational load, the same analysis using TMB was conducted. As depicted in figure 5I, TMB did not seem to be the determining factor for PFS in this patient cohort, indicating that the quality of administered EVX-01 neopeptides is important for clinical benefit.

DISCUSSION

Here, we present the final data from the phase I clinical trial, "Personalized Neo-antigen Vaccine in Advanced Solid Tumors (NeoPepVac)". In this study, patients with metastatic melanoma were treated with a personalized neopeptide vaccine EVX-01-CAF09b in addition to anti-PD-1 therapy. The study addresses the safety,

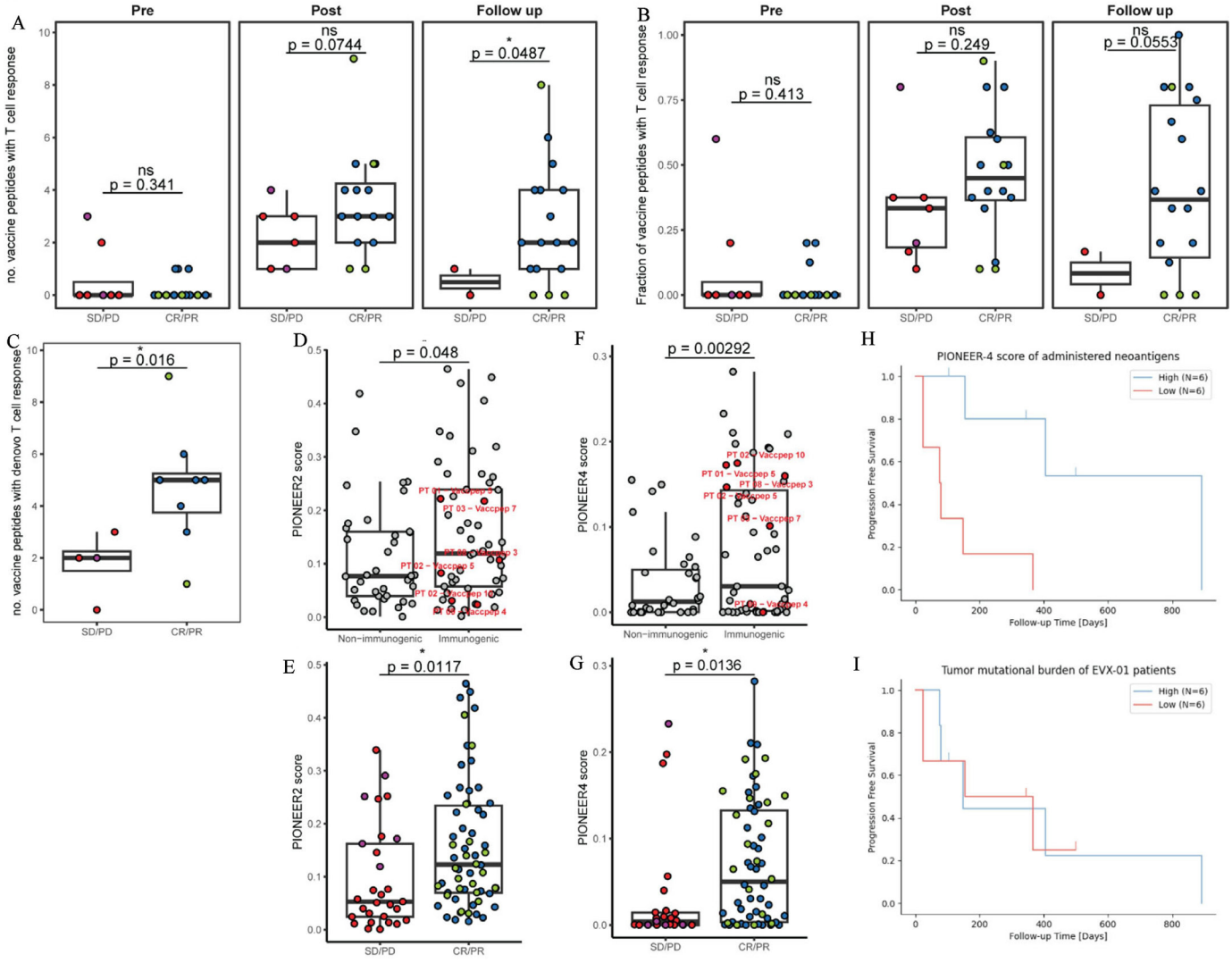

Figure 5 Correlation between clinical responses, neoepeptide immunogenicity and quality scores. Patient clinical BOR was grouped in responders. Immune responses detected before vaccination (Pre), during and after vaccination (Post) and in follow-up samples are grouped and compared with the clinical outcomes of the patients. (A) The number of EVX-01 peptides with T cell responses detected by Elispot was compared with the patients BOR. (B) The fraction of EVX-01 peptides with T cell responses detected by Elispot was compared with the patients BOR. (C) The number of de-novo responses per patient compared with the patients BOR. (D, F) PIONEER quality score for EVX-01 peptides inducing functional responses detected by Elispot (immunogenic) compared with non-immunogenic EVX-01 peptides. Prediction scored for both PIONEER2 and PIONEER4 is shown. EVX-01 responses detected by the pMHC-I multimers in ex vivo PBMCs are marked in red. A t-test was used to test the difference between immunogenic and non-immunogenic EVX-01 peptides' prediction scores (*p<0.05, **p<0.01). (E, G) The PIONEER quality scores for EVX-01 peptides (both from PIONEER2 and PIONEER4) was compared between patients BOR. Means were compared between response groups using t-test. Note that measurement is not completely internal independent as they are confounded based on patient response, hence caution should be taken when interpretating the p values. Significant difference is indicated with asterisks (*p<0.05, **p<0.01). (H) PIONEER quality score impact on progression-free survival (PFS). Kaplan-Meier curves were generated by grouping patients based on the median PIONEER4.0 quality score of administered neoepeptides, in two balanced groups (high and low-quality score) (I) TMB impact on PFS. Kaplan-Meier curves were generated by grouping patients based on tumor mutational burden (TBM) (balanced high/low) calculated from NGS data using FDA guidelines (https://www.accessdata.fda.gov/cdrh_docs/pdf17/P170019B.pdf). BOR, best overall response; CR, clinical response; PD, progressive disease; PR, partial response; SD, stable disease.

immunogenicity, feasibility, and efficacy and includes three escalating dose levels. We found the EVX-01-CAF09b vaccine to be safe at all dose levels with primarily local side effects. Two patients experienced grade 3 systemic immune-related side effects. These did not deviate from what is observed with anti-PD-1 treatment alone and were judged must likely ICI related even though this trial was not designed so that contribution from the vaccine can be ruled out.[28] The level of side effect in this vaccine trial is in contrast to the known high risk of severe toxicity of other ICI combination treatments, for example, anti-PD-1/anti-CTLA-4 (>50% grade 3–4 AEs).[29] Eight of the

12 patients (66%) showed an objective response (CR and PR). Given the low number of patients and that all patients initiated anti-PD-1 treatment prior to the addition of the EVX-01-CAF09b vaccine, it is not possible to conclude on the potential clinical benefit of adding the vaccine from this study. However, responses compare favourably to expected responses by anti-PD-1 treatment alone and peptide-based neoantigen treatments in addition to ICI treatment previously reported by Ott *et al* (59% in 27 patients).[28] Patient 8 had SD at baseline after ICI treatment and developed PR after six vaccinations (subcutaneous target lesion decrease 10mm to 0 mm, subcutaneous abdominal non-target lesion (biopsi) decreased from 10 mm to 0. Only sign of disease was PET positive lymph node, which could indicate that the EVX-01-CAF09b boosted antitumor efficacy. However, further studies are needed to conclude on the clinical benefit of EVX-01.

We found robust EVX-01-specific T-cell responses in all patients, with 58% of administered neopeptides identified as immunogenic in the IFN-γ ELISpot assay. We observed that neopeptide dose correlates with magnitude but not breadth of response. To our knowledge, this is the first study to report on a dose–response correlation between neopeptides and T-cell response. Previously, studies with non-neoantigen or personalized antigens have shown both an increase and decrease in immune response with increasing doses.[30 31]

As T cell response magnitude increased with peptide dose and no dose-related toxicity was observed a recommended phase-2 dose was established as 200 µg/peptide, 2000 µg total peptide load.

While the EVX-01 specific T-cell responses were maintained over time, we observe that the frequency of the T cell response decline with time in the follow-up samples (online supplemental figure 4), with the lowest response observed in the time point before the patients had disease progression. Perhaps because other cancer cell clones where then responsible for the progression of disease. Hence, future clinical trials might benefit from administration of a EVX-01 vaccine, with newly predicted peptides, at later time points to boost the immune response.

To induce a stronger CD8 T cells response, we used the adjuvant CAF09b comprising the TLR3 agonist poly(I:C).[32] Additionally, patients were vaccinated through the IP route for the first three vaccination, as it has previously been described that IP administration route in mouse models elicited stronger CD8+T cell responses than subcutaneous administration.[32] However, we could not confirm an increase in CD8+T cell response after IP administration (T3) compared with IM administration (T4). The trial design was not optimal for delineating the differences between IP and IM immunizations. As IP administration carries higher patient risk future designs could include a design for testing if IP administration add significant increase in T cell responses. This has to some extent been investigated using a cancer-associated antigen peptides and CAF09b in.[19] Furthermore, the immunogenic responses were predominantly CD4+T cell responses, although CD8+T cell responses were observed in 7/12 patients across dose levels. These observations are similar to those from other trials reporting CD4+T cell dominated responses toward peptide-based neoantigen vaccines.[10 11 28 33] CD4+T cells play a role in priming and enhancing CD8+T cells[34 35] and in overcoming ICI resistance.[36] Hence, CD4+T cell responses might augment ICI treatment substantially.

Vaccine-embedded neoepitope reactive CD8+T cells detected by MHC multimer stainings (VaccNARTs) were of low frequency in expanded PBMCs. We were unable to detect VaccNARTs for most immunogenic peptides measured by ELISpot. This might be due to the wrong minimal epitope being investigated. Two vaccine-induced responses were detected in patient 6 by MHC multimer staining's were not detected by ELISpot, which could indicate that responses of smaller magnitude can be detected by multimer staining. A more pronounced CD8+T cell response could potentially also be detected with ELISpot if using a short EVX-01 peptide pool for assay stimulation. However, this was not feasible due to sample limitations.

We examined CD8+T cell responses in PBMCs, to investigate the effect of EVX-01-CAF09b vaccine unbiased by prestimulation. No obvious neoepitope-spreading within non-vaccine-related predicted neopeptides was detected following the vaccination. However, one patient showed an increase in NARTs after initiation of ICI treatment, comparable to a previous study reporting an ICI-induced increase in NARTs in the blood.[37] Importantly, we only had access to blood samples and not tumor samples collected after vaccination for this analysis. Ideally, it would have been beneficial to analyze TILs from tumor samples obtained postvaccination, which would have allowed us to detect potentially new vaccNARTs within the tumor microenvironment. This could better indicate epitope-spreading, as demonstrated in previous studies.[28]

We further compared the clinical outcome, BOR, to the immunogenicity of the EVX-01 neopeptides and found that a higher fraction of, as well as, the number of immunogenic EVX-01 peptides was correlated with CR. It appears that the number of immunogenic peptides might be important for CR and longer PFS, rather than the magnitude of response toward single vaccine-embedded peptides. A broader T-cell response will reduce the risk of tumor escape through antigen loss.[6 38]

Finally, we evaluated the neoantigen quality scores obtained with the AI prediction tool PIONEER used to identify EVX-01 peptides with our immunogenicity results. The newest version of PIONEER (V.4.0) had a larger split between immunogenic and non-immunogenic based on quality scores and better placement of VaccNART-detected EVX-01 peptides. These results, together with a better positive correlation between clinical outcome and prediction score, show that the newer version of PIONEER is better at predicting immunogenic peptides with CD4+and CD8+ T cell responses. A new cohort could,

therefore, benefit from the observed improvement of the vaccine prediction tool.

In conclusion, personalized immunotherapy is a promising approach to cancer treatment. We demonstrated that EVX-01-CAF09b, a personalized neopeptide vaccine at three different dose levels is safe and capable of eliciting T-cell responses in a clinical setting where patients received concurrent standard-of-care immunotherapy. We detected both CD8+ and CD4+ T cell responses toward corresponding vaccine peptides and found that the number of immunogenic peptides and PIONEER epitope quality score are both predictive for good CR and longer PFS. Objective responses were observed in metastatic melanoma patients at all three dose levels, with all patients at the highest dose level achieving a CR. In addition to the beneficial safety profile, EVX-01-CAF09b may be a promising addition to ICI monotherapy. However, larger trials are warranted to confirm these findings and conclude on clinical benefit.

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Contributors SKM: Author acting as guarantor, project administration, investigation, writing–original draft, visualization, resources. SKS: validation, formal analysis, investigation, data curation, writing–original draft, visualization. BA: validation, formal analysis, investigation, writing, review and editing, visualization. AD: validation, formal analysis, writing, review and editing, visualization. KB: validation, formal analysis, writing, review and editing, visualization. MK: investigation, data curation, writing–review and editing. MCWW: validation, formal analysis, writing, review and editing, visualization. JSG: validation, formal analysis, writing, review and editing, visualization. AB: formal analysis, writing–review and editing, data curation. NVP: data curation, writing–review and editing. NT: software, formal analysis, data curation, visualization. ISR: validation, resources, formal analysis, writing, review and editing. LVA: conceptualization, methodology, writing–review and editing, supervision. RBD: resources, writing–review editing. CWY, NN and TL: resources. ABS: methodology, formal analysis, resources, writing–review and editing. DK-K: data curation, writing–review and editing. AJ: data curation. DC: conceptualization, methodology, writing–review and editing. JK: conceptualization, methodology, software, writing–review and editing, supervision, funding acquisition. MD: conceptualization, methodology, writing, review and editing, supervision, funding acquisition. SRH: conceptualization, recources, supervision, project administration, funding acquisition. IMS: project administration, conceptualization, methodology, writing, review and editing, supervision, funding acquisition, validation.

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