Systemic immune dynamics in cancer

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CHAPTER 8

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Abstract

Background

Usual vulvar intraepithelial neoplasia (uVIN) is a premalignancy caused by persistent infection with high-risk types of human papillomavirus (HPV), mainly type 16. Even though different treatment modalities are available (e.g., surgical excision, laser evaporation or topical application of imiquimod), these treatments can be mutilating, patients often have recurrences and 2-8% of patients develop vulvar carcinoma. Therefore, immunotherapeutic strategies targeting the pivotal oncogenic HPV proteins E6 and E7 are being explored to repress carcinogenesis.

Method

In this phase I/II clinical trial, 14 patients with HPV16+ uVIN were treated with a genetically enhanced DNA vaccine targeting E6 and E7. Safety, clinical responses and immunogenicity were assessed. Patients received four intradermal HPV-16 E6/E7 DNA tattoo vaccinations, with a 2-week interval, alternating between both upper legs. Biopsies of the uVIN lesions were taken at screening and +3 months after last vaccination. Digital photography of the vulva was performed at every check-up until 12 months of follow-up for measurement of the lesions. HPV16-specific T-cell responses were measured in blood over time in *ex vivo* reactivity assays.

Results

Vaccinations were well tolerated, although one grade 3 suspected unexpected serious adverse reaction (SUSAR) was observed. Clinical responses were observed in 6/14 (43%) patients, with 2 complete responses (CR) and 4 partial responses (PR). 5/14 patients showed HPV-specific T-cell responses in blood, measured in *ex vivo* reactivity assays. Notably, all 5 patients with HPV-specific T-cell responses had a clinical response.

Conclusions

Our results indicate that HPV-16 E6/E7 DNA tattoo vaccination is a biologically active and safe treatment strategy in patients with uVIN, and suggest that T-cell reactivity against the HPV oncogenes is associated with clinical benefit.

Trial registration number: NTR4607

Keywords

HPV-16, E6, E7, DNA tattoo vaccination, uVIN, phase I/II clinical trial, immune monitoring.

Abbreviations

AIN	Anal intraepithelial neoplasia
APC	Antigen presenting cell
CIN	Cervical intraepithelial neoplasia
CMV	Cytomegalovirus
CR	Complete response
CTCAE	Common Terminology Criteria for Adverse Events
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GMP	Good manufacturing practice
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papillomavirus
IFNγ	Interferon gamma
IL-2	Interleukin-2
NEF	Negative Factor
NR	No response
PADRE	Pan HLA DR epitope
PD-1	Programmed cell death protein 1
PeIN	Penile intraepithelial neoplasia
PBMCs	Peripheral blood mononuclear cells
PR	Partial response
SLP	Synthetic long peptide
SUSAR	Suspected unexpected serious adverse reaction
TLR	Toll-like receptor
TNFα	Tumor necrosis factor alpha
TTFC	Tetanus toxin fragment C
uVIN	Usual vulvar intraepithelial neoplasia
vHSIL	Vulvar high-grade squamous intraepithelial lesion
VIN	Vulvar intraepithelial neoplasia
WBC	White blood cell count

Introduction

Usual vulvar intraepithelial neoplasia (uVIN), also known as vulvar high-grade squamous intraepithelial lesions (vHSIL), is a premalignant chronic skin disorder of the vulva and associated with a persistent infection with high risk types of HPV, mainly HPV type 16[1-3]. Spontaneous regression is rare, restricted to 1-2% of women, and progression to vulvar cancer is observed in 2-8% of cases[4-8]. Current treatment strategies are laser ablation, local excision or topical treatment with the toll-like receptor (TLR) 7-ligand imiquimod. Since patients frequently suffer from recurrent disease, different sequential therapies are often applied over the years[5, 7-9]. Multiple surgical treatments can however be mutilating, and induce psychosexual dysfunction[10, 11]. Also, topical treatment with imiquimod is associated with side effects such as pruritus and pain[12]. In order to avoid the need for debilitating treatments, and prevent relapses and potential malignant transformation, new therapeutic strategies should be explored with a final goal to eradicate transformed, oncoprotein E6 and E7 expressing epithelial cells.

Infection with high-risk genotypes of HPV leads to the expression of the oncogenic HPV proteins E6 and E7. Together, E6 and E7 drive cellular immortalization and maintain the transformed phenotype during tumor progression[13-15]. The E6 and E7 oncoproteins are continuously expressed in transformed cells, consequently enabling presentation of E6 and E7 epitopes by the transformed cells and creating the opportunity for T-cell recognition. Notably, patients with persistent uVIN often have dysfunctional HPV16-specific T-cell responses[16-18], suggesting that immune stimulating therapies that induce or enhance functional HPV16-specific T-cell responses may lead to clinical benefit.

In line with this notion, several HPV-vaccination studies targeting E6 and/or E7 have been performed with some promising immunological and clinical responses, confirming the suitability of the target proteins. Strategies that have been studied included genetic vaccines (DNA/RNA/virus/bacterial), protein-based, peptide-based or dendritic cell-based vaccines[19-22]. To date, these vaccines have not found their way to clinical practice because of little efficacy, high production costs, or cumbersome production processes like dendritic cell-based vaccines which requires a personalized cell product. Also upscaling the cell expansion protocol for adoptive transfer can be complicated and troublesome.

DNA vaccination forms an attractive approach for the induction of cellular immune responses, as these vaccines are easy to produce, very stable, relatively cheap and do not suffer from the drawback of pre-existing immunity or induction of anti-vector immunity, as is the case for most viral vectors[23, 24]. Since subcutaneous administration with adjuvant of peptide-based therapeutic HPV-vaccines can cause significant adverse events (such as local skin swelling)[21] we focused on improving the administration route and optimization of immunogenicity of the vaccine. Therefore, we developed a DNA vaccination strategy based on DNA tattoo vaccination, which demonstrated a 10-100 fold increase in vaccine specific

T-cell responses as compared to classical intramuscular DNA vaccination when tested in non-human primates[25].

Recently, we performed for the first time a phase I clinical trial using the E7 directed DNA vaccine Tetanus Toxin Fragment C (TTFC)-E7SH, which was delivered using the tattooing technique in patients with uVIN[26]. This DNA vaccine was well tolerated and the tattoo-induced skin damage was completely reversible. However, no induction of E7 directed $CD8^+$ responses nor clinical responses could be observed[26].

The aim of the current study is to improve the immunological response and monitor clinical outcome in patients with uVIN. Therefore we developed a novel DNA vaccine that can be administered by DNA tattoo vaccination[27]. Since targeting both E6 and E7 has been reported to have a synergistic effect on HPV infection control[26, 28], both oncogenes are targeted in this new format. With the combined novel DNA vaccines sig-HELP-E6SH-KDEL and sig-HELP-E7SH-KDEL (further referred to as HPV-16 E6/E7 DNA tattoo vaccine), we aim to increase the immunogenicity towards E6 and E7 by inducing $CD4^+$ helper T cells and including signals for enhanced endoplasmic reticulum targeting and retention. Here, we describe the results of a phase I/II clinical trial in which we evaluated the toxicity, clinical response and immunogenicity of this HPV-16 E6/E7 DNA tattoo vaccination in patients with uVIN.

Materials & Methods

Patients

Fourteen female patients with histology and PCR proven HPV16+ uVIN lesions were included between January 2017 and December 2019. Patients needed to have adequate bone marrow function, renal function and liver function. Exclusion criteria were pregnancy/lactation, active infectious disease, autoimmune disease or immunodeficiency. Other exclusion criteria were use of oral anticoagulant drugs or an indication of severe cardiac, respiratory or metabolic disease. Furthermore, patients could not participate if the uVIN was treated with another modality within 6 weeks prior to enrolment, if patients were treated before with therapeutic HPV vaccines, or if patients participated in a study with another investigational drug (for different indications than uVIN) within 30 days prior to enrolment. Patient characteristics are shown in table 1.

The study was approved by the Central Committee on Research Involving Human Subjects (In Dutch: Centrale Commissie Mensgebonden Onderzoek; CCMO) in The Hague, the Netherlands (Number NL46637.000.13) and registered at trialregister.nl (NTR4607). All study protocols were conducted in accordance with the ICH Harmonised Tripartite Guideline for Good Clinical Practice and the principles of the Declaration of Helsinki. All patients provided written informed consent before enrolment.

Table 1: *Baseline characteristics of the study population. uVIN, usual vulvar intraepithelial neoplasia; LE, local excision. All patients were diagnosed with HPV type 16, but patient #10 had a co-infection with HPV type 56 and patient #13 had a co-infection with HPV type 40.*

Patient no.	Age	Multi/ unifocal	Symptoms	Smoker	Previous treatment(s)	First diagnosis uVIN	Lesion size (cm²)
1	51	Uni	Pruritis	No	Laser, LE (2x), imiquimod	2012	1,4
2	64	Multi	Pruritis	Former smoker (stopped in 2016)	Laser, imiquimod	2015	1,3
3	55	Multi	Pruritis	Smoker	None	2017	0,6
4	37	Multi	None	Former smoker (stopped in 2017)	LE	2013	3,5
5	65	Uni	Pain	Former smoker (stopped in 1998)	None	2017	3,5
6	69	Uni	None	Former smoker	Laser (2x), imiquimod	1996	0,9
7	46	Multi	None	Smoker	LE (3x)	2010	3,7
8	45	Uni	None	Former smoker (stopped in 2018)	Imiquimod	2018	3,8
9	41	Multi	Pruritis	Smoker	Laser (3x), LE (3x), imiquimod (3x)	2005	36
10	50	Multi	Pruritis	Smoker	LE (3x), laser (6x), imiquimod	1993	6,8
11	46	Multi	None	Smoker	Laser (2x), imiquimod	2016	1,7
12	61	Multi	Pruritis, pain	Former smoker (stopped in 1995)	Laser, LE, imiquimod	2003	3,5
13	29	Multi	None	Smoker	Imiquimod	2019	0,7
14	36	Multi	Pruritis, pain	Smoker	Laser	2017	2,0

Vaccine composition

The HPV-16 E6/E7 DNA vaccine comprises of sig-HELP-E6SH-KDEL and sig-HELP-E7SH-KDEL, which are plasmid DNA constructs of 4814 and 5240 base pairs respectively (Figure 1a). In this plasmid, the Cytomegalovirus (CMV) promoter drives the continuous expression of E6SH and E7SH. To prevent toxicity and protect against the transforming properties of E6 and E7, coding sequences were rearranged ('shuffled'). To prevent loss of potential immunogenic epitopes, sequences flanking the positions where the coding sequence was cut, were added 3' from the coding regions (Figure 1b). The HPV-16 E6/E7 DNA vaccine includes three CD4 helper sequences: antigenic epitopes of the Negative Factor (NEF) protein from Human immunodeficiency virus (HIV) (39bp)[29], the P30 epitope derived from Tetanus Toxin (63bp)[30] and the universal synthetic, non-natural pan HLA DR epitope PADRE (39bp)[31].

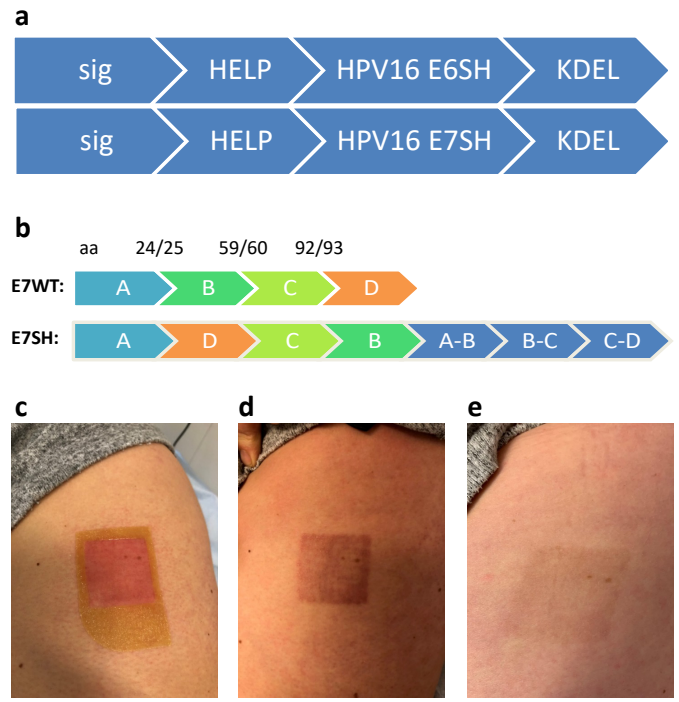

Figure 1: pUMC3 sig-HELP-E6SH-KDEL and pUMVC3 sig-HELP-E7SH-KDEL plasmids used in this trial and administered by tattoo vaccination. a) *schematic representation of the therapeutic region of the plasmid, including 3 helper sequences: Synthetic epitope PADRE (39bp), NEF from HIV (39bp) and P30 from Tetanus Toxin (63bp) for CD4 help. Sig and KDEL for improved ER targeting and retention, resulting in better antigen uptake by DCs, enhanced processing and presentation.* ***b)*** *To prevent toxicity, E6 and E7 coding sequences were shuffled. Splice sites are added at the back of the construct so no potential immunogenic epitopes are lost.* ***c)*** *Picture of the patients' skin immediately after vaccination with HPV-16 E6/E7 tattoo vaccination.* ***d)*** *Picture of the skin two weeks after vaccination.* ***e)*** *Picture of the skin 6 months after last vaccination, demonstrating hardly any visible tissue scar remains.*

By only inserting the relevant CD4 epitopes, and not the full protein domains, the risk of antigenic competition and skewing of the $CD8^+$ T cell response towards the helper epitopes was minimized. The C-terminal KDEL amino acid sequence was included to achieve endoplasmic reticulum targeting and retention, resulting in higher immunogenicity[24, 32].

For the manufacturing of both vaccines, a standard Good Manufacturing Practice (GMP) production process was followed as described earlier[33]. Resulting DNA vaccines were formulated as a lyophilized powder for solution for intradermal injection, using sucrose as stabilizer[33]. Just before administration, 1 mg of sig-HELP-E6SH-KDEL was reconstituted with 0.4 ml Water for Injection and mixed with 1 mg reconstituted sig-HELP-E7SH-KDEL to obtain

2 mg of the combined HPV-16 E6/E7 DNA tattoo vaccine at a concentration of 5mg/ml. For each of the four subsequent vaccinations, 2 mg of the combined HPV-16 E6/E7 DNA tattoo vaccine was used.

Study design

This was a single center, non-randomized phase I/II study, consisting of two cohorts. In the first cohort, 5 patients were treated, followed by an interim analysis that assessed vaccine immunogenicity. Since the criteria for continuation after interim analysis were met (a 2-fold increase in the T cell response compared to baseline in ≥2 out of 5 patients), an additional 9 patients were enrolled. Patients in both cohorts were treated identically. The primary objective of this trial was to study the systemic HPV-specific immune response of patients with HPV16+ uVIN that received the HPV-16 E6/E7 DNA tattoo vaccine. Secondary objectives were the safety and clinical responses. However, to improve the readability of the paper, we will first discuss our clinical findings, followed by the immunogenicity data.

The HPV-16 E6/E7 DNA tattoo vaccine was applied topically on the skin of the upper legs (close to a regional lymph node area) on days 0, 14, 28 and 42 and administered into the skin using a permanent make-up tattoo device (Derm.MT GmbH, Berlin, Germany). Patients received 2 mg of vaccine injected over a skin surface of 16 cm^2. Prior to tattoo vaccination the skin area was treated with an epilating cream (Veet; Reckitt Benckiser Healthcare B.V., Hoofddorp, The Netherlands). Vaccination at day 28 was administered to the same area as vaccination at day 0, and vaccination at day 42 was administered to the same area as vaccination at day 14. Patients were observed during one hour after tattooing. Peripheral blood mononuclear cell (PBMC) isolation was performed at day 0 and day 28 before vaccine administration, and at follow-up on day 56 and day 84. A biopsy of the uVIN was taken before treatment and 3 months after the last vaccination. Patients were seen for follow-up after 3, 6, 9 and 12 months after last vaccination with evaluation of the vulvar lesions including photography and measurement of the size of the lesion(s).

Safety & toxicity

The Common Terminology Criteria for Adverse Events (CTCAE) version 4.03 was used for the assessment of adverse events. All patients that received at least one vaccine dose were included in the evaluation of safety. Vital signs were measured at baseline and at all visiting days. Hematology and biochemistry tests were performed before inclusion, and at day 0, day 28, day 56 and day 84. Unacceptable toxicity was defined as an adverse event of the following types for which the relation to the study treatment was likely or not assessable: non-hematological toxicity of grade 3 or higher, hematological toxicity grade 4, neutropenia grade 3 plus fever, or non-reversible neurotoxicity of grade >2. In case unacceptable toxicity occurred in more than 30% of patients, the study would be discontinued. Local toxicity was scored as CTCAE 'injection site reaction'.

Clinical responses

Lesions were examined and the size was measured bi-dimensionally by an experienced gynecologist and another member of the study team. Drawings were made on a vulvoscopy form in the medical record. Furthermore, the lesions were monitored by digital photography. The total area (in mm^2) of the lesions was determined using ImageJ. A complete response (CR) was defined as a complete disappearance of the lesion(s) and a partial response (PR) defined as at least 50% regression of the lesion. A patient was classified as a Non Responder (NR) if lesion size was reduced by less than 50% compared to the original lesion size, or in case of progressive disease.

Immune monitoring

To assess systemic induction of HPV E6 and E7 specific T cells, PBMCs were collected at baseline (day 0) and at day 28, 56, and 84 after the first HPV-16 E6/E7 DNA tattoo vaccination. PBMCs were isolated from fresh heparinized blood samples by Ficoll density-gradient centrifugation and cryopreserved until further use.

Presence and magnitude of HPV E6 and E7 specific T-cell responses was determined by co-culture of T cells with autologous antigen presenting cells (APCs) loaded with long overlapping peptides for 6 hours (adapted protocol based on method described by Samuels *et al.*[26]). To obtain peptide loaded APCs, PBMCs were thawed and plated in 24 well plates at a concentration of 0.3-1.5 *10^6 cells/mL in T cell mixed media (20% Roswell Park Memorial Institute (RPMI)/ 80% AIM- V medium) without serum. Monocytes were separated by plate adherence, and the non-adherent cells were harvested to be used as T cell input in the co-culture. Monocytes were peptide loaded in T cell mixed media with 800 U/ml GM-CSF (Invitrogen/Thermo Fisher Scientific, California, USA) with 5 different peptide pools. Long overlapping peptides covering the entire E6 protein were split over pool 1 and 2, long overlapping peptides covering the entire E7 protein were combined in pool 3. Pool 4 consisted of all epitopes that arose as a consequence of shuffling E6 and E7 proteins. The full amino acid sequences of the long overlapping peptides from these 4 pools are listed in Supplementary Table 1. Pool 5 consisted of a set of 32 viral epitopes covering multiple HLA-alleles, and served as a positive control to assess immune competence (ICE peptide pool, U-CyTech biosciences, Utrecht, The Netherlands). Because these were short peptides that could be directly presented without processing, the ICE peptide pool was loaded onto the APCs for only 1.5 hrs prior to the start of co-cultures. An unloaded APC condition was taken along in order to determine the background reactivity. Five hours after peptide loading, monocytes were cultured overnight in the presence of 25 µg/ml poly(I:C) (InvivoGen, California, USA), to generate monocyte-derived APCs. The previously harvested non-adherent T cells were rested overnight in T cell mixed media without serum or cytokines. After

overnight incubation, peptide loaded APCs were washed and T cells were added, alongside the CD107a antibody. After 1 hour, 0.7 µl/ml Golgistop and 1 µl/ml Golgiplug was added to each well (BD Biosciences, USA), and cultures were continued for an additional 5 hours. Subsequently, T cells were harvested and stained for surface markers and intracellular cytokines and analyzed by multiparametric flow cytometry (antibody panel listed in Supplementary Table 2). Acquisition of cells was performed using an LSR II flowcytometer (BD Biosciences). FCS files were analyzed using FlowJo software (FlowJo_v10.6.1).

Immunological responses were assessed by measuring intracellular cytokine production (interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and interleukin 2 (IL-2)) and the degranulation marker CD107a (LAMP-1). Gates were placed based on the negative population with the highest MFI and consistent for stimulated and unstimulated conditions. Patients were considered an immunological responder when the frequency of positive cells for one or more readout molecules exceeded that of the unloaded APC control by at least a factor two at any time point. In addition, the magnitude of the response should be greater than 0.1% from respectively the $CD4^+$- or $CD8^+$ T-cell parent population. A T-cell response was considered vaccine induced, when the response was not yet present at baseline.

Blood counts by hemocytometer

Routine blood counts were measured with a hemocytometer at the Clinical Chemistry Department at the Netherlands Cancer Institute. Blood was analyzed on the Xn2000 system (Sysmex). Lymphocyte, neutrophil, eosinophil and monocyte counts were extracted and analyzed from the patient records by the involved study team.

Statistical analysis

For sample size calculation, an optimal Simons two-stage design was implemented, aimed to exclude an immunological response rate of 30% and targeting a response rate of 60%. With alpha = 0.1 and power = 80%, five patients had to be enrolled in the first stage and the vaccine-induced immune response had to be observed in at least two patients to continue to the next stage (second cohort of n=9).

Patients were included in the evaluation of HPV-specific immune responses if they had received at least two doses of the vaccine, and if blood samples were drawn at baseline and at least two during therapy. Fishers exact test was used to test whether responding patients had significantly more immunological responses *ex vivo* compared to non-responding patients.

Blood counts were compared between responders (CR and PR) and non-responders using the non-parametric two-tailed Mann-Whitney U test. Paired analysis of the same patient over two time points was performed using the Wilcoxon signed rank test. *P*-value < 0.05 is * and *p*-value < 0.01 is **.

***Table 2**: Overview of treatment-related adverse events. Grades according to the Common Terminology Criteria for Adverse Events (CTCAE) v4.03.*

Toxicity	Grade	Related	No. of patients
Steven Johnsons Syndrome	3	Unlikely	1
Pruritus	1	Definitely	5
Injection site reaction	1	Definitely	3
Fatigue	1	Possibly	3
Flu like symptoms	1	Possibly	3
Dizziness	1	Possibly	2
Dysgeusia	1	Possibly	2
Local infection after skin biopsy	1	Definitely	1
Hot flushes	1	Possibly	1
Pain of skin	1	Possibly	1

Results

Safety and toxicity

13 patients received all four vaccinations and 1 patient received only two vaccinations due to adverse reactions. All adverse events are listed in table 2. The patient (patient #12) who had to discontinue vaccination was diagnosed (by biopsy of a skin eruption) with a Stevens-Johnson syndrome grade 3, two weeks after the second vaccination. Although she presented with similar symptoms earlier that year during imiquimod treatment and before she received the first vaccination, an effect of the vaccination could not be excluded, and this event was therefore reported as a suspected unexpected serious adverse reaction (SUSAR). This patient fully recovered from the SUSAR, within 4 months after last vaccination all skin lesions had disappeared. Other patients did not have treatment-related adverse events higher than grade 1 (table 2). Pruritus at the injection site after vaccination was the most commonly observed adverse event (36%). A picture of the injected skin immediately after vaccination, 2 weeks after vaccination and 6 months after vaccination is shown in Figure 1 c-e.

Observation of clinical responses after HPV-vaccination in patients with uVIN lesions

In the first cohort we included 5 patients. In this cohort a complete response was observed in 2 patients and a partial response was seen in 1 patient (Figure 2). Both complete responses were seen after 6 months of follow-up and the partial responses after 3 months. The uVIN lesions did not recur after a complete response had been observed for the duration of follow-up (12 months after the last vaccination). Patient #3 showed no clinical response and was treated with laser evaporation 2 years after vaccination. Patient #5 showed no response and started with imiquimod treatment 3 months after the last vaccination. Clinical responses

after the start of new treatments were not taken into account in this study. In the second cohort, 9 patients were included. In this second cohort 3 patients showed a durable partial response during follow-up. An example of a patient showing a partial response is shown in Figure 2a-b. The biopsies of the vulva at 3 months follow-up showed uVIN in all of the vaccinated patients. This correlates with the clinical observation that complete responses were first seen at 6 months after vaccination. Six patients showed no clinical response. One patient (patient #11) was diagnosed with micro-invasive vulvar cancer after 6 months of follow-up for which a local excision was performed. Patient #10 underwent laser treatment. Patient #12 underwent laser excision after 84 days of follow-up. Patient #14 showed no response. An overview of all clinical responses is given in Figure 2c-d. In Figure 2e the pre-treatment size of the lesions per group (NR, PR, CR) is illustrated. The patients with the biggest lesion size (#9 and #10), were both non-responders. These two patients also had received most previous treatments before inclusion in this study, as shown in Table 1.

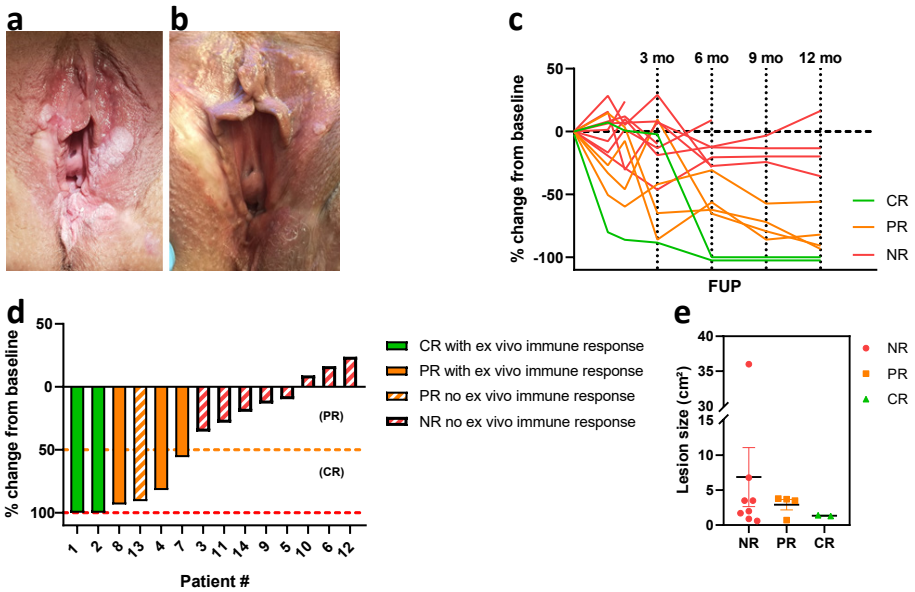

Figure 2: clinical response data of cohort 1 and 2 a) *uVIN lesions visible at screening visit.* ***b)*** *Partial response of uVIN lesions visible at follow-up +12 months after vaccination with HPV-16 E6/E7 tattoo vaccination.* ***c)*** *Overview of uVIN lesion size changes (as percentage change compared with baseline) during follow-up.* ***d)*** *Waterfall plot showing percentage change of uVIN lesion at last follow-up compared with baseline lesion size (= lesion size at screening).* ***e)*** *Lesion size before therapy per response category. Complete responders are depicted in green, partial responders in orange and non-responders in red.*

Phenotypic characterization of systemic T cells

Patient PBMCs from baseline samples, as well as from ~day 28, ~ 56, and ~ 84 after primary vaccination were subjected to basic phenotypic characterization, as determined by multiparameter flow cytometry (see Supplementary Figure 1 for gating strategy). Programmed cell death protein 1 (PD-1) expression on systemic T cells was overall low (<0,4%) and did not show any directionality in terms of response prediction or evaluation (see Supplementary Figure 2a). We also did not uncover an increase in PD-1 expression in $CD4^+$ and $CD8^+$ T cells upon vaccination. The absence of PD-1 expression on circulating T cells does not necessarily reflect expression levels of PD-1 on T cells infiltrating the uVIN lesions. No differences between responders and non-responders could be found in the differentiation state of T cells based on the surface marker expression of CD45RA and CCR7 (see Supplementary Figure 2b).

Systemic HPV-16 specific T-cell responses

The same PBMCs used for phenotypic characterization of T cells were also used to monitor systemic immune responses against the HPV16 E6 and E7 oncoproteins. A patient was considered an immunological responder if the percentage of positive $CD4^+$ and/or $CD8^+$ T cells for one or more of the measured molecules (IFNγ, TNFα, IL-2 and CD107a) was greater than 0.1% and at least two times higher than the background. Furthermore, a response was considered vaccine induced when it was not yet present at baseline. To illustrate an *ex vivo* immune response, expression of IFNγ in $CD4^+$ and $CD8^+$ T cells in the presence or absence of stimulation with peptide-loaded APCs from an immunological responder (patient #8) are displayed in Figure 3a. T-cell responses against E6-1, E6-2 and E7 peptide pool compared to unloaded APCs of all immunological responders are presented in Figure 3b. Table 3 provides an overview of the $CD4^+$ and $CD8^+$ T-cell responses against E6-1, E6-2 and E7 peptide pools from all patients, depicted as the fold change over the unloaded APC background.

The peak of the immunological response in blood was mostly detected at day 56; two weeks after the boost vaccination. From the 14 patients treated in this study, five showed an *ex vivo* immunological response (36% immunological response rate). Four of these immunological responses were not detected at baseline, and one response showed a substantial increase after vaccination (Figure 3b, patient #7 IFNγ). Furthermore, 4 out of 5 of these responses could still be detected at day 84, over a month after the last vaccination that was given at day 42 (namely in patient #1, #2, #7 and #8).

The effector molecule measured in the response varied between patients, but IFNγ was the dominant effector molecule (4/5). Interestingly, both $CD4^+$ and $CD8^+$ T-cell reactivity against all peptide pools was observed (Figure 3b). In all immunological responders (5/5) the response could be detected in both the $CD4^+$ and $CD8^+$ T cell compartments. A Boolean gating strategy was applied to distinguish single, double and triple producing T cells

(combinations of IFNγ, TNFα and IL2), with or without co-expression of degranulation marker CD107a in responding patients. T cells predominantly produced one cytokine (Supplementary Figure 3), indicating suboptimal functionality of the T cells[34, 35]. Time course graphs showing the IFNγ, TNFα, IL-2 and CD107a responses against E6-1, E6-2 and E7 peptide pool of all patients (including the non-responders), can be found in Supplementary Figure 4.

As described in the method section, E6 and E7 coding sequences needed to be shuffled for safety reasons. To assess the immunogenicity of the junction sites of the shuffled proteins, all possible epitopes covering those regions were taken along in a separate pool during the *ex vivo* immune reactivity assays. In Supplementary Figure 5, reactivity from $CD4^+$ and $CD8^+$ T cells against the shuffle points is depicted at day 0 and day 56. $CD8^+$ T cells from patient #1 and patient #8 (both responders) produced IFNγ upon stimulation with the shuffle point peptide pool. For patient #8, shuffle point reactivity seemed vaccine induced and for patient #1 the reactivity was also found in the baseline samples, possibly indicating cross reactivity towards another epitope. The magnitude of the response against the shuffle points was occasionally higher than the magnitude of the response against E6 and E7 epitopes. We do not know the exact reason for this, although we could speculate that this is due to differences in antigen processing and/or presentation between patients. In general, we do not see common reactivity against the shuffle point epitopes and it is important to note that no “on target, of lesion” toxicity was observed in any of the patients.

Reactivity against the ICE peptide pool consisting of 32 viral epitopes covering multiple HLA-alleles was tested to assess differences in immune competence between responding and non-responding patients (see Supplementary Figure 6). In total, $CD8^+$ T cells from baseline samples of 9/14 patients produced cytokines upon culturing with ICE peptide loaded APCs and no CD4 reactivity was measured against the ICE peptide pool (see Supplementary Figure 6). As a positive control we took along 4 healthy donors, which were all responsive towards the ICE peptide pool (see Supplementary Figure 6). Also, all patient samples produced high amounts of cytokines after PMA/ionomycin stimulation (data not shown).

Correlation between T-cell reactivity against the HPV oncogenes and clinical benefit

Notably, all patients who showed *ex vivo* HPV E6 or E7 specific T-cell responses also experienced clinical benefit from the vaccine (Figure 3c). In contrast, such HPV E6 or E7 specific T-cell responses were completely absent in clinical non-responders (0/8). For 1 out of 6 patients that showed a clinical response, no *ex vivo* immune reactivity could be determined (Table 3 and Supplementary Figure 4, patient #13). Collectively, these findings demonstrate that there is a strong correlation between the induction of immune reactivity and clinical response (Fischer’s exact test, p=0.003).

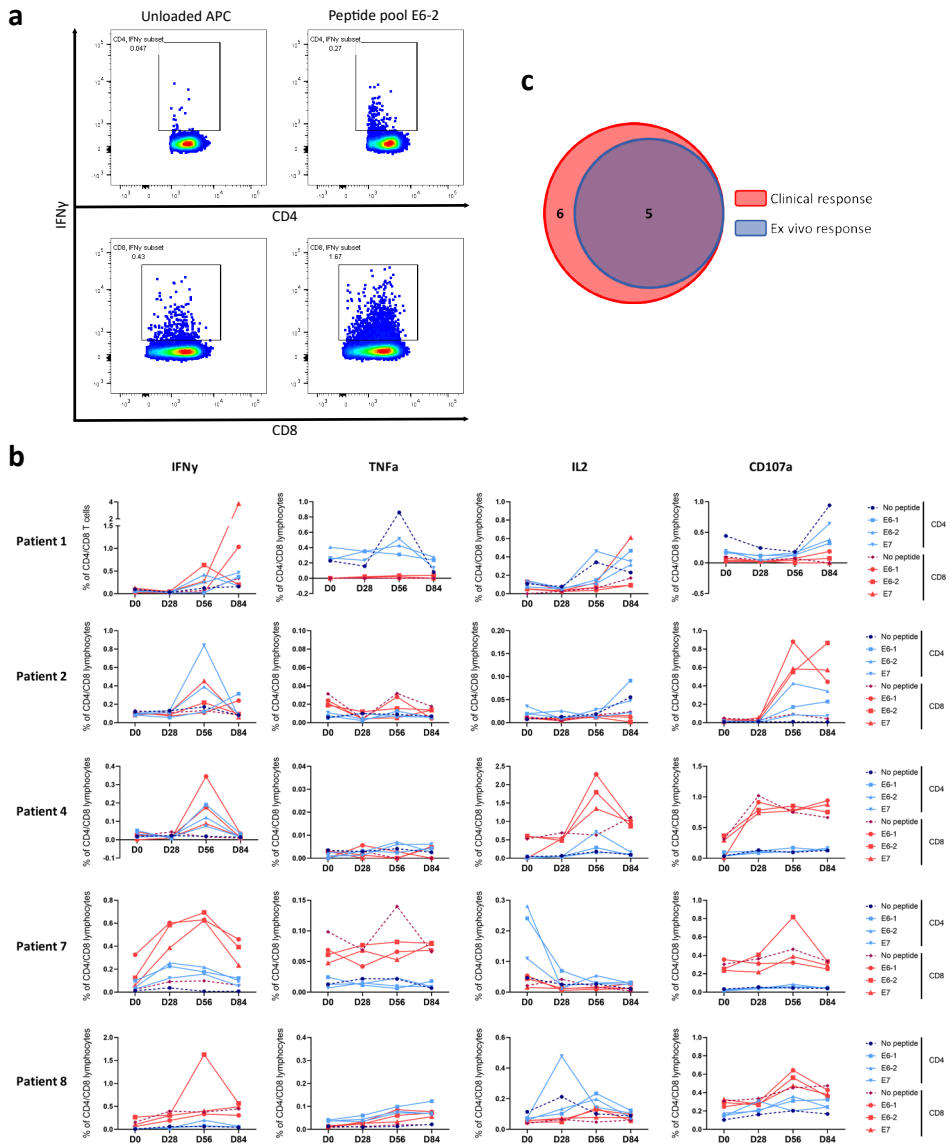

Figure 3: ex vivo reactivity data. a) *Example of an immunological responder (patient #8) at day 56, in which you can appreciate a cloud of IFNγ producing CD4 and CD8 cells, that also meets the fold increase over background requirement.* ***b)*** *Time course of all immunological responders. T cell responses against E6-1, E6-2 and E7 peptide pool are depicted. The dashed line represents the ‘no peptide’ control to visualize background reactivity. Time courses of IFNγ, TNFα, IL-2 and CD107a production for all patients are displayed in Supplementary Figure 4.* ***c)*** *Venn diagram visualizing the overlap between clinical responders (6/14) and immunological responders (5/14) (Fishers exact test, p=0.003).*

Table 3: *overview of immunological responses against E6 and E7 peptide pools.*

Patient	Ex vivo response	Clinical response			E6-1 IFNy	E6-1 TNFa	E6-1 IL2	E6-1 CD107a	E6-2 IFNy	E6-2 TNFa	E6-2 IL2	E6-2 CD107a	E7 IFNy	E7 TNFa	E7 IL2	E7 CD107a
1	Yes	CR	CD4	D0	<0,1%	1,1	<0,1%	0,4	<0,1%	1,8	1,3	0,4	<0,1%	1,2	1,2	0,4
				D56	<0,1%	0,4	0,4	0,7	3,4	0,5	0,3	0,9	2,0	0,6	1,4	0,7
			CD8	D0	<0,1%	n.d.	n.d	<0,1%	<0,1%	n.d.	n.d.	<0,1%	1,5	n.d.	n.d.	<0,1%
				D56	<0,1%	n.d.	<0,1%	<0,1%	8,7	n.d.	<0,1%	<0,1%	3,8	n.d.	2,0	0,0
2	Yes	CR	CD4	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	0,8	<0,1%	<0,1%	<0,1%
				D56	0,7	<0,1%	<0,1%	15,9	2,3	<0,1%	<0,1%	39,9	4,9	<0,1%	<0,1%	<0,1%
			CD8	D0	0,9	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	0,2
				D56	0,8	<0,1%	<0,1%	9,5	1,7	<0,1%	<0,1%	5,9	3,4	<0,1%	<0,1%	6,3
3	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	1,1	<0,1%	<0,1%	<0,1%	1,0	<0,1%
				D56	<0,1%	<0,1%	1,0	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	<0,1%	1,0	<0,1%
			CD8	D0	1,2	<0,1%	0,6	0,8	0,9	<0,1%	1,0	1,2	0,7	<0,1%	1,0	0,8
				D56	<0,1%	<0,1%	1,0	1,7	<0,1%	<0,1%	1,0	1,0	<0,1%	<0,1%	1,0	0,9
4	Yes	PR	CD4	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	10,1	<0,1%	1,6	1,8	6,4	<0,1%	0,9	1,2	<0,1%	<0,1%	4,0	<0,1%
			CD8	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	1,2	1,2	<0,1%	<0,1%	1,1	0,9
				D56	24,2	<0,1%	3,6	1,0	12,4	n.d.	2,8	1,1	<0,1%	<0,1%	2,1	1,0
5	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	1,2	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	<0,1%	0,7
			CD8	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
6	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
			CD8	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
7	Yes, Vaccine enhanced, not induced	PR	CD4	D0	<0,1%	<0,1%	5,2	<0,1%	<0,1%	<0,1%	6,0	<0,1%	<0,1%	<0,1%	2,4	<0,1%
				D56	22,1	<0,1%	<0,1%	<0,1%	27,3	<0,1%	<0,1%	<0,1%	19,8	<0,1%	<0,1%	<0,1%
			CD8	D0	13,8	<0,1%	<0,1%	1,2	5,3	<0,1%	<0,1%	0,8	<0,1%	<0,1%	<0,1%	0,8
				D56	6,4	<0,1%	<0,1%	0,7	7,0	<0,1%	<0,1%	1,7	6,3	<0,1%	<0,1%	0,8
8	Yes	PR	CD4	D0	<0,1%	<0,1%	<0,1%	1,4	<0,1%	<0,1%	<0,1%	1,7	<0,1%	<0,1%	<0,1%	1,4
				D56	<0,1%	<0,1%	2,3	1,5	3,2	<0,1%	1,9	1,8	<0,1%	<0,1%	1,4	1,0
			CD8	D0	<0,1%	<0,1%	<0,1%	0,8	1,7	<0,1%	<0,1%	0,9	0,7	<0,1%	<0,1%	1,1
				D56	0,9	<0,1%	2,6	1,4	4,3	<0,1%	2,9	1,2	1,1	<0,1%	<0,1%	1,0
9	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	1,0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
			CD8	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	<0,1%	<0,1%
10	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	0,6	<0,1%	1,1	<0,1%	1,1	<0,1%	<0,1%	<0,1%	0,7
				D56	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	1,2	<0,1%	<0,1%	<0,1%	<0,1%
			CD8	D0	<0,1%	<0,1%	<0,1%	0,7	<0,1%	<0,1%	<0,1%	1,2	<0,1%	<0,1%	<0,1%	0,8
				D56	<0,1%	<0,1%	<0,1%	1,2	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	<0,1%	1,1
11	No	NR	CD4	D0	<0,1%	<0,1%	1,7	<0,1%	<0,1%	<0,1%	0,7	<0,1%	<0,1%	<0,1%	1,0	1,8
				D56	<0,1%	<0,1%	<0,1%	1,0	<0,1%	<0,1%	0,9	1,0	<0,1%	<0,1%	0,6	0,9
			CD8	D0	<0,1%	<0,1%	<0,1%	0,9	1,1	<0,1%	<0,1%	1,0	1,1	<0,1%	<0,1%	1,1
				D56	1,1	<0,1%	<0,1%	0,9	1,2	<0,1%	<0,1%	0,9	1,1	<0,1%	<0,1%	1,0
12	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	1,0	0,9	<0,1%	<0,1%	1,7	0,7
				D56	<0,1%	<0,1%	<0,1%	1,2	<0,1%	<0,1%	1,4	1,6	<0,1%	<0,1%	1,0	1,3
			CD8	D0	<0,1%	<0,1%	<0,1%	1,0	<0,1%	<0,1%	<0,1%	1,1	<0,1%	<0,1%	<0,1%	<0,1%
				D56	0,7	<0,1%	<0,1%	0,9	0,7	<0,1%	1,8	0,9	0,5	<0,1%	<0,1%	0,9
13	No	PR	CD4	D0	<0,1%	<0,1%	<0,1%	1,8	<0,1%	<0,1%	<0,1%	1,8	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	1,9	<0,1%	<0,1%	<0,1%	1,7	<0,1%	<0,1%	<0,1%	<0,1%
			CD8	D0	<0,1%	<0,1%	<0,1%	0,7	<0,1%	1,0	<0,1%	1,0	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	0,8	<0,1%	0,7	<0,1%	0,6	<0,1%	0,9	<0,1%	<0,1%	<0,1%	1,2
14	No	NR	CD4	D0	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	1,9	<0,1%	<0,1%	<0,1%	1,2	<0,1%	<0,1%	<0,1%	<0,1%
			CD8	D0	<0,1%	<0,1%	0,6	0,8	<0,1%	<0,1%	1,0	0,7	<0,1%	<0,1%	<0,1%	<0,1%
				D56	<0,1%	<0,1%	<0,1%	0,8	<0,1%	<0,1%	<0,1%	0,9	<0,1%	<0,1%	1,1	0,6

Numbers represent the fold change over background (unloaded APCs). n.d.: no positive cells detected. Fold changes greater than two are highlighted in green. <0.1% indicates that the fraction of positive cells was less than 0.1% from its parent ($CD4^+$ or $CD8^+$ T cells). CR = complete response, PR = partial response and NR = no response.

Lymphocyte counts in relation to clinical response

Regular blood cell counts were established at matching time points with the PBMC isolation for *ex vivo* reactivity assays. At baseline, no statistically significant differences in number of circulating lymphocytes, neutrophils, monocytes and eosinophils could be found between responding and non-responding patients (shown in Figure 4). During the vaccinations at day 28, as well as at the peak of the response at day 56, lymphocyte counts were significantly higher in responding patients than in non-responding patients (Figure 4a). Systemic neutrophil to lymphocyte ratio was decreased at the peak of the response compared to baseline in responding patients. No significant change in neutrophil to lymphocyte ratio over time was found in non-responding patients (Figure 4c). The number of circulating neutrophils, monocytes and eosinophils remained similar for responders and non-responders and unaltered compared to baseline levels (Figure 4 b, d, e).

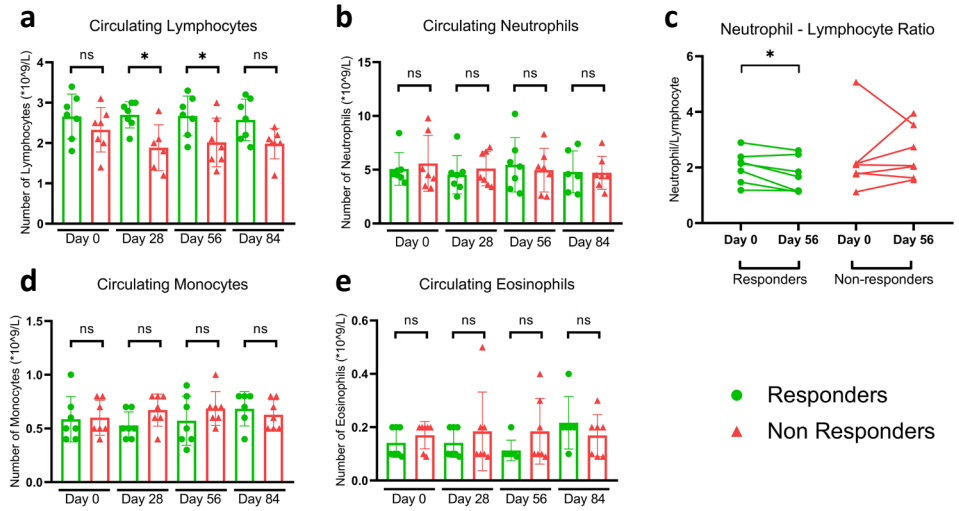

Figure 4: Systemic blood counts reveal differences between responders and non-responders during- and post vaccination a) *Significantly reduced number of circulating lymphocytes in non-responding patients, compared to responding patients. Two tailed Mann-Whitney test D28 p=0,015, D56 p=0,048.* ***b)*** *No statistically significant differences detected between responding and non-responding patients at any time point in neutrophils.* ***c)*** *Responding patients show a decreased Neutrophil to Lymphocyte ratio at the peak the immune response compared to baseline. No significant changes in Neutrophil to Lymphocyte ratio in non-responding patients. Wilcoxon matched-pairs signed rank test p=0,0469. No statistically significant differences detected between responding and non-responding patients at any time point in circulating* ***d)*** *monocytes and* ***e)*** *eosinophils.*

Discussion and Conclusion

Despite a variety of treatment modalities for patients suffering from uVIN, these patients are often confronted with recurrent disease and are at risk to progress to invasive disease. In this study, we have used a therapeutic HPV-16 E6/E7 DNA tattoo vaccine comprising of sig-HELP-E6SH-KDEL and sig-HELP-E7SH-KDEL. In mice, this DNA vaccine has shown to be much more immunogenic than the variants with other helper cassettes (such as TTFC) that were previously used in the clinic[24, 32]. This is the first clinical trial using this optimized DNA vaccine targeting the HPV oncoproteins E6 and E7 in patients with uVIN.

Several HPV-vaccination studies targeting E6 and/or E7 have been performed with varying results. Intramuscular TA-CIN (fusion protein HPV16 E6E7L2) administration preceded by local imiquimod application has been studied, with a clinical response rate of 63% in patients with uVIN, but all patients in this study displayed moderate (n=5, 26%), or severe (n=14, 74%) side effects[22]. TA-HPV, a recombinant vaccinia virus, encoding modified HPV 16 and 18 E6 and E7, has also been successfully applied in uVIN and vaginal intraepithelial neoplasia patients. This was resulting in both a potent clinical responses (8/18 and 5/12 respectively) and immunological responses (13/18 and 6/10 respectively)[16, 36]. However, the use of live vaccinia virus limits the broad application of this therapy. In trials investigating subcutaneously administered HPV16 E6 and E7 synthetic long peptides (SLP), clinical responses were observed after 12 months in 52-79% of women with uVIN[21, 37]. However, grade 1 and grade 2 side effects were reported at very high frequencies and were probably linked to the use of the Montanide ISA51. In our trial, no adverse events higher than grade 1 were reported, (apart from one patient with a grade 3 SUSAR that was probably unrelated as symptoms had occurred before the first vaccination) and at much lower frequencies, suggesting that HPV-16 E6/E7 DNA tattoo vaccination is safe to use. This difference in toxicity and tolerability can likely be explained by the fact that we used the tattoo technique, and no adjuvant or other initial treatment modality such as imiquimod was used in our trial. Since subcutaneous administration of a therapeutic HPV peptide vaccine with adjuvant can cause significant adverse events (such as local skin swelling), we focused on improving the administration route and optimization of immunogenicity of the vaccine.

Our data indicate a 43% clinical response rate. A clinically durable and ongoing complete response was seen in 14% of the patients. Partial responses were observed in 29% of patients and were ongoing at the time of most recent follow-up. Importantly, unlike other treatment modalities (e.g., laser ablation, surgical excision or imiquimod application) in which up to one-third of patients show a recurrence[9, 38], none of the responders in our study had recurrences or increasing lesion size over time. A likely explanation for this difference is that our vaccination strategy targets the cause of the disease, *i.e.* HPV16 E6 and E7 expressing cells, and this is underlined by the fact that 83% of the responding patients showed a clear E6/E7 specific T-cell response in their blood. However, recurrences often

occur over one year after treatment in this patient group and follow-up period in this study was only 12 months. Future studies have to point out whether the recurrence of uVIN is maintained more than a year after therapeutic HPV-vaccination. Furthermore, no HPV-testing at the end of follow-up was performed, which would be interesting to incorporate in follow-up studies to confirm the successful clearance of the virus at the uVIN lesions after vaccination.

Although responses were durable in our study, complete response rates were still low (2/14). Therefore we would like to advocate the combination of our vaccine with for instance immune checkpoint inhibitors such as (locally administrated) anti PD-(L)1 or TLR_agonists, such as poly (I:C) (TLR3 agonist) or Imiquimod (TLR7 agonist). Besides this, it might be beneficial for patients with large uVIN lesions to first decrease lesion size (e.g. by laser or topical therapy), before administering our vaccine, because patients with largest uVIN lesion size at baseline did not show any response to vaccination in this trial. However, since the sample size in this study was quite small, future studies have to reveal whether this effect will still be observed. Interestingly though, Kenter *et al.* also reported that lesions were smaller in the CR group after E6 and E7 synthetic long-peptide vaccination in uVIN patients[21].

Systemic immunological HPV-specific T-cell responses were found in both the $CD4^+$ as well as the $CD8^+$ compartment. These responses were either vaccine induced (4/5) or vaccine enhanced (1/5). Interestingly, 5 out of 6 patients with complete or partial responses showed systemic HPV-specific T-cell responses in *ex vivo* assays. Likewise, patients *without* a clinical response, did *not* show an HPV-specific T-cell response *ex vivo*. Previous HPV targeting vaccines, in the same patient group, observed a similar relationship. Both Kenter *et al.* and Van Poelgeest *et al.* reported a correlation between (the magnitude of) the *ex vivo* response and the clinical outcome of the patients after vaccination with HPV16 E6 and E7 synthetic long peptides[21, 37] However, in a study evaluating the effect of a TA-HPV vaccine against E6 and E7, *ex vivo* responsiveness to the vaccine vector was confirmed in all patients, there was no relation with clinical benefit[20]. The differences between clinical and immunological responses between our study and previous studies could be explained by a different study design, different vaccine, different patient group and a different technique used to identify *ex vivo* immune responses.

At baseline, the number of circulating lymphocytes, neutrophils, monocytes and eosinophils did not differ statistically significant between responders and non-responders. Upon treatment, non-responders had statistically significant fewer circulating lymphocytes than responders, which could potentially be a reflection of a less competent immune system.

Future experiments will tell whether responders will have relatively higher numbers of VIN lesion infiltrating lymphocytes compared to non-responders, and what potential immunosuppressive mechanisms in the lesions might have hampered a T-cell response in the non-responding patients.

Follow-up studies should be performed to determine the effects of this vaccination strategy in a larger cohort of patients with uVIN, as well as patients with other intraepithelial neoplasia caused by HPV 16, such as anal intraepithelial neoplasia (AIN), penile intraepithelial neoplasia (PeIN) and cervical intraepithelial neoplasia (CIN). HPV-16 and HPV-18 CIN2/3 patients have already shown to respond to other types of DNA vaccination targeting E6 and E7 proteins[39].

In conclusion, we found in this phase I/II clinical trial that HPV-16 E6/E7 DNA tattoo vaccination for the treatment of HPV16 positive uVIN is a safe and immunologically effective strategy. Interestingly, in 5 out of 6 clinically responding patients, E6/E7 specific $CD4^+$ and $CD8^+$ T cell reactivity could be detected in blood samples. Such responses were not observed in patients without a clinical response. Therefore, HPV-16 E6/E7 DNA tattoo could possibly be a clinically meaningful treatment strategy in patients with uVIN.

Competing interests

N.A.M.B., J.R., M.v.B., H.J.M.A.A.Z., M.R., S.S., **B.N., E.S.J., G.G.K.** and **N.E.v.T** have no competing interest to declare.

J.H.B. is (part time) employee of Modra Pharmaceuticals and stockholder in Modra Pharmaceuticals. (not related to the manuscript).

T.N.S. is advisor for Adaptive Biotechnologies, Allogene Therapeutics, Merus, Neogene Therapeutics, and Scenic Biotech; is a recipient of research support from Merck KgaA; is a stockholder in Allogene Therapeutics, Merus, Neogene Therapeutics and Scenic Biotech; and is venture partner at Third Rock Ventures, all not related to the current work.

J.B.A.G.H. is advisor to Achilles Therapeutics, Bristol-Myers Squibb, BioNTech USA, Ipsen, Gadeta, Immunocore, MSD, Merck Serono, Molecular Partners, Neogene Therapeutics, Novartis, Pfizer, Roche/Genentech, Sanofi, Seattle Genetics, Third Rock Ventures, is stock holder in Neogene Therapeutics, and is a recipient of grant or research support from Bristol-Myers Squibb, MSD, Novartis and BioNTech USA.

K.E.d.V. reports research funding from Roche and is consultant for Third Rock Ventures, all outside the scope of this work.

T.D.d.G. has served as advisor to TILT Biotherapeutics, LAVA Therapeutics, Macrophage Pharma and DCPrime, is a recipient of a grant from Idera Pharmaceuticals, and is co-founder and shareholder of LAVA Therapeutics.

J.H.v.d.B is a recipient of grant or research support from BioNTech USA and Astra Zeneca, and is currently employed at CellPoint B.V.

Authors' contributions

N.A.M.B. constructed the plasmids and produced DNA vaccines, designed and performed immune monitoring experiments, interpreted data and co-wrote the manuscript.

J.R. vaccinated patients, collected and interpreted clinical data and co-wrote the manuscript.

M.v.B. involved in patient care

H.J.M.A.A.Z. involved in patient care

S.S. involved in writing the study protocol

M.R. vaccinated patients and collected clinical data

B.N. and **J.H.B.** supervised DNA production

T.N.S. conceived the project, designed plasmids and interpreted data and reviewed the manuscript

J.B.A.G.H. conceived the project, designed plasmids and interpreted data

K.E.d.V. interpreted data and reviewed the manuscript

T.D.d.G. interpreted data

E.S.J. interpreted data

G.G.K. conceived the project, interpreted data

J.H.v.d.B conceived the project, interpreted data, designed plasmids, supervised DNA production and co-wrote the manuscript

N.E.v.T. conceived the project, interpreted clinical data and co-wrote the manuscript

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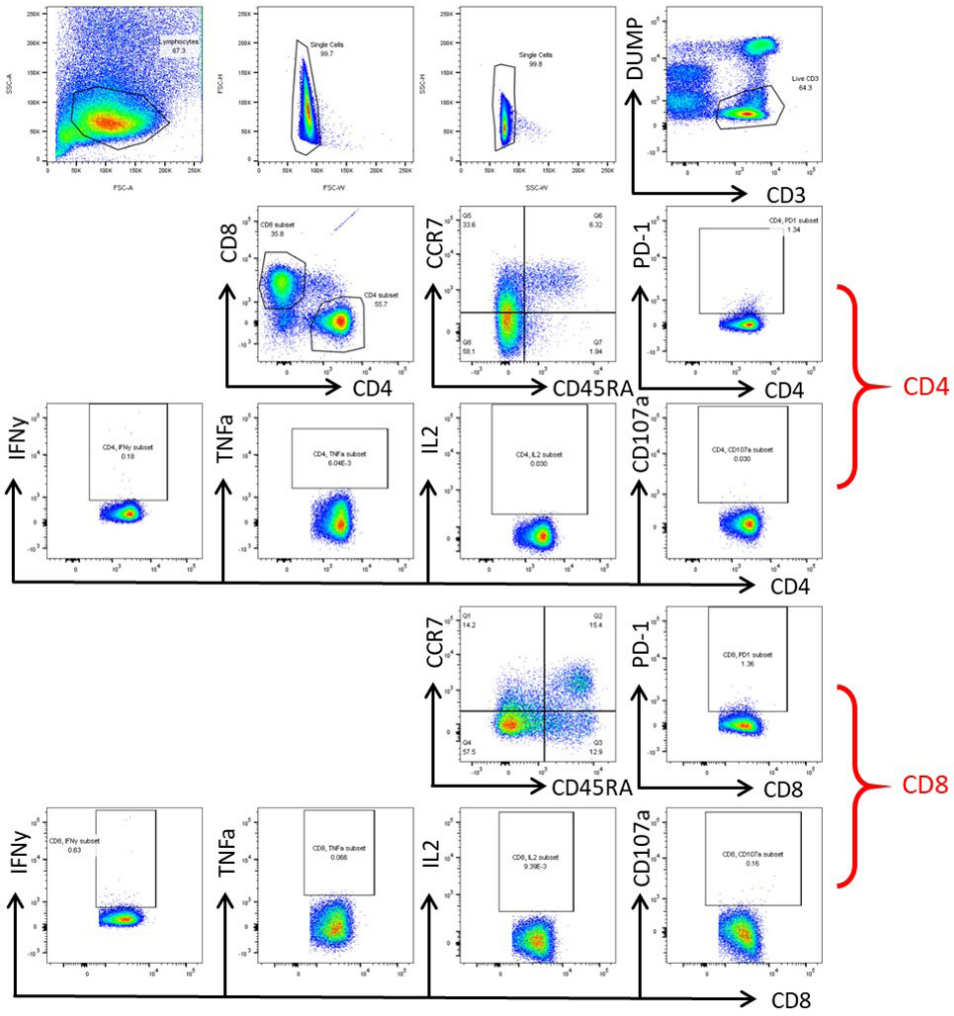
Supplementary Material

***Supplementary table 1**: Overview of peptide pools used in the ex vivo reactivity screens, and the amino acid sequence of each peptide.*

Peptide Pool	Peptide Number	Amino Acid Sequence
Pool 1: E6-1	1	MHQKRTAMFQDPQERPRKLPQL
	2	DPQERPRKLPQLCTELQTTIHD
	3	QLCTELQTTIHDIILECVYCKQ
	4	HDIILECVYCKQQLLRREVYDF
	5	KQQLLRREVYDFAFRDLCIVYR
	6	DFAFRDLCIVYRDGNPYAVCDK
	7	YRDGNPYAVCDKCLKFYSKISE
	8	DKCLKFYSKISEYRHYCYSLYG
Pool 2: E6-2	9	SEYRHYCYSLYGTTLEQQYNKP
	10	YGTTLEQQYNKPLCDLLIRCIN
	11	KPLCDLLIRCINCQKPLCPEEK
	12	INCQKPLCPEEKQRHLDKKQRF
	13	EKQRHLDKKQRFHNIRGRWTGR
	14	RFHNIRGRWTGRCMSCCRSSRT
	15	GRWTGRCMSCCRSSRTRRETQL
Pool 3: E7	16	MHGDTPTLHEYMLDLQPETTDL
	17	YMLDLQPETTDLYCYEQLNDSS
	18	DLYCYEQLNDSSEEEDEIDGPA
	19	SSEEEDEIDGPAGQAEPDRAHY
	20	PAGQAEPDRAHYNIVTFCCKCD
	21	HYNIVTFCCKCDSTLRLCVQST
	22	CDSTLRLCVQSTHVDIRTLEDL
	23	STHVDIRTLEDLLMGTLGIVCP
	24	RTLEDLLMGTLGIVCPICSQKP
Pool 4: Potential epitopes that may have arisen as a consequence of shuffling the protein domains.	25	TDLYCICSQKPKCDSTLRL
	26	GTLGIVCPYEQLNDSS
	27	YNIVTFCCQPETTDLY
	28	HDIILECVNCQKPLCP
	29	GRWTGRCMKCLKFYSK
	30	CDLLIRCIYCKQQLLR
	31	GNPYAVCDSCCRSSRT
	32	RTRRETQLQLCTELQT

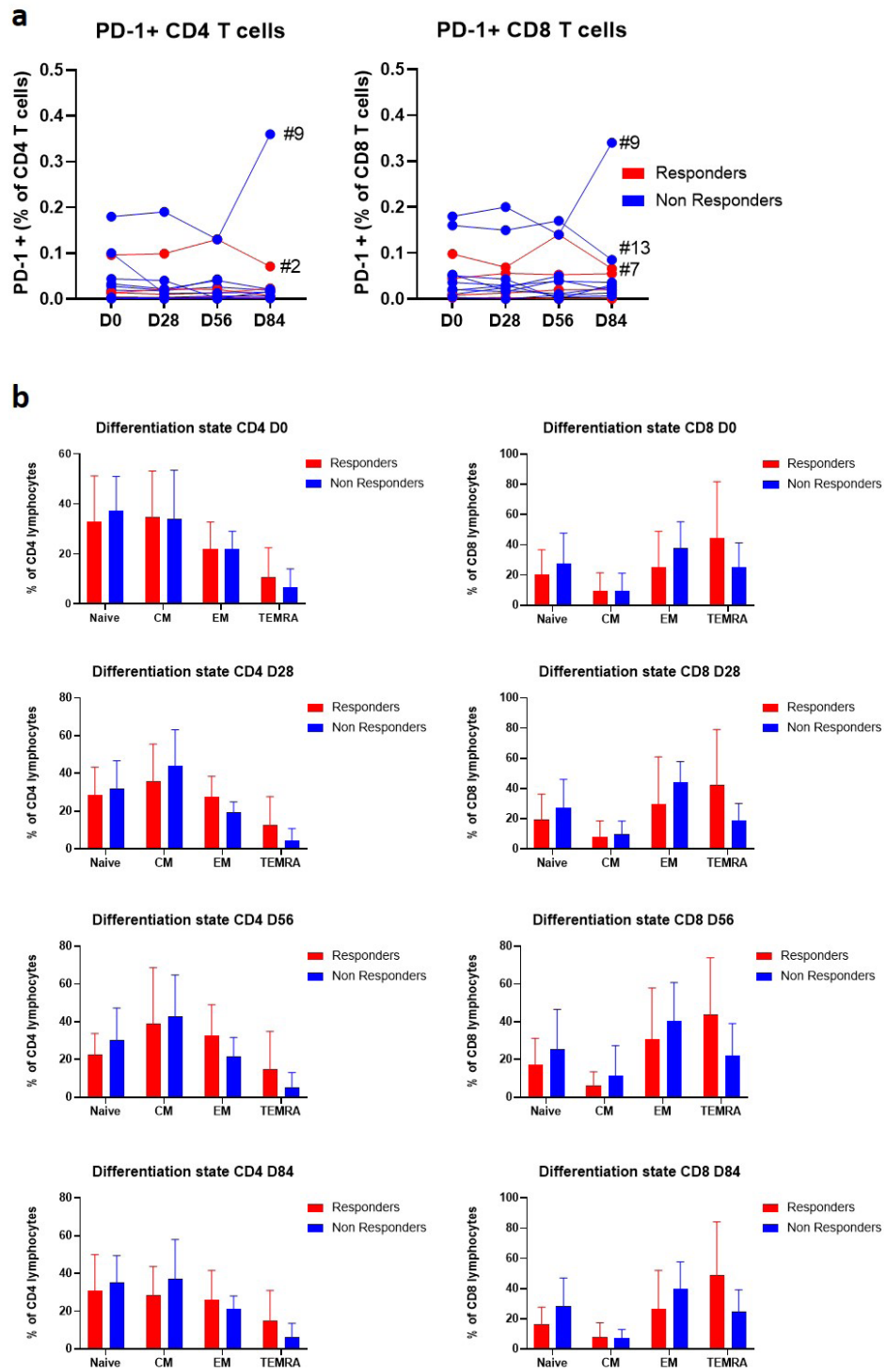
Supplementary table 2: *Antibody panel used for ex vivo reactivity screens.*

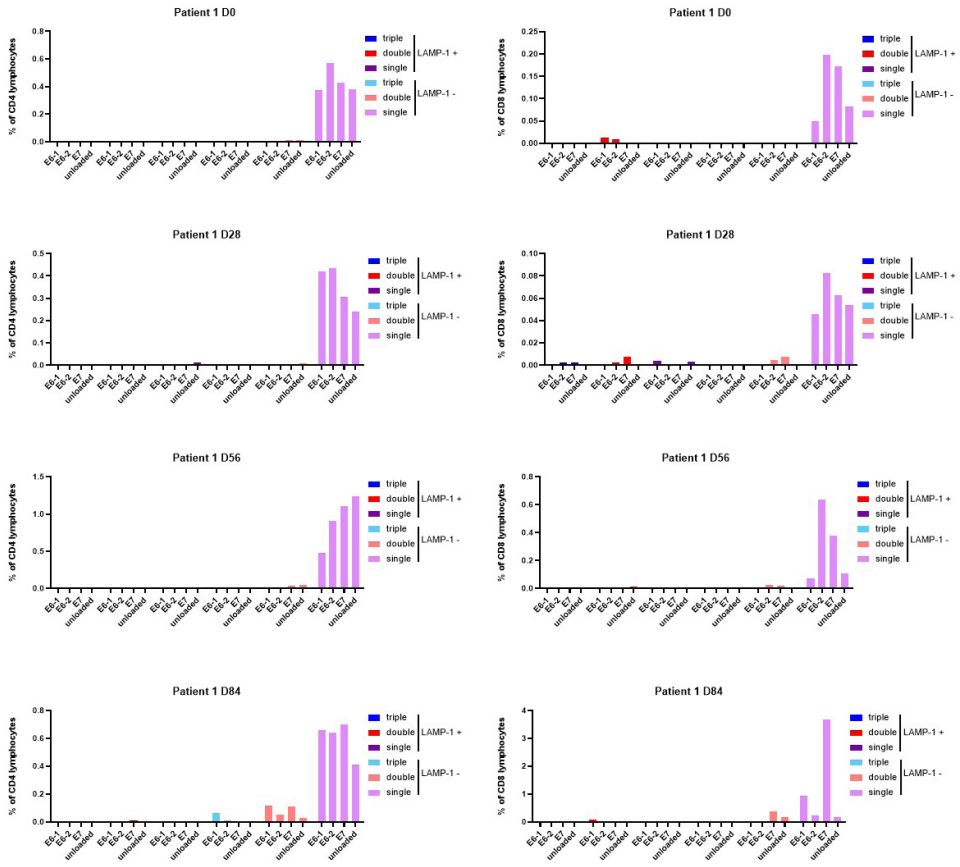
Target + Fluorochrome	Clone	Details	Vendor
CD3-APC-H7	Clone SK7	Mouse IgG1	BD Biosciences
CD14-Pacific Blue	Clone TùK4	Mouse IgG2a	Invitrogen
CD16-Pacific Blue	Clone 3G8	Mouse IgG1	Invitrogen
CD19-Pacific Blue	Clone SJ25-C1	Mouse IgG1	Invitrogen
CD4-PE	Clone S3.5	Mouse IgG2a	Invitrogen
CD8-PerCP-Cy5.5	Clone SK1	Mouse IgG1	BioLegend
CCR7-PE-CF594	Clone 150503	Mouse IgG2a	BD Biosciences
CD45RA-PE-Cy5.5	Clone MEM-56	Mouse IgG2b	Invitrogen
PD-1-eVolve 655	Clone J105	Mouse IgG1	eBiosciences
IFNγ-FITC	Clone B27	Mouse IgG1	BD Biosciences
IL-2-APC	Clone MQ1-17H12	Rat IgG2a	BD Biosciences
TNFα-PE-Cy7	Clone MAb11	Mouse IgG1	BD Biosciences
CD107a-Alexa Fluor 700	Clone H4A3	Mouse IgG1	BD Biosciences
Fixable Violet Dead Cell Stain Kit, 405 nm	Fluorescent reactive dye + DMSO		Invitrogen

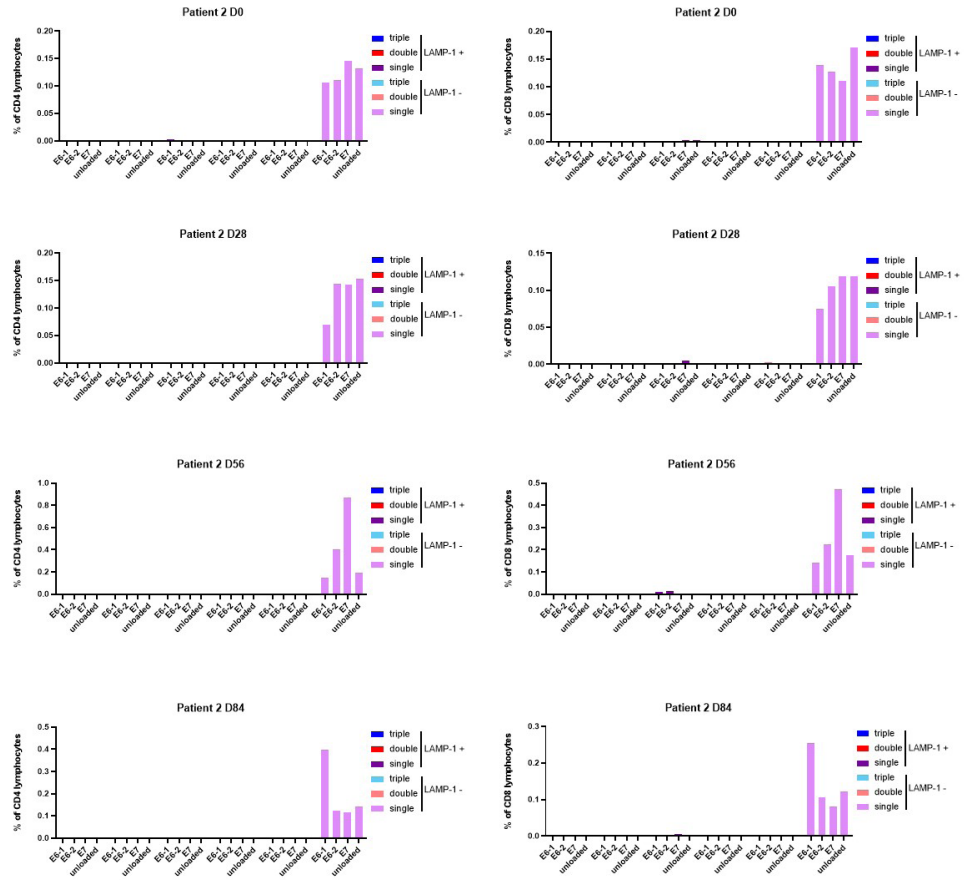


Supplementary figure 1: *Gating strategy example of phenotypic characterization and cytokine production of CD4 and CD8 T cells.*

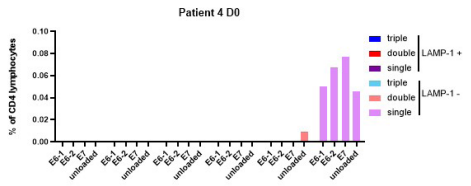
Supplementary figure 2: *Phenotypic characterization of circulating T cells.* ***a)*** *Frequency of PD-1 positive CD4 and CD8 T cells over time.* ***b)*** *Differentiation state of CD4 and CD8 T cells defined by CD45RA and CCR7 surface marker expression. CD45RA+ CCR7+: naïve T cells, CD45RA- CCR7+: central memory T cells, CD45RA- CCR7-: effector memory T cells and CD45RA+ CCR7-: effector T cells. Responding patients are colored red and non-responding patients are colored blue.* ▶▶▶

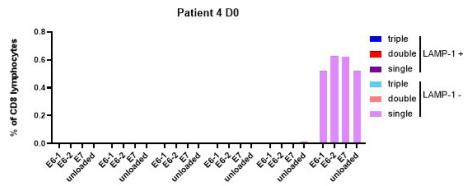


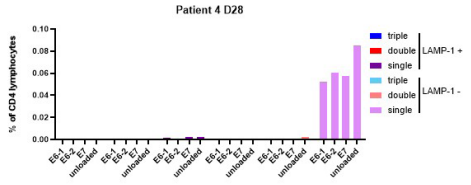


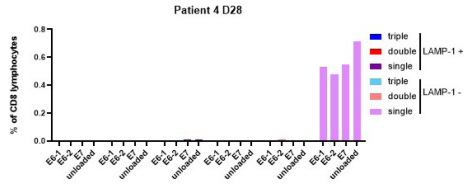


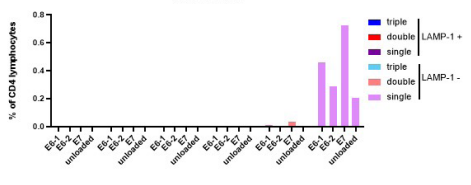
***Supplementary figure 3**: Depicted are the frequencies of single, double and triple cytokine producing CD4 and CD8 T cells, with and without co-expression of degranulation marker CD107a (LAMP-1), determined using Boolean gating. Depicted are immunologically responding patients **a** patient 1, **b** patient 2, **c** patient 4, **d** patient 7 and **e** patient 8.*

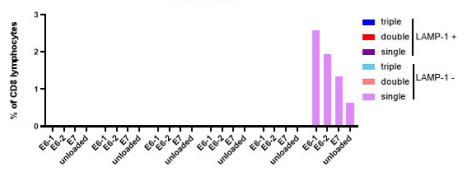

Patient 4 D0
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

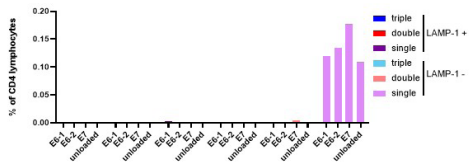

Patient 4 D0
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

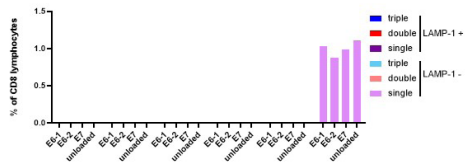

Patient 4 D28
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

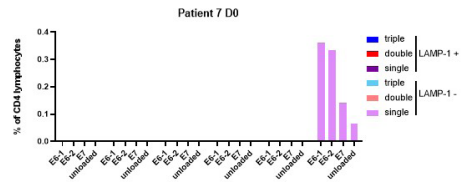

Patient 4 D28
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

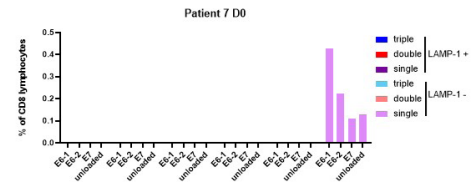

Patient 4 D56
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

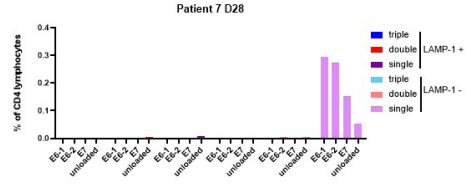

Patient 4 D56
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

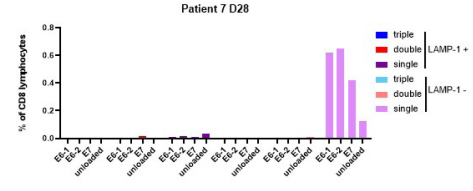

Patient 4 D84
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

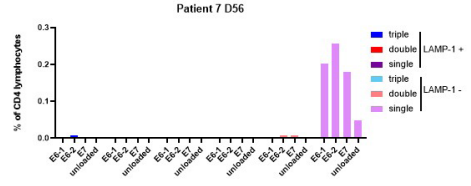

Patient 4 D84
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

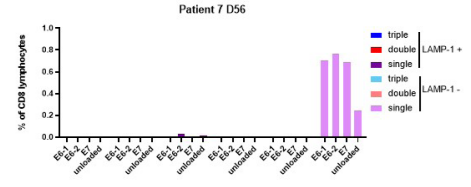

Patient 7 D0
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

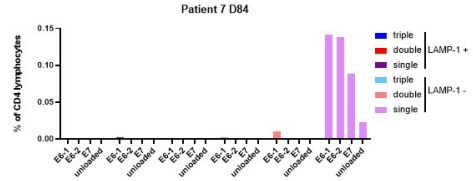

Patient 7 D0
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

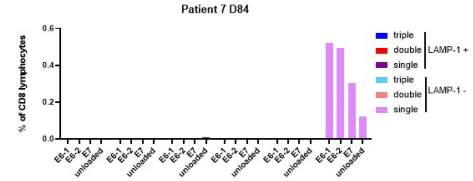

Patient 7 D28
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

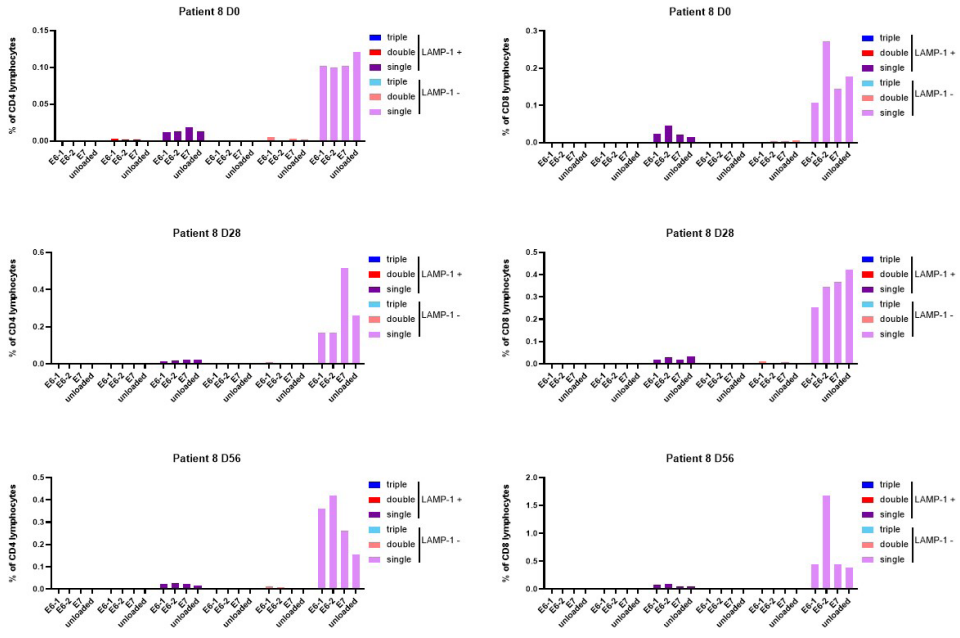

Patient 7 D28
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -


Patient 7 D56
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

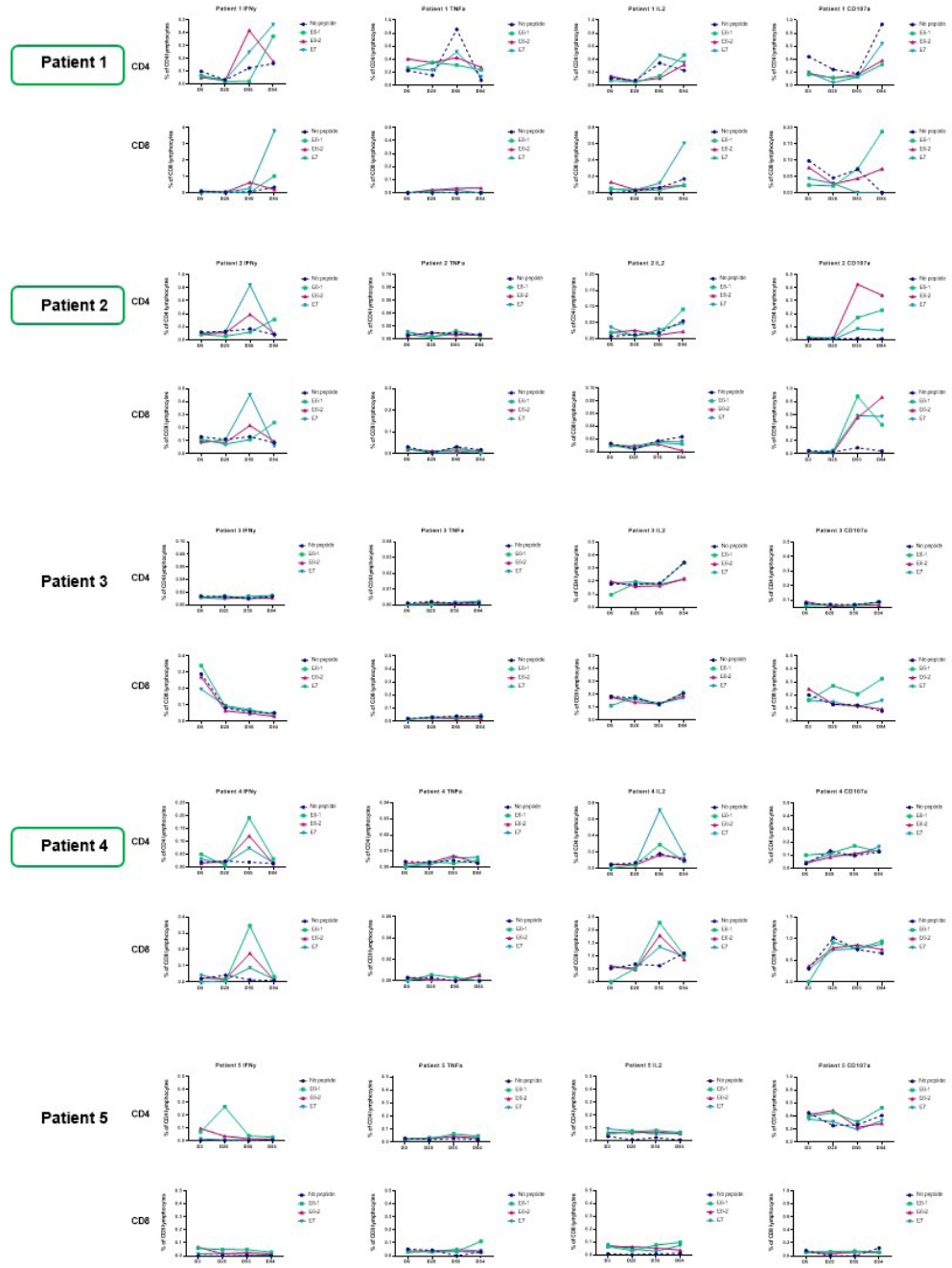

Patient 7 D56
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -


Patient 7 D84
% of CD4 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -

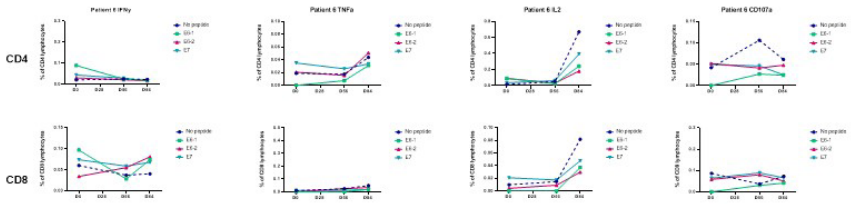

Patient 7 D84
% of CD8 lymphocytes
triple
double
single
LAMP-1 +
LAMP-1 -



Supplementary figure 4: *Time course graphs showing the IFNy, TNFa, IL-2 and CD107a responses against E6-1, E6-2 and E7 peptide pool for each patient. No peptides indicate that T cells were co-cultured with unloaded APCs to access background or aspecific reactivity. The green boxes highlight the patients with an immunological response.* ▶▶▶



Patient 6



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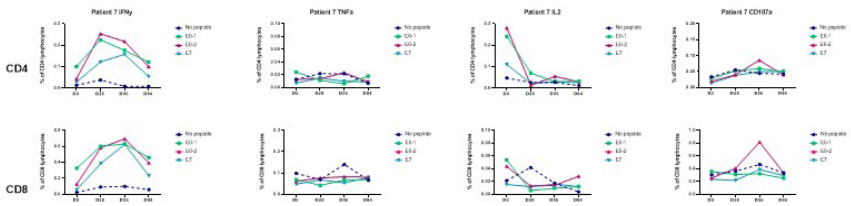

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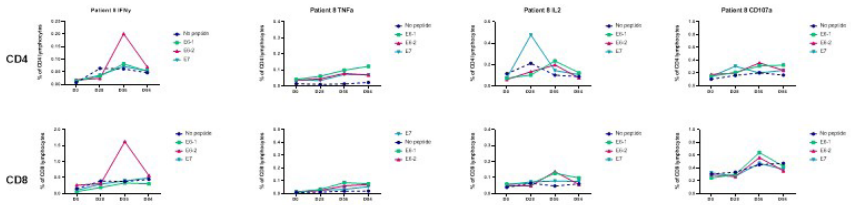

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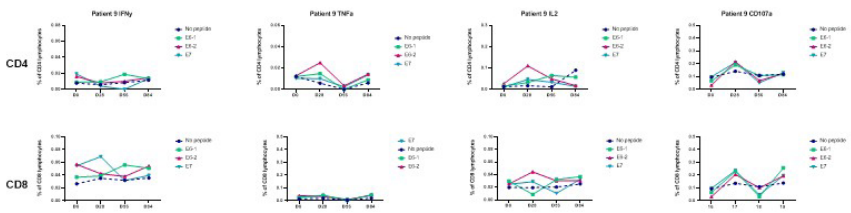
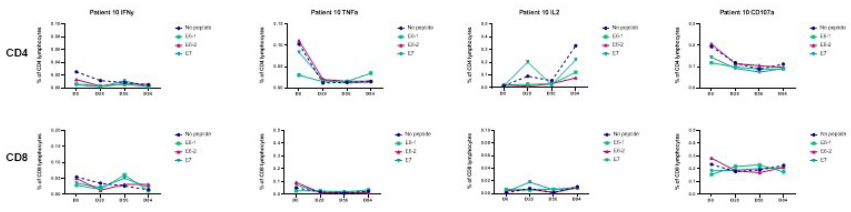
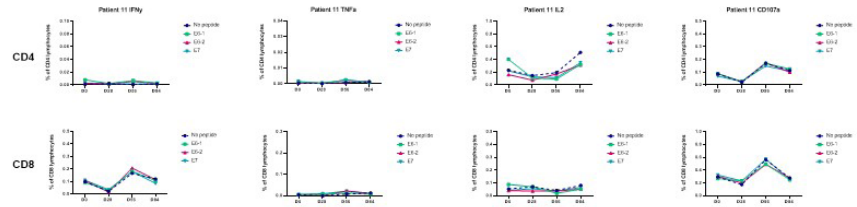


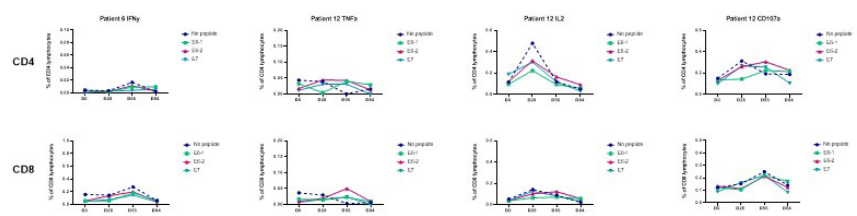
Patient 10



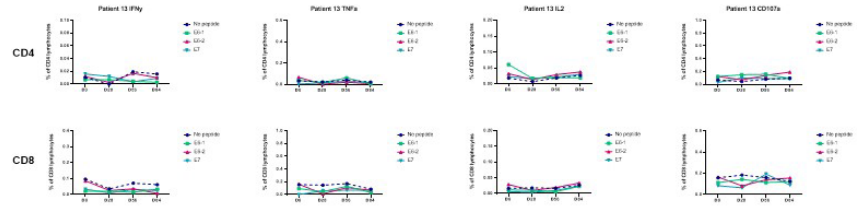
Patient 11



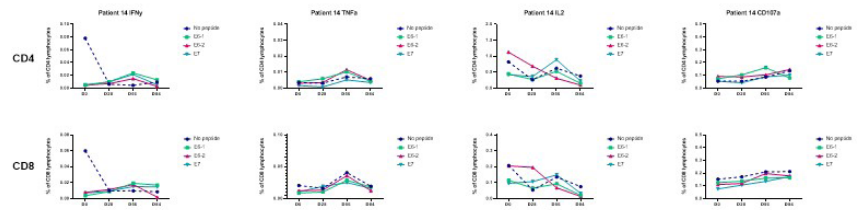
Patient 12

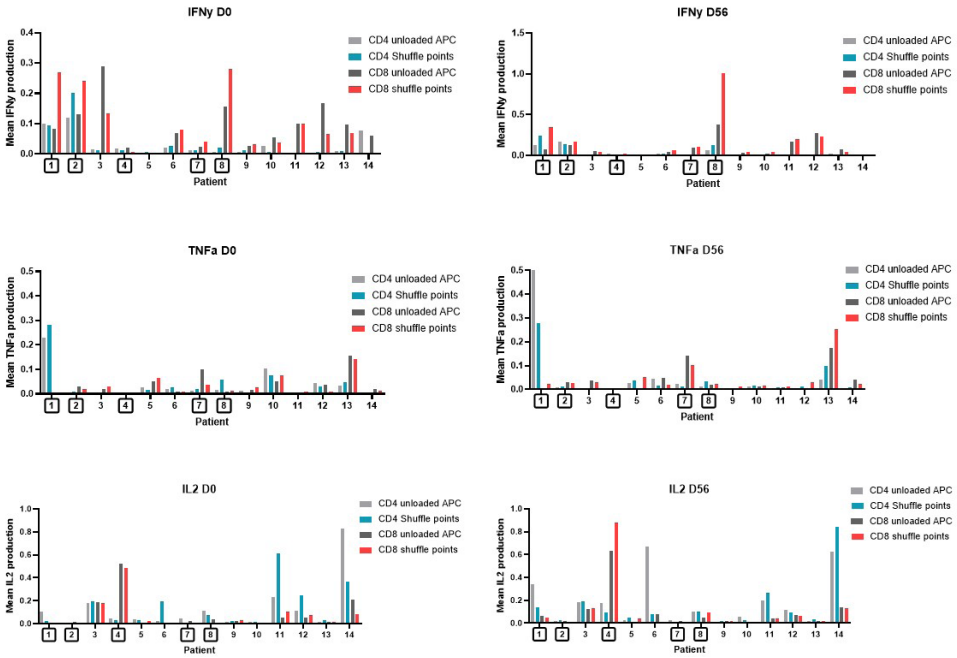


Patient 13

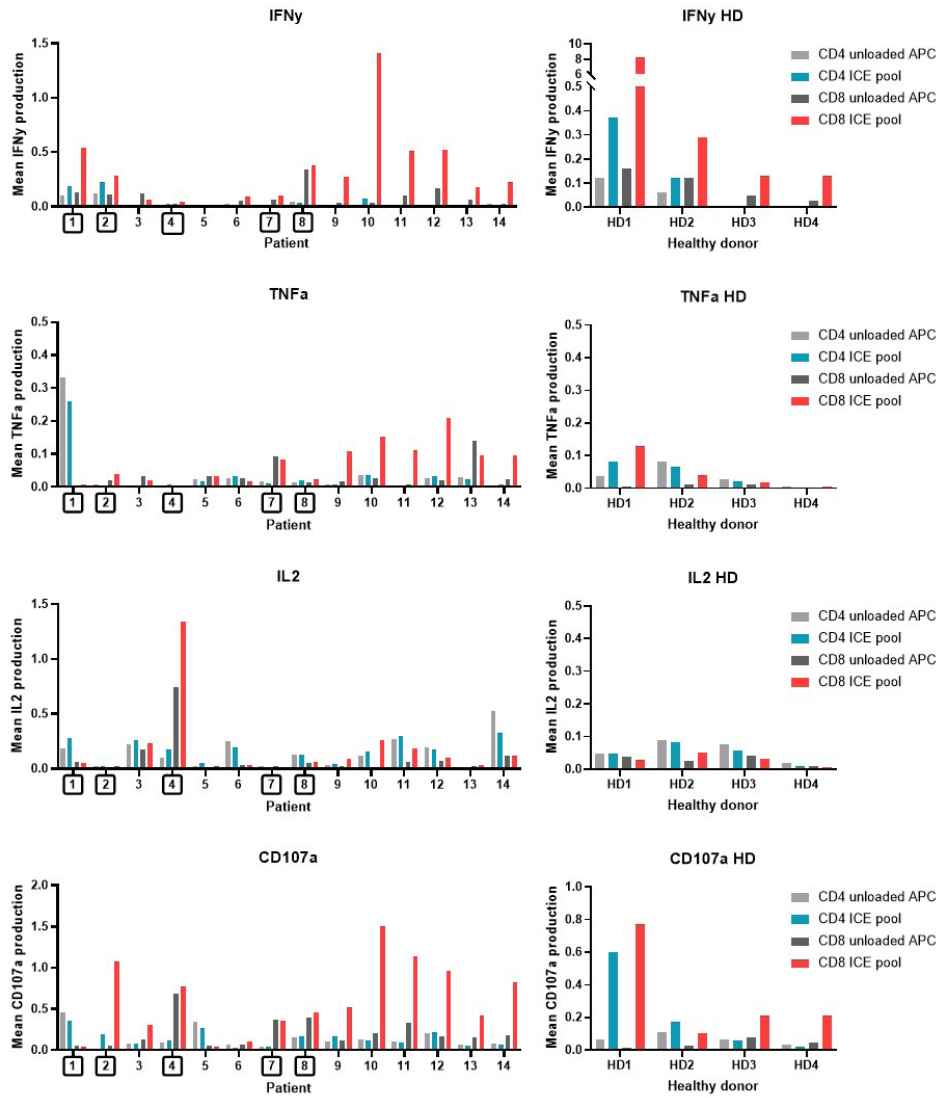


Patient 14





Supplementary figure 5: *Reactivity against the peptides that arose as a consequence of shuffling the E6 and E7 coding sequences. Boxes highlight responding patients.*



Supplementary figure 6*: Reactivity against ICE peptide pool at day 0. On the left, patient responses are depicted and on the right four healthy controls are depicted for the matched cytokines or LAMP-1. The black boxes on the x-axis highlight responding patients.*