Chapter 7

Addition of Interferon-alpha to the p53-SLP® vaccine results in increased production of Interferon-gamma in vaccinated colorectal cancer patients: a phase I/II clinical trial

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ABSTRACT

Purpose: We previously established safety and immunogenicity of a p53 synthetic long peptides (p53-SLP®) vaccine. In the current trial we investigated whether combination of Interferon-alpha (IFN-α) with p53-SLP® is both safe and able to improve the induced p53-specific IFN-γ response.

Experimental design: Eleven colorectal cancer patients successfully treated for metastatic disease were enrolled in this study. Of these, nine patients completed follow up after two injections with p53-SLP® together with IFN-α. Safety and p53-specific immune responses were determined before and after vaccination. Furthermore, cryopreserved PBMCs were compared head-to-head to cryopreserved PBMCs obtained in our previous trial with p53-SLP® only.

Results: Toxicity of p53-SLP® vaccination in combination with IFN-α was limited to grade 1 or 2, with predominantly small ongoing swellings at the vaccination site. All patients harbored p53-specific T cells after vaccination and most patients showed p53-specific antibodies. Compared to the previous trial, addition of IFN-α significantly improved the frequency of p53-specific T cells in IFN-γ ELISPOT. Moreover, in this trial, p53-specific T cells were detectable in blood samples of all patients in a direct *ex vivo* multiparameter flowcytometric assay, opposed to only 2 out of 10 patients vaccinated with p53-SLP® only. Finally, patients in this trial displayed a broader p53-specific immunoglobulin-G response, indicating an overall better p53-specific T-helper response.

Conclusions: Our study shows that p53-SLP® vaccination combined with IFN-α injection is safe and capable of inducing p53-specific immunity. When compared to a similar trial with p53-SLP® vaccination alone the combination was found to induce significantly more IFN-γ producing p53-specific T-cells.

INTRODUCTION

The modest to poor prognosis of colorectal cancer patients treated with curative intent, calls for additional treatment modalities such as immunotherapy [1].

p53 is one of the most frequently used tumor-associated antigens in tumor directed vaccination studies [2]. Due to a mutation, p53 is inactivated and over-expressed in 34-45% of colorectal tumors, while wild-type p53 is expressed at extremely low levels [3]. This provides an appropriate immunological window for T cells, being targeted to p53, to discriminate between tumor cells and normal cells [4].

A clinical-grade p53 synthetic long peptides (p53-SLP®) vaccine was developed that was tested in two parallel phase I/II studies in colorectal and ovarian cancer patients [5-7]. Results from these first trials revealed that in the vast majority of vaccinated cancer patients mainly p53-specific $CD4^+$ T cells were induced [6;7].

The presence of tumor-specific $CD4^+$ T cells in the cancer microenvironment is a prerequisite for support, proliferation, recruitment and cytolytic function of tumor-specific $CD8^+$ T cells, greatly accelerated by the production of IFN-γ and IL-2 [8-10]. Patients with metastatic colorectal cancer vaccinated against the tumor antigen 5T4 were found to have more clinical benefits when 5T4-specific IFN-γ ELISPOT responses were induced [11]. Also, in women with human papillomavirus (HPV) positive vulvar intraepithelial neoplasia, complete responses after vaccination against HPV were positively associated with the induction of IFN-γ-producing and proliferative T-cell responses [12]. Together, these data suggest that clinical responses after vaccination depend on the induction of strong and broad vaccine-specific type 1 T-cell responses. Results from the first two trials with p53-SLP® showed that vaccine-induced type 1 T-helper (Th1) cells produced only low amounts of the key cytokines (i.e. IFN-γ and IL-2), indicating that tumor-induced p53-specific Th-responses are present but not properly polarized [6;7]. Therefore, in order to benefit from the tumor-specific Th cells at the tumor site, the p53-SLP® should be combined with immune modulating adjuvants that specifically induce Th1-cell polarization. A possible candidate adjuvant to achieve this is Interferon-alpha (IFN-α).

IFN-α is used to treat patients suffering from chronic viral hepatitis infection and different malignancies [13]. IFN-α plays a major role in the differentiation of the Th1 subset, the generation of CTL and the promotion of proliferation and survival of T cells [14;15]. Moreover, several studies have shown that type I IFNs promote the differentiation of monocytes into dendritic cells (DC) and enhance DC activity [16-21]. In a murine melanoma model, it was shown that addition of IFN-α to a gp100 peptide, suppressed melanoma growth and increased the accumulation and proliferation of gp100-specific, IFN-γ-secreting $CD8^+$ T cells [22]. Moreover, adoptive transfer of tumor-reactive T cells and daily injections of IFN-α in metastatic melanoma patients can lead

to successful treatment of metastatic melanoma [23]. In humans, peptide vaccination has been combined with IFN-α injections showing that the combination was safe, resulted in a consistent enhancement of vaccine-specific $CD8^+$ T cells and increased the percentage of blood circulating DC precursors [24]. We now report the results of a phase I/II trial addressing safety and immunogenicity in which successfully treated metastatic colorectal patients were subcutaneously vaccinated with p53-SLP® in combination with subcutaneous administration of IFN-α. In addition, we analyzed whether addition of IFN-α close to the vaccine site not only induced a stronger p53-specific but also a better polarized Th1 response by testing and comparing cryopreserved peripheral blood mononuclear cells (PBMCs) and serum samples of the current trial head-to-head to samples obtained in our previous clinical trial, in which a similar group of colorectal cancer patients were vaccinated with the p53-SLP® vaccine only [6].

PATIENTS, MATERIALS AND METHODS

Patients, vaccination scheme and safety and tolerability monitoring

Colorectal cancer patients who were successfully treated with metastasectomy, chemotherapy and/or Radiofrequency Ablation (RFA) for disease metastasis to the liver and/or the lung were accrued during their follow up visits at the surgical oncology out-patient clinic into this phase I/II trial. Primary endpoint of this study was safety and immunogenicity of the p53-SLP® in combination with administration of IFN-α. The secondary endpoint was to assess whether this combination is able to induce an overall significantly stronger p53-specific Th1 response than observed in the group of patients vaccinated in our previous trial [6]. Patient eligibility criteria for in- and exclusion and the study design, including the vaccination schedule (twice vaccinated with a three week interval), were identical to those used in the previously performed clinical trial with p53-SLP® [6], with the exception that in the current study one hour after each vaccination pegylated interferon-alpha-2b (Pegintron, 1 μg/kg body weight, Schering-Plough, the Netherlands) was injected within 10 centimeters proximity to the vaccination site. Furthermore, patients were discharged within one hour after they received their Pegintron injection. The study design was approved by the Central Committee on Research Involving Human Subjects in The Hague, the Netherlands (NL24089.000.08) and by the medical ethical committee of the Leiden University Medical Center. All patients gave their written informed consent before they were enrolled in the study.

Patients were asked to monitor and report any adverse event (AE) including fever (temperature measured at home either orally or anally above $38^{O}C$). Prompted and

spontaneous AEs, injection site reactions, clinical assessments, and clinical laboratory variables were monitored during all visits as reported previously [25]. Injection site reactions were defined as pain, redness, itch and calor on a scale of 0-3 (0 being no reaction, 1 as mild, 2 as moderate and 3 as a severe reaction). Local swelling was measured bi-directionally in cm. Before each vaccination the medical history was taken and blood was drawn (both for safety and immunological assessment). In addition, the patients were physically examined before and after each vaccination.

Vaccine

The clinical-grade peptides (9 peptides of 20-30 amino acids long with an overlap of 5-14 amino acids) were synthesized at the Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center (LUMC), the Netherlands, together representing the part of the p53 protein from amino acid positions 70 to 235. In comparison to our previous trial one long peptide (i.e. the peptide with amino acid sequence 224-248) was not included in the current vaccine mixture, because yields of the synthesis and purification of this peptide were very low. At the day of vaccination, the vaccine was prepared as previously described [6].

Immunohistochemistry and evaluation

The expression of p53 by colorectal tumor cells was determined in the available paraffin-embedded metastatic tissue of the vaccinated patients by standard two-step indirect immunohistochemical staining as described previously [6]. The percentage of tumor cells expressing p53 (nuclear expression), together with internal control, was estimated and categorized into three groups: (1) expression of p53 in <25% of the tumor cells; (2) expression of p53 in ≥25% but <75% of the tumor cells; (3) expression of p53 in ≥75% of the tumor cells.

P53-peptide ELISA for IgG antibodies

Serum samples (pre-vaccination and 3 weeks after the second vaccination) of the colorectal cancer patients from both trials were subjected to a p53-peptide ELISA for detection of p53 peptide-specific immunoglobulin G (IgG). A 96-wells plate (Costar 3590) was coated overnight at 4°C with the individual p53 peptides (30-mers, 14 amino acids overlap; 50µl of 1µg/ml diluted in 0.1M carbonate/bicarbonate coating buffer; Merck, Darmstadt, Germany). Then, the plate was washed 6 times with phosphate buffered saline (PBS; Fresenius Kabi Bad Homburg, Germany) +0.05% Tween (Merck) and blocked for 1 hour at room temperature (RT) in 100µl/well PBS+0.05% Tween+0.1% bovine serum albumin (BSA; Sigma Aldrich, St Louis, MO, USA), which is assigned as blocking buffer. After 6 washings with PBS+0.05% Tween, the serum samples diluted in blocking buffer (1:100) were added to triplicate wells (50 µl/well)

and incubated at RT for 2 hours. The plate was washed again and 50 µl/well of goat anti-human IgG-Horseradish peroxidase (HRP; Southern Biotechnology, Birmingham, AL, USA) (diluted 1:3000 in blocking buffer) was added and incubated for 1 hour at RT. Finally, after the 6 washings tetramethyl-benzidine liquid substrate (50 µl/well TMB, Sigma Aldrich) was added for the colorimetric enzymatic reaction. This reaction was stopped by adding 50 µl/well of 2M H_2SO_4 (Merck) and the plate was read in an ELISA reader at 450 nm. A cut-off value was calculated to define a positive response. For this the average OD-value plus 2xSD of the triplicate wells for all 12 peptides per plate in a pre- or post-vaccination serum sample was calculated. All OD-values above this cut-off value were discarded and again the average+2xSD was calculated with the remaining OD-values. This process was repeated until all OD-values were below the last calculated cut-off value. At least 2 of the 3 OD-values per triplicate peptide test needs to be above this cut-off value, then a peptide was considered to yield a positive response, i.e. is recognized by specific IgG in the serum. A 2-fold increase of the post-vaccination serum sample over that of the pre-vaccination serum sample was considered a vaccine-induced positive response and calculated as fold induction. The average number of vaccine-induced positive responses for the individual peptides was determined in the group of patients from both clinical trials. Subsequently, the number of positive peptide reactions per patient and an overall response rate (the number of positive peptides divided by the total number of tested peptides) was calculated for both groups.

We acknowledge the concept of the Minimal Information About T-cell Assays (MIATA) reporting framework for human T-cell assays [26;27].

Cell samples

Hundred mL of heparine blood was drawn prior to vaccination and 3 weeks after the second vaccination. PBMCs were isolated using Ficoll density gradient centrifugation within 2 hours, washed with PBS, resuspended in cold Fetal Calf Serum (FCS; PAA Laboratories, Pasching, Austria) and cooled on ice for 15 minutes. After drop-wise addition in a 1:1 ratio of freezing medium (80% FCS and 20% DMSO (Sigma Aldrich)), the PBMCs were cryopreserved at 10 million per ml per vial using an automated controlled rate freezer (Cryosolutions, 's Hertogenbosch, The Netherlands), and stored in equal aliquots in a vapor phase liquid nitrogen vessel until use. The handling and storage of the PBMCs were done according to the standard operation procedures (SOPs) of the department of Oncology at the LUMC by trained personnel.

Antigens

Overlapping peptides (30-mers with 14 amino acids overlap) covering the entire p53 protein were synthesized at the department of Clinical Pharmacy and Toxicology,

LUMC, with >95% purity [28], dissolved in DMSO at 50 mg/mL and further diluted in PBS to obtain a concentration 0.5 mg/mL (in PBS/1% DSMO). The clinical-grade peptides of the vaccine were used in the immune monitoring assays. PHA (HA16; Murex BioTech,Kent, UK) and memory response mix [25;29] was taken along as a positive control.

T-cell assays and data acquisition

The PBMCs were tested for p53-specificity by a set of complementary T-cell immune monitoring assays including: IFN-γ ELISPOT, lymphocyte stimulation test (LST) and cytometric bead array (CBA), all as previously described [6]. Fresh PBMCs and T cells cultured out of the vaccination site biopsy were also subjected to the directly *ex vivo* intracellular cytokine staining (ICS) and analyzed as previously described [6]. In this study the cells were stained for the following markers: CD3, CD4, CD8, CD154, CD137, IL-2 and IFN-γ [6;30]. For a fair comparison with the results of our previous trial with metastasized colorectal patients vaccinated with p53-SLP® only, cryopreserved PBMCs from both trials were thawed and subjected to our novel ICS assay [25] under the same conditions. As higher concentrations of the peptides (i.e. 50 µg/mL) were required in this new ICS assay the non-clinical grade peptides covering the complete p53 protein were used.

Data analysis and interpretation

A positive response is predefined per assay and described previously [6]. For all T-cell assays, a vaccine-induced response was defined as at least a 3-fold increase in the response after vaccination when compared to the results before vaccination. Statistical analyses were conducted in SPSS (version 17.0 for Windows; SPSS, Inc). The Fisher's exact test or the Mann-Whitney test were used to evaluate differences in patient characteristics between patients included in the current and the previous trial with the p53-SLP® vaccine. The Mann-Whitney test was also used to evaluate the difference in number of IFN-γ-producing T cells, the level of IFN-γ production and to compare the difference in antibody responses between the two study cohorts.

Laboratory environment

The immunomonitoring assays were performed in the laboratory of the department of Clinical Oncology (LUMC) that operates under research conditions, following SOPs and using trained staff. This laboratory has participated in all proficiency panels of the CIMT Immunoguiding Program (CIP) (http://www.cimt.eu/workgroups/cip/), as well as in IFN-γ ELISPOT panels of the Cancer Immunotherapy Consortium [31;32], to validate its SOPs.

RESULTS

Patient characteristics

Eleven colorectal cancer patients were enrolled in this study, 9 of whom completed all follow-up visits. The clinicopathological characteristics are displayed in Table 1. None of these patients showed evidence of any macroscopic disease at enrollment. Six out of 9 patients were male. The average age of the 9 patients vaccinated twice, was 58 years. Over-expression of p53 in the tumor (i.e. ≥25% of the tumor cells

Table 1. Patient characteristics of patients enrolled

Patient Number	*Sex*	*Age*	*TNM*	*Location Primary*	*Location Metastasis/ Recurrence*	*Treatment*	*Clinical status (months NED)*	*P53 status*
p20	M	62	4	Ascending colon	Liver	PR/CTx/Rli RFA	Alive (15)	3
p21	M	44	1	Rectum	Liver,LR	PR/RTx/CTx/Rlu/ CTxLu	Alive (14)	2
p22*	F	57	4	Sigmoid colon	Liver	PR/CTx/Rli	Withdrew consent	X
p23	M	60	4	Rectum	Lung	PR/RTx/CTx/Rlu/ CTxlu/RFA	Alive (2)	3
p24	F	61	3	Rectum	Liver	PR/RTx/Rli/CTx/ RFA	Alive (12)	2
p25	F	50	4	Sigmoid colon	Liver	PR/Rli/CTx/RFA	Alive (12)	X
p26	M	52	4	Rectum	Liver	PR/Rli/CTx	Alive (2)	3
p27	M	60	4	Cecum	Liver	PR/Rli/CTx/ CTxli/RFA	Death (5)	1
p28*	M	64	4	Rectum	Liver	PR/RTx/CTx	Withdrew consent	X
p29	M	65	4	Rectum	Liver	PR/RTx/CTxli/ RFA/Rli	Alive (7)	1
p30	F	64	3	Ascending Colon	Liver	PR/CTx/Rli	Alive (4)	2

Abbrevations: TNM: Tumor Node Metastasis Stage at time of diagnosis primary tumor. F; female. M; male. Lr: local recurrence. RTx; neo-adjuvant radiotherapy, Pr; primary resection. CTx; (neo) adjuvant chemotherapy. CTxlu; isolated lung perfusion, CTxLi: isolated liver perfusion, Rlu; resection lung lesion. Rli; resection liver lesion. RFA: radiofrequency ablation. NED; no evidence of disease (months between second vaccination and disease recurrence or last follow up date). P53 status immunohistochemistry (IHC). 1: expression of p53 in <25% of the tumor cells; 2: expression of p53 in ≥25% but <75% of the tumor cells; 3: expression of p53 in ≥75% of the tumor cells; X: no material was available for IHC. *Patient number 22 withdrew consent after the first vaccination due to adverse event (Table 2). Patient number 28 withdrew consent before the first vaccination.

express p53) was found in 6 patients, while normal p53 expression (i.e. in <25% of the tumor cells) was observed in 2 patients (p27, p29). Of 3 patients, the p53 status was not determined because no tumor material was available (p25) or because they prematurely withdrew consent (p22, p28).

Safety of the vaccine

The AEs of all vaccinated patients are summarized in Table 2. All patients reported swelling confined to the vaccination site. In the majority of the cases the induration occurred after both vaccinations and was still present at the final check-up visit around 28 weeks after the first vaccination. The average size of these swellings at the first vaccination site was 3.3 cm and at the second site 3.5 cm. Four patients reported fever post vaccination, but it never lasted longer than 1 day. One patient also suffered from flu-like symptoms after both vaccinations for one day. Only two patients reported pain at the vaccination sites. None of the patients reported any pain, swelling or other changes of the skin at the IFN-α injection site. Only patient p22 experienced an AE exceeding grade I toxicity based on the Common Terminology Criteria (CTC) for AE version 4.0. This patient already experienced pain before vaccination in her left arm. After first vaccination, she experienced local swelling classified as an AE grade 1 and pain throughout her entire left arm, classified as an AE grade 2 and she chose to withdraw consent.

We have previously vaccinated 10 patients with the p53-SLP® vaccine but without the administration of IFN-α [6]. Clinicopathological parameters of both trial cohorts were similar (Table 3). In the current trial all patients developed ongoing swelling at either one or both peptide vaccination sites visible at the final check-up visit (28 weeks after first vaccination), which contrasts with the previous trial in which only one patient showed inflammation at the p53-SLP® injection site. In conclusion, addition of IFN-α to the p53-SLP® resulted in prolonged and increased inflammation at the vaccination site, suggesting that addition of IFN-α promotes inflammation at p53-expressing sites after injection of p53-SLP®.

p53-SLP® and IFN-α injection elicit both proliferative and IFN-γ producing p53-specific T cells

Using PBMCs isolated from blood samples taken before and after the second vaccination, three complementary T-cell assays (LST, CBA and IFN-γ ELISPOT) were performed to monitor the immunogenicity of p53-SLP® combined with IFN-α injection. After the two vaccinations, 4 (p25, p26, p29, p30) out of 9 patients showed vaccine-induced proliferative responses as determined by LST. All 4 patients responded against peptide pool 5, whereas for patients p25 and p26 also responses against peptide pool 3 and in the case of p29, against peptide pool 4 were detected (Figure 1A). Based on

Table 2. Adverse Events of patients enrolled

Patient	*AE description*	*Relation*	*Action*	*CTC*	*Type*	*Duration*
p20	-Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p21	-Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
	-Pain at both site 1,2	Possibly	No	Grade I	1	Ongoing
	-Flulike symptoms post both vaccinations, no fever	Probably	No	Grade I	2	1 day
p22	-Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	-Pain at site 1 and trough out left arm	Possibly	Yes*	Grade II	4	Ongoing
p23	-Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p24	-Swelling at site 1	Definitely	No	Grade I	1	1,5 months
	-Fever after vaccination 1	Possibly	No	Grade I	2	1 day
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p25	-Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	-Swelling at site 2	Definitely	No	Grade I	1	3 weeks
p26	-Swelling at site 1	Definitely	No	Grade I	1	Ongoing
	-Fever after vaccination 1	Possibly	No	Grade I	2	2 days
	-Swelling at site 2	Definitely	No	Grade I	1	2 months
p27	-Swelling at site 1	Definitely	No	Grade I	1	1,5month
	-Fever after vaccination 1	Possibly	No	Grade I	2	1 day
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
p29	-Swelling at site 1#	Probably	No	Grade I	1	Ongoing
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
P30	-Swelling site 1	Definitely	No	Grade I	1	Ongoing
	-Swelling at site 2	Definitely	No	Grade I	1	Ongoing
	-Fever after vaccination 2	Possibly	No	Grade I	2	1 day

Note: all adverse events (AE) recorded for each patient included in this trial during the entire follow up period. AE were detected either at site 1 (the site of the first vaccination) or at site 2 (the site of the second vaccination). No AEs were reported with respect to the injection site of either the first or the second IFN-α administration. The heading *AE* provides a description of all the AE reported on in each patient. For each EA it is stated whether there was a plausible relation of the AE to the vaccination and whether the AE required any *actions* of the trial coordinator. All AEs were graded according to the Common Terminology Criteria for Adverse Events v4.0 as published by the EORTC (www.eortc.org). Grade I implicates mild AE defined as asymptomatic or mild symptoms; clinical or diagnostic observations only; intervention not indicated. Grade II implicates moderate AE defined as minimal, local or noninvasive intervention indicated; limiting age-appropriate instrumental activities of daily living. Grade III or IV AEs were not observed during this trial. The heading *Type* reports on how the AE was diagnosed; this was either at the injection site (Type I), as a systemic response (Type 2), in the laboratory (Type 3) or otherwise such as pain which cannot be objectified (Type 4). Finally the *duration* of the AE was listed. The description "Ongoing" implicates that the AE was still present at the final follow up visit. Patient number 28 was not mentioned in this table because consent was withdrawn before vaccination 1 and therefore no AE were recorded.

The swelling did not occur until 1.5 weeks after the first vaccination. In all other cases the swelling post vaccination occurred directly or within 1 hour after vaccination.

* Because of the pain in the arm after the first vaccination p22 withdrew consent to participate in the trial. The patient stated that this exact pain had also been present before the vaccination but because of the swelling and the fear of worsening of the pre-existing symptoms, the consent was withdrawn

Table 3. Comparison of patient characteristics p53 vaccination study with and without IFN-α

Characteristic	p53-SLP® (n=10)	P53-SLP® + IFN-α (n=9)
Sex (%male)	8 (80%)	6 (68%)
Age (average, years)	61	58
TNM (%)		
1/2	0 (0%)	1 (11.1%)
3	5(50%)	2 (22.2%)
4	5(50%)	6 (66.7%)
Location primary (%)		
Cecum	1(10%)	1(11.1%)
Ascending colon	0(0%)	2(22.2%)
Transverse colon	1(10%)	0(0%)
Sigmoid colon	4(40%)	1(11.1%)
Rectum	4(40%)	5(55.6%)
Location 1st metastasis (%)		
Liver	8(80%)	8(88.9%)
Lung	1(10%)	1(11.1%)
Liver+Lung	1(10%)	0(0%)
P53 Status (IHC)*		
1	4(40%)	2(25%)
2	2(20%)	3(37.5%)
3	4(40%)	3(37.5%)
CEA (screening, average)	3,7	2.2

Abbreviations: TNM: Tumor Node Metastasis Stage at time of diagnosis primary tumor. p53 status IHC. 1: expression of p53 in <25% of the tumor cells; 2: expression of p53 in ≥25% but <75% of the tumor cells; 3: expression of p53 in ≥75% of the tumor cells. * in patients vaccinated with p53-SLP® and IFN-α there was insufficient tissue present for IHC in 3 patients. CEA: Carcinoembryonic Antigen.

our cutoff criteria, PBMCs of patient p21 displayed a proliferative response against p53 peptide pools 1 and 6 at baseline that disappeared after vaccination. Patient p27 showed a positive proliferative response against peptide pool 6 after vaccination. However, this response was not induced by vaccination as it may have already been present at baseline, although just below the cut-off. Except for patients p20 and p24, a proliferative response against the recall antigens in the memory response mix was detected both at baseline and after vaccination (data not shown).

Supernatants isolated at day 6 from the cultures of all PBMC samples tested in the LST were used for the analysis of antigen-specific production of cytokines (IFN-γ, TNF-α, IL-2, IL-4, IL-5, and IL-10) by CBA. After vaccination, 6 out of 9 patients (p20, p23, p24, 25, p26, p30) showed detectable induction of IFN-γ (median 55, average 134; range, 26 – 618 pg/mL). IFN-γ production was induced by the vaccine as shown upon stimulation of PBMCs with peptide pool 1 (p26), pool 2 (p24), pool 3 (p20, p24, p25, p26), pool 4 (p24, p30) and/or pool 5 (p20, p23, p25, p26) (Figure 1B). One patient (p21) showed IFN-γ production against peptide pool 5 following vaccination, however, also displayed production of this cytokine already prior to the vaccinations.

Moreover, the IFN-γ production at baseline was mainly found after stimulation with p53 peptide pools 1 or 6 in patients p21 and p25 (median 251, average 220; range, 23 – 505 pg/mL) (Figure 1B). Vaccine-induced production of TNF-α, albeit at very low amounts, was detected in PBMCs of patients p25, p26, and p27 (median 48, average 75; range, 22 – 175 pg/mL). IL-5 production was found in patients p20, p24, p25, p26 (median 33, average 40; range, 22 – 74 pg/mL) and IL-10 in patient p20, p24, p25 and p26 (median 28, average 30; range, 21 – 39 pg/mL) after the vaccinations. No IL-2 was

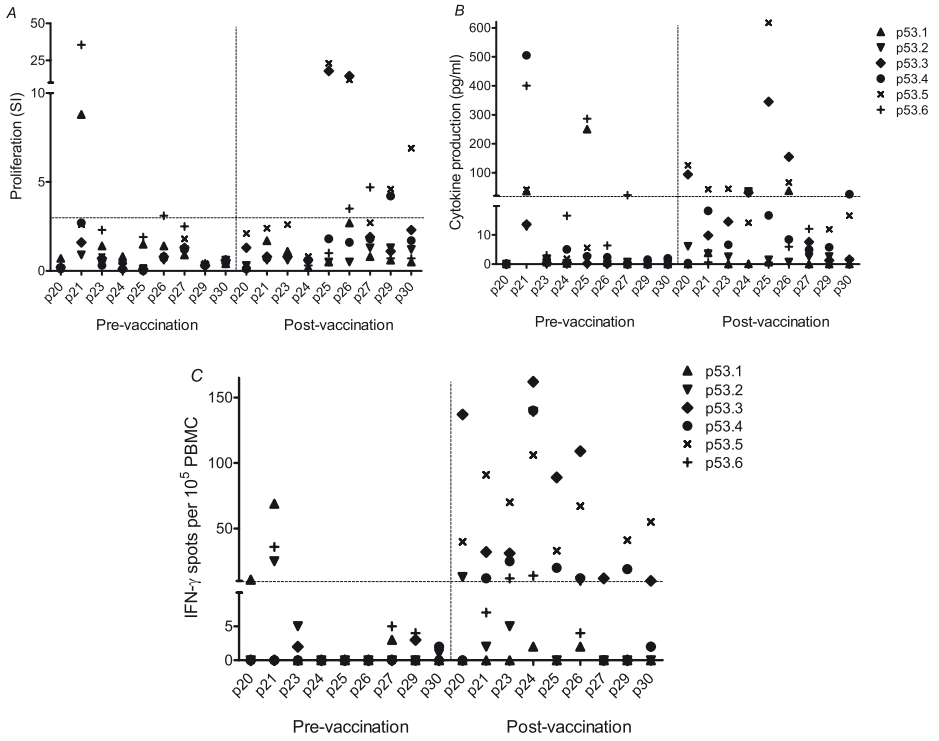

Figure 1. Results of three complementary T-cell assays with either freshly isolated PBMCs: (A) LST and (B) IFN-γ in CBA, or cryopreserved PBMCs: (C), IFN-γ ELISPOT.

Results are depicted for each individual patient (*n* = 9) before vaccination (pre-vaccination; left) and 3 weeks after vaccination (post-vaccination; right); each peptide pool is represented by a symbol. *(A)* Proliferative responses upon stimulation with the indicated peptide pools of p53 are depicted as a stimulation index (SI); a SI ≥ 3 (*indicated line*) was defined as a positive response. *(B)* Concentration of IFN-γ (pg/mL) as measured by CBA in the supernatants isolated at day 6 from the proliferation assay; production of ≥ 20 pg/mL (*indicated line*) was defined as a positive response. *(C)* IFN-γ ELISPOT results; number of T cells per 10^5 PBMCs specifically producing a spot of the cytokine IFN-γ after stimulation with the indicated p53 peptide pools are shown; antigen-specific T-cell frequencies were considered to be positive when specific T-cell frequencies were ≥ 10 of 10^5 PBMCs (indicated line).

detected, most likely because IL-2 was consumed by the cells during the 6 days of culture. In none of the cultures, IL-4 could be detected.

The IFN-γ ELISPOT assay was used to determine the number of IFN-γ producing p53-specific T cells. In all patients, p53-SLP® vaccination combined with IFN-α injection induced p53-specific T-cell responses (Figure 1C). Up to 162 specific spots per 10^5 PBMC against at least one of the vaccine-representing p53 peptide pools were found. Out of 9 patients, 6 patients (p20, p21, p23, p24, p25, p26) displayed an IFN-γ-associated T-cell response to at least 3 or 4 peptide pools that represented the vaccine (Figure 1C). In patients p20 and p21, IFN-γ-producing T cells were detected in the baseline samples mainly against peptide pools 1 and/or 6 that represented peptides outside the vaccine pool of peptides. These responses were not boosted after vaccination. Five patients displayed an IFN-γ-associated T-cell response to the positive control (memory response mix; data not shown). In contrast to patients with p53-negative tumors determined by immunohistochemstry (p27, p29), higher vaccine-induced cytokine levels were found in patients that exhibited p53-positive tumors, as determined by CBA and IFN-γ ELISPOT. In conclusion, the three immune monitoring assays showed that injection of IFN-α in close proximity of the p53-SLP® vaccine induced p53-specific IFN-γ-producing T cells in all cases.

Intracellular cytokine staining (ICS) detects p53-specific activated T-cells capable of producing IFN-γ/IL-2

To phenotype and enumerate p53-specific T cells, freshly isolated PBMCs from blood samples drawn before the first and after the second vaccination were directly *ex vivo* stimulated overnight with p53 peptide pools, followed by analysis of the expression of the T-cell markers: CD3, CD4 and CD8; in combination with the T-cell activation markers: CD137 and CD154; and cytokines: IFN-γ and IL-2 by multiparametric flow cytometry (Figure 2A). In 8 of the 9 vaccinated patients an increase in the percentage of p53-specific $CD4^+$ T cells expressing CD137 and/or CD154 was found after vaccination (Figure 2B). In general, the production of the cytokines IFN-γ (Figure 2C) and/or IL-2 (Figure 2D) in these activated cells was also boosted after vaccination. Notably, the $CD4^+$ T cells of patient p24 displayed CD137 and CD154 expression before vaccination, suggesting that this patient already had a pre-existing response to p53, however, only after vaccination these activated T cells produced IFN-γ and IL-2 (Figures 2B, 2C, 2D). In 1 out of the 9 tested patients (p20) activated $CD8^+$ T cells, which also produced IFN-γ, were detected in the freshly isolated PBMCs obtained after two vaccinations (Figure 2A).

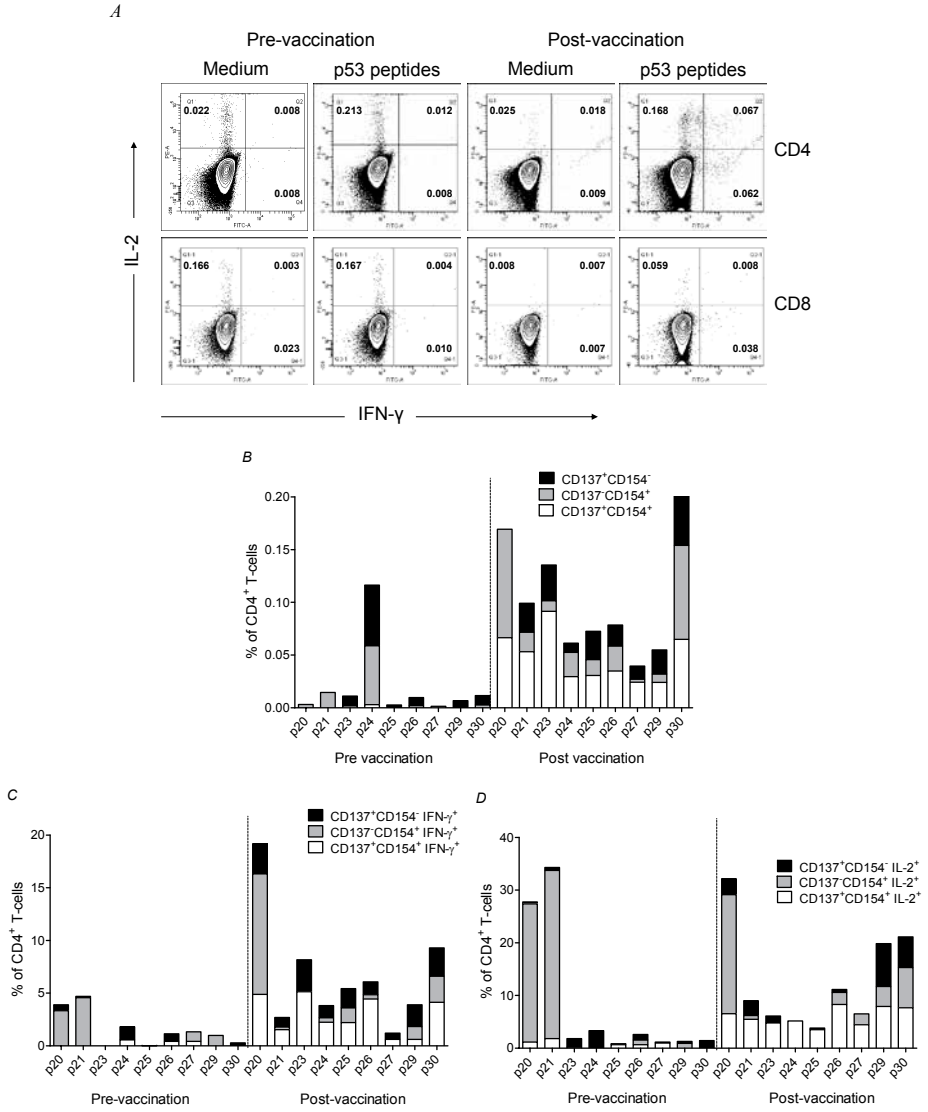

Figure 2. *Ex vivo* measurement of the percentage of p53-specific activated T cells, and their production of IFN-γ and IL-2, as determined by multiparametric flow cytometry.

Freshly isolated PBMCs of patients before the first and three weeks after second vaccination were intracellularly stained directly *ex vivo* after an overnight stimulation with medium or peptide pools p53.2 to p53.5. *(A)* depicts ICS results from patient p20 pre- and post-vaccination. Freshly isolated PBMCs were stained directly *ex vivo* after incubation in medium or stimulation with p53 peptide pools. Depicted are the IL-2 and/or IFN-γ-producing cells in the $CD3^+CD4^+$ (upper) or $CD3^+CD8^+$ (lower) T-cell population. The numbers in the quadrants indicate the percentage (%) of positive cells within this population. *(B)* displays percentages (%) of p53 specific $CD4^+$ T cells stained positively with the activation markers CD154 and/or CD137; results are depicted before (left) and after (right) vaccination. *(C and D)* displays the percentages (%) of IFN-γ^+ *(C)* and $IL2^+$ *(D)* activated $CD4^+$ T cells subdivided for expression of the activation markers CD137 and/or CD154; results are depicted before (left) and after (right) vaccination.

T cells cultured from skin biopsies harbor p53-specific reactivity

From 6 out of 9 skin biopsies taken from the second vaccination site sufficient T cells could be cultured to perform multiparametric flow cytometry using the same markers as described above for PBMCs. The $CD4^+$ T cells from 5 out of these 6 skin biopsy cultures (p25, p26, p27, p29, p30) displayed elevated expression levels (at least twice the non-stimulated sample) of the activation marker(s) upon stimulation with p53-SLP® vaccine-specific peptides (Figure 3A); a median frequency of 21% $CD137^+$ and/or $CD154^+CD4^+$ T cells could be observed in the p53-peptide stimulated samples versus 2.2% in the non-stimulated T-cell culture control. The vast majority of these activated $CD4^+$ T cells produced IFN-γ and/or IL-2 (Figures 3B and 3C). Moreover, in biopsies from 3 patients (p25, p26 and p30) both p53-specific IFN-γ producing $CD4^+$ and $CD8^+$ T cells were found (Figure 3D).

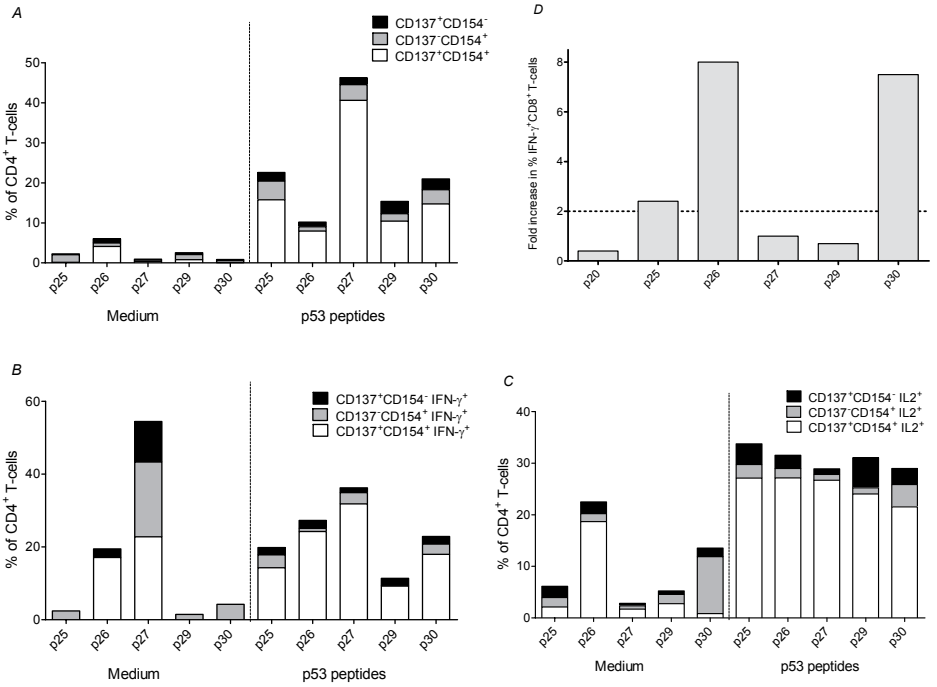

Figure 3. Measurement of the percentage of p53-specific activated T cells cultured from skin biopsies of the p53-SLP® injection site, which produce IFN-γ and/or IL-2, as determined by multiparametric flow cytometry.

These cultured T cells were incubated in medium or stimulated with peptide pools p53.2 to p53.5. *(A)* displays percentages (%) of $CD4^+$ T cells stained positively for the activation markers CD154 and/or CD137. *(B and C)* displays the percentages (%) of IFN-γ^+ *(B)* and $IL2^+$ *(C)* activated $CD4^+$ T-cells subdivided for expression of the activation markers CD137 and/or CD154. *(D)* depicts the ratio of the percentage (%) of IFN-γ^+CD8^+ T cells of p53 peptides stimulated versus medium incubated T cells. The line indicated a 2-fold increase; ≥ 2-fold increase is defined as a positive response.

Addition of IFN-α to p53-SLP® results in increase of IFN-γ producing p53-specific CD4+ T cells

The characteristics of the two patient cohorts vaccinated in the current and our previous trial are generally similar (Table 3), thereby allowing us to compare the p53-specific immune responses after administration of the two different vaccine modalities in terms of their immunogenicity, with the limitation that the two vaccine modalities were not directly compared in the same trial.

To enable comparison of the results with those from the first trial, available cryopreserved PBMCs from patients in the first clinical trial (i.e. p01, p02, p03, p04, p07, p10, p11), vaccinated with p53-SLP® only, and cryopreserved PBMC samples from the patients in the current trial were thawed and subsequently head-to-head tested in a direct ex vivo ICS assay, optimized for detecting both antigen-specific CD4+ and CD8+

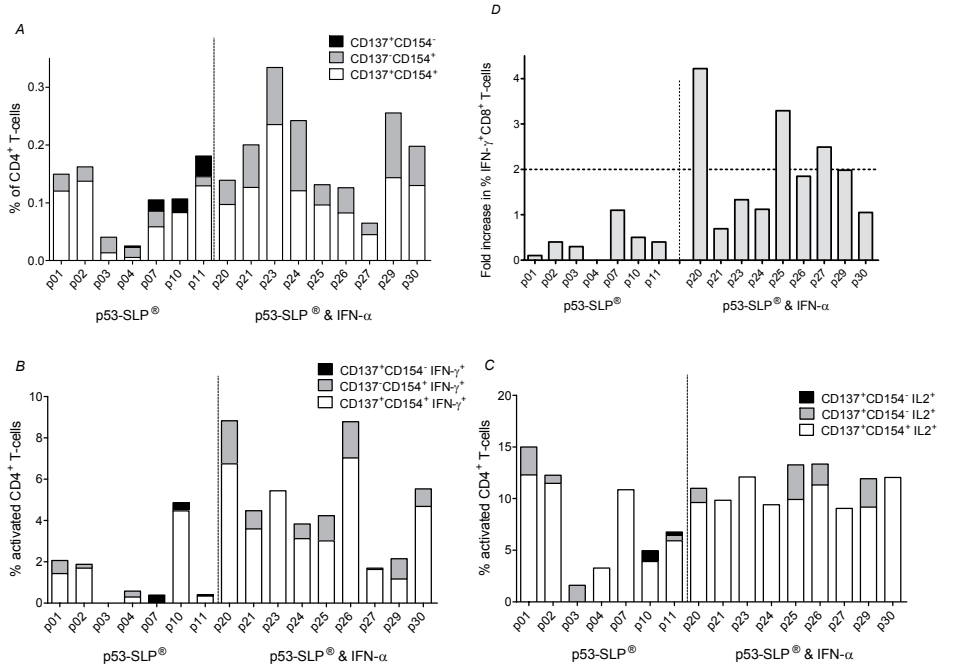

Figure 4. Results from patients injected with p53-SLP® and IFN-α compared to the results from colorectal cancer patients injected with p53-SLP® only.

(A) The bar plot shows a graphical comparison of the percentages of activated CD4+ T-cells (CD137+CD154- in black, CD137-CD154+ in gray and CD137+CD154+ in white) between the two vaccination studies. *(B and C)* displays the percentages (%) of IFN-γ+ *(B)* and IL2+ *(C)* activated CD4+ T-cells subdivided for expression of the activation markers CD137 and/or CD154. *(D)* depicts the ratio of the percentage (%) of IFN-γ+CD8+ T cells of p53 peptides versus medium stimulated PBMC. The line indicated a 2-fold increase; ≥ 2-fold increase is defined as a positive response. *(A-D)*: results of patients vaccinated with only p53-SLP® (left) and vaccinated with p53-SLP® and IFN-α (right)

T-cell responses in one single cryopreserved PBMC sample using long overlapping peptides as antigens [25] (Figures 4A-D). In PBMCs from patients receiving p53-SLP® in combination with IFN-α not only significantly more $CD154^+CD4^+$ were found (p=0.002), but also a significantly higher frequency of these activated $CD4^+$ T-cells produced IFN-γ (p= 0.008), when compared to the activated $CD4^+$ T cells isolated from patients that received the p53-SLP® vaccine only. Importantly, in 4 out of 9 patients (p20, p25, p27 and p29), who received p53-SLP® and IFN-α, also low numbers of IFN-γ-producing $CD8^+$ T-cells were found, while patients vaccinated with p53-SLP® only, showed no p53-specific $CD8^+$ T-cell reactivity (Figure 4D). Of note, cryopreserved PBMCs, stored in the vapour phase of liquid nitrogen, reacted similarly over a period of at least 4 years indicating that the influence of cryopreservation time is unlikely (data not shown).

ELISPOT plates from the previous trial were reanalyzed using the same ELISPOT reader conditions as the current trial to obtain a fair comparison. Addition of IFN-α to the p53-SLP® clearly results in a broader response per vaccinated patient (Figure 5A). Patients that were injected with both p53-SLP® and IFN-α showed a significantly higher median frequency of IFN-γ producing T cells after vaccination (p=0.018) compared to patients that received the p53-SLP® only vaccine (Figure 5B). These data recapitulate the results obtained in the direct ex vivo ICS assay conducted on cryopreserved PBMCs.

Comparison of the IgG responses to p53 in serum of p53-SLP® vaccinated patients with and without IFN-α

In order to analyze whether vaccination also resulted in the induction of a p53 peptide-specific antibody response we developed a p53 peptide-specific ELISA and subsequently analyzed the sera of the patients obtained prior to the first and after the second vaccination. These analyses were performed simultaneously on the sera obtained from patients participating in the current trial and our previous vaccination study. In the current trial p53-specific IgG antibody responses were detected in 7 out of the 8 patients of whom both serum samples (pre- and post-vaccination) were available. In one patient (p29) no antibody response to p53 was detected. On average, the number of p53 peptides to which IgG antibodies were detected in these 8 patients was 2.3 (range 0-5), with an obvious peak in the recognition of those peptides that were present in the vaccine (peptides 9-15; Figure 6). In sera of 3 out of the 9 tested patients from our previous colorectal cancer trial p53-specific IgG responses were detected. Here on average the number of peptides recognized was 0.4 (range 0-2). Patients in the current trial recognized significantly more peptides than those from the first trial (p=0.02). The results of the ELISA therefore indicate a broader p53-specific IgG response by the addition of IFN-α to the p53-SLP® vaccine.

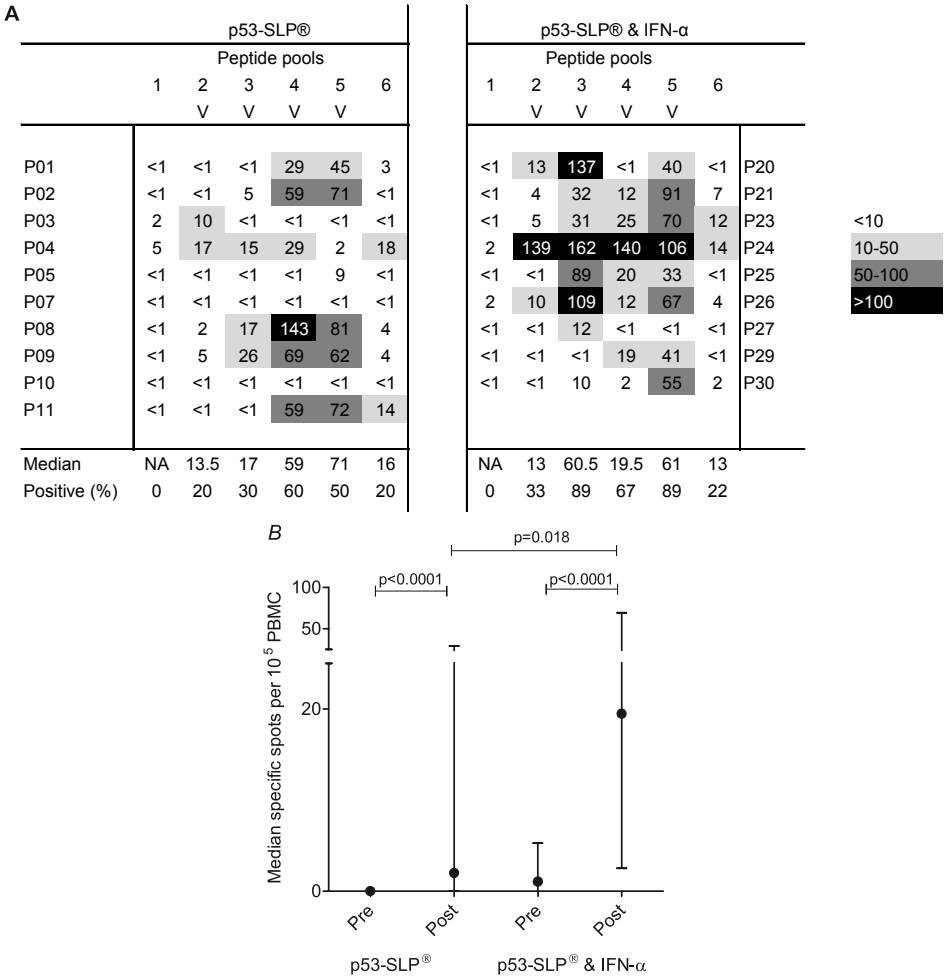

Figure 5. Comparison of IFN-γ ELISPOT results in colorectal cancer patients vaccinated with p53-SLP® only or in combination with IFN-α.

(A) The heat map reflects the IFN-γ ELISPOT results from both trials. The ELISPOT plates of the first trial (p53-SLP® only) were reanalyzed with the same settings of the reader as the current trial (p53-SLP® and IFN-α). The number of positive spots per 10^5 PBMC is given for every patient. Every value is colored in relation to the number of positive spots per 10^5 PBMC. White corresponds with a count of < 10 positive spots 10^5 PBMC, light grey ≥ 10 and < 50, dark grey ≥ 50 and < 100 and black ≥ 100 spots per 10^5 PBMC. On the bottom of the heat map the median and the percentages of the positive responses are given per peptide pool. Peptide pools represented by the vaccine are indicated by a 'V'. *(B)* the median (plus interquartile range) of all specific spots as determined by IFN-γ ELISPOT in the two trials before and three weeks after the second vaccination are compared. Not only the number of specific spots in both cohorts was significantly higher after vaccination compared to pre-vaccination, this increase in specific spots was significantly better in the patients that also received the IFN-α injections besides the p53-SLP® vaccine. *(A-B)*: results of patients vaccinated with only p53-SLP® (left) and vaccinated with p53-SLP® and IFN-α (right).

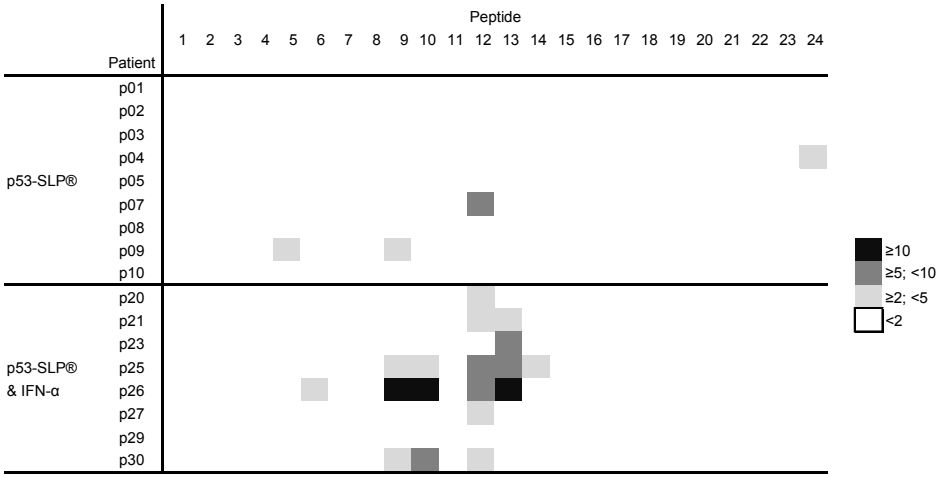

Figure 6. The heat map represents the number of peptides that were recognized by the individual patients based on their specific IgG antibody responses.
The peptides are listed on the x-axis, the individual patients on the y-axis. Peptides are numbered and cover the whole p53 protein sequence. Patients p1-p10 represent the study cohort of the first trial, who received solely p53-SLP® injections, patients p20-p30 represent the study cohort of the second trial in which patients received both p53-SLP® and IFN-α injections. Negative responses are white (< 2); positive responses are displayed in light grey: ≥ 2 and < 5, dark grey ≥ 2 and < 5-fold and black ≥ 10-fold increase of p53-specific IgG response after vaccination compared to prior to vaccination. There is an obvious peak, mainly present in the second cohort, representing the recognition of peptides that were actually covered by the vaccine (amino acids 70 - 235 or 248)

DISCUSSION

Results from previous studies suggest that colorectal cancer vaccines should aim at inducing strong type 1-associated immunity to obtain a clinical response [11;33-35]. Although in patients, vaccination with the p53-SLP® resulted in the induction of p53-specific $CD4^+$ T-cell immunity, the production of Th1-associated cytokines such as IFN-γ and IL-2 was probably too low to become truly effective [6]. Combining vaccines with immune modulating adjuvants should allow polarization of the vaccine-induced immune response. Here we show that the clinical grade p53-SLP® vaccine combined with IFN-α induced p53-specific Type 1-polarized $CD4^+$ and $CD8^+$ T-cell responses in all and 6 of 9 colorectal cancer patients, respectively.

We have previously shown that the p53-SLP® vaccine was safe [6;7]. The addition of IFN-α in the current trial also resulted in no serious AEs. However, in contrast to vaccination with p53-SLP® only [6], this time all vaccinated patients showed long lasting local swelling and inflammation of at least one of the peptide but not the IFN-α injection sites. As the groups of vaccinated patients were highly comparable

(Table 3), this suggests that addition of IFN-α potentiates inflammation at the vaccination sites where p53 antigen is present, thereby improving the antigen presentation conditions and subsequently the priming of T cells.

The vaccine-induced p53-specific antibody and cellular response of patients vaccinated with the combination p53-SLP® and IFN-α were compared with those of patients vaccinated with p53-SLP® only. They were analyzed in a head-to-head comparison of cryopreserved PBMC samples by ICS and serum samples in the peptide ELISA assay. Although, these materials were obtained in two independent trials, they were similarly isolated from successfully treated colorectal cancer patients with highly comparable disease state and preserved under the same conditions. Within these limitations, our results indicate that addition of IFN-α to p53-SLP® induces an immune response against a broader range of peptide pools and also a higher frequency of vaccine-specific activated IFN-γ producing T cells. Addition of IFN- α to p53-SLP® also increased the amount of p53-specific IgG antibodies, indicating the underlying improved Th cell induction.

In the current trial, one of the peptides was excluded from the original p53-SLP® vaccine composition, due to low yield of purified material of this particular long peptide [6]. The peptide not included was the last 13 amino acid overlapping peptide from the C-terminal section of the p53 sequence used in the previous vaccination trial [6]. Our data comparing p53-specific T-cell responsiveness was not focused on the measurement of responses to individual peptides, therefore, it is difficult to estimate how the exclusion of this specific peptide altered the immunogenicity of the vaccine. However, in the current trial the responsiveness after vaccination was significantly increased compared to the previous trial, despite the lack of this one peptide.

In the literature, it has been suggested that the p53-specific $CD8^+$ T-cell repertoire is severely restricted due to self-tolerance [36;37]. Consequently, p53-specific vaccination will result mainly in the induction of p53-specific high affinity $CD4^+$ T-cells and low affinity $CD8^+$ T cells. Our results indicated that addition of IFN-α might have increased the number of p53-specific $CD8^+$ T cells as we were able to detect them in 6 out of 9 patients from the present trial and in none of the patients from the previous trial. The 6 patients with p53-specific $CD8^+$ T cells included the following 4 patients: one patient (p20) who showed a response when PBMC were freshly tested and the others (p25, p26 and p30) displayed p53-specific $CD8^+$ T cells in the cells cultured from the biopsy of the vaccine site. Most $CD8^+$ T-cells responses were found in cryopreserved PBMCs (p20, p25, p27 and p29). The reason why we were better able to detect p53-specific $CD8^+$ T cells in the cryopreserved samples lies in the fact that the ICS assay used for analyzing thawed PBMC is optimized to detect antigen-specific $CD8^+$ T cells by using 10-fold higher concentrations of the long peptides as antigens.

It also differs from the assay used to analyze the fresh PBMC samples by the addition of TLR3 agonist poly I:C to activate the peptide-loaded antigen presenting cells [25].

Together, we have found that combining p53-SLP® with IFN-α injection results in enhanced inflammation, p53-specific type 1-polarized $CD4^+$ and $CD8^+$ T-cell responses. We have not studied the effect on DC activity, therefore we can only speculate on the exact function of IFN-α. However, from literature it is clear that IFN-α improves antigen cross-presentation [38] and enhances survival of activated T cells [39]. A recent study also found a reduction in regulatory T cells following high-dose IFN-α [40].

We can conclude that the addition of IFN-α clearly induces both a qualitatively and quantitatively better p53-specific T-cell response compared to p53-SLP® vaccination alone. These data provide support to the notion of combining cancer vaccines with immune modulating agents such as IFN-α to augment and polarize the vaccine-induced immune response. However, the minimal requirements of a vaccine-induced immune response in order to obtain a clinical response are undefined. Therefore it is tempting to perform an efficacy study with p53-SLP® combined with IFN-α to determine whether the strength and quality of the response are good enough to prevent recurrence or metastasis in stage II and stage III colorectal cancer patients, who have not yet developed any kind of distant metastasis at the time of vaccination.

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