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ABSTRACT

**Purpose:** The tumor-associated self-antigen p53 is commonly over-expressed in cancer, including colorectal cancer, and can serve as a target for immunotherapy. The safety and immunogenicity of a p53 synthetic long peptides (p53-SLP®) vaccine was investigated in patients treated for metastatic colorectal cancer.

**Experimental design:** Ten patients were vaccinated twice with a set of ten overlapping p53-SLP® in a phase I/II trial. Both safety as well as the breadth, magnitude and polarization of vaccine-induced p53-specific T cells was evaluated in blood samples drawn before and after vaccination by IFN-γ ELISPOT, proliferation, cytokine secretion and multi-parameter flow cytometry. The migratory capacity of p53-specific T cells was evaluated by assessing their presence in a biopsy of the second vaccination site.

**Results:** Toxicity was limited to grade I/II, mostly at the vaccination site. P53-specific T-cell responses were induced in 9 out of 10 colorectal cancer patients as measured by IFN-γ ELISPOT, proliferation and cytokine bead array. In 6 out of 9 tested patients, p53-specific T-cell reactivity persisted at least six months. Furthermore, p53-specific T cells isolated from the vaccination site were characterized as CD4+ T cells producing both T-helper (Th) type 1 and Th2 cytokines upon stimulation with p53 peptide and p53 protein. Multi-parameter flow-cytometry revealed that only a minor population of the p53-specific CD4+ T cells was optimally polarized.

**Conclusions:** The p53-SLP® vaccine is safe and capable to induce p53-specific T-cell responses in patients treated for colorectal cancer. New trials should focus on improving the polarization of the p53-SLP® vaccine induced T-cell response.
INTRODUCTION

Colorectal adenocarcinoma is the third most common cancer and the second most frequent cause of death due to cancer. Despite treatment approximately 45% of all colorectal cancer patients die within 5 years. Efforts to improve survival in patients with advanced colorectal cancer have had limited success indicating a high need for new treatment modalities, which may include immunotherapy.

Mutations in either the p53 tumor suppressor gene itself or in genes regulating p53 activity are found in a wide variety of tumors, including colorectal cancers, leading to aberrant expression of p53. Because p53 is not expressed at the cell surface, only p53-specific T-cell immunity is likely to exert therapeutic antitumor effects. Wild-type p53-specific cytotoxic T lymphocytes (CTL) and T-helper (Th)-cells have been detected in PBMC cultures in vitro. In addition, wt.p53-specific proliferative responses were demonstrated in patients with breast cancer, ovarian cancer and colorectal cancer. There are strong indications that the p53-specific CD8+ T-cell repertoire is severely restricted by self tolerance, as high-avidity self-reactive T cells are deleted in the thymus leaving available only CD8+ T cells with a low avidity T-cell receptor. In contrast, the CD4+ T-cell repertoire is not affected, presumably because the low expression levels and rapid breakdown of p53 in the thymus disfavor presentation by MHC class II. Even in the case of MHC class II-negative cancers, the availability of p53-specific CD4+ T cells is important in cancer immunotherapy because IFNγ secreting CD4+ Th1-cells play an important role in orchestrating and sustaining the local immune attack by CD8+ CTL and innate immune effector cells. Indeed, adoptively transferred p53-specific CD4+ Th-cells supported the anti-tumor response against p53 over-expressing tumors. Moreover, Th1-cells can activate peritumoral DC, which generally display an immature phenotype, a requirement for DC to be able to launch an effective CTL response against one or more unique tumor antigens that are present in tumor cells. Analyses of the p53-specific CD4+ T-cell repertoire in patients undergoing colorectal carcinoma resection revealed that these responses were weak and required at least one round of in vitro stimulation. Examination of the cytokines produced by these Th-cells revealed that the majority of the proliferative p53-specific T-cell cultures failed to produce any of the key cytokines (IFNγ, TNFα, IL-4, IL-5 and/or IL-10), indicating that tumor-induced p53-specific Th-responses are not properly polarized. Interestingly, the presence of circulating IFNγ-producing p53-specific CD4+ T cells was associated with a stronger CD8+ T-cell infiltration of the tumor, suggesting that the induction of a strong p53-specific Th1-response may enhance the efficacy of the anti-tumor response.
Several different antigen delivery systems have been tested to immunize patients against p53. In previous studies an adenoviral vector encoding wt.p53, recombinant canarypox virus encoding wt.p53, or adenoviral vector encoding wt.p53 transfected DCs were used. These modalities were safe and capable of stimulating p53-specific T-cell responses in some of the vaccinated patients. Unfortunately, presence and enhancement of anti-vector immunity were found in almost all patients, which may have hampered the induction of a truly effective p53-specific T-cell response. In addition, DC pulsed with known p53 HLA-A2.1 binding peptides have been used and this resulted in safe induction of specific T-cell responses against p53 peptides in some of the treated patients, but has the disadvantage that patients with other HLA types can not be treated.

Recently, we have developed the concept of using synthetic long peptides (SLP) as vaccines. When injected, these SLP are predominantly taken up by DC resulting in the presentation of both helper T-cell epitopes and CTL epitopes that are present in the SLP. The efficacy of SLP vaccines to induce truly strong tumor-specific CD4+ and CD8+ T-cell responses was demonstrated in rodents therapeutically treated for human papillomavirus induced tumors as well as in patients with cervical cancer. In parallel, we have developed a SLP vaccine for the induction of p53-specific T-cell immunity. Injection of p53-SLP® resulted in a strong p53-specific CD4+ T-cell response to three different epitopes in mice. Here, we have performed a phase I/II trial with as primary endpoint the study of the safety and immunogenicity of the p53-SLP® vaccine in patients treated for metastatic colorectal cancer.

PATIENTS, MATERIALS AND METHODS

Patients and vaccination scheme

Patients treated for colorectal cancer metastasis were accrued into this phase I trial between January 2007 and March 2008 after oral and written informed consent. Primary endpoint of this study was safety and immunogenicity, secondary endpoint was tumor reactivity. Based on our previous clinical study, in which 2 out of 5 patients injected with canarypoxvirus with human wt.p53 mounted a T-cell response, and based on our animal studies, in which the p53-SLP® vaccine was able to induce immunity in all mice, as well as on the high number of cancer patients responding in our HPV16-SLP studies, it was expected that sufficient subjects in a group of 10 patients will show a p53 specific immune response to report on safety and immunogenicity. Eligibility required the following criteria: (a) performance status of WHO 0 to 1; (b) pretreatment laboratory findings of leukocytes >3 x 10^9/L, lymphocytes >1 x 10^9/L, platelets >100 x 10^9/L, hematocrit >30%, and hemoglobin >6
mmol/L; (c) no radiotherapy, chemotherapy, or other potentially immunosuppressive therapy administered within four weeks before the vaccination; (d) no history of autoimmune disease or systemic disease which might affect immunocompetence; (e) no other malignancies (previous or current), except adequately treated basal or squamous cell carcinoma of the skin; (f) HIV and hepatitis B seronegative and (g) a life expectancy of more than 6 months. The patient characteristics are summarized in Table 1. The study design was approved by the medical ethical committee of the Leiden University Medical Center and registered to the ISRCTN (ISRCTN43704292). After written informed consent, a screening visit was performed and after enrollment the patients were subcutaneously vaccinated two times with a 3-week interval. At baseline and 3 weeks after the last vaccination, 200 mL blood was drawn for both immunomonitoring and assessment of hematologic values and organ function markers. In addition, 3 weeks after vaccination a biopsy (4 mm) of the second vaccination site was taken. Furthermore, during the trial smaller blood samples (60 mL) were drawn for assessment of hematologic values and organ function markers. Approximately 6-9 months after vaccination a third blood sample was drawn for immunomonitoring. For clinical monitoring a CT-scan was made before and after vaccination and the serum tumor-marker carcinoembryonic antigen (CEA) was determined at several different time points during the whole trial. The vaccination scheme is depicted in figure 1.

![Figure 1. A schematic overview of the vaccination scheme.](image)
electrocardiogram (ECG), case report form (CRF).
<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>TNM stage</th>
<th>Primary treatment</th>
<th>Recurrences</th>
<th>Secondary treatments before vaccination</th>
<th>Clinical status (months after vaccination)</th>
<th>PS3 expression</th>
<th>Adverse events</th>
</tr>
</thead>
<tbody>
<tr>
<td>P01</td>
<td>M</td>
<td>71</td>
<td>4</td>
<td>PR+RLi</td>
<td>1 Lu</td>
<td>RLu</td>
<td>Alive, Rec (17)</td>
<td>4</td>
<td>flu-like symptoms (2X), atrial fibrillation</td>
</tr>
<tr>
<td>P02</td>
<td>M</td>
<td>54</td>
<td>3</td>
<td>PR+Ro</td>
<td>1 Li</td>
<td>RLi</td>
<td>Alive, NED (13)</td>
<td>2</td>
<td>Pain vaccination sites (2X)</td>
</tr>
<tr>
<td>P03</td>
<td>M</td>
<td>62</td>
<td>4</td>
<td>PR+RLi</td>
<td>-</td>
<td>-</td>
<td>Alive, NED (15)</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>P04</td>
<td>F</td>
<td>57</td>
<td>3</td>
<td>PR+A</td>
<td>1 Lu</td>
<td>RFLu</td>
<td>Alive, NED (12)</td>
<td>0</td>
<td>Pain vaccination site (1X)</td>
</tr>
<tr>
<td>P05</td>
<td>M</td>
<td>67</td>
<td>4</td>
<td>PR+Ro</td>
<td>3 Li</td>
<td>RLi (1x); RFLi (2x)</td>
<td>Alive, Rec (11)</td>
<td>1</td>
<td>-</td>
</tr>
<tr>
<td>P07</td>
<td>M</td>
<td>64</td>
<td>4</td>
<td>PR+RLi</td>
<td>3 Li; Li; Lu</td>
<td>RLi+C; RLi+RFLi; RLu</td>
<td>Alive, NED (7)</td>
<td>0</td>
<td>swelling + erythema injection site (2X)</td>
</tr>
<tr>
<td>P08</td>
<td>F</td>
<td>58</td>
<td>3</td>
<td>PR+Ro</td>
<td>1 Li</td>
<td>C+RFLi</td>
<td>Alive, Rec (3)</td>
<td>3</td>
<td>swelling + erythema injection site (2X)</td>
</tr>
<tr>
<td>P09</td>
<td>M</td>
<td>59</td>
<td>3</td>
<td>PR+Ro</td>
<td>2 Li</td>
<td>C+RLi; C+RLi</td>
<td>Alive, Rec (3)</td>
<td>4</td>
<td>flu-like symptoms + swelling + erythema injection site (1X), prostatitis</td>
</tr>
<tr>
<td>P10</td>
<td>M</td>
<td>69</td>
<td>3</td>
<td>PR+AA</td>
<td>2 Li</td>
<td>RLi; RFLi</td>
<td>Alive, Rec (3)</td>
<td>4</td>
<td>Pain + swelling vaccination site (1X)</td>
</tr>
<tr>
<td>P11</td>
<td>M</td>
<td>50</td>
<td>4</td>
<td>C+RLi</td>
<td>-</td>
<td>-</td>
<td>Alive, NED (1)</td>
<td>0</td>
<td>swelling + erythema injection site + itching (2X)</td>
</tr>
</tbody>
</table>

All adverse events of the vaccine were temporarily. The swelling at the injection site was only painful direct after injection, lasted approximately 15 minutes and probably due to the adjuvant Montanide ISA-51 in the vaccine (not in Table). 1 Seven months after 1st follow up lung metastases were found that were resected and treated by isolated lung perfusion; 2 Direct after vaccination two metastases in liver that were resected; 3 PS3 expression 0=absent; 1 >0-25%; 2= 25-50%; 3= 50-75%; 4= >75. The current clinical status is given, and the time period (in months) after enrollment between brackets. Abbreviations: Female (F), Male (M); Primary resection (PR), Adjuvant chemotherapy (A), pre-operative radiotherapy (Ro), Resection tumor lungs (RLu), Resection tumor liver (RLi), RFA lung lesion(s) (RFLu), RFA liver lesion(s) (RFLi), Chemotherapy (C), Liver (Li), Lungs (Lu), No evidence disease (NED), Recurrence (Rec)
Vaccine
The vaccine consisted of 10 overlapping peptides, together representing the p53 protein from amino acid 70 to 248. This region is recognized by T cells of all colorectal patients displaying p53-immunity\textsuperscript{11;12}, and it harbors most of the published MHC class I and class II epitopes (reviewed in \textsuperscript{40}). The clinical-grade peptides (10 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 as previously described \textsuperscript{39}. At the day of vaccination, the 10 peptides (0.3 mg/peptide) were dissolved in Dimethylsulfoxide (DMSO), admixed with Phosphate Buffered Saline (PBS) and emulsified in Montanide ISA-51 adjuvant in a total volume of 2.7 ml (DMSO:PBS:Montanide 20:30:50, v/v/v). The dose of the peptides used and the number and schedule of vaccinations were based on our previous observations in mice \textsuperscript{13;36} and patients vaccinated with an HPV16-SLP vaccine \textsuperscript{38;39}\ The results in the latter two studies indicated that two vaccinations were sufficient to induce a strong T-helper type 1 response in patients with cancer \textsuperscript{38;39}

Safety and tolerability monitoring
At the day of vaccination, the patients were under observation in the hospital until 3 hours after vaccination. After the second vaccination, patients were seen at least once approximately every 4 months as part of their regular follow up visits to the hospital. Prompted and spontaneous adverse events, injection site reactions, clinical assessments, and clinical laboratory variables were monitored. Injection site reactions were defined as induration, erythema, and tenderness. In addition to their medical history, the patients were examined hematologically and physically before and after each vaccination. An electrocardiogram was made before and 3 hours after vaccination. Further vital sign examination included temperature, pulse, blood pressure, oxygen saturation, and respiratory frequency before and at 1, 2 and 3 hours after vaccine administration.

Immunohistochemistry and evaluation
The expression of p53, HLA class I and HLA class II was determined in the available primary and metastatic paraffin-embedded tissue of the vaccinated patients by standard two-step indirect immunohistochemistry, as described previously \textsuperscript{41;42}. The following primary antibodies were used: anti-p53 (clone DO-7, 1:500, DAKO), anti-HLA class I (EMR 8-5, 1:250, MBL) and anti-HLA-DP/DQ/DR (clone CR3/43, 1:100, DAKO). Secondary anti-Mouse HRP EnVision+ (K400111, DAKO) was used. The percentages of the tumor cells expressing p53 (nuclear expression), HLA class I and HLA-DP/DQ/DR (both membranous expression) were estimated in each case. Tissue stroma, lymphocytes and endothelium served as a positive internal control for HLA expression.
Analysis of p53-specific T cells by IFNγ-ELISPOT, lymphocyte proliferation assay and cytokine polarization analysis

T cells from peripheral blood mononuclear cells (PBMC) or skin biopsies were isolated and cultured as previously described \(^{39,43}\) and either directly used or cryopreserved. A set of six pools of long overlapping peptides, indicated by the first and last amino acid in the p53 protein were used for the screening of T-cell responses: p53.1: 1-78, p53.2: 70-115; p53.3:102-155, p53.4:142-203; p53.5:190-248, p53.6:241-393. Peptide pools p53.2-p53.5 represented the area included in the vaccine, while the other two peptide pools p53.1 and p53.6 represented the remaining part of p53. As a positive control, PBMC were cultured in the presence of a recall antigen mixture, the memory response mix (MRM) \(^{44}\). Analysis of p53-specific T-cell responses from PBMC were done using IFNγ enzyme-linked immunospot (ELISPOT), proliferation assay (6 days for PBMC and 3 days for the T-cells cultured out of the skin biopsy) and supernatants isolated on the last day of the proliferation assay were subjected to a Th1/Th2 inflammation cytokine bead array (CBA) kit (BD Biosciences), as previously described \(^{29,39}\). Specific spots in the ELISPOT were calculated by subtracting the mean number of spots + 2 x SD of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were considered to be positive when specific T-cell frequencies were ≥1 of 10,000 PBMC \(^{29,39}\). The average proliferation and SD of the eight medium only wells (negative control) were calculated, the cut-off of the proliferation assay was defined as this average plus 3xSD. The stimulation index was calculated as the average of eight tested wells divided by the average of the medium control wells. A positive proliferative response was defined as a stimulation index of at least 3, and the counts of at least six of the eight wells must be above the cut-off value \(^{39}\). Positive antigen-specific cytokine production as determined by CBA was defined as a cytokine concentration above the cut-off value and >2x the concentration of the medium control \(^{39}\). According to the manufacturer, the proposed detection limit for the CBA was 20 pg/mL for tumor necrosis factor-α (TNFα), interleukin (IL)-10, IL-5, IL-4, and IL-2. We deviated with respect to the cut-off value of IFNγ (set to 50 pg/mL) because the standard curve showed linearity starting at a concentration of 50 pg/mL. A vaccine-induced response was defined as at least a 3-fold increase in response after vaccination compared to the baseline sample.

Detection of IFNγ, IL-2 and IL-5 production by p53-specific T cells using flow cytometry

PBMC were either directly ex vivo used for intracellular cell staining or 10 days presensitized using the peptides in pools p53.2-p53.5 (2.5 μg/peptide/mL) as previously described \(^{43}\). T cells from the biopsy were directly tested ex vivo. Then the cells were stimulated overnight with the indicated antigens while the Golgi-mediated secretion
of cytokines was inhibited by the addition of Brefeldin A (Sigma). After fixation cells were permeabilized and prepared for multicolor flow analysis using the following primary antibodies: anti-CD3 Pacific Blue (clone UCHT1, BD PharMingen), anti-CD8 PerCP (clone SK1, BD PharMingen), anti-CD4 PEcy7 (clone SK3, BD PharMingen), anti-CD154 PEcy5 (clone TRAP1, BD PharMingen), anti-CD137 APC (clone 4B4-1, BD PharMingen), anti-IFN\(\gamma\) FITC (clone 45.B3, BD PharMingen), anti–IL-5 PE (clone JES1-39D10, BD PharMingen) and anti–IL-2 PE (clone MQ1-17H12, BD PharMingen). The presence of p53-specific T cells was considered to be positive when the percentage of p53-peptide stimulated CD4+CD154+ (activated) T cells or CD8+CD137+ (activated) T cells was at least twice the percentage detected in the medium only control, and the responding cells should be visible as a clearly distinguishable population in the plot of the flow cytometer. The percentage of IFN\(\gamma\) and/or IL-2 producing p53-specific T cells was determined by gating on the activated cell population.

**Detection of p53-specific CD4+CD25+Foxp3+ T cells**

The detection of p53-specific CD4+CD25+Foxp3+ T cells was performed as reported previously. Briefly, PBMC (1-2 x 10^6) were cultured for 10 days in medium only or in the presence of pooled p53-peptides (5 μg/peptide/mL). Then, the cells were harvested and 2 x 10^5 cells were stained for the surface markers CD25 (anti-CD25 FITC; clone M-A251, BD Pharmingen), CD4 (anti-CD4-APC; clone RPA-T4, BD Pharmingen) and CD8 (anti-CD8 PerCP; clone SK1, BD Pharmingen) before these cells were fixed, permeabilized, blocked with 2% normal rat serum and then stained with anti-human Foxp3 (PCH101) antibody or rat isotype IgG2a control. As a positive control a previously isolated HPV16-specific CD4+CD25+Foxp3+ regulatory T-cell clone (C148.31) and as negative control a HPV16-specific CD4+CD25+Foxp3+ T-cell clone (C271.9) were used. The fluorescence intensity of these two control clones was used to set the gates for the other samples in which the Foxp3 positivity of the stimulated polyclonal T-cell populations was analyzed. An antigen-induced up regulation of Foxp3 or CD25 was defined as at least twice the percentages of Foxp3 or CD25 positive cells in the medium only control, and a vaccine-induced increase in Foxp3 positive cells was defined as at least a 3-fold increase compared to the percentages of the baseline sample for the same condition.
RESULTS

Safety of the vaccine

A total of ten patients were vaccinated at least one month after their last treatment for metastatic colorectal cancer. The patients showed no macroscopic tumor lesions in abdomen or thorax at enrollment. The average age of the patients was 61 years (Table 1). Analyses of HLA and p53-expression in both primary tumor and metastases revealed the expression of HLA class I in at least 50% of all tumor cells and the complete absence of HLA class II on tumor cells. Over-expression of p53 was <25% of all tumor cells in patients p04, p05, p07, and p11 (Table 1). All patients completed the vaccination regimen of two injections. One patient (p06) did not meet the inclusion criteria and was therefore not enrolled in the study. The adverse events did not exceed grade II toxicity and were transient. All patients experienced the vaccination as mildly painful. The pain vanished within 10 to 15 minutes after injection. Flu-like symptoms, lasting <1 day (2 of 10 vaccinated patients), swelling and/or redness of the injection site (5 of 10 patients), pain and/or itching of the injection site (four of ten patients), were observed but did not exceed grade II toxicity of the common terminology criteria (Table 1). Interestingly, (re)activation of loco-regional inflammatory events at the prior injection site was frequently observed after the second vaccination. Two patients experienced grade II systemic adverse events (prostatitis and atrial fibrillation) during the trial but these were unlikely to be caused by the vaccination. The first event resolved after treatment with antibiotics and the second conversed spontaneously within a half an hour into a sinus rhythm (this patient was familiar with paroxysmal atrial fibrillation). The time of follow-up and the clinical status are given in Table 1. Cancer recurrences were detected in patient seven out of ten patients during follow up as shown in Table 1.

Induction of p53-specific IFNγ-producing circulating p53-specific T cells

To determine the effect of the vaccine on the immune system, PBMC isolated before and after vaccination were analyzed for the presence of p53 specific T cells by IFNγ ELISPOT. No IFNγ-producing T cells were detected in the baseline samples against either one of the six different tested long peptide pools. After vaccination, up to 220 specific spots per 10^5 PBMC against at least one of the vaccine-representing p53 peptide pools were observed in six out of the nine tested patients (Table 2). Patient p07 only showed a positive response against peptide pool p53.6, which represents the C-terminal part of the p53 protein and is not included in the vaccine and, therefore, this response was not regarded as a direct vaccine-induced response. Due to a low number of isolated PBMC we were not able to perform an ELISPOT assay for p11. Figure 2A shows a typical response in patient p01 and p08. More frequently and
Figure 2. Vaccination with the p53 synthetic long peptides vaccine elicits strong T-cell responses in patients.

(A) Two typical examples of the IFN-γ-ELISPOT results are shown: p01 (left) and p08 (right). Columns indicate the number of T cells per 10⁵ PBMC specifically producing a spot of the cytokine IFN-γ after stimulation with the indicated six peptide pools covering the p53 protein; responses before vaccination (white columns), after two vaccinations (black columns). (B) Two typical examples of the proliferation assay of p01 and p08, before (white columns), 3 weeks after vaccination (black columns) and at least 6 months after last vaccination (grey columns); columns indicate proliferation mean (cpm), error bars – SD. (C) Proliferative responses upon stimulation with the indicated peptide pools of p53 are depicted as a stimulation index for each individual patient before, 3 weeks after vaccination (n=10) and at least 6 months after last vaccination (n=9). From patient p11 no late follow-up blood sample was obtained. Each patient is represented by a symbol. A stimulation index (SI) above 3 (indicated line) was defined as a positive response.
somewhat stronger responses were observed against p53 peptide pools p53.4 and p53.5 (Table 2). T-cell frequencies were increased up to 1 per 454 PBMC (p53.4) and up to 1 per 694 (p53.5). Only five patients (p02; p03; p07; p08; p09) displayed an IFN-γ-associated T-cell response to the positive control antigen mixture (MRM; data not shown). In conclusion, the synthetic long p53 peptide vaccine induced a p53-specific immune response in six out of nine vaccinated patients as detected by IFN-γ ELISPOT.

Table 2. IFN-γ Elispot analysis before and after two p53-SLP vaccinations

<table>
<thead>
<tr>
<th>Patient</th>
<th>Pre vaccination</th>
<th>Post vaccination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PS3.1</td>
<td>PS3.2</td>
</tr>
<tr>
<td>P01</td>
<td>2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>P02</td>
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</tr>
<tr>
<td>P10</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

PBMC were tested against six different peptide pools. The numbers indicate the number of T cells per 10⁵ PBMC specifically producing a spot of the cytokine IFN-γ after stimulation with the indicated pool of peptides; in bold the positive responses (definition is described in material and methods); V followed by number indicates the amino acid stretch in the peptide pools as represented in the p53-SLP vaccine.

Vaccine induced p53-specific T cells proliferate but produce low amounts of cytokines

To analyze the proliferative capacity of p53-specific T cells before and after vaccination as well as during follow-up, PBMC were tested in a lymphocyte stimulation test. Based on our cut-off criteria, the PBMC of patient p03 displayed a proliferative response against p53 peptide pools 1 and 5, p04 against pool 6, p05 against pool 5 and p11 against pool 4 at baseline. All other patients did not show a p53-specific proliferative response before vaccination. None of the pre-existing proliferative responses was boosted (>3-fold increase) after vaccination. The p53-specific responses detected in patients p05 and p11 were approximately at the same level after vaccination and those of patients p03 and p04 had disappeared. After vaccination, seven out of ten patients displayed vaccine-induced p53-specific reactivity to at
least one of the four pools of p53 peptides present in the vaccine (Figure 2B, C). Two patients showed positive responses against one peptide pool (p04 and p09), while five patients showed positive responses for \(\geq 2\) different peptide pools (p01, p02, p07, p10 and p11; Figure 2B, C). Notably, due to a higher background response (medium control) the calculated response of patients p08 and p09 was low. When compared to peptide pool p53.1, which is not present in the vaccine, p08 displays positive responses against peptide pools p53.3, p53.4 and p53.5 (figure 2B), while p09 not only would show a positive response against p53.5 but also against p53.4. In nine patients (p01-p10), we were able to obtain a follow-up blood sample approximately six months after the last vaccination. Even then, strong proliferative p53-specific T-cell responses were observed in six patients (Figure 2C). Except for patient p04 and p09, a proliferative response against the antigens in the MRM could be detected both at baseline and post vaccination (data not shown). Supernatants isolated from the cultures of all PBMC samples tested in the lymphocyte stimulation test were used for the analysis of antigen-specific production of cytokines (IFN\(\gamma\); IL-2; TNF\(\alpha\); IL-10; IL-5; IL-4) by cytometric bead array. In a minority of the patients (p01, p04, p08, p10 and p11), vaccine-induced p53 specific proliferation coincided with the detectable production of IFN\(\gamma\) (mean 228; range 35 - 1521 pg/mL). TNF\(\alpha\) was produced in PBMC of patients p04, p07 and p08 (mean 137; range 20 - 254 pg/mL). IL-5 was found in patients p01, p02, p04, p10 and p11 (mean 90; range 24 - 204 pg/mL) and IL-10 was only induced in patient p02 (28 pg/mL). Production of IL-2 or IL-4 could not be detected. These data indicate that the p53-SLP\(^\circ\) vaccine can induce a strong and sustained p53-specific T-cell reactivity in the majority of cases but also that these responses are not associated with the production of high amounts of cytokines.

Only CD4\(^+\) p53-specific T cells are detected after vaccination
In order to gain more insight in the p53-SLP\(^\circ\)-induced T-cell response, patient-derived PBMC were stimulated, directly \textit{ex vivo} as well as after a 10-day pre-sensitization period, with p53 peptides and recombinant p53 protein and analyzed simultaneously for the following T cell markers (CD3, CD4 and CD8), activation markers (CD137 and CD154) and cytokines (IFN\(\gamma\), IL-2 and IL-5) by multi-parameter flow cytometry. The antigen-induced upregulation of the activation markers allowed us to assess the percentage, phenotype and cytokine-polarization of p53-specific T cells (e.g. CD3\(^+\)CD4\(^+\)CD154\(^+\) and CD3\(^+\)CD8\(^+\)CD137\(^+\) for p53-specific CD4\(^+\) and CD8\(^+\) T cells, respectively). In two cases (p01 and p08) we were able to detect p53-specific CD4\(^+\) T-cell responses directly \textit{ex vivo} (Figure 3A). Analysis of the pre-sensitized PBMC samples revealed the presence of circulating p53 specific CD4\(^+\) T cells against at least one of the peptide pools in five patients (p01, p02, p08-p10) (Figure 3B, C). Most of the detected responses displayed mixed cytokine profiles with varying percentages
Figure 3. Only p53-specific CD4+ T cells are induced.
Measurement of the percentage of p53-specific activated T cells, which produce IFNγ and IL-2, as determined by flow cytometry either directly ex vivo as well as after a 10-day pre-sensitization period. (A) Left two panels show directly ex vivo stained PBMC isolated after vaccination and overnight stimulation with medium or peptide pool p53.2 – p53.5. CD4+ T cells were plotted against the activation marker CD154. Numbers indicate percentage CD3+CD4+CD154+ T cells of patient p01 (left panels) and of patient p08 (right panels). No p53-specific T cells could be detected in the other patients. (B) CD4 and CD154 expression after 10 days pre-sensitization in post vaccination PBMC of p01 stimulated with the indicated antigens; numbers indicate percentage CD3+CD4+CD154+ T cells. (C) Pie-plots indicating the percentage of CD3+CD4+CD154+ T cells in pre-sensitized post-vaccination PBMC of p01, p02, p08, p09 and p10 after stimulation with the indicated peptide pool as determined by multi-parameter flow cytometry. Pies indicate the fraction of IFNγ (white), IL-2 (shaded), both IFNγ and IL-2 (grey) and neither IFNγ nor IL-2 (black) producing CD3+CD4+CD154+ T cells. IL-5 was not detected in these cultures. (D) Shows directly ex vivo stained T cells isolated from the biopsy and overnight stimulation with medium or peptide pool p53.2 – p53.5. In the upper panels are CD3+CD4+ T cells plotted against the activation marker CD154, numbers indicate percentage CD3+CD4+CD154+ T cells; IFNγ and IL-2 expression in CD3+CD4+CD154+ T cells is shown in the lower two panels, numbers indicate the percentage cells per quadrant.
of IFNγ and IL-2 producing p53-specific T cells. Notably, in most cases a high percentage of p53-specific T cells was observed which neither produced IFNγ nor IL-2. The p53-specific production of IL-5 was never observed. Importantly, no CD8+ T-cells reactive to p53 could be detected in any of these samples.

**Vaccination does not result in the induction of p53-specific CD4+CD25+Foxp3+ T cells**
Recently, we observed that vaccination of cancer patients may result in the induction of circulating CD4+CD25+Foxp3+ T cells, which presumably may have regulatory activity

In six cases (p01-p04, p08, p09) we were able to isolate sufficient numbers of PBMC to analyze the presence of vaccine-induced p53-specific CD4+CD25+Foxp3+ T cells before the first and after the last vaccination. PBMC were stimulated with p53 peptides and rested for 10 days, as this allows the measurement of stably Foxp3 expressing T cells which are specific for p53. As a control, PBMC were cultured without antigen. The induction of cell surface expression of CD25 on vaccine-induced p53-specific CD4 T cells varied between the subjects, with a high percentage of CD4+ T cells being CD25-positive in p01 (28.7%) and p02 (8.8%), intermediate percentage in p04 (3.6%) and lower percentage in patients p08 (1.1%) and p09 (1.5%) after vaccination, reflecting the magnitude of the response observed in the proliferation assays. No overt induction of p53-specific CD4+CD25+Foxp3+ T cells was found (mean 0.3%, range 0-0.9%).

**T cells cultured from skin biopsies display p53 specificity**
From four (p01, p07, p08, p09) of the ten vaccinated patients we obtained enough T cells from the skin biopsy of the second vaccine site to allow further examination. In two cases (p07 and p08) the biopsies contained p53-specific T cells able to proliferate when stimulated with p53 peptide or protein pulsed APC (Figure 4A). Analysis of the supernatants with proliferation-associated production of cytokines revealed the presence of large quantities of all cytokines (Figure 4B). Noteworthy, the vaccination sites of these patients showed the clearest signs of inflammation, which made it easier to take a biopsy from inflamed tissue. In order to characterize the p53-specific T-cell population, the vaccine-site infiltrating T cells were stimulated with peptide pools and analyzed by multi-parameter flow cytometry. Of the vaccine-site infiltrating T cells of p07, 10% responded to p53 peptide and 5% to p53 protein. Analysis of the cytokine profile confirmed our data obtained from the PBMC cultures of p07 in that the vaccine-infiltrating cells did produce IL-2 but no IFNγ (data not shown). Of p08, 35% of the infiltrating cells responded to peptide and 10% also to protein pulsed APC. Similarly, the majority of the p53-specific cell population produced IL-2 after stimulation but only 2.6% of these cells were able to produce IFNγ (Figure 3D). IL-5 production was not tested by flow cytometry. All responses were confined to the CD4+ T-cell subset.
In this phase I/II study, immunotherapy with synthetic long peptides representing the sequence of the most immunogenic part of the p53 protein in patients with colorectal cancer in formulation with Montanide ISA-51 adjuvant has proven to be safe and highly immunogenic. The maximum toxicity seen was grade II according to the common terminology criteria and mainly consisted of discomfort and swelling at the vaccination sites. The application of several complementary assays revealed that the p53-SLP® vaccine had induced p53-specific immunity in nine of ten vaccinated patients, which was sustained for up to at least 6 months after vaccination. In contrast to patients vaccinated with a Human Papillomavirus Type 16-SLP, the p53-SLP® induced only p53-specific CD4+ T cells. This was to be expected as the p53-specific CD8+ T cell but not the CD4+ T cell repertoire is severely restricted by self tolerance and might only consist of lower affinity p53-specific CD8+ T cells. Notably, the detection of p53-specific Th1/Th2 cytokine producing CD4+ T cells, able to recognize both p53 peptide and p53 protein pulsed APC in the site of vaccination, suggests that the p53-SLP® vaccine is capable of inducing functionally active p53-specific T cells which can migrate to areas where antigen is present. Most p53-specific

**DISCUSSION**

Figure 4. Vaccine-site infiltrating T cells are p53-specific. (A) T cells from the skin biopsy of patients p07 (left) and p08 (right) were tested for their capacity to proliferate upon stimulation with peptides or protein pulsed monocytes. Phytohemagglutinin (PHA) served as a positive control, while stimulation with HPV16 protein or medium served as negative control. The columns indicate the mean and SD of the proliferation. (B) Concentration of the concomitantly produced cytokines (pg/mL) as measured in the supernatants isolated from the proliferation test by cytometric bead array.
responses were found against peptide pools p53.4 and p53.5 indicating that the C-terminal part of the vaccine is most immunogenic. These responses appeared to be restricted by multiple HLA class II molecules since no particular HLA type was found to be present in these responding patients (data not shown).

Previous studies, in which subjects were vaccinated by different antigen delivery systems including canarypoxvirus 29, adenovirus 30 or peptide loaded autologous dendritic 31 cells, described varying results with regard to induction of p53-specific immunity. Two studies induced a p53-specific response in only a very low percentage of patients 27;29 while in another study 57% of vaccinated patients mounted a p53-specific immune response 30, with – based on IFN\(\gamma\) ELISPOT - a comparable magnitude as in our trial. Therefore, the p53-SLP® vaccine induces the highest response rate, at least in colorectal cancer patients. This is probably attributable to the fact that the T-cell epitopes in SLP are efficiently processed and presented by DC, do not have to compete with dominant epitopes present in viral vectors and that the response induced by this vaccine is not restricted to one HLA type 32;33.

In at least five out of the seven patients p53-specific proliferation was associated with the production of detectable amounts of IFN\(\gamma\). However, the levels of IFN\(\gamma\) (mean 242 pg/mL) were rather low when compared to what we observed in a trial in which cervical cancer patients were treated with a HPV16-SLP vaccine and in which the levels ranged from 250 pg/mL to more than 5000 pg/mL 39. In fact, the overall production of pro-inflammatory cytokines by the p53-SLP® vaccine-induced T-cell population was low and this seems to be reflected by the vaccine-sites, most of which showed no clear signs of inflammation (Table 1), while this was the case in the majority of vaccinated cervical cancer patients 38. Assessment of all p53-activated CD4+ T cells, by gating on the CD4+CD154+ T-cell population by multi parameter flow cytometry, revealed that only in some cases the IFN\(\gamma\) producing population of T cells was the major subset among the vaccine-induced p53-specific T-cell response. As such, the polarization of the p53-specific immune response induced by p53-SLP® vaccine strongly resembles the spontaneous p53-specific immune response in colorectal cancer patients 11;12.

The vaccine dose and injection scheme used in the current study was based on the results obtained with an HPV16-SLP vaccine in patients with cervical cancer, of which our studies indicated that the CD4+ T-cell response was not different between two and four vaccinations 38;39 as well as on our studies in mice which showed that the same peptide dose used to stimulate HPV16-specific immunity 36 was also able to stimulate p53-specific immune responses 13. In patients with metastasized colorectal cancer, however, two injections with p53-SLP® only seems insufficient to activate a strong Th1-response. Recently, it was described that prolonged antigen presentation could elicit full expansion, effector cytokine production and memory cell differentiation, even in the absence of DC maturation signals 36;48. Notably, in some of the
HPV16-SLP vaccinated end-stage cervical cancer patients also four injections were required to obtain a strong IFNγ-associated E7-specific T-cell response. As such, a prolonged vaccination scheme (i.e., multiple instead of two injections) may result in a stronger polarized Th1 response and possibly in the expansion of p53-specific CD8+ CTL previously observed in patients with cancer but which display a low affinity for p53. In addition, one could make use of immunomodulatory adjuvants, of which chemotherapeutics form an interesting group. A recent study showed that patients with advanced colorectal cancer, who developed late signs of autoimmunity after treatment with the Golfig chemoimmunotherapy regimen (chemotherapy, GM-CSF and IL-2), showed a prolonged time to progression and survival. In the PBMC of these patients a progressive increase in lymphocyte and eosinophil counts, amplification in central memory, a marked depletion of immunosuppressive regulatory T cells and activation of colon cancer-specific cytotoxic T cells was found. Another study combined a cancer vaccine with chemotherapy in patients with extensive stage small cell lung cancer showing a trend with induction of immunologic response to vaccination and clinical response to subsequent chemotherapy. These studies provide evidence that combining chemotherapeutics with cancer vaccines might lead to better treatment results in colorectal cancer patients.

Finally, our results fit with the safety and immunogenicity experience gathered thus far with vaccines consisting of long peptides dissolved in Montanide adjuvant, showing only low-grade toxicity and strong immunogenicity. The p53-SLP® vaccine is able to enhance the number of p53-specific CD4+ T cells to a broad array of epitopes in approximately 90% of all vaccinated patients, while no p53-specific CTLs are induced. Despite the induction of p53-specific T-cell immunity in vaccinated patients, the p53-specific Th1 responses are probably too weak to become truly effective. Most likely this is due to the fact that the p53-SLP® vaccine did not contain a compound able to activate a Th1-promoting DC population. Consequently, the addition of a strong Th1-inducing adjuvant to the p53-SLP® vaccine is required to obtain strong p53-specific Th1 immunity which, even in the absence of HLA class II positive tumor cells, is vital to coordinate a local anti-tumor immune attack of innate effector cells and CTL directed against unique tumor-specific antigens that are cross-presented by dendritic cells. A new trial with p53-SLP® in combination with a Th1 enhancing compound has been initiated.

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REFERENCES


