Induction of p53-Specific Immunity by a p53 Synthetic Long Peptide Vaccine in Patients Treated for Metastatic Colorectal Cancer

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Abstract

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

Experimental Design: Ten patients were vaccinated twice with a set of 10 overlapping p53-SLP in a phase I/II trial. Both the safety and 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 enzyme-linked immunospot, proliferation, cytokine secretion, and multiparameter 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 1/2, mostly at the vaccination site. p53-specific T-cell responses were induced in 9 of 10 colorectal cancer patients as measured by IFN-γ enzyme-linked immunospot, proliferation, and cytokine bead array. In 6 of 9 tested patients, p53-specific T-cell reactivity persisted at least 6 months. Furthermore, p53-specific T-cells isolated from the vaccination site were characterized as CD4+ T-cells producing both T-helper types 1 and 2 cytokines on stimulation with p53 peptide and p53 protein. Multiparameter 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.

Colorectal adenocarcinoma is the third most common cancer and the second most frequent cause of death due to cancer (1). Despite treatment, ~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 either in the p53 tumor suppressor gene itself or in genes regulating p53 activity are found in a wide variety of tumors, including colorectal cancers (2, 3), 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 CTL and T-helper (Th) cells have been detected in peripheral blood mononuclear cell (PBMC) cultures in vitro (4–8). In addition, wild-type p53-specific proliferative responses were shown in patients with breast (9), ovarian (10), and colorectal (11, 12) cancers. There are strong indications that the p53-specific CD8+ T-cell repertoire is severely restricted by self-tolerance (13–16), as high-avidity self-reactive T cells are deleted in the thymus (17), leaving available only CD8+ T cells with a low-avidity T-cell receptor. In contrast, the CD4+ T-cell repertoire is not affected (13), presumably because the low expression levels and rapid breakdown of p53 in the thymus disfavor presentation by MHC class II (18). Even in the case of MHC class II-negative cancers, the availability of p53-specific CD4+ T cells is important in cancer immunotherapy because IFN-γ-secreting CD4+ Th1 cells play an important role in orchestrating and sustaining the local immune attack by CD8+ CTL and innate immune effector cells (19–21). Indeed, adoptively transferred p53-specific CD4+ Th cells supported the antitumor response against p53-overexpressing tumors (13, 22). Moreover, Th1...
Colorectal cancer is an important cause of death due to cancer worldwide. There is a high need for new treatment modalities to improve the outcome of this disease. Cancer vaccines inducing CTL as well as Th-helper (Th) responses to tumor-associated antigens may represent one such approach. One of such tumor-associated antigens is p53. Although the p53-specific CTL repertoire is severely restricted by central tolerance, this is not the case for p53-specific CD4+ Th cells. Importantly, p53 is expressed in a significant proportion of cancer patients and many types of tumors, suggesting that p53 may act as a universal Th vaccine. Recent studies showed the high immunogenicity of synthetic long peptide (SLP) vaccines in cancer patients. Here, the safety and immunogenicity of a p53-SLP vaccine were tested. The data reveal that the p53-SLP induces only low-grade toxicity and a p53-specific CD4+ Th-cell response in the majority of patients.

Translational Relevance

Colorectal cancer is an important cause of death due to cancer worldwide. There is a high need for new treatment modalities to improve the outcome of this disease. Cancer vaccines inducing CTL as well as Th-helper (Th) responses to tumor-associated antigens may represent one such approach. One of such tumor-associated antigens is p53. Although the p53-specific CTL repertoire is severely restricted by central tolerance, this is not the case for p53-specific CD4+ T cells. Importantly, p53 is expressed in a significant proportion of cancer patients and many types of tumors, suggesting that p53 may act as a universal Th vaccine. Recent studies showed the high immunogenicity of synthetic long peptide (SLP) vaccines in cancer patients. Here, the safety and immunogenicity of a p53-SLP vaccine were tested. The data reveal that the p53-SLP induces only low-grade toxicity and a p53-specific CD4+ Th-cell response in the majority of patients.

SLP vaccines to induce truly strong tumor-specific CD4+ and CD8+ T-cell responses was shown in rodents therapeutically treated for human papillomavirus (HPV)-induced tumors (36, 37) as well as in patients with cervical cancer (38, 39). 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 (13). 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 of 5 patients injected with canary poxvirus with human wild-type p53 mounted a T-cell response (28, 29), and based on our animal studies, in which the p53-SLP vaccine was able to induce immunity in all mice (13), as well as on the high number of cancer patients responding in our HPV16-SLP studies (38, 39), 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 × 10^9/L, lymphocytes >1 × 10^9/L, platelets >100 × 10^9/L, hematocrit >30%, and hemoglobin >6 mmol/L; (c) no radiotherapy, chemotherapy, or other potentially immunosuppressive therapy administered within 4 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 >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 International Standard Randomised Controlled Trial Number (ISRCTN43704292). After written informed consent, a screening visit was done, 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 to 9 months after vaccination, a third blood sample was drawn for immunomonitoring. For clinical monitoring, a computed tomographic scan was made before and after vaccination and the serum tumor-marker carcinoembryonic antigen was determined at several different time points during the whole trial. The vaccination scheme is depicted in Fig. 1.

Vaccine. The vaccine consisted of 10 overlapping peptides, together representing the p53 protein from amino acids 70 to 248. This region is recognized by T cells of all colorectal patients displaying p53 immunity (11, 12), and it harbors most of the published MHC class I and II epitopes (reviewed in ref. 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 described previously (39). At the day of vaccination, the 10 peptides (0.3 mg/peptide) were dissolved in DMSO, admixed with PBS, and emulsified in Montanide ISA-51 adjuvant in a total volume of 2.7 mL (DMSO/PBS/Montanide ISA-51 20:30:50, v/v/v). The dose of the peptides used and the number and...
schedules of vaccinations were based on our previous observations in mice (13, 36) and patients vaccinated with a HPV16-SLP vaccine (38, 39). The results in the latter two studies indicated that two vaccinations were sufficient to induce a strong Th1 response in patients with cancer (38, 39).

Safety and tolerability monitoring. At the day of vaccination, the patients were under observation in the hospital until 3 h 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 h after vaccination. Further vital sign examination included temperature, pulse, blood pressure, oxygen saturation, and respiratory frequency before and at 1, 2, and 3 h after vaccination.

Immunohistochemistry and evaluation. The expression of p53 and HLA class I and 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 (41, 42). The following primary antibodies were used: anti-p53 (clone DO-7; 1:500; DAKO), anti-HLA class I (EMR 6-5; 1:250; MBL), and anti-HLA-DP/DQ/DR (clone CR3/43; 1:100; DAKO). Secondary anti-mouse horseradish peroxidase 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 IFN-γ enzyme-linked immunospot, lymphocyte proliferation assay, and cytokine polarization analysis. T cells from PBMC or skin biopsies were isolated and cultured as described previously (39, 43) and either directly used or cryopreserved. A set of six pools of long overlapping peptides, indicated by the first and last amino acids in the p53 protein, was used for the screening of T-cell responses: p53.1: 190-248; and p53.6: 241-393. Peptide pools p53.2 to p53.5 represented previously (39, 43) and either directly used or cryopreserved. A set of six pools of long overlapping peptides, indicated by the first and last amino acids in the p53 protein, was used for the screening of T-cell responses: p53.1: 190-248; and p53.6: 241-393. Peptide pools p53.2 to p53.5 represented the area included in the vaccine, whereas the other two peptide pools p53.1 and p53.6 represented the remaining part of p53. As a positive control, PBMCs were cultured in the presence of a recall antigen mixture, the memory response mix (44). Analysis of p53-specific T-cell responses from PBMC were done using IFN-γ enzyme-linked immunospot (ELISPOT) and 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 kit (BD Biosciences) as described previously (29, 39). Specific spots in the ELISPOT were calculated; the cutoff of the proliferation assay was 2 spots (ELISPOT) and 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 kit (BD Biosciences) as described previously (29, 39). Specific spots in the ELISPOT were calculated by subtracting the mean number of spots + 2 × SD of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were calculated by subtracting the mean number of spots + 2 × SD of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were calculated by subtracting the mean number of spots + 2 × SD of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were calculated by subtracting the mean number of spots + 2 × SD of the medium control from the mean number of spots in experimental wells. Antigen-specific T-cell frequencies were calculated by subtracting the mean number of spots + 2 × SD of the medium control from the mean number of spots in experimental wells. 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**Table 1.** Patient characteristics and adverse events observed

<table>
<thead>
<tr>
<th>Patient</th>
<th>Gender</th>
<th>Age</th>
<th>Metastasis stage</th>
<th>Primary treatment</th>
<th>Recurrences</th>
<th>Secondary treatments before vaccination</th>
<th>Clinical status (months after vaccination)</th>
<th>p53 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 (2×), atrial fibrillation sites (2×)</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 site (1×)</td>
</tr>
<tr>
<td>p03</td>
<td>M</td>
<td>62</td>
<td>4</td>
<td>PR + RLi</td>
<td>—</td>
<td>RFLu</td>
<td>Alive, NED (15)</td>
<td>4</td>
<td>Pain vaccination site (1×)</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>—</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 (1×); RFLu (2×)</td>
<td>Alive, Rec (11)</td>
<td>1</td>
<td>—</td>
</tr>
<tr>
<td>p06</td>
<td>F</td>
<td>64</td>
<td>4</td>
<td>PR + RLi</td>
<td>3 Li; Li; Lu</td>
<td>RLI + C; RFLi</td>
<td>Alive, NED (7)</td>
<td>0</td>
<td>Swelling + erythema injection site (2×)</td>
</tr>
<tr>
<td>p07</td>
<td>F</td>
<td>64</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 (2×)</td>
</tr>
<tr>
<td>p08</td>
<td>M</td>
<td>59</td>
<td>3</td>
<td>PR + RLi</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 (1×), prostatitis</td>
</tr>
<tr>
<td>p09</td>
<td>M</td>
<td>69</td>
<td>4</td>
<td>PR + A</td>
<td>2 Li</td>
<td>RLi; RFLi</td>
<td>Alive, Rec (3)</td>
<td>4</td>
<td>Pain + swelling vaccination site (1×)</td>
</tr>
<tr>
<td>p10</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 (2×)</td>
</tr>
</tbody>
</table>

NOTE: All adverse events of the vaccine were temporary. The swelling at the injection site was only painful direct after injection, lasted ~15 min, and was probably due to the adjuvant Montanide ISA-51 in the vaccine (not in table).

Abbreviations: F, female; M, male; PR, primary resection; A, adjuvant chemotherapy; Ro, preoperative radiotherapy; RLi, resection tumor lungs; RFLu, resection tumor liver; RFLu, RFA lung lesion(s); RFLu, RFA liver lesion(s); C, chemotherapy; Li, liver; Lu, lungs; NED, no evidence disease; Rec, recurrence.

*P53 expression: 0, absent; 1, >0% to 25%; 2, 25% to 50%; 3, 50% to 75%; 4, >75%. The current clinical status is given, and the period (months) after enrolment is between brackets.

† Direct after vaccination two metastases in liver that were resected.
defined as this average plus $3 \times SD$. 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 cutoff value (39). Positive antigen-specific cytokine production as determined by cytokine bead array was defined as a cytokine concentration above the cutoff value and $>2$ times the concentration of the medium control (39). According to the manufacturer, the proposed detection limit for the cytokine bead array was 20 pg/mL for tumor necrosis factor-$\alpha$, IL-10, IL-5, IL-4, and IL-2. We deviated with respect to the cutoff value of IFN-$\gamma$ (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 with the baseline sample.

Detection of IFN-$\gamma$, IL-2, and IL-5 production by p53-specific T cells using flow cytometry. PBMCs were either directly ex vivo used for intracellular cell staining or 10 days presensitized using the peptides in pools p53.2 to p53.5 (2.5 $\mu$g/peptide/mL) as described previously (43). T cells from the biopsy were directly tested ex vivo. Then, the cells were stimulated overnight with the indicated antigens, whereas 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 (43) 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-CD1/54 PECy5 (clone TRAP1; BD Pharmingen), anti-CD137 antigen-presenting cells (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) 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 done as reported previously (39). Briefly, PBMCs (1–2 $\times$ 10$^7$) were cultured for 10 days in medium only or in the presence of pooled p53 peptides (5 $\mu$g/peptide/mL). Then, the cells were harvested and 2 $\times$ 10$^5$ cells were stained for the surface markers CD25 (anti-CD25 FITC; clone M-A251; BD Pharmingen), CD4 (anti-CD4 antigen-presenting cells; 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. A previously isolated HPV16-specific CD4$^+$CD25$^+$Foxp3$^+$ regulatory T-cell clone (C148.31) as a positive control and a HPV16-specific CD4$^+$CD25$^+$Foxp3$^+$ T-cell clone (C271.9) as negative control (45) 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$^+$ cells in the medium-only control, and a vaccine-induced increase in Foxp3$^+$ cells was defined as at least a 3-fold increase compared with the percentages of the baseline sample for the same condition (39).

Results

Safety of the vaccine. A total of 10 patients were vaccinated at least 1 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. Overexpression 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 2 toxicity and were transient. All patients experienced the vaccination as mildly painful. The pain vanished within 10 to 15 min 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), and pain and/or itching of the injection site (4 of 10 patients), were observed but did not exceed grade 2 toxicity of the common terminology criteria (Table 1).

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>p53.1 p53.2 1-78</td>
<td>V70-115</td>
</tr>
<tr>
<td>p01</td>
<td>2 2 &lt;1 &lt;1 1 1</td>
<td>1 1 1</td>
</tr>
<tr>
<td>p02</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
<td>&lt;1 3 9 113 137</td>
</tr>
<tr>
<td>p03</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
<td>&lt;1 4 20</td>
</tr>
<tr>
<td>p04</td>
<td>7 4 4 2 4</td>
<td>4 12 31 63 14 35</td>
</tr>
<tr>
<td>p05</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
</tr>
<tr>
<td>p07</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
<td>&lt;1 4 16 56 126 110 7</td>
</tr>
<tr>
<td>p08</td>
<td>7 1 2 4 4</td>
<td>4 3 7 42 220 144 8</td>
</tr>
<tr>
<td>p09</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
<td>&lt;1 4 16 56 126 110 7</td>
</tr>
<tr>
<td>p10</td>
<td>&lt;1 &lt;1 &lt;1 &lt;1 &lt;1</td>
<td>&lt;1 4 &lt;1 3 2 2 1</td>
</tr>
<tr>
<td>Total no. positive reactions</td>
<td>0 0 0</td>
<td>0 0 0</td>
</tr>
</tbody>
</table>

NOTE: PBMCs were tested against six different peptide pools. The numbers indicate the number of T cells per 10^5 PBMCs specifically producing a spot of the cytokine IFN-γ after stimulation with the indicated pool of peptides; the positive responses (definition is described in Materials and Methods) are in bold; 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, PBMCs were tested in a lymphocyte stimulation test. Based on our cutoff 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 preexisting 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, 7 of 10 patients displayed vaccine-induced p53-specific reactivity to at least one of the four pools of p53 peptides present in the vaccine (Fig. 2B and C). Two patients showed positive responses against one peptide pool (p04 and p09), whereas 5 patients showed positive responses for two or more different peptide pools (p01, p02, p07, p10, and p11; Fig. 2B and C). Notably, due to a higher background response (medium control), the calculated response of patients p08 and p09 was low. When compared with 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 (Fig. 2B), whereas p09 would show a positive response not only against p53.5 but also against p53.4. In 9 patients (p01–p10), we were able to obtain a follow-up blood sample ~ 6 months after the last vaccination. Even then, strong proliferative p53-specific T-cell responses were observed in 6 patients (Fig. 2C). Except for patients p04 and p09, a proliferative response against the antigens in the memory
response mix could be detected both at baseline and after 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-γ, IL-2, tumor necrosis factor-α, IL-10, IL-5, and IL-4) by cytometric bead array (46). In a minority of the patients (p01, p04, p08, p10, and p11), vaccine-induced p53-specific proliferation coincided with the detectable production of IFN-γ (mean, 228; range, 35-1,521 pg/mL). Tumor necrosis factor-α 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 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. To gain more insight in the p53-SLP-induced T-cell response, patient-derived PBMCs were stimulated, directly ex vivo as well as after a 10-day presensitization 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-γ, IL-2, and IL-5) by multiparameter flow cytometry. The antigen-induced up-regulation 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

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**Fig. 2.** Vaccination with the p53-SLP vaccine elicits strong T-cell responses in patients. A, two typical examples of the IFN-γ ELISPOT results: p01 (left) and p08 (right). Columns, number of T cells per 10⁶ PBMCs 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) and after two vaccinations (black columns). B, two typical examples of the proliferation assay of p01 and p08 before vaccination (white columns), 3 wk after vaccination (black columns), and at least 6 mo after last vaccination (gray columns). Columns, mean proliferation (cpm); bars, SD. C, proliferative responses on stimulation with the indicated peptide pools of p53 are depicted as a stimulation index for each individual patient before vaccination, 3 wk after vaccination (n = 10), and at least 6 mo 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.
CD8+ T cells, respectively). In 2 cases (p01 and p08), we were able to detect p53-specific CD4+ T-cell responses directly ex vivo (Fig. 3A). Analysis of the presensitized PBMC samples revealed the presence of circulating p53-specific CD4+ T cells against at least one of the peptide pools in 5 patients (p01, p02, and p08-p10; Fig. 3B and C). Most of the detected responses displayed mixed cytokine profiles with varying percentages of IFN-γ- and IL-2-producing p53-specific T cells. Notably, in most cases, a high percentage of p53-specific T cells was observed which produced neither IFN-γ 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 (39). In 6 cases (p01-p04, p08, and 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. PBMCs were stimulated with p53 peptides and rested for 10 days, as this allows the measurement of stably Foxp3-expressing T cells (47), which are specific for p53. As a control, PBMCs 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+ 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%).

**Discussion**

In this phase I/II study, immunotherapy with SLPs representing the sequence of the most immunogenic part of the p53 protein in patients with colorectal cancer (11, 12) in formulation with Montanide ISA-51 adjuvant has proven to be safe and highly immunogenic. The maximum toxicity seen was grade 2 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 9 of 10 vaccinated patients, which was sustained for up to 9 months.
least 6 months after vaccination. In contrast to patients vaccinated with a HPV16-SLP (39), the p53-SLP induced only p53-specific CD4+ T cells. This was to be expected as the p53-specific CD8+ T-cell repertoire 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 (13, 14). Notably, the detection of p53-specific Th1/Th2 cytokine-producing CD4+ T cells, able to recognize both p53 peptide and p53 protein-pulsed antigen-presenting cells 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 responses were found against peptide pools p53.4 and p53.5, indicating that the COOH-terminal part of the vaccine is most immunogenic. These responses appeared to be restricted by multiple HLA class II molecules because 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 canary poxvirus (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), whereas, 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 dendritic cells and 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 5 of the 7 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 with 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 to >5,000 pg/mL (39). In fact, the overall production of proinflammatory 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), whereas 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 multiparameter 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 were based on the results obtained with a 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 dendritic cell 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 IFN-\(\gamma\)-associated E7-specific T-cell response (38). As such, a prolonged vaccination scheme (multiple instead of two injections) may result in a stronger polarized Th1 response and possibly in the expansion of p53-specific CD8+ CTL observed previously in patients with cancer (49) but which display a low affinity for p53 (14). 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, granulocyte-macrophage colony-forming units, and IL-2), showed a prolonged time to progression and survival (50). 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 (50). 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 (30). 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 ISA-51 adjuvant, showing only low-grade toxicity and strong immunogenicity (38, 51). The p53-SLP vaccine is able to enhance the number of p53-specific CD4+ T cells to a broad array of epitopes in ~90% of all vaccinated patients, whereas 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 dendritic cell 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 antitumor immune attack of innate effector cells and CTL directed against unique tumor-specific antigens that are cross-presented by dendritic cells (19–21). A new trial with p53-SLP in combination with a Th1-enhancing compound has been initiated.

Disclosure of Potential Conflicts of Interest

The LUMC, Leiden, the Netherlands, holds a patent on long peptide vaccines (US 7,202,034) on which S.H. van der Burg and C.J.M. Melief are named as inventors, C.J.M. Melief is partly (75%) employed as of January 20\(^{th}\) 2008, by ISA Pharmaceuticals, which exploits this long peptide vaccine patent. F. Essahsah is also employed by ISA Pharmaceuticals.

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