Anti-colorectal cancer immunity: Control ‘the force’!

Frank Speetjens

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Cover: The universal symbol of colorectal cancer: the Blue Star. It is a combination of a star and a ribbon, reflecting power, hope, and awareness. This symbol is filled with representative figures from chapters 2-7.

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CONTENTS

Chapter 1

General introduction

1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
14.
15.
16.
17.
18.
19.
20.
21.
22.
23.
24.
25.
26.
27.
28.
29.
30.
31.
32.
33.
34.
35.
36.
37.
38.
39.

INTRODUCTION

Main objective of this thesis is to explore factors involved in especially the T-cell mediated anti-tumor immune response and to understand and control the force of the immune system to effectively search and destroy tumor cells.

COLORECTAL CANCER

Colorectal adenocarcinoma is the third most common cancer and accounts for a significant number of cancer deaths worldwide [1-3]. Colorectal cancer has a lifetime risk of about 5-6% with a peak incidence in the 7th decade. Surgery is treatment of choice when the disease is only confined to the bowel wall. However, 30–40% of patients have loco-regionally advanced or metastatic disease on presentation which cannot be cured by surgery alone [4]. Adjuvant radiation therapy, chemotherapy, or both are beneficial in selected patients [4-6]. Despite intended curative therapy still a large proportion of the patients eventually die of their disease leaving room for new treatment modalities such as T-cell mediated immunotherapy [7].

CANCER AND THE IMMUNE SYSTEM

Both spontaneous and therapeutic induced tumor specific immune responses require induction of cell-mediated immunity, to attack and eliminate tumor cells. This calls for close collaboration between cells of the innate immune system, in particular antigen presenting Dendritic Cells (DCs), and cells of the adaptive immune system, notably B-cells, CD4$^+$ T-helper cells (T_H) and CD8$^+$ cytotoxic T cells (CTL). Despite scientific progress, the interaction between the immune system and cancer remains elusive. Growth of tumor cells that escaped the immune system may implicate selective pressure of the immune system. These mechanisms include active down-regulation of immune responses by the tumor by producing immunosuppressive agents, altered expression of major histocompatibility complex (MHC) and/or tumor-associated antigens (TAAs) by tumor cells, altered expression of adhesion molecules by tumor and/or DCs, and the use of host immune responses to the advantage of the cancer. Better understanding of mechanisms of tumor immune evasion may improve immunotherapeutic strategies.

TUMOR INFILTRATED LEUKOCYTES REPRESENT THE PRESENCE OF AN ONGOING ANTI-TUMOR RESPONSE

Presence of both myeloid and lymphoid cells in different intra- and peri-tumoral compartments in colorectal cancer represents one of the most evident witnesses of an active involvement of the immune system in cancer growth and progression. Immunohistochemical techniques comprise one of the most frequent techniques used to study infiltration of leukocytes in colorectal tumors. These studies determined the clinical impact of many different leukocyte subpopulations such as dendritic cells, macrophages and different (sub-)populations of lymphocytes [8-23]. However, there is still a lot unclear about the exact type and role of leukocytes that infiltrate into tumors. Only the infiltration of intra-tumoral or more precise intra-epithelial located CTLs is without doubt associated with good prognosis in colorectal cancer patients [8;10;12;16;18;19;23]. In addition, several studies showed that intra-epithelial compared to stromal $CD8^+$ T-cells express more molecules involved in target cell killing such as higher expression of Granzyme B and TIA-1 and showed higher proliferative activity, suggesting that intra-epithelial $CD8^+$ T-cells are active effectors [8;12;24]. Limitation of most immunohistochemical techniques is that in general per staining only one antigen is identified. Unfortunately most leukocytes characterized with one antigen fulfill different and even opposing functions. This is one of the explanations why it is difficult to assess the clinical impact of leukocytes using immunohistochemical techniques. Studies using different techniques revealed that especially tumor-specific $CD4^+$ T_H1 cells are associated with a supportive cancer microenvironment that is beneficial to the prognosis of colorectal cancer patients [25-27]. It has been well documented that $CD4^+$ T-cells not only license the priming of $CD8^+$ T-cells but are important to sustain their fitness [28], and also enhance $CD8^+$ T-cell proliferation and cytolytic function [29]. Expression of the IL-17-associated genes in colorectal cancer patients correlated with poor prognosis [30]. The expression of T_H2- and regulatory T cells has no or opposing effects on clinical outcome [30-33].

ROLE OF HUMAN LEUKOCYTE ANTIGEN CLASS I IN COLORECTAL CANCER

Expression of MHC class I, for humans also called Human Leukocyte Antigen (HLA) class I, presenting TAAs on the tumor cell surface, is considered as a prerequisite for effective T-cell mediated immunity [34]. As a consequence, tumor cells with down-regulated HLA class I expression might escape this immune response, resulting in a selective outgrowth of these tumor cells. HLA class I molecules comprise the classical (class Ia) HLA-A, -B, and -C alleles, and the non-classical (class Ib) HLA-E, -F, and –G alleles. In this section we focus on the role of classical HLA class I molecules.

They form a trimolecular complex consisting of a highly polymorphic heavy chain, a peptide antigen, and the non-polymorphic ß2-microglobulin (ß2m) light chain [35]. The heavy chain molecules are encoded by genes located within the HLA region on chromosome 6, whereas ß2m is encoded by a gene mapped on chromosome 15. HLA class I is constitutively expressed by many cells, although the intensity of expression varies between different tissue types. Peptides presented in the context of HLA class I molecules are generated from degraded proteins by the antigen processing machinery. After processing, the peptide is associated with the heavy chain and ß2m and expressed on the cell surface to present the antigen to CTL.

In addition to T cell-induced tumor cell killing, tumor cell lysis can also be induced by activated NK cells. NK cell activation is regulated by a balance between signals mediated through activating and inhibitory receptors [36]. HLA class I is a ligand for inhibitory receptors on NK cells. Loss or down-regulation of HLA class I is a possible strategy to escape T cell control [37], and is frequently found in colorectal cancer [38;39]. Loss or down-regulation of HLA class I might however activate NK cells and induce tumor cell lysis [40]. Defects in one of the processes that are involved in antigen presentation, will lead to loss of expression of HLA class I molecules on the cell surface. Complete loss of HLA class I is usually caused due to loss of ß2m expression or TAP deficiency [41;42]. This is mostly found in microsatellite unstable (MSI-H) tumors when compared to microsatellite stable (MSS) tumors [41;42]. Loss of one of the HLA heavy chains (A, B or C alleles) is usually caused by chromosomal aberrations of chromosome 6 [43]. Only limited studies have reported on the clinical impact of HLA class I expression in colorectal cancer using mixed cohorts of genetic instability and reporting contrasting results [44-47]. None of these studies determined the prognostic inpact of HLA class I expression with regard to genetic instability.

LEUKOCYTE TRAFFICKING IS COORDINATED BY CHEMOKINES

Chemokines are a superfamily of small secreted cytokines that were initially characterized through their ability to coordinate trafficking of leukocytes [48]. Chemokines bind to specific cell surface transmembrane receptors coupled with G proteins, whose activation leads to formation of intracellular signaling cascades that prompt migration toward the chemokine source. To date, studies have identified in humans, more than 50 chemokines and 20 chemokine receptors [48-50]. Chemokines coordinate migration of all types of cells including tumor cells, influencing tumor development and organ selective metastases [51-53]. The role of chemokines in gastrointestinal disorders and cancer has been extensively reviewed [49;54]. As described, high T-cell infiltration in colorectal cancer is associated with good prognosis and might protect from tumor growth. Chemokines regulate trafficking of immune cells and might

therefore represent an important factor in coordinating an anti-tumor immune response. This concept that (over-)expression of specific chemokines causes tumor infiltration by distinct leukocyte subsets, resulting in tumor regression and tumor specific immunity has been described in several tumor models [55-61]. However, understanding this complex network of factors involved in trafficking of leukocytes in the cancer microenvironment remains further exploration [62].

T-CELL MEDIATED IMMUNOTHERAPY

In search for new treatment options to cure patients from colorectal cancer, much effort has been put in exploiting the immune system and evoking tumor-specific immune responses using T-cell-mediated immunotherapy. The unique advantage of this type of treatment is that theoretically the immune system is able to specifically target and destroy tumor cells. Despite great progress in the field of tumor immunology, clinical application of T-cell-mediated immunotherapy yielded only limited success [63]. So far cellular immunotherapy is not part of the clinical routine to treat colorectal cancer patients. However, recent studies have revealed the dawn of a new era in which the activation of tumor-specific T-cells starts to make a difference. Sipuleucel-T is the first therapeutic cancer vaccine to demonstrate effectiveness in Phase III clinical trials by prolonging the life of advanced or late stage metastatic, asymptomatic hormone refractory prostate cancer patients (HRPC) [64;65]. The vaccine was approved by the U.S. Food and Drug Administration to treat patients with HRPC [66]. Treatment with Ipilimumab, a monoclonal antibody that targets the immune regulatory molecule CTLA-4 represents the first modality that had a significant impact on the overall survival of patients with metastatic melanoma [67]. These results are the first positive demonstration that blockade of a T-cell activity inhibitory pathway can be an effective cancer treatment. Also adoptive T-cell therapy (ACT) has been found to be effective in the treatment for metastatic melanoma patients [68-70]. Last but not least, vaccination with a synthetic long-peptide (SLP) vaccine against the HPV-16 oncoproteins E6 and E7 resulted in the complete regression of human papillomavirus-16-positive, grade 3 vulvar intraepithelial neoplasias in 47% of the patients [71]. Complete responses in this study were correlated with the strength of HPV-16-specific immunity [71]. These encouraging results in patients with different types of carcinomas positively stimulate research on immunotherapy of colorectal cancer patients.

FRAMESHIFT-MUTATED GENE PRODUCT-DERIVED PEPTIDES, A CLASS OF TUMOR-SPECIFIC ANTIGENS

Despite many years of work, the number of antigens recognized by tumor infiltrated lymphocytes (TILs) of colorectal cancer identified is limited [40;72-74]. Consequently, vaccines so far have been developed on the basis of proteins that are selectively expressed by tumor cells. A possible unique group of TAAs comprises MSI-H tumors that, due to numerous of frameshift mutations in microsatellites express neo-antigens (Figure 1). MSI-H is a molecular feature of tumors associated with the familial Lynch syndrome also known as hereditary non-polyposis colorectal cancer (HNPCC) syndrome, accounting for approximately 5% of all colorectal cancer cases [75-77] and for approximately 15% of all sporadic colorectal, gastric and endometrial cancers, as well as at lower frequencies for various other sporadic cancers [78-82]. MSI-H colorectal tumors are predominantly localized in the proximal colon, comprising 50% of all proximal colon tumors [83;84]. Since frameshift-mutated products (FSPs) are foreign to the immune system, they represent a unique group of tumor-specific antigens. As no tolerance and consequently strong T-cell responses are expected against the non-self-segment encoded by sequences downstream of the mutation, they are considered promising candidates for prophylactic vaccination of subjects with Lynch syndrome or HNPCC, or as adjuvant therapy in combination with surgery for patients

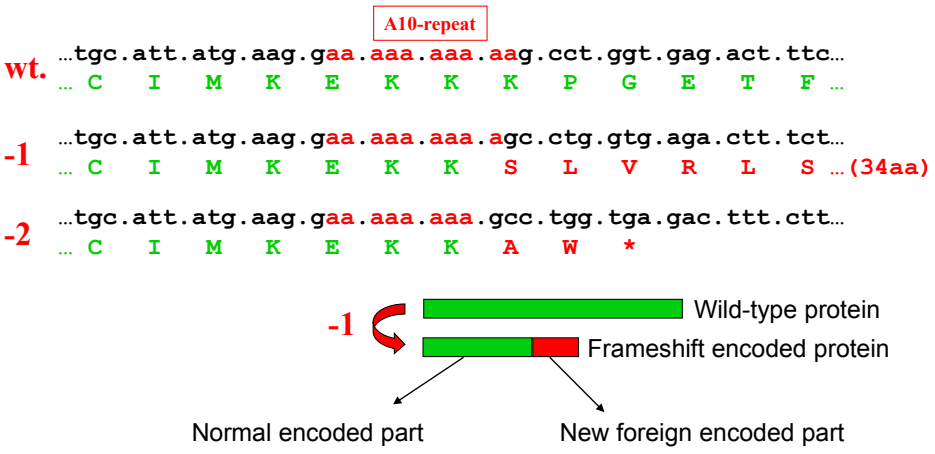

Figure 1. Insertion or deletion of mutations in microsatellites result in frameshift mutations and if 'translated' in proteins with a 'foreign' part.
A part of the TGFβR2 gene and corresponding amino-acid translation is depicted of the wild-type (wt.), and containing a -1 or -2 deletion in the microsatellite (red). As shown a -1 deletion in the microsatellite results in a new foreign encoded part after the frameshift mutation and a new stop after 34 amino acids (aa), while a -2 deletion results in a frameshift mutation and a new stop 2 amino acids after the microsatellite.

with (sporadic) MSI-H tumors. Unfortunately, relatively little is known on the immunogenic behavior of most of the FSPs [40].

VACCINES TARGETING P53-OVEREXPRESSING COLORECTAL TUMORS

Defined antigens to be used as vaccine candidates should ideally be overexpressed in the context of HLA at the cell surface of tumor cells and not (or at very low) levels by other cells of the human body. FSPs are a unique example of tumor specific antigens. Unfortunately only a minority of the colorectal tumors comprises MSI-H tumors that express these FSPs. The majority of the colorectal cancers are chromosomal unstable (CIN). CIN tumors lack tumor specific antigens to be used in vaccination trials. Antigens used in vaccination studies for colorectal cancer comprise TAA and consequently are likely to be expressed by normal cells [85-87]. Different TAA such as: p53, CEA, MUC1, Sialyl-Tn, 5T4, SART3, MAGE have been applied in clinical trials to vaccinate colorectal cancer patients [85-89]. The use of antigens potentially expressed by normal cells bears the risk of immune tolerance. Indeed, tolerance too many TAA such as p53, CEA and MUC1 has been found [90-96]. These results indicate that tolerance forms a potential hurdle for immunotherapies of cancer when using TAA.

One of the TAA frequently used in cancer vaccination trials and much studied in the Leiden University Medical Center comprises p53. Due to a mutation, p53 is overexpressed, while wildtype (wt) p53 in normal cells is not or in very low levels expressed [97-100]. The most common way to disrupt the p53 pathway is through a point mutation that inactivates its capacity to bind specifically to its cognate recognition sequence, and often results in overexpression of p53 [101]. The aberrant expression of the p53 protein in tumor cells versus the low expression in non-tumor cells provides an immunological window for the use of p53 as a tumor antigen for immunotherapeutic intervention against cancer [102]. P53 is mutated and overexpressed in approximately 34-45% of all colorectal cancers [103].

The presence of humoral and proliferative immunity against p53 in the blood of humans has been described for a long time. Both IgM and IgG type antibodies against p53 have frequently been detected in the sera of cancer patients, including patients with colorectal cancer [104;105]. Because p53 is not expressed at the cell surface, only p53-specific T-cell mediated immunity is likely to exert therapeutic antitumor effects. T-helper responses have been described in humans especially in cancer patients [25;106-108]. However, there are strong indications that the p53-specific $CD8^+$ T-cell repertoire is severely restricted by self-tolerance [90;91;109], as high-avidity self-reactive T cells are suspected to be deleted in the thymus [110]. Most of the described human p53-specific CTLs have been generated after *in vitro* culture [111-115]. Although vaccination

against p53 might mainly induce p53-specific CD4+ T cells, these are important in cancer immunotherapy because IFNγ secreting CD4+ T_H1-cells play an important role in orchestrating and sustaining the local immune attack by CD8+ CTL and innate immune effector cells [116-118].

Several different antigen delivery systems have been tested to immunize patients against p53. In previous studies adenoviral vector encoding wt.p53 [119], recombinant canarypox virus encoding wt.p53 [108;120], and adenoviral vector encoding wt.p53 transfected DCs [121] 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 [122]. This concept but has the disadvantage that patients with other HLA types cannot be treated [109].

Synthetic long peptides (SLP®) can also be used as vaccines [28;123]. 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® [124;125]. A SLP® vaccine for the induction of p53-specific T-cell immunity was developed. Injection of p53-SLP® resulted in a strong p53-specific CD4+ T-cell response to three different epitopes in mice [91]. This p53-SLP® vaccine is to be tested for its safety and immunogenicity cancer patients.

OPTIMIZATION OF VACCINATION STUDIES RESULTS IN CLINICAL SUCCESS

The most recent vaccine developments suggest that some of the current vaccine strategies do harbor the capacity to induce immune responses in cancer patients even to self-antigens. However, lack of clinical results in phase I/II trials in colorectal cancer patients suggests that the vaccine-induced T-cell responses against these antigens are at this point not robust enough or of sufficient quality to confidently progress to efficacy trials. A stronger focus should be put on how to induce the strongest and best qualified leukocyte population by vaccination. A clear positive relation between survival of colorectal cancer patients and high expression of a type 1 response has been established [30]. 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 [29;126]. This unique function of the tumor-specific CD4+ T cells is greatly accelerated by production of IFN-γ and IL-2 [25;29]. For example, patients with metastatic colorectal cancer receiving chemotherapy and vaccinations

against the tumor antigen 5T4 were found to have more clinical benefits when 5T4-specific IFN-γ ELIspot responses were induced. [127]. Altogether, these data suggest that clinical responses after vaccination not only depend on the induction of vaccine-specific responses, but merely require the induction of a strong and broad type 1 T-cell response. Therefore, in order to benefit from the local effect of tumor-specific T cells, vaccines should be combined with immune modulating adjuvants that specifically induce polarization of the induced immune response into a type 1 response.

A possible candidate adjuvant might be Interferon-alpha (IFN-α) as it plays a major part in the differentiation of the Th1 subset, as well as in the generation of CTL and the promotion of the *in vivo* proliferation and survival of T cells [128]. Moreover, several studies have shown that type I IFNs promote the differentiation of monocytes into DC *in vitro* and can markedly enhance DC activities [129-134]. Only one study in humans has combined IFN-α injections with peptide vaccination [135]. This study showed that the concomitant combination of a peptide-based vaccine with IFN-α was safe, resulted in a consistent enhancement of vaccine-specific $CD8^+$ T cells and yielded a general increase of the percentage of blood circulating DC precursors/ $CD14^+$ monocytes [135]. It would be interesting to study if addition of IFN-α to the p53-SLP® vaccine not only induced a stronger p53-specific but also a better polarized Th1 response.

THESIS OUTLINE

The studies described in this thesis aim to increase the knowledge on the interaction between the immune system and colorectal tumor cells, with final purpose, the design of effective T-cell mediated immunotherapy. As there are strong indications that presence of intra-tumoral $CD8^+$ T cells is associated with prognosis of colorectal cancer patients and most tumor associated antigens comprise intracellular proteins and might therefore not be accessible for antibodies, this thesis primarily focuses on T-cell mediated anti-tumor immunity.

Conflicting results have been described for the association between expression of HLA class I and prognosis in colorectal cancer patients, possibly due to the use of cohorts with mixed types of genetic instability [44-47]. Therefore in **chapter 2** we evaluated the association between HLA class I expression and prognosis in patients curatively operated for rectal cancer consisting of mainly MSS cancers. The infiltration of diverse types of NK and T-cells in the different types of tumor compartments is carefully assessed and stratified, especially in relation to HLA class I down-regulation in **chapter 3**.

Interaction of chemokines with their cognate receptors allows attraction of immune cells into a tumor, but also influences migration of disseminated tumor cells. In **chapter 4**, a specific chemokine, CXCL5 that in rats was found to be associated with aggressive growth, was studied for its association to survival and T-cell infiltration in rats and humans.

MSI-H tumors are characterized by mutations in microsatellites that result in the expression of frameshift-mutated proteins. In **chapter 5** the use of an expression system to systematically analyze the characteristics and immunogenic properties of proteins encoded by frameshift mutated genes that are commonly found in MSI-H cancers is described.

In **chapter 6** the results of a phase I trial are presented, studying both safety and immunogenicity of a vaccine consisting of a pool of synthetic long p53 peptides in patients treated for metastasized colorectal cancer. **Chapter 7** describes the results from a phase I trial that studied if addition of IFN-α to the p53-SLP® vaccine enables polarization of the induced p53 $CD4^+$ T-cell response into a strong Th1 response.

Finally, **chapter 8** provides a summary and discussion of this thesis.

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Chapter 2

Clinical impact of HLA class I expression in rectal cancer

Frank M. Speetjens[1,*], Elza C. de Bruin[2,*], Hans Morreau[3], Eliane C.M. Zeestraten[1], Hein Putter[4], J.Han van Krieken[5], Maaike M. van Buren[1], Monique van Velzen[2], N.Geeske Dekker-Ensink[1], Cornelis J.H. van de Velde[1], Peter J.K. Kuppen[1']

Departments of [1]Surgery, [2]Clinical Oncology, [3]Pathology and [4]Medical Statistics and Bioinformatics, Leiden University Medical Center, Leiden, the Netherlands
[5]Department of Pathology, Radboud University Nijmegen Medical Center, Nijmegen, the Netherlands
[*]These authors contributed equally to this work

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ABSTRACT

Purpose: To determine the clinical impact of Human Leukocyte Antigen (HLA) class I expression in irradiated and non-irradiated rectal carcinomas.

Experimental design: Tumor samples in tissue micro array format were collected from 1135 patients. HLA class I expression was assessed after immunohistochemical staining with two antibodies (HCA2 and HC10).

Results: Tumors were split into two groups: 1) tumors with >50% of tumor cells expressing HLA class I (high) and 2) tumors with ≤50% of tumor cells expressing HLA class I (low). No difference in distribution or prognosis of HLA class I expression was found between irradiated and non-irradiated patients. Patients with low expression of HLA class I (15% of all patients) showed an independent significantly worse prognosis with regard to overall survival and disease free survival. HLA class I expression had no effect on cancer specific survival or recurrence free survival.

Conclusions: Down-regulation of HLA class I in rectal cancer is associated with poor prognosis. In contrast to our results, previous reports on HLA class I expression in colorectal cancer described a large population of patients with HLA class I negative tumors, having a good prognosis. This difference might be explained by the fact that a large proportion of HLA negative colon tumors are microsatellite instable (MSI). MSI tumors are associated with a better prognosis than microsatellite stable (MSS). As rectal tumors are mainly microsatellite stable (MSS), our results suggest that it is both, oncogenic pathway and HLA class I expression, that dictates patient's prognosis in colorectal cancer. Therefore, to prevent confounding in future prognostic analysis on the impact of HLA expression in colorectal tumors, separate analysis of MSI and MSS tumors should be performed.

INTRODUCTION

The immune system is thought to have an important function in controlling tumor growth and eliminating metastasizing tumor cells. Expression of Human Leukocyte Antigen (HLA) class I, presenting tumor-associated antigens on the tumor cell surface, is considered as a prerequisite for an effective T cell immune response [1]. As a consequence, tumor cells with down-regulated HLA class I expression might escape this immune response, resulting in a selective outgrowth of these tumor cells.

Many studies described HLA class I expression in cancer [2-5]. Only limited studies have reported on the clinical impact of HLA class I expression in colorectal cancer with contrasting results. Some studies found no significant correlation between staining intensity and survival [6-8], while others found a prognostic correlation between HLA expression and survival [9;10]. The latter two studies had in common that total absence of HLA class I resulted in a favorable prognosis compared to patients with down-regulated expression HLA class I of tumor cells. Discrepancy between the two studies is, that one described high expression of HLA class I in tumor cells to result in a better prognosis compared to partial down-regulation of HLA class I [9], while the other found the opposite [10]. These studies both analyzed a mixed population of colon and rectal cancer patients. For rectal cancer patients, the clinical impact of HLA class I expression is still unknown. Since HLA class I expression is often absent in microsatellite instable (MSI) tumors [11;12] and MSI is more frequently observed in right-sided colon tumors than in rectal tumors [13], results obtained from a mixed population of colon and rectal cancer patients might not hold true for rectal cancer patients.

Purpose of this study was to analyze the clinical relevance of HLA class I expression for rectal cancer patients. In addition to determine the impact of MSI on HLA class I expression, tumors most at risk for MSI i.e. HLA negative tumors were examined for MSI by determining the expression of the mismatch repair proteins, mismatch mutL homolog 1 (MLH1) and postmeiotic segregation increased 2 (PMS2), that are most absent in sporadic MSI tumors [14;15]. Radiotherapy has been described recently to increase cell surface expression of Major Histocompatibility Complex (MHC) class I molecules in a murine colon adenocarcinoma cell line [16]. Therefore, our study also evaluated the effect of radiotherapy on HLA class I expression in rectal cancer patients. For these purposes, HLA class I expression was evaluated in a set of 1135 formalin-fixed paraffin-embedded rectal cancer specimens. The tumors studied were obtained at time of surgery from patients of a prospective multicenter trial, who were randomized between standardized pre-operative radiotherapy treatment followed by surgery or surgery alone [17].

MATERIAL EN METHODS

Study population

Patients were obtained from the Dutch TME trial, a multicenter trial that evaluated total mesorectal excision (TME) surgery with or without preoperative radiotherapy (5x5 Gray) [17]. Radiotherapeutical, surgical and pathological procedures were standardized and quality-controlled [17;18]. Tumor staging was determined using the Tumor Node Metastasis (TNM) classification [19]. Patients with the hereditary Lynch syndrome also known as hereditary non-polyposis colorectal cancer (HNPCC) were excluded from the TME trial. Sufficient formalin-fixed paraffin-embedded tumor material was available for 1135 Dutch patients. Three 2mm cores of each tumor sample were arrayed into tissue microarrays (TMA) as previously described [20].

Immunohistochemistry and microscopic analysis

TMAs [20] were immunohistochemically stained for HLA class I using the mAb antibodies HCA2 and HC10 and the rabbit anti-β2m polyclonal Ab (A 072; DAKO Cytomation, Glostrup, Denmark). The HCA2 and HC10 antibodies were applied in immunohistochemistry as hybridoma culture supernatant, kindly provided by Prof. J.J. Neefjes from the Netherlands Cancer Institute (Amsterdam, The Netherlands) The reactivity spectrum of HCA2 includes HLA-A (except HLA-A24), HLA-B73 and HLA-C molecules as well as HLA-E, HLA-F and HLA-G antigens [21-23]. HC10 reacts with HLA-B and HLA-C molecules and HLA-A10, -A28, -A29, -A30, -A31, -A32 and -A33 heavy chains [23-26]. The immunohistochemical procedures are described in detail elsewhere [10]. All tumor specimens were stained simultaneously to avoid intra-assay variation. Microscopic analysis was assessed by two independent observers (M.M. v. B. and M. v. V.) in a blinded manner. HCA2, HC10 and β2m stainings were scored in 6 categories. Essentially, the scoring was divided into quartiles but for tumors with less than 25% stained cells there was a distinction made between those with 6%–25% positively stained tumor cells, those with approximately 1%-5% positively stained cells and those with absolute no positively stained tumor cells. [27;28]. Where discrepancies arose between the staining of cores from the same tumor, an average of the scores was taken, with confirmation by 2 observers using a double-headed microscope with a consensus decision taken in all cases. Tissue stromal cells, normal epithelium or lymph follicles served as positive internal controls to ascertain the quality of the staining. Patients were excluded if stromal cells of tumor were not stained for HCA2 or HC10. Twenty five tumors with negative staining of the stromal cells for HCA2 were excluded. HC10 showed in all tumors staining of the stromal cells. Also TNM stage 0 patients, tumors lost due to technical failure and ineligible patients were excluded, leaving 1092 tumors in which HC10 and 1035 in which HCA2 could be evaluated. Combining

results for HCA2 and HC10 staining resulted in 1008 eligible stage I-IV rectal cancer patients for analyses of clinical impact of HLA class I expression.

Tumors negatively stained for HCA2 and/or HC10 were stained for mismatch repair proteins MLH1 and PMS2. MLH1 and PMS2 are deficient in sporadic MSI tumors. Therefore, expression of these proteins was used to differentiate between MSI and MSS rectal cancers. Tissue stromal cells, normal epithelium or lymph follicles served as positive internal controls when analyzing MLH1, PMS2 expression. Expression of MLH1 and PMS2 was scored positive if tumor cells showed expression and scored negative if tumor cells showed no expression of either MLH1 or PMS2 and tissue stromal cells did show expression, indicating respectively Microsatellite Stable (MSS) and Microsatellite Instable (MSI) tumors [14].

Statistical analyses

All analyses were performed with SPSS statistical software (version 12.0 for Windows, SPSS Inc, Chicago, USA). Mann-Whitney U, T-test and χ^2-tests were used to compare variables. Kaplan-Meier analyses were performed to analyze patient survival. The entry date for the survival analyses was the time of surgery of the primary tumor. Events for time to local recurrence, distant recurrence, cancer specific survival, disease free and overall survival were defined as follows; from time of surgery to time of local disease relapse (for local recurrence), time of distant disease relapse (for distant recurrence), time of disease relapse or death by disease (for cancer specific survival), time of disease relapse or death (for disease free survival) and time of death, respectively, (for overall survival). Non-irradiated and irradiated patients were first separately analyzed in univariate analysis and second, variables with a p-value of $\leq$0.10 in the univariate analyses were subjected to a multivariate analysis. Multivariate analysis was performed on the whole group of irradiated and non-irradiated patients with the following variables: HLA class I, randomization for radiotherapy, TNM and circumferential margin. Cox' regression analyses were used to calculate Hazard Ratios (HR) with 95% confidence intervals (CI).

RESULTS

Scoring methods

Several methods are described to analyze HLA class I expression in cancer. The standard is defined by the International HLA and Immunogenetics Workshop (IHIW) [27;28]. A recent paper describing HLA class I expression in colorectal cancer used an adjusted form of this scoring method [9]. Our scoring was primarily adapted from IWIH, i.e. division into quartiles, but for tumors with less than 25% stained cells a

distinction was made between those with 6%–25% positive tumor cells, those with approximately 1%-5% positive tumor cells and those with absolute no HLA class I positive stained tumor cells. After scoring and analyzing this method we found that patients in the groups with absolute no, 1%-5%, 6%-25% and 26%-50% HLA class I expression of tumor cells did not differ in prognosis but had a worse prognosis compared to patients with HLA class I expression in groups with 50-75% and >75% of tumor cells expressing HLA class I. Therefore, we distinguished two categories. These two categories were 1) 0%-50%; and 2) >50%-100% of tumor cells expressing HLA class I.

HCA-2 and HC10 staining in rectal cancer

Immunohistochemical staining with HCA2 and HC10 antibodies demonstrated strong positive membrane staining of stromal cells and tumor-infiltrating inflammatory cells, indicating the success of the staining. A total of 1035 and 1092 tumors were evaluated with HCA2 and HC10. 324 (65%) irradiated tumors and 312 (58%) non-irradiated tumors showed at least 50% of all tumor cells positive for HCA2. Staining with HC10 resulted in 403 (76%) irradiated tumors and 436 (77%) non-irradiated tumors that showed more than 50% positive tumor cells. Complete results are shown in Table 1. Representative examples of the immunohistochemical stainings of tumors are displayed in Figure 1A-F. These results show that about 35% of irradiated and 42% of non-irradiated patients showed in less than 50% of the tumor cells expression of HCA2. HC10 is expressed in less than 50% of the tumor cells in about 25% of both irradiated and non-irradiated rectal cancer patients.

Table 1. Most rectal tumors have high numbers of tumor cells positive for HCA2 or HC10.

		Irradiated patients N (%)	Non-irradiated patients N (%)
HCA2	High	324 (65%)	312 (58%)
	Low	142 (28%)	174 (32%)
	Absence	31 (6.2%)	52 (9.7%)
HC10	High	403 (76%)	436 (77%)
	Low	117 (22%)	116 (21%)
	Absence	8 (1.5%)	12 (2.1%)

Numbers (N) of patients are indicated with percentages shown in parentheses, showing: expression of HCA2 and HC10 in more than 50% of the tumor cells (High), expression in less than 50% of the tumor cells (Low) and total absence (Absence).

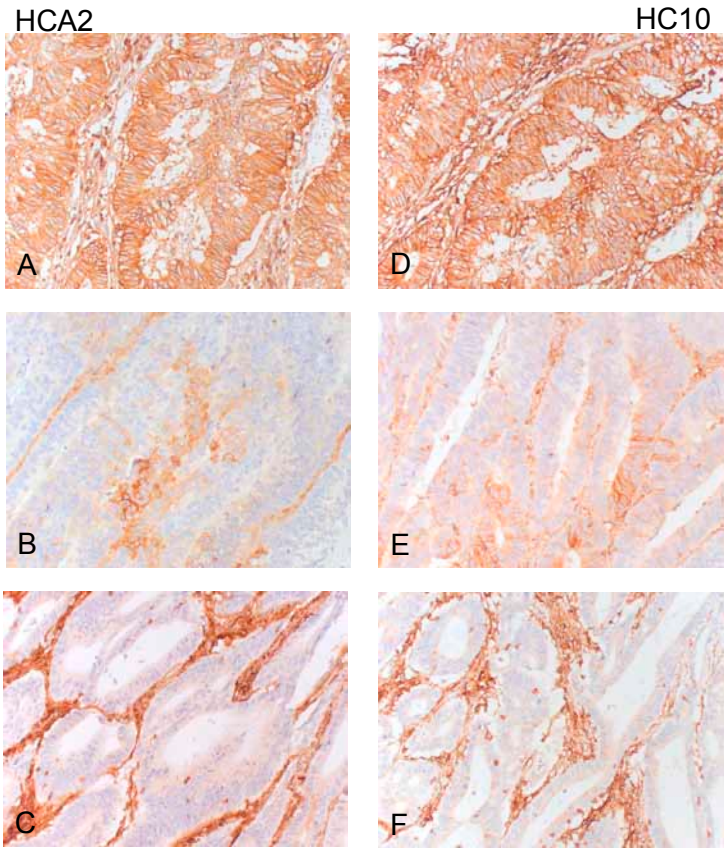

Figure 1. Examples of HCA2 and HC10 immunohistochemical staining of rectal tumors; (A-C) HCA2, (D-F) HC10 expression.

(A, D) expression of HLA class I in >50% tumor cells; (B, E) expression of HLA class I in <50% tumor cells; *(C, F)* epithelial cells show total absence for HCA2 or HC10 and only stromal and infiltrative cells show positive staining for HCA2 or HC10; Original magnification x20.

Analysis of HLA class I expression in rectal tumors

Together, the results obtained with HCA2 and HC10 are expected to reflect HLA class I expression in rectal cancer. In a group of 64 tumors it was studied whether an additional staining for β2m would better define HLA class I expression. The results of the addition of β2m to HCA2 and HC10 were comparable with those obtained with HCA2 and HC10, i.e. only 1 of 64 tumors was differently classified. Therefore, β2m was not scored in the whole cohort and HLA class I expression was assessed by combining HCA2 and HC10.

A total of 406 (85%) irradiated and 445 (84%) non-irradiated tumors exhibited expression of at least one of the two markers showing >50% positive staining of all tumor cells (further referred to as 'the HLA class I high expression group'). A total of 70 (15%) irradiated and 87 (16%) non-irradiated tumors showed reduced numbers (≤50%) of HLA class I positive tumor cells. Only 3 (0.6%) irradiated tumors and 8

(1.5%) non-irradiated tumors showed total loss of HLA class I (negative for both HCA2 and HC10). Survival results of patients with total absence of HLA class I on tumor cells did not show significant difference from patients with reduced numbers of HLA class I positive tumor cells. Therefore, these groups were combined and will be further referred to as 'the HLA class I low expression group'. Complete results are shown in Table 2. The number of patients in the group of the HLA class I high expression group and the HLA class I low expression group was equally distributed between irradiated and non-irradiated tumors (χ^2=0.519, p=0.471), indicating that irradiation had no effect on HLA class I expression in these patients.

Table 2. Expression of HLA class I in rectal cancer using HCA2 and HC10 antibodies.

	HCA2	HC10		HLA class I
		High (N)	Low + Absence (N)	N (%)
Irradiated	High (N)	270	37	406 (85%)
	Low + Absence (N)	99	70	70 (15%)
Non-irradiated	High (N)	277	32	445 (84%)
	Low + Absence (N)	136	87	87 (16%)

Expression of results of HCA2 and HC10 staining in a cross table for numbers (N) of irradiated and non-irradiated patients; expression of HCA2 and HC10 in more than 50% of the tumor cells (High) versus expression in less than 50% of the tumor cells (Low) is shown. A significant correlation was noted between HCA2 and HC10 staining for both irradiated (χ2 = 53.947, p<0.001) and non-irradiated patients (χ2 = 61.257, p<0.001). The right side of the table displays HLA class I expression estimated on HCA2 and HC10 expression. A total of 406 (85%) irradiated and 445 (84%) non-irradiated tumors exhibited expression of at least one of the two markers showing >50% positive staining of all tumor cells. A total of 70 (15%) irradiated and 88 (16%) non-irradiated tumors showed reduced numbers (≤50%) of HLA class I positive tumor cells. The number of patients in the group of the HLA class I high expression group and the HLA class I low expression group was equally distributed between irradiated and non-irradiated tumors (χ2 = 0.519, p = 0.471).

HLA class I negative cells and microsatellite instability

It has been described that a majority of MSI colorectal tumors do not express HLA class I, while only a minority of MSS tumors do not express HLA class I [12;29]. Therefore, HLA class I negative rectal tumors are most at risk to be MSI tumors. To evaluate the numbers of sporadic MSI tumors in our study, HCA2 or HC10 negative tumors were analyzed for the expression of PMS2 and MLH1. PMS2 and MLH1 are mismatch repairs proteins that are most frequently absent in MSI sporadic tumors [30]. Of the HLA class I negative tumors only one out of 11 tumors did not express PMS2 and MLH1. In the tumor group negative for only one of the two HLA class I markers, 2 of 81 tumors displayed no PMS2 and MLH1 staining. These results indicate that HLA class I down-regulation is not associated with MSI in rectal cancer and are in accordance with previous findings that only a very small minority of rectal tumors is MSI [31;32].

HLA class I expression and clinicopathological parameters

The relationship between HLA class I expression and patient/tumor characteristics was assessed (Table 3). HLA class I expression levels were distributed equally in non-irradiated and irradiated patients with regard to most clinical and pathological parameters. Three significant differences were observed. For the non-irradiated patients, significantly more men appeared in the HLA class I low expression group (p=0.03). The group of irradiated tumors with HLA class I low expression contained significantly more stage III and IV tumors (p=0.01) and also more patients with a tumor positive circumferential resection margin (p=0.02) when compared with tumors with high HLA class I expression.

Table 3. Clinicopathological characteristics of irradiated and non-irradiated patients with high or low numbers of HLA class I positive tumor cells.

	Non-irradiated patients		p-value	Irradiated patients		p-value
	High N=445	Low N=87		High N=406	Low N=70	
Gender						
Male, (%)	63	75	**0.03**	65	66	0.90
Age						
median years	65	68	0.32	65	65	0.99
TNM stage, (%)						
I	31	24	0.52	33	24	**0.01**
II	27	30		30	21	
III	36	38		32	40	
IV	5	8		5	14	
Circumferential margin						
Negative, (%)	83	77	0.28	86	74	**0.02**
Distant from anal verge, (%)						
≥ 10 cm	28	33	0.17	27	32	0.30
5-10 cm	41	31		46	36	
< 5 cm	31	36		27	32	
Operation type, (%)						
Low anterior resection	66	61	0.77	65	66	0.89
Abdomino-perineal resection	29	33		29	30	
Hartmann	5	6		6	4	

Number (N) of patients with expression of total HLA class I expression in more than 50% of the tumor cells (High) and expression in less than 50% of the tumor cells (Low).
* Statistical significant p-values are in bold

Expression of HLA class I and clinical prognosis

Because radiotherapy might influence local tumor recurrences [17], irradiated and non-irradiated tumors were analyzed separately in order to evaluate the impact of HLA class I expression on tumor recurrence and patient survival. HLA class I expression was not related with distant or local recurrence rates. Patients with low expression of HLA class I had a worse overall survival and disease free survival when compared to patients with HLA class I high expression, irrespective of treatment (Fig 2; overall survival: p=0.008 and p=0.01; disease free survival: p=0.01, p=0.006 in irradiated and non-irradiated patients respectively). Irradiated patients with low HLA class I expression also had a worse cancer specific survival (p=0.003). For non-irradiated patients, HLA class I expression had no significant effect on cancer specific survival (Fig 2). All results of univariate analysis are

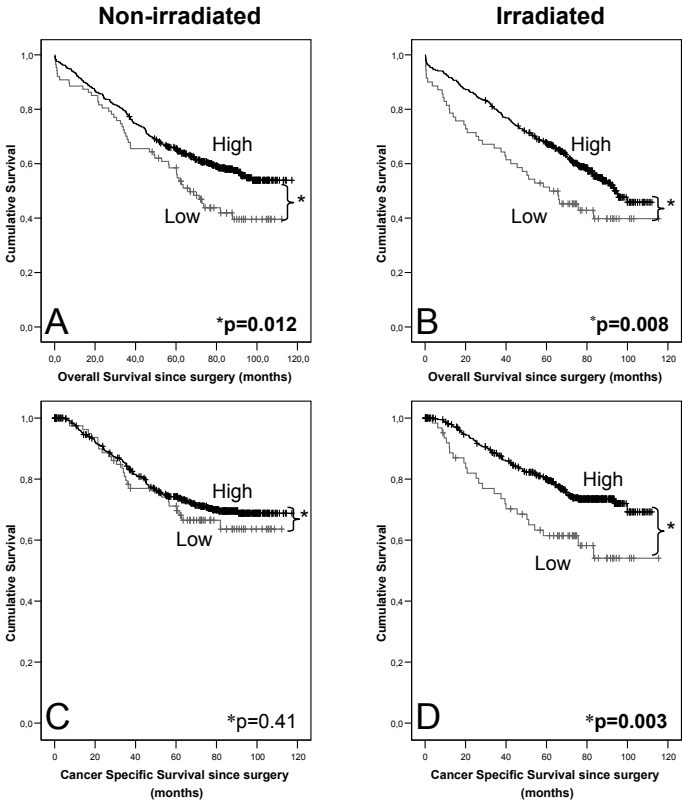

Figure 2. Examples of Kaplan-Meier curves showing overall survival and cancer specific survival for irradiated and non-irradiated patients.

Kaplan-Meier curves for overall survival *(A, B)* and cancer specific survival *(C, D)*; curves show prognosis for non-irradiated *(A, C)* and irradiated patients *(B, D)* for HLA class I expression in more than 50% of the tumor cells (High) versus expression in less than 50% of the tumor cells (Low). P-value is based on univariate log rank analyses; statistical significant p-values are in bold.

shown in table 4. Univariate analysis showed a better outcome for overall survival and disease free survival in patients with high HLA class I expression.

Table 4. Both irradiated and non-irradiated patients with high expression of HLA class I have a better overall -, and disease free survival.

	Non-irradiated patients			Irradiated patients		
	High	Low	p-value	High	Low	p-value
Overall survival	65.5%	58.5%	**0.012**	67.5%	51.3%	**0.008**
Disease free survival	62.2%	53.5%	**0.015**	62.2%	48.3%	**0.006**
Cancer specific survival	74.3%	71.4%	0.41	80.1%	61.8%	**0.003**
Local recurrence	8.9%	13.7%	0.22	4.7%	3.2%	0.72
Distant recurrence	26.7%	28.7%	0.88	24.7%	29.3%	0.34

Survival and recurrence rates indicated in percentages after 5-years of follow-up for non-irradiated and irradiated patients for HLA class I expression in more than 50% of the tumor cells (High) versus expression in less than 50% of the tumor cells (Low). P-value is based on univariate log rank analyses for overall survival, disease free survival, cancer specific survival, local recurrence and distant recurrence.
* Statistical significant p-values are in bold

Multivariate analysis

Multivariate analysis was performed to identify factors with independent prognostic significance and to calculate hazard ratios (HR). Analyses included TNM, circumferential margin, randomization for pre-operative radiotherapy and HLA class I expression (low versus high HLA class I positive tumor cells) (table 5). Advanced pathological (TNM) stage and tumor positive circumferential resection margins retained their strength as independent prognostic factors in these survival analyses. HLA class I expression showed independent prognostic value for overall survival and disease free survival (HR: 1.3, p=0.042 and HR: 1.4, p=0.006 respectively), but not for cancer specific survival.

Table 5. Multivariate analysis confirms independent better overall -, and disease free survival for rectal cancer patients with high expression of HLA class I.

	Overall survival		Disease free survival		Cancer specific survival	
	HR (95% CI)	p-value	HR (95% CI)	p-value	HR (95% CI)	p-value
HLA						
High	**1**	**0.042**	**1**	**0.006**	1	0.653
Low	**1.3 (1.0 – 1.6)**		**1.4 (1.1 – 1.8)**		1.1 (0.8 – 1.5)	
Randomization						
TME	1	0.632	1	0.214	1	0.282
TME + RT	1 (0.8 – 1.2)		0.9 (0.7 – 1.1)		1.1 (0.9 – 1.5)	
TNM						
I	**1**		**1**		**1**	
II	**2.2 (1.7 – 3.0)**	**<0.001**	**2.1 (1.6 – 2.8)**	**<0.001**	**3.5 (2.0-6.1)**	**<0.001**
III	**3.1 (2.4 – 4.1)**	**<0.001**	**3.1 (2.3 – 4.0)**	**<0.001**	**9.0 (5.4 – 14.9)**	**<0.001**
IV	**11.8 (8.1 – 17.1)**	**<0.001**	**--**	**--**	**50.3 (28.5 – 89.1)**	**<0.001**
CRM						
negative	**1**	**<0.001**	**1**	**<0.001**	**1**	**<0.001**
positive	**1.3 (1.1 – 1.5)**		**1.8 (1.4 – 2.2)**		**1.3 (1.1 – 1.5)**	

Multivariate analysis for cancer specific, overall and disease free survival was performed to identify factors with independent prognostic significance and to calculate hazard ratios (HR) with 95% confidence intervals (CI) shown in parentheses. HLA class I expression in more than 50% of the tumor cells (High) versus expression in less than 50% of the tumor cells (Low); Total mesorectal excision (TME); Radiotherapy (RT); circumferential margin (CRM); p-value is based on Cox' regression analyses.
* Statistical significant p-values are in bold

DISCUSSION

We showed that rectal cancer patients from the HLA class I low expression group had an independent worse overall and disease free survival when compared to patients from the HLA class I high expression group. These data imply that expression of HLA class I in tumor cells predicts survival for rectal cancer patients. Although, significant better cancer specific survival for irradiated patients with high HLA class I was found in univariate analysis, the predictive value was lost in multivariate analysis. This observation can be explained by the fact that the group with low HLA class I included significant more stage III/IV and more patients with a positive circumferential margin compared to the group patients with high expression of HLA class I. Also no predictive value of HLA class I expression was found with regard to recurrence free survival of these patients. Therefore, we have no indications that support the notion that better survival of high HLA class I expression is due to the better antigen presenting function of these tumor cells, as has been suggested [9;10].

In our study, no difference was found between irradiated and non-irradiated patients for HLA class I expression in tumor cells. It has been described that γ-irradiation induces enhanced peptide production and surface expression of MHC

class I in a colorectal mouse tumor cell line [16]. The fact that we could not find more HLA class I expression in irradiated tumors than in non-irradiated tumors indicates that radiotherapy does not induce HLA class I expression *in vivo*. Immunohistochemistry, however, is less suitable to measure subtle expression changes. Therefore additional research is required to determine the impact of radiotherapy on expression levels of HLA class I in human tumors.

In our study, more tumors showed HLA class I down-regulation after immunohistochemical staining using HCA2 than using HC10. This difference might be due to differences in reactivity spectrum of both antibodies (see 'materials and methods') or to the fact that HLA alleles are differently affected in colorectal cancer. If the latter is the case, our results suggest that HLA A alleles preferentially show down-regulation in rectal cancer.

Previous reports evaluated HLA class I expression in mixed patient populations of colon and rectal cancer patients [9;10]. Watson et al also found in a large group of colorectal cancer patients that patients with low expression of HLA class I had a poor prognosis [9]. However, in contrast to our results, both studies described a substantial population of patients with tumors showing absence of HLA class I. In addition, they described that absence of HLA class I was associated with better prognosis compared to tumors expressing reduced numbers of HLA class I positive tumor cells. A relatively low number (1.1%) of HLA class I negative tumors was observed in our cohort of rectal cancer patients only. These patients showed no survival advantage when compared with patients with reduced numbers of HLA class I positive tumor cells. There are several explanations for the discrepancy in number of HLA class I negative tumors between the study of Watson et al. and ours, like different definition for HLA class I expression, differences in staining techniques, different patient cohort and number of MSI tumors.

We showed that tumors that do not stain HC10 can stain positive for HCA2 and thus are still able to present antigens. Therefore, an explanation for the differences with the results of Watson et al. is that we used strict criteria to classify tumors as HLA class I absent (defined as both HCA2 and HC10 negative) compared to Watson *et al.* (defined as negative for HC10 or negative for beta2M instead of negative for both). Another important explanation is that we examined HLA class I expression in a relative more homogeneous population of patients with a rectal tumor, while the other cohorts are more heterogeneous, consisting of both colon and rectal cancer. Although combining results from colon and rectum is generally accepted when predicting prognosis, this might influence results [33].

In colon cancer patients, approximately 50% of all proximal colon tumors show MSI, whereas almost all distal colon and rectal cancers are MSS tumors [13;34]. Loss of HLA class I has been described in at least 60% of all sporadic right-sided MSI

colorectal tumors but in only 17% of MSS right-sided colon tumors loss of HLA class I is found [12;29]. In our cohort, only one out of 11 HLA negative tumors and two out of 81 tumors negative for HCA2 or HC10 did not express MLH1 and PMS2 and were thus likely MSI tumors. This indicates that rectal cancers are mainly MSS tumors, as has previously been described [31-33]. Of the multiple mechanisms that have been shown to underlie defects in HLA class I expression in colorectal cancer (mutations in the individual HLA class I genes, mutations in β2- microglobulin (β2m) [12], and defects in components of the HLA class I-associated antigen processing machinery (APM) [12;35]), only the first will result in allele-specific aberrancies while the other affect total HLA class I expression and may result in total absence in a tumor cell.

These observations imply that a population of colorectal tumors with total absence of HLA class I probably contains a disproportionate large number of MSI tumors when compared with colorectal tumors expressing HLA class I. In addition, MSI colorectal tumors have a better prognosis when compared with MSS colorectal tumors [34;36]. Therefore, HLA class I negative tumors are more likely to be MSI tumors with a different clinical behavior compared to MSS colorectal tumors. It is likely that MSI influences prognostic results when considering HLA class I expression in colorectal tumors.

Our results show that HLA class I expression in rectal cancer affects patient's prognosis. We hypothesize that both, oncogenic pathway and HLA class I expression, dictates clinical tumor progression. We suggest that in future prognostic studies, analyzing expression of HLA class I or other biomarkers in colorectal cancer, impact of MSI should be considered.

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Chapter 3

Natural Killer cells infiltrating colorectal cancer and MHC class I expression

M.H. Sandel[1], F.M. Speetjens[1], A.G. Menon[1], P.A. Albertsson[2], P.H. Basse[3], M. Hokland[4], J.F. Nagelkerke[5], R.A.E.M. Tollenaar[1], C.J.H. van de Velde[1], P.J.K. Kuppen[1]

[1] Department of Surgery, Leiden University Medical Center, Leiden, the Netherlands
[2] Department of Oncology, Sahlgrenska University Hospital, Göteborg, Sweden
[3] University of Pittsburgh Cancer Institute, Pittsburgh, USA
[4] Institute of Medical Microbiology and Immunology, University of Aarhus, Aarhus, Denmark
[5] Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, the Netherlands

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ABSTRACT

Purpose: A majority of colorectal adenocarcinomas displays diminished MHC class I expression, making them particularly vulnerable for NK cell-mediated killing. Generally, these tumors also show a substantial inflammatory infiltrate. Most inflammatory cells, however, reside in the tumor stroma, where they do not have direct contact with tumor cells in the tumor epithelium. In this study we investigated the correlation between colorectal tumor MHC class I aberrations and infiltration of NK cells.

Experimental design: We studied 88 tumor specimens obtained from 88 colorectal cancer patients for locus specific HLA aberrations and correlated these data to infiltration of CD4, CD8 and CD56 positive lymphocytes. The lymphocyte markers were individually combined with laminin as a second marker to facilitate quantification in the different tumor compartments, i.e. tumor epithelium and tumor stroma.

Results: Locus specific partial -or total HLA class I loss was detected in 72% of the tumors studied. Twenty-eight percent had no HLA loss at all. Mean overall intra-epithelial infiltration of CD56 positive lymphocytes was 7 cells per mm^2 compared to 76 cells per mm^2 for CD8 and 19 cells per mm^2 for CD4 positive lymphocytes. Locus specific partial or total loss of tumor cell MHC class I expression was positively correlated with intra-epithelial infiltration of CD8 positive cells ($p = 0.01$), but not with CD4 or CD56 positive lymphocytes. Triple immunofluorescence staining showed that these cells were CD8 -and granzyme-B positive T-lymphocytes.

Conclusions: Our data showed that colorectal tumors are sparsely infiltrated by CD56 positive cells compared to CD8 positive T-cells and that loss of MHC is associated with T cell infiltration instead of NK cell infiltration. Considering the fact that MHC loss is quite common in colorectal cancer and that, due to local absence of NK cells, it is unlikely that there has been selection for NK-escape variants, improvement of the intra-epithelial infiltration/migration of NK cells may be an important basis for the development of an effective adjuvant NK-based immunotherapy of colorectal cancer.

INTRODUCTION

Colorectal cancer is the most common gastrointestinal cancer in the world and potentially curable with surgical resection of the primary tumor. The clinical problem of colorectal cancer, however, is the spread and outgrowth of metastases. Once the disease has spread to distant organs, treatment options are limited to aggressive systemic therapies with high treatment-related morbidity and/or mortality, while a cure can rarely be obtained. Development of new adjuvant therapeutic strategies focuses on more selective and safer therapeutic options, including immunotherapy. The major advantage of using immune-based adjuvant therapeutic strategies is the potentially high selective focus of immune effector cells on malignant cells, which may limit treatment-related morbidity and/or mortality. However, there are still questions unanswered concerning the immunological mechanisms in an anti-tumor response. A classisal way to gain insight in the immunological mechanisms in the host defense against malignant cells, is to evaluate the histopathology of the host' natural infiltration patterns [1].

The leukocyte infiltrate of primary colorectal tumors is presumed to represent the natural defensive activity of the host against the tumor. A majority of infiltrating leukocytes consists of T-lymphocytes infiltrating the stromal compartment of the tumor. These infiltrating immune cells do not seem to interact directly with the tumor cells, since they are separated by stromal structures (i.e. basal membrane-like structures) that seemingly form a physical barrier preventing interaction [2]. Colorectal tumors show a varying amount of leukocytes in direct contact with tumor cells, i.e. in the tumor-epithelial compartment. This leukocyte fraction consists mainly of CD8 positive lymphocytes [3]. It is generally presumed that these cells are specific anti-tumor CTL. However, as different types of leukocytes may express CD8, among which NK cells, the exact background of these cells still remains unclear.

In vitro, NK cells function well as effector cells against tumor target cells [4-6]. *In vivo* however, NK cells migrating from the bloodstream into a solid tumor only form a minor fraction of the total tumor-infiltrating leukocyte population [3]. Their exact loco-regional function in relation to the tumor is still obscure. One major significance of NK cells is believed to lay in the clearance of tumor cells lacking classical MHC class I surface molecules. Several studies have shown that 70-90% of colorectal tumors show aberrant MHC class I expression [7-10].

To investigate the possible relationship between loss of MHC class I expression and NK cell infiltration in colorectal cancer, we evaluated a series of 88 colorectal tumors obtained from 88 patients for their MHC class I expression and type of leukocyte infiltration using immunohistochemistry and immunofluorescence.

MATERIALS AND METHODS

Patients

A randomly selected group of 88 colorectal cancer patients from a previously described database of consecutive colorectal cancer patients was analyzed [11]. Clinical -and histopathological data are shown in Table 1. Tumors were evaluated for differentiation grade and lymphocytic infiltration according to Jass' criteria [12] on hematoxylin-eosin stainings. Patient follow-up was completed until January 2003.

Table 1. Patient and Tumor Characteristics

	n
Gender	
Female	36
Male	52
Age	
0-50	12
>50	76
Location*	
Right-sided	33
Left-sided	55
Tumor stage	
Stage II	38
Stage III	50
Differentiation	
Poor	54
Moderate	20
Well	11
Unassessable	3
Recurrences	
No	58
Yes	30

Patient and tumor characteristics of 88 curatively resected colorectal cancer patients. Parameters were assessed according to standard clinical and pathology protocols.
*Right-sided location: caecum – flexura lienalis

Immunohistochemistry

Tissue sections were stained as described by Menon et al [3;11]. Either one of the following monoclonal antibodies (culture supernatant) for immunohistochemical staining was used: mouse anti-human HLA-A (Clone HCA2, isotype IgG1, generously provided by dr. J. Neefjes, NKI, Amsterdam), mouse anti-human HLA-B/C (Clone HC10, isotype IgG2a, generously provided by dr. J. Neefjes, NKI, Amsterdam) [13], mouse

anti-human CD4 (Clone 1F6, isotype IgG1, Novocastra Ltd, Newcastle, UK), mouse anti-human CD8 (Clone 4B11, isotype IgG2b, Novocastra Ltd, Newcastle, UK) and mouse anti-human CD56 (Clone 123C3, isotype IgG1, Zymed Inc, San Francisco, USA). Four micrometers thick paraffin sections were mounted on aminopropylethoxysilane (APES) coated slides, and dried overnight at 37°C. Tissue sections were de-paraffinized and rehydrated. Endogenous peroxidase was blocked for 20 minutes in 0.3% hydrogen-peroxide methanol. Antigen retrieval was achieved by boiling in 10mM citrate buffer (pH = 6.0) for 10 minutes in a microwave oven. After washing in PBS the slides were incubated overnight at room temperature with primary antibodies. Sections for HCA2 and HC10 were washed in PBS and incubated with biotinylated rabbit-anti-mouse (1:200, DAKO, Glostrup, Denmark) for 30 minutes, washed again with PBS, and incubated with Streptavidin-Biotin-Complex (DAKO, Glostrup, Denmark) for 30 minutes. Sections for other antibodies were washed and incubated with mouse Envision labeled with Horse Radish Peroxidase (m-Envision^{HRP}) for 30 minutes, washed in PBS and rinsed in 0.05M Tris-HCl buffer (pH 7.6) for 5 minutes. Sections were washed and developed in 3,3-di-amino-benzidine (DAB) tetrahydrochloride substrate solution containing 0.002% hydrogen-peroxid, for 10 minutes, resulting in a brown staining. HCA2 and HC10 stained sections were counterstained with hematoxylin and mounted with pertex (Histolab, Götenborg, Sweden). Lymphocyte stained sections were subsequently incubated in 0.01% trypsin in 0.1 mM $CaCl_2$ for 10 minutes. After washing in demineralized water, sections were incubated overnight with a rabbit polyclonal antibody against laminin (Sigma-Aldrich, Zwijndrecht, The Netherlands). After washing, sections were incubated with a swine-anti-rabbit conjugate labeled with biotin (DAKO) for 30 minutes. Subsequently, sections were incubated for 30 minutes with Streptavidin-Biotin-complex (DAKO) labeled with alkaline phosphatase. Sections were developed in a NBT/BCIP solution, resulting in a blue signal.

For additional immunofluorescent staining we selected 6 tumors with absent HLA-A and B/C expression. Sections were initially treated as described above except that the endogenous peroxidase blocking step was replaced for a 20 minute incubation with 10% human AB serum followed by a 10 minute incubation with 0.1% cationic BSA (Aurion, Wageningen, The Netherlands). Antigen retrieval was performed by boiling in 1 mM EDTA for 10 minutes. First overnight antibody incubation was done with an antibody directed against cytokeratin 8 (DAKO). Next we incubated for 2 hours with ultra-small gold (USG) labelled Goat-anti-Mouse (Aurion) diluted in 0.1% w/v BSAc in PBS. After washing three times with MQ a 40 minute silver enhancement was performed using a silver enhancement kit (Aurion). After washing, sections were consecutively incubated overnight with a mixture of two monoclonal antibodies:

mouse anti-human CD56 (Clone 123C3, Zymed Inc, San Francisco, USA), mouse anti-human Granzyme-B (Clone GRB-7, Monosan, Uden, The Netherlands) and one rabbit polyclonal: anti-CD3 (rabbit polyclonal, Abcam). Next slides were incubated with a mix of three matching fluorescent conjugates: goat-anti-rabbit-IgG2a-Alexa-546, goat-anti-mouse-IgG1-Alexa-488 and goat-anti-mouse-IgG2a-Alexa-647 (Molecular Probes Inc, Leiden, The Netherlands), for 1 hour followed by washing in PBS. Slides were mounted in Mowiol mounting medium and stored in the dark at 4°C until scanning.

Microscopic evaluation of tumor sections

Pictures of the immunofluorescent slides were taken with a confocal Laser Scanning Microscope (Zeiss LSM510; Zeiss, Jena, Germany) in a multi-track setting. For the detection of the silver stain we used bright field microscopy using the 633nm laser in a very low intensity to prevent excitation of the Alexa-647 fluorochrome. Microscopic analysis was performed separately by two observers who had no knowledge of the clinical outcome of the patients. The percentage of the tumor cells expressing HLA-A and HLA-B/C was estimated in each case. Eventually in all cases a consensus was met. Normal HLA expression was defined as a situation in which all tumor cells expressed HLA. HLA expression was defined as reduced when tumor cells showed partial absence of either HLA-A or B/C. Total loss of HLA expression was noted when no tumor cell expressed HLA-A or B/C. The tumor stroma (fibroblasts, lymphocytes, endothelial cells) served as an internal positive control.

Statistical analysis

All statistical analyses were done using the SPSS software package (SPSS, Chicago, Illinois, USA). Disease-free survival data were analyzed using Kaplan-Meier survival estimation and the log-rank test was used for comparison of the survival curves. Statistical analyses between groups were performed using the Chi-squared test for comparing proportions and Krukas Wallis test for comparing means. P values less than 0.05 were considered significant.

RESULTS

Patient characteristics

A panel of 88 primary tumors of colorectal origin was investigated. The patients' characteristics and clinicopathological parameters are given in Table 1. The panel consisted of about equal numbers of stage II (Dukes B; n=38, 43%) and stage III (Dukes C; n=50, 57%) tumors. The average age of the patients was 66.9 years (range:

26.0 – 85.0 years). As expected, tumor stage significantly correlated inversely with the time of disease-free survival (p=0.002, data not shown). None of the other patient -or tumor characteristics as described in Table 1 correlated with DFS.

HLA-I expression

Locus specific down-regulation was detected in 63 (72 %) of 87 colorectal tumors, using antibodies against HLA-A and HLA-B/C (resp. HCA2 and HC10). One case could not be evaluated due to the absence of HLA-A signal on repeated evaluations. In 6 (7 %) tumors HLA expression was absent, i.e. there was no signal detectable for either HLA-A or B/C. Twenty-four tumors (28 %) had no HLA loss at all. HLA-A expression correlated significantly with a longer disease-free survival (p = 0.02).

Tumor-infiltrating lymphocytes

Mean overall intra-epithelial infiltration of CD56 positive lymphocytes was 7 cells per mm^2 versus 76 cells per mm^2 for CD8 and 10 cells per mm^2 for CD4 positive lymphocytes. Immuno-histochemical analysis of primary tumor sections revealed that locus specific MHC aberrations significantly correlated with the intra-epithelial infiltration of CD8 positive cells (p = 0.02), but not with CD4 or CD56 positive lymphocytes (Table 2).

We performed an additional immunofluorescent triple staining on the total HLA-I down-regulated tumors and found that the majority of the intra-epithelial infiltrating cells consisted of CD3 -and Granzyme-B positive lymphocytes (Figure 1). These results show that not NK cells, but T cells preferentially infiltrate colorectal tumor specimens that show down-regulated MHC class I expression.

Table 2. Tumor-infiltrating lymphocytes according to HLA class I aberrations

	HLA class I expression						
	normal		**reduced**		**absent**		**p-value**
	[mean cells per mm^2]	n	[mean cells per mm^2]	n	[mean cells per mm^2]	n	
Intra-epithelial lympocytes							
CD4	8	21	9	56	27	2	0.13
CD8	18	19	79	56	203	2	***0.01***
CD56	5	21	8	54	6	2	0.30

The number of intra-epithelial lymphocytes according to the HLA-I status of the tumor cells. Nine tumor specimens were not evaluated for infiltration by CD4-positive cells and 11 cases for both CD8- and CD56-positive cells.

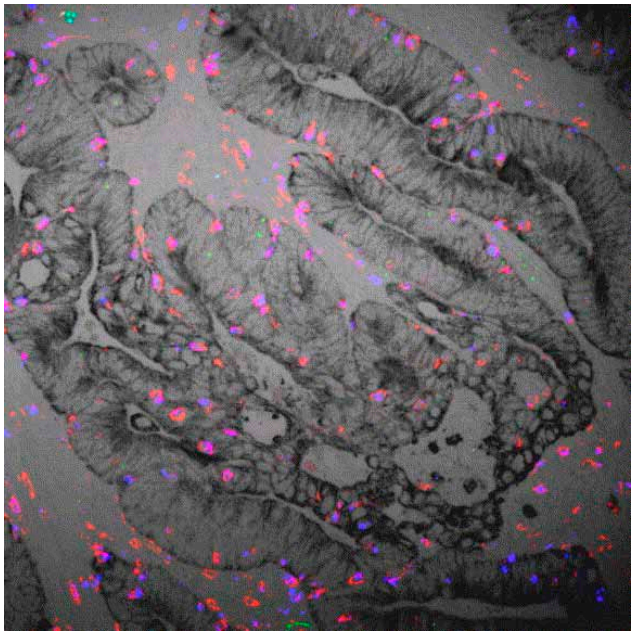

Figure 1. Tumor-infiltrating lymphocytes in a HLA-I down-regulated colorectal tumor. Combined silver-gold immunofluorescence staining of a HLA-I down-regulated colorectal tumor. Picture shows both stromal and intra-epithelial infiltrating CD3 (red) and granzyme B (blue) positive lymphocytes. Only few CD56 positive cells (green) are present in the tumor stroma. Bright-field microscopy reveales the cytokeratin labeled (dark-grey) tumor epithelial fields.

DISCUSSION

Ample studies have shown an accumulation of inflammatory cells in the direct vicinity of solid tumors [3;14-16]. In most studies, patients with a relatively dense infiltrate performed better in the clinical outcome. Detailed analysis of this infiltrate in various adenocarcinomas showed that only cells infiltrating the tumor-epithelium, i.e. cells in direct contact with tumor cells, contributed to this survival benefit [3;14;17]. NK cell infiltration in colorectal cancer has not been studied extensively in the past. Several studies found that the infiltration of NK cells in malignant tumors was associated with a favorable outcome [18-20]. However, they used an antibody against CD57 to identify NK cells, which is not an exclusive NK cell marker, since it is also expressed on a subset of T-lymphocytes. We recently found that the majority of infiltrating CD57 positive cells in colorectal tumors also expressed the T-cell receptor (data not shown). We therefore used the marker CD56, which however is not an exclusive NK cell marker, since it is also expressed on a subset of T lymphocytes, but in combination with CD3 we were able to distinguish these subpopulations.

Other studies have shown that loss of MHC class I expression is quite common in colorectal cancer and that these patients show a survival benefit [3;7-9;11;21;22]. We asked ourselves whether this survival benefit could be due to specific infiltration in tumors showing loss of MHC class I expression. We found that both CTL as well as NK cells infiltrate colorectal tumors, but the NK cell fraction is relatively small, especially in the tumor-epithelium. Furthermore, our staining demonstrated that tumors showing loss of MHC class I expression were more vigorously infiltrated by CD8 positive lymphocytes. At first we hypothesized that these were CD8 positive NK cells, but our triple immunofluorescence staining surprisingly revealed that the majority of these intra-epithelial infiltrating CD8 cells also carried the T-cell receptor CD3 and did not express CD56. It remains unclear why HLA-I aberrant tumors contain significantly more intra-epithelial CD8 positive T-lymphocytes than HLA intact tumors. Further characterization of these cells, as to their exact T cell receptor ($\alpha\beta$ or $\gamma\delta$), is necessary to illuminate their role and function. It is possible that in these tumors, due to T cell-mediated tumor cell killing, there have been a selection for MHC class I negative variants and that these tumors have maintained the capacity to attract T cells.

In a previous study we found that patients with primary tumors showing loss of MHC class I expression developed fewer distant metastases. Therefore, it is possible that the survival benefit is due to the fact that metastasizing tumor cells in these patients are efficiently cleared by NK cells in the circulation. This hypothesis is further supported by the observation that in a paired series of primary colorectal tumor and distant metastases from the same patient, less loss of MHC class I was observed than in a random series of primary colorectal cancer [23]. These data suggest that NK cells play an important role in the prevention of metastatic spread rather than locally in the primary tumor.

We hypothesize that if NK cells are capable of infiltrating solid tumors, they may kill tumor cells that show loss of MHC class I expression, and thus may contribute to treatment of colorectal cancer. The success of such NK based immunotherapy will depend on the ability of NK cells to infiltrate the tumor-epithelium. If this can be attained, for example through activation of NK cells by specific pro-infiltration chemokines/ cytokines, NK cells potentially form an effective immunotherapeutic basis.

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Chapter 4

Disrupted expression of CXCL5 in colorectal cancer is associated with rapid tumor formation in rats and poor prognosis in patients

Frank M. Speetjens[1], Peter J.K. Kuppen[1], Maro H. Sandel[1], Anand G. Menon[1], Danny Burg[2], Cornelis J.H. van de Velde[1], Rob A.E.M. Tollenaar[1], Hans J.G.M. de Bont[2], J.Fred Nagelkerke[2]

[1]Department of Surgery, Leiden University Medical Center, Leiden, the Netherlands
[2]Division of Toxicology, Leiden/Amsterdam Center for Drug Research, Leiden University, Leiden, the Netherlands

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ABSTRACT

Purpose: We isolated a sub-line (CC531M) from the CC531S rat colon carcinoma cell line, which grows and metastasizes much more rapidly than CC531S. We found, using RNA expression profiling that one of the major changes in the CC531M cell line was a 5.8-fold reduction of the chemokine CXCL5. The purpose of this study was to determine the impact of CXCL5 expression on colorectal tumor growth and metastasis.

Experimental design: CC531 clones were generated with either knock-down or restored expression of CXCL5. These clones were inoculated in the liver of rats. In addition, in two independent cohorts of colorectal cancer patients, the level of CXCL5 expression was determined and associated to clinical parameters.

Results: Knock-down of CXCL5 expression in CC531S resulted in rapid tumor growth and increased number of metastasis, while restored expression of CXCL5 in CC531M resulted in a return of the 'mild' tumor growth pattern of the parental cell line CC531S. *In vitro* no difference was found in proliferation rate between clones with either high or low expression of CXCL5, suggesting that environmental interactions directed by CXCL5 determine tumor outgrowth. Finally, the importance of our findings was established for patients with colorectal cancer. We found that low expression of CXCL5 was significantly associated with poor prognosis for colorectal cancer patients. CXCL5 showed a trend ($p=0.05$) for a positive correlation with intra-tumoral $CD8^+$ T-cell infiltration, suggesting a possible explanation for the observed poorer prognosis.

Conclusions: Our results show that CXCL5 is important in growth and development of colorectal cancer, implicating a future role in both cancer therapy and diagnosis.

INTRODUCTION

Colorectal cancer is one of the three leading causes of cancer-related death among men and women in the western world [1;2]. Despite curative surgical resection of the primary tumor, 40 to 50 percent of the patients ultimately die of metastases [3]. Tumor growth and metastasis result from a complex cascade of biological processes. Therefore, knowing key factors in these processes is crucial to design new treatment modalities.

In a previous paper we reported the *in vivo* selection of an aggressive rat colorectal cell line (CC531M) from the well described CC531S cell line [4;5]. The present study was initiated to identify factors that contribute to rapid growth and metastatic capacity of CC531M. In this study we focus on the chemokine CXCL5.

CXCL5 is a member of the subfamily of lipopolysacharide (LPS)-inducible ELR$^+$ CXC chemokines [6]. It functions, mainly through interaction with the CXCR2 receptor, both as a chemoattractant and as an angiogenic factor [7-10]. CXCL5 is expressed in the epithelial cells of the colon and over-expressed in colorectal cancer [11;12]. It has been reported that CXCL5 plays a role in development and metastasis of several cancer types [13-15]. CXCL5 contributes to the *in vivo* growth and angiogenic potential of non-small cell lung cancer (NSCLC). Homogenates of human NSCLC specimens were angiogenic in the rat corneal micropocket assay, and the development of vasculature can be blocked by antibodies that neutralize CXCL5 [14]. The role of CXCL5, produced by colorectal tumors, in relation to cancer progression and prognosis is poorly understood.

In this study, we investigated expression of CXCL5 on tumor growth and metastasis in a colorectal tumor rat model. CC531 cells, expressing different levels of CXCL5, were inoculated in the livers of syngenic rats and both tumor formation and metastasis were determined. CXCL5 expression was determined in two different independent large panels of human colorectal tumors and correlated with clinical follow-up and T-cell infiltration data.

EXPERIMENTAL DESIGN

CC531S and CC531M cell lines and culture conditions

The rat colon carcinoma cell line CC531S was originally developed using dimethylhydrazin in Wag/Rij rats [5]. The aggressive CC531M was isolated from CC531S using an *in vivo* selection protocol [4;16]. Cells were cultured at 37°C and 5% CO_2, in cell culture flasks (Corning, NY, USA) containing culture medium, composed of RPMI1640

(Gibco, Paisley, Scotland), supplemented with 10% heat-inactivated FCS, 100 µg/ml streptomycin, 100 IU/ml penicillin and 2 mM L-glutamine (all Gibco, Paisley, Scotland).

Development of CXCL5 knock-down and CXCL5 expressing CC531 clones

RNAi techniques were used to generate CC531S CXCL5 knock-down clones. A 19-nucleotide sequence (AACGGAGCTACGCTGTGTT), separated by a 9-nucleotide non-complementary spacer (TTCAAGAGA) from the reverse complement of the 19-nucleotide sequence, was cloned and sequenced after digestion with BglII and HindIII and inserted into the pSUPER backbone (OligoEngine, Seattle, USA), using standard procedures. To obtain stably transfected CC531S CXCL5-knock-down and control clones, the pSUPER-CXCL5 siRNA or empty vectors were co-transfected with the pcDNA3 vector, using Lipofectamine2000 (Invitrogen, California, USA). Three CXCL5-knock-down CC531S clones ($S1^{CXCL5-}$, $S2^{CXCL5-}$ and $S3^{CXCL5-}$) and three control clones ($S4^{Control}$, $S5^{Control}$ and $S6^{Control}$) were selected.

To restore CXCL5 expression in CC531M clones, CXCL5 was amplified by routine PCR using cDNA derived from CC531S. Forward and reversed primers were designed, using the first or last complementary 20 base pairs in addition of a HindIII or EcoRI sequence respectively. In front of the initial ATG code a KOZAK sequence was placed. PCR products were directly cloned with the pGEM-T-Easy cloning kit (Promega, Wisconsin, USA) and sequenced. Expression plasmids for CXCL5 were obtained, through unidirectional cloning of the sequence into the mammalian expression vector pcDNA3 (Invitrogen, California, USA). To obtain stably transfected CC531M CXCL5-expressing and control clones, CC531M cells were transfected with pcDNA3-CXCL5 or control empty vector. Two CC531M clones expressing CXCL5 ($M1^{CXCL5+}$ and $M2^{CXCL5+}$) and two control clones ($M3^{Control}$ and $M4^{Control}$) were selected. Selection was based upon expression of CXCL5 as indicated by immunostaining. Stably transfected clones were grown under selective pressure, in culture medium supplemented with 200µg/ml G418 (Sigma, St. Louis, MO, USA).

Immunocytochemistry

Cells were cultured on 12-mm glass coverslips and stained as previously described [17], using the primary antibody rabbit anti-murine LIX [18] (Peprotech EC Ltd, London, UK) and Cy3-conjugated goat anti rabbit secondary antibody (Jackson, Suffolk, UK) in TBP (1h RT). Finally, cells were stained with 2 µg/ml Hoechst 33258 (Invitrogen, California, USA). Cells were analyzed using a Nikon Eclipse E600 fluorescence microscope with a 40x plan fluor Nikon objective (Nikon, Tokyo, Japan). The percentage CXCL5 positive tumor cells and the CXCL5 pixel intensity per cell were determined. For every clone or cell line at least 5 randomly chosen fields were analysed to determine the percentage of CXCL5 positive cells.

Cell proliferation

DNA content was used to determine the proliferation rate of CC531 cells and clones by a method previously described [19]. In short: 25000 cells were seeded into a 24 well plate. Medium was replaced daily. From 24h up to 144h after the start of seeding cells, each day plates were removed, rinsed with PBS, and stored at -80°C until assayed. On the day of assay, cells were thawed, 200 µl of distilled water was added (1h at 37°C). The plates were frozen at -80°C and thawed. From each well 50µl was taken and placed into a 96 well plate. DNA content was determined after addition of 50µl of 20µg/ml Hoechst 33258 fluorochrome (Invitrogen, California, USA) and measured on a fluostar optima platereader (BMG Labtech GmbH, Offenburg, Germany).

Rat experiments

All animal experiments were approved by the animal experiment committee of Leiden University. Animals were kept in our own animal facilities. Male Wag/Rij rats (Charles River, Zeist, The Netherlands) were anesthetized with halothane, underwent laparotomy, and were double blind randomized for induction of a liver tumor. A suspension of $5*10^4$ viable CC531 tumor cells in 50µl was injected sub-capsulary at four sites into the liver. Per clone, four rats were inoculated. Rats were sacrificed by abdominal bleeding under halothane anesthesia, liver and tumor were removed and weight was determined. To determine the number of lung metastasis, lungs were removed and filled with an ink solution, as previously described [20;21].

RT-PCR of CXCL5 in a patient cohort

Tumours from a cohort of 70 patients that were curatively treated by surgery for colorectal cancer, between 1990 and 2001, were used to associate level of CXCL5 RNA expression with prognosis. Fifty percent of the patients were female. The mean age at the time of surgery was 67.2 years. Tumors were staged according to the American Joint Committee on Cancer (AJCC) criteria [22]: 47 (67%) stage I/II; 23 (33%) stage III. At the time of censoring 41 (59%) had died of whom 22 (54%) died from their disease, and 29 patients were still alive; four of them were alive with recurrence of the tumor. Mean follow up was 99 months (range 50-172 months). Patient material was obtained with approval of local medical ethics committee. RNA from snap-frozen tumors, containing at least 60% tumor cells as determined by a pathologist, was isolated using RNeasy columns (Qiagen Sciences, Germantown, MD, USA). Quantative reverse transcriptase PCR (RT-PCR) primers for the detection of housekeeping genes (Cleavage and polyadenylation specificity factor subunit 6 (CPSF6), Heterogeneous nuclear ribonucleoprotein M (HNRPM) and TATA-binding protein (TBP) and CXCL5 were designed in PRIMER Express (Applied Biosystems, Foster City, CA, USA) and span at least one exon-exon boundary). The primers used were:

CPSF6, 5′-AAGATTGCCTTCATGGAATTGAG-3′, 5′-TCGTGATCTACTATGGTCCCTCTCT-3′; HNRPM, 5′-GAGGCCATGCTCCTGGG-3′, 5′-TTTAGCATCTTCCATGTGAAATCG-3′, TBP, 5′-CACGAACCACGGCACTGAT-3′, 5′-TTTTCTTGCTGCCAGTCTGGAC-3′ CXCL5, 5′- ctgt-gttgagagagctgcgt-3′, 5′-gttttccttgtttccaccgtc-3′. RT-PCR reactions were performed on an ABI Prism 7900ht (Applied Biosystems) using the SybrGreen RT-PCR core-kit (Eurogentec, Seraing, Belgium). Cycle conditions were 10 minutes at 94°C followed by 40 cycles of 10 s at 94°C and 1 minute at 60°C. Cycle threshold extraction was performed using the SDS software (version 2.2.2, Applied Biosystems). For all PCRs, a standard curve was generated using a five-step, five-fold dilution of pooled cDNA from the HCT81 colorectal cancer cell line. Relative concentrations of mRNA for each gene were calculated from the standard curve. After RT-PCR, dissociation curves were made to check the quality of the reaction. Reactions with more than one peak in the dissociation curve were discarded. For normalization, the expression values for each gene were divided by the normalization factor of the gene (the average of the three house keeping genes).

Immunohistochemistry of CXCL5 in a patient cohort

In a second independent cohort of 58 patients, curatively operated for colorectal cancer was used to associate protein level of CXCL5 to prognosis. The cohort comprised 43% females; mean age at the time of surgery was 66.2 years; 29 stage I/II (50%) and 29 (50%) stage III colorectal tumors. At the time of censoring 46 (79%) had died, mean follow up was 49 months (range 1.2-162 months). Standard two-step, indirect immunohistochemistry was performed on 4-µm paraffin tissue sections, including blockage of endogenous peroxidise, EDTA antigen retrieval (not for CXCL5 detection) and di-aminobenzidine development. To be able to distinguish intra-epithelial from stromal infiltration, an additional staining for laminin was performed on CD4 and CD8 stained sections, including trypsin antigen retrieval and development using NBT/BCIP solution, as previously described [23]. The following primary antibodies were used: the mAb anti- CXCL5 (clone MAB254, R&Dsystems, Minneapolis, USA), the mAb anti-CD4 (clone 1F6, Novocastra, UK), the mAb anti-CD8 (clone 4B11, Novocastra, UK) and rabbit anti-human laminin polyclonal Ab (Sigma-Aldrich, USA). Secondary reagents used were anti Mouse HRP EnVision+ (K400111, Dako, USA), biotinylated swine anti-rabbit IgG antibodies (DAKO Cytomation, Denmark), and biotinylated-peroxidase streptavidin complex (SABC; DAKO Cytomation, Denmark). CXCL5 expression was scored by microscopically assessing the percentage CXCL5 positive tumor of the whole section. Infiltration in the tumor tissue was scored in three compartments of the tumor tissue, i.e. intra-epithelially, intra-stromally and in the advancing tumor margin. Method of scoring has previously been described [23].

Statistical analysis

All analyses were performed with SPSS statistical software (version 12.0 for Windows, SPSS Inc, Chicago, IL). Mann-Whitney U test was used to compare variables. Kaplan-Meier analyses were performed to analyze patient survival. The entry date for the survival analyses was the time of surgery of the primary tumor. Events for time to disease free survival were defined as follows: from time of surgery to time of disease relapse or death. Events for time to cancer specific survival were defined as follows: from time of surgery to time of disease relapse or death by disease. Cox' regression analyses were used to calculate Hazard Ratios (HR) with 95% confidence intervals (CI). Variables with a p-value of $\leq$0.10 in the univariate analyses were subjected to a multivariate analysis. Pearson's product-moment correlation was used to analyze correlations between level of CXCL5 expression and T-cell infiltration.

RESULTS

Expression of CXCL5 is lost in CC531M compared to CC531S cells

We showed previously that subcapsular inoculation of the colorectal cell line, CC531M in the liver of rats resulted in rapid tumor growth and induction of larger number of metastases as compared to inoculation of the parental CC531S [4]. Affymetrix micro array analysis was performed in triplicate to determine differences in gene expression between the parental cell line CC531S and CC531M. The major change was a 5.8-fold (SD=0.7) reduction of CXCL5 RNA content in CC531M cells as compared to expression in CC531S. To confirm RNA expression data, cells were stained for the presence of CXCL5 protein using immuno-fluorescence techniques. In CC531S cells a strong cytoplasmic staining was found, while CC531M cells hardly showed any staining (figure 1A). The percentage of CXCL5 positive cells was significantly ($p<0.0001$) higher in CC531S cells compared to CC531M cells (figure 1B). *In vitro*, CC531S and CC531M showed the same proliferation rate (figure 1C).

Knock-down of CXCL5 expression results in aggressive tumor growth in vivo

To study the contribution of CXCL5 to tumor outgrowth and metastatic potency of CC531S cells, RNAi technology was used to knock-down CXCL5 in this cell line. Three CXCL5 siRNA transfected CC531S clones ($S1^{CXCL5-}$, $S2^{CXCL5-}$ and $S3^{CXCL5-}$) and three control clones ($S4^{Control}$, $S5^{Control}$ and $S6^{Control}$) transfected with the empty vector, were selected. Immuno-fluorescence staining for CXCL5 expression showed a significant ($p<0.0001$) CXCL5 down-regulation in $S1^{CXCL5-}$, $S2^{CXCL5-}$ and $S3^{CXCL5-}$ compared to $S4^{Control}$, $S5^{Control}$ and $S6^{Control}$ (figure 2A). *In vitro*, no significant difference in mean proliferation rate was found between CC531S CXCL5 knock-down clones and control

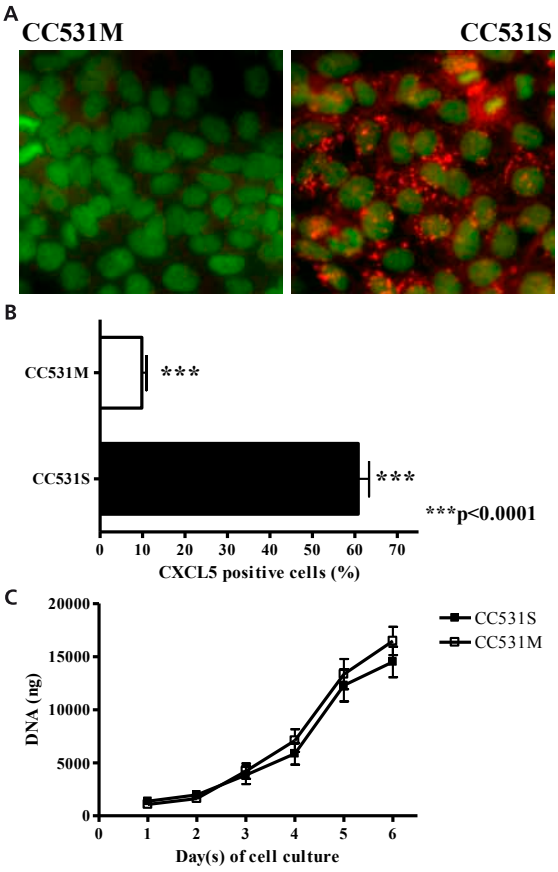

Figure 1. CXCL5 expression is reduced in CC531M cells.
(A) Immuno-fluorescent staining of CXCL5 expression of CC531S and CC531M (CXCL5 – red; nuclei – green). *(B)* Displays the percentage CXCL5 positive CC531S and CC531M cells; columns - mean; error bars - standard error mean (SEM). *(C)* Represents *in vitro* proliferation rate of CC531S and CC531M cells on different time points; error bars - SEM. Statistically significant differences are marked (*).

clones (figure 2B). To study the effect of loss of CXCL5 in CC531S on tumor formation, each individual clone was subcapsularly injected in the liver of four rats. At sacrifice, rats injected with clones S1^{CXCL5-} or S2^{CXCL5-} showed large tumors over-growing the whole liver and, in addition, also large tumor masses in the peritoneal cavity and lungs were found. Due to the massive tumor outgrowth it was impossible to determine weight and surface of the individual tumors of rats inoculated with clones S1^{CXCL5-} or S2^{CXCL5-}. Therefore, the weight of both tumor and liver of all rats was determined. The third clone, CC531S clone S3^{CXCL5-}, showed somewhat less aggressive outgrowth: 4 solitary liver tumors at the site of inoculation were found at sacrifice. Only one of

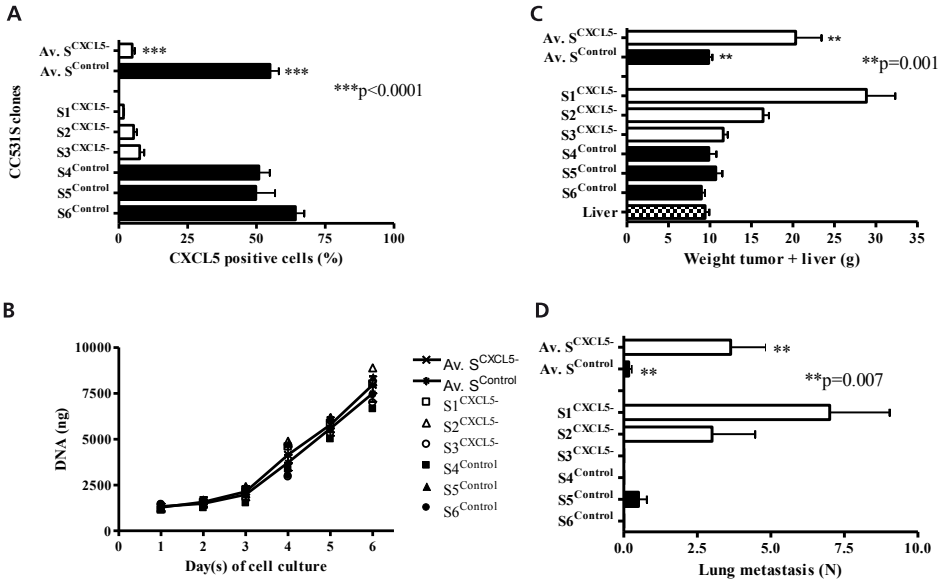

Figure 2. Knock-down of CXCL5 expression results in massive tumor outgrowth and formation of metastasis *in vivo.*

(A) The percentage CXCL5 positive cells of all knock-down and control clones was determined. Top bars represent the average (Av.) number of CXCL5 positive cells, error bars - SEM. *(B) In vitro* growth rate of all knock-down and control CC531S clones on several time points connected by a line. *(C)* Represents the average liver and tumor weight of both control clones versus the knock down clones after inoculation in the liver of rats at sacrifice. Top bars show the average (Av.) of the knock-down versus the control clones. *(D)* The number of lung metastases found after inoculation of the knock-down and control clones in the liver. Statistically significant differences are marked (*).

the 3 control clones, $S5^{Control}$, showed 4 small tumors at the place of inoculation, the others ($S4^{Control}$, $S6^{Control}$) did not show any tumor formation in the liver. The average weight of tumor and liver of the three CXCL5 knock-down clones was significant (p=0.001) higher than the control clones (figure 2C). Furthermore, injection of CXCL5 knock-down clones resulted in significant more lung metastases compared to the control CC531S clones (p=0.007) (figure 2D). Images of *in vivo* tumor growth are displayed in figure 3. Together, these results show that while knock-down of CXCL5 *in vitro* did not result in difference in proliferation rate; *in vivo* CXCL5 knock-down in CC531S resulted in aggressive tumor growth accompanied with increased formation of metastases.

Restoration of CXCL5 expression results in less aggressive tumor growth in vivo

Stable transfection of CXCL5 into CC531M cells was used to study whether restored expression of CXCL5 would inhibit tumor growth and metastasizing capacity of CC531M *in vivo*. Two CXCL5 transfected clones ($M1^{CXCL5+}$ and $M2^{CXCL5+}$) and two

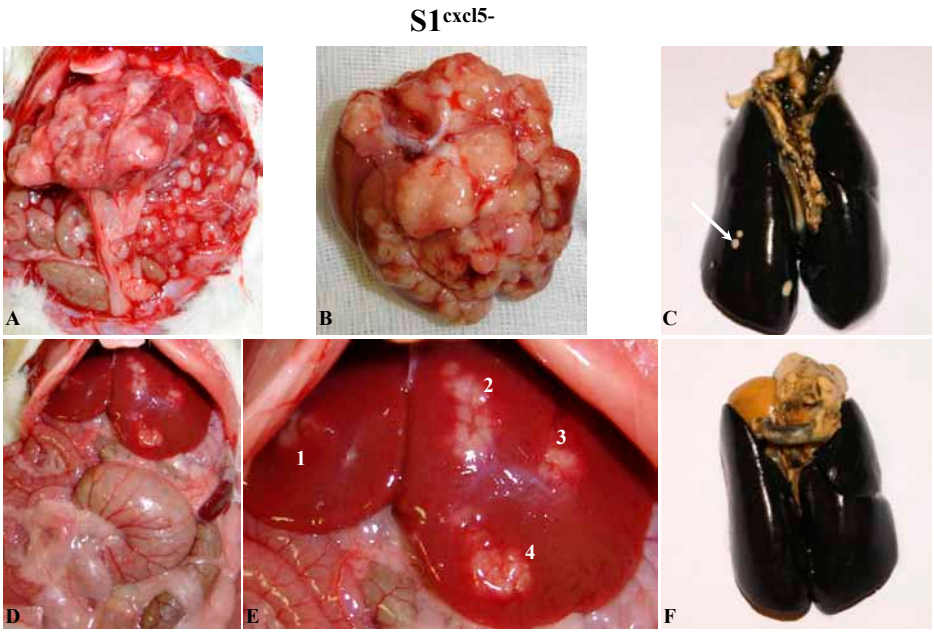

Figure 3. Examples of tumor outgrowth in the rat of a CXCL5 knock-down and a control CC531S clone.
Top panels *(A-C)* display tumor outgrowth of CXCL5 knock-down clone $S1^{cxcl5-}$ after inoculation of the clone in the liver of a rat at sacrifice. *(A)* Displays the peritoneal cavity with tumor throughout the liver and peritoneal metastasis. *(B)* Shows the same liver after resection, overgrown with tumor mass, individual sites of inoculation cannot be distinguished. *(C)* Displays the lungs of this rat after ink injection, with 4 metastases (one is indicated by an arrow). Bottom panels *(D-F)* display tumor growth after inoculation with CXCL5 positive CC531S control clone $S5^{control}$. *(D)* Tumor is only found in the liver at the four sites of inoculation and not in other places in the peritoneal cavity. *(E)* Shows the liver in detail, with 4 individual tumors (numbered 1-4). *(F)* No lung metastases were found in this rat.

control clones ($M3^{Control}$ and $M4^{Control}$) transfected with the empty vector were used. Analysis showed significant ($p<0.0001$) up-regulation of CXCL5, in CXCL5-transfected clones (figure 4A). Restoration of CXCL5 expression had no significant impact on *in vitro* proliferation rate (figure 4B). To determine the *in vivo* growth capacity of the different clones, each individual clone was inoculated in the liver of four rats. All clones showed solitary tumors at the site of inoculation, as determined at sacrifice. Tumors were enucleated from the liver and tumor weight was determined. The mean tumor weight of the CXCL5-transfected clones was significantly less compared to the tumor weight of the control clones ($p=0.005$) (figure 4C). Only very few lung metastases were found, not differing among clones. These results demonstrated that CXCL5 reconstitution in CC531M resulted in inhibition of tumor growth *in vivo*.

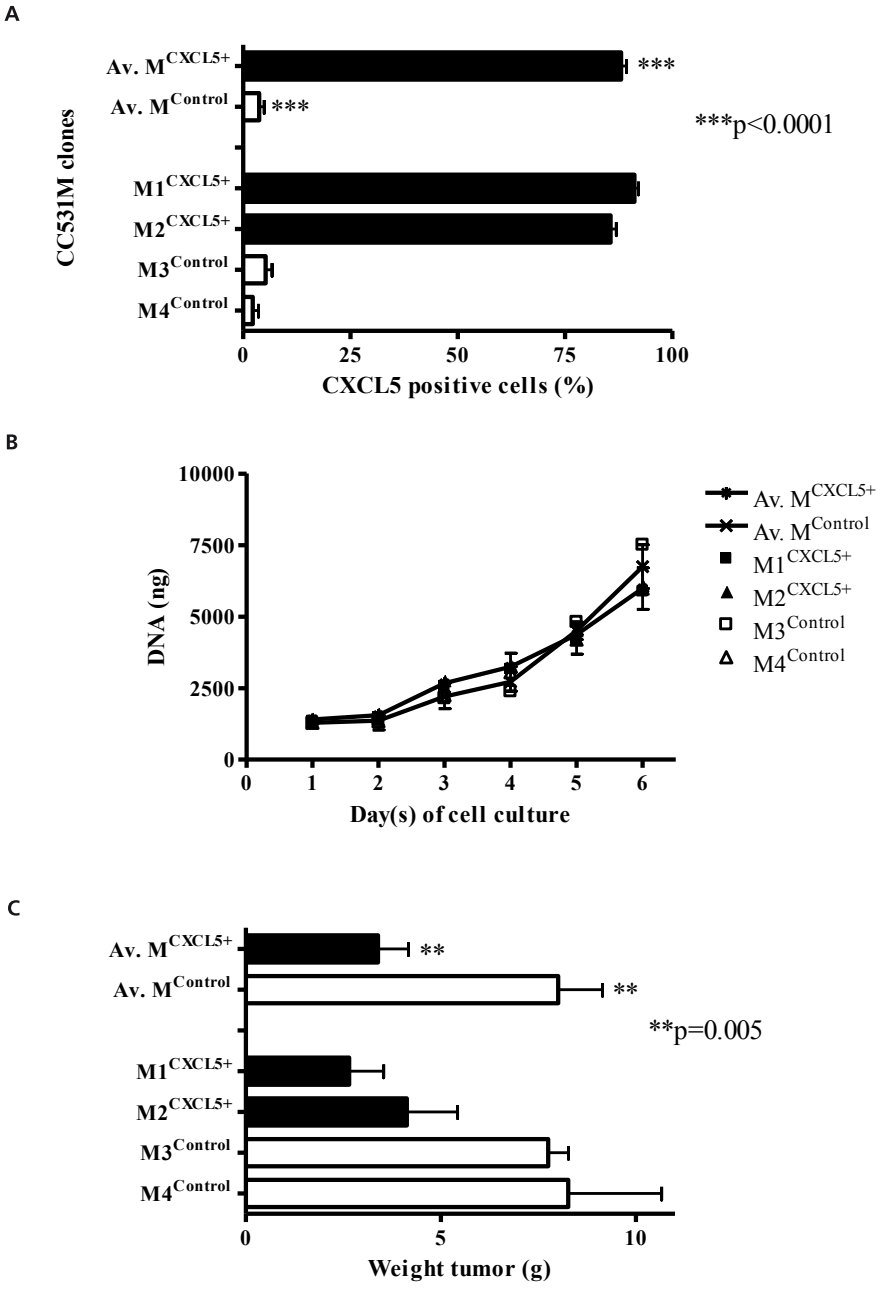

Figure 4. Restoration of CXCL5 expression inhibits tumor growth, *in vivo*.
(A) CXCL5 expression of the CXCL5 and control CC531M clones; columns - mean; bars - SEM. *(B) In vitro* growth rate of all CXCL5 transfected versus control clones at several time points. *(C)* The mean weight of the total tumor mass per clone in four rats at sacrifice is shown. The two columns on top represent the average weight of the CXCL5 transfected versus the control clones; columns - mean; bars - SEM. Statistically significant differences are marked (*).

Low expression of CXCL5 in human colorectal cancer is associated with decreased survival

The relation between expression of CXCL5 in human colorectal tumors and prognosis was studied in two cohorts of colorectal cancer patients, using different techniques to determine the level of CXCL5 expression. The RNA level of expression of CXCL5 in the first cohort was determined using quantitative RT-PCR and linked to clinical follow-up data. The impact of high versus low expression of CXCL5 was assessed using the 25th percentile as cut off point, leaving 53 patients with high expression of CXCL5 (11.2±2.1; mean±sd) and 17 patients with low expression (7.1±1.3) of CXCL5. CXCL5 expression levels were distributed equally with regard to clinical and pathological parameters (table 1). Univariate cox regression analyses were performed to identify prognostic factors for disease free survival. Advanced patient age, advanced pathological stage, and low CXCL5 expression proved to be significant predictors of poor prognosis in the univariate analyses (table 1). The Kaplan Meyer curve for disease free survival is shown for low versus high CXCL5 expression (figure 5) and revealed that low expression was associated with a significantly worse prognosis (p=0.016). Parameters, significant in univariate analysis, were subjected to Cox multivariate analysis. Patient age above the median (HR: 2.3, C.I.: 1.2-4.2, p=0.01), advanced pathological stage (HR: 3.1, C.I.: 1.6-5.7, p<0.001), and low CXCL5 expression (HR: 2.3, C.I.: 1.2-4.4, p=0.016) all retained their strength as independent prognostic factors for disease free survival (table 1).

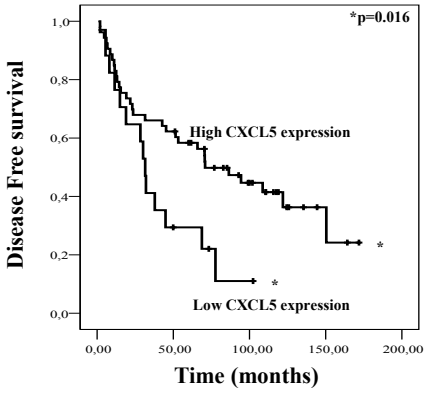

Figure 5. Correlation between disease free survival and expression of CXCL5 assessed by RT-PCR in a cohort of colorectal cancer patients.
Kaplan Meier survival curve is displayed, patients with low expression of CXCL5 have a significant (p=0.016) decreased disease free survival compared to patients with high expression of CXCL5.

Table 1. RNA level of CXCL5 in relation to clinicopathological and prognostic parameters

	CXCL5 expression		Relation CXCL5 to:	Disease Free Survival		
			M-W	Univariate analysis	Multivariate analysis	
	High N (%)	Low N (%)	p-value	p-value	HR (95% CI)	p-value
Gender						
Male (%)	27 (51%)	8 (47%)	0.78	0.78	---	---
Location tumor						
Proximal (%)	29 (55%)	7 (41%)	0.34	0.51	---	---
Median age at diagnosis (years)						
<68.5	27	8	0.78	**0.006**	1	**0.010**
>68.5	26	9			2.3 (1.2 – 4.2)	
Stage						
I and II	36 (68%)	11 (65%)	0.81	**0.0001**	1	**<0.001**
III	17 (32%)	6 (35%)			3.1 (1.6 – 5.7)	
Pathway						
MSI	1 (6%)	11 (21%)	0.16	0.60	---	---
MSS	16 (94%)	42 (79%)				
CXCL5						
High	53 (76%)	---	---	**0.016**	1	**0.016**
Low	---	17 (24%)			2.3 (1.2 – 4.4)	

NOTE: Table 1 displays level of CXCL5 in a panel of colorectal cancer patients determined by quantitative RT-PCR. The 25th percentile was used to define high versus low expression of CXCL5. On the left side of both tables, the distribution of high versus low expression of CXCL5 with respect to clinical and pathologic characteristics and the relation of CXCL5 to clinicopathologic factors are displayed. On the right side of the table, prognostic factors are displayed. Univariate Cox regression analyses were done to identify prognostic factors for survival. All factors with a *P*-value of $\leq$0.10 were subjected to Multivariate Cox regression analysis. Statistically significant *P*-values are in bold.

Abbreviations: MSS, microsatellite stable; MSI, microsatellite instable.

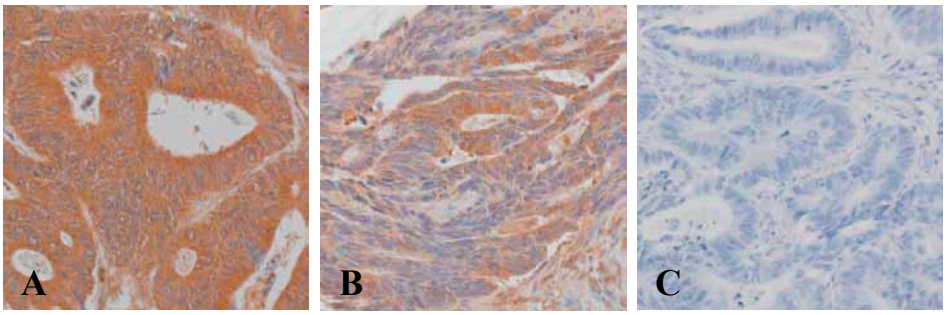

Figure 6. Examples of CXCL5 immunohistochemical staining of human colorectal tumors.
(A) displays CXCL5 expression in almost colon cancer cells; *(B)* shows heterogeneous expression of CXCL5 in a colorectal tumor; *(C)* displays a negative PBS control; original magnification x200

In a second independent cohort of colorectal cancer patients, the protein level of CXCL5 expression was determined using immunohistochemical staining of tissue sections. After staining, the percentage positive tumor cells was scored. Staining confirmed previous data showing that tumor cells displayed increased expression of CXCL5 compared to normal colon epithelium (figure 6) [11;12]. Fifty tumors showed CXCL5 expression in more than 50% of the tumor cells, while 8 tumors showed expression of CXCL5 in less than 50% of the tumor cells. CXCL5 levels were distributed equally to clinicopathological parameters (table 2). Univariate analysis showed that CXCL5 (p=0.009) and stage (p=0.03) both predicted prognosis. Cox multivariate analysis confirmed the value of low level of CXCL5 (HR: 3.6, C.I.: 1.3-9.9, p=0.01) as independent predictor of poor prognosis in addition to advanced pathological stage (HR: 2.6, C.I.: 1.1-6.3, p=0.04) (table 2). Of the latter cohort also the CD4 and CD8 infiltration was scored in three compartments of the tumor (intra-epithelial, stromal and advancing margin). Using Pearson's product-moment correlation a trend was found for significant positive correlation between level of CXCL5 and intra-epithelial and stromal infiltration of $CD8^+$ T-cells (r=0.21, p=0.12; r=0.26, p=0.05 respectively), (table 3). Neither $CD4^+$ T cell infiltration, nor $CD8^+$ T-cell Infiltration, scored at the border of the tumor (advancing margin) was correlated with expression of CXCL5. Low CXCL5 expression was an independent predictor of decreased disease free survival in colorectal cancer patients, showing a trend for a positive correlation for level of CXCL5 and intra-tumoral cytotoxic T-cell infiltration.

Table 2. Protein level of CXCL5 in relation to clinicopathological and prognostic parameters

	CXCL5 expression		Relation CXCL5 to:	Cancer Specific Survival		
			M-W	Univariate analysis	Multivariate analysis	
	High N (%)	Low N (%)	p-value	p-value	HR (95% CI)	p-value
Gender						
Male (%)	27 (54%)	6 (75%)	0.27	0.15	---	---
Median age at diagnosis (years)						
<68.5	25	4	1.0	0.83	---	---
>68.5	25	4				
Stage						
I and II	24 (48%)	5 (62%)	0.45	**0.03**	1	**0.04**
III	26 (52%)	3 (38%)			2.6 (1.1 – 6.3)	
CXCL5						
High	50 (86%)	---	---	**0.009**	1	**0.01**
Low	---	8 (14%)			3.6 (1.3 – 9.9)	

NOTE: Table 2 displays the results after immunohistochemical staining and scoring the percentage of CXCL5-positive tumor cells. For immunohistochemical staining, high was defined as <50% of tumor cells showing CXCL5 expression and low was defined as <50% of tumor cells showing CXCL5 expression. On the left side of both tables, the distribution of high versus low expression of CXCL5 with respect to clinical and pathologic characteristic and the relation of CXCL5 to clinicopathologic factors are displayed. On the right side of the table, prognostic factors are displayed. Univariate Cox regression analyses were done to identify prognostic factors for survival. All factors with a *P*-value of $\leq$0.10 were subjected to Multivariate Cox regression analysis. Statistically significant *P* values are in bold.

Table 3. Correlation between expression of CXCL5 and infiltrative T-cell markers

Location infiltrate	$CD8^+$		$CD4^+$	
	Pearson correlation (r)	p-value	Pearson correlation (r)	p-value
Intra-epitelial	0.21	0.12	0.12	0.38
Stromal	0.26	0.05	0.15	0.27
Advancing margin	-0.02	0.87	-0.93	0.50

NOTE: a trend for positive correlation between expression of CXCL5 and intratumoral T-cell infiltration was found. T-cell infiltration was scored in different compartments of the tumor: intraepithelial, stromal, and at the advancing margin. Infiltration in each of these different compartments was associated to protein expression of CXCL5 using Pearson's product-moment correlation.

DISCUSSION

Many chemokines play a pivotal role in colorectal cancer [24]. We decided to study CXCL5 because our initial rat experiments indicated that the absence of this chemokine was associated with an aggressive tumor phenotype. CXCL5, an important homeostatic factor in the colon, is mainly produced in epithelial cells and is in general more highly expressed in cancer tissue compared to normal tissue [11;12]. This pattern, higher expression in tumor tissue than in normal tissue was also found in our experiments. However, absence of CXCL5 expression in tumor tissue was correlated with poor prognosis. To our knowledge, the only report describing mechanisms by which CXCL5 expression is abrogated is provided by Dimberg *et al.* showing that CXCL5 gene variants are related to expression of CXCL5 protein in colorectal cancer [12]. Besides (epi-)genetic explanations, other mechanisms influencing CXCL5 expression might be involved in the nuclear factor-kappaB (NF-κB) that controls expression of CXCL5 [25]. Functions of CXCL5 include chemo-attraction and promotion of angiogenesis, mainly by interaction with the CXCR2 receptor [7;8]. Our data indicate that CXCL5 is involved in growth and development of colorectal cancers. The importance of CXCL5 for tumor formation *in vivo* was confirmed by comparing the growth of transfected CC531 clones that expressed either high or low levels of CXCL5. Importantly, our findings in the rat proved to be relevant for colorectal cancer patients as in two different tumor tissue cohorts of these cancer patients, low expression of CXCL5 was associated with shorter survival.

Well established is the chemo-attraction of neutrophils into inflamed regions after CXCL5-CXCR2 interaction [9;26]. Antagonists to the CXCR2 receptor prevent neutrophil attraction and reduce the inflammatory response [27;28]. CXCR2 is also involved in chemokine-induced migration of NK and T-cells [28;29]. CXCL5 produced by tumor cells may attract CXCR2 expressing leukocytes as T-cells, NK cells and neutrophils, triggering an anti-tumor immune response. A trend for positive correlation between level of CXCL5 and intratumoral cytotoxic T-cell infiltration was found. This trend was not found for infiltration in the advancing border of the tumor, suggesting that CXCL5 indeed especially contributes to intratumoral infiltration of cytotoxic T-cells. High tumor infiltration of these inflammatory immune cells is positively associated with good prognosis in colorectal cancer [30-32]. This concept that over-expression of specific chemokines causes tumor infiltration by distinct leukocyte subsets, resulting in tumor regression and tumor specific immunity, has also been described for other chemokines [33-41]. Thus, CXCL5 may contribute to an anti-tumor response.

Another mechanism by which CXCL5 may be involved in colorectal tumor growth is based on the fact that the CXCR2 receptor has been found on colorectal tumor cells [42-44]. Expression of CXCR2 has also been found in CC531 cells (unpublished data).

This may indicate that CXCL5 functions as an autocrine growth inhibitory factor. This is in contrast with other reports that described a positive effect of CXCR2 ligands on tumor growth [43;44]. In our results the presence or absence of CXCL5 expression had no influence *in vitro* on proliferation rate of any of our cell lines. Moreover, we found *in vivo* that low expression of CXCL5 promotes tumor growth. These data indicate that the effect of CXCL5 is not very likely to depend on an autocrine signaling pathway.

CXCL5 may play opposing roles in tumor formation in general. On the one hand CXCL5 may induce an anti-tumor response by chemo-attraction of immune cells; on the other hand it may promote angiogenesis that supports tumor growth. Our results indicate that in colorectal cancer formation, the anti-tumor response is dominant. In support of our results for head and neck squamous cell carcinoma HNSCC higher expression of CXCL5 was also found in mortal tumors associated with a better prognosis compared to immortal tumors having a poorer prognosis [45]. In other cancers a tumor promoting role for CXCL5 has been reported [13-15;46]. Arenberg *et al.* found a strong correlation between levels of CXCL5 and the level of vascularitzation in human NSCLC. In addition they showed, using a SCID mouse model that expression of CXCL5 in developing human NSCLC correlated with tumor growth [14]. The data presented by Arenberg and others may seem in contrast with our findings. However, in addition to the different tumor type studied, our findings were derived from a syngeneic rat model for colorectal cancer, with a competent immune system, while their results were obtained in immune deficient mouse models. Therefore, in the study by Arenberg *et al.* the potential effect of a CXCL5-dependent immune response on tumor development would not have been manifested.

In conclusion, our results show that CXCL5 is an important factor in growth and development of colorectal cancer. Our data suggest that expression of CXCL5 by tumor cells enhances the recruitment of tumor infiltrating lymphocytes thereby bringing about better prognosis in colorectal cancer patients. Therefore CXCL5 should be further studied for its potential role as a therapeutic target and prognostic biomarker in colorectal cancer.

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Chapter 5

Prediction of the immunogenic potential of frameshift-mutated antigens in microsatellite instable cancer

Frank M. Speetjens[1,*], Marjolein M. Lauwen[2,*], Kees L. Franken[2], Connie M. Janssen-van Rhijn[1], Suzanne van Duikeren[2], Sandra A. Bres[2], Cornelis J.H. van de Velde[1], Cornelis J.M. Melief[2], Peter J.K. Kuppen[1], Sjoerd H. van der Burg[3], Hans Morreau[4] and Rienk Offringa[2]

Departments of [1]Surgery, [2]Immunohematology and Blood Transfusion, Tumor Immunology Group, [3]Clinical Oncology and [4]Pathology, Leiden University Medical Center, Leiden, the Netherlands
Note: [*]These authors contributed equally to this work

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ABSTRACT

Purpose: Microsatellite instable (MSI) cancers express frameshift-mutated antigens, the C-terminal polypeptides of which are foreign to the immune system. Consequently, these antigens constitute a unique pool of tumor-specific antigens that can be exploited for patient diagnosis and selective, immune-mediated targeting of cancers. However, other than their sequence, very little is known about the characteristics of the majority of these proteins.

Experimental design: We therefore developed a methodology for predicting their immunogenic behavior that is based on a gene expression system in which each of the proteins was fused to a short C-terminal polypeptide comprising two epitopes that can be readily detected by T-cells and antibodies respectively. In this manner, accumulation of the antigens, and processing of peptides derived thereof into MHC, can be monitored systematically. The antigens that accumulate in the cells in which they are synthesized are of primary interest for cancer immunotherapy, because peptide epitopes derived thereof can be presented by dendritic cells in addition to the tumor cells themselves. As a result, these antigens constitute the best targets for a coordinated immune response by both $CD8^+$ and $CD4^+$ T-cells, which increases the likelihood that tumor-induced immunity would be detectable against these antigens in cancer patients, as well as the potential value of these antigens as components of anti-cancer vaccines.

Results and conclusions: Our data indicate that, of fifteen frameshift-mutated antigens examined in our present study, four (TGFβR2-1; MARCKS-1; -MARCKS-2; CDX2-2) are of primary interest and four additional antigens (TAF1B-1; PCNXL2-2; TCF7L2-2; Baxα+1) of moderate interest for further tumor immunological research.

INTRODUCTION

A high frequency of microsatellite instability (MSI-H) is a molecular feature of tumors associated with the Hereditary Non-Polyposis Colorectal Cancer (HNPCC) syndrome [1;2]. Furthermore approximately 15% of sporadic colorectal, gastric and endometrial cancers, as well as lower frequencies of various other sporadic cancers, are characterized by widespread MSI [3-7]. Microsatellites are repetitive nucleotide sequences of different length, distributed throughout the human genome that are prone to small insertion/deletion mutations, caused by DNA polymerase slippage during DNA replication [8]. Usually, these errors are corrected by the inherent proofreading capacity of a group of proteins involved in mismatch repair [9;10]. Defects in mismatch repair, like in MSI-H tumors, allow the accumulation of errors in microsatellites. Deletion or insertion of one or two base pairs in a coding gene results in a shift of the reading frame downstream of the mutation and thereby translation of an abnormal protein product.

These frameshift-encoded products constitute "foreign" antigens for the immune system and therefore represent an unique pool of tumor specific antigens [11]. Specific T-cell and antibodies responses have indeed been found against a limited number of frameshift products [12-14]. Furthermore, MSI-H tumors are associated with several treats that point at immune surveillance, such as increased lymphocyte infiltrate, increased incidence of MHC class I loss, and better patient survival prognosis as compared to microsatellite stable tumors. [15-17]. Therefore, these frameshift-mutated proteins, in particular the non-self segment encoded by sequences downstream of the mutation, are considered promising candidates for preventive vaccination of subjects with HNPCC, or as adjuvant therapy in combination of surgery for patients with sporadic MSI-H tumors. The potential of these antigens for development of anti-cancer vaccines is further supported by the notion that mutations in several genes are found at high (>50%) frequencies in MSI-H cancers [18-22].

Unfortunately, little is known about the immunogenic properties of most proteins encoded by these commonly mutated genes. In order to predict their potential for inclusion in vaccines, it is of particularly interest to know whether they accumulate in tumor cells or are rapidly degraded. Accumulation of stable frameshift mutated antigens in tumor cells can result in cross-presentation of antigen-derived peptides by "professional" antigen presenting cells (APCs), in particular dendiritic cells (DCs), in the tumor-draining lymphoid tissues and, thereby, in pre-existing antigen-specific T-cell responses in cancer patients. Furthermore, vaccination-induced $CD4^+$ T-cells are in this case expected to provide 'help' to the anti-tumor response, because they will encounter their cognate antigen on DCs in the peritumoral area. In contrast, instable antigens that do not reach significant steady-state levels are unlikely to become

cross-presented [23-25]. Consequently, neither 'spontaneous' priming of anti-tumor T-cell immunity, nor efficacy of vaccine-induced $CD4^+$ T-helper cells against such antigens are to be expected. Nevertheless, break down of these instable antigens through the ubiquitin-proteasome pathway can result in peptides that may be presented in the context of MHC class I at the tumor cell surface. In that case, vaccine-induced $CD8^+$ T-cells recognizing these peptides could be used to target the tumor.

In the present study, we made use of an expression system to systematically analyze the characteristics and immunogenic properties of proteins encoded by a selection of frameshift mutated genes that are commonly found in MSI-H cancers. This inventory provided important information on the manner in which these antigens should be used for further studies concerning patient diagnosis and cancer immunotherapy.

MATERIAL AND METHODS

Tumor cell lines

The cell lines B3Z, HeLa and HeLa-K^b were used in experiments. HeLa-K^b is a stable transfectant of the human HeLa cell line, expressing the mouse H-$2K^b$ MHC class I molecule [26]. All cells were cultured at 37°C and 5% CO2, in cell culture flasks (Corning, NY, USA) containing culture medium, composed of IMDM (BioWhitaker), supplemented with 10% heat-inactivated FCS, 100µg/ml streptomycin, 100IU/ml penicillin and 4mM L-glutamine (all from Gibco).

Selection of frameshift products

Twelve genes, containing a microsatellite, were selected. Selection was based on known high mutation frequency in MSI-H colon cancer [18;19;22], previously described immunogenicity [12-14] or combinations of these factors. Insertion of 1 base pair or a deletion of two base pairs (-2/+1) result in the same reading frame but differ one amino difference in length. The same is valid for an insertions of 2 base pairs or a deletion of one base pair (-1/+2). Therefore only one type of a -2 or +1 and -1 or +2 mutation was used and only if the mutation resulted in a stretch of more than 4 new amino acids behind the microsatellite. Table 1 summarizes selected genes, mutation frequency in different types of cancer, whether -2/+1, -1/+2 or both sequences were constructed (15 in total), function and other characteristics.

Construction of frameshift products and transfection

Sequences of selected frameshift products were amplified by routine PCR using cDNA derived from several human colorectal MSI-H cell lines or were synthetically synthesized. Primers, containing a restriction site, were designed for start and end

Table 1. Characteristics of frameshift-mutated antigens included in study.

Gene	Locus	Mutation incidence (%)			Microsatellite repeat	Function	Mutations studied (length)
		Colorectal cancer	Gastric cancer	Endometrial cancer			
FTO	U79260	82	6.7	42	14T	Unknown	-1 (51)
TGFßRII	M85079	75	63	12	10A	Signal transducer	-1 (34)
TAF1B	L39061	75	87	58	11A	Transcription factor	-1 (25)
MARCKS	D10522	73	60	25	11A	Motility, phagocytosis, membrane trafficking and mitogenesis	-1 (11) & -2 (27)
PCNXL2 / FLJ11383	AK021445	66.0	---	---	10A	Unknown	-2 (18)
ACVR2	M93415	62	44	---	8A	Growth factor receptor	-2 (18)
C14orf106 / FLJ11186	AK002048	49	---	---	11A	Unknown	-1 (23) & -2 (7)
Caspase 5	U28015	46	47	14	10A	Apoptosis	-1 (25)
TCF7L2 / TCF-4	Y11306	45	12	3.8	9A	Transcription factor	-1 (22) & -2 (7)
Baxα	L22473	43	36	24	8G	Apoptosis	-1 (18) & +1 (31)
CDX2	Y13709	1.8	---	---	7G	Proliferation & differentiation	-2 (29)

The majority of frameshift-mutated antigens included in this study were selected on basis of high incidence (>40%) in at least one type of cancer and a minimal length of the non-self amino acid stretch, C-terminal of the mutation, of more than 4 residues. Antigen CDX2 was included despite its low mutation incidence, because antibody responses were found against this antigen in a cancer patient [14], indicating that it could be employed as a positive control for a stable, accumulating antigen in our assays.
Names of genes and loci are based on GenBank nomenclature. Incidence of frameshift mutations, and (putative) functions are based on previously published data [18;19;22]. The mutations studied are indicated with the length of the foreign amino acid sequences between parentheses. The -1 and -2 mutations were chosen as representative sof the two types of frameshift mutations that can occur.

of the sequence. A KOZAK sequence was added before the initial ATG code of each fragment. PCR products were directly cloned with the pGEM-T-Easy cloning kit (Promega) and sequenced, using standard procedures. Expression plasmids for each of the selected frameshift were obtained, through unidirectional cloning of the sequences concerned into the mammalian expression vector, pcDNA3-OVA/Flag containing an in-frame OVA/Flag tag. The tag was located downstream of the sequence direct behind the restriction site containing the H-2K^b restricted CD8$^+$ T-cell epitope of chicken ovalbumine (OVA: SIINFEKL) and the Flag epitope (DYKDDDDK),

which were spaced by a triple alanine sequence to allow efficient processing of the OVA T-cell epitope. These plasmids were transiently transfected into cells using Lipofectamine 2000 (Invitrogen) in 6 wells plate. Per well 2µg DNA was added. In some experiments 0.5µg eGFP-pcDNA3 vector was added to the transfection medium to determine transfection efficiency. Cells were harvested 48 h after transfection and used in experiments.

Detection of localization of frameshift products

Immunofluorescence was used to detect the localization of the different frameshift products. Cells were grown on Laboratory-Tech 8 well culture slides (Nalge-Nunc) precoated with poly-L-lysine (Sigma-Aldrich). Two days after transfection, cells were fixed with 1% paraformaldehyde for 5 minutes, permeabilized with methanol at –20°C for 10 min and incubated with a mouse monoclonal anti Flag antibody M2 (F3165, Sigma-Aldrich) for 1h at room temperature followed by incubation for 1h with Alexa-546-conjugated goat anti mouse secondary antibodies (Invitrogen). Stained cells were examined using a laser scanning confocal microscope (LSM510, Zeiss).

Expression of frameshift products

The expression of frameshift products was determined by flow cytometry with the PE labeled α-Flag antibody M2 (F3165, Sigma-Aldrich). Briefly, cells were harvested 48 h after transfection with one of the sequences and in addition of some eGFP to detect transfection efficiency. Cells were fixed in 1% paraformaldehyde for 5 minutes on ice, permeabilized by incubation in methanol for 10 minutes at -20^{o}C and stained with monoclonal anti-Flag antibody M2 (Sigma-Aldrich) followed by PE-conjugated Goat anti mouse IgG1 polyclonal antibody (PickCell Laboratories BV). Expression was measured on a flowcytometer LSRII (BD Biosciences) equipped with the FACSDIVA software (BD Biosciences). Approximately 10,000 single cell events (as predicted by size) were analyzed per sample. The green (eGFP) and the red (PE) fluorescence were measured using a 530/30 nm and a 575/26 nm band pass filter respectively. Compensation was set using single positive stained controls. Analysis was performed using Winlist 5.2 software (Verity Software House). Expression was found positive when fluorescent signal was shifted to the right side. Experiments were performed in duplicate.

Direct class I MHC presentation

Hela-K^{b} cells were plated in a 96-well flat-bottom plate and after overnight incubation transfected. Two days after transfection, B3Z hybridomas were added to the transfected Hela-Kb cells in a final concentration of 50,000 B3Z cells/well in 200µl.

B3Z is a T-cell hybridoma that recognizes the SIINFEKL peptide in the context of H-2K^b and expresses ß-galactosidase (ß-Gal) upon activation [27]. After 24h at 37°C, the medium was replaced with 100 µl lysis buffer (PBS, 100 mM 2-mercaptoethanol, 9 mM MgCl2, 0.125% NP-40, and 0.15 mM chlorophenol red-ß-D-galactopyranoside (Calbiochem) per well. Following color change, the absorbance at 590 nm was read using a 96 well plate reader. Per frameshift product 4 wells were transfected and analyzed. Experiments were done in duplo.

Mice

C57BL/6 mice and OT-1 TCR-transgenic mice (specific for OVA$^{257-264}$/H2-K^b on Rag -/-CD45.1$^+$ background) were bred in our own animal facilities (Leiden, The Netherlands) but were originally obtained from the Jackson Laboratory (Maine, USA). The experiments were approved by the animal experimental committee (UDEC) of Leiden University.

Cross presentation of frameshift products studied in a mouse model

Transfected HeLa cells were harvested, lethally irradiated (4000 rad) and washed twice in PBS. C57BL/6 mice (n=3-4) received an intra peritoneal injection of 2*10^6 irradiated cells suspension or 50µg OVA peptide. After three days T cells freshly isolated from spleen and lymph nodes of OT-1 TCR-transgenic mice were labelled with 5µM CFSE (InVitrogen, California, USA) and injected intravenous via the tail vein at a final concentration of 2*10^6 cells in PBS. After three days mice were sacrificed and mesenteric lymph nodes were isolated. Single cells suspensions of lymph nodes were prepared by mechanical disruption and prepared for flow cytometric detection CFSE intensity of OT-1 cells using PerCP conjugated α-CD8α (53-6.7), APC conjugated α-CD45.1 (A20) antibodies. FACS data were analyzed using CellQuest software. All antibodies and analysis software were purchased from BD Pharmingen (New Jersey, USA). Calculations of average fluorescent intensity of groups of mice (n=3-4) and Student's T test statistical analyses were performed by comparing all groups to the 'mock transfected' group using GraphPad software (GraphPad software Inc.).

RESULTS

Validation of a fusion gene expression system for assessment of immunogenic properties of potential tumor antigens

For many of the genes that are frequently mutated in MSI-H cancers no information is available with respect to the immunogenic properties of these proteins. Because no knowledge on T cell epitopes, nor specific T cell clones for *in vitro* tests were

at hand to enable analysis of the expression and immunogenicity of the individual antigens, we designed a gene expression system in which each of these proteins can be fused to a short C-terminal polypeptide comprising sequences that can readily be detected by T-cells and antibodies respectively: the H-2K^b-restricted CD8$^+$ T-cell epitope of chicken ovalbumin (OVA; SIINFEKL) [28] and the Flag tag epitope (DYKDDDDK) [29] (Fig. 1A). To validate this screening methodology, we chose to insert the coding sequences of wild-type (wt) and mutated (V143A) p53 into the expression vector, as these constitute prime examples of proteins (in fact, versions of the same protein) with very different expression characteristics [30]. Whereas wt.p53 has a very short half life, resulting in very low nuclear expression levels, mutations in p53 result in accumulation of this protein in both nucleus and cytoplasm of the cell. Furthermore, the degradation of p53 through the ubiquitin/proteasome pathway can result in efficient presentation of peptides derived thereof into MHC class I, even at the surface of cells that do not exhibit detectable intracellular levels of this protein [31].In accordance with the above, cells transiently transfected with the mutated p53 gene construct displayed high levels of p53 that are detected in nucleus and cytoplasm, while wt.p53-transfected cells displayed exclusive nuclear localization of this antigen (Fig 1B). Flow cytometry, used to further quantify the level of expression, confirmed that mutated p53 accumulated in a major fraction of the transfected cells. Transfection of wt.p53 resulted in positive staining in a considerably smaller fraction of cells, even though transfection efficiencies as determined by co-transfection of an eGFP-encoding gene construct were comparable (Fig. 1C). The high levels of antigen detected in a minor fraction of the wt.p53 transfected cells (Fig 1B, C) is probably due to the uptake of massive amounts of DNA by the cells concerned and the very high synthesis of the protein resulting from this. As shown in Figure 1D, optimal distinction between accumulation of wt and mutated p53 is obtained when cells are transfected with 1-2 µg of p53-encoding DNA.

To evaluate processing and presentation of p53 degradation products into MHC class I, the recognition of the transfected cells by OVA-specific T-cell hybridoma cells (B3Z) was evaluated. Because the octamer peptide recognized by this T-cell constitutes only a very small part of the fusion proteins tested, we deem it highly conceivable that the behavior of the OVA T-cell epitope in the context of the fusion protein reflects that of putative T-cell epitopes within the sequences of the wt and mutated p53 sequences. In concordance with its accumulation in a large fraction of transfected cells, expression of the fusion protein comprising mutated p53 resulted in efficient presentation of the OVA T-cell epitope in MHC class I (Fig. 1E). However, also transfection of the fusion gene comprising wt.p53 resulted in clearly detectable epitope presentation. This is in line with our previous observation that accumulation

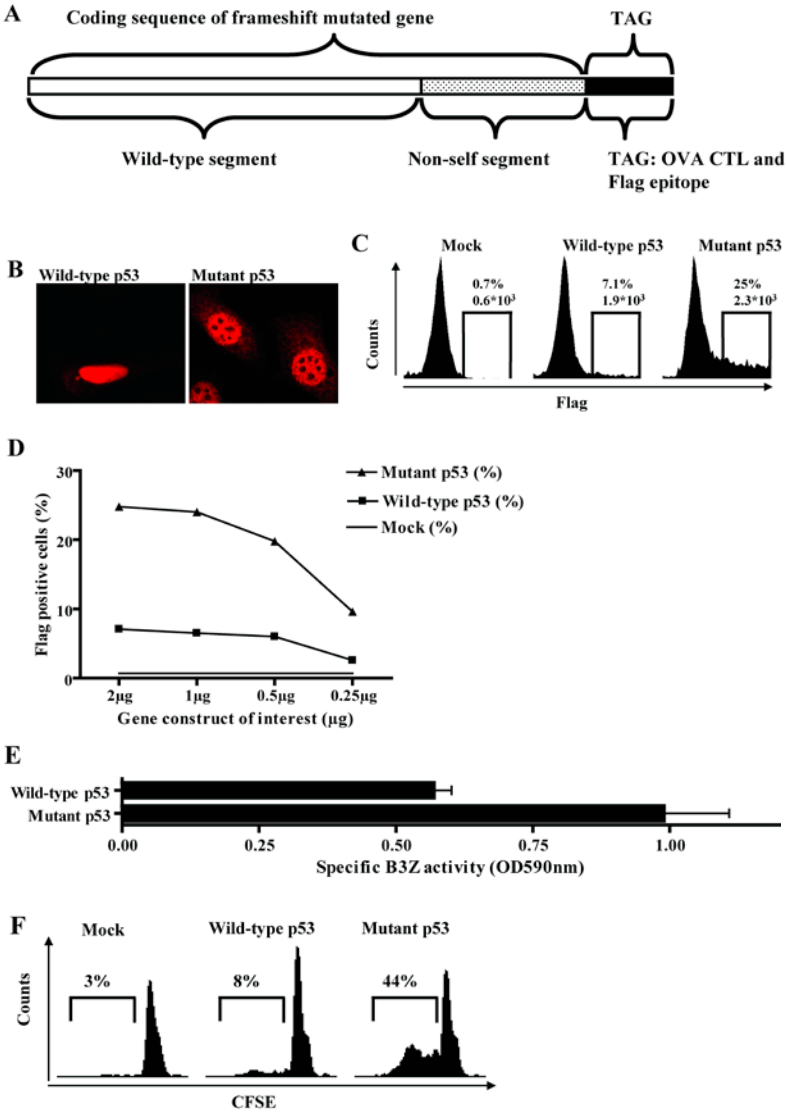

Figure 1. Design of screening methodology and validation of assays on basis of characteristics wild type and mutant p53.

(A) Full length coding sequences of frameshift mutated genes that lack stop codons were fused in frame to a sequence encoding the H-2K^b-restricted CD8$^+$ T-cell epitope of chicken ovalbumin (OVA; SIINFEKL, [28]) and the FLAG tag epitope (DYKDDDDK, [29]), which were spaced by a triple alanine sequence to allow efficient processing of the OVA T-cell epitope [40]. *(B)* Detection of tagged fusion gene products comprising wild-type (wt) and mutant (V143A) human p53 by means of immunofluorescence in paraformaldehyde-fixed HeLa cells that were transfected with the indicated constructs 48 hrs. prior to analysis. *(C)* Detection of p53 antigen by flow cytometry in wt.p53, V143A p53 and mock transfected HeLa cells. Percentage of cells with FLAG-tag-specific staining above background and mean fluorescence intensity (MFI) of positive fraction are indicated.

(D) Percentage of positive cells of positive fraction in relation to amount of DNA transfected is plotted. Comparable transfection efficiency between samples was ensured by co-transfection of DNA encoding eGFP. *(E)* Direct MHC class I-restricted presentation of the OVA (SIINFEKL) epitope in fusion-gene transfected HeLa-K^b cells, as measured by reactivity of B3Z T-cell hybridoma cells. Magnitude of response is measured on base of β-galactisodase activity in stimulated B3Z cells and expressed as absorbance (OD 590nm) of converted substrate in quadruplicate samples. *(F) In vivo* cross-presentation of the OVA epitope derived from fusion-gene transfected HeLa cells (lacking H-2Kb expression) cells, as determined by proliferation of SIINFEKL-specific OT-1 TCR-transgenic CD8+ T-cells. Hela cells transfected with wt.p53, V143A p53 or control DNA were injected in mice that received naïve, CFSE-labeled OT-1 cells. Proliferation of OT-1 on basis of dilution of CSFE was assessed three days later. Percentage of divided CFSE cells is indicated.

of p53 is not required for direct MHC-restricted presentation of CTL epitopes derived from this antigen [31].

Cross-presentation of cell-derived antigens by professional APC does depend on the availability of these antigens in sufficient amounts [23-25]. To test whether our fusion gene expression system can also be used to address the behavior of antigens in cross-presentation, we transfected human cells with genes comprising wt or mutated p53 and injected these xenogeneic cells into mice that were infused with naïve, CSFE-labeled T-cells (OT-1) that recognize the OVA CTL epitope. The use of this xenogeneic system is justified by the notion that the antigen processing machinery is highly conserved between mouse and human [32]. Fig. 1F shows that *in vivo* challenge of OT-1 through injection of cells transfected with a fusion gene comprising mutated p53 elicited strong proliferation of OT-1, while injection of cells transfected with the wt.p53 gene construct induced only modest OT-1 proliferation.

In conclusion, the wt.p53 OVA/FLAG fusion construct encoded a protein with short half life that failed to accumulate in the majority of transfected cells and was therefore not available for efficient uptake and cross-processing by professional APC, while the turn over of this protein nevertheless resulted in direct MHC class I-restricted presentation by the antigen-expressing cell. In contrast, the OVA/FLAG fusion gene comprising mutated p53 encoded a stable protein that accumulated in transfected cells, and that could serve as a basis for both direct and cross-presentation. Taken together these results demonstrate that the behavior of these fusion proteins properly reflects that of the antigens under examination, and that our expression system is a suitable tool for evaluating the characteristics of additional antigens, such as those encoded by the frameshift mutated genes commonly found in MSI-H cancers.

Steady state expression levels of frameshift-mutated antigens

After validation of our expression system, we cloned multiple frameshift mutated genes into the OVA/FLAG gene cassette. All but one of genes to be examined in

our proof of concept study were selected on basis of two criteria. First the high prevalence of a given frameshift mutation in MSI-H cancers as reported in previous studies, in particular a reported frequency of at least 40% in at least one type of MSI-H cancer. Second, we focused on frameshift mutations that gave rise to foreign sequences, downstream of the frameshift mutation, of at least 5 amino acids in length, because shorter sequences are unlikely to render immunogenic T-cell epitopes. An overview of the selected frameshift-mutated genes is shown in Table 1. The mutated CDX2 gene was included in our studies despite its low incidence, because antibody responses were found against its gene product in a cancer patient [14], arguing that this antigen could be used in our screening as a positive control for a stable, accumulating antigen.

Steady state expression levels and intracellular localization of the selected frameshift product were initially determined by performing immunocytochemistry on transiently transfected cells, using the FLAG tag-specific antibody. The resulting data showed that only four of the gene constructs (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2) gave rise to high protein levels in a large fraction of the cells (Figure 2). Expression of the different frameshift proteins was further quantified by means of flow cytometry. These experiments confirmed that only 4 of the gene constructs tested (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2) encoded antigens that accumulated in a large fraction of the transfected cells (Figure 3A, B). Five additional gene products (TAF1B-1; PCNXL2-2; TCF7L2-1; -2; Baxα+1) were found expressed in a modest fraction of transfected cells, while expression of the remaining genes did not surpass that of background levels. Transfection efficiencies were comparable for all assays, as determined by co-transfection of an eGFP-encoding gene construct.

Direct antigen processing and presentation into MHC class I

As shown for wt.p53, failure of antigens to accumulate does not necessarily preclude processing of peptides derived thereof into MHC class I (Fig 1E). Accordingly, recognition of the OVA CTL epitope by the B3Z hybridoma on transfected cells was not limited to the 4 gene constructs that encode stable antigens (figure 3B). In addition, efficient CTL epitope recognition was observed for cells transfected with gene constructs encoding PCNXL2-2, Caspase5-1, Baxα-1 and Baxα+1, while transfection of four other constructs (FTO-1, C14orfl06-2, TCF7L2-1 and TCF7L2-2) resulted in moderate T-cell recognition (Figure 3C). These results confirm that accumulation of protein is not a prerequisite for direct processing and presentation of epitopes into MHC class I.

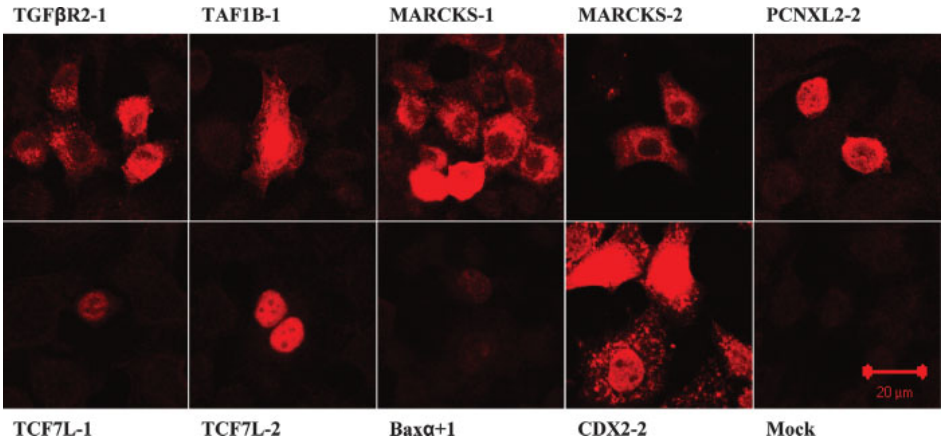

Figure 2. Steady state expression of frameshift antigens.
Detection of tagged fusion gene products by means of immunocytochemistry in fixed HeLa cells, transfected with the indicated constructs 48 hrs. prior to analysis. Cells shown in pictures are representative for positive cells obtained after transfection with indicated gene construct. Frequency of positively staining cells depends on gene construct used (see Fig. 3C). Images were made under standardized conditions, using 40x magnification and the same exposure.

In vivo cross-presentation of antigen-derived epitopes

Whereas T-cell action at the effector level requires direct presentation of epitopes by the target cell, the prevailing view on priming of T-cell responses is that successful activation of T-cell immunity requires uptake, processing and presentation of antigen by dendritic cells. We therefore analyzed the capacity of the frameshift-mutated antigens to give rise to cross-presented T-cell epitopes by immunizing mice with transiently transfected xenogeneic (human) cells. Presentation of the OVA CTL epitope was monitored by analyzing the antigen-specific proliferation of CSFE-loaded OT-1 cells that were infused into the mice. The capacity of gene constructs to give rise to *in vivo* cross-presentation of the OVA epitope correlated with the accumulation of the corresponding antigen in transfected cells (figure 4A and 4B). Most efficient cross-presentation was restricted to the four gene constructs that gave rise to accumulating antigen in a large fraction of transfected cells (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2). Four of the five gene constructs that gave rise to protein accumulation in a modest fraction of transfected cells (Figure 3B), resulted in low but detectable levels of cross-presentation, (BAXα+1, PCNXL2-2, TCF7L2-2 and TAF1B-1). No *in vivo* cross-presentation above background was found for the fifth of this set of genes (TCF7L2-1). This can most likely be explained by our finding that protein accumulation in transfected cells for this gene was lower than for the other four genes (Fig. 3B).

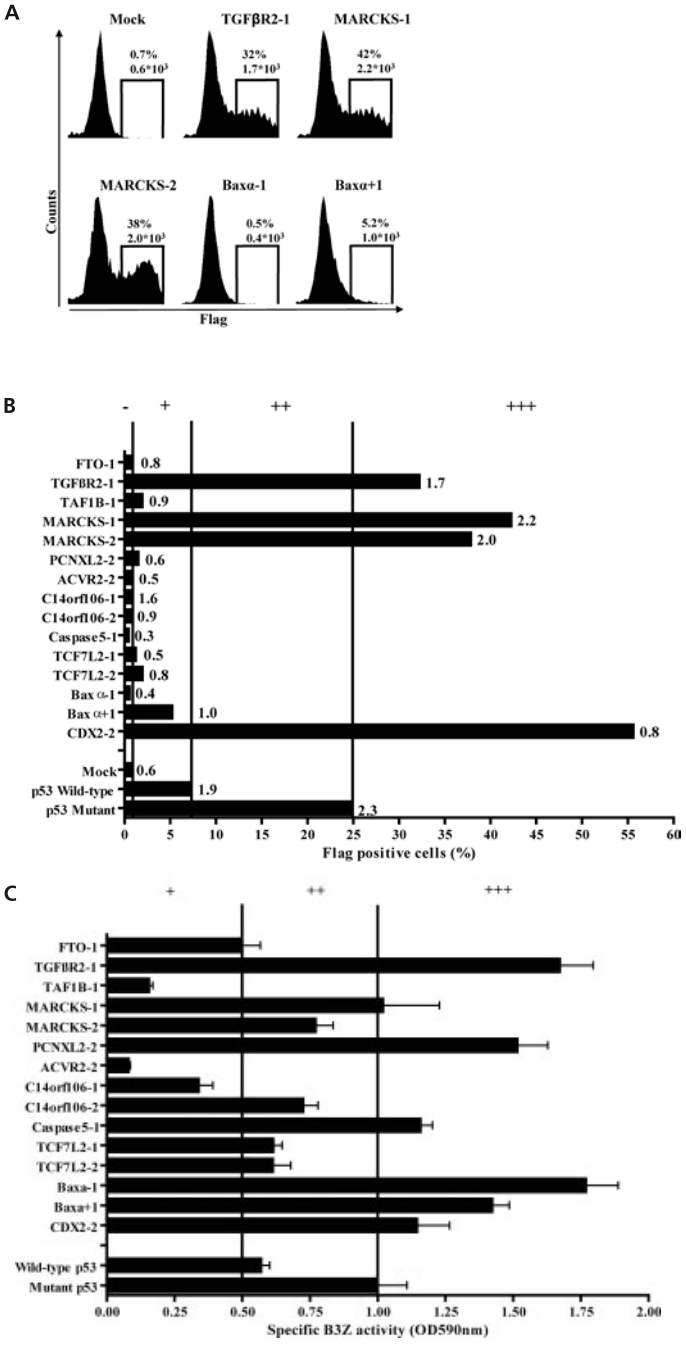

Figure 3. Quantitative evaluation of steady state expression and direct processing into MHC class I.

(A) Flow cytometric analyses of HeLa cells transfected with 2μg gene constructs encoding the indicated frameshift proteins. Results for several of the proteins tested are shown. Percentages

of cells with FLAG-tag-specific staining and MFI of positive fraction are indicated. *(B)* Summary of the flow cytometric analyses for all antigens tested. The bars represent percentages of Flag positive cells. Number behind each bar indicates the MFI of the Flag-positive cells. These analyses were performed twice with very similar outcome; data from one experiment are shown. Expression of frameshift products is categorized as strong (+++, > mutant p53), moderate (++, > wt.p53 - < mutant p53), low (+, > Mock - < wt.p53) and negative (-, - < Mock). Comparable transfection efficiency between samples was ensured by co-transfection of DNA encoding eGFP (not shown). *(C)* Overview of direct MHC class I-restricted presentation of the OVA SIINFEKL epitope in fusion-gene transfected HeLa-Kb cells, as measured by reactivity of B3Z T-cell hybridoma cells. B3Z responses against transfected antigens are categorized as strong (+++, >1.0), moderate (++, 0.5-1.0) and low (+, <0.5). Error bars indicate SD.

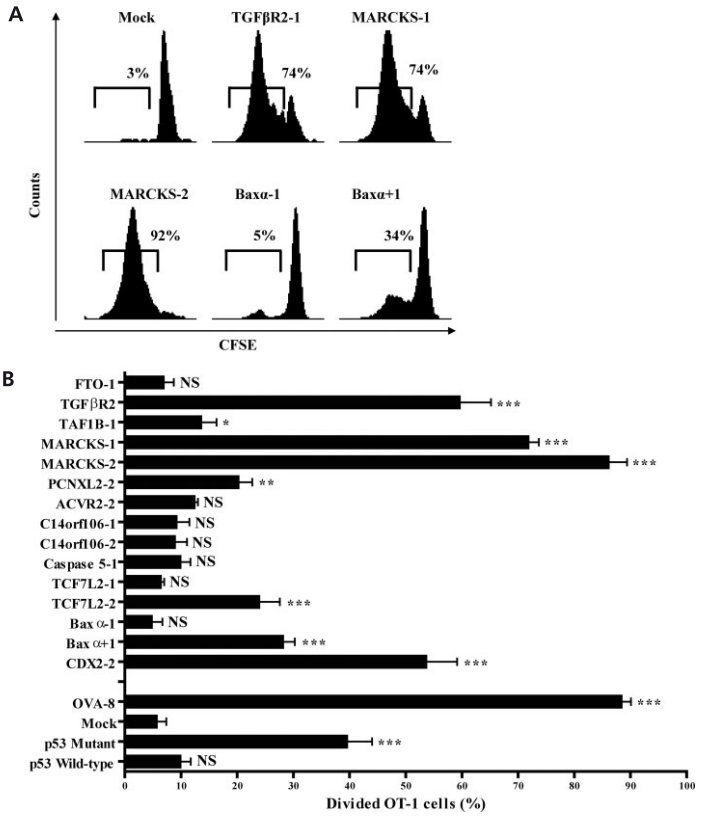

Figure 4. *In vivo* processing of frameshift mutated antigens.
(A) Hela cells transfected with indicated frameshift gene constructs were injected in mice that received naïve, CFSE-labeled OT-1 cells. Proliferation of OT-1 on basis of dilution of CSFE was assessed three days later. Percentage of divided CFSE cells is indicated in each graph (examples shown). *(B)* Summary of the outcome of *in vivo* cross-presentation experiments for all gene constructs studied. Dashed line indicates mean % of CFSE-low OT-1 cells in mice injected with mock-transfected HeLa cells plus the SEM of these values. T-test was used to calculate significant difference from mock transfected cells. Samples are categorized as strong (***, p<0.001), moderate (**, p<0.01-p>0.001), low (*, p<0.05-p>0.01) and not significant different from mock (NS, p>0.05).

DISCUSSION

We performed a series of experiments aimed at predicting the immunogenic behavior of proteins encoded by a selection of frameshift-mutated genes that are frequently found in MSI-H cancers. Our data demonstrate that fusion proteins comprising eight of the antigens examined (TGFβR2-1; MARCKS-1; -MARCKS-2; CDX2-2; BAXα+1, PCNXL2-2, TCF7L2-2 and TAF1B-1) gave rise to direct epitope presentation by the cell expressing the antigen, as well as to cross-presentation by DCs. (Table 2). Our proof of concept study provides important guidelines for further research concerning this antigen family in the context of cancer immunotherapy. The antigens that, in view of their capacity to accumulate in tumor cells, are cross-presented by DCs, can become visible to naïve T-cells in the tumor-draining lymphoid tissues. Consequently, one can expect cancer patients to display 'tumor-induced' T-cell or IgG-type immunity against these antigens. Indeed, such responses have been found against the frameshift-mutated TGFβR2-1 and CDX2-2 proteins [13;14;33]. Our results argue that further evaluation of spontaneous immunity against frameshift mutated antigens should be focused on the eight antigens mentioned above and on defining additional antigens with similar characteristics. Moreover, our data indicate that antigens belonging to this subclass are most valuable as components of vaccines that are intended to raise anti-tumor immunity by both $CD4^+$ and $CD8^+$ T-cell subsets. Vaccination-induced $CD4^+$ T-cells can only provide efficient 'help' to the immune response if they encounter their cognate antigen on cross-presenting DCs in the tumor microenvironment and/or draining lymphoid tissue. In addition, they may contribute to the effector response by recognizing their antigen on MHC class II-expressing colorectal tumors [34-36]. On basis of our experiments with fusion gene constructs, such class II MHC-restricted presentation by professional APCs or by tumor cells, the latter of which lack efficient MHC class II processing, is less likely to occur for the seven remaining, non-accumulating antigens (Table 2). Therefore, the impact of $CD4^+$ T-cells raised by vaccination against these latter antigens will be limited to the vaccination phase. Due to the lack of their cognate antigen in the peritumoral area, these $CD4^+$ T-cells will fail to contribute to the effector phase. For the latter antigens (FTO-1; ACVR2-2; C14orf106-1; -2; Caspase5-1; TCF7L2-1; Baxα-1), breakdown did result in class I MHC-restricted epitope presentation by the antigen-expressing cells, indicating that the $CD8^+$ T-cell arm of a vaccine-induced response against these antigens could be used to target tumors (Table 2). Whether MHC class I-restricted epitope presentation by the tumor truly occurs in a given human subject will, of course, depend on the compatibility between the proteolytic fragments generated and the repertoire of class I molecules comprised within the subject's HLA-type. The available HLA-specific peptide-binding motifs can be used for further prediction of such epitopes within the antigens concerned.

The degree at which the fusion proteins comprising frameshift mutated antigens accumulate in transfected cells correlates well with the efficiency by which these antigens give rise to *in vivo* cross-presentation after injection of transfected cells (compare Figures 3B and 4B). Protein accumulation and cross-presentation are high for fusion proteins containing TGFβR2-1, MARCKS-1, -MARCKS-2 or CDX2-2, while being modest for fusion proteins comprising BAXα+1, PCNXL2-2, TCF7L2-2 or TAF1B-1. Notably, the fusion protein comprising wt.p53, although accumulating at considerably higher efficiency than the latter four fusion constructs, did not give rise to detectable cross-presentation (Figure 4B). This suggests that factors other than steady state levels, such as efficiency of antigen uptake or intracellular routing after uptake, may impact on the handling of antigens by cross-presenting DCs. We would like to emphasize that the methodology described in our present paper should be regarded as part of a multifaceted selection procedure that also involves evaluation of the immunogenicity of these antigens by means of *in vitro* human T-cell cultures using lymphocytes from MSI-H cancer patients and healthy subjects. Furthermore, the potential impact of frameshift mutations on mRNA stability through nonsense-mediated RNA decay (NMD) should be taken into account, as this might preclude efficient synthesis of gene products [37]. NMD was reported not to impact on 7 of the 8 frameshift mutations that we found to encode accumulating gene products [38;39]. Whether NMD affects expression of the remaining gene product (PCNXL2-2) still needs to be determined.

In conclusion, the frameshift-mutated antigens expressed by MSI-H cancers are promising antigens for selective, immune-mediated targeting of cancers, because they comprise sequences foreign to the immune system. However, other than their sequence, very little is known about the immunogenic characteristics of the majority of these proteins. By providing a methodology for predicting the immunogenic behavior of frameshift-mutated proteins, our study constitutes a valuable step towards the systematic selection of target antigens for immune intervention against MSI-H cancers.

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Table 2. Summary of expression characteristics of frameshift-mutated antigens and their expected relevance for evaluation of spontaneous T-cell immunity and vaccine design.

Coding sequence and type of mutation		Experimental data			Predicted immunogenic profile	
		Accumulation	Presentation in MHC class I	Cross-presentation	Tumor induced immunity	Vaccine component
1	TGFßR2-1	+++	+++	+++		
2	MARCKS-1	+++	+++	+++	CD4 & CD8	CD4 & CD8
3	MARCKS-2	+++	++	+++		
4	CDX2-2	+++	+++	+++		
5	Baxα+1	+	+++	+++		
6	TCF7L2-2	+	++	+++	Possibly	
7	PCNXL2-2	+	+++	++	CD4 & CD8	(CD4 &) CD8
8	TAF1B-1	+	+	+		
9	Caspase5-1	-	+++	-		
10	Baxα-1	-	+++	-		
11	FTO-1	-	++	-		
12	C14orf106-2	-	++	-	-	CD8
13	TCF7L2-1	+	++	-		
14	ACVR2-2	-	+	-		
15	C14orf106-1	-	+	-		

The results of the experiments concerning steady state protein expression (Fig 3B), direct presentation in MHC class I (Fig. 3C) and *in vivo* cross-presentation (fig 4B) have been quantitated as described in the figures. On basis of these data, the top 8 antigens, in particular (TGFβR2-1, MARCKS-1, MARCKS-2, CDX2-2), are of interest with respect to evaluation of anti-tumor immunity in non-vaccinated patients, as well as for inclusion into vaccines that elicit potentially effective anti-tumor immunity through both $CD8^+$ and $CD4^+$ T-cell subsets.

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Chapter 6

Induction of p53-specific immunity by a p53 synthetic long peptide vaccine in patients treated for metastatic colorectal cancer.

Frank M. Speetjens[1], Peter J.K. Kuppen[1], Marij J.P. Welters[2], Farah Essahsah[3], Anne Marie E.G. Voet van den Brink[1], M. Graziella Kallenberg Lantrua[1], A. Rob P.M. Valentijn[4], Jaap Oostendorp[4], Lorraine M. Fathers[4], Hans W. Nijman[5], Jan W. Drijfhout[2], Cornelis J.H. van de Velde[1], Cornelis J.M. Melief[2, 3], Sjoerd H. van der Burg[6]

[1]Departments of Surgery, [2]Immunohematology and Blood Transfusion, [4]Clinical Pharmacy and Toxicology, [6]Clinical Oncology, Leiden University Medical Center, Leiden, the Netherlands
[3]ISA Pharmaceuticals B.V. Bilthoven, the Netherlands
[5]Department of Gynecology, University Medical Center Groningen, Groningen, the Netherlands

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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 [1]. 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 [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 (wt.p53)-specific cytotoxic T lymphocytes (CTL) and T-helper (Th)-cells have been detected in PBMC cultures *in vitro* [4-8]. In addition, wt.p53-specific proliferative responses were demonstrated in patients with breast cancer [9], ovarian cancer [10] and colorectal cancer [11;12]. 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 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 [19-21]. Indeed, adoptively transferred p53-specific $CD4^+$ Th-cells supported the anti-tumor response against p53 over-expressing tumors [13;22]. Moreover, Th1-cells can activate peritumoral DC, which generally display an immature phenotype [23;24], 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 [25;26]. Analyses of the p53-specific $CD4^+$ Th-cell repertoire in patients undergoing colorectal carcinoma resection revealed that these responses were weak and required at least one round of *in vitro* stimulation [11]. 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 [11]. Interestingly, the presence of circulating IFNγ-producing p53-specific $CD4^+$ T cells was associated with a stronger $CD8^+$ T-cell infiltration of the tumor [12], 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 [27], recombinant canarypox virus encoding wt.p53 [28;29], or adenoviral vector encoding wt.p53 transfected DCs [30] 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 [31], but has the disadvantage that patients with other HLA types can not be treated [14].

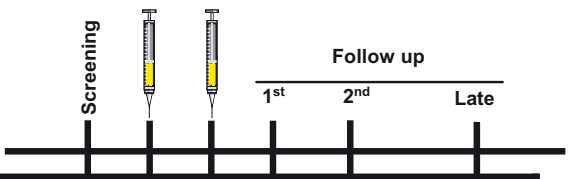
Recently, we have developed the concept of using synthetic long peptides (SLP) as vaccines [32;33]. When injected, these SLP are predominantly taken up by DC resulting in the presentation of both helper T-cell epitopes and CTL epitopes that are present in the SLP [34;35]. The efficacy of SLP vaccines to induce truly strong tumor-specific $CD4^+$ and $CD8^+$ T-cell responses was demonstrated in rodents therapeutically treated for human papillomavirus induced tumors [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 out of 5 patients injected with canarypoxvirus with human wt.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 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.



Week(s)	-1	0	3	6	12	±28
Vaccination		X	X			
Immuno monitoring						
Blood sample	X			X		X
Biopsy				X		
CRF	X	X	X	X	X	
ECG		X	X			
Physical Examination	X	X	X	X	X	
Inspection vaccination site		X	X	X	X	
Biochemistry/CEA/ Hematology	X	X	X	X	X	X
CT scan	X				X	X

Figure 1. A schematic overview of the vaccination scheme.
electrocardiogram (ECG), case report form (CRF).

Table 1. Patient characteristics and adverse events observed

Patient	Gender	Age	TNM stage	Primary treatment	Recurrences	Secondary treatments before vaccination	Clinical status (months after vaccination)	P53 expression[3]	Adverse events
P01	M	71	4	PR+RLi	1 Lu	RLu	Alive, Rec (17)	4	flu-like symptoms (2X), atrial fibrillation
P02	M	54	3	PR+Ro	1 Li	RLi	Alive, NED[1] (13)	2	Pain vaccination sites (2X)
P03	M	62	4	PR+RLi	-	-	Alive, NED[2] (15)	4	-
P04	F	57	3	PR+A	1 Lu	RFLu	Alive, NED (12)	0	Pain vaccination site (1X)
P05	M	67	4	PR+Ro	3 Li	RLi (1x); RFLi (2x)	Alive, Rec (11)	1	-
P07	M	64	4	PR+RLi	3 Li; Li; Lu	RLi+C; Rli+RFLi; RLu	Alive, NED (7)	0	swelling + erythema injection site (2X)
P08	F	58	3	PR+Ro	1 Li	C+RFLi	Alive, Rec (3)	3	swelling + erythema injection site (2X)
P09	M	59	3	PR+Ro	2 Li	C+RLi; C+RLi	Alive, Rec (3)	4	flu-like symptoms + swelling + erythema injection (1X), prostatitis
P10	M	69	3	PR+A	2 Li	RLi; RFLi	Alive, Rec (3)	4	Pain + swelling vaccination site (1X)
P11	M	50	4	C+RLi	-	-	Alive, NED (1)	0	swelling + erythema injection site + itching (2X)

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]P53 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 [11;12], and it harbors most of the published MHC class I and class II epitopes (reviewed in [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 [39]. At the day of vaccination, the 10 peptides (0.3 mg/peptide) were dissolved in Dimethylssulfoxide (DMSO), admixed with Phosphate Bufferd 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 [13;36] and patients vaccinated with an HPV16-SLP vaccine [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 [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 [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 pre-sensitized 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 [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-CD154 PEcy5 (clone TRAP1, BD PharMingen), anti-CD137 APC (clone 4B4-1, BD PharMingen), anti-IFNγ 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 IFNγ 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 [39]. 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) [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 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 [39].

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

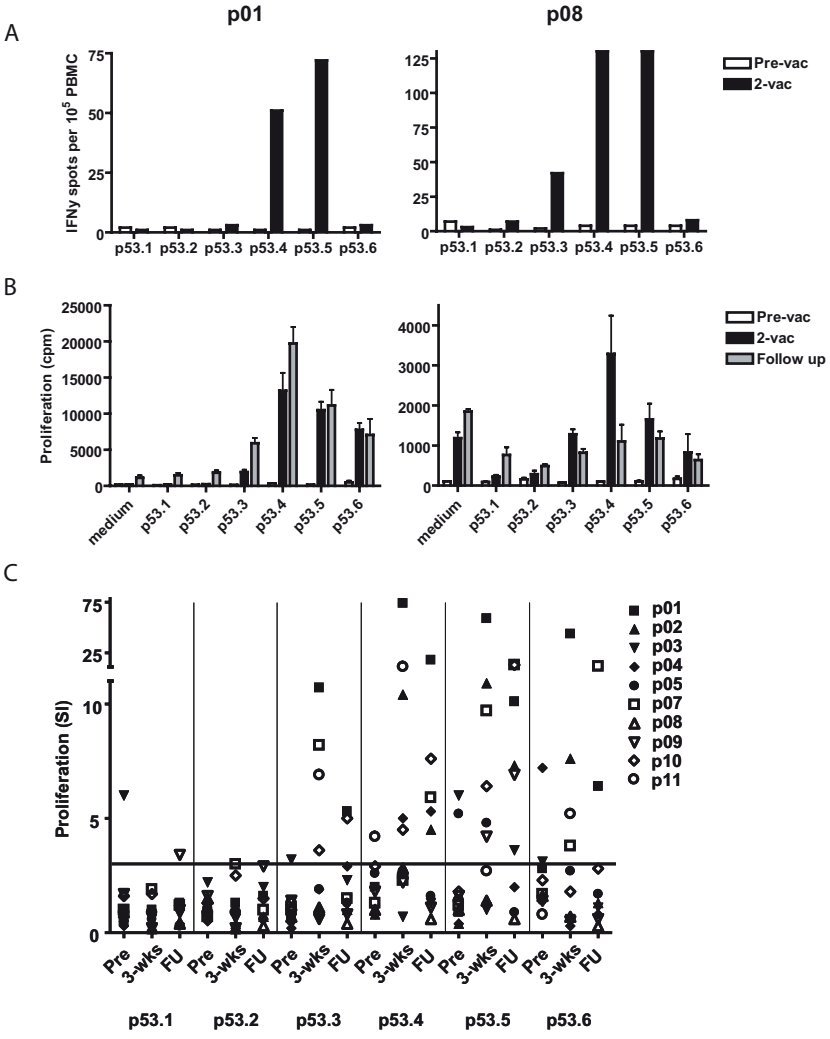


Figure 2. Vaccination with the p53 synthetic long peptides vaccine elicits strong T-cell responses in patients.

(A) Two typical examples of the IFNγ-ELISPOT results are shown: p01 (left) and p08 (right). Columns indicate the number of T cells per 10^5 PBMC specifically producing a spot of the cytokine IFNγ 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 IFNγ-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 IFNγ ELISPOT.

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

Patient	Pre vaccination						Post vaccination					
	P53.1	P53.2	P53.3	P53.4	P53.5	P53.6	P53.1	P53.2	P53.3	P53.4	P53.5	P53.6
	1-78	V70-115	V102-155	V142-203	V190-248	241-393	1-78	V70-115	V102-155	V142-203	V190-248	241-393
P01	2	2	<1	<1	1	2	<1	<1	3	**51**	**72**	3
P02	<1	<1	<1	<1	<1	<1	<1	3	9	**111**	**137**	<1
P03	<1	<1	<1	<1	<1	<1	4	**20**	<1	<1	8	<1
P04	7	4	4	2	4	2	12	**31**	**26**	**63**	**14**	**35**
P05	<1	<1	<1	<1	10	<1	<1	<1	<1	<1	<1	<1
P07	<1	4	<1	<1	<1	<1	<1	<1	<1	<1	<1	**22**
P08	7	1	2	4	4	4	3	7	**42**	**220**	**144**	8
P09	<1	<1	<1	<1	4	<1	4	**16**	**56**	**126**	**110**	7
P10	<1	<1	<1	<1	<1	<1	4	<1	3	2	2	1
Total number of positive reactions												
	0	0	0	0	0	0	0	3	3	5	5	2

PBMC were tested against six different peptide pools. The numbers indicate the number of T cells per 10^5 PBMC specifically producing a spot of the cytokine IFNγ 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 ≥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 (IFNγ; IL-2; TNFα; IL-10; IL-5; 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 IFNγ (mean 228; range 35 - 1521 pg/mL). TNFα 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

In order to gain more insight in the p53-SLP®-induced T-cell response, patient-derived PBMC were stimulated, directly *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 (IFNγ, 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 *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

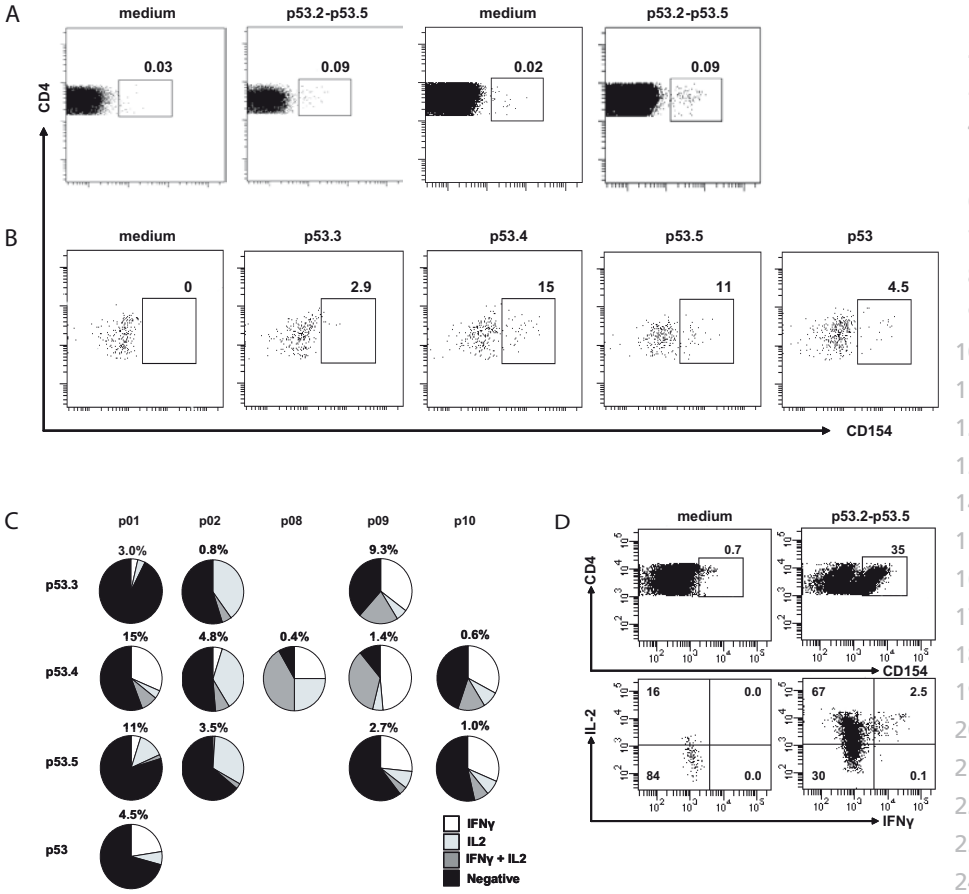


Figure 3. Only p53-specific CD4+ T cells are induced.

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 [39]. 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 [47] 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.

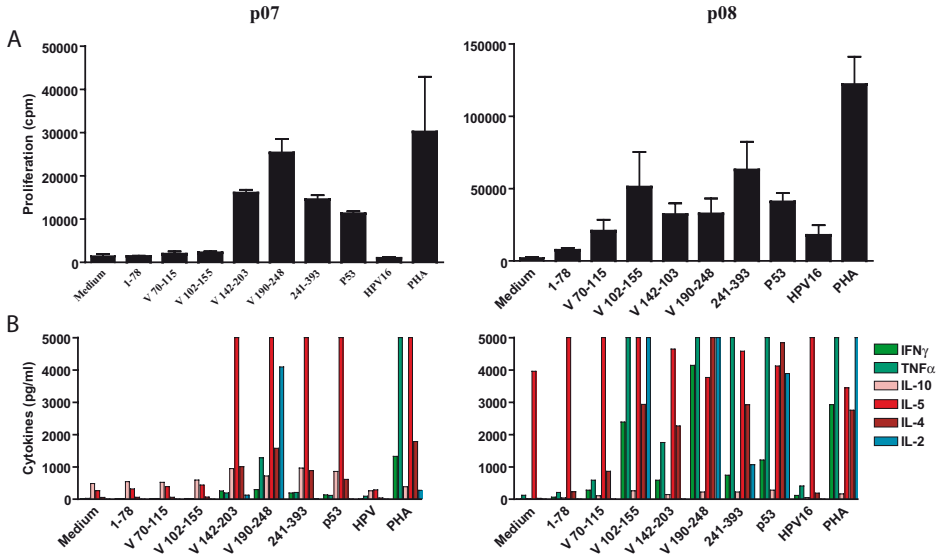


Figure 4. Vaccine-site infiltrating T cells are p53-specific.
(A) T cells from the skin biopsy of patients p07 (left) and p08 (right) were tested for their capacity to proliferate upon stimulation with peptides or protein pulsed monocytes. Phytohemagglutinine (PHA) served as a positive control, while 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.

DISCUSSION

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 [11;12] 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 [39], 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 [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 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

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 IFNγ 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 IFNγ. However, the levels of IFNγ (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 IFNγ 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 [38].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 [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, GM-CSF 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 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 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 [19-21]. A new trial with p53-SLP® in combination with a Th1 enhancing compound has been initiated.

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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

Frank M. Speetjens[1 *], Eliane C.M. Zeestraten[1 *], Marij J.P. Welters[2 *], Sepideh Saadatmand[1], Linda F.M. Stynenbosch[2], Rogier Jongen[2], Ellen Kapiteijn[2], Hans Gelderblom[2], Hans W. Nijman[3], A. Rob P.M. Valentijn[4], Jaap Oostendorp[4], Lorraine M. Fathers[4], Jan W. Drijfhout[5], Cornelis J.H. van de Velde[1], Peter J.K. Kuppen[1], Sjoerd H. van der Burg[2], Cornelis J.M. Melief[5, 6]

[1]Departments of Surgery, [2]Clinical Oncology, [4]Clinical Pharmacy and Toxicology, [5]Immunohematology and Blood Transfusion, Leiden University Medical Center, Leiden, the Netherlands
[3]Department of Gynecology, University Medical Center Groningen, Groningen, the Netherlands
[6]ISA Pharmaceuticals B.V. Leiden, the Netherlands.
Note: [*]These authors contributed equally to this work

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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

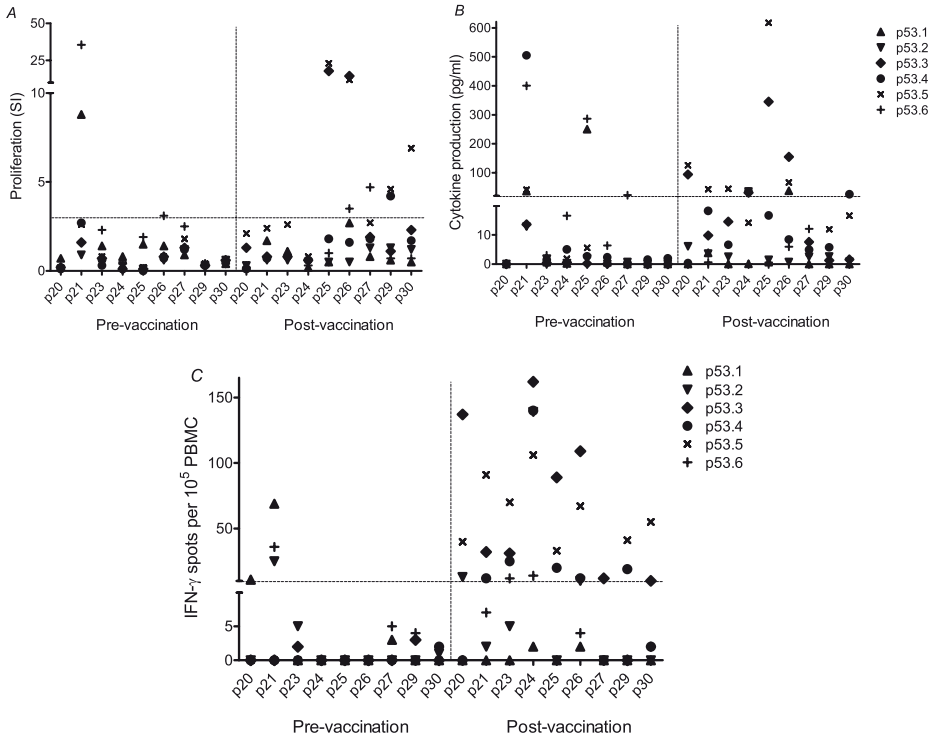

Figure 1. Results of three complementary T-cell assays with either freshly isolated PBMCs: (A) LST and (B) IFN-γ in CBA, or cryopreserved PBMCs: (C), IFN-γ ELISPOT.

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).

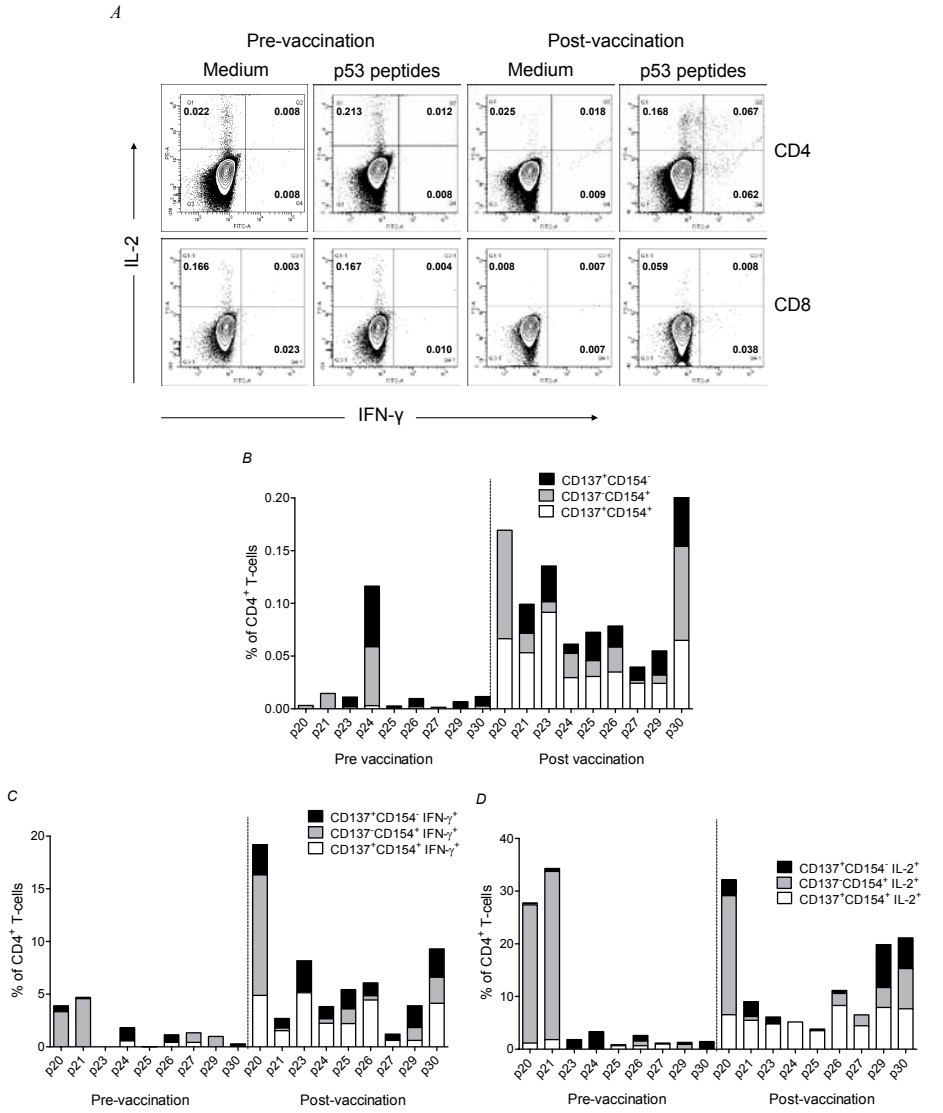

Figure 2. *Ex vivo* measurement of the percentage of p53-specific activated T cells, and their production of IFN-γ and IL-2, as determined by multiparametric flow cytometry.

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).

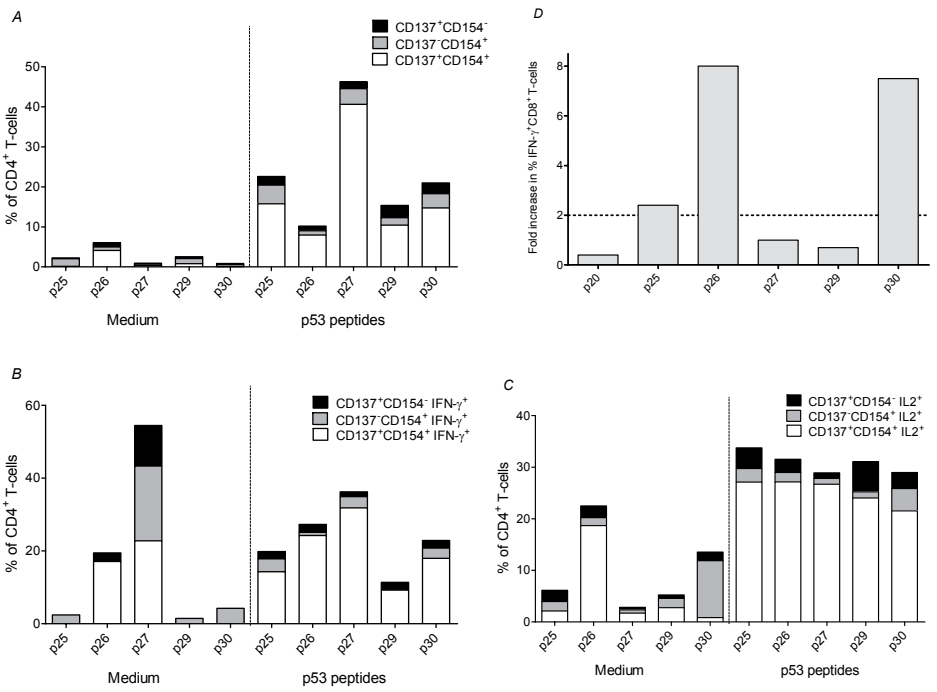

Figure 3. Measurement of the percentage of p53-specific activated T cells cultured from skin biopsies of the p53-SLP® injection site, which produce IFN-γ and/or IL-2, as determined by multiparametric flow cytometry.

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+

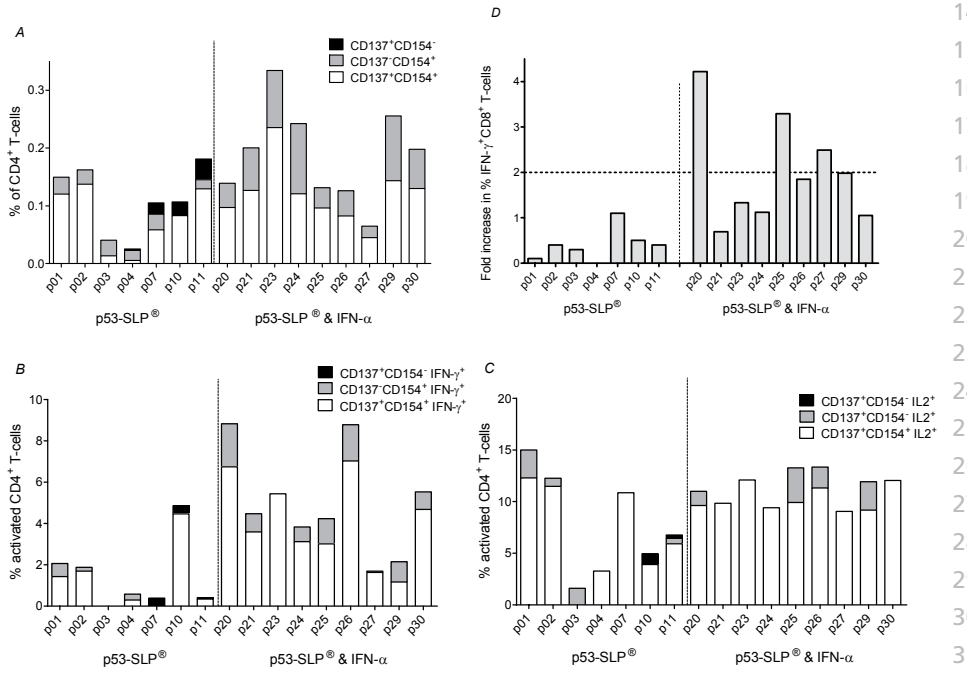

Figure 4. Results from patients injected with p53-SLP® and IFN-α compared to the results from colorectal cancer patients injected with p53-SLP® only.

(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.

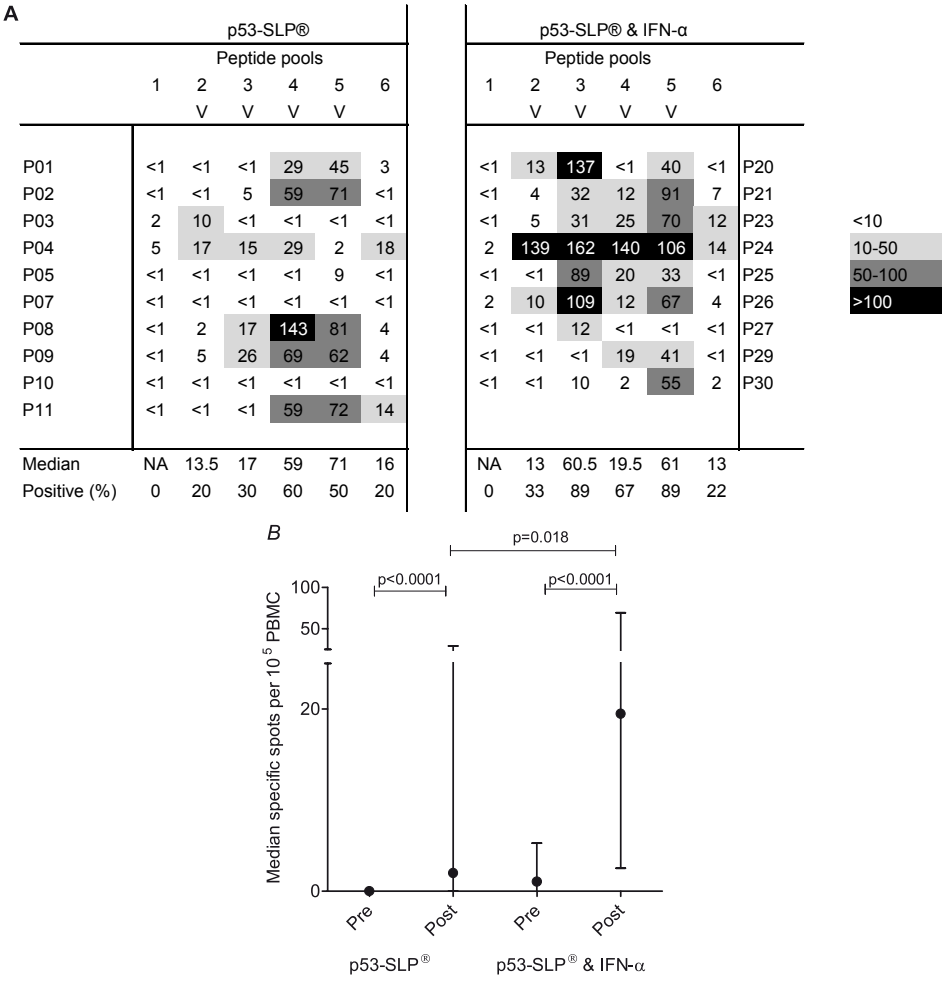

Figure 5. Comparison of IFN-γ ELISPOT results in colorectal cancer patients vaccinated with p53-SLP® only or in combination with IFN-α.

(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).

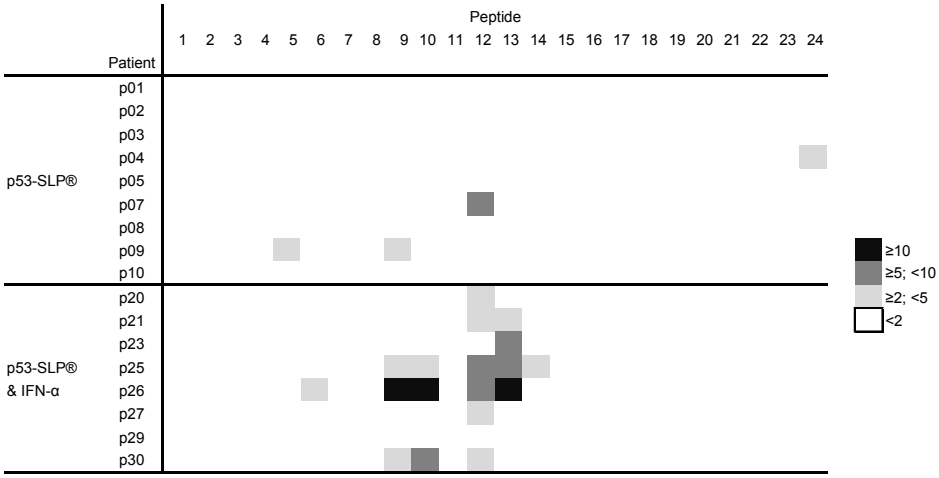

Figure 6. The heat map represents the number of peptides that were recognized by the individual patients based on their specific IgG antibody responses.
The peptides are listed on the x-axis, the individual patients on the y-axis. Peptides are numbered and cover the whole p53 protein sequence. Patients 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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Chapter 8

Summary and future perspectives

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SUMMARY

In this dissertation, the triad immune system, colorectal cancer and immunotherapy was explored to understand how they interact, to develop immunotherapeutic approaches and to improve prognosis of colorectal cancer patients in the future.

Immune cell infiltration and HLA class I expression in colorectal tumors

Many factors present on tumor cells and in the cancer microenvironment influence the function of the immune cells and enable tumor cells to escape from immunity. One of these might consist of down-regulation of human leukocyte antigen (HLA) class I on tumor cells, thereby prohibiting presentation of tumor antigens to cytotoxic T lymphocytes (CTL), and keeping CTL from tumor cell lysis. There are strong indications that complete absence of HLA class I expression in colorectal tumors is particularly found in colon tumors with a high level of microsatellite instability (MSI-H) [1;2]. Unfortunately previous studies that evaluated the prognostic impact of HLA class I expression in colorectal cancer, used cohorts consisting of both colon and rectal cancer patients including both microsatellite stable (MSS) and MSI-H tumors [3;4]. These studies described a survival advantage for patients with HLA class I negative tumors, probably being the MSI-H tumors [3;4]. To study the prognostic impact of HLA class I loss in MSS tumors, we decided to determine HLA class I expression in a population of rectal cancer patients, as rectal tumors mainly consist of MSS tumors [5-7]. In **chapter 2** our results indicated that low expression of HLA class I in rectal tumors was associated with poor overall and disease free survival of rectal cancer patients [8]. Therefore these results indicated that rectal cancer cells down-regulate expression of HLA class I molecules to escape CTL mediated immunity. Our results in rectal tumors might be extrapolated to patients with a MSS colon tumor. The clinical impact of HLA class I expression remains to be established for patients with a MSI-H colon tumor.

It is generally accepted that of all immune cell markers, especially presence of the T-cell markers CD3 and CD8 is positively associated with prognosis of colorectal cancer patients [9]. Down-regulation of HLA class I surface molecules is generally thought to be a tumor immune escape mechanism aimed at evading CTL cell recognition and elimination [3;4;8]. Cytotoxic activity of Natural Killer (NK) cells is regulated by a balance of activating receptors and inhibitory receptors [10;11]. The most prominent inhibitory receptor in humans being: HLA class I. Consequently down-regulation of HLA class I potentially activates Natural Killer (NK) cells. Previously it has been shown that presence of $CD8^+$ lymphocytes in colorectal cancer cells correlated with absence of HLA class I [12]. Whether intratumoral $CD8^+$ cells represented CTL, NK or NK-T cells remained to be determined. Obviously, patients with tumors lacking HLA class I

expression would benefit most if these $CD8^+$ T-cells represented NK cells. In **chapter 3** we showed that NK cells form only a minor fraction of the total tumor-infiltrating leukocyte population in all colorectal tumors, using CD56 to detect NK cells [13]. A potential pitfall is formed by the expression of CD56. Two subpopulations of NK cells exist i.e.: $CD56^{dim}$ NK cells appear to be primarily cytotoxic effector cells while $CD56^{bright}$ NK cells have predominately regulatory functions [14]. A possible explanation for the low number of NK cells might be that immunohistochemical techniques are not capable to detect $CD56^{dim}$ cells. Therefore, a four-color-immunofluorescence staining technique was applied [15], demonstrating that tumors showing loss of MHC class I expression were more vigorously infiltrated by $CD3^+CD8^+$Granzyme B^+ positive T-cells, confirming that tumors are poorly infiltrated with NK cells. A possible explanation for the lack of intratumoral NK cells might be that the main function of NK cells is on a systemic level, where they may be able to eliminate metastasized malignant cells [16;17].

Migration of leukocytes into the cancer microenvironment

Effective anti-tumor immunity requires contact between cells of the immune system and tumor cells. Immune effector cells that developed in lymphoid organs and entered the circulation have to leave the vasculature and enter the cancer microenvironment. Homing of activated effector T-cells into the tumor consists of different steps. At the site of the tumor, endothelial cells are activated to express ligands for leukocyte adhesion. Once leucocytes attach to these ligands they have to pass the endothelium and enter the extravascular cancer microenvironment. From here, depending on their function, they have to migrate into the nests of tumor cells. The mechanisms governing homing of effector cells into tumors remain poorly understood, but this whole process is affected and coordinated by cytokines. One group of cytokines influencing the migration of leukocytes comprises of chemokines. In **chapter 4** we showed, using a rat tumor model that low expression of the chemokine CXCL5 in tumor cells resulted in rapid tumor growth and increase in the number of metastases, while *in vitro* no difference was found in proliferation rate between clones with either high or low expression of CXCL5 [18]. The relevance of these results for humans was confirmed, as low expression of CXCL5 in cancer cells was significantly associated with poor prognosis in a population of colorectal cancer patients. Finally a positive correlation between expression of CXCL5 and presence of intra-tumoral $CD8^+$ T-cell infiltration in humans was found. These results indicated that expression of CXCL5 is associated with intraepithelial infiltration of specific leukocyte subtypes, resulting in tumor regression, tumor specific immunity and better prognosis [18]. This concept has also been described for other chemokines in various types of tumors [19-27]. Together, these data argue that tumor cells themselves play a

key role in shaping the tumor-immune microenvironment and thereby clinical course of patients [28]. To finally influence the type of immune cells trafficking towards tumor cells it is important to determine the correlation between colorectal cancer phenotype and type of immune cell infiltrate in the cancer microenvironment.

Colorectal cancer vaccines

One of the most unique features of the immune system consists of its capacity to specifically search and destroy targets. As such, many have discussed if tumor cells represent one of the regular targets of the immune system and in addition if the patient's own immune system can be used to specifically destroy tumor cells once tumor cells escaped immune surveillance [29]. Subsequently, many have tried to reinforce the immune system to cure cancer patients, using different approaches. Here we focused on induction of tumor specific T-cells against predefined antigens. Distinction should be made between MSI-H and MSS tumors for immunotherapeutic purposes, as MSI-H colon tumors express neo-antigens "foreign" to the immune system while immunotherapy against MSS colorectal tumors depends on tumor associated "self"-antigens.

MSI-H tumors: frameshift mutated products, a unique class of tumor-specific antigens

Despite many years of work, the number of antigens recognized by TILs of colorectal cancer identified is limited [30-33]. Consequently, vaccines so far have been developed on the basis of proteins that are selectively expressed by tumor cells but for which immunity can be blunted or may lead to autoimmunity [34;35]. The exception comprises MSI-H tumors that, due to numerous of frameshift mutations in microsatellites express neo-antigens. MSI-H is a molecular feature of tumors associated with the familial Lynch or hereditary non-polyposis colorectal cancer (HNPCC) syndrome, accounting for approximately 5% of all colorectal cancer cases and for approximately 15% of all sporadic colorectal cancers [36-45]. Since frameshift mutated protein products (FSPs) are foreign to the immune system, they represent a unique group of tumor-specific antigens. No tolerance and consequently strong T-cell responses are expected against these FSPs. A few studies have been performed to predict the immunogenic behavior of a selection of frameshift mutated genes which are frequently detected in MSI-H cancers [33;46;47]. Unfortunately, relatively little is known on the immunogenic behavior of most of the FSPs [33]. Therefore we developed a methodology, described in **chapter 5** for predicting their immunogenic behavior that is based on accumulation and MHC class I presentation [46]. Our data indicated that, out of the 15 FSPs examined in our study, 4 (TGFβR2-1, MARCKS-1, MARCKS-2 and CDX2-2) are of primary interest [46]. Four additional antigens (TAF1B-1, PCNXL2-2,

TCF7L2-2 and Baxα+1) are of moderate interest for further tumor immunological research [46]. The data of others suggested that FSP-specific T-cells may be present in the circulation of patients with MSI-H colorectal cancer, healthy HNPCC syndrome mutation carriers, but not in patients with microsatellite stable (MSS) colorectal cancer or in healthy donors [47;48]. In general, most FSPs consist of a relatively small number of amino acids downstream of the frameshift mutation, suggesting that the FSPs may contain a sequence that can only be presented by a limited number of HLA class I or HLA class II molecules. In order to treat patients, knowledge on which HLA class I and II molecules can present epitopes comprised by the FSPs should be obtained. Although MSI-H tumors comprise only about 15% of all colorectal tumors, patients with a MSI-H tumor are very interesting vaccination candidates because: 1) strong effector responses are expected after vaccination using non-self-antigens; 2) colorectal cancer is one of the major cancers in the western world; and 3) many families with Lynch or HNPCC syndrome at risk for a MSI-H tumor have been identified. The latter group may be amenable for prophylactic vaccination to prevent the outgrowth of MSI-H tumors. Hence, a rapid identification of the immunogenic non-self-segment of the frameshift products is required.

MSS tumors: p53 vaccination in colorectal cancer patients

In **chapter 6** the safety and immunogenicity of a p53 synthetic long peptides (p53-SLP®) vaccine were investigated in patients treated for metastatic colorectal cancer [49]. The vaccine proved to be safe and highly immunogenic. However, mainly p53-specific $CD4^+$ T cells were induced after vaccination. Since the p53-specific $CD8^+$ T-cell, but not the $CD4^+$ T-cell repertoire is known to be severely restricted by self-tolerance and might only consist of lower affinity p53-specific $CD8^+$ T cells, these results confirmed previous studies [34;50]. The presence of tumor-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 effector cells [51-54]. Unfortunately, the overall production of pro-inflammatory cytokines such as IFNγ by the p53-SLP® vaccine-induced T-cell population in our trial was low. Therefore a new study was designed (**chapter 7**) to modulate the induced p53-specific $CD4^+$ T-cells by combining the p53-SLP® vaccine with Interferon-alpha (IFNα). This study clearly illustrated that addition of an adjuvant such as IFN-α injection to the vaccine safely modified both the vaccine-induced p53-specific humoral and T-cell responses. Addition of IFN-α to the p53-SLP® vaccine significantly improved p53-immune response against a broader range of peptide pools and also induced a larger number of vaccine-specific IFN-γ^+ T-cells. These results confirmed that IFN-α is able to modulate a vaccine-induced Th1 response.

FUTURE PERSPECTIVES

Altogether this dissertation reports on the relation between the immune system, colorectal cancer and immunotherapy. This knowledge can be used to further optimize immunotherapeutic strategies to treat cancer patients. For colorectal cancer only a few trials focused on clinical efficacy, this comprised phase III trials using irradiated tumor samples [55;56]. These trials suggested some clinical benefit in selected subpopulations but overall results were rather disappointing [55;56]. Most of the vaccination trials for colorectal cancer patients have been designed to test safety and immunogenicity but have yet not resulted in the design and execution of phase III trials [57]. Although in most trials no serious vaccine related adverse events were noted, lack of clinical results suggests that the vaccine-induced T-cell responses against these antigens are at this point not robust enough or of sufficient quality to confidently progress to efficacy trials. The most recent vaccine developments suggest that some of the current cancer vaccine strategies do harbor the capacity to induce strong immune responses in cancer patients even to self-antigens [49;58-64]. While these vaccines may still have to be optimized, the data suggest that the vaccine-induced activation of tumor-specific T-cell reactivity is no longer an issue of concern. However, other relevant questions remain:

- What are the tumor antigens recognized by tumor-infiltrating T-cells, and which antigens would be most appropriate in colorectal cancer?
- Which immune cells are to be induced during vaccination and does vaccination only enhance effector T-cells or also suppressive T-cells?
- Which adjuvants should be combined with vaccines to optimize the induced vaccine response?
- Do vaccine-induced tumor-specific leukocytes migrate to the tumor and mediate an antitumor effect?
- Which cancer patients are most likely to benefit from immunotherapy?

What are the tumor antigens recognized by tumor-infiltrating T-cells, and which antigens would be most appropriate in colorectal cancer?

New vaccine strategies have resulted in vaccines that are able to efficiently induce vaccine specific immune responses. However, vaccine strategies in colorectal cancer still suffer from a lack of antigens that may be used for vaccination. Whereas for other types of tumors the reactivity of tumor-infiltrating T-cells validate the choice of antigen used in the vaccines for that type of cancer [65], this is still limited in colorectal cancer and calls for more in-depth studies on the specificity of T-cells infiltrating the tumor or present in metastatic lymph nodes. In view of the increasing knowledge on

the role of role of CD4+ T-cell help to the induction, sustainment and migration of CD8+ T-cells, it is advisable to screen not only for tumor-specific CD8+ T-cell responses but also for tumor-specific CD4+ T cells.

Which immune cells are to be induced during vaccination and does vaccination only enhance effector T-cells or also suppressive T-cells?

The history of constant interactions between tumor and immune system shapes both tumor and the immune system of an individual patient in a way that is difficult to mimic in animal tumor models. It is of utmost importance that vaccines only boost the reactivity of immune cells that mediate an antitumor effect and not that of immune cells that support tumor growth. As most tumor associated antigens are intracellular proteins and results from observational studies show that especially presence of intra-epithelial activated CD8+ T-cells has a positive impact on prognosis [9], immunotherapeutic strategies start by inducing tumor-specific CD8+ T-cell responses. The activation of cytotoxic T-cells depends on a network of collaborating leukocytes. Consequently vaccines should create a CD8+ T-cell friendly and supportive cancer microenvironment. Indeed data from different studies indicate that especially a Th1 associated type of cancer microenvironment is beneficial to the prognosis of cancer patients [66-68].

From immunohistochemical studies it is clear that colorectal cancers are amongst others infiltrated by both CD4+ and CD8+ Foxp3+ T-cells [69;70]. The number of Foxp3+ Regulatory T-cells (Tregs) correlates with disease stage and survival in colorectal cancer in several studies [70-72]. Notably, the analyses of the antigens recognized by colorectal cancer infiltrating Tregs revealed that they recognized colorectal cancer-associated antigens, in particular Mucin, Her-2/neu, and CEA [31]. Hence, therapeutic vaccination with these antigens may not only boost CD4+ and CD8+ effector T-cells but also the Treg population. Vaccine-induced expansion of such antigen-specific Tregs has been observed previously in a mouse tumor model [73] and also in humans [58]. In the p53-SLP® vaccination trial, strong p53-specific CD4+ T-cell responses were found but this did not coincide with the expansion of p53-specific CD4+Foxp3+ T-cells [49]. This fits with the observation that the T-cell response to p53 in colorectal cancer patients is not under control of Tregs [31].

Which adjuvants should be combined with vaccines to optimize the induced vaccine response?

It is not likely that colorectal cancer vaccines are able to induce the desired clinical responses on their own, but need to be combined with other modalities that target regulatory mechanisms in order to improve the local microenvironment. The current wealth of preclinical and clinical information predicts a future strategy in which

therapeutic vaccines, blockers of immunosuppressive mechanisms and conventional therapies are applied jointly to overcome immunological tolerance and promote tumor regression. In general, a stronger focus should be put on how to induce the strongest and best qualified leukocyte population by vaccination. Vaccines should be combined with adjuvants to induce a vaccine specific type 1 polarized response and suppress a type 2 response. At the moment many candidate adjuvants are available. Also chemotherapeutics and monoclonal antibodies comprise strong immune modulating agents that can be used to polarize a response after vaccination. Various mechanisms may explain the reported synergistic effects of chemotherapy and T-cell restricted immunotherapy. Direct effects of chemotherapy on tumor or host environment, such as induction of tumor cell death, elimination of regulatory T cells, and/or enhancement of tumor cell sensitivity to lysis by CTL may account for enhancement of immunotherapy by chemotherapy. On the other hand, immunotherapy may directly modulate the tumor's sensitivity to chemotherapy [74]. Indeed, results have suggested that a vaccine encoding the tumor antigen 5T4 can be layered on top of chemotherapy regimens in patients with metastatic colorectal cancer without any evidence of enhanced toxicity or reduced immunological or therapeutic efficacy [75].

Monoclonal antibodies are designed to interfere with specific signaling pathways. Recently, the CTLA-4 blocking antibody Ipilimumab has been successfully used in the treatment of melanoma patients [76]. In human beings several approaches have been used to delete Tregs [77]. Notably, decreases in $CD4^+CD25^+Foxp3^+$ cells have been detected when patients with hepatocellular cancer were treated with low cyclophosphamide [78], as well as in metastatic melanoma patients treated with the anti-CD25 antibody Daclizumab [79], or after using denileukin diftitox [80].Based on their mechanisms of action it is highly likely that these antibodies will synergize with vaccines as they will block the negative feed-back on vaccine-induced tumor-specific T cells.

Do vaccine-induced tumor-specific leukocytes migrate to the tumor and mediate an antitumor effect?

We showed that expression of CXCL5 by tumor cells was positively related with both strong intra-epithelial infiltration of the tumor cell nests by $CD8^+$ T cells and a better clinical prognosis of colorectal cancer patients. Indeed chemokine expression as well as that of endothelial adhesion molecules and extracellular matrix has been associated with the migration of leukocytes into colorectal carcinoma [18;81-83]. This suggests that tumor cells themselves play a key role in shaping the tumor-immune microenvironment. The tumor phenotype, i.e. the status of tumor gene expression that attracts, activates or inhibits immune defense, determines the magnitude and type of immune infiltration and thereby clinical course of patients and represents a target for innovative diagnostic and therapeutic strategies. A profound understanding of

how the trafficking of these different cell populations is coordinated can be exploited for the development of successful immunotherapeutic strategies. One can start by comparing gene profiles of colorectal tumors with a high number of tumor infiltrating leukocytes versus those with a low number of tumor infiltrating leukocytes.

Optimization of vaccination studies may result in clinical success

To gain a thorough understanding of the immunological events occurring in patients in vaccination trials it is crucial to comprehensively perform immune monitoring during vaccination trials. Results from immune monitoring make it possible to understand possible clinical effects, to guide the optimization of vaccination strategies and may even encourage investigators to move a product forward into phase III trials [84]. Unfortunately, most immunotherapeutic vaccine trials mostly report on one particular aspect of the desired immune response (e.g. HLA-multimer+ cells, IFN-γ-producing cells). They do not include more detailed analyses of the total vaccine-modulated immune response [57]. Therefore implementation of assays that allows correlation of a broad array of immune cells with disease parameters is a prerequisite.

Although many studies determined the induced immune response after immunization, no gold standard has been set to define clinical response after vaccination [85]. Many different bioassays have been developed for immune monitoring: enzyme-linked immunosorbent spot (ELISA), carboxyfluorescein succinimidyl ester-based proliferative assays, HLA peptide multimer staining and flow cytometry-based tests. Unfortunately substantial variability in results among laboratories prohibits data reproducibility and prevents meaningful comparison among studies. Therefore initiatives have been put up to standardize immune monitoring and harmonize cellular immune assays. Harmonization will establish the use T-cell-based assays as a reproducible gold standard for immunotherapy and reliable parameter to determine the correlation between induced T-cell responses and clinical events [86-88].

Which cancer patients are most likely to benefit from immunotherapy?

An important question that remains is which cancer patients are best candidates to study clinical endpoints once safety and immunogenicity of a therapeutic vaccine strategy have been established. So far most trials have included end-stage patients only. Although regression of tumor mass can be very convincing and objectively measured, vaccination of end-stage patients may present with several drawbacks that negatively influence the immunotherapeutic effect. Major drawbacks are the suppressed immune status, the general short survival period that may obscure clinical effects of therapy at later time points [89], a large immunosuppressive tumor mass, variety of treatments before vaccination, and co-morbidity. Therefore clinical endpoints might be best studied in an adjuvant rather than an end-stage setting. These

patients, who have no measurable tumor mass and a relatively normal functioning immune system are expected to respond optimally to immunization.

FINAL

There is a clear role for tumor-specific T-cell immunity in the final clinical outcome of colorectal cancer patients. Immune escape variants of tumor cells indicate the selective force of the immune system. A continued effort will be put to exploit this force in the development of vaccines and vaccine strategies against colorectal cancer. Despite that some of the current vaccines are able to induce strong antigen-specific immune responses in the absence of serious adverse events, there is hardly any evidence generated to show the clinical impact of these vaccines in patients with colorectal cancer. It is not likely that colorectal cancer vaccines are able to induce the desired clinical responses on their own, but need to be combined with immune modulating modalities to redirect the force of the immune system into an effective anti-tumor response *in vivo*. Studies suggest that these modalities should primarily induce a type 1 polarized immune response and suppress a type 2 response. As chemotherapeutics are already used in the treatment of cancer patients, they should be the first tested for their immune modulating capacity. For current vaccination studies it is of utmost important to monitor and link the type of induced immune response after vaccination to clinical cancer effect, to know which immune are to be induced after vaccination. To obtain proof-of-concept, the immunotherapy of colorectal cancer may want to first concentrate on the treatment of tumors with microsatellite instability as they are known to be heavily infiltrated by T cells and express tumor-specific antigens that are derived from frameshift-mutated gene products.

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Chapter 9

Nederlandse samenvatting

List of Publications

Curriculum Vitae

Dankwoord

1.
2.
3.
4.
5.
6.
7.
8.
9.
10.
11.
12.
13.
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15.
16.
17.
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SAMENVATTING

Een van de meest unieke kenmerken van het afweersysteem bestaat uit het vermogen om specifieke doelen te herkennen en deze eventueel te vernietigen. Er is uitgebreide discussie of tumorcellen een doel vormen voor het afweersysteem en vervolgens of het afweersysteem gebruikt kan worden om tumorcellen te vernietigen. In dit proefschrift werd de interactie tussen het afweersysteem en dikkedarm- / rectumkanker onderzocht met als uiteindelijk doel T-cel gemedieerde immuuntherapie van colorectale tumoren verder te ontwikkelen.

In **hoofdstuk 2** werd de klinische impact van (verminderde) expressie van Humaan Leukocyten Antigeen (HLA) klasse I onderzocht voor patiënten behandeld aan rectumkanker. HLA klasse I moleculen presenteren antigenen aan cytotoxische T lymfocyten (CTL). Hierdoor kunnen CTL, cellen herkennen die een bedreiging vormen voor het lichaam om ze daarna eventueel vernietigen. Verminderde expressie van HLA klasse I op tumorcellen maakt het mogelijk dat tumorcellen kunnen ontsnappen aan de CTL. Eerdere studies toonden dat verminderde expressie, in het bijzonder volledige afschakeling van HLA klasse I moleculen in dikkedarm- en rectumkanker geassocieerd is met een betere overleving van de patiënt. Echter, het lijkt erop dat volledige afschakeling van HLA klasse I expressie in colorectale tumoren voornamelijk voorkomt in tumoren met een hoge graad van microsatellietinstabiliteit (MSI-H). Patiënten met een MSI-H tumor zouden een betere overleving hebben t.o.v. patiënten met een microsatellietstabiele (MSS) tumor. In eerdere studies naar het prognostische effect van HLA klasse I afschakeling, werden patiëntcohorten gebruikt welke bestonden uit patiënten geopereerd aan dikkedarm- of rectumkanker zonder onderscheid te maken tussen MSS en MSI-H tumoren. Omdat in deze studies niet gecorrigeerd werd voor de prognostische impact van microsatelliet-instabiliteit, kunnen de statistische berekeningen dus tot een onzuivere schatting van het overlevingsvoordeel van patiënten met HLA klasse I afgeschakelde tumoren hebben geleidt. Zoals in hoofdstuk 2 wordt aangetoond, bestaan rectumtumoren bijna geheel uit MSS tumoren. Onze resultaten tonen dat afschakeling van HLA klasse I expressie in rectumtumoren is geassocieerd met onafhankelijk slechtere overleving van rectumkankerpatiënten. Volledige afschakeling van HLA klasse I werd nauwelijks teruggevonden in rectumtumoren. Deze resultaten suggereren dat afschakeling van HLA klasse I een manier is voor tumorcellen om aan CTL-gemedieerde immuniteit te ontsnappen.

In **hoofdstuk 3** werd de infiltratie van natural-killer (NK) cellen in dikkedarmtumoren bestudeerd. De HLA klasse I negatieve tumorcellen kunnen een doelwit voor NK cellen vormen omdat NK cellen in staat zijn om cellen met verminderde

HLA klasse I te elimineren. Onze studie toonde aan dat er relatief weinig NK cellen dikkedarmtumoren infiltreerden. Dit gold ook voor tumoren zonder HLA klasse I expressie. NK cellen lijken dus geen rol te spelen in een tumor, maar hypothetisch kunnen zij wel een rol spelen in het bestrijden van systemische uitzaaiingen van HLA klasse I negatieve tumorcellen.

Effectieve antitumor T cel immuniteit vereist niet alleen presentatie van antigenen aan CTL, een andere voorwaarde is dat T cellen naar de tumor migreren en contact maken met de tumorcellen. Migratie van leukocyten naar en in de tumor is een gecompliceerd en slecht begrepen proces waarin chemokines een sleutelrol vervullen. In **hoofdstuk 4** werd gevonden dat lage expressie van een specifiek chemokine: CXCL5, in een rat-tumormodel, resulteerde in snelle tumorgroei en toename van het aantal van metastasen, terwijl *in vitro* geen verschil in o.a. delingssnelheid werd gevonden tussen klonen met hoge of lage expressie van CXCL5. Hoge expressie van CXCL5 bleek in twee verschillende cohorten van patiënten met colorectale kanker geassocieerd te zijn met een betere prognose. Daarnaast werd een positieve correlatie gevonden tussen expressie van CXCL5 en het aantal in de tumor gelokaliseerde $CD8^+$ T cellen. Deze gegevens pleiten ervoor dat tumorcellen zelf een belangrijke rol kunnen spelen in de migratie van immuuncellen en op deze wijze de antitumorimmuunreactie beïnvloeden.

In het laatste deel van het proefschrift wordt de versterking van de tumorspecifieke T cel reactie m.b.v. vaccinatie tegen vooraf gedefinieerde antigenen beschreven. Voor colorectale tumoren moet onderscheid worden gemaakt tussen MSI-H- en MSS tumoren voor immuuntherapeutische doeleinden. MSI-H tumoren hebben als unieke eigenschap dat zij vele nieuwe antigenen tot expressie brengen die als lichaamsvreemd door het immuunsysteem kunnen worden gezien. De afweerreactie tegen MSS tumoren berust veel meer op het herkennen van lichaamseigen antigenen waarvoor het afweersysteem mogelijk tolerant is. Tot nu toe is T cel-gemedieerde immuuntherapie geen standaardbehandeling voor colorectale kankerpatiënten. Slechts in enkele subanalyses van studies zijn er aanwijzingen dat immuuntherapeutische strategieën een klinische impact kunnen hebben voor colorectale kankerpatiënten. Notabene de meeste van deze studies zijn uitgevoerd als fase I en/of fase II. Deze studies tonen wel aan dat vaccins goed in staat zijn een tegen het vaccin gerichte afweerreactie te kunnen induceren.

In **hoofdstuk 5** werd een methode beschreven om het afweeractiverende vermogen van frameshiftgemuteerde eiwitten, zoals die voorkomen in MSI-H tumoren, te voorspellen. MSI-H tumoren brengen, door mutaties in frameshifts, unieke tumorspecifieke antigenen tot expressie die als lichaamsvreemd door het immuunsysteem worden aangemerkt. Helaas is relatief weinig bekend over de capaciteit om een

afweerreactie op te wekken voor de meeste van de frameshift gemuteerde eiwitten. Wij ontwikkelden daarom een selectiemethode voor de identificatie van frameshift gemuteerde eiwitten die van belang kunnen zijn voor immuuntherapeutische doeleinden. Deze methode is gebaseerd op accumulatie van eiwitten en Major Histocompatibility Complex (MHC) klasse I presentatie. Onze gegevens tonen dat 8 van de 15 frameshiftgemuteerde eiwitten onderzocht in onze studie, verder bestudeerd moeten worden. In het bijzonder 4 antigenen die zeer sterk tot expressie komen en goed gepresenteerd worden in MHC klasse I, lijken zeer relevant te zijn voor immuuntherapie van kanker. Hoewel MSI-H tumoren slechts ongeveer 15% van alle colorectale tumoren omvatten, zijn patiënten met een MSI-H tumor zeer interessante vaccinatiekandidaten omdat: 1) een sterke effectieve afweerreactie wordt verwacht na vaccinatie met lichaamsvreemde antigenen; 2) colorectale kanker één van de meest voorkomende kankersoorten in de westerse wereld is; en 3) vele families met genetische belasting voor het Lynchsyndroom, die bijna allemaal MSI-H tumoren krijgen, zijn geïdentificeerd. De laatstgenoemde groep zou profylactisch gevaccineerd kunnen worden om uitgroei van MSI-H tumoren te voorkomen.

In **hoofdstuk 6** werd van een p53 synthetische lange peptide (p53-SLP®) vaccin de veiligheid en de capaciteit om een afweerreactie op te roepen onderzocht bij patiënten behandeld aan gemetastaseerd colorectaal kanker. Het vaccin bleek veilig en zeer immunogeen te zijn. Voornamelijk p53-specifieke $CD4^+$ T cellen werden geactiveerd m.b.v. het vaccin. Het ontbreken van p53-specifieke $CD8^+$ T cellen, lijkt resultaten van eerdere studies te bevestigen die suggereren dat het p53-specifieke $CD8^+$ T cel repertoire ernstig verstoord is door tolerantie van het afweersysteem voor lichaamseigen eiwitten. IFN-γ producerende $CD4^+$ Th1 cellen spelen een belangrijke rol in het coördineren en ondersteunen van de lokale afweerreactie door $CD8^+$ CTL en andere immuuncellen. Helaas blijkt de totale productie van cytokinen zoals IFN-γ door de p53-SLP® vaccin geactiveerde T cel populatie in onze studie erg klein te zijn. In **hoofdstuk 7** werd daarom bestudeerd of het combineren van p53-SLP® vaccin met Interferon-alfa (IFN-α) injecties tot een verbetering van de p53-specifieke IFN-γ productie kon leiden. Deze studie illustreert duidelijk dat toevoeging van een adjuvans zoals IFN-α injectie aan een vaccin veilig is. Tevens bleek dat toevoeging van IFN-α aan het p53-SLP® vaccin de IFN-γ geassocieerde p53-specifieke T cel reactie verbeterde.

TOT BESLUIT

Er is een duidelijke (toekomstige) rol voor tumorspecifieke T cel therapie in de behandeling van colorectale kankerpatiënten. Dit proefschrift doet verslag over de

relatie tussen het immuunsysteem en colorectale kanker op basis van zowel observationele - als interventiestudies. Het is niet waarschijnlijk dat alleen het gebruik van vaccins voor de behandeling van colorectale kanker tot het gewenste klinische effect zullen leiden. Meer waarschijnlijk is dat zij gecombineerd zullen moeten worden met immuunmodulerende modaliteiten om een effectieve antitumorreactie *in vivo* te genereren. Belangrijk hierbij zal zijn om de resultaten van observationele studies te gebruiken voor het ontwerpen van nieuwe interventiestudies met uiteindelijk doel de kracht van het immuunsysteem te controleren en in te zetten tegen colorectale tumorcellen.

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CURRICULUM VITAE

The author of this thesis was born on October 3, 1976 in The Hague, the Netherlands. After graduating Athenaeum high school at the Aloysius College in The Hague, he started to study Biomedical Sciences at the Leiden University in 1995. He performed research for his master's thesis at the departments of Molecular Biology and Immunology at University North Texas Health Science Center, Fort Worth, Texas, USA and at department of surgical oncology at the Leiden University Medical Center, under supervision of respectively Prof. dr. R.H. Goldfarb and dr. P.J.K. Kuppen and graduated in 2002. From 1998 he started medical school at the same university. After receiving his medical degree in 2003, he worked as a surgical resident at the Diaconessenhuis, Leiden. In 2005 he started his PhD research project that resulted in the current thesis at the department of surgical oncology at the Leiden University Medical Center, under supervision of prof. dr. C.J.H. van de Velde, dr. P.J.K. Kuppen and prof. dr. S.H. van der Burg. In 2008 he obtained a grant from The Netherlands' Organization for Health Research and Development (NWO-AGIKO stipendium), enabling him to combine his research activities with his residency training in internal medicine at Rijnland Hospital, Leiderdorp (Dr. M.J. Janssen) and Leiden University Medical Center (prof. dr. J.T. van Dissel). The author of this thesis is living together with Irene C. Notting and has two children: Friso and Loek.

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