Dendritic cell vaccination of patients with metastatic colorectal cancer

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THE 3 ORIGINAL PAPERS ARE

1. Burgdorf SK, Fischer A, Claesson MH, Kirkin AF, Dzhandzhugazyan KN, Rosenberg J. Vaccination with melanoma lysate-pulsed dendritic cells, of patients with advanced colorectal carcinoma: report from a phase I study. J Exp Clin Cancer Res 2006; 25:201-6.

2. Burgdorf SK, Fischer A, Myschetzky PS, Munksgaard SB, Zocca MB, Claesson MH, Rosenberg J. Clinical responses in patients with advanced colorectal cancer to a dendritic cell based vaccine. Oncol Rep 2008; 20:1305-11.

3. Burgdorf SK, Claesson MH, Nielsen HJ, Rosenberg J. Changes in cytokine and biomarker blood levels in patients with colorectal cancer during dendritic cell-based vaccination. Acta Oncol 2009; 48:1157-64.

INTRODUCTION

Colorectal cancer

Colorectal cancer (CRC) is one of the leading causes of death related to cancer in the Western World [1]. The incidence of new cases of colorectal cancer per year in Denmark alone is more than 4.300 [2]. This is the second highest incidence of cancer in Denmark, in men only exceeded by prostate cancer, and in women only exceeded by breast cancer [2]. Many of the patients with colorectal cancer will develop advanced disease, primarily with metastases in liver- and lungs, and the median survival time for these patients is between six and nine months without treatment. In Denmark patients with stage III and IV disease are offered systemic chemotherapy. Addition of bevacizumab to the standard first-line regimens FOLFOX (5-FU/LV with oxaliplatin) and FOLFIRI (5-FU/LV with irinotecan) for metastatic CRC has resulted in a life-prolonging effect with a median survival around 20 months [3]. For many years, the 3 major treatment modalities, surgery, radiotherapy and chemotherapy have been the only standardized treatment options and surgery has been considered, and is still considered, the only curative treatment. Radiotherapy has especially been used in rectal cancer and is effective in down staging the tumor and making it eligible for curative surgery. Chemotherapy has evolved during the last years and apart from 5-FU there are now more active chemotherapy agents available and newer approaches like regional chemotherapy for liver metastases have been tested, but the prognosis for advanced colorectal cancer is still poor [3]. Advanced disease without possibility for radical surgery is considered fatal and second and third line chemotherapy or the newer biological therapies have not produced high response rates and have not shown impressive survival benefits. Many of the potentially life prolonging oncological treatment modalities are in many cases complicated by adverse effects and poor quality of life [3-7]. Development of new and even more effective and less toxic treatment options are therefore of major importance, especially for the patients with cancers that are resistant to conventional chemotherapeutics.

Immunotherapy

The theory of immune surveillance was first postulated by Burnet and Thomas in the 1950s [8-10]. Immune surveillance in cancer is the ability of the immune system to detect transformed cells and eradicate these via immunologically processes. The hypothesis was that T-cells recognised changes on the surface of the transformed cells. This theory was, partly because studies in the 1970s showed that athymic mice did not evolve cancer [11,12], controversial, but widely accepted after studies in the 1990s showed that IFN-γ and IFN-γ receptor knockout mice evolved more tumors than wildtype mice [13,14] and that patients in immunosuppressive therapy presented with higher incidences of tumors [15,16]. Finally and most important, the discovery of TA and the finding of immune responses against tumors in patients with cancer have confirmed the theory of immune surveillance [9].

Immunotherapy is often divided into three distinctive subgroups: non-specific, passive, and active specific immunotherapy [17] (Figure 1). Initially immunotherapy was non-specific but as the knowledge about immune-competences improved and especially with the discovery of TA, immunotherapy has evolved to a more specific and active treatment. The DC was discovered in 1973 by Steinman and Cohn [18]. This discovery has resulted in the ability to manipulate immune responses and direct them in certain manners. DCs can be cultured and primed in vitro and pulsed with antigens specific for the patient's tumor.

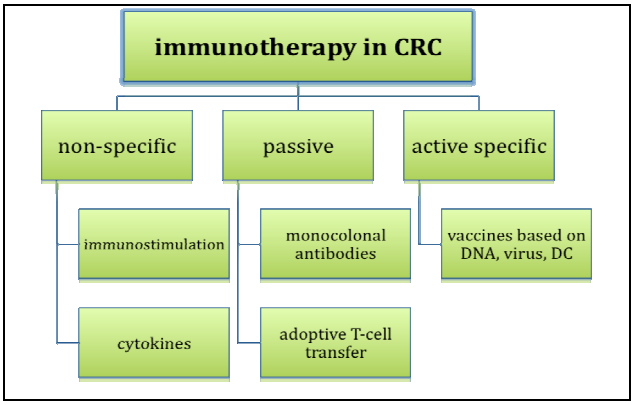

Figure 1
Immunotherapy in colorectal cancer can be divided into the following subgroups: non-specific, passive and active specific.
CRC = colorectal cancer, DC = dendritic cell

Non specific immunotherapy
Agents such as cytokines and interferons can stimulate parts of the immune competences. Various cells of the immune systems like macrophages and NK cells may also be stimulated by a variety of agents. William Coley's toxin, which had an immune stimulatory effect via activation of TLR 4, was especially used in the treatment of sarcoma. Treatment with Coley's vaccine resulted in a cure rate of more than 10% [19]. The most studied non-specific immune stimulant is probably BCG, which in a non-specific way activates parts of the immune system. The immune stimulatory effect of BCG does also happen via TLR 4 [20]. BCG is still being used as a standard treatment for certain bladder cancers [21,22]. In many cases the non-specific treatment of cancer was associated with intolerable toxicity and limited efficacy. As a consequence, research has been carried out intending to explore the mechanisms for bacterial infections leading to immune stimulation. Numerous studies have investigated the effects of bacterial components or genetically modified bacteria [23-25]. In colon cancer the studies with BCG alone or in combination with chemotherapy have shown limited clinical effects, and some of the studies have even shown deleterious effects [25].

Cytokines
Research in this field has shown that cytokines can either enhance or inhibit tumor growth [26-28]. Interferons have shown antineoplastic activity indirectly by changes in the immune response (activates macrophages and up regulates MHC molecules), but also in direct action on the tumor (promotion of CTL lysis) [24]. A variety of tumors express immunosuppressive cytokines, e.g. TGF-β and IL-10, which may help the cancer cells escape immune surveillance [28]. The cytokines have mainly been tested in animal studies, but there are also some human clinical trials, mainly with IFNs, TNFs, IL-2, IL-3, IL-4, IL-6, IL-12 and GM-CSF. In most of these cases the side effects were unacceptable and the clinical efficiency very limited [24,25,29-34]. Treatment with high or moderate doses IL-2 has shown clinical responses in patients with malignant melanoma and renal cell carcinoma [35-37]. The objective clinical response rates were 15% for melanomas and 19% for renal cell carcinoma [30] with an estimated 5-year survival rate of around 16% of patients with metastatic renal cell carcinoma [35]. Studies with IL-2 in patients with CRC have not yet shown useful clinical responses [24,33]. Cytokine inhibition such as TNF-α therapy remains a potential therapeutic option in CRC as recently demonstrated in a mouse model [34].

The anticipated effects of non-specific immunotherapy are still unknown, and there are still concerns of adverse effects. It is not expected that this therapy will advance to standard treatment in CRC as single therapy, but maybe in combination with other treatment modalities. Efficiency in patients with CRC has been shown in combination studies with IL-2 and 5-FU [38-40]. It was shown in a small pilot study that addition of IL-2 in low doses subcutaneously and standard 5-FU improved time to progression with 7.5 months compared to a historical control group treated with a similar 5-FU regimen [40].

Passive immunotherapy
Monoclonal antibodies
Extensive research has resulted in new therapeutic modalities for metastatic CRC. Bevacizumab (Avastin®) and Cetuximab (Erbitux®) were in 2004 approved for treatment of CRC in both Europe and the United States. Panitumumab (Vertibix®) has later also been approved for treatment of CRC [27]. Cetuximab and Panitumumab block the EGFR, and Bevacizumab is an antibody to VEGF. VEGF stimulate cellular responses by binding to VEGF receptors resulting in increased migration and mitosis of endothelial cells leading to angiogenesis. Theoretically angiogenesis plays an important role in all cancers since tumor growth is dependent on establishment of new blood vessels to secure nutrition and oxygen supplies [26,27,41-44]. Trastuzumab (Herceptin®) which has been approved for treatment of breast cancer has due to low overexpression rate of Human Epidermal growth factor Receptor-2 (HER-2/neu) in advanced CRC not shown valuable in the treatment of CRC [45]. Inhibition of the binding to these receptors may lead to diminished tumor growth or even shrinkage of established tumors.

All of these approved monoclonal antibodies for the treatment of CRC have been used in combination with chemotherapy, and have resulted in improved time to progression by 3 to 4 months and overall survival by 4 to 6 months [27]. There is still ongoing research trying to identify responding patients and clarifying reasons for non-responding patients. An intrinsic and a required resistance to anti-angiogenic drugs have been described [46], and in addition such treatment modalities may also lead to serious side-effects. Toxicity to the VEGF inhibitor Avastin increases the risk of arterial thromboembolic events, increases the risk of bleeding, carries a small risk of gastrointestinal perforation, and approximately 10% of the patients experienced hypertension grade 3 – 4 [47]. The side effects from the antibodies against EGFR are acne-like rashes in more than 80% of the patients, nausea, diarrhoea and hypomagnesaemia [27,48].

An ongoing concern regarding monoclonal antibodies is the excessive rise in the costs of treating patients with metastatic CRC. Cost-effectiveness analyses have questioned the recommendation of such antibodies [27]. Prediction of responders and non-responders, and competition on the market may limit the future costs to a reasonable level.

Adoptive T-cell transfer
Transfusion of allogeneic or autologous lymphocytes, preferentially T-cells, to patients is called adoptive T-cell therapy. Preliminary results from murine and some early human studies have been promising, but currently there are no FDA-approved therapies for cancer [49]. Trials have included infusion of tumor infiltrating lymphocytes, cytotoxic T-lymphocytes, T-helper cells, T-regulatory cells and genetically modified T-cells. Adoptive T-cell

therapy is assumed to enhance anti tumor immunity to malignant transformed cells [49-51]. In patients with metastatic melanoma adoptive cell therapy with autologous tumor-infiltrating lymphocytes have shown objective response rates of approximately 50% [52,53]. In spite of promising results from preliminary murine CRC studies, the effects in human studies have been limited and with major variations in achieved results from study to study. Hitherto, results from clinical trials with adoptive T-cell transfer in human CRC have not been published.

Some of the technical difficulties in producing tumor specific T-cells are still concerns. Apart from the highly specialized laboratory facilities, consuming of time, money, and labour required for the production are still obstacles that need to be dealt with before implementation can take place [49,54]. Toxicity, which especially has been a problem with the genetically engineered T-cells is also a difficulty that has to be solved [49]. Whether adoptive T-cell transfer in the future will play a part in treatment of cancer is still unclear, but it may get a role in combination with other treatment modalities.

In patients with leukaemia there has been extensive research in the use of immunotherapeutic modalities. When patients have received allogeneic stem cell transplantation it is evident that a critical step is transfer of donor immunity to the treated patient. Adoptive T cell therapy after stem cell transplantation in patients with leukaemia has turned out to be very effective with response rates of up to 70-80% [55].

With immunotherapy there is a constant risk of inducing autoimmunity. Cancer immunotherapy is preferentially aimed to target tumor cells alone without targeting healthy cells that is without inducing autoimmunity. There is a fine balance between immunotherapeutic tumor rejection and the harmful and unwanted self-directed immunological activities [56]. Since cancer cells arise from self-cells these express many of the same antigens on the cell surface, even though specific or upregulated antigens are expressed on cancer cells (see tumor antigens below). Different immune regulators and checkpoints in the induction of immune responses have been described and are thought to be important in the prevention of autoimmune responses. It seems clear though, that some of these checkpoints need to be blocked (e.g. anti-CTLA-4) in order to generate a sufficient immunologic response that may lead to cure of cancer [56].

Active specific immunotherapy

The aim of active specific immunotherapy is to establish a highly selective and potent cellular immune response, specifically directed against the patient's cancer cells. Thus, responses are based on the cellular components of the immune system. The T-cells are the most prominent effector cells and are those that need to be activated and directed against cancer cells. Tumor specific Th-cells seem to be most important especially in generation of CTL activity. The specificity of the T-cells are highly dependent on specific TAAs. The activity of the T-cells is dependent on the APCs, which present TAA derived peptides of both HLA class I and class II molecules resulting in Th/CTL activation, co-stimulatory molecules, and the local cytokine environment. The T-cells are activated in the T-cell zone of the lymph nodes. After activation they leave the lymph node via the efferent lymphatic vessel and carry out the immunological effects (Figure 2). Of great importance in cancer immunotherapy is the activation of tumor specific CD8+ CTLs, which are the active cells in Th1 dominant

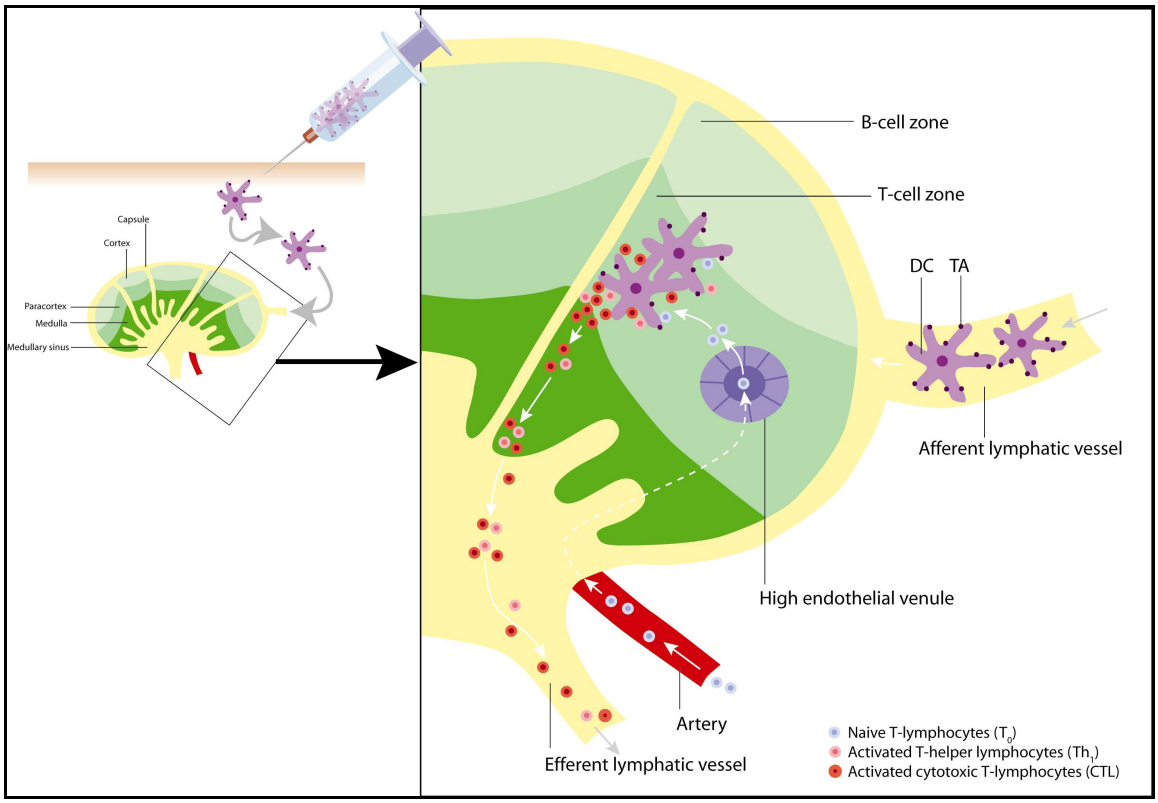

Figure 2

Immunological mechanisms in the lymph node after intradermal injection of DC's loaded with TA. The antigen presenting DC migrates to the draining lymph nodes, where it on MHC-molecules presents TA for T-lymphocytes, which in the presence of co-stimmulation is activated. The activated T-cell leaves the lymph nodes to carry out its immunological effects.

DC = dendritic cells, TA = tumor-associated antigens.

responses [57]. Immunological responses are balanced between Th1 and Th2 responses (see below), both in relation to cellular and humoral responses [57-64]. Polarisation of T-cells requires three distinctive signals. The first signal is the interaction between the T-cell receptor and the antigen presented in combination with MHC molecules by APCs. The second signal is interaction via co-stimulatory molecules between the T-cell and the APC (CD28-CD80/86). The third and final signal is the one directing the polarisation of T-cells, including the secretion of cytokines [61]. The T-cells are polarised in a Th1 response by primarily IFN-γ, TNF-α, IL-2, and IL-12 whereas the cytokines IL-4, IL-5, IL-6 and IL-10 directs the Th2 response [61].

In 2006 the clinical and immunological responses to active specific cancer vaccines in human colorectal cancer were reported in a meta-analysis [17], which was based on 527 patients in 32 trials. The rate of clinical responses (complete and partial responses, graded after WHO criteria) was limited to 0.9%. The rate of immunologic responses was also evaluated in some of the studies. Of the evaluated patients 59% showed antibody responses against the vaccine or tumor, and 44% showed cellular responses, primarily assessed by lymphoproliferation and ELISPOT analyses. Since the immunologic responses are results of different tests, these have to be interpreted with caution. When comparing the rate of clinical responses with the rate of immunologic responses it seems clear that many patients achieve immunologic responses without influence on the clinical response. Further investigation is needed to explain the differences, and if possible to direct immunologic responses into clinical responses.

Active specific immunotherapy appears to be less toxic than radio- and chemotherapy, which also affect proliferating normal cells especially in the bone marrow and intestine. This fact in combination with promising preliminary results in murine and in some human trials encourages further investigation and possible optimization of the active specific immunotherapy.

Tumor antigens

A variety of TAs and TAAs have been identified and the list is still expanding [65]. The antigens are traditionally divided into four distinctive categories: 1) up regulated antigens which are antigens that are present in normal cells, but which are up regulated in cancer cells. Examples of these are CEA, telomerase and survivin. 2) Tissue specific antigens, which are present in the tissue from which the tumor is derived and in the tumor itself. Examples are melanocyte derived GP-100 and MART-1. 3) Tumor specific antigens, which are unique for the specific tumor, as a result of mutations in the tumor cell DNA. Examples are p53 and RAS. 4) Cancer testis antigens (CT-antigens), which are expressed on many different cancer cells due to their dedifferentiation, but in the adult only expressed by germinal cells in testis and ovary, where cells are protected from cellular immune responses by blood barriers. Examples of CT-antigens are the melanoma associated antigens named MAGE-A, -B and -C groups, GAGE-groups, BAGE-groups and others [66].

The optimal tumor antigen for immunotherapeutic targeting is an antigen that is present and highly expressed on all of the patient's cancer cells and not expressed on normal healthy cells, an antigen that is necessary for the cancer cell's proliferation and survival, and an antigen that is well recognized by T-cells [67,68]. Using tumor lysates as antigens in vaccines results in a larger spectrum of targeted TAs and therefore to a broader T-cell immune-response potentially being directed against proteins necessary for tumor survival, but this might also increase the risk of inducing autoimmunity resulting in targeted attacks on normal healthy cells [56].

Dendritic cells

The dendritic cell is known as the most potent APC. It was first described in 1973 [18]. The cell has the unique ability to process and present peptide fragments on its MHC class I and II molecules, known as cross presentation [69]. After maturation the dendritic cell migrates to the draining lymph node, where it interacts with naïve T-cells, leading to their activation via the three distinctive signals previously described (Figure 2).

From peripheral blood monocytes immature DCs can be generated *in vitro* in the presence of GM-CSF and IL-4 [70,71]. The immature dendritic cell is efficient in capturing and processing antigens, but inefficient when it comes to co-stimulation and activation of T-cells [72-75]. When the antigen has been captured and is being processed, the maturating DC becomes less efficient in capturing and processing further antigens. The mature DC is on the other hand efficient in T-cell activation and co-stimulation.

Migration of activated DCs to lymph nodes is a crucial step in activating specific immune responses. It is know that chemokines play an important role in controlling the DC migration [76]. Studies have explored the DC migration with injection of immature and mature ^{111}Indium-labeled DCs and scintigraphic imaging [77,78]. Those studies have concluded that mature DCs are more efficient in reaching the lymph nodes than immature DCs.

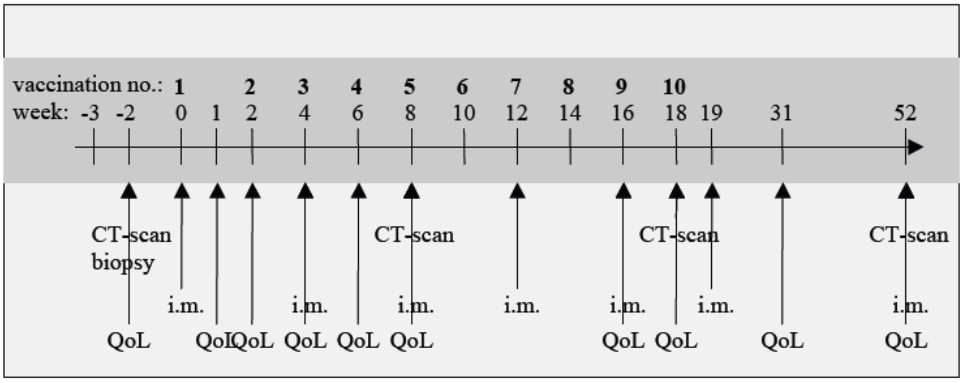

Figure 3
Vaccination schedule.
i.m. = immune monitoring, QoL = quality of life

Administration of vaccines
Since the interaction between the APC and the T-cell takes place in the lymph node it seems obvious, that direct injection of mature antigen pulsed DCs in the lymph node may be effective. In spite of that, studies have shown that intradermal injections may be just as effective and more effective than subcutaneous or intravenous administration [79]. Vaccinations are administered more frequently than anti-viral/toxin vaccines namely every one to four weeks, firstly, because TAA compared to antigens derived from toxins or virus are only weakly antigenic and secondly since studies have shown that the number of TAA-specific CTLs reaches a maximum around 7 days after vaccination and returns to pre-vaccine levels around day 28 [80].

Immune monitoring
Immune monitoring has become an important part of cancer vaccine trials. The monitoring is essential in exploring immunological responses during treatment, explore various responses in different patients, enhance knowledge about immune-competences, being able to identify and predict responders and non-responders, and ultimately optimizing treatment. Monitoring is often distinguished between humoral and cellular immune responses. Humoral responses are recognized by measuring cyto- and chemokines in serum and plasma. The methods used for these measurements are ELISA and also in Luminex systems with multiplex assays. Cellular responses are monitored with DTH, tetramer-analyses, lymphoproliferation, FACS and ELISPOT assays [17,81].

Dendritic cell vaccination trials in patients with CRC
Active specific immune therapy has been tested in patients with CRC in many smaller trials [17,23,24,66,79,82-85]. The previous mentioned meta-analysis by Nagorsen et al. [17] showed that the rate of CR, PR, MR, and SD for patients treated with DC based vaccines were 17% in the 70 patients treated with DC vaccines. A positive cellular response was observed in 20 of 38 patients (53%) treated with DC based vaccines [17].

Aims
The aim of the present Ph.D.-study was to establish and perform a clinical trial, testing a DC based cancer vaccine in patients with advanced CRC. The first objective was to perform a phase I trial to test toxicity and safety of this DC-vaccination therapy. Secondly, the aim was to evaluate the effectiveness assessed in clinical responses and quality of life in a phase II trial, and finally study the humoral responses following treatment with a cancer vaccine based on DCs pulsed with an allogenic tumor cell lysate.

MATERIAL AND METHODS
Study design
The study was designed as a classic phase I/II trial. A standard schedule was used for vaccinations, observations, and all measurements related to the trial. The treatment regimen consisted of a total of 10 vaccines administered as intradermal injections on the proximal thighs. Each of the vaccines consisted of 3-5 x 10^6 DCs (Figure 3). Since the included patient had no other conventional treatment offers, patients were invited to continue in the trial in spite of clinical progression at the evaluations.

Primary and secondary endpoints
The primary endpoints were safety and toxicity related to the treatment monitored with the National Cancer Institute's common toxicity criteria, and clinical responses determined by changes in sizes of tumors/metastases assessed by CT-scans and graded after the RECIST criteria. Secondary endpoints were changes in quality of life, changes in cytokines and biomarker blood levels during treatment, and overall survival.

Patients, inclusion and exclusion criteria
Six patients were included in the phase I trial and continued after interim analyses in the phase II trial, which included a total of 20 patients. A total of 36 patients were screened for eligibility and the 16 patients, who were not accomplishing the inclusion criteria, were primarily rejected because of low performance status.

The inclusion criteria were the following: biopsy verified colonic or rectal carcinoma with distant metastases, no indication for further conventional oncological or surgical therapies, age at inclusion between 25 and 75 years, 6 weeks prior to inclusion no radio- or chemotherapy, performance status ≥ 2 (WHO performance status scale). More than 4 months expected survival at inclusion, at inclusion adequate hepatic, renal, haematopoietic and blood coagulation function tests verified by blood samples, preserved pulmonary function and normal ECG. The exclusion criteria were the following: use of immune suppressive treatment (e.g. systemic corticosteroid) the last 2 months before inclusion, serious uncontrolled infections, participation in other clinical trials during the last 6 weeks, pregnancy, or lactation.

After inclusion needle biopsies were collected of tumors from all patients. Biopsies were placed in RNAlater (Ambion, Austin, TX, USA) and samples were analysed for expression of MAGE-A1, -A3, -A4, -A6, -A10 and –A12 with reverse transcriptase poly chain reaction (RT-PCR). Methods are described in detail in paper I.

Preparation of vaccine
DC cultures, tumor lysate production and lysate pulsing of DC to generate the full vaccine, MelCancerVac, were performed at DanDrit Biotech, Copenhagen, Denmark. DCs were cultured in vitro from 200 ml freshly drawn peripheral blood. By centrifugation over lymphoprep (Medinord A/S, Roskilde, Danmark) mononuclear cells were isolated and intensively washed in PBS/EDTA (Cambrix Bio Science, Versene, Belgium). Mononuclear cells were re-suspended in 2 ml RPMI 1640 medium (Cambrix Bio Science) and supplemented with 1% of autologoues plasma. Cell suspensions were left in non-treated T75 tissue culture flasks for 1 hour. Plastic adherent fractions were further cultured over night. GM-CSF (100 ng/ml) and IL-4 (50 ng/ml) (CELL Genix, Freiburg, Germany) were added. The same concentrations of GM-CSF and IL-4 were added on day 3. The melanoma cell lysate (MCL) (see below) was added to DCs at day 5 of culture at a final concentration of 10% (0.3 mg protein).The culture was supplemented with TNF-α (20 ng/ml) (CELL Genix) on day 6. Non-adherent and adherent fractions were harvested on day 7 and washed in Dulbecco's PBS and the number of cells with diameters > 14 microns was determined in a coulter counter system. The DC phenotype was confirmed by FACS analyses. A total of 3-5 x 10^6 DCs were re-suspended in 1 ml saline supplemented with 1% autologous plasma. In portions of 3-5 x10^6 DCs the remaining cells were frozen in 10% DMSO, 30% autologous plasma and 60% of RPMI 1640 medium. Before injection, frozen cells were thawed, washed twice in saline supplemented with 1% plasma and re-suspended in 1 ml physiological saline supplemented with 1% of autologous plasma.

Melanoma cell line and lysate

From an original melanoma cell line, FM3 [86,87] a melanoma cell clone DDM-1.13 was established, from which the MCL was prepared. The FM3 cell line originates from a melanoma patient that went through surgical removal of the tumor in 1992. The patient had a long disease free survival supposedly as a consequence of high immunogenicity of the tumor cells. The DDM-1.13 cell clone was established by selecting for high expression of MAGE-A antigens and for not expressing melanocyte differentiation antigens. Melanoma cells were expanded in RPMI 1640 medium (Cambrix Bio Science) with 2% human AB-serum (Cambrix Bio Science). Cells were cultured in serum free medium PC-1 (Cambrix Bio Science) and analysed for expression of MAGE antigens. Subsequent to harvesting by Versene (Cambrix Bio Science) the cells were washed twice in RPMI 1640 medium and re-suspended at a concentration of 10^7 cells/ml in RPMI 1640 medium. The suspension was frozen (liquid nitrogen) and thawed (37° C water bath five times). By centrifugation, first 10 minutes at 400 G, then 30 minutes at 10.000 G, the supernatant from the lysate containing approximately 3 mg protein/ml was filtrated through 0.2 µm filter. Finally, the lysate was stored in aliquots at -80°C until use.

Vaccines were administered no more than 3 hours after final re-suspension in the process of preparation.

Monitoring of adverse events and toxicity

At every visit the patient were weighed, had temperature, pulse-rate, and blood pressure measured, had general blood samples taken, had urine analysed for glucose, protein and blood, had oxygen saturation measured by pulse oximetry, and finally went through a general clinical examination. Adverse events were graded according to the National Cancer Institutes common toxicity criteria and monitored throughout the entire study period.

Monitoring of clinical response

Before start of treatment (baseline values), all patients had a CT-scan of the chest and abdomen with contrast medium. Evaluation CT-scans were performed after five given vaccines, after all ten vaccines and finally six months after end of the vaccination schedule. CT-scans were evaluated by the same senior radiologist and the responses were graded according to the RECIST criteria [88].

At the baseline CT-scan the number of measurable target lesions were counted and target metastasis with a diameter above 1 cm were selected. Non-target lesions (lesions with a diameter smaller than 1 cm, peritoneal carcinomatosis etc.) were also registered. The sum of the longest diameters of all the target lesions was calculated (baseline sum of the longest diameters). At evaluation CT-scans the sum of the longest diameters of all target lesions were calculated again and any new tumors were registered. Complete response (CR) is disappearance of all target and non-target lesions and normalisation of tumor marker levels; partial response (PR) is at least 30% reduction in the sum of the longest diameters of target lesions and no new lesions; progressive disease (PD) is at least 20% increase in the sum of the longest diameters or appearance of new lesions; and stable disease (SD) is between 30% decrease and 20% increase of the sum of the longest diameter and no new lesions. Reference to all of the evaluation measures is the baseline sum of the longest diameters [88].

Monitoring of quality of life

As an assessment of quality of life and especially development in quality of life the SF-36 (”short form” with 36 questions) was used in a validated Danish version [89,90]. The patients independently filled out the questionnaire at baseline and nine times later through the study period (Figure 3), and every time before they talked to the investigation physician. The following eight categories are covered by the SF-36 questionnaire: ‘physical function’, ‘physical role limitation’, ‘bodily pain’, ‘general health perceptions’, ‘vitality’, ‘social function’, ‘emotional role limitation’, and ‘mental health’. According to the SF-36 manual all answers were scored and electronically transformed to a scale ranging from 0 to 100 with higher scores indicating better status.

Monitoring of humoral and biomarker responses

Plasma and serum samples were collected by drawing peripheral blood into endotoxin-free tubes with EDTA as anticoagulation agent (plasma) and tubes with no anticoagulation agent (serum) (Becton Dickenson, NJ, USA) before treatment and six times throughout the study period. After collection the samples were left at room temperature for up to one hour. Separation of plasma and serum from blood cells was done by centrifugation at 2500 x G for 10 minutes at room temperature and the supernatants were stored in cryo-tubes (Thermo Fischer Scientific, Roskilde, Denmark) at -80° C. To avoid intra assay variations all samples from a specific patient were analysed on one ELISA plate or at the same run in the Luminex. Plasma levels of GM-CSF, IL-2, IL-6, TNF-α, IFN-γ, IL-4, IL-8, IL-1β, IL-5, IL-10, IL-12, MIP-1β, IP-10 and Eotaxin were analysed in a multiplex platform (Luminex 100 TM). All samples were run in triplicates with human extracellular protein buffer reagent kits (Invitrogen corp., CA, USA) set-up according to instructions from manufacturer. Serum levels of CEA were measured with a commercially available ELISA-platform (IBL, Immuno Biological Laboratories, Minneapolis, MN, USA). This assay determines concentrations between 0.25 ng/ml and 75.0 ng/ml. Plasma levels of tissue inhibitor of metalloproteinase-1 (TIMP-1) were analysed with a validated TIMP-1 ELISA [91]. A sheep polyclonal antibody was used to coat microtitre plates. A monoclonal antibody (MAC-15) and a secondary alkaline phosphatase-coubled antibody (Dako, Glostrup, Denmark) were used to detect TIMP-1.

STATISTICAL CONSIDERATIONS

In the phase I trial statistics were purely descriptive. In the phase II trial changes in quality of life were assessed with Friedman’s test. Overall survival time was estimated with Kaplan-Meier test and comparison of overall survival between patients with PD and SD was tested with Breslow’s (generalized Wilcoxon’s test) test of equality. In the third paper about immunologic responses to the dendritic cell treatment changes in levels of cytokines/chemokines/proteins were assessed with Friedman’s test. Comparisons of pre-vaccine levels were tested with the Mann Whitney U-test. Overall, statistical significance was determined at the $p<0.05$ level. Continuous variables were reported as medians (range). All statistical calculations were performed using SPSS software version 15.0 (SPSS Inc., Chicago, USA). Patients achieving SD were tested against patients with PD to show differences in survival, cytokine, and biomarker levels.

ETHICAL CONSIDERATIONS

Before inclusion all patients received written and oral information about the trial. All included patients gave their signed informed consent. The study was approved by The Local Ethics Committee (KA 04097gs), the Danish Health Authorities (2612-1970), the GCP Unit at Copenhagen University Hospital and by The Danish Data

Protection Agency (2004-41-4262). The study was performed at The Department of Surgical Gastroenterology at Gentofte University Hospital, Hellerup, Denmark according to international conference on harmonisation (ICH) guidelines for good clinical practice (European Directive on GCP 2001/20/EC). The study was registered at www.clinicaltrials.gov with the study identification number: NCT 00311272.

Table 1
Patient characteristics at baseline (n=20)

Characteristics	number of patients	%
Sex		
Female	10	50
Male	10	50
Median age (range)	60 (40-72) years	
WHO performance status at inclusion		
0	10	50
1	9	45
2	1	5
Expression of MAGE antigens (n=19)		
0	4	21
1	1	5
2	0	0
3	0	0
4	1	5
5	4	21
6	9	47
Median (range)	5 (0-6)	
Median time from initial diagnosis to inclusion (range)	36.4 (11.6-73.0) months	

RESULTS

Patient characteristics

Characteristics for all included patients are summarized in Table 1. Out of the 20 included patients 17 received intervention, two were included in violation with the protocol (one had primary lung cancer and one used corticosteroids) and one died before receiving any vaccines. Fourteen of the 17 treated patients received 5 vaccines and went through the first evaluation CT-scan. Two patients dropped out because of weakness before the first evaluation CT-scan, and one patient died before going through the CT-scan. Since these three patients did not go through an evaluation CT-scan their responses can not be assessed with the RESIST criteria, but it was assumed that they dropped out of the study due to disease progression, and therefore these three patients are considered progressive in the overall analysis of response rate. Eight patients received all ten allocated vaccinations and went through the second evaluation CT-scan. Only one patient completed the third evaluation CT-scan six months after the last vaccine (Figure 4).

Main results from study I

The phase I study consisted of six included patients. None of the patients had any serious adverse events related to the vaccine. There were no significant changes in the performance status, general well-being, blood pressure, temperature, BMI or general clinical appearance. One of the patients reported a one day temporary mild fatigue after each vaccine, a few times accompanied by a feeling of mild fever.

Conclusively, all of the patients tolerated the vaccine well and the treatment was considered non-toxic and safe.

Main results from study II

At the first evaluation CT-scan four of the 14 patients had achieved stable disease. Two of these remained stable throughout the rest of the period of treatment. Results from RECIST analysis of the CT-scans of these 4 patients are shown in table 2. One of the patients experienced a minor decrease in the sum of the longest diameters from baseline CT-scan to the first evaluation CT-scan, but was graded as SD since the decrease in the sum of the longest diameter was less than 30% (RECIST criteria). Images from these 2 CT-scans are shown in Figure 5. According to the RECIST criteria the best observed response was SD.

Kaplan-Meier curves were constructed for overall survival both from inclusion and from time of initial diagnosis to death (Figure 6 A+B). Median survival from initial diagnosis was 43.1 month (range 11.6 – 73.0 months, 95% CI 29.9 – 56.3 months). At initial diagnosis 50% of the 20 included patients had disseminated disease. Median survival from inclusion was 5.3 months (range 0.2 – 29.2 months 95% CI 4.3 – 6.3 months). Dividing the patients into two subgroups one with patients with SD and one with patients with PD and comparing the overall survival showed that patients with SD lived significantly longer (p = 0.038) than patients with PD (Figure 6C).

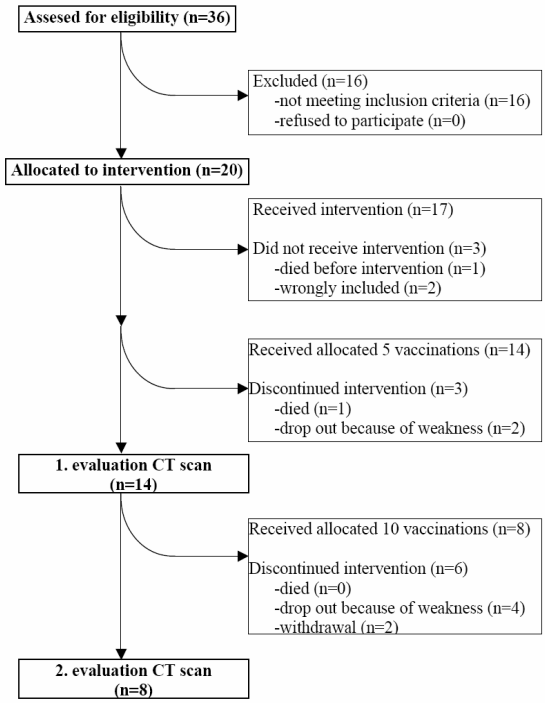

Figure 4
Study profile.

Variances in the quality of life showed no significant changes in the 'physical function' (p = 0.872), 'physical role limitation' (p = 0.965), 'bodily pain' (p = 0.079), 'social function' (p = 0.649), 'emotional role limitation' (p = 0.252), 'and mental health' (p = 0.626). Regarding the categories 'general

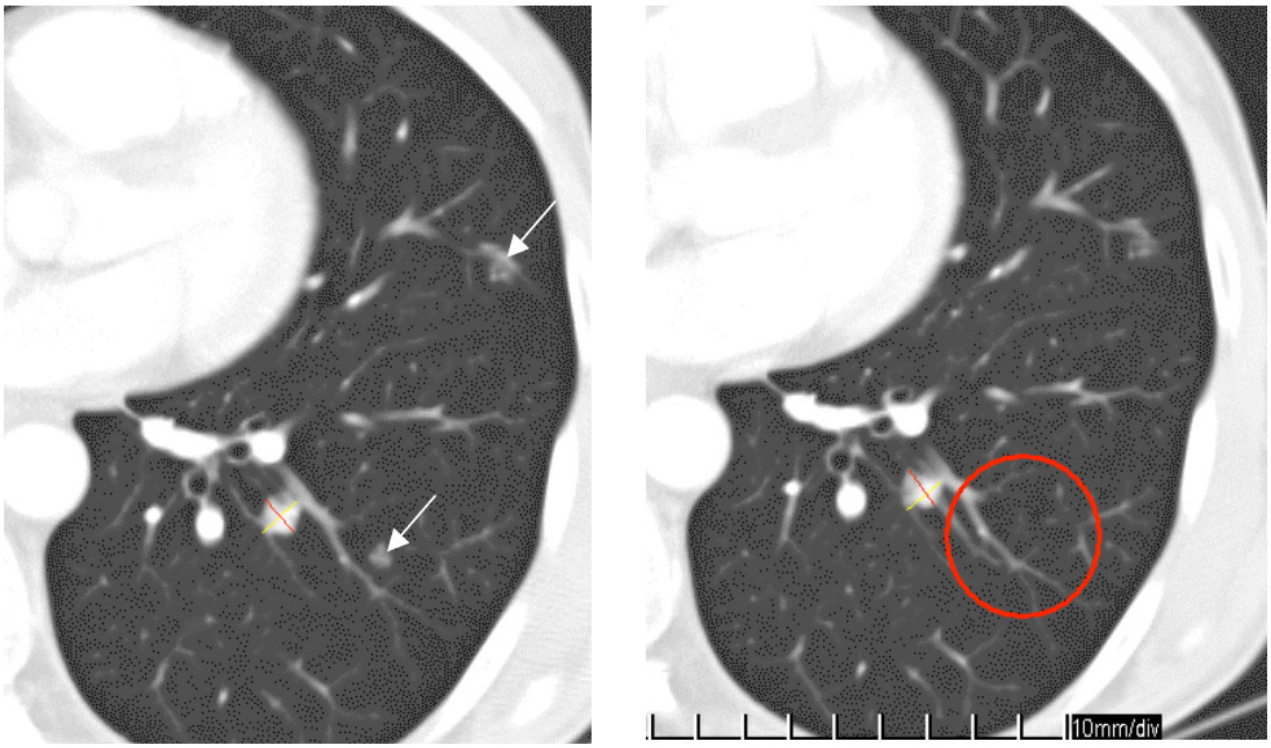

Figure 5
Baseline and first evaluating CT scan of a patient with stable disease.
Left: Baseline CT scan showing a target lesion measured with a longest diameter of 1.07 cm and two non-target lesions (arrows).
Right: First evaluation CT scan showing the same target lesion measured with a longest diameter of 0.95 cm and disappearance of one of the non-target lesions (circle).

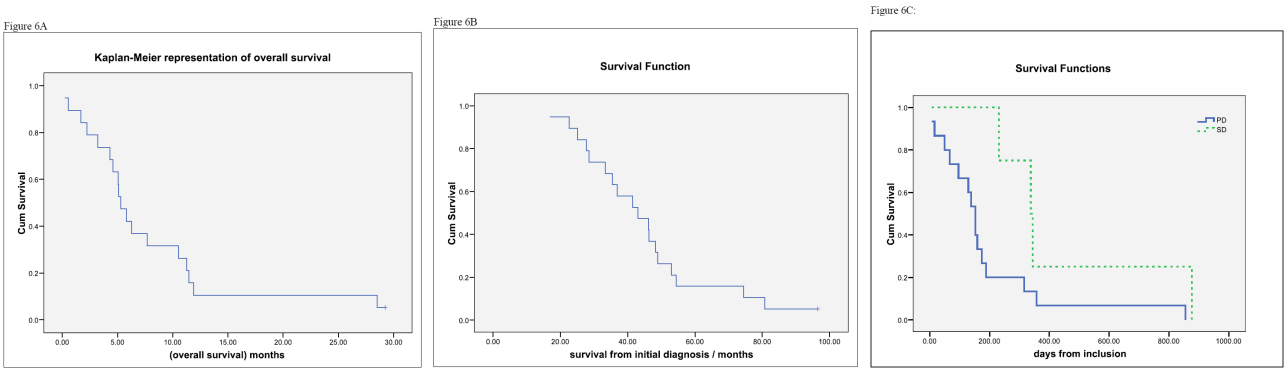

Figure 6
6A: Kaplan-Meier curve of median survival from inclusion.
6B: Kaplan-Meier curve of median survival from initial diagnosis, where 50% of the patients had disseminated disease.
6C: Kaplan-Meier curve of median survival from inclusion for patients with progressive disease (PD) and patients with stable disease (SD).

health perception' (p = 0.006) and 'vitality' (p = 0.011) there were significantly lower scores towards the end of the study. All vari ances were tested with Friedman's test. Variances in the self-reported quality of life assessed by the SF-36 questionnaire are shown in Figures 7A and 7B, respectively physical and mental quality of life.

Main results from study III
There were significant variances in TNF-α, IFN-γ, IL-2, IL-5, IL-10, IL-1β, and CEA for the entire cohort during treatment. These results are shown in Table 2.

There were no significant differences in pre-vaccine levels of cytokines between patients subsequently achieving SD versus PD nor between MAGE+ versus MAGE-. However, IL-6 levels among the patients achieving PD were significantly higher com

Figure 7A:

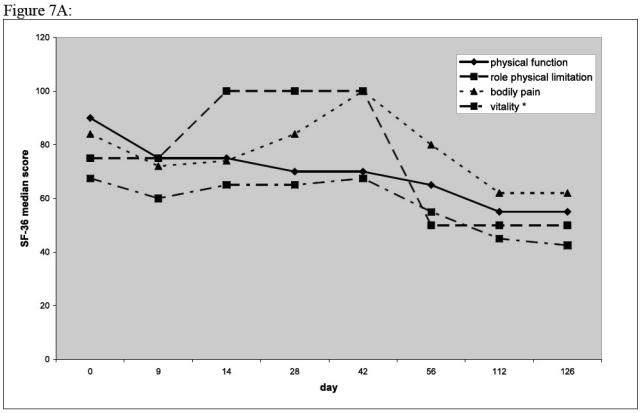

Figure 7B:

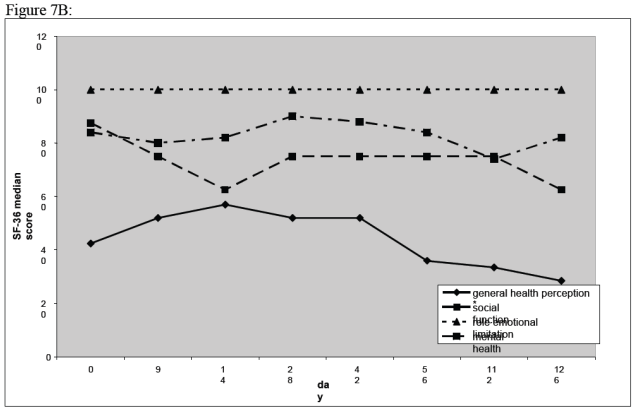

Figure 7

Changes in quality of life during the study period estimated with Friedman's statistical analysis. The median values are shown in the plots. There were no significant changes in the patients' "physical function" (p=0.872) (fig. 7A), "physical role limitation" (p=0.965) (fig. 7A), "bodily pain" (p= 0.079) (fig. 7A), "social function" (p=0.649) (fig. 7B), "emotional role limitation" (p=0.252) (fig. 7B) and "mental health" (p=0.626) (fig. 7B). There were a significant lower score at the end of the study concerning "general health perception" (p=0.006) (fig. 7B) and "vitality" (p=0.011) (fig. 7A).

pared with patients achieving SD (median 18 vs 10 pg/ml, p = 0.036).

Patients that achieved SD had significant variation in GM-CSF (Figure 8A), TNF-α (Figure 8B), IFN-γ (Figure 8C), IL-5 (Figure 8D), and IL-2 (Figure 8E). Patients who were graded as PD had significant increases in CEA levels (p<0.001) (Figure 8F), and TIMP-1 (p=0.011) (Figure 8G). Patients who were graded as SD had only slight increases in CEA levels (p=0.027) (Figure 8F), and no significant change in TIMP-1 levels. There was no significant difference in CEA levels between patients graded as PD versus SD. Patients expressing one or more MAGE antigens (MAGE+) showed significant increases in CEA levels (p<0.001), whereas MAGE- patients did not show any change in CEA levels throughout the study period (figure 9). Pre-vaccine levels of TIMP-1 were not significantly different between patients graded as PD or SD. No significant variation in TIMP-1 levels was observed in the subgroup of MAGE+ and MAGE- patients and there were no significant difference in pre-vaccine levels.

Table 2

Results from RECIST analysis of the four patients with stable disease
SD = stable disease, PD = progressive disease

Patient	Baseline CT scan	first evaluable CT scan			second evaluable CT scan		
	sum of longest diameters	sum of longest diamenters	new lesions	status	sum of longest diameters	new lesions	status
6	2.41 cm	2.55 cm	no	**SD**	3.18 cm	yes	PD
8	19.48 cm	19.64 cm	no	**SD**	21.87 cm	no	**SD**
10	6.19 cm	6.72 cm	no	**SD**	8.42 cm	yes	PD
19	1.07 cm	0.95 cm	no	**SD**	0.95 cm	no	**SD**

DISCUSSION

Results from the studies in the present Ph.D.-thesis have given interesting insights in the field of treating patients with advanced CRC with a DC-based cancer vaccine. First of all, the studies have proven that it was feasible to establish and run a scheduled vaccine therapy regimen to such patients. This was proven unproblematic in spite of different geographic locations for the actual treatment of the patients, and the production of the vaccines. Secondly, the studies strongly confirmed that the treatment was non-toxic and safe. Treatment was well tolerated by all patients. Clinical response rates were, according to the RECIST criteria, limited. We did not observe any complete or partial responses. A total of four patients achieved SD and two of these remained stable throughout the entire study period. Thus, a clinical benefit rate of 24% (4/17) was achieved. Regarding self-reported quality of life assessed by the SF-36 questionnaire analyses regarding physical quality of life showed that 'physical function', 'physical role limitation', and 'bodily pain' remained high and stable while there was a significant decrease in the patients' 'vitality'. Regarding mental quality of life, 'social function', 'functional role limitation', and 'mental health' remained high and stable, while there was a significant decrease in 'general health perception'. In addition to that we found significant changes in TNF-α, IFN-γ, IL-2, IL-5, IL-10, IL-1β, and CEA for the entire cohort during treatment with the vaccine and detailed analyses indicated a polarisation towards a Th1 response in patients achieving SD. With respect to CEA levels there was a major increase in patients with PD and only a slight increase in patients with SD with no difference between the pre-vaccine levels. Regarding TIMP-1 levels we registered increasing levels in patients with PD, and no changes in patients with SD, with no difference in pre-vaccine levels.

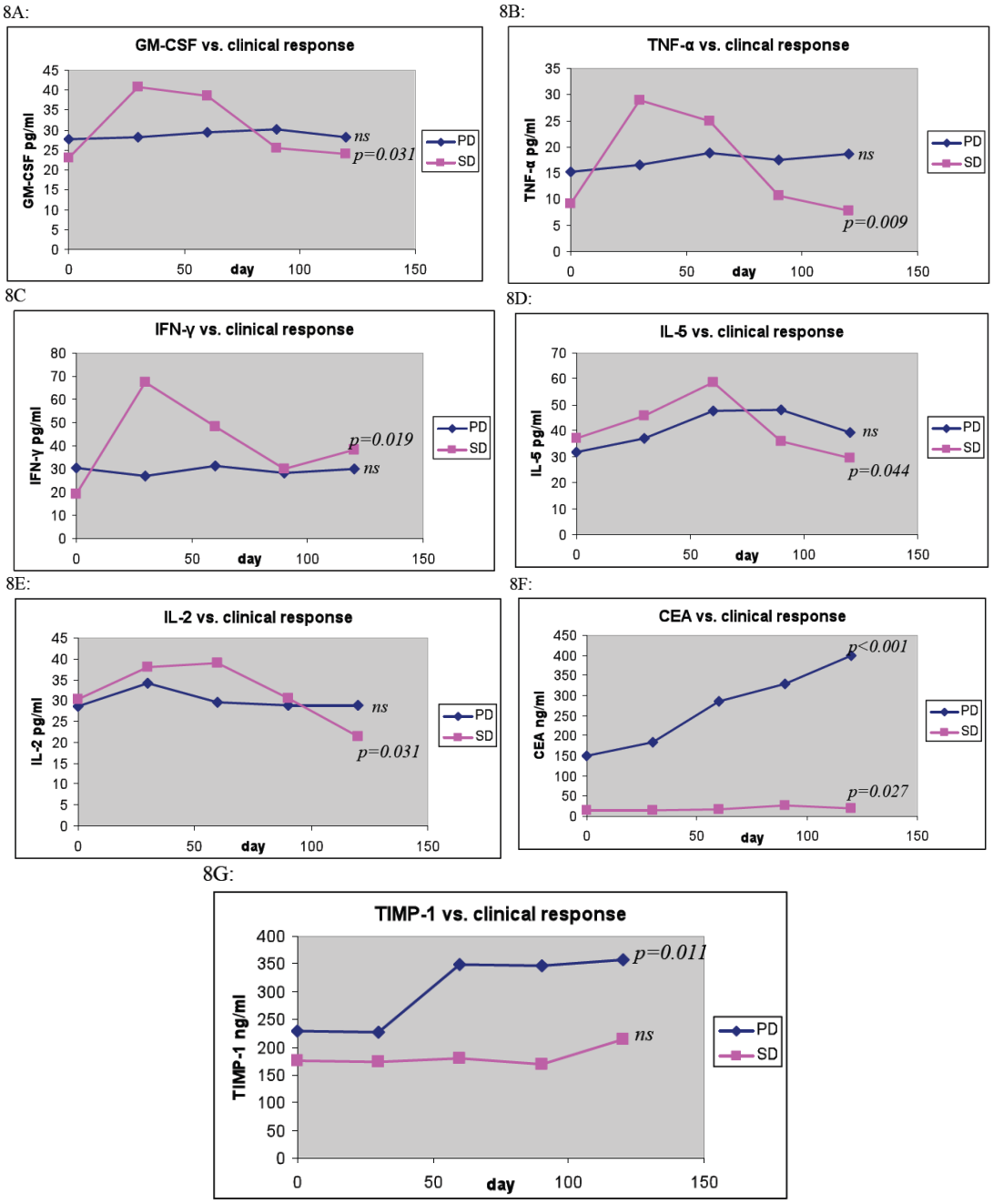

Figure 8

For patients with SD there were significant changes in GM-CSF (Fig. 8A), TNF-α (Fig. 8B), IFN-γ (Fig. 8C), IL-5 (Fig. 8D), and IL-2 (Fig 8E), whereas patients with PD did not show any significant changes during the study period.

For patients with both PD and SD there were significant changes in the CEA-levels (Fig. 8F), although the levels were numerically higher in patients with PD (Fig. 8F). There was not significant difference in pre-vaccine CEA levels between SD and PD.

For patients with PD TIMP-1 (Fig. 8G) changed significantly, whereas patients with SD did not experience significant changes during the study period.

PD = progressive disease, SD = stable disease, ns = non-significant

Immune responses versus clinical responses

As mentioned earlier immune responses are not always followed by clinical responses. Nagorsen and Thiel published in 2006 a meta-analysis on clinical and immunologic responses in patients with CRC during treatment with active specific cancer vaccines [17]. A total of 527 patients with advanced CRC were reported in 32 trials. The proportion of patients treated with a dendritic cell based vaccine was 13% (70/527) in 8 different trials. A new term called clinical benefit rate was introduced and consisted of com

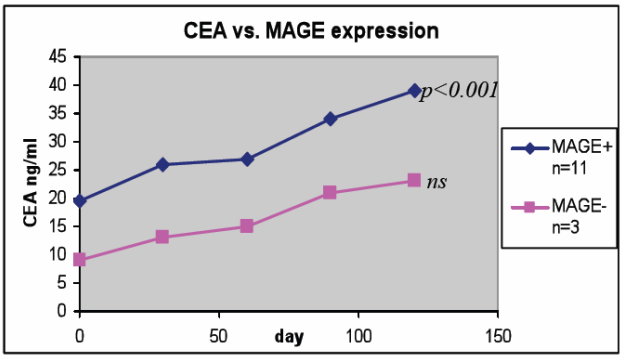

Figure 9
For MAGE+ patients there was significant change in CEA, p<0.001, while there was no significant change for MAGE- patients.

plete responses, partial responses, mixed responses, and stable disease and was introduced to avoid the risk of not detecting small clinical benefits that may adapt to relevant responses. The clinical benefit rate in patients treated with a dendritic cell based cancer vaccine was 17% (12/70). Humoral immune responses were not applied to the patients treated with dendritic cells, but 38 of the patients were monitored for cellular responses and 20 of these were positive (53%). It is obvious from these results that the rate of immunologic responses is higher than the corresponding clinical responses. The reasons for the lack of clinical responses in spite of supposedly good immunologic responses are not fully understood. One explanation could be that the immunologic responses may be directed against the vaccine, but not against the tumor, since the cancer cells might express other antigens than the ones the vaccine is directed against. Another explanation could be that immune competences in patients with large disseminated tumor burdens are diminished and therefore not sufficient to eradicate enough tumor cells to result in a clinical response [92-94]. It has been suggested that patients with end-stage disease do not present with as effective immunological responses as patients with earlier stage disease [85,95,96]. Another explanation could also be that large tumors are more heterogeneous and as a consequence of that a sub-fraction of the tumor cells may not express the antigens the vaccine is directed against.

Optimization of treatment

There is still plenty of room for improvement and optimization of immunotherapy, also when it comes to cancer vaccines based on DCs. The optimal route of administration and administration intervals are steps that already have been considered [79,80]. All the crucial steps from selection of targeted TAA (maybe multi-epitopes), including generation, pulsing, and maturation of DCs, and administration of vaccine and homing of DC to lymph nodes and interaction with T-cells (with all the necessary co-stimulatory molecules and the specific cytokine mircorenvironment), followed by homing of effective cytotoxic T-cells to all locations with malignant cells and finally to destruction of tumor cells, need to be clarified and ensured. If somehow the vaccines had proven to be efficient and showed good clinical responses in a considerable part of the patients, detailed determination of the evolved process could for practical reasons be considered less relevant, but since clinical responses are limited, thoroughly knowledge about all the steps are necessary in order to optimize treatment. This may require new methods for monitoring responses at the different crucial steps mentioned above. Clarification of the detailed processes combined with sufficient knowledge about immunological mechanisms may ultimately lead to interventional possibilities to overcome the inhibitory or limiting obstacles. For instance, lack of polarisation towards a Th1 response may be overcome by artificially establishing a favourable cytokine environment. Another option is to supply the immunotherapy with an adjuvant that either enhances the immunologic response or prevents it from being down regulated.

Levels of biomarkers in the blood may become a useful tool in identifying those patients, who will respond to immunotherapeutic treatments. The only recommended biomarker in CRC is CEA [97,98]. CEA levels are elevated as a consequence of de-differentiation of CRC cells (associated with disease activity). It is widely used, especially in the follow-up after treatment for CRC, in the daily clinical practise [99-103]. We have in the present study shown increasing levels of serum CEA in non-responding patients during treatment with the DC vaccine. We also saw an increase, though much smaller, in patients achieving SD. Since CEA measurements have some limitations, many other biological markers have been developed and tested, with TIMP-1 being one of interest. TIMP-1 has shown promising perspectives, especially in combination with CEA [99,104-113]. We have in the present study shown increasing levels of plasma TIMP-1 in patients, who did not respond to treatment with the DC vaccine, whereas patients achieving SD showed stable levels during treatment. These observations support the use of TIMP-1 as a biomarker in CRC during treatment with DC based cancer vaccines. The pre-vaccine levels of both CEA and TIMP-1 were lower (but not significantly) in patients subsequently achieving SD than in those with PD. The question whether CEA and TIMP-1 can be used as predictors of subsequently responding patients needs to be addressed in larger clinical trials.

In both humoral and cellular immune responses there is a balance between Th1 and Th2 responses [57-64]. Depending on the type of pathogen a DC encounters and presents, and especially the cytokine microenvironment determines whether the immune response will be Th1 or Th2 dominated, respectively. A Th1 dominant immune response directed against the tumor is favourable in cancer immune therapy [57]. The Th1 response promotes activation of CD8+ CTLs. Activated CTLs are considered the effector cells of cellular immunotherapy and are important for the specific killing of cancer cells.

Optimization of immune monitoring

The many new trials with various immunotherapeutic approaches to treat different cancer types have resulted in a substantial need for monitoring the patients. Effective monitoring is an absolute necessity in order to recognize responders early during treatment. Clinical responses, in terms of regression of tumor masses determined by CT-scan, may not be the best way of determining responders, since it may take some time to establish an effective immunological response and through that achieve clinical response, especially in patients with large tumor masses. More than that, lack of clinical response does not explain the specific immunological reasons for the lacking response. Ultimately, effective immune monitoring will be able to detect limiting/inhibitory steps in the immunological processes from administration of treatment and all the way to killing of the cancer cells. Clarification of the problematic steps in the patients might allow for applying a precise directed immune adjuvant to overcome the obstacle.

Earlier cancer stages

There are well-established therapies for most types of cancer, including CRC. A main problem concerning immunotherapy in this scenario is that so far all trials performed are purely experimental and therefore most trials are conducted in patients who have gone through various surgical and oncological treatments without responding or with subsequent relapse. These patients all have end-stage disease when they enter the immunotherapy trials, and many of the patients may have impaired immune competence due to the advanced disease and as a consequence of the received surgical and oncological treatments [92-94]. Studies suggest that a better immunologic response can be mobilized in patients with earlier cancer stages than in patients with end-stage disease [85,95,96]. More than that, it may, as mentioned earlier, take some time to establish a full immunologic response and turn this response into a clinical response [114]. Patients with end-stage disease may not have the time and immunologic capacity to generate a full immunologic response. Furthermore, it is most likely that the tumor burden in some patients may be so massive that even a strong immunologic response directed against the tumor cells might not be sufficient enough to kill the billons of cancer cells.

Combinations of treatments

Much of the research in immunotherapeutic treatment of cancer suggests that immunotherapy alone may not be sufficient to overcome the disease, especially not in advanced cases. In order to gain efficiency from immunotherapy it may be necessary to stimulate the immune system with adjuvants.

Since chemotherapy and immunotherapy act in very different ways it seems reasonable to assume that these treatment modalities cannot be combined. Chemotherapy and immunotherapy have often been thought of as merely counteracting, since many chemotherapeutics depress the immune competences, while the whole idea of immunotherapy is to establish and maintain a strong immunologic response. In spite of this, recent studies have suggested additive effects of combining treatments [25,43,49,66,85,115]. A recent review by Zhang and Herlyn [115] have suggested some of the beneficial effects of combining immunotherapy with chemotherapy in the treatment of cancer. Some chemotherapeutics like cyclophosphamide may down regulate Tregs [115]. Tregs are also called suppressor T cells and are known to suppress activity of the immune system, thereby maintaining immune homeostasis and tolerance to self-antigens. Down regulation of Tregs activity are therefore preferable in active immunotherapy. Chemotherapeutics have also in some studies resulted in tumor cells being more sensible to attack from CTL [115]. On the other hand some studies have indicated that the tumor sensitivity to chemotherapy has been enhanced after treatment with immunotherapy [115]. In addition, tumor cell death caused by chemotherapy may increase levels of TAA presented on APC and thereby stimulating immune cells and initiating an active specific immunologic response against the remaining tumor cells [115]. Combinational therapies with chemotherapy and immunotherapy have only been applied in patients with CRC in very few trials. In a recent phase II trial a viral vector encoding a TAA was administered to patients with metastatic CRC before, during, and after treatment with cycles of 5-FU, folinic acid, and oxaliplatin [116]. Addition of the vaccine to chemotherapeutic treatment was not associated with any toxicity, and specific cellular and/or antibody responses were detected in all evaluable patients. Out of 11 patients, 5 achieved partial and 1 complete responses and the authors conclude, that the addition of this vaccine to chemotherapy may provide additional clinical benefit [116]. However, these uncontrolled data must be validated in randomised studies with sufficient statistical power.

Integrated multi-disciplinary cancer settings

In many aspects treatment of cancer becomes more and more complicated. The armamentarium is expanding, therapies are becoming more specific, and treatment regimens existing of surgery, chemotherapy, radiotherapy, and maybe immunotherapy are becoming tailored to individual patients. This requires an intensive collaboration between the different specialities. Beyond the therapeutic treatments there is also an escalating use of symptomatic therapies with anti-emetics, analgesics, etc.. Integrated multi-disciplinary settings, as instituted in most Danish hospitals taking care of CRC patients, with teams of oncologists, surgeons, radiologists, and pain specialists are probably the preferred constellation in order to optimize treatment for each individual patient.

Limitations of the studies

Depending on the objectives there are several limitations to the studies in this PhD thesis. The main objective of the phase I trial was to evaluate safety and toxicity related to treatment with the DC based cancer vaccine in patients with advanced CRC. In 2003 more than 1000 patients had been treated with DC based vaccines without severe toxicity [83]. No dose escalation was done in this study, since toxicity most likely is not linked to the number of DCs administered. It is possible that the efficiency of the vaccine is better with more DCs in each vaccine. The limitation in the number of DC cells is related to the in *vitro* generation from PBMC; some patients have many PBMCs, while others have a limited amount, and there is even variability of the subgroup of DCs in such patients.

Limitations in the phase II trial are primarily the limited number of included patients. The study is well proportioned as a "proof-of-principle" trial [117], including tests for toxicity and vaccine activity, but far from sufficient to determine efficacy of the treatment. Controlled phase III studies are needed to study efficacy. The main objective of a "proof-of-principle" trial is, apart from safety-analysis, induction of biological activity in terms of clinical or immunological responses [117]. We have in our phase II study shown a clinical benefit rate of 24% (SD achieved in four out of 17 evaluable patients). We have also shown that the DC vaccine induced immunologic responses with polarization towards a Th1 response. A limiting factor in the analysis of responding patients is the complete lack of patients achieving PR or CR, even though this should not always be expected in "proof-of-principle" trials [117], particularly not in patients, who have received all known therapy before inclusion in the present protocol. It is questionable whether the four patients achieving SD did so as a consequence of treatment with the DC based vaccines or merely as a fact of slow progressing disease. Three of the four patients graded with SD had on the evaluation CT-scans minor increases in the sum of the longest diameters of the tumors. Two of these patients were at the following CT-scan graded with PD. Theoretically these two patients are suspected to having slow growing disease, but objectively it seems the slow progression in tumor-size is slower during the first five vaccine cycles than during the last five vaccine cycles, where new metastases are diagnosed (table 2). One patient had a minor decrease in tumor-size and remained with this lower tumor-mass throughout the study period. The fact that all of the patients had end-stage disease, had relapsed or progressed on previous therapies, and had no further

indication for conventional oncological or surgical therapies argue for that the SD was induced by the vaccine. It is noteworthy that the patients' quality of life remained high during treatment. Most of the patients were able to maintain their daily activities and a large proportion of them were still working.

CONCLUSIONS AND FUTURE PERSPECTIVES

The objective of this Ph.D.-study was to establish and perform a clinical trial, testing a DC based cancer vaccine in patients with advanced CRC. In conclusion the results have shown that:

- Treatment with our DC based cancer vaccine was non-toxic and safe.
- Four patients (24%) achieved SD, and two of these remained stable throughout the entire study period.
- Median survival from inclusion was 5.3 months (range 0.2-29.2 months).
- Quality of life remained high and stable for the parameters 'physical function', 'physical role limitation', 'bodily pain', 'social function', 'emotional role limitation', and 'mental health'.
- The DC based vaccine initiated favourable anti-cancer responses of the immune system, indicated by increases in TNF-α, IFN-γ, and IL-2 (Th1 response) in patients achieving SD.

Perspectives for DC based cancer vaccines in the future are promising, also in patients with CRC. Still, surgery is the most effective and the only established curative therapy of CRC. The future holds many challenges in treating the 20-60% of the patients with stage II-III CRC, who subsequently are relapsing [3,118]. Immunotherapeutic treatments need to be optimized in all involved processes from generation of DCs through elimination of cancer cells. Personalised regimens with conventional oncological therapy and newer immunotherapeutic agents may improve the efficacy of treatments. Earlier detection and prediction of responding and non-responding patients may result in reduced rates of relapses, improved overall survival and improved quality of life during treatment. These advancements can only be achieved through more knowledge about immunological mechanisms and interaction between therapeutic modalities gained through larger controlled clinical trials with solid clinical and robust immunological monitoring.

LIST OF ABBREVIATIONS

5-FU = 5-fluorouracil
APC = antigen presenting cell
BCG = Bacille Calmette-Guérin
CR = complete response
CRC = colorectal cancer
CT antigens = cancer testis antigens
CTL = cytotoxic T-lymphocyte
DC = dendritic cell
DMSO = dimethyl sulfoxid
DTH = delayed type hypersensitivity
EGFR = epidermal growth factor receptor
ELISA = enzyme-linked immuno-sorbent assay
ELISPOT = enzyme-linked immuno-sorbent spot
FACS = fluorescence-activated cell sorting
GM-CSF = granulocyte macrophage colony stimulating factor
IL = interleukin
IFN = interferon
LV = leucovorin
MCL = melanoma cell lysate
MHC = major histocompatibility complex
MR =mixed response
NK = natural killer
PD = progressive disease
PR = partial response
RECIST = response evaluation criteria in solid tumors
SD = stable disease
TA = tumor antigens
TAA = tumor associated antigens
TGF = transforming growth factor
TLR = Toll Like Receptor
TNF = tumor necroses factor
Treg = regulatory T cell
VEGF = vascular endothelial growth factor

SUMMARY

Colorectal cancer is with more than 4000 new cases every year the third most common cancer in Denmark. Metastases are most often found in the liver, and 20-25% of the patients have synchronous metastases to the liver at time of primary diagnosis. Other frequent sites for metastases are lungs and lymph nodes. Without treatment the median survival for patients with metastatic colorectal cancer is 7-9 months. Patients receiving systemic or regional chemotherapy now have a median survival of approximately 20 months. Up to 40% of the patients undergoing intended curative surgery subsequently relapse with local or distant disease, and approximately 80% of the relapses appear within the first 3 years. If the cancer metastasises, and the chances of radical surgery are eliminated, the prognosis is poor.

The aim of the present study was to evaluate the clinical and immunological effects of treating patients with disseminated colorectal cancer with a dendritic cell based cancer vaccine (MelCancerVac). The vaccine consisted of dendritic cells generated from autologous mononuclear cells pulsed with an allogeneic tumor cell lysate, selected for its high expression of cancer associated antigens.

A clinical phase I study evaluating tolerability and toxicity of the treatment was established. Six patients with progressive disease were included and the analysis revealed that the treatment was well tolerated and not associated with toxicity.

A subsequent clinical phase II study evaluating the activity of the treatment with CT-scan based measurements of tumors (RECIST), self reported quality of life (SF-36), and clinical evaluation was established. Out of twenty included patients with progressive disease, seventeen received intervention with the vaccine. Stable disease was achieved in four patients and two of these remained stable throughout the entire study period. Quality of life remained for most parameters included in the evaluation high and stable.

The immunological consequences of the treatment were evaluated with plasma- and serum-levels of inflammatory and non-inflammatory markers (the following 10 cytokines: GM-CSF, INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, and TNF-α, and in addition the inflammatory chemokines MIP-1β, Eotaxin and IP-10) and biomarkers CEA and TIMP-1. These analyses showed that the vaccine induced increasing levels of Th1 cytokines such as GM-CSF, TNF-α, IFN-γ, and IL-2 in patients achieving stable disease. Patients with progressive disease had increasing levels of CEA and TIMP-1, while patients achieving stable disease maintained relatively stable levels.

Conclusively, treatment with this dendritic cell based cancer vaccine was non-toxic and safe, clinical response in terms of stable disease was achieved in 24% of the patients, and the pa-

tients maintained a high quality of life during treatment. The immunological analyses indicated that the treatment resulted in favourable anticancer responses in the patients' immune system in terms of polarisation towards a Th1 dominated response potentially directed against tumor cells. Since no partial or complete responses were observed and since the number of patients was relatively low these results have to be interpreted with caution. Moreover, phase II study designs do not lead to final conclusions regarding clinical efficacy, which must be validated in larger prospective, randomised and controlled studies.

REFERENCES

1. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC et al. Cancer statistics, 2005. CA Cancer J Clin 2005; 55:10-30.
2. Cancerregisteret 2005 og 2006. Nye tal fra Sundhedsstyrelsen. 2008.
3. Goldberg RM. Advances in the treatment of metastatic colorectal cancer. Oncologist 2005; 10 Suppl 3:40-48.
4. Ju JH, Chang SC, Wang HS, Yang SH, Jiang JK et al. Changes in disease pattern and treatment outcome of colorectal cancer: a review of 5,474 cases in 20 years. Int J Colorectal Dis 2007; 22:855-62.
5. Arndt V, Merx H, Stegmaier C, Ziegler H, Brenner H. Restrictions in quality of life in colorectal cancer patients over three years after diagnosis: a population based study. Eur J Cancer 2006; 42:1848-57.
6. McWilliams RR, Goetz MP, Morlan BW, Salim M, Rowland KM et al. Phase II trial of oxaliplatin/irinotecan/5-fluorouracil/leucovorin for metastatic colorectal cancer. Clin Colorectal Cancer 2007; 6:516-21.
7. Nakata B, Sowa M, Tsuji A, Kamano T, Sasaki K et al. Continuous infusion of 5-fluorouracil with versus without low-dose, consecutive administration of cisplatin in advanced colorectal cancer. A prospective randomized phase II study. J Exp Clin Cancer Res 2007; 26:51-60.
8. Burnet M. Cancer: a biological approach. III. Viruses associated with neoplastic conditions. IV. Practical applications. Br Med J 1957; 1:841-7.
9. Kim R, Emi M, Tanabe K. Cancer immunoediting from immune surveillance to immune escape. Immunology 2007; 121:1-14.
10. Thomas L. On immunosurveillance in human cancer. Yale J Biol Med 1982; 55:329-33.
11. Rygaard J, Povlsen CO. The mouse mutant nude does not develop spontaneous tumours. An argument against immunological surveillance. Acta Pathol Microbiol Scand [B] Microbiol Immunol 1974; 82:99-106.
12. Stutman O. Tumor development after 3-methylcholanthrene in immunologically deficient athymic-nude mice. Science 1974; 183:534-6.
13. Dighe AS, Richards E, Old LJ, Schreiber RD. Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. Immunity 1994; 1:447-56.
14. Street SE, Cretney E, Smyth MJ. Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. Blood 2001; 97:192-7.
15. Birkeland SA, Storm HH, Lamm LU, Barlow L, Blohme I, Forsberg B et al. Cancer risk after renal transplantation in the Nordic countries, 1964-1986. Int J Cancer 1995; 60:183-9.
16. Pham SM, Kormos RL, Landreneau RJ, Kawai A, Gonzalez-Cancel I et al. Solid tumors after heart transplantation: lethality of lung cancer. Ann Thorac Surg 1995; 60:1623-6.
17. Nagorsen D, Thiel E. Clinical and immunologic responses to active specific cancer vaccines in human colorectal cancer. Clin Cancer Res 2006; 12:3064-9.
18. Steinman RM, Cohn ZA. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 1973; 137:1142-62.
19. Wiemann B, Starnes CO. Coley's toxins, tumor necrosis factor and cancer research: a historical perspective. Pharmacol Ther 1994; 64:529-64.
20. Yuk JM, Shin DM, Yang CS, Kim KH, An SJ et al. Role of apoptosis-regulating signal kinase 1 in innate immune responses by Mycobacterium bovis bacillus Calmette-Guerin. Immunol Cell Biol 2009; 87:100-7.
21. Lamm DL, Thor DE, Harris SC, Reyna JA, Stogdill VD et al. Bacillus Calmette-Guerin immunotherapy of superficial bladder cancer. J Urol 1980; 124:38-40.
22. Shelley MD, Court JB, Kynaston H, Wilt TJ, Fish RG et al. Intravesical Bacillus Calmette-Guerin in Ta and T1 Bladder Cancer. Cochrane Database Syst Rev 2000;CD001986.
23. Rosenberg SA. Development of effective immunotherapy for the treatment of patients with cancer. J Am Coll Surg 2004; 198:685-96.
24. Huber CH, Wolfel T. Immmunotherapy of cancer: from vision to standard clinical practice. J Cancer Res Clin Oncol 2004; 130:367-74.
25. Yip D, Strickland AH, Karapetis CS, Hawkins CA, Harper PG. Immunomodulation therapy in colorectal carcinoma. Cancer Treat Rev 2000; 26:169-90.
26. Saif MW, Kang SP, Chu E. Treatment of metastatic colorectal cancer: from cytotoxic agents to molecular agents and multitargeted strategies. Oncology 2006; 20:11-9.
27. Pfeiffer P, Qvortrup C, Eriksen JG. Current role of antibody therapy in patients with metastatic colorectal cancer. Oncogene 2007; 26:3661-78.
28. Halak BK, Maguire HC, Jr., Lattime EC. Tumor-induced interleukin-10 inhibits type 1 immune responses directed at a tumor antigen as well as a non-tumor antigen present at the tumor site. Cancer Res 1999; 59:911-7.
29. Rosenberg SA. A new era for cancer immunotherapy based on the genes that encode cancer antigens. Immunity 1999; 10:281-7.
30. Rosenberg SA. Progress in the development of immunotherapy for the treatment of patients with cancer. J Intern Med 2001; 250:462-75.
31. Aulitzky WE, Huber C, Peschel C. Cytokine therapy of neoplastic and inflammatory disease. Int Arch Allergy Immunol 1993; 101:221-6.
32. Hill HC, Conway TF, Jr., Sabel MS, Jong YS, Mathiowitz E et al. Cancer immunotherapy with interleukin 12 and granulocyte-macrophage colony-stimulating factor-encapsulated microspheres: coinduction of innate and adaptive antitumor immunity and cure of disseminated disease. Cancer Res 2002; 62:7254-63.
33. Holcombe RF, Li A, Stewart RM. Levamisole and interleukin-2 for advanced malignancy. Biotherapy 1998; 11:255-8.
34. Wilson JA. Tumor necrosis factor alpha and colitis-associated colon cancer. N Engl J Med 2008; 358:2733-4.

35. Donskov F, von der MH. Impact of immune parameters on long-term survival in metastatic renal cell carcinoma. J Clin Oncol 2006; 24:1997-2005.
36. Donskov F, Middleton M, Fode K, Meldgaard P, Mansoor W et al. Two randomised phase II trials of subcutaneous interleukin-2 and histamine dihydrochloride in patients with metastatic renal cell carcinoma. Br J Cancer 2005; 93:757-62.
37. McDermott DF. The application of high-dose interleukin-2 for metastatic renal cell carcinoma. Med Oncol 2009; 26:13-7.
38. Gebbia V, Testa A, Majello E, Cannata G, Tirrito ML et al. Subcutaneous low-dose interleukin-2 and intravenous 5-fluorouracil plus high-dose levofolinic acid as salvage treatment for metastatic colorectal carcinoma. Anticancer Drugs 1996; 7:386-91.
39. Mitchell MS. Combinations of anticancer drugs and immunotherapy. Cancer Immunol Immunother 2003; 52:686-92.
40. Nicolini A, Carpi A, Ferrari P, Sagripanti A, Anselmi L. A multistep therapy with subcutaneous low dose recombinant interleukin-2, 5-fluorouracil and leucovorin prolongs the response of metastatic colorectal cancer patients: a pilot study. Biomed Pharmacother 1998; 52:311-6.
41. Prat A, Casado E, Cortes J. New approaches in angiogenic targeting for colorectal cancer. World J Gastroenterol 2007; 13:5857-66.
42. Moosmann N, Heinemann V. Cetuximab plus oxaliplatin-based chemotherapy in the treatment of colorectal cancer. Expert Rev Anticancer Ther 2008; 8:319-29.
43. Mahtani RL, Macdonald JS. Synergy between cetuximab and chemotherapy in tumors of the gastrointestinal tract. Oncologist 2008; 13:39-50.
44. Wiedmann MW, Caca K. Molecularly targeted therapy for gastrointestinal cancer. Curr Cancer Drug Targets 2005; 5:171-93.
45. Ramanathan RK, Hwang JJ, Zamboni WC, Sinicrope FA, Safran H et al. Low overexpression of HER-2/neu in advanced colorectal cancer limits the usefulness of trastuzumab (Herceptin) and irinotecan as therapy. A phase II trial. Cancer Invest 2004; 22:858-65.
46. Kerbel RS. Tumor angiogenesis. N Engl J Med 2008; 35:2039-49.
47. Scappaticci FA, Skillings JR, Holden SN, Gerber HP, Miller K et al. Arterial thromboembolic events in patients with metastatic carcinoma treated with chemotherapy and bevacizumab. J Natl Cancer Inst 2007; 99:1232-9.
48. Saltz LB, Meropol NJ, Loehrer PJ, Sr., Needle MN, Kopit J et al. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol 2004; 22:1201-8.
49. June CH. Adoptive T cell therapy for cancer in the clinic. J Clin Invest 2007; 117:1466-76.
50. Knutson KL, Almand B, Mankoff DA, Schiffman K, Disis ML. Adoptive T-cell therapy for the treatment of solid tumours. Expert Opin Biol Ther 2002; 2:55-66.
51. Finn OJ. Cancer immunology. N Engl J Med 2008; 358:2704-15.
52. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer 2008; 8:299-308.
53. Dudley ME, Yang JC, Sherry R, Hughes MS, Royal R et al. Adoptive cell therapy for patients with metastatic melanoma: evaluation of intensive myeloablative chemoradiation preparative regimens. J Clin Oncol 2008; 26:5233-9.
54. Riddell SR. Engineering antitumor immunity by T-cell adoptive immunotherapy. Hematology Am Soc Hematol Educ Program 2007 :250-256.
55. Loren AW, Porter DL. Donor leukocyte infusions for the treatment of relapsed acute leukemia after allogeneic stem cell transplantation. Bone Marrow Transplant 2008; 41:483-93.
56. Korman AJ, Peggs KS, Allison JP. Checkpoint blockade in cancer immunotherapy. Adv Immunol 2006; 90:297-339.
57. Nishimura T, Nakui M, Sato M, Iwakabe K, Kitamura H et al. The critical role of Th1-dominant immunity in tumor immunology. Cancer Chemother Pharmacol 2000; 46:S52-S61.
58. Huang H, Hao S, Li F, Ye Z, Yang J et al. CD4+ Th1 cells promote CD8+ Tc1 cell survival, memory response, tumor localization and therapy by targeted delivery of interleukin 2 via acquired pMHC I complexes. Immunology 2007; 120:148-59.
59. Dranoff G. Cytokines in cancer pathogenesis and cancer therapy. Nat Rev Cancer 2004; 4:11-22.
60. Nembrini C, Abel B, Kopf M, Marsland BJ. Strong TCR signaling, TLR ligands, and cytokine redundancies ensure robust development of type 1 effector T cells. J Immunol 2006; 176:7180-8.
61. Gutcher I, Becher B. APC-derived cytokines and T cell polarization in autoimmune inflammation. J Clin Invest 2007; 117:1119-27.
62. Moingeon P. Strategies for designing vaccines eliciting Th1 responses in humans. J Biotechnol 2002; 98:189-98.
63. Tatsumi T, Kierstead LS, Ranieri E, Gesualdo L, Schena FP et al. Disease-associated bias in T helper type 1 (Th1)/Th2 CD4(+) T cell responses against MAGE-6 in HLA-DRB10401(+) patients with renal cell carcinoma or melanoma. J Exp Med 2002; 196:619-28.
64. Dredge K, Marriott JB, Todryk SM, Dalgleish AG. Adjuvants and the promotion of Th1-type cytokines in tumour immunotherapy. Cancer Immunol Immunother 2002; 51:521-31.
65. Novellino L, Castelli C, Parmiani G. A listing of human tumor antigens recognized by T cells: March 2004 update. Cancer Immunol Immunother 2005; 54:187-207.
66. Mocellin S, Rossi CR, Lise M, Nitti D. Colorectal Cancer Vaccines: Principles, Results, and Perspectives. Gastroenterology 2004; 127:1821-37.
67. Segal NH, Parsons DW, Peggs KS, Velculescu V, Kinzler KW et al. Epitope landscape in breast and colorectal cancer. Cancer Res 2008; 68(3):889-892.
68. Mocellin S, Mandruzzato S, Bronte V, Lise M, Nitti D. Part I: Vaccines for solid tumours. Lancet Oncol 2004; 5:681-9.
69. Rodriguez A, Regnault A, Kleijmeer M, Ricciardi-Castagnoli P, Amigorena S. Selective transport of internalized antigens to the cytosol for MHC class I presentation in dendritic cells. Nat Cell Biol 1999; 1:362-8.
70. Romani N, Gruner S, Brang D, Kampgen E, Lenz A et al. Proliferating dendritic cell progenitors in human blood. J Exp Med 1994; 180:83-93.
71. Thurner B, Roder C, Dieckmann D, Heuer M, Kruse M et al. Generation of large numbers of fully mature and stable dendritic cells from leukapheresis products for clinical application. J Immunol Methods 1999; 223:1-15.
72. Jiang W, Swiggard WJ, Heufler C, Peng M, Mirza A et al. The receptor DEC-205 expressed by dendritic cells and thymic epithelial cells is involved in antigen processing. Nature 1995; 375:151-5.
73. Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by

granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 1994; 179:1109-18.
74. Adema GJ, Hartgers F, Verstraten R, de VE, Marland G et al. A dendritic-cell-derived C-C chemokine that preferentially attracts naive T cells. Nature 1997; 387:713-7.
75. Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998; 392:245-52.
76. Whiteside TL, Odoux C. Dendritic cell biology and cancer therapy. Cancer Immunol Immunother 2004; 53:240-8.
77. de V, I, Krooshoop DJ, Scharenborg NM, Lesterhuis WJ, Diepstra JH et al. Effective migration of antigen-pulsed dendritic cells to lymph nodes in melanoma patients is determined by their maturation state. Cancer Res 2003; 63:12-7.
78. Mackensen A, Krause T, Blum U, Uhrmeister P, Mertelsmann R, Lindemann A. Homing of intravenously and intralymphatically injected human dendritic cells generated in vitro from CD34+ hematopoietic progenitor cells. Cancer Immunol Immunother 1999; 48:118-22.
79. Cranmer LD, Trevor KT, Hersh EM. Clinical applications of dendritic cell vaccination in the treatment of cancer. Cancer Immunol Immunother 2004; 53:275-306.
80. Chakraborty NG, Chattopadhyay S, Mehrotra S, Chhabra A, Mukherji B. Regulatory T-cell response and tumor vaccine-induced cytotoxic T lymphocytes in human melanoma. Hum Immunol 2004; 65:794-802.
81. Nagorsen D, Scheibenbogen C, Thiel E, Keilholz U. Immunological monitoring of cancer vaccine therapy. Expert Opin Biol Ther 2004; 4:1677-84.
82. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004; 10:909-15.
83. Ridgway D. The first 1000 dendritic cell vaccinees. Cancer Invest 2003; 21:873-86.
84. Kavanagh B, Ko A, Venook A, Margolin K, Zeh H et al. Vaccination of metastatic colorectal cancer patients with matured dendritic cells loaded with multiple major histocompatibility complex class I peptides. J Immunother 2007; 30:762-72.
85. Mosolits S, Ullenhag G, Mellstedt H. Therapeutic vaccination in patients with gastrointestinal malignancies. A review of immunological and clinical results. Ann Oncol 2005; 16:847-62.
86. Kirkin AF, thor SP, Hansen MR, Barfoed A, Dzhandzhugazyan KN et al. Establishment of gp100 and MART-1/Melan-A-specific cytotoxic T lymphocyte clones using in vitro immunization against preselected highly immunogenic melanoma cell clones. Cancer Immunol Immunother 1999; 48:239-46.
87. Kirkin AF, Petersen TR, Olsen AC, Li L, thor SP et al. Generation of human-melanoma-specific T lymphocyte clones defining novel cytolytic targets with panels of newly established melanoma cell lines. Cancer Immunol Immunother 1995; 41:71-81.
88. Therasse P, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS et al. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. J Natl Cancer Inst 2000; 92:205-16.
89. Bjorner JB, Thunedborg K, Kristensen TS, Modvig J, Bech P. The Danish SF-36 Health Survey: translation and preliminary validity studies. J Clin Epidemiol 1998; 51:991-9.
90. Ware JE, Jr., Sherbourne CD. The MOS 36-item short-form health survey (SF-36). I. Conceptual framework and item selection. Med Care 1992; 30:473-83.
91. Holten-Andersen MN, Murphy G, Nielsen HJ, Pedersen AN, Christensen IJ et al. Quantitation of TIMP-1 in plasma of healthy blood donors and patients with advanced cancer. Br J Cancer 1999; 80:495-503.
92. Klein G, Klein E. Surveillance against tumors--is it mainly immunological? Immunol Lett 2005; 100:29-33.
93. Yu P, Lee Y, Wang Y, Liu X, Auh S et al. Targeting the primary tumor to generate CTL for the effective eradication of spontaneous metastases. J Immunol 2007; 179:1960-8.
94. Reiman JM, Kmieciak M, Manjili MH, Knutson KL. Tumor immunoediting and immunosculpting pathways to cancer progression. Semin Cancer Biol 2007; 17:275-87.
95. Vermorken JB, Claessen AM, van Tinteren H, Gall HE, Ezinga R et al. Active specific immunotherapy for stage II and stage III human colon cancer: a randomised trial. Lancet 1999; 353:345-50.
96. Cubas R, Li M, Chen C, Yao Q. Colorectal cancer: new advances in immunotherapy. Cancer Biol Ther 2007; 6:11-7.
97. Duffy MJ, van DA, Haglund C, Hansson L, Holinski-Feder E et al. Tumour markers in colorectal cancer: European Group on Tumour Markers (EGTM) guidelines for clinical use. Eur J Cancer 2007; 43:1348-60.
98. Locker GY, Hamilton S, Harris J, Jessup JM, Kemeny N et al. ASCO 2006 update of recommendations for the use of tumor markers in gastrointestinal cancer. J Clin Oncol 2006; 24:5313-27.
99. Nielsen HJ, Christensen IJ, Brunner NA. [Serological markers of colorectal cancer]. Ugeskr Laeger 2005; 167:4163-7.
100. Ferroni P, Palmirotta R, Spila A, Martini F, Formica V et al. Prognostic value of carcinoembryonic antigen and vascular endothelial growth factor tumor tissue content in colorectal cancer. Oncology 2006; 71:176-84.
101. Takagawa R, Fujii S, Ohta M, Nagano Y, Kunisaki C et al. Preoperative serum carcinoembryonic antigen level as a predictive factor of recurrence after curative resection of colorectal cancer. Ann Surg Oncol 2008; 15:3433-9.
102. Kosmider S, Stella DL, Field K, Moore M, Ananda S et al. Preoperative Investigations for Metastatic Staging of Colon and Rectal Cancer across Multiple Centres- What is Current Practice? Colorectal Dis 2009; 11:592-600.
103. Tan E, Gouvas N, Nicholls RJ, Ziprin P, Xynos E et al. Diagnostic precision of carcinoembryonic antigen in the detection of recurrence of colorectal cancer. Surg Oncol 2009; 18:15-24.
104. Holten-Andersen MN, Christensen IJ, Nielsen HJ, Stephens RW, Jensen V et al. Total levels of tissue inhibitor of metalloproteinases 1 in plasma yield high diagnostic sensitivity and specificity in patients with colon cancer. Clin Cancer Res 2002; 8:156-64.
105. Moller SN, Vejgaard S, I, Ornbjerg WS, Schrohl AS, Dowell B et al. Biology and potential clinical implications of tissue inhibitor of metalloproteinases-1 in colorectal cancer treatment. Scand J Gastroenterol 2008; 43:774-86.
106. Hilska M, Roberts PJ, Collan YU, Laine VJ, Kossi J et al. Prognostic significance of matrix metalloproteinases-1, -2, -7 and -13 and tissue inhibitors of metalloproteinases-1, -2, -3 and -4 in colorectal cancer. Int J Cancer 2007; 121:714-23.
107. Kim TD, Song KS, Li G, Choi H, Park HD et al. Activity and expression of urokinase-type plasminogen activator and matrix metalloproteinases in human colorectal cancer. BMC Cancer 2006; 6:211.

108. Hammer JH, Basse L, Svendsen MN, Werther K, Brunner N et al. Impact of elective resection on plasma TIMP-1 levels in patients with colon cancer. Colorectal Dis 2006; 8:168-72.
109. Holten-Andersen MN, Hansen U, Brunner N, Nielsen HJ, Illemann M et al. Localization of tissue inhibitor of metalloproteinases 1 (TIMP-1) in human colorectal adenoma and adenocarcinoma. Int J Cancer 2005; 113:198-206.
110. Oberg A, Hoyhtya M, Tavelin B, Stenling R, Lindmark G. Limited value of preoperative serum analyses of matrix metalloproteinases (MMP-2, MMP-9) and tissue inhibitors of matrix metalloproteinases (TIMP-1, TIMP-2) in colorectal cancer. Anticancer Res 2000; 20:1085-91.
111. Nielsen HJ, Brunner N, Frederiksen C, Lomholt AF, King D et al. Plasma tissue inhibitor of metalloproteinases-1 (TIMP-1): a novel biological marker in the detection of primary colorectal cancer. Protocol outlines of the Danish-Australian endoscopy study group on colorectal cancer detection. Scand J Gastroenterol 2008; 43:242-8.
112. Sorensen NM, Bystrom P, Christensen IJ, Berglund A, Nielsen HJ et al. TIMP-1 is significantly associated with objective response and survival in metastatic colorectal cancer patients receiving combination of irinotecan, 5-fluorouracil, and folinic acid. Clin Cancer Res 2007; 13:4117-22.
113. Wagenaar-Miller RA, Gorden L, Matrisian LM. Matrix metalloproteinases in colorectal cancer: is it worth talking about? Cancer Metastasis Rev 2004; 23:119-35.
114. Copier J, Ward S, Dalgleish A. Cell based cancer vaccines: regulatory and commercial development. Vaccine 2007; 25 Suppl 2:B35-B46.
115. Zhang T, Herlyn D. Combination of active specific immunotherapy or adoptive antibody or lymphocyte immunotherapy with chemotherapy in the treatment of cancer. Cancer Immunol Immunother 2009; 58:475-92.
116. Harrop R, Drury N, Shingler W, Chikoti P, Redchenko I et al. Vaccination of colorectal cancer patients with modified vaccinia ankara encoding the tumor antigen 5T4 (TroVax) given alongside chemotherapy induces potent immune responses. Clin Cancer Res 2007; 13:4487-94.
117. Hoos A, Parmiani G, Hege K, Sznol M, Loibner H et al. A clinical development paradigm for cancer vaccines and related biologics. J Immunother 2007; 30:1-15.
118. Tebbutt NC, Cattell E, Midgley R, Cunningham D, Kerr D. Systemic treatment of colorectal cancer. Eur J Cancer 2002; 38:1000-15.