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ORIGINAL ARTICLE

Changes in cytokine and biomarker blood levels in patients with colorectal cancer during dendritic cell-based vaccination

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Abstract
Introduction. Immunotherapy based on dendritic cell vaccination has exciting perspectives for treatment of cancer. In order to clarify immunological mechanisms during vaccination it is essential with intensive monitoring of the responses. This may lead to optimization of treatment and prediction of responding patients. The aim of this study was to evaluate cytokine and biomarker responses in patients with colorectal cancer treated with a cancer vaccine based on dendritic cells pulsed with an allogeneic melanoma cell lysate. *Material and methods.* Plasma and serum samples were collected prior to vaccination and continuously during treatment. GM-CSF, IL-2, IL-6, TNF-α, IFN-γ, IL-4, IL-8, IL-1b, IL-5, IL-10, IL-12, MIP-1b, IP-10 and Eotaxin were analyzed in a multiplex assay with a Luminex 100™ instrument. CEA and TIMP-1 were analysed on ELISA platforms. *Results.* Patients achieving stable disease showed increasing levels of plasma GM-CSF, TNF-α, IFN-γ, IL-2, and IL-5. Patients with progressive disease showed significant increase in CEA and TIMP-1 levels, while patients with stable disease showed relatively unaltered levels. *Conclusion.* The increased levels of key pro-inflammatory cytokines in serum of patients who achieved stable disease following vaccination suggest the occurrence of vaccine-induced Th1 responses. Since Th1 responses seem to be essential in cancer immunotherapy this may indicate a therapeutic potential of the vaccine.

Immunotherapeutic approaches to the treatment of cancer have evolved during the last decades. Since patients with colorectal cancer (CRC) have shown natural T-cell responses against their tumor, it is assumed that CRC might be targeted by immunotherapy. Such therapy has evolved from non-specific immunotherapy to highly specific passive and active therapies [1]. Of the different approaches proposed, immunotherapy based on dendritic cells loaded with tumor antigens has shown to be promising. Numerous vaccine trials based on dendritic cells have been carried out in CRC [2] as well as in a variety of other cancers [3]. Encouraging results have been shown, primarily in malignant melanoma and non-Hodgkin's lymphoma. CRC is potentially curable by surgery alone, while oncological treatment modalities appear to prolong life for a large part of the patients. Unfortunately, 20–60% of patients with stage II–III CRC that have undergone intended curative surgery will subsequently relapse [4,5]. If the disease disseminates extensively and eliminates the chance for additional curative surgery, only palliative treatment modalities remain. The overall survival rate for disseminated CRC is less than 10% [6]. The armamentarium of oncological therapies has expanded during recent years, and has become more effective and less toxic. In spite of this, the many cases with limited treatment efficacy complicated by adverse effects and poor quality of life as a consequence of oncological treatments, are still unsolved issues [6–11]. This demand for further investigation in improved and maybe combined therapeutic options. Immunotherapy may play an important role in this setting, and cancer vaccines based on dendritic cells may have the potential as one of the modalities in future treatment options.

In order to optimize immunotherapy, in this case vaccines based on dendritic cells, and in order to

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select the right treatment for the specific patient it is necessary with an extensive registration and immune monitoring during investigational treatment. Immune monitoring can roughly be divided into cellular and humoral monitoring. Cellular immune responses can be monitored by delayed type hypersensitivity (DTH) test against the injected vaccine, tetramer analysis, lymphoproliferation, flow cytometry (FACS,) and enzyme-linked immunosorbent spot (ELISPOT) of T cells reacting with or responding against vaccine antigens and/or tumor cells [2,12]. Humoral immune responses can be monitored by detection of cytokines in plasma- and serum samples by conventional enzyme-linked immunosorbent assay (ELISA) and ELISPOT assays and also in multiplex assays in Luminex systems.

Hitherto, carcinoembryonic antigen (CEA) is the only recommended soluble biological marker in CRC [13,14]. CEA has a variety of limitations however, that requires development of new and more specific biomarkers to be included in the overall treatment of CRC. Tissue inhibitor of metalloproteinase (TIMP)-1 determined in tissue or in plasma is one such biomarker that has shown promising potential (especially in combination with CEA) in early detection of CRC. In particular, TIMP-1 may also be valuable in prediction of treatment response and monitoring of the efficacy of a given treatment [15,16].

The aim of the present study was via analyses of cytokines and tumor-related biomarkers to evaluate the immune response during treatment with a cancer vaccine based on dendritic cells pulsed with an allogeneic melanoma cell lysate in patients with CRC.

Material and methods

Plasma and serum samples were collected from our clinical phase 1 and 2 trials including patients with advanced progressive colorectal carcinoma. These trials were previously reported including detailed description of the vaccine (MelCancerVac, DanDrit Biotech, Copenhagen, Denmark), trial set-up, toxicity, safety, and clinical results [17,18]. MelCancerVac is based on dendritic cells generated from peripheral blood mononuclear cells. The dendritic cells are pulsed with a selected allogeneic tumor cell lysate expressing high levels of cancer-testis antigens also expressed by the majority of colorectal carcinomas (see expression of MAGE antigens below). Vaccines were administered biweekly without pauses (see Figure 1) and each vaccine contained $3–5 \times 10^6$ dendritic cells. Of 20 patients allocated to intervention 17 received treatments, but only 14 patients received the full first cycle of

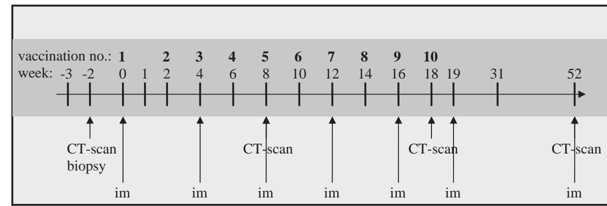

Figure 1. Vaccination schedule.
im = immune monitoring.

five vaccinations and went through the first evaluation CT scan. Eight of these patients completed the entire ten allocated vaccinations. The present study is based on the 14 patients, receiving five or more vaccinations and completing the first evaluation CT scan. Clinical responses were graded according to the response evaluation criteria in solid tumors (RECIST) [18]. At inclusion, needle biopsies (0.9 mm) were collected from distant metastases of all patients and analysed with RT-PCR for expression of six tumor associated MAGE antigens (MAGE A-1, A-3, A-4, A-6, A-10, and A-12). This procedure has previously been described in detail [17]. In the present study patients with expression of at least one of the MAGE antigens were considered MAGE+.

Peripheral blood was drawn into endotoxin-free EDTA and dry collection tubes (Becton Dickinson, NJ, USA) before and during treatment (see Figure 1). Blood samples for immune monitoring were collected before start of treatment, before the third, fifth, seventh, and finally after the tenth vaccine. A last blood sample was scheduled for week 52 (six months after the final vaccination), but since only one patient reached this point this measure is omitted. The samples were left at room-temperature for 0.5–1 h after collection. Plasma and serum were separated from blood cells by centrifugation at room temperature at $2\,500 \times G$ for 10 min, and stored in cryo tubes (Thermo Fisher Scientific, Roskilde, Denmark) at $-80°C$. After collection of all samples these were thawed and analyzed. It was ensured that all samples from a specific patient were analysed on one ELISA plate or at the same run in the Luminex analyser. This ensured absence of interassay variations in the analysis of samples from a specific patient.

The study was performed according to ICH Guidelines for Good Clinical Practice (European Directive on GCP 2001/20/EC). The study was registered at ClinicalTrials.gov (identification number: NCT00311272). Before inclusion, all patients gave their signed informed consent according to Danish law and Good Clinical Practice (GCP). The study was approved by the local ethics committee, the Danish Health Authorities, the GCP unit at

Copenhagen University Hospital, and by the Danish Data Protection Agency.

Cytokine analyses

The cytokines GM-CSF, IL-2, IL-6, TNF-α, IFN-γ, IL-4, IL-8, IL-1b, IL-5, IL-10, IL-12, MIP-1b, IP-10 and Eotaxin were analyzed in plasma using a multiplex platform (Luminex 100™). Human extra-cellular protein buffer reagent kits (Invitrogen Corp., CA, USA) were used and set up according to instructions from manufacturer. All samples were analysed in triplicates.

The Luminex multiplex assay has made it possible to analyse several cytokines in a rather quick and sensitive way. The Luminex assay is in many concerns comparable to conventional ELISA. The major difference is that the multiplex capture antibodies are attached to polystyrene beads that covalently can be bound to different antibodies in the same multiplex assay enabling the sandwich immunoassay to be read in the Luminex machine. Consequently numerous cytokines can be analysed on one plate. For measuring cytokines the Luminex multiplex assay has proven to be a quick and valid alternative to ELISA [19].

Carcinoembryonic antigen (CEA) analyses

Levels of CEA were determined in serum using a commercially available ELISA platform (IBL, Immuno Biological Laboratories, Minneapolis, MN, USA). The assay determines concentrations between 0.25 ng/ml and 75.0 ng/ml. The intra-assay and inter-assay variations are below 10%.

Tissue inhibitor of metalloproteinase-1 (TIMP-1) analyses

Levels of TIMP-1 were determined in plasma using an in-house, rigorously validated TIMP-1 ELISA [20]. Microtitre plates were coated with a sheep polyclonal antibody and detection of TIMP-1 was done with a monoclonal antibody (MAC 15) and a secondary alkaline phosphate-coupled antibody (Dako, Glostrup, Denmark). The method has previously been described in detail [20].

Statistics

Continuous variables were reported as medians (range). Changes in cytokine/protein levels of GM-CSF, IL-2, IL-6, TNF-α, IFN-γ, IL-4, IL-8, IL-1b, IL-5, IL-10, IL-12, MIP-1b, IP-10, Eotaxin, CEA, and TIMP-1 from pre-vaccination to during treatment for the entire cohort and for the four subgroups of MAGE+, MAGE−, responders (stable disease – SD), and non-responders (progressive disease – PD) were tested with Friedmans test. Differences in pre-vaccine levels were tested with the Mann-Whitney U test. Graphs were made for all analyses that showed significant changes for a subgroup during treatment. P-values less than 0.05 were considered significant. All calculations were performed using SPSS 15.0.

Results

Of the 14 patients included in this study four achieved stable disease and two of these remained stable throughout the entire study period. No complete or partial responses were achieved. Of the 14 patients 11 were considered MAGE+ and three considered MAGE−. Three of the patients achieving SD and eight of the patients with PD were MAGE+, thus one of the patients achieving SD was MAGE−.

Results from all analyses are shown in Table I. For the entire cohort there were significant increases in TNF-α, IFN-γ, IL-2, IL-5, IL-10, IL-1b, and CEA during treatment with the dendritic cell-based cancer vaccine. The general trends were initial increases after start of treatment and after day 60 decreases to levels that approximate pre-vaccine levels or lower. Exception to this trend was observed in plasma TIMP-1 levels, where a stepwise increase was shown.

There was significant difference in pre-vaccine levels of IL-6 between patients subsequently achieving SD (10 pg/ml (4–16)) *versus* patients with PD (18 pg/ml (8–40)), $p=0.036$. For all other cyto- and chemokines there were no significant differences in pre-vaccine levels neither between patients achieving SD *versus* PD nor between MAGE+ *versus* MAGE−.

For the subgroups of patients with SD there were significant changes in GM-CSF (Figure 2a), TNF-α (Figure 2b), IFN-γ (Figure 2c), IL-5 (Figure 2d), and IL-2 (Figure 2e). For patients with PD there were major increases in CEA levels ($p<0.001$) (Figure 2f) and TIMP-1 ($p=0.011$) (Figure 2g), while patients with SD had slight increases in CEA levels ($p=0.027$) (Figure 2f). The pre-vaccine levels of CEA were not significantly higher for patients with subsequent PD than for patients with subsequent SD. MAGE+ patients had significant increases in CEA ($p<0.001$), while there was no change in CEA levels for MAGE− patients (Figure 3). The initial level of CEA was higher, although not significant, for MAGE+ patients compared with MAGE− patients. TIMP-1 levels increased in patients with PD ($p=0.011$) (Figure 2g), but did not change in patients with SD. There was no significant difference

Table I. Median (range) values for the entire cohort (n = 14) and all analyses in the study. Variances in the analysed parameters are tested with Friedmans test.

	day 0 n = 14	day 30 n = 14	day 60 n = 14	day 90 n = 11	day 120 n = 11	p-value
GM-CSF (pg/ml)	27 (10–85)	34 (15–64)	33 (18–85)	30 (3–74)	28 (0–72)	ns
IL-6 (pg/ml)	16 (4–40)	21 (8–173)	28 (12–83)	20 (9–49)	24 (7–53)	ns
TNF-α (pg/ml)	14 (4–27)	21 (8–64)	19 (8–63)	17 (6–60)	17 (1–30)	0.005
IFN-γ (pg/ml)	29 (7–44)	30 (13–78)	34 (13–108)	28 (7–100)	30 (0–67)	0.017
IL-2 (pg/ml)	29 (23–50)	34 (20–51)	33 (25–52)	29 (24–53)	29 (13–50)	0.046
IL-4 (pg/ml)	55 (30–81)	47 (30–111)	50 (37–86)	51 (31–63)	44 (14–78)	ns
IL-5 (pg/ml)	33 (9–64)	37 (7–73)	52 (7–69)	45 (15–66)	37 (22–56)	0.010
IL-8 (pg/ml)	136 (60–271)	141 (30–257)	179 (38–288)	95 (52–238)	114 (57–303)	ns
IL-10 (pg/ml)	18 (12–58)	25 (10–42)	26 (12–50)	24 (9–48)	22 (9–45)	0.036
IL-12 (pg/ml)	12 (0–280)	13 (0–188)	15 (0–175)	15 (0–238)	15 (0–319)	ns
IL-1b (pg/ml)	2 (0–8)	4 (0–10)	3 (0–10)	4 (0–10)	6 (0–18)	0.007
IP-10 (pg/ml)	41 (26–88)	30 (16–119)	32 (16–101)	35 (19–63)	43 (23–84)	ns
MIP-1b (pg/ml)	29 (13–63)	26 (14–268)	28 (13–53)	32 (19–53)	31 (16–47)	ns
Eotaxin (pg/ml)	51 (19–238)	59 (21–228)	49 (23–139)	52 (16–220)	54 (17–257)	ns
TIMP-1 (ng/ml)	211 (127–378)	214 (133–802)	316 (115–1469)	289 (121–576)	332 (131–833)	ns
CEA (ng/ml)	48 (9–1260)	70 (8–1260)	93 (8–1260)	38 (10–973)	36 (9–1203)	<0.001

in pre-vaccine levels of TIMP-1 between PD and SD patients, although the tendency was that TIMP-1 was lower in patients achieving SD.

Discussion

Immune monitoring is an important step in conduction of trials dealing with the effect of immunotherapy, including trials in patients with malignant diseases. In the present study we have examined responses in blood cytokines and key proteins during treatment with a cancer vaccine based on dendritic cells pulsed with an allogeneic melanoma cell lysate. We found significant changes in TNF-α, IFN-γ, IL-2, IL-5, IL-10, IL-1b and CEA during treatment with the vaccine. Furthermore, there were differences in cytokine and protein levels during treatment in patients with stable compared with progressive disease.

General trends in the cytokine levels were initial increases from before the first vaccination to day 60 and followed by a decrease in the levels after day 60. Due to withdrawal and mortality the number of patients remaining in the calculations was reduced from 14 to 11 after day 60. The three patients, who did not proceed in the study, were all categorised as PD and MAGE+, thus the numbers of patients in the subgroups of SD and MAGE − did not change.

In the immune homeostasis of healthy individuals, both cellular and humoral immunological responses are tightly balanced between Th1 and Th2 responses [21–28]. In cancer immunotherapy the general concept is that a Th1-dominant response directed against the tumor is favourable [26]. The Th1 response may lead to activation of tumor specific CD8+ cytotoxic T-lymphocytes (CTL) capable of killing or impairing proliferation of tumor cells. The Th1 polarization of T-cells is driven by cytokines, primarily IFN-γ, TNF-α, IL-2 and IL-12 [24] whereas the cytokines related to unfavourable Th2 responses are IL-4, IL-5, IL-6, and IL-10. The polarization of T-cells induced by antigen presenting cells, of which the dendritic cell is the most potent, requires three distinctive signals. The first signal is represented by the presentation of antigen on MHC molecules to T-cell receptors. The second signal is the co-stimulatory interaction between the antigen presenting cell and the T-cell (CD80/86 – CD28). The third signal is the secretion of cytokines, that directs the polarization of T-cells [24].

Detailed analyses of the different patient subgroups with SD *versus* PD and MAGE+ *versus* MAGE − showed a large initial increase in IFN-γ and TNF-α in the subgroup of patients with SD. For IFN-γ there was a 3.5 fold increase from pre-vaccine level to day 30 and for TNF-α there was a three fold increase from pre-vaccine level to day 30. TNF-α is known to be part of the systemic inflammation and a stimulator of acute phase reactions. The primary role of TNF-α seems to be regulation of other immune cells. However, TNF-α is also considered to play an important role in induction of apoptosis via the MAPK pathways [29]. Thus TNF-α by itself might reduce survival of the tumor. TNF-α is currently used in the treatment of locally advanced soft tissue sarcomas and metastatic melanomas [30]. IFN-γ is known to be one of the most important cytokines of Th1 cells and is also secreted by the dendritic cells and NK-cells. IFN-γ has immuno-regulatory and anti-tumor properties and it upregulates MHC class I molecules thereby potentially increasing the tumor antigen targets for Th1

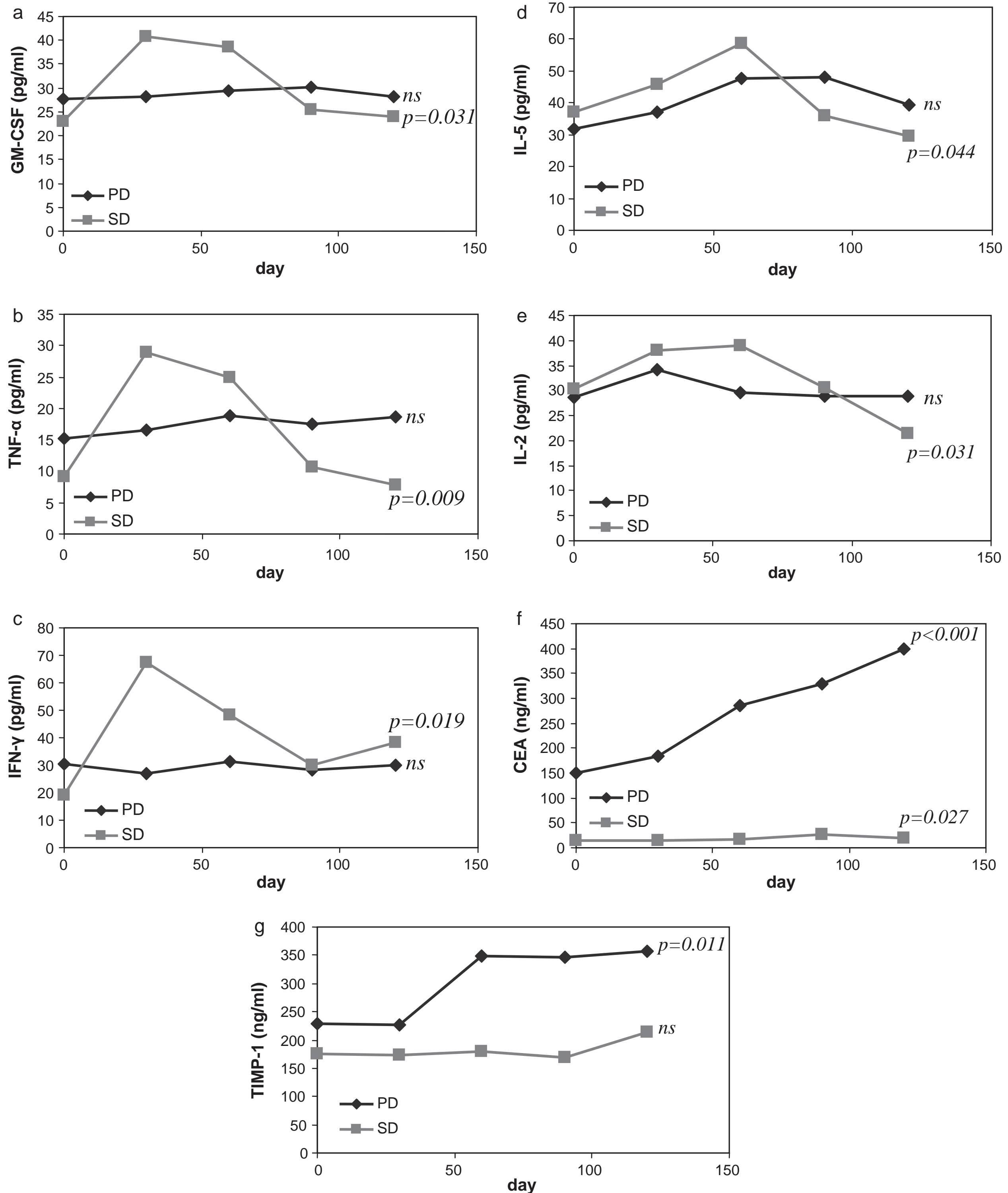

Figure 2. Patients with advanced colorectal cancer were treated with dendritic cells pulsed with allogeneic tumor cell lysate. Vaccinations were administered biweekly without pauses and with a total of 10 vaccines. Of 14 patients four achieved stable disease. Blood samples for immune monitoring were collected before start of treatment, before the third, fifth, seventh, and finally after the tenth vaccine. For patients with SD there were significant changes in GM-CSF (Figure 2a), TNF-α (Figure 2b), IFN-γ (Figure 2c), IL-5 (Figure 2d), and IL-2 (Figure 2e), 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 (Figure 2f), although the levels were numerically higher in patients with PD (Figure 2f). There was not significant difference in pre-vaccine CEA levels between SD and PD. For patients with PD TIMP-1 (Figure 2g) changed significantly, whereas patients with SD did not experience significant changes during the study period.
PD = progressive disease, SD = stable disease, ns = non-significant.

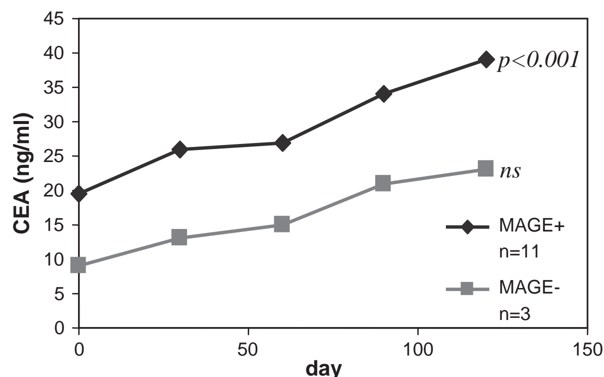

Figure 3. Patients with advanced colorectal cancer were treated with dendritic cells pulsed with allogeneic tumor cell lysate. Vaccinations were administered biweekly without pauses and with a total of 10 vaccines. Of the 11 MAGE+ patients, three achieved stable disease and out of the three MAGE− patients, one achieved stable disease, thus, the remaining nine patients had progressive disease. For MAGE+ patients there was significant change in CEA, $p < 0.001$, while there was no significant change for MAGE− patients.

driven CTLs. Some of the effects are suppression of Th2 cell activity, enhancement of NK-cell activity, and enhanced lysosome-activity in macrophages [31]. The initial increases in IFN-γ and TNF-α could indicate a therapeutic potential of the vaccine because they promote a Th1-dominant immune response with cytotoxic activity. However, due to the small number of patients and since no partial or complete responses were observed among the patients, these results have to be interpreted with caution. The decreases in both IFN-γ and TNF-α after day 60 could indicate that sufficient persistency of the immunological response are not achieved. A small, but significant change has been observed in IL-5 for patients achieving SD. IL-5 is associated with a Th2 response.

MAGE expression is normally only expressed by embryonic cells, spermatogonias and oocytes and CEA is normally only expressed by embryonic cells. Expression of MAGE and CEA in neoplastic cells reflects cellular de-differentiation which is in line with the observation that CEA levels are higher in MAGE+ patients. During treatment, CEA-levels increased significantly in patients with PD and SD, but to a smaller extend in the latter group of patients. CEA increased significantly in MAGE+ but not in MAGE− patients and the pre-vaccine level of CEA tended also to be higher in MAGE+ than in MAGE− patients. In addition, our CEA data suggest that expression of MAGE antigens is associated with larger tumor masses, since the CEA level is high in MAGE+ patients. These data suggest that our vaccine therapy may be more effective in patients with large MAGE+ tumor masses than in patients with little or no MAGE expressing tumor masses. Only three of the 11 patients with MAGE+ tumors achieved SD; thus MAGE positivity does not necessarily lead to a response. This DC vaccine employed is based on an allogeneic tumor cell lysate expressing MAGE antigens and presumably a multitude of other tumor associated antigens of relevance for triggering an anti-tumor immune response. The expression of MAGE by tumors may therefore more indicate de-differentiation of tumor cells than their ability to be recognized by vaccine-induced cytotoxic T-cells.

TIMP-1 has an inhibitory role against most of the known matrix metalloproteinases (MMPs), which are a family of extracellular matrix degrading enzymes that are involved in all stages of tumor progression and play a central role in tumor invasion and metastases [32,33]. TIMP-1 seems to have an anti-apoptotic function, presumably both as a result of the inhibition of MMPs and independently of the MMPs [16,34]. There was no difference in TIMP-1 levels between MAGE+ and MAGE− patients, and there was no significant difference in the pre-vaccine level of TIMP-1 between patients achieving SD or PD, respectively.

CEA is a known tumor marker in colorectal cancer [35–39]. It is widely used in daily clinical practise, especially in the follow-up after treatment to show activity (a de-differentiation of cells) in the disease. TIMP-1 has been proposed as a new tumor marker, maybe in combination with CEA [15,16,33–35,40–45]. We have shown that TIMP-1 levels before vaccination tend to be higher, though insignificantly, in patients subsequently achieving PD, and that TIMP-1 levels increased significantly during treatment with this dendritic cell- based cancer vaccine in patients with PD, while it remained stable in patients achieving SD. This observation supports that TIMP-1 might be used in the immunomonitoring during treatment with dendritic cell based vaccines, but these results need to be confirmed in larger trials and in trials with patients responding clinically with partial or complete responses.

In conclusion, the present study indicates that this dendritic cell based cancer vaccine initiates favourable anti-cancer responses of the immune system. The patients, who achieved SD showed simultaneously increased levels in peripheral blood of TNF-α, IFN-γ, and IL-2, which reflect polarization towards Th1 responses that potentially are directed against tumor cells.

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