Cancer Immunol Immunother (2007) 56:2003–2016
DOI 10.1007/s00262-007-0299-y

ORIGINAL ARTICLE

Induction of tumor-specific T-cell responses by vaccination with tumor lysate-loaded dendritic cells in colorectal cancer patients with carcinoembryonic-antigen positive tumors

Ayala Tamir · Ernesto Basagila · Arash Kagahzian · Long Jiao · Steen Jensen · Joanna Nicholls · Paul Tate · Gordon Stamp · Farzin Farzaneh · Phillip Harrison · Hans Stauss · Andrew J. T. George · Nagy Habib · Robert I. Lechler · Giovanna Lombardi

Received: 2 June 2006 / Accepted: 7 February 2007 / Published online: 23 February 2007

Abstract
Background Dendritic cells (DCs) are the most effective antigen-presenting cells. In the last decade, the use of DCs for immunotherapy of cancer patients has been vastly increased. High endocytic capacity together with a unique capability of initiating primary T-cell responses have made DCs the most potent candidates for this purpose. Although DC vaccination occasionally leads to tumor regression, clinical efficacy, and immunogenicity of DCs in clinical trials has not been yet clarified. The present study evaluated the safety and effectiveness of tumor-lysate loaded DC vaccines in advanced colorectal cancer (CRC) patients with carcinoembryonic antigen (CEA) positive tumors.
Results Six patients HLA-A*0201-positive were vaccinated with autologous DCs loaded with tumor lysates (TL) together with tetanus toxoid antigen, hepatitis B, and influenza matrix peptides. Two additional patients were injected with DCs that were generated from their sibling or parent with one haplotype mismatch. All patients received the vaccines every 2 weeks, with a total of three intra-nodal injections per patient. The results indicated that DC vaccination was safe and well tolerated by the patients. Specific immune responses were detected and in some patients, transient stabilization or even reduction of CEA levels were observed. The injection of haplotype mismatched HLA-A*0201-positive DCs resulted in some enhancement of the anti-tumor response in vitro and led to stabilization/reduction of CEA levels in the serum, compared to the use of autologous DCs.
Conclusion Altogether, these results suggest that TL-pulsed DCs may be an effective vaccine method in CRC patients. Elimination of regulatory mechanisms

A. Tamir · H. Stauss · A. J. T. George
Department of Immunology,
Imperial College at Hammersmith Hospital, London, UK

E. Basagila · A. Kagahzian · L. Jiao · S. Jensen · J. Nicholls · N. Habib
Liver Surgery Section, Department of Surgical Oncology and Technology, Imperial College at Hammersmith Hospital, London, UK

P. Tate
Department of Radiology,
Imperial College at Hammersmith Hospital, London, UK

G. Stamp
Department of Histopathology, Faculty of Medicine,
Imperial College at Hammersmith Hospital, London, UK

F. Farzaneh
Department of Molecular Biology, King's College London,
Guy's Hospital Campus, London, UK

P. Harrison
Department of Medicine,
King's College London,
Guy's Hospital Campus, London, UK

R. I. Lechler · G. Lombardi (✉)
Immunoregulation Laboratory,
Department of Nephrology and Transplantation,
Guy's Hospital, King's College London,
Guy's King's and St. Thomas School of Medicine,
5th Floor Thomas Guy House, SE1 9RT London, UK
e-mail: giovanna.lombardi@kcl.ac.uk

Present Address:
A. Tamir
Research Department,
Puget Sound Blood Center,
Seattle, WA 98104, USA

as well as adjustment of the vaccination protocol may improve the efficacy of DC vaccination.

Keywords Dendritic cells · Carcinoembryonic antigen (CEA) · Immunotherapy

Introduction

Colorectal cancer (CRC) is a common type of cancer, with one million cases diagnosed each year. It has a poor prognosis with 60% survival rate at 5 years. CRC is treated by surgery, chemotherapy/radiotherapy, and with anti-proliferative drugs [1]. However, if these treatments fail there are no effective alternative therapies. Over the past few years, there has been increased understanding of the progression of CRC from adenoma, a benign epithelial tumor, to carcinoma, a malignant tumor, thereby enabling the development of novel therapeutic strategies [2]. Among these are immunotherapy methods, which aim to target CRC-related tumor antigens [3].

The most commonly expressed tumor-associated antigen on gastrointestinal carcinomas is the carcinoembryonic antigen (CEA). This glycosylated oncofetal protein plays a key role in differentiation and maintenance of cells and promotes intracellular adhesion. CEA is found in high levels at early stages of the embryonic period (9–14 weeks) and is expressed at low levels on a variety of normal human cells. However, it is found at abnormally high levels in a variety of malignant tumors, including cancer of the colon, stomach, small intestine, rectum, pancreas and liver as well as breast, and non-small lung carcinomas [4, 5]. In addition, expression of CEA has been correlated with metastasis [6–8]. CEA has been used as a target for immunotherapy not only because it is highly expressed on tumor cells, but also because it contains multiple epitopes that can bind to numerous HLA class-I molecules [9, 10].

Dendritic cells (DCs) play a crucial role in immunity, as they are highly specialized in the capture and presentation of antigen to T cells [11, 12]. These special properties of DCs enable them to initiate or modulate immune responses against microbial as well as tumor and self-antigens [13–15]. During the last decade, DCs have been increasingly used in immunotherapy trials for the treatment of different types of cancer. In vitro, they were shown to effectively process and present tumor antigens via the distinctive mechanism of cross-priming. Under some circumstances, they display efficient effector functions against cancer cells and activate resting T cells to release IFN-γ and to trigger anti-tumor cytolytic activity [16–18]. Several DC-based clinical trials have demonstrated potent immunological and some clinical responses [19–21]. A variety of DC vaccines have been tested against CEA expressing tumors, among which are DCs pulsed with CEA peptides (CEApep) [21–23], and CEA-mRNA-loaded or transfected DCs [24–26]. Although tumor and peripheral blood-based immune responses were demonstrated in these studies, the clinical responses were minor. In order to enhance the diversity of the immune response and to engage multiple CD4 and CD8 epitopes, tumor lysate (TL)-pulsed DC-based cancer vaccines have been used for the treatment of various types of cancer [27–30]. Recently, application of this approach to the treatment of CEA-expressing tumors was tested both in vitro and in vivo [31, 32], however, the later studies mostly monitored clinical rather than antigen-specific responses.

In the present study, the safety and efficacy of TL-loaded DCs vaccination in CRC patients with CEA-expressing tumors was evaluated. Autologous and HLA mismatched DCs were used. DCs were injected into the patient's uninvolved inguinal lymph nodes, every 2 weeks, for a total of three vaccinations. Immune responses as well as clinical outcomes were evaluated before, during and after the administration of the vaccines.

Materials and methods

Patients

Eight patients, HLA-A*0201 positive were enrolled in this study and were recruited from Hammersmith Hospital, London, UK. All patients had histologically proven colorectal liver metastases, which were unsuitable for any further radiotherapy, chemotherapy, or surgery. In addition, all patients demonstrated evidence of progressive disease, with over-expression of CEA in the blood serum. Availability of a healthy sibling or parent was required for patients who received haplotype mismatched DCs. Patients with evidences of autoimmune disease, active infection, acute or chronic hepatitis or human immunodeficiency virus (HIV) were excluded from the study. Patients who have had no Hepatitis B (HB) were defined negative for this disease, however, it was not recorded whether they were vaccinated against it or not. The selected patients were chemotherapy or radiotherapy free at least 6 weeks before the start of vaccination. The protocol was approved by the local ethics committee. Signed informed consent was obtained from each patient and donor

(when appropriate). The list of all patients who participated in this study with their clinical data is presented in Table 1.

Generation of clinical graded DCs

An amount of 150 ml of peripheral blood was taken from the patient or the donor at the start of the study and heparinized. A further 20 ml of non-heparinized blood was also taken from the patients, for the preparation of serum. DCs were generated from the adherent population of peripheral blood mononuclear cells (PBMCs) and cultured in X-VIVO 15 (BioWhittaker, Walkersville, MD, USA) and Gentamycin (20 μg/ml) (Hoechst Marion Roussel, Kansas City, MO, USA) supplemented with 2% autologous plasma and with GM-CSF and IL-4 (20 ng/ml each, both from First-Link, Brierley Hill, UK). All the reagents were good manufacturing practice (GMP)-approved. On day 5, DCs were harvested and pulsed overnight with TL (100 μg/ml) and 0.08 IU/ml tetanus toxoid (TT) (Adsorbed Tetanus Vaccine, Evans Vaccines Ltd., Liverpool, UK). On the following day, DCs were pulsed for 2 h with 2.5 μg/ml HB HLA-A2-binding peptide (FLLSLGIHL) and 2.5 μg/ml influenza virus matrix HLA-A2 binding peptide (MP) (GILGFVFTL) (both from Bachem Ltd., St. Helens, Merseyside, UK). The cells were then divided into three cryovials (one for each vaccination) and stored at −140°C.

Preparation of tumor lysate

A sample of liver metastases was obtained from the patients using ultrasound guided transcutaneous biopsy. The biopsy cores were divided into minute pieces. Small volume of normal saline was added and the mixture was passed through a 19G needle, attached to a 5 ml syringe, several times, until the passage of the mixture occurred without difficulty. The process was repeated with 21G, 23G, and if possible 25G needle. The entire mixture was placed in liquid nitrogen until frozen, and then thawed in a water bath at 42°C. The freezing and thawing was repeated for a total of five times. The sample was passed through another 23G or 25G needle to disperse any clumps. The tube was sealed and irradiated at 10,000 rads, then stored at −140°C.

Vaccination protocol and patients monitoring

Antigen-pulsed autologous or haplotype-mismatched DCs were thawed, extensively washed and suspended in serum free medium X-VIVO 15 (Bio Whittaker) containing 1% autologous plasma. The vaccine was injected under ultrasound guidance into a lymph node in the inguinal region. Patients were vaccinated at three time points normally 2 weeks apart between each vaccination (days 0, 14, and 28). Whole blood (20 ml) was collected from the patients before each injection and after 42 days from the start of the trial, to evaluate immune response before the trial, and after the first, second, and third vaccinations. PBMCs were purified from these blood samples and cryopreserved until further use. For the immunological monitoring, DCs were generated in serum free medium supplemented with 2% human serum (HS) in the presence of GM-CSF and IL-4 (20 ng/ml each, both from FirstLink). On days 5 and 6, DCs were pulsed with the various peptides/antigens and TL as described in the preparation of the vaccine section above. Thirty-six hours after pulsing, DCs were analyzed or used in functional assays. In each assay, all samples from each patient were tested together. In addition, serum was generated at each time point from 10 ml of non-heparinized patient's blood. CEA Elisa kit was used to measure periodical CEA levels in the serum (Oxford Biosystems, Wheatley, UK). Clinical monitoring included evaluation of a tumor biopsy that was taken under computerized tomography (CT) scan before and after vaccination.

Table 1 Baseline patient data

Patient number	Gender	Age	CEA level on entry (ng/ml)	Albumin (g/l)/bilirubin levels (μmol/l) on entry	Hb (g/dl) /WCC (WCC/l) levels on entry	Allo/auto
P02	Female	49	209	32/7	11/7	Auto
P05	Male	62	296	37/9	16/7	Auto
P06	Male	72	1,041	32/32	10/13	Auto
P04	Female	56	101	38/13	15/6	Allo
P08	Male	75	116	38/8	16/11	Auto
P19	Male	56	5,965	36/17	13/8	Auto
P09	Male	64	3,899	33/12	12/8	Allo
P20	Female	62	271	34/9	12/10	Auto

Pre-vaccination data of the patients in the present study

Flow cytometry analysis

Dendritic cells were stained on day 6 of culture with various antibodies for 30 min at 4°C. Analysis was performed by using FACSCalibur flow cytometer and CellQuest Software (Becton Dickinson, Oxford, UK). The following mouse-anti human Fluorescein Isothiocyanate (FITC)-labeled monoclonal antibodies were used: anti-CD11c (DAKO, Glostrup, Denmark), anti-HLA-DR (Sigma, St Louis, MO, USA) anti-CD86, and anti-CD83 (both from Caltag, Burlingame, CA, USA). Isotype-matched-irrelevant antibodies: anti-IgG1 (Sigma) and anti-IgG2α (Pharmingen, San Diego, CA, USA) were used as negative controls.

Evaluation of antigen uptake by DCs

The capacity of DCs to capture antigen via the mannose receptor or by macro-pinocytosis was measured by incubating DCs (10^5 cells per sample) for different time periods at 37 and 4°C (negative controls) with either FITC-conjugated Dextran (FD) or Lucifer Yellow (LY) (both from Sigma, Dorset, UK) at a final concentration of 1 mg/ml. Endocytosis was terminated by washing cells in ice-cold PBS-containing 0.1% sodium azide and 1% FCS. Cells were then analyzed by flow-cytometry as above. The staining results of samples incubated with FITC-Dextran (FD) or LY at 4°C were subtracted from the results of samples stained at 37°C.

ELISpot

Day 5 DCs (5×10^4 cells per well) that were obtained before, during and after the vaccination period were separately pre-pulsed in tubes with 10 μg/ml rhCEA antigen (CEAag) (Protein Sciences Corporation, Meriden, CT, USA), 100 μg/ml CEApep (Gift of Prof. J Schlom, NIH, USA), TL, TT antigen, MP peptide, and HB peptide (concentrations indicated above) or were left unpulsed, and incubated at 37°C. The cells were then transferred to multi-screen ELISpot filtration plates (Millipore, Bedford, MA, USA), which were pre-coated with anti-IFN-γ or IL-4 mAb (Mabtech, Nacka, Sweden). Autologous or allogeneic T cells (2×10^5 cells per well) were then added (as indicated) to the DCs. Unstimulated T cells were used as negative control and positive controls were T cells stimulated with IL-2 (10 U/ml) and phytohaemagglutinin (PHA) (2 μg/ml). After incubation for 36 h at 37°C, cells were washed extensively and stained with a biotinylated anti-IFN-γ or IL-4 mAb (Mabtech) for 2 h followed by addition of streptavidin-alkaline phosphatase for 45 min. After extensive washing, the substrate solution BCIP/NBT (BioRad, Richmond, CA, USA) was added to allow color development. Spots were counted using an AID ELISpot reader system (Autoimmun Diagnostika, Strassberg, Germany). The numbers of spots obtained with unpulsed DCs were subtracted from the numbers of spots obtained with the corresponding DCs pulsed with antigens or peptides.

Tetramer staining

The following HLA-A*0201 tetramers (PE) were used in this study: MP (GILGFVFTL) (A*0201 58–66), HB (FLLSLGIHL) (A*0201 573–581), and two epitopes of the CEA: C-1 (YLSGANLNL) (A*0201 571–579) and C-2 (GVLVGVALI) (A*0201 694–702) (all from Proimmune, Oxford, UK). T cells (10^6 cells per staining) were incubated with the tetramers for 20 min at room temperature. Following washing with FACS buffer, cell were co-stained with mAbs specific for CD3 (APC) and CD8 (FITC), incubated on ice for 30 min, washed and analyzed by flow cytometry. The percentage of tetramer positive cells has been calculated in respect to $CD3^+/CD8^+$ T cells.

Results

Characterization of patient DCs

The efficacy of DC-based immunotherapy is dependent on the functional activity of DCs. We assessed the function of DCs generated from blood monocytes of eight cancer patients before and during vaccination. DCs were evaluated 6 days after isolation for the expression of CD11c, CD86, HLA-DR, and CD83 (Fig. 1a). $CD11c^{high}CD83^{low}$ DCs were generated from the patients before the start of vaccination and had a similar phenotype to DCs generated from healthy individuals (Fig. 1b). DCs obtained from patients following the first and second vaccinations generally displayed a more mature phenotype, with increased expression of CD86 and HLA-DR, similar to healthy individual LPS-stimulated DCs. In contrast, DCs generated after the third vaccination displayed a phenotype similar to the DCs obtained before vaccination or from healthy controls. As the number of DCs generated was very limited, phenotype was obtained for two patients only.

In the preparation of the vaccine, DCs were pulsed in vitro with autologous tumour lysate before being injected into the patients. Patient DCs at day 5 of culture were analyzed by flow cytometry to assess their ability to capture antigen using either FITC-dextran (Fig. 2a) or Lucifer-Yellow (LY, Fig. 2b). Patient DCs

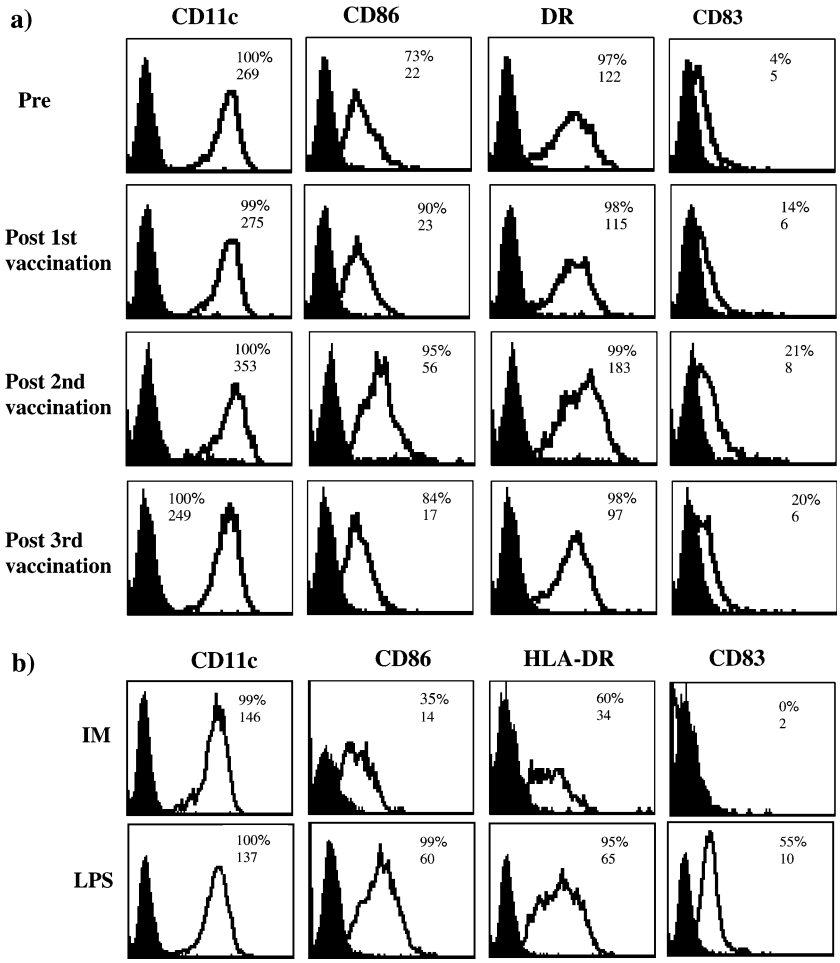

Fig. 1 Phenotype of patients DC. DCs were cultured as described in Materials and methods. On day 6, DCs were stained with mAbs specific for the listed cell surface molecules. Expression of these molecules was analyzed by flow cytometer. *Filled curves* represent staining with isotype-matched controls while *unfilled curves* represent staining with specific antibodies. *Upper numbers* represent percentages of positive cells and lower numbers represent mean fluorescence. **a** DCs were derived from patient number 2 and the results are representative of experiments with two patients. **b** DCs were derived from a healthy individual and are representative of at least five experiments

were as efficient at antigen capturing as DCs from healthy individuals, as shown in Fig. 2.

Evaluation of vaccination-related toxicity

All the patients were monitored for toxicity or autoimmunity, during and following the vaccination period. Our evaluation of toxicity was based on the NCI recommendations [33]. In each patient, body temperature, blood pressure, heart rate, and general well-being were monitored. Patients were also monitored for the appearance of signs typical of common autoimmune malignancies such as Sjogren's syndrome or Systemic Lupus Erythematosus (SLE). There was no indication of toxicity or autoimmune reactions in any of the patients, during and following the trial.

Evaluation of the immune response

We monitored the immune response in the patients before, during and following DC vaccination. HB-negative HLA-A*0201-positive CRC patients were vaccinated with either autologous (patients 2, 5, 6, 8, 19, and 20) or allogeneic (patients 4 and 9) DCs, pulsed overnight with TL and TT antigen and then for 2 h with HLA-A2 binding peptides. Patient PBMCs were obtained before the first vaccination (pre) and 2 weeks after each vaccination (post-first, post-second, and post-third vaccinations) and then cryopreserved. In patients 4 and 9, who had stable disease, PBMCs were also obtained 3 months from the start of the study. Results from patient 2, post-second vaccination were not obtained due to a technical problem. Frozen blood samples from each patient at different time points were analyzed on the same day.

Analysis of cytokine production during the vaccination by Elispot

Cytokine production (IFN-γ and IL-4) were measured by ELISpot, in order to assess whether DC vaccination in CRC patients could induce specific immune responses. Patient DCs were pulsed separately with three antigenic preparations (TL, CEA, and TT) and three synthetic peptides (MP, CEA, and HB) as described in Materials and methods (that was described

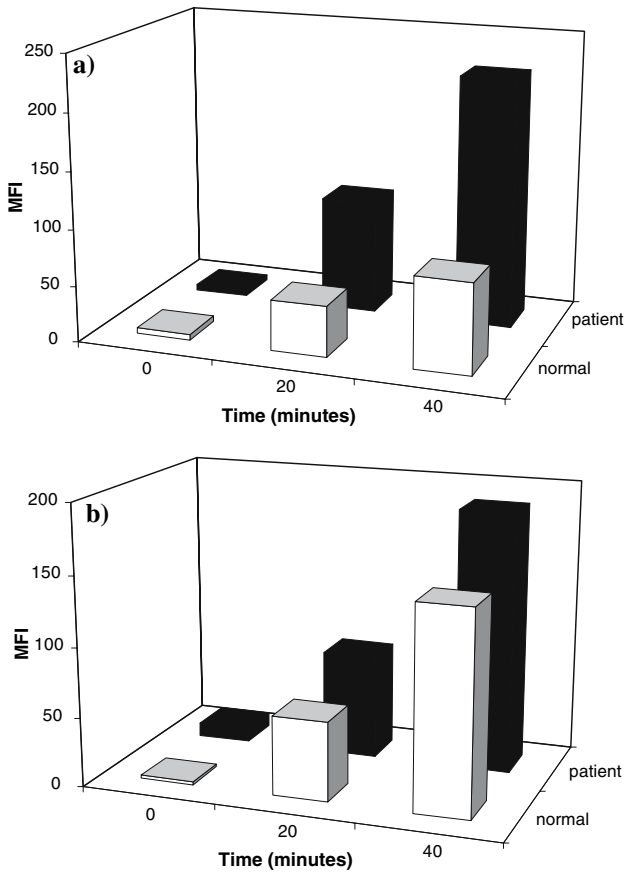

Fig. 2 Patient DCs were capable of capturing antigen. DCs were generated from a patient and from a healthy individual. On day 5, DCs were harvested and incubated with FD (**a**) and LY (**b**) for different periods of time, at 37 and 4°C. DCs were then acquired by FACS for the analysis of antigen uptake, presented by mean florescence intensity (*MFI*). The results obtained with FITC-Dextran or Lucifer Yellow staining at 4°C were subtracted from the staining results at 37°C. The data are representative of experiments with two patients and of at least five experiments with healthy individuals

already). As expected, the positive controls displayed the maximum numbers of IFN-γ and IL-4 spots (data not shown) (part of this text was added to figure legends). ELISpot results of IFN-γ (Fig. 3) and IL-4 (Fig. 4) production show that for all patients (except for patients 5 and 19 for whom no ELISpot data were obtained for technical reasons), IFN-γ production was observed in response to one or more of the antigen/peptide-pulsed-DC conditions. IL-4 was undetectable in patients 4 and 20 to any of the antigenic preparations. In response to TT-pulsed DCs (Figs. 3a, 4a), a significant IFN-γ production was detected in three patients (patients 2, 4, and 9), but IL-4 release was observed in patient 8 only (Fig. 4a). Interestingly, two of the three patients that responded to TT, received DCs from an allogeneic source (patients 4 and 9). Stimulation with MP-pulsed DCs induced production of IFN-γ (Fig. 3b) and IL-4 (Fig. 4b) in the same patients (patients 2 and 6.) but at different levels. In contrast, HB-pulsed DCs predominantly induced IL-4 production (Fig. 4c) which was about eight times higher in patients 6 and 8 compared to their IFN-γ production (Fig. 3c).

In response to TL-pulsed DCs, IFN-γ production was observed in only two patients (patients 2 and 6) (Fig. 3d) while four patients (patients 2, 6, 8, and 9) produced IL-4. However, in patient 9, IL-4 production was already present before vaccination (Fig. 4d). The enhanced response to tumor antigens displayed by IFN-γ production was further confirmed in patient 2 in response to CEAag-pulsed DCs (Fig. 3e) and in patient 6 to both the CEAag and CEApep (Fig. 3f). In patient 6 only, IL-4 was produced after vaccination in response to both CEA antigen and peptides (Fig. 4e, f). In most patients, though, the antigen-specific response was down-regulated toward the end of the trial.

Analysis of tetramer positive T cells during the vaccination

T cells derived from each patient and after each vaccination were also stained with four different tetramers (MP, HB, and two CEA peptide sequences) and analyzed by flow cytometry. Figure 5 represents the complete tetramer staining for patient 6. In this patient, $CD8^+$ T cells were detected in staining with C-1 tetramer before the trial and their number increased during the vaccination period. $CD8^+$ T cells positive for C-2, MP, and HB tetramers were also detected after the first vaccination but the response was down-regulated over subsequent vaccinations.

In Fig. 6, the response to all antigenic preparations is presented as percentage of positive cells calculated. In some patients, no positive tetramer staining was obtained in some stages during the trial. Three of eight patients (patients 2, 4, and 5) had a high level (2.5, 3.3, and 4.2%, respectively) of MP tetramer positive cells before the start of the trial (Fig. 6a). While during the trial the percentage of tetramer positive T cells increased in patients 2 and 4 (32 and 22%, respectively), in patient 5 it diminished. The most striking response was seen in patient 19 in whom, after the second vaccination, the population of MP tetramer positive cells was sixfold higher than before vaccination. However, after the third vaccination, these levels fell to background levels. Patient 20 had an essentially unaltered number of MP-tetramer positive T cells up to the third immunization. Similar to MP tetramer staining, many patients had a detectable percentage of T cells specific for HB-tetramer before the trial (Fig. 6b). Only

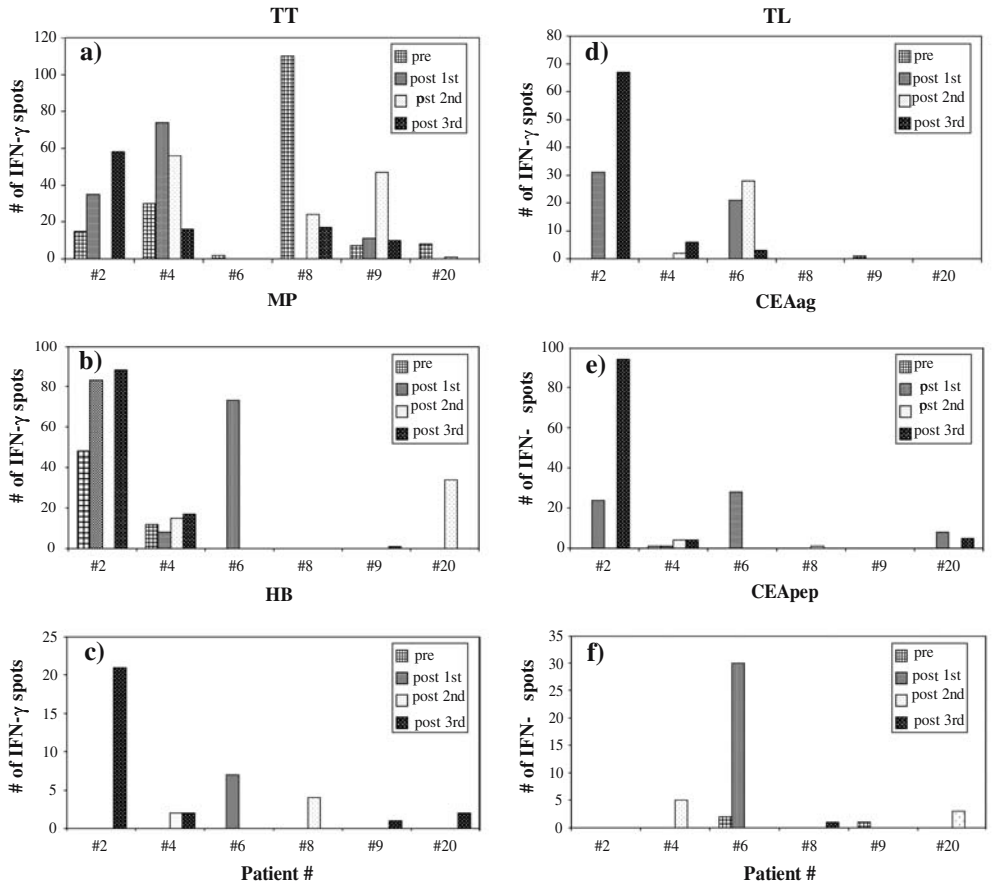

Fig. 3 Summary of IFN-γ production in ELISpot from T cells stimulated with antigen-pulsed DCs. Day 5 DCs were pulsed separately with tumor lysates (*TL*), tetanus toxoid antigen (*TT*), CEA antigen (*CEAag*), CEA peptide (*CEApep*), hepatitis B (*HB*) peptide, and matrix peptide (*MP*) as described in Materials and methods. Some DCs were left unpulsed (cDCs). DCs ($5 - 6 \times 10^4$) were then co-cultured in ELISpot plates with autologous T cells (2×10^5 T cells/well). Background control of cytokine production from unpulsed cells was subtracted from the data obtained with the corresponding peptides/antigens. Where the adjusted values were below zero, the values were expressed as zero in the figures

patient 4 exhibited enhanced HB-positive staining at the end of the trial (0.72%) while a similar trend was seen in patients 2, 9, 19, and 20 but up to the second vaccination only.

Tetramer staining to detect tumor specific T-cell responses was performed with tetramers expressing two different CEA epitopes, GVLVGVALI (C-1) and YLSGANLNLI (C-2). C-2 is considered to be the immunodominant epitope and it has been previously used for the generation of CEA-specific cytotoxic T cells in vitro [34] as well as in vaccination trials in vivo [21, 35]. This epitope (C-2) was used in this study as the synthetic peptide in the ELISpot assays. Staining was more consistent among the different patients with C-1 tetramer (Fig. 6c). Some T cells specific for C-1 and C-2 were detected before the trial with the highest level of C-1 positive staining seen in patient 2, which was further elevated during the trial. Similarly, in patient 4 and 6, the highest percentages of T cells specific for C-1 were observed at the end of the trial. In patient 9, a threefold increase was found up to the second vaccination only, with a major down-regulation afterward. Finally, patient 19 and 20 exhibited a substantial decrease in C-1-tetramer positive T cells during the trial. As for C-2 staining, only patients 2 and 4 demonstrated high levels of positive cells. Patient 2 had 4.54% positive cells after the first vaccination, but this response was twofold lower after the last vaccination, further confirming the ELISpot responses to CEA antigen and peptide. In contrast, patient 4 maintained high levels of C2-positive staining but these were lower than the pre-trial levels. A summary of the data for all patients is given in Table 2.

Evaluation of clinical response

Elevations of circulating levels of CEA in blood serum were shown to correlate with disease progression

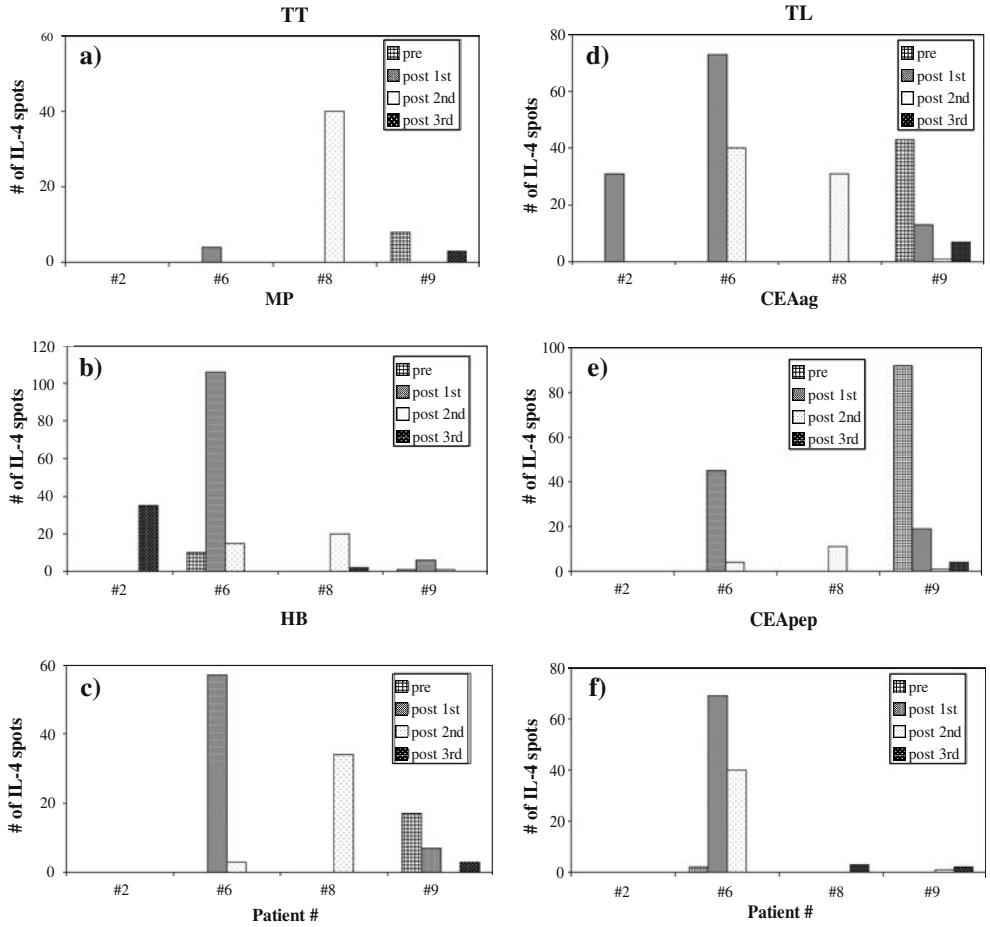

Fig. 4 Summary of IL-4 production in ELISpot from T cells stimulated with antigen-pulsed DCs. Day 5 DCs were pulsed or left unpulsed as described in the legend of Fig. 3. DCs were then washed and co-cultured in ELISpot plates with autologous T cells. Number of IL-4 spots obtained is per 2×10^5 T cells tested

[36–38]. Stabilization or partial stabilization of this marker levels in the blood serum may well indicate treatment effectiveness [39–41]. In healthy individuals, CEA is found in the blood serum in small amounts (around 5 ng/ml) and it is elevated in smokers and elderly persons [42, 43]. To see whether the immunological monitoring results correlated with the clinical outcomes, serum CEA levels were measured in each patient. The first evaluation was conducted 14 days before the trial (pre) and then following each immunization. In patients 2 and 5, there was a partial stabilization of CEA levels at different stages during the trial, between the first and second and between the second and third vaccination, respectively (Table 3). It is interesting to note that although only two patients in this study (patients 4 and 9) received allogeneic haplotype mismatched DCs, both demonstrated stabilization/reduction in their CEA levels during and/or following the vaccination period. Patient 4 had stable levels of CEA during the vaccination (but not after) and in patient 9, an initial amplification of CEA expression was demonstrated up to the second vaccination but was then decreased by 25 and 32% after the third vaccination and post-42 days, respectively. In all other patients, augmentation of CEA levels in the serum was shown. Finally, CT scans of patient tumors were conducted before and after vaccination. Progressive disease was indicated in all patients before and after the vaccination period.

Discussion

In the present study, TL-pulsed DCs were administered to CRC patients with CEA expressing tumors. Autologous DCs were used in six patients and allogeneic DCs in two patients. The vaccination was performed three times into the patient's uninvolved inguinal lymph nodes, every 2 weeks. Immune responses as well as clinical outcomes were evaluated before and after the administration of the vaccines.

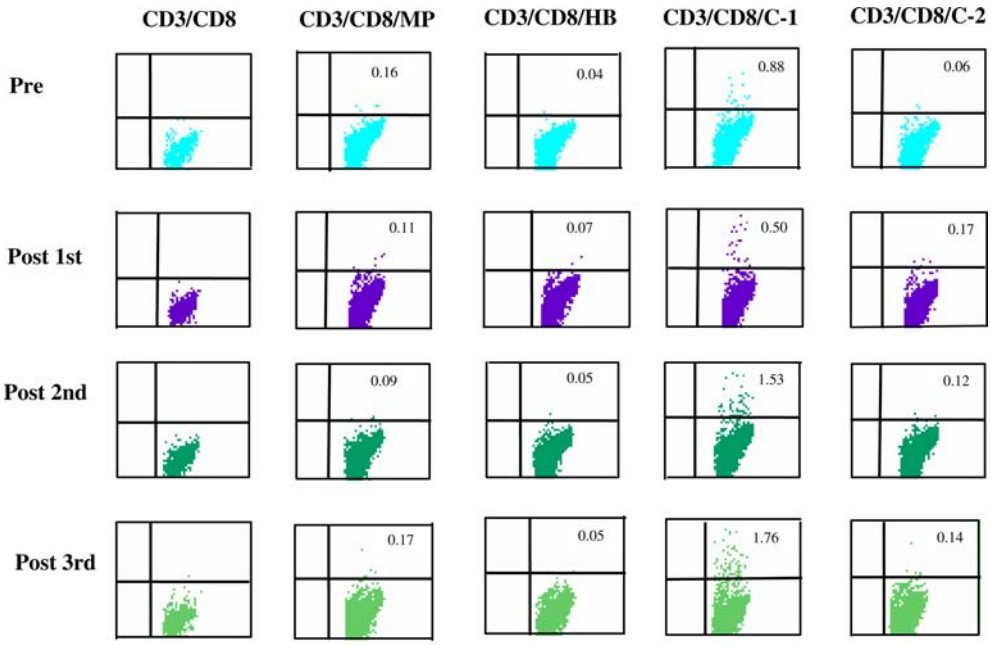

Fig. 5 Patient T cells could generate specific immune responses as was indicated by tetramer staining. T cells were generated from blood withdrawn at different stages of the trial and were co-stained with mAb specific for CD3 (FITC), CD8 (APC), and with a tetramer specific either for MP, HB C-1 or C-2. First, bright $CD3^{+}CD8^{+}$ cells were selected and gated. This population was then used to compare staining with the different tetramers (PE). The cells were acquired and analyzed by FACS. Negative controls were T cells stained with anti-CD3/CD8 only. A positive control for MP staining was a MP-specific cell line (not shown). The results in this figure were obtained in patient 6

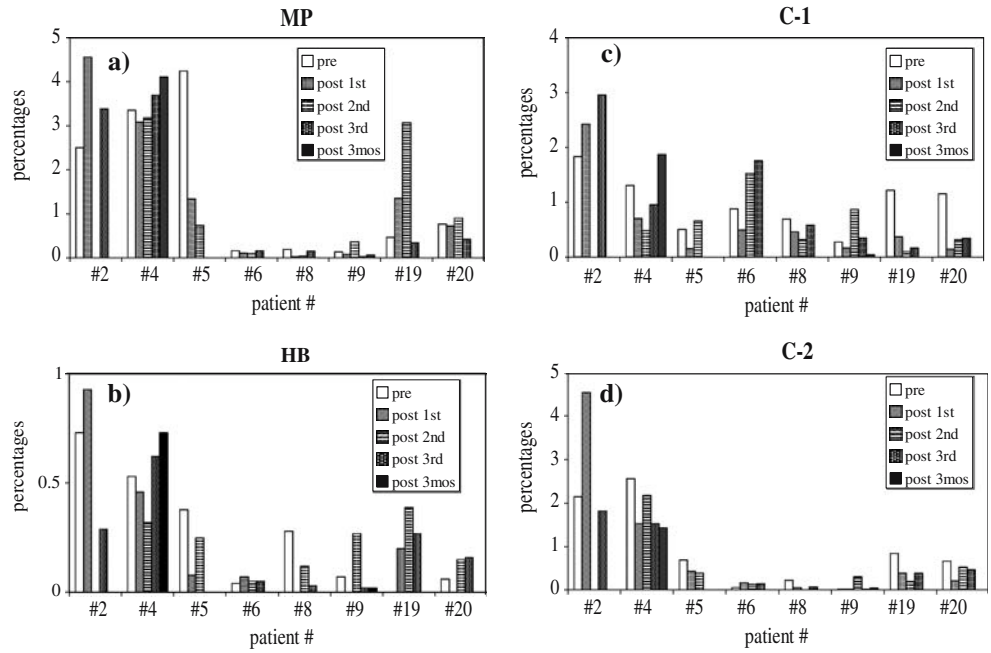

Fig. 6 Summary of T-cell staining by tetramers. Patient T cells were co-stained with specific mAb and tetramers as indicated in Fig. 5. The figure provides a summary of tetramer staining for each tetramer separately, in all the patients that were tested

First, the feasibility and safety of the DC-vaccination procedure was evaluated. The in vitro preparation of the DC vaccine was performed in a laboratory approved under the Good Laboratory Practice (GLP) regulations. No toxicity or significant side effects were reported by the patients during the trial or after it. This conclusion has been recently confirmed in another clinical trial, where TL-pulsed DCs were injected to

Table 2 Summary of all results obtained in the study

Patient number		IFN-γ ELISpot							IL-4 ELISpot							Tetramer staining				CEA
		up	ca	tl	fl	tt	hb	cp	up	Ca	tl	fl	tt	hb	cp	fl	hb	c-1	c-2	
P02	Pre				++	+			+							++	+	+	++	=
	Post-first		+	+	+	+			=							++	+	+	++	=
	Post-third	+	++	++	++	++	++		=	+	+	++				+	–	+	-	+
P05	Pre															++	+	+	+	+
	Post-first															-	–	–	-	+
	Post-second															-	-	=	-	=
	Post-third															=	-	-	-	-
P06	Pre	+			+	+							+			+		+		+
	Post-first	+	+	+	++	=	+	++	=	+	+	=	++	+	+	-	=	-	+	+
	Post-second	+	=	+	=	=	=	+	+	-	=	=	–	-	-	-	=	+	+	+
	Post-third	++	=	++	=	=	=	++	++	=	-	=	-	-	-	=	=	++	+	+
P08	Pre						+							+	+	+	+	+		
	Post-first	++					-									-	-	-	-	+
	Post-second						–		+	++	++	++	++	+	+	-	-	-	-	+
	Post-third						–	+	+	+	=	+	+	+	+	=	-	=	-	+
P19	Pre															+		+	+	
	Post-first															+	+	-	-	+
	Post-second															++	++	–	–	+
	Post-third															=	+	–	-	+
P20	Pre	+			+	++	+	+								+		+	+	+
	Post-first	++	++		+	=	+	+								=		–	–	+
	Post-second				++	=		+								+	+	-	-	+
	Post-third					=										-	+	-	-	+
P04	Pre				+											++	+	+	++	+
	Post-first				=	+										–	=	-	–	=
	Post-second				=	++										=	-	-	-	=
	Post-third				=	+										+	+	-	–	=
	Post-three mos															++	+	+	–	
P09	Pre					+				++	+					+		+		++
	Post-first					=				-	-					=		=	=	+
	Post-second					++				–	–					+	++	++	++	-
	Post-third					=				–	–					-	-	+	=	-
	Post-three mos															-	-	–	=	+

Summary of results from ELISpot (IFN-γ and IL-4), tetramer staining and assessment of CEA levels in serum. Symbols are as follow: *up* unpulsed, *ca* CEA antigen, *tl* tumor lysates, *fl* flu matrix, *tt* tetanus toxoid, *hb* hepatitis B, *cp* CEA peptide, (**+**) increased levels, (**-**) decreased levels, (**=**) no changes, double sign (++ or –) means stronger changes

Table 3 CEA levels in the patient sera

Patient	Pre-vaccination	Post-first vaccination	Post-second vaccination	Post-third vaccination	Post-42 days
2	280	420	430	710	
4	200	180	160	200	750
5	370	500	750	730	
6	200	360	600	780	
8	260	500	780	860	
9	400	540	700	550	480
19	100	160	240	400	
20	1,150	1,300	1,500	1,670	

Serum was separated from non-heparinized blood of patients. CEA levels were measured by Elisa and expressed as ng/ml

hepatocellular carcinoma patients [32]. Monitoring any potential development of autoimmunity has been of particular importance in this work because of the use of TL. TL contain multiple antigens, among which are self-antigens expressed on normal tissues, therefore a response against self-antigens could have been induced. Autoimmune syndromes such as Syogren's syndrome, which involves decreased saliva and consequently dry eyes, or SLE, which is indicated by renal failure, were not detected in these patients. Indeed, in

other studies in mice or in humans, vaccination with shared antigens has not resulted in induction of autoimmunity [19, 20, 44].

In this study, T-cell responses to tumor and control antigens were monitored by ELISpot and by tetramer staining. IFN-γ ELISpot has been a common way to assess $CD8^+$ and $CD4^+$ responses following DC vaccination against tumor antigens [45–47]. IFN-γ has been shown to be produced by peptide-specific $CD8^+$ T cells that were able to lyse target cells pulsed with tumor peptide [48]. Release of IL-4 has also been shown to contribute to immune responses against tumors [49, 50] as was observed in our study in response to TL-DCs in three of four patients. Finally, several studies have demonstrated a correlation between cytokine production in ELISpot and positive clinical outcomes [51–53].

The addition of highly immunogenic antigens has been suggested to improve the efficacy of cancer vaccines [54]. This idea has been further confirmed by Banchereau et al. who have previously reported that DC vaccination in metastatic melanoma patients rapidly induced KLH and MP specific responses that were detectable within 2 weeks, after the first DC vaccination [53]. Similarly, after the same period of time, a response against melanoma antigens was found in 5 of 11 patients. In our study, a rapid increase in tetramer positive T cells specific for tumor antigens was observed in some patients.

The efficacy of vaccination with haplotype mismatched DCs was also investigated in this study. The hypothesis behind the use of allogeneic DCs was that the alloantigens expressed by DCs would have stimulated a CD4 helper response. Unfortunately, after screening for HLA-A2 positive patients and applying inclusion/exclusion criteria among the selected patients and donors, it was possible to find suitable donors for two patients only. Interestingly, these two patients (patients 4 and 9) responded only to TT in ELIspot. TT has been broadly used in immunotherapy trials against cancer [55, 56] and in particularly against CEA-expressing tumors [57, 58]. It was found that TT-specific IFN-γ secretion was predominantly mediated by $CD4^+$ T cells and it was HLA class II-restricted [59]. In a recent trial, allogeneic DCs were used to vaccinate against metastatic renal cell carcinoma. Although allogenic immunotherapy was feasible and well tolerated, the immunogenicity of allogeneic DCs was less pronounced than that of autologous DCs [60]. However, this study demonstrates that the allogeneic-DC vaccinated patients produced antigen-specific immune responses to some of the antigens/peptides used. Moreover, stabilization/reduction of CEA levels in the sera of these two patients was observed.

Expression of CEA in the patient serum was used as a factor that defines clinical outcomes in these patients as it has been previously reported that CEA expression positively correlates with disease progression [61]. In four patients (2, 5, 4, and 9), partial or complete stabilization of CEA expression was shown. However, lack of any other clinical benefit, even partial, may indicate that this parameter cannot be regarded as a clinical factor on its own.

The main setback in this study was the down-regulation of the immune responses to tumor as well as to control antigens toward the end of the trial. This conclusion is mostly based on tetramer staining results, although a reduction in T-cell responses was also observed in ELISpot. Interestingly, in almost all cases where no $CD8^+$ T cells were detected against a certain peptide (mainly against HB) before the trial, they could be detected following vaccinations. This finding may indicate that primary responses could be induced even in patients with progressive disease. However, existing specific $CD8^+$ T cells were more likely to be down-regulated within the trial. Perhaps, DC injection every 2 weeks while being optimal for the induction of primary T-cell responses, may be too frequent for the maintenance of an existing response. Different protocols of DC vaccination have been recently used in cancer patients, however with limited success [32, 62].

The tumor microenvironment induced in progressive diseases may impose regulatory mechanisms. Terabe et al. have shown that NKT cells down-regulated anti-tumor immunity by mediating IL-13 and IL-4R-STAT6 pathways [63]. In addition, others have shown that regulatory T cell (Tr1 and $CD4^+CD25^+$ T cells) suppress immune responses against tumors [64–66]. A promising strategy in vaccination trials has been recently aimed on the transient block of regulatory T cells, by using antibodies, such as anti-CTLA-4 [67, 68] or anti-IL-10 [69, 70]. However, in such trials, signs of autoimmunity should be monitored carefully. Finally, there are increasing evidences in mice and in humans that leptin affects immunity and that in the absence of leptin, the immune response is down-regulated [71–73]. Leptin levels in the patient sera were measured and displayed a rapid decrease during the vaccination period (data not shown).

Altogether, results from this phase I study are encouraging. Because of high levels of immunosuppression normally observed in late-stage cancer patients, this vaccination method may be more beneficial in cancer patients with less advanced malignancy. Also, because our current vaccine was unable to induce long-lasting effective immune responses in the patients, we have proposed a follow-up study, which involves

removal of regulatory T cells pre-therapy with adoptive T-cell therapy in CRC patients in combination with preceded TL-pulsed autologous DC injections. This method may enable a better control of T-cell stimulation by DCs and of antigen-specific $CD8^+$ T-cell expansion.

Acknowledgments This study was supported by the Pedersen Charity Foundation.

References

1. Rougier P, Mitry E (2003) Epidemiology, treatment and chemoprevention in colorectal cancer. Ann Oncol 14(Suppl 2):ii3–ii5
2. Nicum S, Midgley R, Kerr DJ (2003) Colorectal cancer. Acta Oncol 42(4):263–275
3. Indar AA, Maxwell-Armstrong CA (2003) Active specific immunotherapy for colorectal cancer. Expert Rev Anticancer Ther 3(5):685–694
4. Goldenberg DM (1976) Oncofetal and other tumor-associated antigens of the human digestive system. Curr Top Pathol 63:289–342
5. Grunert F, et al (1983) Comparison of colon-, lung-, and breast-derived carcinoembryonic antigen and cross-reacting antigens by monoclonal antibodies and fingerprint analysis. Ann NY Acad Sci 417:75–85
6. Kodera Y, et al (1993) Expression of carcinoembryonic antigen (CEA) and nonspecific crossreacting antigen (NCA) in gastrointestinal cancer; the correlation with degree of differentiation. Br J Cancer 68(1):130–136
7. Ilantzis C, et al (2002) Deregulated expression of the human tumor marker CEA and CEA family member CEACAM6 disrupts tissue architecture and blocks colonocyte differentiation. Neoplasia 4(2):151–163
8. Duxbury MS, et al (2004) CEACAM6 gene silencing impairs anoikis resistance and in vivo metastatic ability of pancreatic adenocarcinoma cells. Oncogene 23(2):465–473
9. Nukaya I, et al (1999) Identification of HLA-A24 epitope peptides of carcinoembryonic antigen which induce tumor-reactive cytotoxic T lymphocyte. Int J Cancer 80(1):92–97
10. Tsang KY, et al (1995) Generation of human cytotoxic T cells specific for human carcinoembryonic antigen epitopes from patients immunized with recombinant vaccinia-CEA vaccine. J Natl Cancer Inst 87(13):982–990
11. Banchereau J, et al (2000) Immunobiology of dendritic cells. Annu Rev Immunol 18:767–811
12. Lanzavecchia A, Sallusto F (2001) Regulation of T cell immunity by dendritic cells. Cell 106(3):263–266
13. Macatonia SE, et al (1989) Primary stimulation by dendritic cells induces antiviral proliferative and cytotoxic T cell responses in vitro. J Exp Med 169(4):1255–1264
14. Fong L, Engleman EG (2000) Dendritic cells in cancer immunotherapy. Annu Rev Immunol 18:245–273
15. Dhodapkar MV, et al (2000) Mature dendritic cells boost functionally superior CD8(+) T-cell in humans without foreign helper epitopes. J Clin Invest 105(6):R9–R14
16. Subklewe M, et al (2001) Dendritic cells cross-present latency gene products from Epstein-Barr virus-transformed B cells and expand tumor-reactive CD8(+) killer T cells. J Exp Med 193(3):405–411
17. Herr W, et al (2000) Mature dendritic cells pulsed with freeze-thaw cell lysates define an effective in vitro vaccine designed to elicit EBV-specific CD4(+) and CD8(+) T lymphocyte responses. Blood 96(5):1857–1864
18. Fernandez NC, et al (1999) Dendritic cells directly trigger NK cell functions: cross-talk relevant in innate anti-tumor immune responses in vivo. Nat Med 5(4):405–411
19. Nestle FO, et al (1998) Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. Nat Med 4(3):328–332
20. Murphy GP, et al (1999) Phase II prostate cancer vaccine trial: report of a study involving 37 patients with disease recurrence following primary treatment. Prostate 39(1):54–59
21. Morse MA, et al (1999) A Phase I study of active immunotherapy with carcinoembryonic antigen peptide (CAP-1)-pulsed, autologous human cultured dendritic cells in patients with metastatic malignancies expressing carcinoembryonic antigen. Clin Cancer Res 5(6):1331–1338
22. Fong L, et al (2001) Altered peptide ligand vaccination with Flt3 ligand expanded dendritic cells for tumor immunotherapy. Proc Natl Acad Sci USA 98(15):8809–8814
23. Itoh T, et al (2002) Immunotherapy of solid cancer using dendritic cells pulsed with the HLA-A24-restricted peptide of carcinoembryonic antigen. Cancer Immunol Immunother 51(2):99–106
24. Nair SK, et al (1999) Induction of carcinoembryonic antigen (CEA)-specific cytotoxic T-lymphocyte responses in vitro using autologous dendritic cells loaded with CEA peptide or CEA RNA in patients with metastatic malignancies expressing CEA. Int J Cancer 82(1):121–124
25. Morse MA, et al (2002) The feasibility and safety of immunotherapy with dendritic cells loaded with CEA mRNA following neoadjuvant chemoradiotherapy and resection of pancreatic cancer. Int J Gastrointest Cancer 32(1):1–6
26. Morse MA, et al (2003) Immunotherapy with autologous, human dendritic cells transfected with carcinoembryonic antigen mRNA. Cancer Invest 21(3):341–349
27. Arroyo JC, et al (2004) Immune response induced in vitro by CD16- and CD16+ monocyte-derived dendritic cells in patients with metastatic renal cell carcinoma treated with dendritic cell vaccines. J Clin Immunol 24(1):86–96
28. Asavaroengchai W, Kotera Y, Mule JJ (2002) Tumor lysate-pulsed dendritic cells can elicit an effective antitumor immune response during early lymphoid recovery. Proc Natl Acad Sci USA 99(2):931–936
29. Griffioen M, et al (2004) Analysis of T-cell responses in metastatic melanoma patients vaccinated with dendritic cells pulsed with tumor lysates. Cancer Immunol Immunother 53(8):715–722
30. Zhao X, Wei YQ, Peng ZL (2001) Induction of T cell responses against autologous ovarian tumors with whole tumor cell lysate-pulsed dendritic cells. Immunol Invest 30(1):33–45
31. Correale P, et al (2005) Dendritic cell-mediated cross-presentation of antigens derived from colon carcinoma cells exposed to a highly cytotoxic multidrug regimen with gemcitabine, oxaliplatin, 5-fluorouracil, and leucovorin, elicits a powerful human antigen-specific CTL response with antitumor activity in vitro. J Immunol 175(2):820–828
32. Lee WC, et al (2005) Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial. J Immunother 28(5):496–504
33. US department of Health and Human Services, N.I.H National Cancer Institute (1998) Cancer therapy evaluation program, common toxicity criteria. Vol. Version 2.0. DCTD, NCI, NIH, DHHS, Bethesda
34. Tsang KY, et al (1997) Phenotypic stability of a cytotoxic T-cell line directed against an immunodominant epitope of

human carcinoembryonic antigen. Clin Cancer Res 3(12 Pt 1):2439–2449
35. Cole DJ, et al (2000) Phase I study of recombinant carcinoembryonic antigen (CEA) vaccinia virus vaccine with post vaccination carcinoembryonic antigen peptide (CAP-1) boost. Clin Lung Cancer 1(3):227–229
36. Kosugi S, et al (2004) Clinical significance of serum carcinoembryonic antigen, carbohydrate antigen 19–9, and squamous cell carcinoma antigen levels in esophageal cancer patients. World J Surg 28(7):680–685
37. Tendler A, Kaufman HL, Kadish AS (2000) Increased carcinoembryonic antigen expression in cervical intraepithelial neoplasia grade 3 and in cervical squamous cell carcinoma. Hum Pathol 31(11):1357–1362
38. Pellegrini P, et al (2000) Simultaneous measurement of soluble carcinoembryonic antigen and the tissue inhibitor of metalloproteinase TIMP1 serum levels for use as markers of pre-invasive to invasive colorectal cancer. Cancer Immunol Immunother 49(7):388–394
39. Ward U, et al (1993) The use of tumour markers CEA, CA-195 and CA-242 in evaluating the response to chemotherapy in patients with advanced colorectal cancer. Br J Cancer 67(5):1132–1135
40. Tsavaris N, et al (1993) Carcinoembryonic antigen (CEA), alpha-fetoprotein, CA 19.9 and CA 125 in advanced colorectal cancer (ACC). Int J Biol Markers 8(2):88–93
41. Yamao T, et al (1999) Tumor markers CEA, CA19-9 and CA125 in monitoring of response to systemic chemotherapy in patients with advanced gastric cancer. Jpn J Clin Oncol 29(11):550–555
42. Fischbach W, Kiel HJ (1987) Follow-up of moderately elevated serum CEA levels in "healthy patients". Cancer Detect Prev 10(1–2):109–112
43. Engaras B, et al (1999) Standard serum concentrations and normal fluctuations of CEA, CA 50 and CA 242 during twelve months in men and women aged 60–64 years without malignant disease. Eur J Surg 165(2):110–116
44. Gong J, et al (1998) Reversal of tolerance to human MUC1 antigen in MUC1 transgenic mice immunized with fusions of dendritic and carcinoma cells. Proc Natl Acad Sci USA 95(11):6279–6283
45. Morse MA, et al (2005) Phase I study of immunization with dendritic cells modified with fowlpox encoding carcinoembryonic antigen and costimulatory molecules. Clin Cancer Res 11(8):3017–3024
46. Palucka AK, et al (2005) Boosting vaccinations with peptide-pulsed CD34+ progenitor-derived dendritic cells can expand long-lived melanoma peptide-specific CD8+ T cells in patients with metastatic melanoma. J Immunother 28(2):158–168
47. Schmittel A, et al (2001) Application of the IFN-gamma ELISPOT assay to quantify T cell responses against proteins. J Immunol Methods 247(1–2):17–24
48. Lee PP, et al (1999) Characterization of circulating T cells specific for tumor-associated antigens in melanoma patients. Nat Med 5(6):677–685
49. Chakrabarti R, et al (2004) Plasmids encoding membrane-bound IL-4 or IL-12 strongly costimulate DNA vaccination against carcinoembryonic antigen (CEA). Vaccine 22(9–10):1199–1205
50. Conticello C, et al (2004) IL-4 protects tumor cells from anti-CD95 and chemotherapeutic agents via up-regulation of antiapoptotic proteins. J Immunol 172(9):5467–5477
51. Marshall JL, et al (2000) Phase I study in advanced cancer patients of a diversified prime-and-boost vaccination protocol using recombinant vaccinia virus and recombinant nonreplicating avipox virus to elicit anti-carcinoembryonic antigen immune responses. J Clin Oncol 18(23):3964–3973
52. Wang F, et al (1999) Phase I trial of a MART-1 peptide vaccine with incomplete Freund's adjuvant for resected high-risk melanoma. Clin Cancer Res 5(10):2756–2765
53. Banchereau J, et al (2001) Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res 61(17):6451–6458
54. Jager E, Jager D, Knuth A (2002) Clinical cancer vaccine trials. Curr Opin Immunol 14(2):178–182
55. Slingluff CL Jr, et al (2001) Phase I trial of a melanoma vaccine with gp100(280–288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes. Clin Cancer Res 7(10):3012–3024
56. King CA, et al (1998) DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma. Nat Med 4(11):1281–1286
57. Lund LH, et al (2003) Signal sequence deletion and fusion to tetanus toxoid epitope augment antitumor immune responses to a human carcinoembryonic antigen (CEA) plasmid DNA vaccine in a murine test system. Cancer Gene Ther 10(5):365–376
58. Rice J, et al (2001) DNA fusion vaccine designed to induce cytotoxic T cell responses against defined peptide motifs: implications for cancer vaccines. J Immunol 167(3):1558–1565
59. Mayer S, et al (2002) Analysis of the immune response against tetanus toxoid: enumeration of specific T helper cells by the Elispot assay. Immunobiology 205(3):282–289
60. Holt L, et al (2005) Allogeneic dendritic cell vaccination against metastatic renal cell carcinoma with or without cyclophosphamide. Cancer Immunol Immunother 54(7):663–670
61. Mitchell EP (1998) Role of carcinoembryonic antigen in the management of advanced colorectal cancer. Semin Oncol 25(5 Suppl 11):12–20
62. Maier T, et al (2003) Vaccination of patients with cutaneous T-cell lymphoma using intranodal injection of autologous tumor-lysate-pulsed dendritic cells. Blood 102(7):2338–2344
63. Terabe M, et al (2000) NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. Nat Immunol 1(6):515–520
64. Terabe M, Berzofsky JA (2004) Immunoregulatory T cells in tumor immunity. Curr Opin Immunol 16(2):157–162
65. Roncarolo MG, et al (2001) Type 1 T regulatory cells. Immunol Rev 182:68–79
66. Levings MK, et al (2002) Human CD25+CD4+ T suppressor cell clones produce transforming growth factor beta, but not interleukin 10, and are distinct from type 1 T regulatory cells. J Exp Med 196(10):1335–1346
67. Hodi FS, et al (2003) Biologic activity of cytotoxic T lymphocyte-associated antigen 4 antibody blockade in previously vaccinated metastatic melanoma and ovarian carcinoma patients. Proc Natl Acad Sci USA 100(8):4712–4717
68. Phan GQ, et al (2003) Cancer regression and autoimmunity induced by cytotoxic T lymphocyte-associated antigen 4 blockade in patients with metastatic melanoma. Proc Natl Acad Sci USA 100(14):8372–8377
69. Carbonneil C, et al (2004) Dendritic cells generated in the presence of interferon-alpha stimulate allogeneic CD4+ T-cell proliferation: modulation by autocrine IL-10, enhanced T-cell apoptosis and T regulatory type 1 cells. Int Immunol 16(7):1037–1052
70. Carbonneil C, et al (2004) Defective dendritic cell function in HIV-infected patients receiving effective highly active antiretroviral therapy: neutralization of IL-10 production and

depletion of CD4+CD25+ T cells restore high levels of HIV-specific CD4+ T cell responses induced by dendritic cells generated in the presence of IFN-alpha. J Immunol 172(12):7832–7840
71. Howard JK, et al (1999) Leptin protects mice from starvation-induced lymphoid atrophy and increases thymic cellularity in ob/ob mice. J Clin Invest 104(8):1051–1059
72. Lord GM, et al (2002) Leptin inhibits the anti-CD3-driven proliferation of peripheral blood T cells but enhances the production of proinflammatory cytokines. J Leukoc Biol 72(2):330–338
73. Lord GM, et al (1998) Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression. Nature 394(6696):897–901