Cancer Immunol Immunother (2002) 51: 663–668
DOI 10.1007/s00262-002-0325-z

ORIGINAL ARTICLE

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Calcitonin-specific antitumor immunity in medullary thyroid carcinoma following dendritic cell vaccination

Received: 18 February 2002 / Accepted: 1 August 2002 / Published online: 29 October 2002

Abstract In this study, we investigated the immune response following immunotherapy with calcitonin-pulsed dendritic cells (DC) in 7 patients with metastasized medullary thyroid carcinoma. After immunization with 1–5×10^6 autologous DC, significant calcitonin-specific T cell proliferation was detectable in 3 patients. Measurement of cytokine release from T lymphocytes demonstrated high post-treatment interferon-γ (IFN-γ) secretion after stimulation with calcitonin in 5 patients, one of whom experienced significant tumor regression. In contrast, antigen-specific interleukin-4 (IL-4) production was only slightly increased in 4 patients. All 7 patients developed a strong delayed-type hypersensitivity (DTH) skin reaction, which was confirmed to be mediated by infiltrating $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T cells in all 3 patients who underwent skin biopsy. This is the first study to show that a polypeptide hormone can be used to develop a DC vaccination strategy for the immunotherapy of highly malignant endocrine cancers.

Keywords Calcitonin · Dendritic cell immunotherapy · Medullary thyroid carcinoma · Peptide hormone · Vaccination

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Abbrevations *CEA* Carcinoembryonic antigen · *CTL* Cytotoxic T lymphocytes · *DC* Dendritic cells · *DTH* Delayed-type hypersensitivity · *MTC* Medullary thyroid carcinoma · *PBMC* Peripheral blood mononuclear cells

Introduction

Several endocrine carcinomas including medullary thyroid carcinoma (MTC), a neoplasm of the calcitonin-producing parafollicular C cells of the thyroid, fall under the subgroup of tumors with limited response to conventional chemo- and radiotherapy. Since complete surgery is the only established treatment for this condition, residual and recurrent disease or systemic metastases remain a major problem. To treat patients with metastasized MTC we developed a novel immunotherapeutic strategy based on vaccination with dendritic cells (DC) [20]. Activated DC are the most potent antigen-presenting cells (APC) that are able to initiate a strong immune response by the copresentation of antigen and costimulatory molecules and the secretion of T cell activating cytokines [1, 14]. Several animal studies and clinical trials have used the unique abilities of DC to induce an immune response against autologous tumors. Promising results have been reported for metastasized melanoma [9, 13], advanced prostate cancer [11], B cell lymphoma [7], renal cell carcinoma [16], as well as breast and ovarian cancer in humans [2]. DC vaccines have been generated using tumor-associated peptides or tumor lysate [11, 13, 18, 19], DNA or RNA encoding specific antigens [6] or fusion of tumor cells with DC [44]. These immunotherapeutic interventions have led to the generation of potent anti-tumor cytotoxic T cells and disease regression in some clinical studies.

Treatment of MTC is complicated by the fact that no tumor-specific antigens have been identified thus far. However, MTC cells express the carcinoembryonic antigen (CEA) and the cell-specific polypeptide hormone calcitonin which are both established, sensitive tumor markers used in postoperative care to monitor persistent

disease. In our study we used a specific CEA peptide for the presentation of a single epitope in the context of HLA-A2. Previous in vitro studies have demonstrated that the DC presenting this peptide can induce cytotoxic T lymphocytes (CTL) in patients with CEA-expressing malignancies [12]. In addition, DC were pulsed with full-length calcitonin to broaden the spectrum to a second antigen and to mediate the presentation of multiple epitopes on HLA class II molecules. Recently, we have shown that this vaccination strategy results in a clinically measurable response in some treated patients [20]. However, the underlying immunological mechanisms by which these clinical responses are mediated are still unknown.

The aim of the present study was to investigate the cellular immune response against calcitonin before and after administration of the DC vaccine. Our data suggest that DC immunization breaks the state of tolerance against calcitonin and stimulates autologous T helper 1 $CD4^+$ T cells and $CD8^+$ lymphocytes.

Materials and methods

Patients and treatment

Patients with histologically proven MTC (Table 1) and postoperative elevated plasma calcitonin levels >100 pg/ml (normal range <5–12 pg/ml) were included into the study, as described recently [20]. Antigen-pulsed DC ($1–5\times10^6$ DC for each vaccination) were administered by subcutaneous (s.c.) injections in the upper arm. Patients nos. 1 and 3–7 received DC pulsed with calcitonin and CEA peptide (HLA-A2 positive), whereas patient no. 2 was only immunized with calcitonin-loaded DC (HLA-A2 negative). The first 4 treatments were administered weekly, while the following vaccinations were given at intervals of 4–8 weeks. Clinical responses were evaluated by the measurement of the tumor markers calcitonin and CEA at each visit and morphology imaging (CT scan, ultrasound) at intervals of 3 to 6 months. The mean follow-up was 13.1 months (range: 9–18 months). The trial was designed and conducted in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethical Review Board.

Preparation of dendritic cells

Mature DC were generated from peripheral blood mononuclear cells (PBMC) as described previously [17]. Briefly, PBMC were isolated by Ficoll-Hypaque density centrifugation, resuspended in RPMI 1640 medium with 10% fetal calf serum (FCS; Biochrom, Berlin), penicillin (100 U/ml) and streptomycin (100 μg/ml) (RPMI medium) and allowed to adhere to plastic dishes (Corning Costar, Bodenheim, Germany) for 2 h at 37°C. Adherent cells were cultured for 6 days in RPMI medium with granulocyte-macrophage colony-stimulating factor (GM-CSF; 800 U/ml) and interleukin-4 (IL-4; 500 U/ml; PromoCell, Heidelberg, Germany) (DC-medium). On day 3, cells were fed with GM-CSF (800 U/ml) and IL-4 (500 U/ml). On day 6, DC were stimulated with tumor necrosis factor-alpha (TNF-α; 1,000 U/ml; PromoCell) and pulsed with 10 μg of the HLA-A2 restricted CEA-peptide (YLSGANLNL) and 100 μg calcitonin per 1×10^6 DC/ml medium. For the first 2 vaccinations, DC were also pulsed with keyhole limpet hemocyanin (KLH; 100 μg/ml) which served as a T helper cell antigen. Antigen-loaded DC were washed three times and resuspended in 100 μl NaCl 0.9%. Flow-cytometric analysis demonstrated high-quality DC preparations with purity levels between 80–90% [17].

Analysis of T cell proliferative response

T cell proliferation was measured using PBMC cryopreserved in liquid nitrogen before treatment and after finishing the vaccination period. Pre- and posttreatment PBMC were resuspended in RPMI medium supplemented with 1% Lymphocult-T (Biotest, Dreieich, Germany) and 100 μg/ml calcitonin or 100 μg/ml CEA for 5 days. Thereafter, 1×10^5 cells were cultured in round-bottomed 96-well tissue culture plates (Corning) in triplicate in the presence of calcitonin (1–100 μg/ml) or ovalbumin (1–100 μg/ml) for an additional 5 days. Eighteen hours before harvesting, 100 μl medium were removed and stored at –80°C for cytokine measurement followed by the addition of 1 μCi [^{3}H]thymidine per well (Amersham, Braunschweig, Germany). Thymidine incorporation was assessed using a microscintillation counter (Canberra Packard, Dreieich, Germany). Cellular proliferation was expressed in stimulation indices (SI): mean cpm incorporated in the presence of antigen divided by the mean cpm incorporated in the presence of ovalbumin. PBMC responses before and after DC vaccination were evaluated in the same assay.

Determination of cytokine secretion

Interferon-gamma (IFN-γ) and IL-4 secretion of calcitonin and ovalbumin treated PBMC were determined in the T cell assay supernatants by ELISA (IFN-γ, detection limit: 15–800 pg/ml; IL-4, detection limit: 6–800 pg/ml; both from Roche Diagnostics, Mannheim, Germany). The assays were performed as described by the manufacturer. Samples above the upper detection limit were diluted 1:10 in isotonic NaCl and recounted. All samples were measured in duplicate.

Delayed-type hypersensitivity and immunohistochemistry

Delayed-type hypersensitivity (DTH) skin tests were performed with antigen-pulsed DC. DC were injected intradermally into the upper arm. A positive skin-test reaction was defined as >5 mm diameter erythema and induration 24 h after intradermal injection. A biopsy (diameter 5 mm) of the DTH site was taken from 3 patients 24 h after injection. Serial cryostat sections were stained with

Table 1 Patient characteristics, and status before and after DC vaccination

Patient no.	Age, sex	HLA type	Tumor type	No. of vaccinations	Calcitonin (pg/ml)	
					Before therapy	After therapy
1	53 F	A1, 2; B7, 8 DRB1	S	14	1,6800	16,000
2	62 M	A1, 30; B8, 13 DRB1	S	14	1,068	1,030
3	37 M	A2, 11; B35, 44 DRB1	MEN 2	12	8,895	2,500
4	32 F	A2, -; B44, 62 DRB1	S	10	2,17	193
5	58 F	A1, 2; B44, 62 DRB1	MEN 2	11	1,697	1,650
6	31 M	A2, 11; B44, 62 DRB1	MEN 2	11	174	268
7	38 F	A2, 26; B38, 39 DRB1	S	7	4,900	12,660

Tumour type: *S* sporadic MTC; *MEN 2* multiple endocrine neoplasia type 2

monoclonal antibodies (mAb) against CD4 and CD8 (BD Pharmingen, San Diego, Calif.) and visualized with anti-mouse IgG alkaline-phosphatase-labeled antibodies and fast red staining (Dako, Hamburg, Germany). To distinguish an antigen-specific from an FCS-specific DTH, the skin test was performed once (patient no. 3) with DC (with and without antigen pulsing) generated in the presence of 1% plasma instead of FCS.

Statistical analysis

The results were analyzed for statistical significance by paired *t*-test using Prism computer software (GraphPad Software, San Diego, Calif.).

Results

T cell proliferation and cytokine secretion

As illustrated in Figs. 1 and 2, none of the patients had a measurable T cell proliferation or a specific cytokine secretion against calcitonin before vaccination (SI: 0.86–1.23). However, patients nos. 3, 4 and 5 developed a dose-dependent T cell response towards calcitonin (SI: 1.51–2.74) as measured at the end of the DC vaccination period. Four patients (nos. 1, 2, 6, 7) failed to develop a detectable T cell proliferative response after stimulation with calcitonin (Fig. 1). There was no specific immunity against the control antigen ovalbumin.

After the treatment, lymphocytes from 5 patients (nos. 1–5) secreted high amounts of IFN-γ ($6{,}706 \pm 1{,}519$ pg/ml; range: 4,530–7,900 pg/ml) when they were stimulated with calcitonin ($P < 0.001$; Fig. 2a). IL-4 production was only slightly increased (33.5 ± 19.8 pg/ml; range: <6–63 pg/ml) in these patients. Among these, patient no. 3 experienced a significant regression of liver and pulmonary metastases together with a progressive decrease of the serum tumor markers. After 16 months, however, progression of metastases was observed. Patients no. 6 developed only a low IFN-γ secretion which was, however, still significant compared to pretreatment values ($P < 0.01$). Patient no. 7, who failed to respond to calcitonin in the cytokine assays, developed rapid tumor progression. The mean IFN-γ/IL-4 ratio of 2,883 (range: 161–7,900) of patients nos. 1–6 suggests the induction of a T-helper 1 (Th1)-polarized immune response. There was no significant difference in the cytokine levels when the PBMC were stimulated with ovalbumin (IFN-γ: 2.8–10.4 pg/ml).

Delayed-type hypersensitivity and immunohistochemistry

To study the in vivo immune response, we performed intradermal injections with antigen-pulsed DC and analyzed the DTH skin reaction. After the 3rd to 5th vaccination, all patients developed a significant DTH reaction characterized by the appearance of erythema and induration at the injection site. To analyze the infiltrating cells we performed immunohistochemical staining of skin biopsies from patients nos. 1, 2 and 3. Twenty-four hours after injection of antigen-pulsed DC there was a strong perivascular and epidermal infiltration with $CD4^+$ T helper cells and $CD8^+$ T lymphocytes (Fig. 3). To distinguish an antigen-specific from an FCS-specific response, DTH reactivity was tested once (patient no. 3) with antigen-pulsed DC prepared in the presence of autologous plasma (without FCS). This resulted in a DTH response with erythema and induration. In contrast, non-antigen-pulsed DC, which were also prepared with autologous plasma, did not lead to a DTH skin response.

Discussion

The rationale of our clinical trial was to develop a novel immunotherapy based on the unique immunostimulatory capacity of DC to improve the survival of patients with metastasized MTC. The major problem in MTC is that no tumor cell-specific antigen has been identified

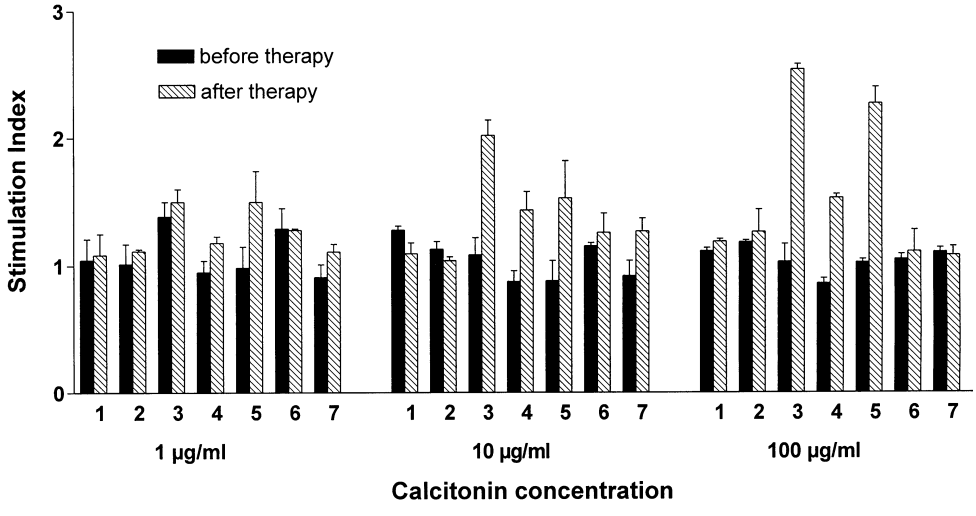

Fig. 1 Proliferative response of peripheral blood mononuclear cells against calcitonin. 1×10^5 PBMC were stimulated with calcitonin (1–100 µg/ml); ovalbumin (1–100 µg/ml) served as control protein. Results are demonstrated before (*black bars*) and after (*hatched bars*) the last immunization (vaccination numbers ranging from 7–14). Data are expressed as stimulation indices ± SEM ($P < 0.05$ for patient nos. 3, 4, 5 at 100 µg/ml)

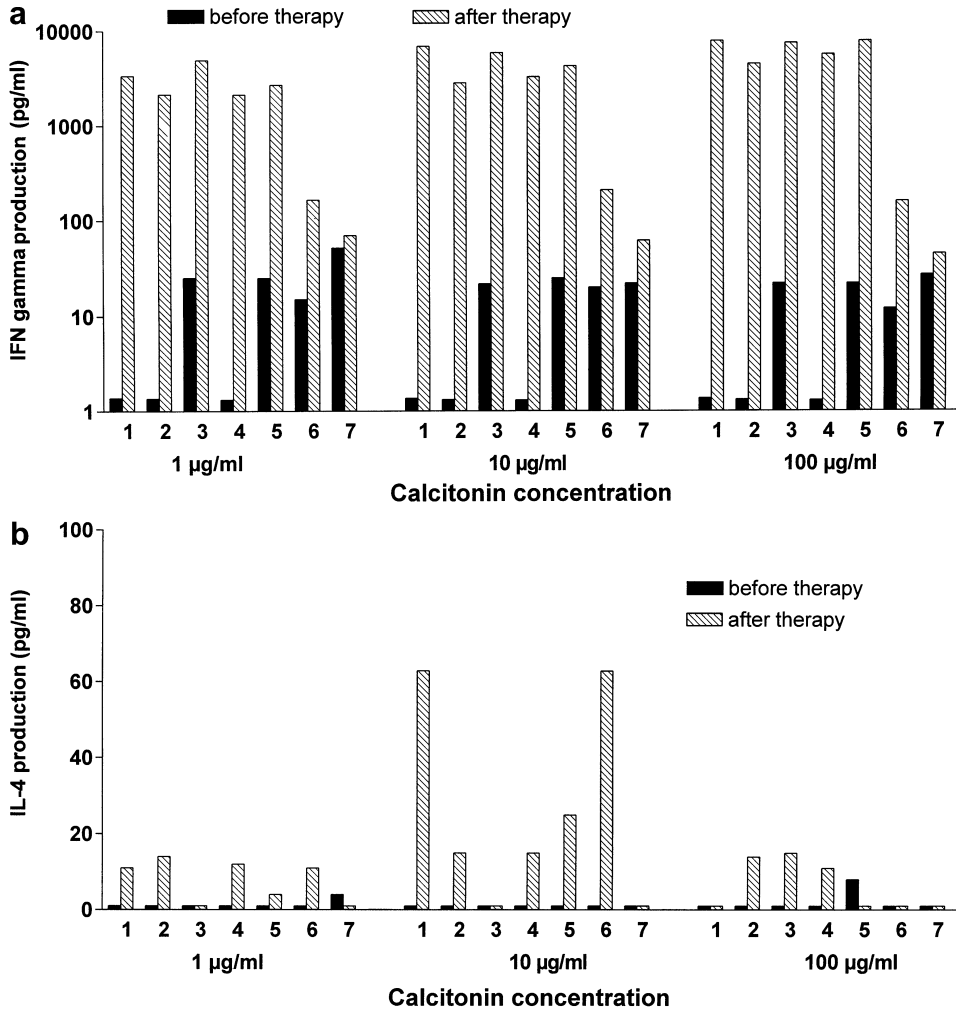

Fig. 2 Cytokine release assay in response to calcitonin before and after DC vaccination. 1×10^5 PBMC were stimulated with calcitonin (1–100 µg/ml) for 4 days. Then supernatants were harvested and assayed for IFN-γ (**a** $P<0.001$ for patients nos. 1–5) and IL-4 (**b** $P<0.01$ for patients nos. 1 and 6)

thus far. Therefore, we used two proteins known to be expressed in malignant C cells, the polypeptide hormone calcitonin and a CEA peptide, to induce an anti-tumor response. While the HLA class I-restricted CEA peptide has been successfully used for DC vaccination in some CEA-expressing tumors [12], the ability of polypeptide hormones to serve as specific targets in anti-tumor therapies has not been investigated thus far.

We here demonstrate that vaccination with calcitonin-loaded DC results in the generation of an antigen-specific T cell response. Dose-dependent T cell proliferation was observed in 3 out of 7 patients after stimulation of PMBC with calcitonin. Moreover, among the 7 treated patients, in 5 cases huge amounts of IFN-γ and only low levels of IL-4 were released after challenge with calcitonin. These findings demonstrate that DC vaccination is effective in stimulating autologous T cells against the self-antigen calcitonin. The potential of activated DC to break a state of tolerance is in agreement with previous studies using prostate-specific antigen (PSA) [5], idiotypic determinants [8] and CEA [12] in animal models and humans. The cytokine patterns suggest that our immunization protocol preferentially induces Th1 cells, which can stimulate APC and provide help to the terminal activation of cytotoxic T cells [15]. The difference between the T cell proliferation and the cytokine release assays may be explained by a lower sensitivity of the proliferation assay, or the induction of subpopulations of T cells which possess a low proliferation but a high cytokine secretion capacity [3, 10].

In addition to the in vitro studies, we also investigated cell-mediated immunity in vivo by the assessment of type IV hypersensitivity reaction. Interestingly, all 7 patients developed a strong DTH reaction after 3–5 immunization cycles, suggesting the generation of antigen-specific lymphocytes. Analyses of skin biopsies revealed that the infiltrating cells were $CD4^+$ and $CD8^+$ T cells. Although we did not directly assess CTL in cytotoxicity assays (due the lack of HLA-restricted target cells), immunohistochemical data and the partial clinical response in some patients strongly suggests that CTL may be induced in at least some patients.

We here clearly demonstrate that our vaccination protocol has the potential to induce a calcitonin-specific cellular immune response. Since all but one patient (no. 2) were treated with DC pulsed with a combination of calcitonin and an HLA class I (A2)-restricted CEA peptide, the clinical effectiveness cannot be attributed to

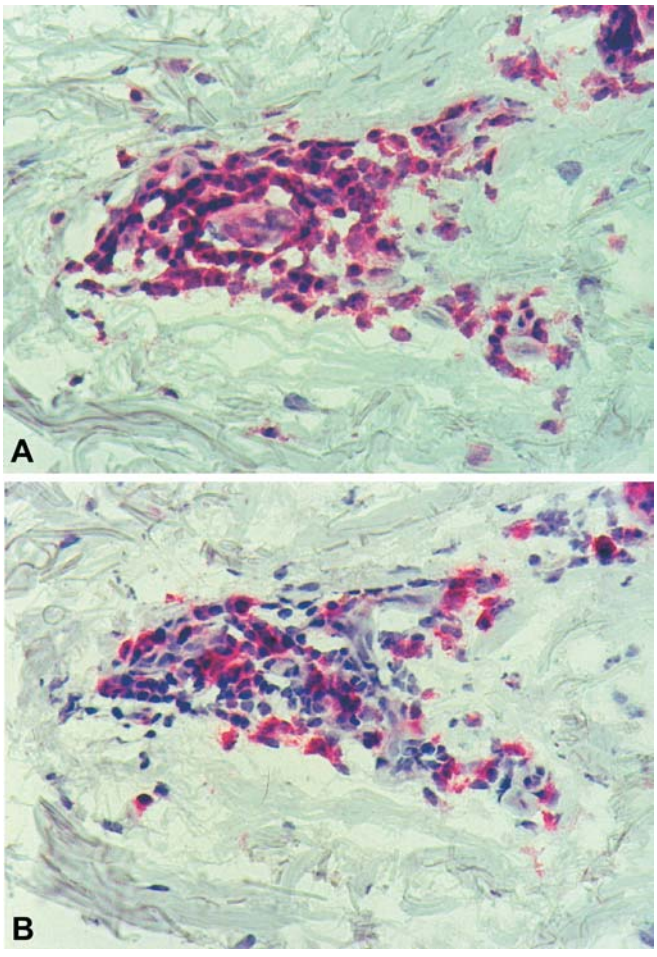

Fig. 3 DTH skin reaction. Skin biopsy demonstrates strong perivascular and epidermal infiltration with $CD4^+$ T lymphocytes (**A**) and $CD8^+$ cytotoxic T cells (**B**) after DC vaccination of patient no. 3. Cryostat sections were incubated with mAb against CD4 (**A**) or CD8 (**B**) and stained with fast red

only one antigen. However, the detection of calcitonin-specific $CD4^+$ lymphocytes which recognize antigens bound to HLA class II molecules strongly suggests that the immune reaction against calcitonin may play a major role in the in vivo anti-tumor response. The patient (no. 3) with a partial regression of pulmonary and liver metastases also had the highest T cell proliferation together with a strong IFN-γ release after stimulation with calcitonin. This patient had the largest tumor burden suggesting that, in line with previous reports, the presentation of minimal or advanced disease does not allow the prediction of vaccination success [13]. Interestingly, patient no. 7, who failed to respond in the T cell proliferation and cytokine release assays, developed progression of liver metastases within 10 months after the start of immunization. However, there was no obvious difference in cellular immunity in responders as compared to those subjects who had stable disease, suggesting that the activation of Th1 cells was not sufficient to eradicate tumor cells in vivo. Further clinical trials varying the antigen pulsing, DC preactivation and time schedule of vaccination are needed to improve the clinical efficacy of treatment for patients with MTC.

The application of calcitonin for tumor therapy may have several advantages compared to other approaches where no tumor antigen has been identified. First, human calcitonin is available in high purity at low cost from pharmacological preparations for the treatment of osteoporosis. Second, calcitonin is specifically expressed in thyroid C cells, which may limit the induction of potentially harmful side effects. Indeed, administration of calcitonin-loaded DC was well tolerated, with only minor side effects such as swelling or erythema at the injection side and low-grade fever. No severe side effects were observed, suggesting that autoimmunity may not represent a major problem in our approach. The third advantage may be the relatively high susceptibility of endocrine tissues to (auto)immune attack, as evidenced by the high prevalence of thyroid, pancreas and adrenal autoimmune diseases. This could make it easier to break tolerance and induce cell-specific cytotoxic immunity as compared to other tissues. The later hypothesis clearly needs further investigation, and the inclusion in further trials of patients with endocrine active carcinomas of the pancreas or the adrenal gland.

In this study, we show for the first time that DC vaccination using calcitonin as target protein can generate a Th1-dominated cellular immune response in patients with metastasized MTC. These findings suggest that it may be feasible to use polypeptide hormones as specific targets for immunotherapeutic strategies in patients with advanced endocrine malignancies. The clinical benefit for these patients has to be determined in future clinical trials using an optimized protocol and larger cohorts of patients.

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