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ORIGINAL RESEARCH

The Role of T Lymphocytes in Cancer Patients Undergoing Immunotherapy with Autologous Dendritic Cells

Cláudia M. Rodrigues[1], Bruna F. Matias[1], Eddie F.C. Murta[1,2] and Márcia A. Michelin[1,3]

[1]Research Institute for Oncology-IPON, Federal University of the Triângulo Mineiro-UFTM, Uberaba, Minas Gerais, Brazil. [2]Titular Professor in the Discipline of Gynecology and Obstetrics, UFTM, Uberaba, Minas Gerais, Brazil. [3]Associated Professor in the Discipline of Immunology, UFTM, Uberaba, Minas Gerais, Brazil.
Corresponding author email: michelinimuno@dcb.uftm.edu.br

Abstract:
Introduction: Cancer stems from mutations in specific genes that induce uncontrolled cell proliferation. Dendritic cells (DCs) are important immunologic cells and play a crucial role in the induction of an antitumour response.
Patients and methods: We examined the immune response mediated by T lymphocytes, helper T cells, cytotoxic T cells, and regulatory T cells, as well as the cytokines [interleukin (IL)-2, IL-12, interferon (IFN)-γ, tumour necrosis factor (TNF)-α and IL-10], produced by these cell populations, in cancer patients (N = 7) undergoing immunotheraphy with autologous DCs.
Results: We observed an initial increase in T helper cells (CD4+) expressing IL-2, IFN-γ, IL-12, TNF-α, and IL-10 after initiation of treatment, with statistically significant for the cytokines IL-2, TNF-α and IL-10. A similar significant effect was observed for IL-2-expressing cytotoxic T cells (CD8+). The percentage of total T cells (CD3+) remained elevated throughout immunotherapy. Regulatory T cells (CD25+/FOXP3+) only showed high percentage of their maximum value when analyzed the pretreatment levels, with statistically significant.
Conclusion: Immunotherapy with DCs stimulated the immune response, as evidenced by an increase in percent fluorescence of most cell populations investigated during the specified treatment period.

Keywords: dendritic cells, T cells, cytokines, immunotherapy, cancer

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Introduction

Cancer represents a set of genetic diseases caused by several mutations in specific genes, leading to genomic instability and consequently to accelerated and uncontrolled cell proliferation.[1] The changes occurring in cancerous cells culminate in the production of "danger signals" resulting in the expression of altered proteins that can be recognized as foreign to the immune system, generating an antitumour response.[2]

The immune system consists of two main lines of response: innate immunity and adaptive or acquired immunity. Innate immunity is composed of cells such as macrophages, dendritic cells (DCs), natural killer (NK) cells, neutrophils, basophils, eosinophils, and mast cells, which can be characterized by their relatively fast response, however, with absence of induction of immunologic memory.[3,4]

Stimulation of the innate immune response leads to activation of acquired immunity, composed of cytotoxic T cells (CD8+), T helper cells (CD4+), and B cells. These cells are characterized by presenting a wide variety of antigen receptors and their ability to generate immunologic memory.[3,5]

DCs are considered to be the most important antigen-presenting cell. Through a cross-presentation mechanism, they can activate cytotoxic T lymphocytes (CD8+) as well as T helper cells (CD4+), with T helper cells being critical in the production of cytokines involved in acquired immunity.[6–8]

Although the main focus of studies involving the antitumour immune response have been aimed mainly at cytotoxic T lymphocytes, there have been reports that T helper cells (CD4+) can also exhibit a wide range of functions in this type of immune response. Some epitopes derived from human tumour antigens recognized by human CD4+ T cells have already been described.[9,10]

Tumour cells can also suppress DC function, thereby causing them to be incapable of stimulating and activating T cells efficiently.[11,12] Given the importance of these cells, clinical studies related to immunotherapy involving DCs have been performed for various tumour types, with noteworthy results[13–15] and it was shown that the vaccine was generally well tolerated without significant adverse effects. Hence, immune responses elicited by immunotherapy with DCs have been observed.

Therefore, given the complexity of inducing an antitumour response, it is critical that we improve our understanding of immune cell behaviour, particularly T lymphocytes, so that possible immunological mechanisms responsible for tumour regression can be inferred. Furthermore, such research can enable the development of new and effective immunotherapeutic protocols for the treatment of cancer.

This study aimed to elucidate the immune response of T lymphocytes in cancer patients during immunotherapy with autologous DCs. For this purpose, we evaluated peripheral blood samples from the patients before and during immunotherapy for total T lymphocytes, T helper cells, cytotoxic T cells, and the cytokines produced by them [interleukin (IL)-2, IL-12, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, and IL-10. We also assessed levels of the transcription factor FOXP3 and the cell surface marker CD25, which is essential for identification of regulatory T cells.

Methods

Patients

Seven female patients diagnosed with cancer were selected at the Research Institute for Oncology (IPON)/Discipline of Gynecology and Obstetrics at UFTM. All individuals involved were fully informed about the intentions of the research study and signed a consent form to confirm their participation (case number 683-2006), in accordance with the research ethics committee requirements.

Some of the participating patients had previously been treated with conventional methods, such as chemotherapy and/or radiation therapy. As such, in order to participate in the current study and undergo immunotherapy with DCs, such patients were required to wait at least 2 months prior to starting the new treatment. Table 1 shows a summary of the general characteristics for each patient participating in the study.

Acquisition of mononuclear cells

Mononuclear cells were obtained from peripheral blood samples from the patients recruited to participate in the study. The mononuclear cells were isolated from peripheral blood cells by density gradient and centrifugation in Ficoll-Hypaque (BD *Biosciences*) solution. The cells were re-suspended in 15 ml of IMDM, enriched with 5% fetal bovine serum,

Table 1. Patient characteristics based on age, tumour type, stage, and previous treatments.

Patient	Age (years)	Tumour type	Stage (TNM)*	Previous treatments
1	76	Vaginal cancer	IIIB (T3N1M0)	–
2	77	Vaginal melanoma	IIC (T4NxMx)	–
3	48	Vaginal cancer	0 (tumour in situ)	Radiation therapy, surgery, IFN
4	66	Breast cancer	IV (T4dN2M1)	Chemotherapy, radiation therapy, surgery.
5	39	Cervical cancer	IVB (T2bN0M1)	–
6	80	Breast cancer	IIIC (T4dN3Mx)	Chemotherapy, radiation therapy, surgery.
7	27	Breast cancer	IV (T2N1M1)	Chemotherapy, radiation therapy, surgery.

Notes: *TNM = classification of cancer staging, where T refers to the size of the tumour, N refers to any involved lymph nodes, and M refers to the presence of metastasis.

1% penicillin/streptomycin, and 1% gentamicin. Cellular viability was assessed using Trypan blue. All procedures were conducted under sterile conditions.

DC vaccines

Mononuclear cell cultures were maintained in vitro at 37 °C and 5% CO_2 for 7 days in GM-CSF and IL-4 to support DC differentiation. The tumor antigens were obtained by tumor biopsy from each patient. The neoplastic cells were lysed by cycles of freezing and used at a concentration of 100 ug/ml. Differentiated cells were placed in contact with tumour antigens, obtained from each patient, and subjected to electroporation. DCs activated with tumour antigens were infused by subcutaneous injection into the forearm ($5\sim10 \times 10^6$ cells autologous DCs/patient) and the procedure was repeated at a mean interval of 15 days.

Flow cytometry

Over the course of the study, peripheral blood samples were drawn from the patients and cells were evaluated by flow cytometry (BD FACS Calibur cytometer and cell sorter). Cytometry protocols were deployed in accordance with those suggested by the manufacturer (BD *Biosciences*). The peripheral blood cells were collected for the following markers in all patients: total T (CD3+), helper T (CD4+), cytotoxic T cells (CD8+), and regulatory T cells (CD25+/FOXP3+). The procedure was performed prior to initiating therapy with DCs (pre-treatment analysis) and further analysis was performed every 15 days.

Leukocytes were isolated from peripheral blood samples via centrifugation at 4 °C using a standard cell lysing protocol (BD *Biosciences*—FACS™ *Lysing Solution*), in accordance with manufacturer's instructions.

For T cell tagging, initially cells were re-suspended in PBS for extracellular tagging. For tagging, α-CD3 PE antibodies were used for total T cells, α-CD4 PE for T helper cells, α-CD8 PE for cytotoxic T cells, and α-CD25 PE for regulatory T cells. After extracellular tagging was completed, cells were incubated at 4 °C for 30 min, and then rinsed twice by centrifugation with PBS.

Following the rinses, a fixation and permeabilization solution was added (BD Cytofix/Cytoperm™) for duration of 20 min at 4 °C. The cells were rinsed twice again with *Perm/wash* (BD *Biosciences*) buffer prior to the second tagging. For intracellular tagging α-IL-2 FITC antibodies were utilized for cytotoxic T cells and T helper cells. T helper cells were also tagged with α-IL-12 FITC, α-IL-10 FITC, α-IFN-γ FITC and α-TNF-α FITC antibodies. α-FOXP3 FITC antibodies were used to tag regulatory T cells. Following intracellular tagging, cells were incubated once more at 4 °C for a 30 min duration. Finally, cells were re-suspended in 500 μL of PBS for cytometric analysis using BD FACSCalibur™. For an accurate determination of the cells, corresponds to lymphocytes and not other cell types, we determine the region to be analysed by constructing a gate according to a chart control for relative size (Forward Scatter FSC) and granularity and complexity (Side Scatter SSC)

in each experiment and for each patient. Figure 1 is an illustrative example of how the determination was performed by flow cytometry data.

Statistical analysis

The data are shown as median values, with error bars representing the range from the minimum value (lower bar) to the maximum value (upper bar). The results were analyzed using the Kruskal-Wallis nonparametric test (ANOVA). Statistical analysis and graphing were performed using GraphPad Prism version 5.0 (GraphPad Software, Inc.). Results were considered statistically significant at $P < 0.05$.

Results

Safety and toxicity of the vaccine

Immunotherapy with DCs was shown to be safe without any significant side effects or toxicity. Only patient 2, who was in treatment for metastatic vaginal melanoma showed worsening of a pre-existing condition (vitiligo) after commencing treatment. However, it was not possible to determine whether this worsening occurred as a result of the action of the vaccine or as a result of systemic activation of an immune response.

Cell population analysis using flow cytometry

A high percentage of T lymphocytes (>60%) were observed to be CD3+ (fluorescently labelled) in the pre-treatment analysis and that percentage increased following initiation of treatment until the twelfth analysis, after which a rapid reduction of fluorescent labelling was observed ($P = 0.3986$). This finding is contrary to the results observed for the other cell types investigated as CD4+, CD8+ and CD25+FOXP3+ (described below), which fell to nearly to zero (Fig. 2A).

CD4+ and CD8+ T lymphocytes showed similar patterns of IL-2 expression, with an initial increase in the percentage of fluorescently labelled cells after initiation of treatment with DCs. The early increase persisted until the seventh and tenth analysis, respectively, and then was followed by a reduction. These treatment effects were highly significant for both cell populations ($P = 0.0044$; $P = 0.0191$, respectively) (Fig. 2B and 2C).

CD4+ T lymphocytes showed a trend toward an increase in percentage of IFN-γ-positive (fluorescent labelled) cells upon initiation of immunotherapy, until the seventh analyse and that upward trend was followed by a reduction ($P = 0.5688$) (Fig. 3A).

Similar patterns of results were obtained for the expression of TNF-α and IL-12 by CD4+ T cells (Fig. 1), with a rapid initial increase that was significant for TNF-α ($P = 0.0419$) (Fig. 3B), but only a weak trend for IL-12 ($P = 0.6774$) (Fig. 3C). These cells exhibited a significant increase in IL-10 expression, as evidenced by the percentage fluorescent cells, after treatment initiation ($P = 0.0111$), followed by a tendency toward reduction after the eighth analysis (Fig. 3D).

With regard to the percentage of regulatory T cells (CD25+, FOXP3+), no considerable stimulation was observed ($P = 0.0278$) (Fig. 3E).

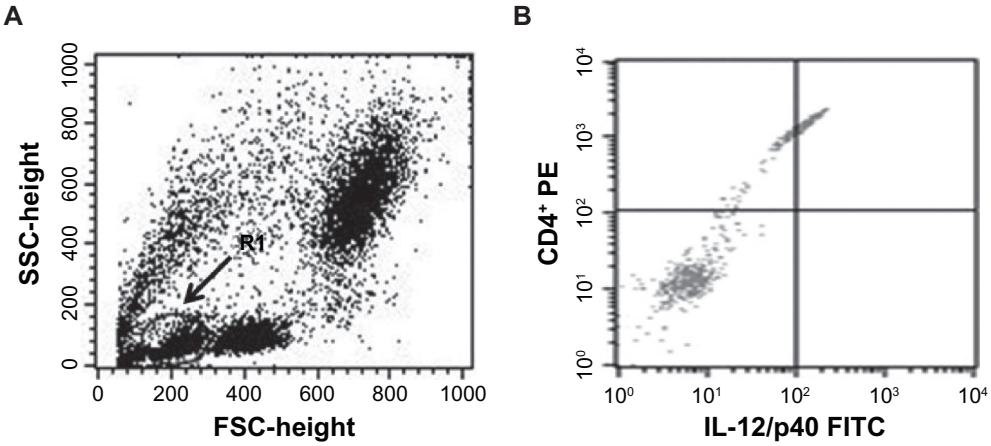

Figure 1. A) Flow cytometry of peripheral blood of patient 2. Gate 1 (arrow) in lymphocytes. **B**) shows double labeling CD4+/IL-12+ lymphocytes in gate 1.

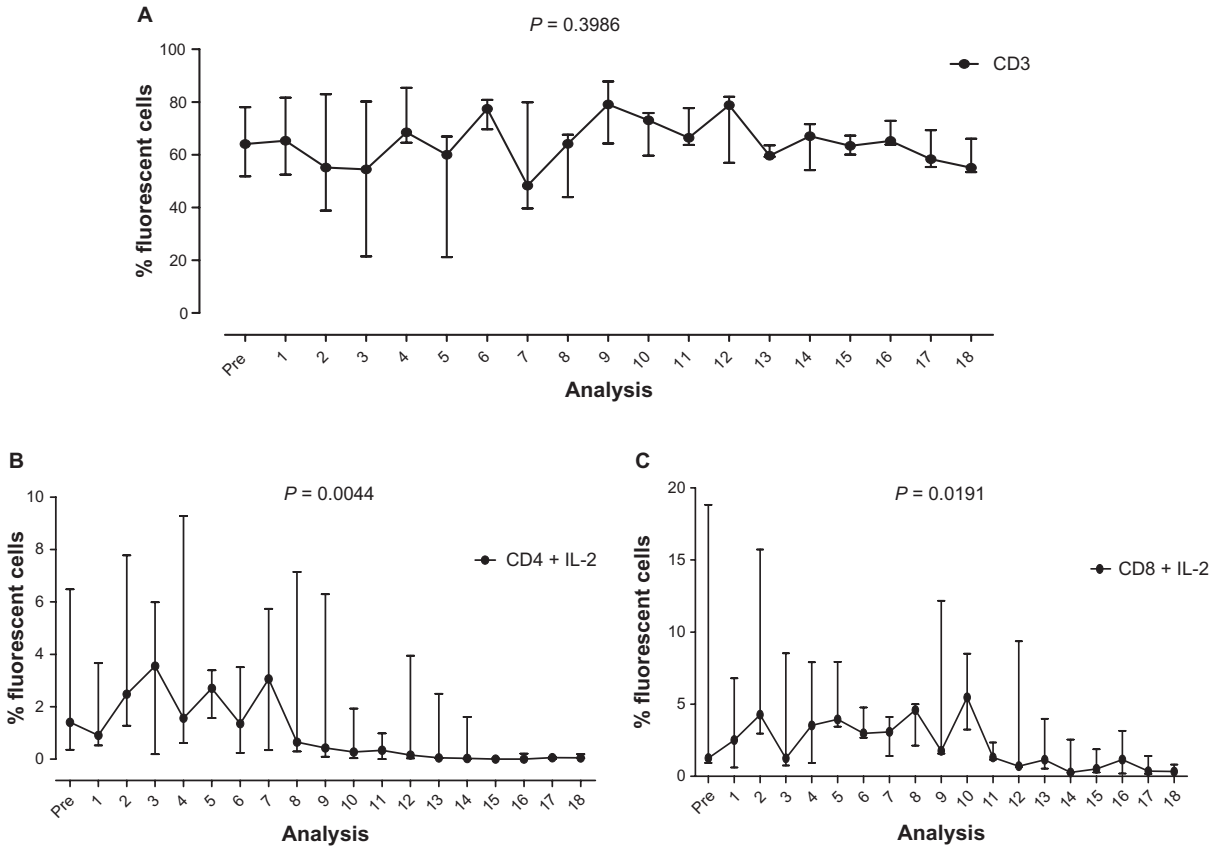

Figure 2. Quantification of total T cell percentage (CD3+) (**A**) and of CD4+ (**B**) and CD8+ (**C**) T lymphocytes expressing cytokine IL-2 (median, maximum, and minimum values), prior to and during immunotherapy with DCs.

Discussion and Conclusion

In recent years, DCs' immunogenic potential for immunotherapy has been the focus of a multitude of studies. The effects of these cells on the host's immunity hold the key to further development of this work.

The study of immune response in cancer patients is difficult to be carried by several factors. The immune response is already a complex factor being studied in humans because this system is highly dependent on the genetics of each individual. The selection of patients depends on that all therapies have been used without success, the consent of the patient and/or family and a good general physical condition. Another point is the variation in the levels of cytokines in humans, which is very variable, however, likely to demonstrate statistical significance the examination in some tests.

The immunotherapy using autologous DCs developed in this study was well tolerated, and no significant side effects were observed to the vaccines. Only one patient experienced a mild worsening of a pre-existing case of vitiligo, which is known to be a common autoimmune response against melanocytes during treatment of melanoma.[16,17]

With regards to the population of T helper cells (CD4+) expressing IFN-γ, it was observed an increased percentage of fluorescently labelled IFN-γ-positive cells, after the start of immunotherapy with DCs. The present study also showed that CD4+ T cells do exhibit a significant increase in TNF-α expression after stimulation with DC immunotherapy. Similar results have been described previously in the literature. Fong et al[18] assessed the effects of DC vaccines via different routes of administration (intradermal, intravenous and intralymphatic routes) and detected the production of IFN-γ by intradermal and intralymphatic routes. However, they found low TNF-α expression levels in 1/6 patients treated with an intravenous DC vaccine.[18] Mumberg et al[19] deduced that cytokine IFN-γ plays a central role in the elimination of tumour cells by CD4+ T cells, however, this effect seemed to be indirect, since the tumour cells remained negative for MHC class II molecules after stimulation with IFN-γ.

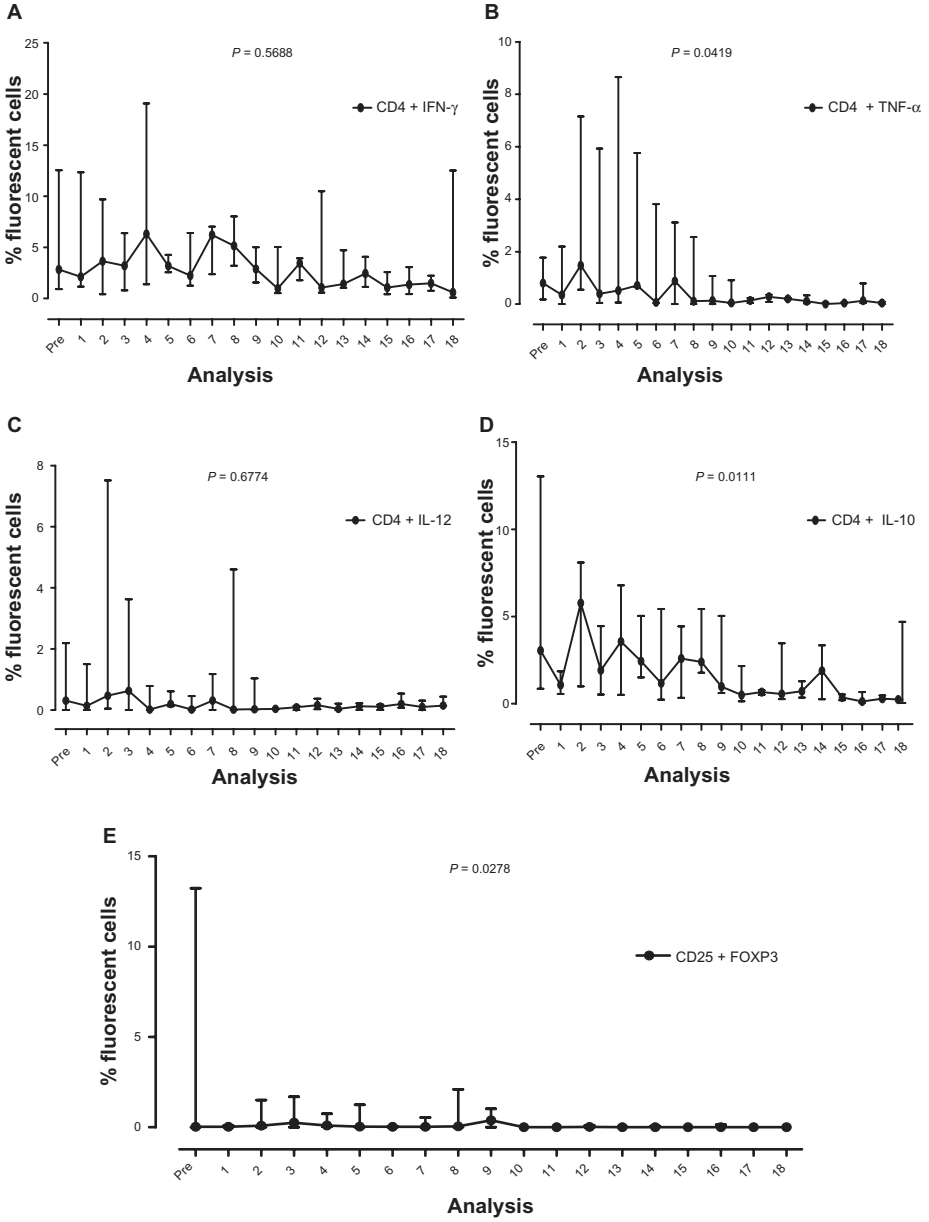

Figure 3. Quantification of T helper cell percentage (CD4+) expressing cytokines IFN-γ (**A**), TNF-α (**B**), IL-12 (**C**), and IL-10 (**D**), and regulatory T cells (CD24+FOXP3+) (**E**), (median, maximum, and minimum values), prior to and during immunotherapy with DCs.

TNF-α is a multifunctional cytokine and a major mediator of inflammation. It is involved in cell survival, proliferation, and apoptosis. It can be detected in stromal and malignant cells in several types of cancer.[20] Chronic production of this cytokine in the neoplastic micro-environment can induce tumour development and spread. In contrast, TNF-α is also important in cell destruction mediated by NK cells and CD8+ T cells.[21,22] Hence, TNF-α is functionally pleiotropic, as it is able to engage in responses that are tumour promoting or tumour suppressing depending on the stimuli received by the cells.

An interesting finding of this work and unpublished in the literature is that we observed a slight stimulation of IL-12 expression by CD4+ T lymphocytes after the onset of DC immunotherapy. Both the IL-12 and IFN-γ cytokines are important for stimulation of the Th1 immune response.[23] In addition, the IL-12 induces cytotoxic effects in NK and CD8+ cells, aside from stimulating their production of IFN-γ.[24,25]

IL-10, in turn, showed a considerable increase after commencement of the treatment. IL-10 can be secreted by a variety of different cells,[26,27] and

although it is most often described in terms of its immunosuppressive action, there are reports describing its effector function on CD8+ T cells.[28] IL-10, generally considered to be a Th2 cytokine, is capable of stimulating B cell function. On the other hand, there is evidence suggesting that it may be produced by Th1 cells,[29,30] and that it may also exhibit an antitumour function.[31,32]

Saraiva et al[33] demonstrated that in vitro differentiation of IL-10-expressing Th1 cells was dependent upon the activation of transcription factor STAT-4-induced by cytokine IL-12, through a powerful activation via T-cell receptors (TCRs) and phosphorylation of extracellular-signal-regulated kinases (ERK) 1 and 2. These findings further demonstrate the multifunctional role of IL-10 and its dependence on IL-12 for its production by Th1 cells.

In the current study, total T cells demonstrated elevated expression of the CD3+ marker practically throughout the entire period of treatment with DCs. Our assessment of IL-2 expression by CD4+ and CD8+ T cells also yielded similar results for these two cell types: an early increase in expression after initiation of immunotherapy followed by a decline to nearly zero by the end of the analysis.

These findings demonstrate that activation of DCs in vitro can enhance immune effector responses in T cells upon vaccination, while inducing a significant increase in IL-2 expression. IL-2 is considered to be an important factor in proliferation of T lymphocytes against antigenic stimulation and plays a central role in the regulation of T cells. Furthermore, its expression is considered to be indicative of cellular activation.[34]

Our findings corroborate the literature. In a study in which autologous DC vaccination was administered to patients with metastatic breast cancer and renal cancer, Avigan et al[35] observed an increased in the percentage of CD4+ and CD8+ T cells that was related to increased expression of the cytokine IFN-γ, which apparently led to induction of antitumour activity. In a study examining advanced stage breast and ovarian cancer patients who were vaccinated with autologous DCs, Brossart et al[36] described observing a specific antigenic response in 5/10 patients, as evidenced by IFN-γ production by cytotoxic T lymphocytes.

The tendency for there to be a sharp reduction in IL-2-tagged CD4+ and CD8+ T cells after a certain time in treatment, despite sustained high levels of total T cell marker (CD3+) expression, suggests that there may be another CD3+ cell population involved. Two cells types can likely be Tγ/δ[37–39] and NK T-cells.[40,41] Both populations are identified as having regulatory and protective functions in cancer.[42–46] Thus, investigating these cell types may help explain the reduced immune response that is observed after some time of immunotherapeutic stimulation, as well as the conservation of marker CD3+.

We did not observe evidence of stimulation of CD25+/FOXP3+ regulatory cells in this study. There was only a higher maximum value in the pre-therapy analysis, relative to the maximum values observed during treatment. Thus, we can conclude that although a decrease in immune response was observed vis-à-vis the assessed markers, which suggests that immunosuppression did occur, these cell types were not responsible for the findings.

On the contrary, others have demonstrated an induction of regulatory T cells after immunotherapy with DCs. According to Berntsen et al,[47] significantly increased levels of CD4+/CD25+/FOXP3+ Treg cells (vs. pretreatment levels) were found in peripheral blood samples of patients with metastatic renal carcinomas treated with a DC vaccine combined with cytokine IL-2.

Our results indicate that there is a stimulation of the response of CD4+ T cells expressing IL-2, IFN-γ, and TNF-α, as well as of IL-2-expressing CD8+ T cells, suggesting that immunotherapy with DCs has the ability to induce a Th1 response. As such, the immune system is probably acting as an effector against tumour cells.

In the present study, some of the patients involved were treated for a prolonged period of time and given the vaccine and immunological stimulation on several occasions. One explanation for the diminished response over the course of treatment could be due to selection for tumour cell variants with immunoresistant phenotypes.[48] Additionally, after a certain period of vaccination, there could have been a loss in expression of MHC class I molecules by tumour antigens, a major mechanism of immune system evasion.[49] The maturation stage of DCs is also a determining factor for directing a Th1 response, as immature DCs secrete IL-10 leading to a Th2 response.[50,51] As such, it is important to continuously review the therapeutic protocol being used and to seek innovative strategies to prevent the development of new tumour escape

mechanisms in order to maintain a good response during the treatment period.

It should be noted that the present study was based on the analysis of the percentage of fluorescently labelled cells for each assessed marker, and that the analysis of fluorescence intensity was important. Thus, although there may be a numerical decline in tagged cells after a certain period of treatment, it is possible that there could still have been an increase in the numbers of assessed markers. In other words, even though there may be a smaller number of cells, each cell might be expressing a larger number of surface markers or producing a larger quantity of cytokines. Given the important implications of this possibility, follow up studies are already underway.

In conclusion, upon assessing immunologic response before and during treatment, we observed that immunotherapy with DCs was capable of stimulating the immune system in cancer patients. This finding was evidenced by observations of increased percentages fluorescently labelled cells for markers of the major cell populations studied.

Upon immunotherapeutic intervention with DCs, activated in vitro and pulsed with tumour specific antigens for each patient, an increase in the percent fluorescently labelled cells was observed for the majority of cell populations assessed. The increases were particularly significant for T helper cells expressing TNF-α, IL-2, IL-10, and for cytotoxic T cells expressing IL-2. The findings of this study indicate that after a certain period of immunologic stimulation against tumour antigens, there is a reduction in the immunologic response.

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Disclosures

This manuscript has been read and approved by all authors. This paper is unique and not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material. Written consent was obtained from the patients for publication of this study.

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