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Evaluation of serum levels of tumour necrosis factor-alpha (TNF-α) and soluble IL-2 receptor (sIL-2R) and CD4, CD8 and natural killer (NK) populations during infrared pulsed laser device (IPLD) treatment

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SUMMARY

The purpose of this study was to evaluate serum levels of TNF-α, sIL-2R and distribution of peripheral leucocyte subsets in patients with advanced neoplastic disease undergoing IPLD treatment. Fifteen cancer patients with evidence of persistent disease were further divided in two groups according to outcome at the end of the period of clinical evaluation: group 1 patients were still alive and group 2 patients had died. Our results show: (i) an increase in the initial level of TNF-α in both groups; (ii) a decrease in TNF-α levels during the follow up of group 1 patients; (iii) a significant increase in serum levels of sIL-2R in patients in group 2 compared with those in group 1; (iv) a progressive and constant increase in TNF-α levels in group 2; (v) a decrease in $CD4^+CD45RA^+$ subpopulation in both groups; (vi) an increase in $CD25^+$ cells; (vii) an increase in $CD4^+$, $CD4^+CD45RA^+$ and $CD25^+$ cells during the follow up of group 2 patients. The data generated here form the basis for further investigations on the use of IPLD as a single agent and in combination with other biological response modifiers in cancer patients.

Keywords cancer laser cytokines tumour necrosis factor-alpha sIL-2R cytofluorometry

INTRODUCTION

Neoplastic diseases cause a sustained and complex alteration of the immune system, including cytokine secretion. Cytokines are a group of polypeptides which play a major role in the modulation of host immune responses, and possibly also in immunopathologic mechanisms [1].

For example, studies with recombinant TNF-α have revealed numerous cell regulating activities of this cytokine, indicating that it is a very important mediator of inflammation and immunity. TNF is cytotoxic/cytostatic for several tumour cell lines *in vitro* [2–4], but can also be cytotoxic for normal cells under certain *in vitro* conditions [5]. Moreover, the majority of tumour cell lines are TNF-resistant [3], and there is evidence that endogenous production of TNF by tumours may be linked to their growth mestastasis, while the neutralization of TNF activity may be of potential therapeutic benefit [6].

IL-2 is an essential cytokine which plays a central role in the immune response [7] and is released after activation of T lymphocytes. In addition to the expression of IL-2 receptors on the cell surface, a soluble form of the IL-2 receptor (sIL-2R) can be released in the serum [8]. Abnormally high levels of sIL-2R have been described in different diseases [9], and it has been proposed as a marker of the host response in patients with neoplasms.

Several studies have also defined functionally distinct subsets of human lymphocytes that display a variety of regulatory and effector functions [10]. The major division of these subsets is between cell populations which bear CD4 and CD8 antigens (helper or cytotoxic/suppressor cells respectively), but also significant are CD16 and CD56 markers (natural killer cells) and CD19 antigen (mature B cells) [10]. The quantification of these subpopulations in cancer patients is an important parameter for evaluating responses induced by the neoplasm.

The bioeffects of laser radiation have been widely reported in the medical literature [11,12]. These are generally manifested as biochemical, physiological or proliferative phenomena. The cell type most widely used in these studies has been the lymphocyte, in which laser radiation induces changes at the level of DNA and RNA synthesis, expressed by acceleration of proliferation and, consequently, stimulation of the cellular immune response [13].

The present study was designed, first, to undertake a sequential examination of immunological parameters (serum levels of TNF-α and sIL-2R, fluorocytometric evaluation of peripheral lymphocytes) in patients with advanced neoplastic

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Table 1. Diagnosis, stage, previous therapeutic experience and evolution in patients with advanced tumours treated with IPLD ($n=15$)

Diagnosis	TNM	Previous treatment	IPLD treatment (months)	Outcome
ADC colon	IV	SUR, RT, CHT	19	Alive
ADC colon	IV	SUR, CHT	10	Alive
Malignant meningioma	IV	SUR, RT, CHT	33	Alive
Chondrosarcoma	III	SUR, RT	17	Alive
Osteoblastoma		—	SUR	17 alive
ADC parotid	IV	SUR, RT	22	Alive
Transitional meningioma	II	SUR	14	Alive
ADC gallbladder		IV	SUR	12 deceased
ADC oesophageal	IV	SUR	7	Deceased
VIPoma	IV	SUR, CHT	4	Deceased
Breast cancer	IV	SUR, RT, CHT	12	Deceased
Breast cancer	IV	SUR, RT, CHT	8	Deceased
Breast cancer	IV	SUR, RT, CHT	6	Deceased
ADC colon	IV	SUR, RT, CHT	8	Deceased
Cancer of the lung	IV	RT	13	Deceased

ADC, Adenocarcinomas; SUR, surgery; CHT, chemotherapy; RT, radiotherapy.

disease, undergoing IPLD treatment, and second, to demonstrate a possible correlation between pretreatment immunological values and the response to IPLD.

PATIENTS AND METHODS

Study subjects

A pilot study included 15 patients with histologically confirmed cancer refractory to conventional therapy, eight females and seven males with an age range of 25–59 years. Table 1 shows the diagnosis, TNM stage, previous therapeutic experience, time of treatment and evolution of the patients after IPLD. Group 1 ($n=7$) consisted of five neoplasias with metastatic disease (two adenocarcinomas (ADC) of the colon, one malignant meningioma, one ADC of the parotid), two neoplasias with a low degree of malignity (one chondrosarcoma of the posterior cranial space and one transitional meningioma), and one benign tumour (one osteoblastoma of the cervical column). Group 2 patients ($n=8$) had neoplasias with metastatic disease (three breast cancer, one ADC of the colon, one ADC of the gallbladder, one ADC of the oesophagous, one cancer of the lung, one VIPoma). All patients had been previously treated by surgery, chemotherapy and/or radiotherapy, either combined or alone. When they were accepted into the protocol, all had active and progressive disease, with a mean of 8 months (range 6–14 months) since their last treatment by radio and/or chemotherapy. For those who had undergone surgery, this had taken place a mean of at least 10 months (range 2–24 months) before the beginning of IPLD treatment. All requirements of the Helsinki protocol were fulfilled. The criteria of selection included: a Karnowsky status of >40, a life expectancy greater than 3 months, no mental disturbance, no steroid treatment, no depressive state or inadequate family cooperation. The patients were divided into two groups according to their status at the end of the period of clinical evaluation: group 1 patients were still alive, while group 2 patients had died during the course of the protocol. Clinical studies of the effects of IPLD were commenced before the initiation of immunological evaluation in all of the patients, and thus pre-therapy values were not available for them. Patients were evaluated several times during the course of the protocol. We arbitrarily grouped results according to the period of time of treatment: initial evaluation (before IPLD treatment when available), 1–4 months, 5–12 months, 12–30 months and >30 months. Informed consent was obtained from patients before entry in the study, in accordance with individual institutional policies.

A group of 45 healthy donors were also evaluated; these included 29 women and 18 men with an age range of 25–50 years.

Therapeutic laser apparatus

The therapeutic laser apparatus [14] has been designed for treating neoplasias and other systemic diseases through the delivery of infrared laser radiation via the biological circuitry of the patient. Briefly, the pulse train is selected at between 0·5 MHz and 7·5 MHz, with a relatively low duty cycle to avoid thermal energy from making the process uncomfortable. The laser beam is applied perpendicularly to the surface of the patient's skin in close proximity to the vascular interstitial closed circuit (VICC) (biological circuit which most efficiently carries laser energy to the target tissue). This procedure is repeated periodically and the clinical response monitored through conventional nuclear magnetic resonance images or other accepted methods.

Cytokine assays

sIL-2R and TNF-α were measured by an ELISA technique. Peripheral blood from patients and controls was collected by venepuncture without anti-coagulant and left to coagulate for 1 h at room temperature. The serum was collected and kept frozen at $-80°C$ until assay. Although there is some controversy concerning the measurements of cytokines on sera, and since several proteases can be released during clotting events, we decided to use sera because most previous work has been done

Table 2. Levels of TNF-α (pg/ml) in sera of patients with advanced cancer during treatment with IPLD

Group	Patients (*n*)	Months 0–1	2–4	5–12	12–30	>30
1	7	33·2±15·1† (6·0–74·0)*	49·1±13·1‡ (6·0–122·0)	145·6±26·2‡ (6·0–400·0)	102·3±16·4‡ (6·0–300·0)	35·0±5·0
		NS§	NS	NS	NS	
2	8	16·4±2·6¶ (6·0–25·0)	81·5±211·4‡ (6·0–320·0)	166·9±27·2‡ (13·0–320·0)		
Controls	47	11·5±2·5‡ (6·0–40·0)				

* Range of values.

† Statistically significant differences ($P<0·05$) in patients from group 1 according to period of evaluation: 0–1 month *versus* 5–12 months and period 2–4 months *versus* 5–12 months and 12–30 months.

‡ Statistically significant differences ($P<0·05$) between patients in groups 1 and 2 according to period of treatment *versus* controls.

§ Comparison by Student's *t*-test between groups 1 and 2. NS, Not significant.

¶ Statistically significant differences ($P<0·05$) in patients from group 2 according to period of evaluation: 0–1 month *versus* 5–12 months and period 2–4 months *versus* 5–12 months.

on sera. The TNF-α kit was provided by Genzyme (Boston, MA) and sIL-2R by Immunotech A.S. (Marseille, France). For TNF-α evaluation, monoclonal anti-TNF-α was diluted in coating buffer and 100 μl added to each well of 96-immunoplate wells. After overnight incubation at 4°C, the plate was washed three times with washing buffer. Serum samples and appropriate negative and positive controls were added in duplicate wells and incubated for 2 h at 37°C. After four washings, polyclonal rabbit anti-TNF-α was added to each well of a 96-plate well (1 h, 37°C). Alkaline-phosphatase-conjugated goat anti-rabbit immunoglobulin was dispensed into each well and incubated for 1 h at 37°C. After four washings, substrate reagent was added and after incubation at room temperature, the plate was read on a standard ELISA reader at 405 nm. For sIL-2R measurements, plates precoated with rat anti-IL-2R MoAb (11H2) were washed four times with washing buffer and incubated with standard or serum samples (40 μl) and a rat anti-IL-2R MoAb conjugated to peroxidase (33B3, which recognizes an epitope distinct from that recognized by 11H2) for 2 h at 37°C with shaking. After four more washings, plates were incubated for 30 min with substrate buffer and read at 490 nm on a standard ELISA reader. Cytokines were considered as detectable when TNF-α was > 12 pg/ml and sIL-2R > 70 pM/ml. When patients were studied several times, individual serum samples were always included on the same plate to avoid variation between plates.

Cytofluorometric analysis

Peripheral blood (100 μl) was incubated with 5 μl of different MoAbs coupled to either FITC, rhodamine (RD-1) or PE. Those employed for all patients were CD4 (T4), CD8 (T8), coupled to RD-1, CD3 (T3), CD25, 4B4 (CD29), 2H4 (CD45RA), coupled to FITC (Coultronics, Hialeah, FL) and Leu-11c (CD16) coupled to PE (Becton Dickinson, MA). After 30 min of incubation at room temperature, the blood cells were washed twice and the erythrocytes lysed (Immunolyse, Coultronics). Cell counts were performed in an 'EPICS 753' (Coultronics) equipped with a 5 W argon dye laser, and fluorescence was displayed on a logarithmic scale. For two-colour analysis, a 530 nm short-pass filter for FITC and 590 nm long-pass filter for PE were employed. Green (FITC) and red (PE) fluorescence were measured on lymphocytes gated at forward and right-angle scatters. Results were expressed as per cent of total lymphocytes.

Statistical analysis

The statistical significance of the differences between the mean±s.d. of the values of the two groups of patients and between the different periods of evaluation within a group was estimated by the normal or paired Student's *t*-test respectively. The Spearman rank correlation coefficient was used to determine the significance of the different correlations calculated. The proportions of patients with positive results for the different evaluations were compared by the χ^2-test.

RESULTS

Serum levels of cytokines in normal controls and cancer patients

The serum levels of TNF (Table 2) at the beginning of IPLD treatment were low and not statistically different between the two groups of patients and the normal controls. However, 60% of patients in group 1 and 83% in group 2 had detectable TNF-α levels (> 12 pg/ml), compared with 21% in the control group. The difference between group 2 and the controls was statistically significant ($P<0·005$).

A significant increase ($P<0·05$) in TNF-α was found in group 1 after 5–12 months of treatment, compared with both their initial values and values at 2–4 months of treatment. This difference was still observed at 12–30 months of treatment. However, upon the last evaluation of two patients from this

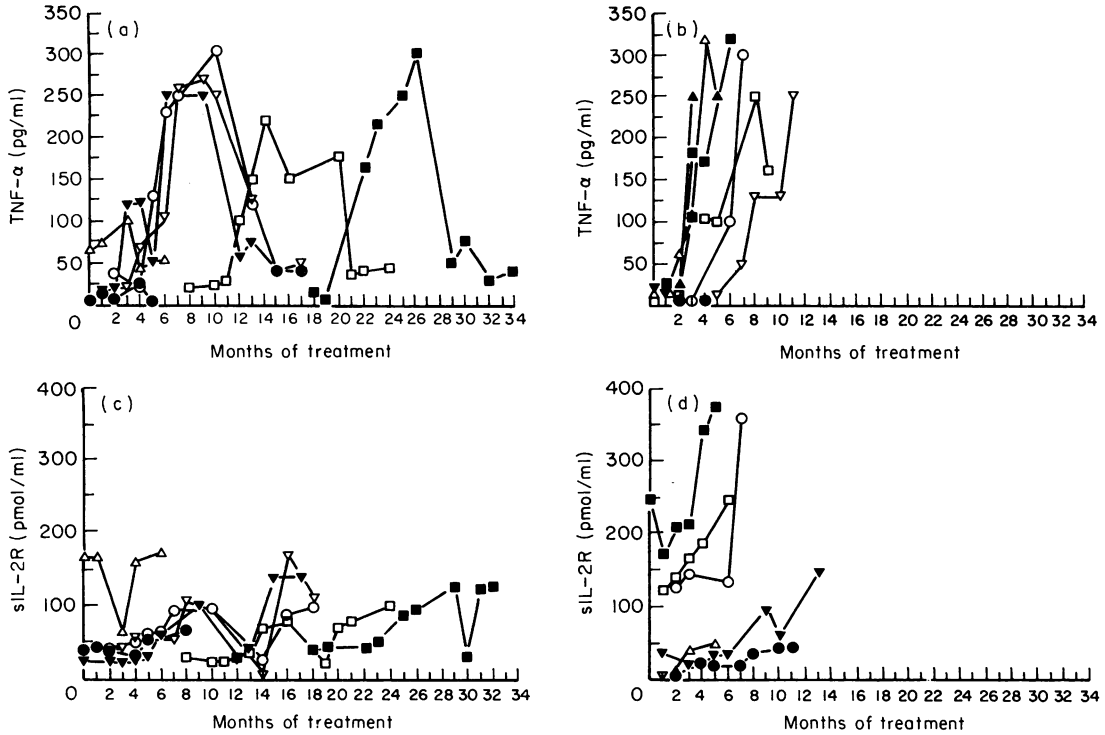

Fig. 1. Evolution of TNF-α and sIL-2R levels during IPLD treatment. Patients were divided in two groups according to outcome: survivors ($n=7$, (a), (c)) and patients who died ($n=7$, (b), (d)). For each patient evolution of serum levels is shown. The months of IPLD treatment are indicated. (a) TNF in surviving patients. (b) TNF in patients who died. (c) sIL-2R in surviving patients. (d) sIL-2R in patients who died.

Table 3. Levels of sIL-2R (pM) in sera of patients with advanced cancer during treatment with IPLD

Group	Patients (*n*)	Months 0–1	2–4	5–12	12–30
1	7	87·9±31·7† (24·6–165·0)*	49·3±11·7 (23·7–160·0)	66·9±8·3 (23·9–171·0)	66·9±8·3 (6·0–166·0)
		NS‡	$P<0·05$	NS	
2	8	116·2±43·8†§ (6·0–246·0)	117·8±24·6 (6·0–340·0)	118·2±32·7 (19·0–375·0)	
Controls	47	37·2±4·2§ (6·0–40·0)			

* Range of values.

† No statistically significant differences ($P>0·05$) in group 2 patients at the different periods of treatment.

‡ Comparison by Student's *t*-test between groups 1 and 2. NS, Not significant.

§ Statistically significant differences between group 2 patients and controls at initial evaluation.

group who had more than 30 months of treatment, values had decreased to normal.

We also observed a significant increase ($P<0·05$) in TNF-α values in group 2 after 2–4 months of treatment; this increase continued to the time of their deaths, when the values were significantly higher ($P<0·05$) compared with earlier times of treatment.

Individual results of the different evaluations performed in patients from the two groups are shown in Fig. 1. In patients from group 2, we observed a progressive and constant increase in TNF-α up to the time of death. In contrast, in patients from group 1, the levels of TNF-α increased steadily from their initial evaluation, but then decreased during the course of treatment.

In contrast to TNF values, the evaluation of sIL-2R (Table 3) demonstrated high levels of this marker upon initial evaluation of both groups of patients when compared with controls, although this difference was statistically significant only for group 2. Moreover, the percentage of patients with significant

Table 4. Comparison of surface markers in cancer patients and controls

Cell subset	Patients ($n=15$)	P	Controls ($n=11$)
CD4	34·14±8·1	<0·01	42·24±6·0
CD8	21·77±7·7	NS	22·5±5·9
CD3	68·36±12·4	NS	62·67±9·8
CD25	6·17±6·36	<0·01	1·94±1·22
CD16	8·10±7·6	NS	6·51±4·9
$CD4^+CD29^+$	24·67±10·0	NS	24·28±7·7
$CD4^+CD45RA^+$	12·7±6·4	<0·05	18·79±9·7

Results are expressed as mean±s.d. per cent of positive cells among total lymphocytes.

values of sIL-2R (>70 pM) was higher in group 1 (40%) and group 2 (60%) when compared with controls (0%), with this difference also statistically significant between group 2 and controls. When we compared the sIL-2R levels between patients from group 1 and group 2 at different periods of evaluation, the general tendency showed higher values for group 2 than for group 1 at all times, but this difference was statistically significant ($P<0·05$) only at 2–4 months of treatment.

During treatment (Fig. 1) we observed an increase in serum values of sIL-2R in the second group of patients, while group 1 had only minor variations. An analysis of the Spearman correlation coefficient between TNF and sIL-2R for patients from both groups demonstrated a positive and significant ($r=0·436$; $P=0·008$) correlation for patients from group 2, but not for patients from group 1 ($r=0·09$; $P=0·47$).

Cytofluorometric analysis of lymphocyte subsets

Table 4 shows the percentages of different lymphocyte subsets in controls and in cancer patients from both groups when studied at the beginning of the protocol. We found a significant decrease in $CD4^+$ and in $CD4^+CD45RA^+$ in cancer patients when compared with normal controls ($P<0·01$). In contrast, $CD25^+$ cells were significantly increased in the patients ($P<0·05$). No significant differences were found in CD8, CD3, CD16 and $CD4^+CD29^+$ populations between patients and controls.

The lymphocyte subsets were followed in five patients from group 1 during IPLD treatment (results not shown). A tendency toward an increase occurred in all five patients evaluated for $CD4^+$, and in three patients who were evaluated for $CD4^+CD45RA^+$ and $CD25^+$. No consistent changes were found for CD8, and in all five patients a slight decrease in $CD16^+$ cells was observed.

An analysis of the Spearman correlation coefficient shows a positive and highly significant ($r=0·769$; $P=0·009$) correlation between the serum levels of sIL-2R and the expression of CD25 marker at the surface of lymphoid cells, measured by fluorocytometry, in patients from group 1, but not in patients from group 2.

DISCUSSION

This study was designed to determine immunological effects in patients with advanced neoplastic disease undergoing IPLD treatment. Neoplastic disease is known to have a profound influence upon immune responses. Growing tumours have been associated with decreasing immunocompetence, including disturbances in cells concerned with immunoregulation [15].

In the present report, we evaluated TNF-α and sIL-2R in the sera of cancer patients treated by IPLD. It should be noted that controversy exists concerning the validity of both biological and immunological methods for the detection of cytokines. The advantage of biological methods lies in the fact that, by definition, they measure the biological activity of the studied cytokine. However, it cannot be excluded that other cytokines also present in the biological sample may influence the results. Immunological methods specifically measure the cytokine in question, but do not provide information about its biological activity. Given the wide application and acceptance of the immunological methods, we chose to employ ELISA in the present study.

The role of TNF during immunological responses against tumour cells is still unclear. Early production of TNF could select TNF-resistant tumour cells, and TNF might be secreted by cancer cells themselves [16,17]. Moreover, late production of TNF has been associated with poor outcome for patients, deleterious inflammation-related phenomena and poor response to treatment [6]. This is in agreement with our results, since we demonstrated that the levels of TNF increased progressively in patients from group 2 until the moment of death. In contrast, the decrease in TNF levels observed in patients from group 1 was associated with a better clinical course for these patients. These results are consistent with the hypothesis that an inactive and necrotic tumour that is regressing is not able to produce TNF or to stimulate TNF production by other cells. Similar results have been obtained in patients with malignancies in which complete remission has been achieved [6].

This argues in favour of the possibility that IPLD can play a role in reestablishing regulatory mechanisms of the immune response that were altered by growing tumours. We speculate, therefore, that serum levels of TNF may be of potential value in the follow up of cancer patients during IPLD treatment and that a decrease in TNF-α could be indicative of a better clinical response.

We also studied the serum levels of sIL-2R. IL-2, which is a pivotal cytokine in T cell differentiation and activation, acts via an interaction with high affinity cell membrane receptors (IL-2R). The high affinity receptor consists of two chains of 75 kD and 55 kD. The CD25 antibody detects 55-kD sIL-2R which is a circulatory form of the 55-kD chain. CD25 has been reported to be increased in several clinical situations, with an imbalance of immune homeostasis [18–20].

Our results showed that serum levels of sIL-2R were significantly increased in three of these patients in group 2 compared with those in group 1. Moreover, the level of sIL-2R was significantly higher at initial evaluation in most patients in group 2, and this tended to increase even more in four of the six patients before their deaths. The mechanism responsible for the increased release of sIL-2R in patients with disseminated solid tumours is still obscure. However, it could be due to unknown factors produced by cancer cells themselves, capable of affecting the normal expression of IL-2R at the cell surface [21]. It has been suggested that high levels of sIL-2R may contribute to the poor prognosis of cancer patients by blocking IL-2-mediated cytotoxic activities, since sIL-2R is capable of binding to free IL-2 [22].

Similarly, various authors have reported that a favourable response to treatment in patients with advanced cancer is associated with a reduction in the serum levels of sIL-2R [20]. In our patients, levels of this marker were somewhat high during the course of IPLD treatment in patients from group 1. This might reflect a cellular immune process in the region of the tumour or activation of the immune system. Indeed, we found a significant and strongly positive correlation between the serum level of this receptor and $CD25^+$ cells in patients from group 1. It has been well documented that expression of this receptor by cells of the lymphoid system (CD4 and CD8) is a sign of cellular activation [10]. Thus, this correlation suggests the possibility that the soluble receptor measured in the serum could be, in part, produced by activated T cells, and could thus reflect the immune response of the host against its tumour. The production and regulation of IL-2 and its receptor are closely related. Sharma *et al.* [23] were unable to correlate sIL-2R levels with CD25 expression on lymphocytes in women with breast cancer, but they found a significant negative correlation between this marker and lymphocytes within the tumours in this same group of patients. This indicates that sIL-2R exerts an immunomodulatory effect on blood lymphocytes by preventing their infiltration into the tumour tissue.

Our results suggest that high levels of sIL-2R at the beginning of the treatment could have a prognostic value in determining the response of the patients to IPLD. It is possible that treatment with IPLD produces variations in the host–tumour relationship, due to a decrease in tumour load in those patients who reacted positively to it. In contrast, the exaggerated production of this receptor could be a reflection of an imbalance in T cell subset homeostasis, in such a way that treatment with IPLD is unable to control it.

Expression of surface markers has revealed differences between advanced cancer patients at the beginning of evaluation, and controls in percentages of $CD4^+$,$CD4^+$ CD45RA and $CD25^+$ cells. Various authors have reported a decrease in CD4 cells in progressive and metastatic cancer patients [21,24]. Our results support the concept that the decrease in CD4 cells is due to a decrease in $CD45RA^+$ cells which are inducers of suppression [25]. This indicates that immune dysfunction existed in our patients before they started IPLD treatment.

When we followed a small group of patients by fluorocytometry during IPLD treatment, it was possible to demonstrate an increase in $CD4^+$ and $CD4^+CD45RA^+$ cells. Our results are in agreement with others [26] who reported a significant increase in $CD4^+$ cells which reached normal levels after radiation therapy for cancer of the uterine cervix only in disease-free patients, whereas these levels remained low in those patients with active disease.

Because of the wide variability in the parameters measured, further research, including the follow up of a larger group of patients, is needed to confirm whether the differences we observed between the groups can be used to predict the response and outcome of cancer patients undergoing IPLD treatment.

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