Systemic inflammatory response and downmodulation of peripheral CD25⁺Foxp3⁺ T-regulatory cells in patients undergoing radiofrequency thermal ablation for lung cancer

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Abstract
Radiofrequency thermal ablation (RFTA) is a local tumor-destructing technique that can potentially modulate the host immune response through mechanisms that are not clearly defined. We assessed whether RFTA could affect multiple systemic inflammatory and immunological parameters, including CD25⁺Foxp3⁺ cells, in patients with primary or metastatic lung tumors. Three days after RFTA, a moderate and temporary systemic inflammatory response developed, as demonstrated by the increase in peripheral neutrophils and monocytes and in plasma levels of proinflammatory chemokines (MIP-1α, MIP-1β, eotaxin, and interleukin(IL)-8) and acute phase reactants (complement C3 and C4, serum amyloid, α1 antichymotrypsin, and C-reactive protein). Moreover, we found a concomitant release of the anti-inflammatory factor IL-10. Thirty days after RFTA, a significant reduction in CD25⁺Foxp3⁺ counts with an increase in CD4⁺ T-cell proliferation and number of interferon-γ-secreting cells was observed. The reduction in CD25⁺Foxp3⁺ cells lasted up to 90 days after treatment. The use of RFTA in lung cancer patients has an immunomodulatory activity: it induces a self-limiting systemic inflammation early and later a reduction of circulating CD25⁺Foxp3⁺ Tregs. In addition to tumor ablation, downmodulation of this regulatory subset might be an important mechanism involved in the long-term clinical efficacy of RFTA.

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1. Introduction
Radiofrequency thermal ablation (RFTA) is a minimally invasive technique that has proved to be effective in the treatment of nonsurgical patients with liver cancer [1,2]. Ongoing experiences indicate that RFTA yields high proportions of sustained complete responses in properly selected patients with pulmonary tumors and is associated with acceptable morbidity [3–8]. This treatment destroys tumor tissue by delivering a high-frequency alternating current with ionic agitation and frictional heating. As a consequence, cell membrane alteration, protein denaturation, and necrosis around the electrode are produced and large amounts of tumor and tissue debris are released, which can potentially modulate the host’s immune response [9,10].

Studies in animal models have provided some evidence that RFTA, as well as producing direct antitumor effects, can also exert immunomodulatory effects. One study in a rabbit tumor model demonstrated that RFTA treatment was followed by a marked local inflammatory response with a dense T-cell infiltrate caused by the generation of an antigen-specific T-cell response [11]. Furthermore, den Brok and collaborators [12] demonstrated in a mouse tumor model that RFTA-induced immune response was enhanced by the coadministration of anticytotoxic T-lymphocyte-associated antigen 4 blocking antibodies. Blocking of anticytotoxic T-lymphocyte-associated antigen 4 leads to the abolishment of the function of natural-occurring CD4⁺CD25⁺Foxp3⁺ T-regulatory cells (Tregs) [13,14].

Few studies exist on the effects of RFTA on immunological parameters in humans. Sanchez-Ortiz and collaborators [15] reported neutrophilia and monocytosis in a patient who had a spontaneous regression of a pulmonary metastasis from renal cell carcinoma following successful RFTA treatment of the primary tumor. Ruggehi and colleagues [16] confirmed these data, demonstrating that RFTA induced fever and increased neutrophil counts in the peripheral blood of patients with liver metastasis. The capacity of RFTA to induce the release of proinflammatory mediators remains a source of ongoing controversy. Whereas one study claimed that RFTA does not affect the production of cytokines [17], others demonstrated significant release of interleukin (IL)-6, tumor necrosis factor receptor 1, C-reactive protein (CRP), hepatocyte growth factor, vascular endothelial growth factor, and secretory phospholipase A2 in
plasma or sera from patients with liver tumors [18–20]. Finally, Kawakami and collaborators [21] demonstrated, through the use of a high-resolution matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer, the up- or downregulation of some acute-phase reactants (APRs) in serum samples from patients with RFTA-treated hepatocellular carcinoma (HCC).

With regard to the modulation of T-cell responses, Zerbini and collaborators [22] demonstrated the presence of an increased number of peripheral tumor-specific T cells and activated T and natural killer (NK) cells, as well as a late enhanced T-cell response to tumor antigens in patients with HCC treated with RFTA. In accordance with these data, Hansler and colleagues [23] demonstrated significant tumor-specific cytotoxic T-cell stimulation with a statistically increased tumor-specific cytolytic activity of CD8+ T cells in patients with HCC or colorectal liver metastases. Although the mechanisms involved in the immunomodulatory effect of RFTA remain to be elucidated, a recent study in HCC patients [24] demonstrates that this effect depends at least in part on the maturation of dendritic cells (DCs) driven by cellular debris released after RFTA.

In this study, we evaluated whether RFTA is associated with a systemic inflammatory response (SIR) and whether the treatment can affect the entity and/or the function of peripheral CD25+Foxp3+ Tregs in a group of patients with inoperable primary or metastatic lung cancer. No study has yet been performed to assess the immunomodulatory activity of RFTA in lung cancer patients, and no data have been published thus far on the possible effect of treatment on CD25+Foxp3+ Tregs.

Soon after the RFTA procedure, a moderate and self-limiting SIR was observed in lung tumor patients, as indicated by the increase in neutrophils and monocytes and plasma levels of multiple proinflammatory mediators. Later after RFTA, there was a significant and long-lasting reduction of the peripheral CD25+Foxp3+ Treg pool with an improvement in patient T-cell reactivity. Downregulation of this regulatory subset might represent one of the mechanisms involved in the long-term clinical efficacy of RFTA.

2. Subjects and methods

2.1. Patients

Between January 2005 and April 2007, 20 patients scheduled for percutaneous RFTA for inoperable primary (n = 14) or metastatic (n = 6) lung cancers were enrolled in the study. All patients had been evaluated jointly by the anesthesiology and surgical staff of Fondazione IRCCS Policlinico San Matteo, University of Pavia. In all cases, the patients’ tumors were considered potentially resectable from an anatomic point of view, but patients were excluded from surgical option on the basis of surgical risk [25,26] and/or on the patient’s refusal to undergo surgery. Patients underwent spiral computed tomography (CT)-guided RFTA after an overnight fast, without a general anesthetic or conscious sedation, as previously reported [3]. Intravenous antibiotic coverage (1 g cefotaxime t.i.d.) was started 3 h prior to the procedure and continued through to the third postoperative day. Patients underwent clinical–radiological–immunological follow-up for 3 months. Spiral CT was performed at the end of the RFTA procedure (for gross assessment of results and the detection of possible complications) and repeated 30 and 90 days later. The presence at the tumor site of a nonenhancing area larger than the treated area on the 30-day CT scan was considered radiological evidence of successful treatment, whereas the presence of enhancing tissue was regarded as indicative of incomplete treatment. Six patients were excluded from the final evaluation because they did not complete the immunological follow-up in an appropriate amount of time. Demographics and diagnoses of the 14 patients evaluated are described in Table 1. Patients had not received any drug that could affect immune responses in the 2 months preceding the enrollment and gave their written informed consent to participate in the study. Twenty normal healthy controls (NHCs; mean age 57.5 ± 17.6 years old; 15 males and 5 females) were used as a control group for immunological parameters.

2.2. Blood collection

Peripheral blood was withdrawn immediately before RFTA (T0) and 3 (T3), 30 (T30), 60 (T60), and 90 (T90) days after treatment for the assessment of whole blood counts (WBCs), routine hematology, and lymphocyte phenotyping. An aliquot of plasma and peripheral blood mononuclear cells (PBMCs) was separated from each blood specimen and stored at −80°C until analysis. Total and differential WBC counts were performed at the Clinical Chemistry Laboratories of Fondazione IRCCS Policlinico, San Matteo, Pavia.

2.3. Flow cytometric analysis

Flow cytometry was performed on a Becton Dickinson FACSscan using CellQuest software (Becton Dickinson Biosciences, Franklin Lakes, NJ). Briefly, 50 µl of fresh whole blood was incubated with the appropriate amounts of fluorochrome-labeled monoclonal antibodies at 4°C for 30 min and appropriate mouse immunoglobulin isotypes as a control (all purchased from BD Biosciences). To assess the putative CD4+ Treg subset, 50 µl of fresh whole blood was incubated with the appropriate amounts of fluorochrome-labeled monoclonal antibodies (CD4+ Cy5.5, CD69PE, CD25FITC) at 4°C for 30 min and the appropriate mouse immunoglobulin isotypes. Lymphocyte subsets were gated in the dot plot obtained from side scatter and CD4+ Cy5.5, and the percentages of total CD4+ CD25-Foxp3+ cells (as percentage of lymphocytes) were evaluated, as previously described [30]. The CD4+CD25+ fraction was determined by isolating the tail of the histogram of CD4+ CD25+ cells with the same setting used in all patients (by fluorescence intensity of 2.5 × 101), as previously established [28].

2.4. Cytokine plasma profile

Chemokines and cytokines were assayed simultaneously in frozen plasma samples by means of the Pierce Endogen SearchLight assay (Pierce Biotechnology Waburn, MA). We used the human chemokine array 1 to measure levels of MIP-1α, MIP-1β, MCP-1, RANTES, I-309, TARC, eotaxin, MDC, and IL-8 and the inflammatory cytokine array 3 for IL-1β, IL-6, IL-10, IL-12p70, and tumor necrosis factor-α. The assays were run according to the instructions provided by the manufacturer. Briefly, diluted plasma samples or cal-

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Table 1

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Primary lung cancer</th>
<th>Pulmonary metastases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>71</td>
<td>NSCLC (IA)</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>67</td>
<td>NSCLC (IA)</td>
<td>Colorectal cancer</td>
</tr>
<tr>
<td>3</td>
<td>Male</td>
<td>68</td>
<td>NSCLC (IA)</td>
<td>Urothelima of the upper urinary tract</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>65</td>
<td>NSCLC (IA)</td>
<td>Renual papillary carcinoma</td>
</tr>
<tr>
<td>5</td>
<td>Male</td>
<td>51</td>
<td>NSCLC (IA)</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Female</td>
<td>61</td>
<td>NSCLC (IA)</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Male</td>
<td>58</td>
<td>NSCLC (IB)</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Female</td>
<td>74</td>
<td>NSCLC (IA)</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Male</td>
<td>83</td>
<td>NSCLC (IA)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Female</td>
<td>75</td>
<td>NSCLC (IA)</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Male</td>
<td>66</td>
<td>NSCLC (IA)</td>
<td></td>
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<tr>
<td>12</td>
<td>Male</td>
<td>60</td>
<td>NSCLC (IB)</td>
<td></td>
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<tr>
<td>13</td>
<td>Male</td>
<td>61</td>
<td>NSCLC (IB)</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Male</td>
<td>68</td>
<td>NSCLC (IA)</td>
<td></td>
</tr>
</tbody>
</table>

Patients were excluded from surgical option on the basis of surgical risk or on the patient’s refusal to undergo surgery. The TMN staging was performed on the basis of Mountain’s revised classification [27]. A single pulmonary node was present in 11 patients (7 with NSCLC and 4 with pulmonary MET), whereas the other 3 patients with NSCLC had two nodes. The mean tumor diameter was 22 ± 11 mm. NSCLC, non-small cell lung carcinoma.
2.5. Plasma APR profile

Variations in the plasma content of APRs [29] were assessed by a proteomic approach based on bidimensional gel electrophoresis (2-DE). The details of the 2-DE protocol were as previously described [30]. Briefly, for each experiment the same amount of plasma (10 μl) was loaded onto a 3–10 nonlinear immobilized pH gradient IPG gel strip (GE Healthcare, Buckinghamshire, UK). Isoelectric focusing was performed using the Ettan IPGphor system. In the second dimension, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (PAGE) was carried out in a Protean II xi 2-D cell apparatus (Bio-Rad, Richmond, CA). Each sample was assessed in triplicate. Gels were stained with ammoniacal silver nitrate (VWR, International LLC, West Chester, PA) or colloidal Coomassie G-250 (Bio-Rad), scanned on a Versadoc Imaging System Model 3000 (Bio-Rad) and images were then imported into PDQuest 7.1 software (Bio-Rad). Initially, we compared the synthetic gels generated by the PDQuest software with only the spots present in each patient before and 3 days after RTFA. Thereafter, we analyzed individual samples in the same system. The total density of a defined spot in a gel image was used as a measure of spot abundance. Spots that differed in density after RTFA with respect to baseline values (ratio post/before: ≥2 and p < 0.05, as assessed by paired Student’s t test) were regarded as differentially expressed. The APR protein spots were identified by gel matching with published Swiss-2DPAGE plasma maps and then confirmed by immunoblotting. In addition, levels of CRP were also assessed by means of a standard turbidimetric method at the Clinical Chemistry Laboratories of Fondazione IRCCS Policlinico, San Matteo, Pavia.

2.6. Isolation of T-cell subsets

We purified total CD4+1, the putative CD4+CD25+ Treg fraction, and its counterpart (CD4+CD25−) from frozen aliquots of PBMCs by magnetic cell sorting (Miltenyi Biotech GmbH, Bergisch Gladbach, Germany) using the CD4+CD25− regulatory T-cell isolation kit (Miltenyi Biotech GmbH) according to the manufacturer’s instructions. In the first step, total CD4+ were negatively purified; in the second step, CD4+CD25− were obtained by negative selection and CD4+CD25+ cells were positively selected (eluted column–retained cells). The artificial activation of CD4+CD25− cells was excluded by the analysis of surface expression of CD69 that was less than 0.5%. The purity of CD4+CD25− and CD4+CD25+ subsets was greater than 90%, and vitality was greater than 99% by the Trypan blue exclusion test.

2.7. Proliferation and suppression assays

Proliferation was performed on total CD4+1 and purified CD4+CD25+ cells in the presence or absence of CD4+CD25− (ratio 1:1). Briefly, cells (5 × 10^4/well) were stimulated in triplicate with T-cell-depleted (by negative selection with anti-CD3 magnetic cell sorting MicroBeads) and irradiated (3000 RADS) fully mismatched allogenic antigen–presenting cells (APCs; 5 × 10^5/well), cultured in 96-well round-bottom plates for 5 days, and then pulsed for an additional 18 h with 1 μCi of [3H]thymidine/well (Amersham, Pharmacia Biotech, Uppsala, Sweden). Cells were harvested and the radioisotope uptake was measured by scintillation counting. [3H]thymidine incorporation by unstimulated control wells was always subtracted.

2.8. Real-time polymerase chain reaction

To assess Foxp3 gene expression, real-time polymerase chain reaction was performed with an ABI prism 7000 apparatus (Applied Biosystems, Foster City, CA) using the Assays on Demand kit for Foxp3 (Applied Biosystems), as previously described [28]. Raw data were analyzed using ABI Prism SDS software (Applied Biosystems), and Foxp3 mRNA expression data were normalized to 18s RNA content.

2.9. Enzyme-linked immunosorbent spot assay

To measure the cytokine profile of all potentially responding cells, we assessed the frequency of interferon (IFN)−γ producing cells after they were challenged with mitogen. Briefly, ImmunoSpot plates (Polyfiltrons, Rockland, MA) were coated with capture antibody specific for IFN−γ (Endogen, Woburn, MA) and incubated overnight at 4°C. A total of 3 × 10^5 PBMCs were added to each well with or without phytohemagglutinin A (PHA, Sigma, St. Louis, MO). After 24 h of incubation at 37°C, biotinylated detection antibody (Endogen) was added. The spots were visualized and counted by a computer-assisted image analyzer (A-EL-VIS GmbH, Hannover, Germany). The number of IFN−γ producing cells was calculated by averaging the number of spots of triplicate wells. Background spot formation was always subtracted from PHA-stimulated cultures.

2.10. Statistical analysis

Data are presented as means ± SD because all variables were normally distributed. Statistical significance of the differences between groups was assessed by a two-tailed Student t test. When comparisons were performed at multiple time-points, one-way analysis of variance followed by Bonferroni’s posttest was used. A p value < 0.05 was considered statistically significant. Calculations were performed using commercial software (GraphPad Prism, version 4, for Windows; GraphPad Software, San Diego, CA).

3. Results

3.1. Clinical data

Fourteen patients with pulmonary malignancies (10 with non-small-cell lung cancer [NSCLC] and 4 with pulmonary metastasis [MET]) underwent spiral CT-guided RFTA without a general anesthetic or conscious sedation. A single pulmonary node was present in 11 patients (78.5%), whereas the other 3 patients (21.5%) had 2 nodes. Each of the 17 lung nodules (mean diameter ± SD, 22 ± 11 mm) had between 10 and 44 WBCs. Results are presented means ± SD, 22 ± 11 mm) has been excluded by the analysis of surface expression of CD69 that was less than 0.5% (ratio post/before: ≥2 and p < 0.05, as assessed by paired Student’s t test) were regarded as differentially expressed. The APR protein spots were identified by gel matching with published Swiss-2DPAGE plasma maps and then confirmed by immunoblotting. In addition, levels of CRP were also assessed by means of a standard turbidimetric method at the Clinical Chemistry Laboratories of Fondazione IRCCS Policlinico, San Matteo, Pavia.

### Table 2

<table>
<thead>
<tr>
<th>WBCs (10^5/μl)</th>
<th>T0</th>
<th>T3</th>
<th>T5</th>
<th>T10</th>
<th>T30</th>
<th>T90</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>5.48 ± 1.92</td>
<td>7.72 ± 2.03*</td>
<td>6.20 ± 2.30</td>
<td>5.98 ± 1.83</td>
<td>6.02 ± 2.30</td>
<td>5.98 ± 1.83</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.16 ± 1.35</td>
<td>5.33 ± 1.97**</td>
<td>3.73 ± 1.60</td>
<td>3.51 ± 1.40</td>
<td>3.73 ± 1.60</td>
<td>3.51 ± 1.40</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.58 ± 0.62</td>
<td>1.53 ± 0.49</td>
<td>2.00 ± 1.20</td>
<td>1.98 ± 0.80</td>
<td>2.00 ± 1.20</td>
<td>1.98 ± 0.80</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.49 ± 0.17</td>
<td>0.71 ± 0.31*</td>
<td>0.57 ± 0.28</td>
<td>0.52 ± 0.25</td>
<td>0.57 ± 0.28</td>
<td>0.52 ± 0.25</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>0.21 ± 0.18</td>
<td>0.19 ± 0.17</td>
<td>0.25 ± 0.21</td>
<td>0.21 ± 0.18</td>
<td>0.25 ± 0.21</td>
<td>0.21 ± 0.18</td>
</tr>
<tr>
<td>Basophils</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.02</td>
<td>0.03 ± 0.01</td>
<td>0.03 ± 0.02</td>
</tr>
</tbody>
</table>

WBCs = white blood cells. T0: before RTFA; T3: 3 days; T5: 30 days, and T90: 90 days post RTFA treatment. Results represent means ± SD.

*p = 0.01; **p < 0.001.
Fig. 1. Effects of RFTA on plasma chemokines. Nine chemokines were simultaneously assessed by means of the human chemokine array 1, before (T₀) and 3 (T₃) and 30 (T₃₀) days post RFTA treatment. All samples were assessed in triplicate. Results represent the mean concentration (pg/ml) ± SD. p values (T₃ vs. T₀) are illustrated.
mm) was treated in a single RFTA session using a single electrode insertion. The minimum duration time for the procedure was 18.5 minutes. In 12 patients the procedure was painless or associated with slight discomfort; in the remaining 2 patients, mild to moderate pain was controlled with a single intravenous injection of tramadol (100 mg). Mild fever (≤38°C) was observed in 6 patients (42.8%) in the initial 24–48 hours after treatment. Posttreatment CT revealed the development of pneumothorax in 2 cases (14%), but both were self-limiting and did not require drainage. These results, which are consistent with those of previous studies [3,4,6], confirm the safety of the procedure in lung cancer. Results of 30-day follow-up CT scans revealed complete radiological necrosis of all lung nodules (100% successful treatment). All patients were alive at the 90-day follow-up: 12 (85.7%) were apparently disease free at CT scan and 1 patient with NSCLC and 1 with pulmonary MET (both with a single node) experienced a new lung nodule at a site other than the treated site and a new extrapulmonary spread, respectively.

3.2. Effects of RFTA on SIR parameters

We considered total and differential WBCs, plasma levels of chemokines, cytokines, and APRs as SIR parameters. A significant increase in the number of total WBCs, particularly neutrophils and monocytes, was observed 3 days post-RFTA treatment compared with pretreatment values (Table 2). Thereafter, the parameters returned to baseline values.

By means of multiple enzyme-linked immunosorbent assays (ELISA), we assessed the chemokine and cytokine plasma profile before (T₀) and 3 (T₃) and 30 (T₃₀) days after RFTA (Figs. 1 and 2). Baseline levels of these factors were in the range of those measured in a group of six NHCs (data not shown). Except for MCP-1 and MDC, whose levels did not vary, the levels of all other factors were slightly enhanced 3 days posttreatment. However, the increase was significant (p < 0.05) only for the proinflammatory chemokines (MIP-1α, MIP-1β, eotaxin, and IL-8; Fig. 1) and for the anti-inflammatory cytokine IL-10 (Fig. 2). Thereafter, all of these factors returned to baseline levels.

Furthermore, we assessed whether RFTA treatment could affect plasma-positive and -negative APRs. Fig. 3 illustrates a representative 2-DE gel image of the plasma protein profile before (Fig. 3A) and 3 days (Fig. 3B) after RFTA treatment. Spot quantity of four positive APRs (i.e., the complement proteins C3 and C4, serum amyloid component, and α1-anti-chymotrypsin) significantly increased 3 days after RFTA compared with pretreatment values (Table 3). In addition, a significant increase in CRP was observed 3
Fig. 3. A representative 2-DE gel image of plasma protein profile before (A) and 3 days (B) after RFTA. Acute phase reactants were identified by gel matching with published plasma 2-DE images and subsequently confirmed by immunoblotting. Spot number refers to proteins listed in Table 3.
3. Effects of RFTA on peripheral CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs

No significant variation in either the percentage (data not shown) or the absolute number of CD3<sup>+</sup>, CD4<sup>+</sup>, and CD8<sup>+</sup> T lymphocytes, B cells, and NK cells was found after the RFTA procedure compared with baseline values (Table 4). In contrast, we observed a significant reduction in the percentage (data not shown) and numbers of CD4<sup>+</sup> CD25<sup>+</sup> CD69<sup>+</sup> cells over time (Fig. 4). Fig. 4A illustrates a representative FACS staining illustrating the decrease in peripheral CD4<sup>+</sup> CD25<sup>+</sup> CD69<sup>+</sup> cells after RFTA, whereas Fig. 4B shows the time course of this cell subset in patients and in a group of 10 NHCs. Variation in CD4<sup>+</sup> CD25<sup>+</sup> CD69<sup>+</sup> counts over time in NHCs was always less than 15%. Although individual variation in CD4<sup>+</sup> CD25<sup>+</sup> CD69<sup>+</sup> was present, pretreatment values were significantly higher in patients with respect to NHCs (p < 0.001). No significant variation in this cell subset was observed early after RFTA, but 30 days posttreatment a significant reduction (from 30 to 80%) in these cells occurred. Moreover, the decrease persisted up to 90 days post RFTA, maintaining cell counts in the range of those measured in NHCs.

In vitro functional studies performed on CD4<sup>+</sup> CD25<sup>+</sup> cells purified from patients before and 30 days after RFTA demonstrated that these cells had functional features of Tregs. They were hyporesponsive to T-cell receptor (TCR) stimulation compared with conventional T cells (Tcon) (mean [3H]thymidine uptake/5 × 10<sup>4</sup> cells was 479 ± 364 vs. 6850 ± 2869 at T<sub>0</sub> and 539 ± 415 vs. 5389 ± 2650 at T<sub>30</sub>; p < 0.01) suppressed the proliferation of Tcon in cocultures (percentage of inhibition, 67.9 ± 14.7 and 65.8 ± 13.88 at T<sub>0</sub> and T<sub>30</sub>, respectively) and expressed constitutively higher levels of Foxp3 mRNA compared with Tcon (ΔΔCT vs. 18s RNA, 87.59 ± 25.70 vs. 1.15 ± 0.55 and 89.35 ± 21.57 vs. 1.99 ± 0.58 at T<sub>0</sub> and T<sub>30</sub>, respectively).

To evaluate whether a reduction in CD25<sup>+</sup> Foxp3<sup>+</sup> could influence patient’s immune competence, we measured the degree of proliferation of total CD4<sup>+</sup> T cells and the number of IFN-γ-secreting cells before and 30 days after RFTA. Ten NHCs were used as a control group. Before treatment, a significant reduction in the proliferation of total CD4<sup>+</sup> T cells (in response to allogeneic stimulation), as well as in the number of IFN-γ-secreting cells (in response to mitogen), was observed in patients compared with NHCs (Fig. 5A and B, respectively). However, 30 days post RFTA both of these parameters were significantly improved compared with the baseline values, reaching values in the range of those obtained in NHCs.

4. Discussion

This study provides the first experimental evidence that RFTA can produce multiple early and late effects on systemic immune reactions in lung cancer patients and, more notably, that it can induce a significant and long-lasting reduction of the peripheral CD25<sup>+</sup> Foxp3<sup>+</sup> Treg pool. Shortly after pulmonary RFTA, a moderate and self-limiting systemic inflammatory reaction develops. An increase in blood neutrophil and monocyte counts, a significant release in plasma of proinflammatory chemokines, such as MIP1α, MIP 1β, eotaxin, and IL-8, and an increase in plasma levels of some positive APRs, including the complement proteins C3 and C4, the amyloid serum component, the protease inhibitor α1-anti-chymotrypsin, and CRP, were observed 3 days post RFTA. Thereafter, these parameters returned to baseline values. These findings strengthen and extend those made by other investigators in malignant liver tumors [17-21]. Systemic acute phase response is a component of innate immunity and a consequence of local or systemic inflammation. APRs are largely produced by hepatocytes [29]; thus, variation in their content after liver manipulation could represent a marker of local inflammation and leakage from damaged liver tissue rather than actual SIR parameters. No experimental evidence has been provided so far on the possible synthesis of APRs in the lung under an acute inflammatory stimulus. In our study, we used 2-DE to assess variation in plasma APRs. The 2-DE method is semiquantitative and can yield a global picture of protein variations in biological samples, but is not suitable to measure slight differences (as we observed for CRP levels) or the true protein levels.

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**Table 3**

Effects of RFTA treatment on plasma acute phase reactants assessed by bidimensional gel electrophoresis

<table>
<thead>
<tr>
<th>Positive APRs (spot number)</th>
<th>Post/pre RFTA spot density</th>
<th>Negative APRs (spot number)</th>
<th>Post/pre RFTA spot density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complement proteins C3 (4)</td>
<td>2.26 ± 1.64*</td>
<td>Albumin (1)</td>
<td>1.05 ± 0.35</td>
</tr>
<tr>
<td>C4 (10)</td>
<td>2.08 ± 1.60*</td>
<td>Transferrin (2)</td>
<td>0.92 ± 0.28</td>
</tr>
<tr>
<td>Major proteins</td>
<td></td>
<td>Transferytin (14)</td>
<td>0.94 ± 0.23</td>
</tr>
<tr>
<td>CRP (11)</td>
<td>1.17 ± 0.45</td>
<td>α2-HS glycoprotein (6)</td>
<td>1.01 ± 0.27</td>
</tr>
<tr>
<td>Serum amyloid component</td>
<td>2.01 ± 1.27*</td>
<td>Serum retinol-binding protein (13)</td>
<td>1.04 ± 0.54</td>
</tr>
<tr>
<td>Coagulation proteins</td>
<td></td>
<td>Apolipoprotein A1 (12)</td>
<td>1.13 ± 0.12</td>
</tr>
<tr>
<td>Fibrinogen (3)</td>
<td>1.22 ± 0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metal-binding proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haptoglobin (9)</td>
<td>1.36 ± 0.30</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemopexin (x)</td>
<td>1.09 ± 0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other proteins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α1-acid glycoprotein (5)</td>
<td>1.32 ± 0.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The protein profile of plasma was evaluated by 2-DE before (pre RFTA) and 3 days after treatment (post RFTA). All samples were assessed in triplicate. The APR spots were identified by gel matching with published Swiss-2DPAGE plasma maps and then confirmed by immunoblotting. The total spot density was assessed by PDQuest software (Bio-Rad). Results represent means ± SD of the ratio between spot densities obtained 3 days after RFTA and baseline values. APRs = acute phase reactants. *p < 0.05.

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**Table 4**

Effects of RFTA on peripheral lymphocyte subsets

<table>
<thead>
<tr>
<th>Lymphocyte subsets</th>
<th>T&lt;sub&gt;0&lt;/sub&gt;</th>
<th>T&lt;sub&gt;1&lt;/sub&gt;</th>
<th>T&lt;sub&gt;30d&lt;/sub&gt;</th>
<th>T&lt;sub&gt;90d&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; (10&lt;sup&gt;4&lt;/sup&gt;/µL)</td>
<td>1.07 ± 0.41</td>
<td>1.05 ± 0.35</td>
<td>1.40 ± 0.95</td>
<td>1.20 ± 0.35</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt; (10&lt;sup&gt;4&lt;/sup&gt;/µL)</td>
<td>0.62 ± 0.23</td>
<td>0.56 ± 0.27</td>
<td>0.72 ± 0.39</td>
<td>0.68 ± 0.32</td>
</tr>
<tr>
<td>CD3&lt;sup&gt;+&lt;/sup&gt; (10&lt;sup&gt;4&lt;/sup&gt;/µL)</td>
<td>0.45 ± 0.30</td>
<td>0.43 ± 0.24</td>
<td>0.55 ± 0.18</td>
<td>0.56 ± 0.26</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; (10&lt;sup&gt;4&lt;/sup&gt;/µL)</td>
<td>0.16 ± 0.12</td>
<td>0.14 ± 0.12</td>
<td>0.22 ± 0.13</td>
<td>0.21 ± 0.09</td>
</tr>
<tr>
<td>CD56&lt;sup&gt;+&lt;/sup&gt;/CD16&lt;sup&gt;+&lt;/sup&gt; (10&lt;sup&gt;4&lt;/sup&gt;/µL)</td>
<td>0.26 ± 0.16</td>
<td>0.30 ± 0.20</td>
<td>0.33 ± 0.24</td>
<td>0.35 ± 0.31</td>
</tr>
<tr>
<td>CD4&lt;sup&gt;+&lt;/sup&gt; (CD8&lt;sup&gt;+&lt;/sup&gt;)/CD4&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2.23 ± 1.78</td>
<td>1.76 ± 1.33</td>
<td>1.98 ± 1.55</td>
<td>2.22 ± 1.82</td>
</tr>
</tbody>
</table>

Results represent means ± SD. No significant difference was observed with respect to baseline values (p > 0.05). T<sub>0</sub>: baseline values; T<sub>1</sub>: 3 days; T<sub>30d</sub>: 30 days; T<sub>90d</sub>: 90 days post RFTA treatment.
Unexpectedly, we observed a concomitant secretion of IL-10, which is thought to be an important negative regulator of proinflammatory gene expression in mononuclear cells [31]. The role of this anti-inflammatory mediator in RFTA-induced systemic inflammation remains unknown. On the basis of the biological function of IL-10 and the lack of major complications observed in patients in the immediate post RFTA period, we can infer that IL-10 release might represent one of the mechanisms involved in the downregulation of the systemic inflammatory reaction induced by RFTA. However, this point deserves further elucidation.

More importantly, we provided the first experimental evidence that RFTA can affect the peripheral CD25<sup>+</sup> Foxp3<sup>+</sup> Treg pool. Indeed, 30 days posttreatment, the number, but not the functional activity, of these cells was significantly reduced, reaching values in the range of those measured in NHCs. Downregulation of CD25<sup>+</sup> Foxp3<sup>+</sup> cells was present, even if at a variable extent, in all 14 patients who achieved complete radiological necrosis upon 30-day CT scan. Furthermore, in the majority of patients (12/14) this effect lasted for at least 90 days after the treatment. On the other hand, recent studies emphasize the role of Treg cells in tumor acceptance both in animal tumor models and in human beings [for review see 32–35]. Interestingly, in the two patients who experienced a new lung nodule and an extrapulmonary spread at 90-day follow-up CT scan, we observed a less marked decrease in CD25<sup>+</sup> Foxp3<sup>+</sup> cells at 30-day follow-up (35 and 30% reduction compared with pretreatment values, respectively) and a trend toward a slight increase thereafter (data not shown). These findings, which must be confirmed in a larger cohort of cancer patients, suggest a possible relationship between the downregulation of CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs and the clinical response to RFTA.

The mechanisms by which RFTA may influence circulating CD25<sup>+</sup> Foxp3<sup>+</sup> Tregs remain to be determined. Pulmonary RFTA is supposed to induce a coagulative necrosis [4], although evidence of an apoptotic mechanism has been recently provided [36]. Necrotic cell death is associated with an inflammatory response inducing the release of proinflammatory signals, such as heat shock proteins and HMGB1, that can play an important role in the initiation of antitumor immunity by inducing DC stimulation and maturation [37–39]. Recent studies [11,12,22] demonstrate that RFTA activates and enhances antitumor immune responses in experimental animals and in HCC patients, and this immunostimulatory effect can depend at least in part on DC maturation driven by cellular debris released after RFTA [24]. On the basis of the relationship between DC maturation and the generation of Tregs [40,41], it is likely that DC differentiation, in addition to directly improving tumor-specific TCR, might be involved in the downregulation of the Treg pool, which in turn might further improve patient’s immune responses.

We demonstrated that RFTA causes a nonspecific CD4<sup>+</sup> immune activation, but whether the procedure also improves CD8 T-cell reactivity and, more importantly, tumor-specific TCR was not tested because of the lack of adequate biological samples for CD8 and tumor antigen purification. Current evidence indicates that Tregs can affect proliferation, activation, and functional activity of both responsive CD4<sup>+</sup> and CD8<sup>+</sup> T cells [42]. Moreover, previous studies in liver cancers have demonstrated that RFTA might in-
increase tumor-specific TCR [22] and the cytolytic activity of CD8+ T cells [23].

Although the mechanism involved in the long-lasting antitumor effect of RFTA remains mostly unknown, our findings suggest that the small but significant reduction of the peripheral CD25+ Foxp3+ Treg pool might be of functional importance in improving host T-cell reactivity. Further analysis on a larger series of cancer patients will be carried out to corroborate these preliminary results, to correlate levels of this Treg subset to the degree and duration of RFTA-related clinical responses, and eventually to elucidate the underlying mechanisms.

Acknowledgments

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References


sure to antigen extract from hepatocellular carcinoma after radiofrequency thermal ablation. J Immunother 2008;31:271–82.


