RESEARCH ARTICLE

Inflammatory protein profile during systemic high dose interleukin-2 administration

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Systemic interleukin-2 (IL-2) administration induces an assortment of downstream effects whose biological and therapeutic significance remains unexplored mostly because of the methodological inability to globally address their complexity. Protein array analysis of sera from patients with renal cell carcinoma obtained prior and during high-dose IL-2 therapy had previously revealed extensive alterations in expression of the soluble factors analyzed, whose discovery was limited by the number of capture antibodies selected for protein detection. Here, we expanded the analysis to SELDI-TOF-MS and quantitative protein analysis (nephelometry). All cytokines/chemokines detected by protein arrays were below the SELDI detection limit, while novel IL-2-specific changes in expression of acute-phase reactants and high-density lipoprotein metabolites could be identified. Serum amyloid protein A (SAA) and C-reactive protein expression were consistently up-regulated after four doses of IL-2, while other proteins were down-regulated. These findings were confirmed by SELDI immunoaffinity capture and nephelometry. Immunoaffinity capture revealed different, otherwise undetectable, isoforms of SAA. A linear correlation between peak area by SELDI and protein concentration by nephelometry was observed. Overall distinct yet complementary information was obtained using different platforms, which may better illustrate complex phenomena such as the systemic response to biological response modifiers.

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Abbreviations: **α1AT**, alpha$_1$-anti trypsin; **Apo**, apolipoprotein; **C3dg**, complement factor C3-C3dg fragment; **CRP** C-reactive protein; **CR**, creatinine; **Cys-C**, cystatin-C; **IL-2**, interleukin-2; **RBP**, retinol binding protein; **RCC**, renal cell carcinoma; **SAA**, serum amyloid protein A; **TFN**, transferrin; **TTR**, transthyretin

1 Introduction

Interleukin-2 (IL-2) has been approved by the Food and Drug Administration as single agent for the treatment of patients with metastatic melanoma and advanced renal cell cancer (RCC) [1], because it can induce durable responses in a consistent number of patients [2]. High-dose IL-2 treatment, however, is limited by significant toxicity [3, 4]. The mechanism(s) responsible for IL-2-mediated cancer regression and toxicity remain(s) largely unknown. It has been postulated that IL-2 acts through the *in vivo* expansion and activation of cytotoxic

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T lymphocytes [1]. However, recent analyses from our group have suggested that IL-2 may facilitate tumor-infiltrating T cell effector function through the secondary production of bioactive factors capable of promoting T cell migration and activation *in situ* through the maturation of antigen-presenting cells [5]. It is also unknown whether the substantial toxicity associated with IL-2 administration is mediated through pathways common or distinct from those mediating its anti-cancer effects [6].

This surprising lack of knowledge about the therapeutic and toxic mechanisms of an approved anti-cancer drug is mostly largely due to the complexity of its biological effects and the lack, until recently, of methods capable of addressing such complexity in its globality. While, sporadically, significant correlations have been reported between serum cytokine and acute-phase reactant levels in patients receiving recombinant IL-2 [7–11], a global analysis of such relationships and its clinical relevance have not been addressed.

In an attempt to estimate the complexity of the problem and identify tools that could be used in future clinical studies to comprehensively correlate clinical with biological data, we previously measured the concentration of 68 different chemokines, cytokines and soluble factors in serum samples obtained from RCC patients before and during systemic IL-2 administration using a protein array platform [12]. This pilot study demonstrated that the expression of most soluble serum factors analyzed were significantly and dramatically altered during therapy. Furthermore, these serum components could be separately grouped according to their kinetics of appearance in the circulation. This study underlined the broadness of the cytokine storm induced by systemic IL-2 administration, and suggested that several other alterations might have escaped detection simply because no capture antibodies were present in the array platform. In an attempt to extend the analysis to additional proteins, using a high throughput tool not biased by the known identity of capture elements, we complemented the information obtained with the array protein profiling by testing the same serum samples tested in the pilot study by SELDI-TOF-MS. In addition, we validated output data by SELDI immunoaffinity capture and nephelometry. Although the detection of the previously identified cytokines was below the threshold of detection of SELDI, we identified novel protein expression patterns relevant to therapeutic IL-2 administration that may serve in the future as markers of therapeutic effectiveness or dose limiting toxicity. In addition, the low sensitivity of SELDI-TOF-MS underlines the limitations of this method when exploring factors present in low yet biologically relevant concentrations.

2 Materials and methods

2.1 Patients and serum collection

Ten patients with metastatic RCC were recruited at the Carolinas Medical Center (Charlotte, NC, USA) to receive systemic high-dose (720 000 IU/kg every 8 h) IL-2 administration (Proleukin, Chiron, Emeryville, CA, USA). Serum was collected prior to treatment (PRE) and 3 h after the first (POST 1) and fourth (POST 4) dose (24 h after the first dose) and aliquoted and stored at −80°C until testing [12]. Serum from nine granulocyte-colony stimulating factor (G-CSF)-treated normal volunteers was similarly collected before and after a 5-day course of G-CSF (10 μg/kg daily, Filgrastim, Amgen, Thousand Oaks, CA, USA). All tested aliquots were subjected to only one freeze-thawing cycle.

2.2 Serum fractionation

Anion exchange fractionation was performed prior to SELDI analysis using the Profiling Fractionation Kit (Ciphergen Biosystems Inc., Fremont, CA, USA) according to manufacturer's instructions. Samples were fractionated using the robotic liquid handling system, Biomex FX (Beckman Coulter, Fullerton, CA, USA) and separated into five different fractions (pH 9, pH 7, pH 5, pH 3 and organic).

2.3 SELDI TOF-MS analysis

All mass spectra were recorded in the positive-ion mode on a PBS II ProteinChip reader (Ciphergen Biosystems). External calibration of the instrument was performed using the all-in-1 peptide molecular mass standard (Ciphergen Biosystems). All samples were analyzed using an automated data collection protocol within the ProteinChip Software (version 3.0), collecting data to a maximum of 80 kDa.

Two consecutive readings (optimized for low and high molecular weight ranges) were obtained using different acquisition modes. In the first, laser intensity was set to 235, detector sensitivity to 4 and high focus mass to 30 000 Da to detect proteins/peptides in the 1000–30 000 Da range. Fifty-five laser shots were collected on average. In the second, laser intensity was set to 250, detector sensitivity to 5, high focus mass to 80 000 Da to detect proteins ranging from 30 000 to 80 000 Da in mass. Among the various chip chemistries evaluated (hydrophobic, anionic, cationic, and metal binding), SAX2 strong anionic exchange chips provided the best resolution in serum protein profile and, therefore, subsequent protein profiling was conducted using the eight-spot format SAX2 ProteinChip Array (Ciphergen Biosystems) at pH 8 (binding buffer, 50 mM Tris-HCl). Each array was pre-equilibrated 2 × 5 min in binding buffer before sample addition. One microliter of original serum diluted 1:20 or 1 μL of fractionated serum (pH 3–9 and organic) was added to 4 μL of binding buffer and incubated on the spot surface for 1 h in a humidity chamber. After incubation, the sample was removed; each spot was washed with binding buffer, rinsed in water and air dried.

Prior to SELDI-TOF MS analysis, 0.8 μL of saturated sinapinic acid (SPA) solution (in 0.1% TFA and 50% aqueous ACN) was added to each of the SAX2 ProteinChip spots.

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2.4 ProteinChip immunoaffinity capture

Immunoaffinity capture was performed with the use of a protein G array (Ciphergen Biosystems). Monoclonal anti-serum amyloid protein A (SAA) antibody (Abcam, Cambridge MA, USA), and Polyclonal anti CRP antibody (DAKO corporation, Carpinteria, CA, USA) were diluted to a concentration of 0.2 μg/μL in PBS. Protein G arrays were placed in a bioprocessor (Ciphergen Biosystems) and rehydrated in PBS for 30 min before antibody binding to protein G. The capture of each protein was performed by placing 100 μL of the appropriate antibody on a single spot of the array followed by incubation for 1 h at room temperature in a humidified chamber. Unbound monoclonal antibodies were removed by washing twice with 500 μL of wash buffer (0.05% Tween 20 in PBS) and twice with PBS. Serum samples (100 μL diluted 1:10 with PBS) were subsequently placed on the spots. Chips were incubated for 2 h at room temperature, washed twice with PBS, rinsed in HEPES buffer (Ciphergen Biosystems) and air dried. Prior to SELDI-TOF MS analysis, 0.8 μL of saturated SPA solution was added to each spot.

2.5 Quantitative analysis of serum components

The concentration of SAA, C-reactive protein (CRP), transthyretin (TTR), alpha$_1$-anti-trypsin (α1AT), apolipoprotein (Apo) A II, haptoglobin (HPT), transferrin (TFN), retinol binding protein (RBP), cystatin c (Cys-C), Apo A-I and Apo B were determined in serum samples obtained before (PRE), after one (POST 1) and after four (POST 4) doses of IL-2. administration. These samples were analyzed by nephelometry utilizing either the BNII analyzer (Dade Behring, Newark, DE, USA) or the IMMAGE analyzer (Beckman-Coulter, Brea, CA, USA) and protein-specific antibodies provided by the nephelometer manufacturer. Several serum components were measured spectrophotometrically using a variety of clinical methods on an LX-20 analyzer (Beckman-Coulter). Total cholesterol (chol) and high-density lipoprotein cholesterol (HDL-chol) were measured by a direct method, albumin by a bromocresol purple dye-binding, total protein by biuret method, and creatinine by a rate method following reaction with alkaline picrate.

2.6 Intra- and inter-chip reproducibility analysis

The reproducibility of SELDI spectra, *i.e.*, mass location and intensity from array to array on a single chip (intra assay) and between chips (inter assay), was determined according to Adam *et al.* [13]. Pooled PRE and POST 4 samples were spotted in 2 eight-spot format SAX2 ProteinChip Array. Seven protein peaks in the range of 6000–30 000 Da (6427.2; 6625.4; 11 666.1; 13 826.0; 15 109.0; 17 232.1; 28 038.7) with *m/z* area values ranging between 36 and 7249 were randomly selected, and used to calculate the CV for intra chip evaluation according to the formula:

CV_{p1} = [(SD of TOF area value or *m/z* value across 8 spots for a specific protein, p1)/(average of TOF area value or *m/z* value across 8 spots for p1)] × 100

For inter chip evaluation the following formula was used:

CV_{p1} = [(SD of TOF area value or *m/z* value across 16 spots for a specific protein, p1)/(average of TOF area value or *m/z* value across 16 spots for p1)] × 100

The seven peak CV ($CV_{p1–7}$) were then averaged.

2.7 Statistical analysis

Preliminary evaluation of serum spectra obtained from SAX2 chips were matched according to molecular weight (MW) identified by the Ciphergen software with that of proteins reported in the Expasy database (GuessProt program: http://us.expasy.org/tools/tagident.html), in the literature, or our unpublished data obtained by MS (Ultraflex Bruker Daltonics, Billerica, MA 01821, USA)

Values corresponding to *m/z* area were transformed in natural log (LN) values, average corrected across experimental samples and displayed according to the central method using a normalization factor as recommended by Ross [14]. Among a total of 36 peaks detected from 6000 to 70 000 Da (all of them with a signal to noise ratio >5), 19 peaks were selected for further analysis on the basis of their close match with the MW of known serum proteins. Relatedness in the expression patterns of the proteins among PRE and POST IL-2 samples across all patients was tested with unsupervised analysis by applying Eisen's hierarchical clustering to the data set. [15]. This tool ranks experiments (patient samples) according to their proximity to each other taking into account the entire data set of *m/z* area values. A similar procedure was followed to display the relatedness in expression of the 19 selected serum components measured by nephelometry or spectrometry.

3 Results

3.1 Preliminary identification of proteins in serum samples

Serum samples obtained from RCC patients before and after one and four doses of high-dose IL-2 were analyzed on SAX2 strong anionic exchange chips encompassing a (*m/z*) range between 6000 and 70 000 Da. To assess the general protein profile of whole sera, samples (PRE and POST 4) were pooled and analyzed (Fig. 1). The majority of peaks were identified in a (*m/z*) range between 6000 and 30 000 Da. A list of dominant peaks that were consistently demonstrated in all patients, and the tentative identification of these peaks by average molecular weight matching to serum proteins in the Expasy database, literature and our unpublished data, are indicated in Table 1.

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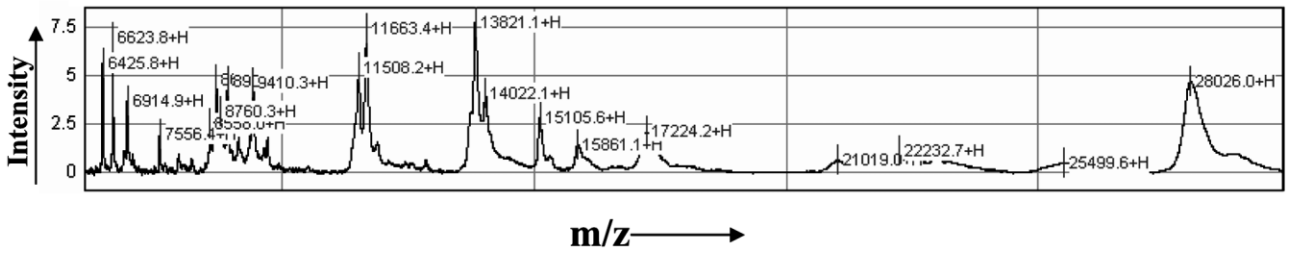

Figure 1. Preliminary detection of proteins in pooled serum samples. Serum samples obtained from RCC patients before and after four systemic administration of high-dose IL-2 were pooled and analyzed on SAX2 strong anionic exchange chips. The SELDI spectra show only peaks identified in the range (*m/z*) between 6000 and 30 000 Da.

3.2 Assessment of reproducibility

The reproducibility of SELDI spectra, *i.e.*, mass location and intensity from array to array on a single chip (intra assay) and between chips (inter assay) was determined using the pooled samples described above. Seven protein peaks in a range between 6000 and 30 000 Da were randomly selected and used to calculate the CV. The intra assay and inter assay CV for peak location (*m/z*) was 0.03% and the intra-assay and inter-assay CV for TOF area were 17% and 10%, respectively (data not shown).

3.3 Protein profiling of sera by SELDI-TOF MS reveals proteins associated with IL-2 treatment

Protein profiling of samples obtained before and after initiating IL-2 treatment was performed in parallel using SAX2 arrays. This analysis revealed two peaks at *m/z* ~11 500 and 11 700 Da that were exclusively expressed in samples obtained after initiating IL-2 therapy (Fig. 2A, B). These *m/z* ratios closely matched the molecular weight of SAA isoforms (Table 1). Analysis of fractionated serum samples on different surfaces on SAX2 chips revealed an additional protein at

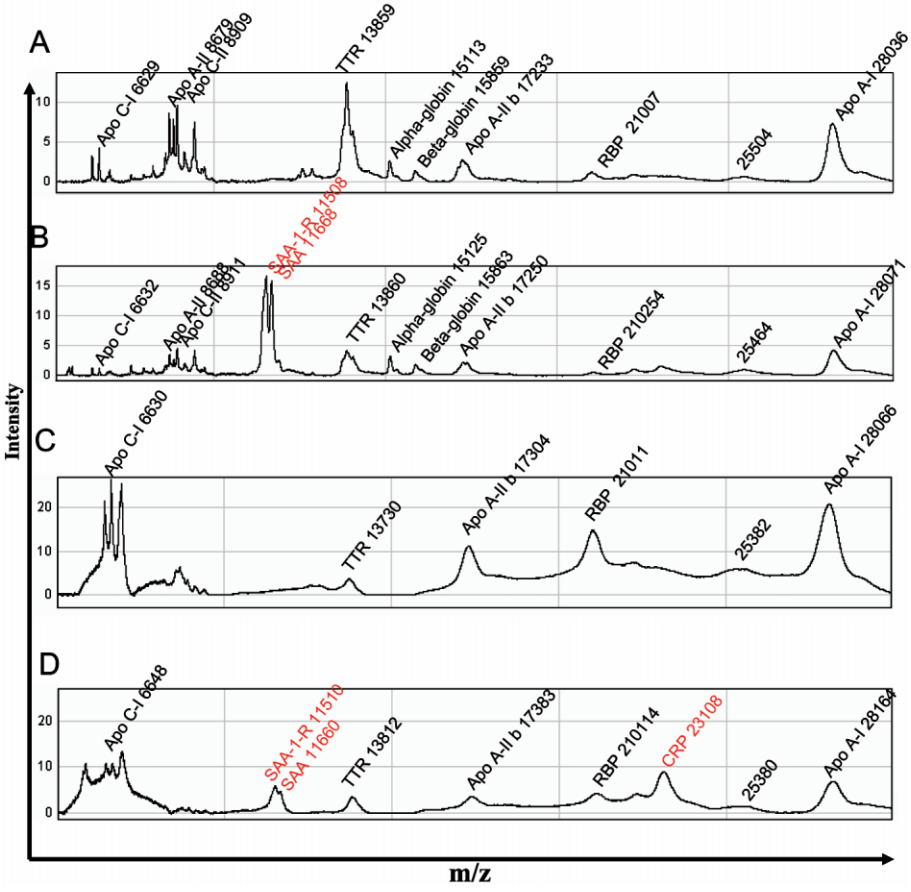

Figure 2. Differential identification of SELDI peaks in serum samples obtained before and after four doses of high-dose IL-2. Patient P3 spectra are shown as representative of all patients. The SELDI profiles of unfractionated serum samples obtained before (A) and 3 h after the administration of the fourth dose of IL-2 (B) are shown that were analyzed on SAX2 strong anionic exchange chips. Also shown are SELDI profiles of fractionated serum samples (pH 3 fraction) before (C) and after (D) four doses of IL-2. The names of proteins found in the Expasy database with molecular masses that potentially match specific peaks are indicated above the peak. Peaks detected only after four doses are indicated in red.

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Table 1. Dominant peaks according to SELDI spectra and tentative annotation[a)]

Averaged (*m/z*)	Expasy closest molecular mass (Da)	Tentative identification	Literature data
6427.22		Des Thr-Val Apo C-I[c)]	
6625.40	6630.58	Apo C-I	
6937.71		Unk 693	
7566.00		Unk 7566	
7934.45		Unk 7934	
8205.00		Unk 8205	
8576.00		Unk 8576	
8680.90	8707.91	Apo A-II	
8804.82		Apo A–II+ Cys (8811 Da)[b)]	
8905.95	8914.92	Apo C-II	
9162.31		Apo C-III (9,126 Da)[b)]	
9411.96		Apo C-III (9417 Da)[b)]	
9707.00		Apo C-III (9709 Da)[b)]	
11 463.27		SAA-1-RS	[16]
11 509.17		SAA-1-R	[16]
11 666.14	11 682.70	SAA	[16]
11 895.00		Unk 11895	
12 573.00		Unk 12573	
12 846.77		Truncated form of TTR	[52]
13 826.02	13 761.41	TTR	[16]
14 025.43		Cys TTR (13 946 Da)[b)]	
15 109.07	15 126.36	Apha-globin	[16]
15 865.23	15 867.22	Beta-globin	[16]
17 232.14		Apo A-IIb	[53]
21 023.77	21 071.60	RBP	[54]
25 476.02	25 982.56	Unk 25476	
28 038.71	28 068.72	Apo A-I	[52]
36 872.00		A_1 Acid glycoprotein (35 287 Da)[b)]	
37 654.00		Unk 37654	
39 624.94	39 673.15	Unk 39624	
42 000.00		A_2 HS-glycoprotein (42,415 Da)[b)]	
44 421.39	44 324.55	B_2 Glycoprotein I (44,944 Da)[b)]	
47 353.89		Unk 47353	
50 510.38		A_1 Antitrypsin (50,303 Da)[b)]	
59 050.69	51 676	Hemopexin	
66 230.90	66 462.21	Albumin	

a) List of dominant peaks obtained from a screening of SELDI-SAX2 spectra of all patients. Tentative identification of peaks was determined by matching their average mass to the molecular weight of serum proteins listed in the Expasy GuessProt database and/or the literature. Unk, unknown.

b) Tentative assignment based on abundance in plasma and assessed by MS (unpublished data).

c) Des Thr-Val Apo C-I = Apo C-I devoid of threonine and valine.

m/z ~23 000 Da in the pH 3 fraction that was again present only after initiating IL-2 treatment (Fig. 2C, D) and that closely matched the molecular weight of CRP. Other peaks were down-regulated after IL-2. The down-regulated peaks included Apo C-I, Apo A-II, TTR, RBP and Apo A-I (Fig. 2, Table 1). To determine whether the changes observed in patients' sera were specific to IL-2 therapy, we used the same method to analyze the protein profiles of sera collected from 9 normal donors undergoing a 5-day hematopoietic progenitor cell mobilization by G-CSF. G-CSF-treated patients offered an alternative and very low inflammatory type of immunotherapy treated samples, and were considered an appropriate negative control (manuscript submitted). No differences were observed in the serum of individuals collected before and after treatment with G-CSF. Furthermore, the SELDI spectra obtained both before and after G-CSF were similar to those observed in PRE-IL-2 samples (data not shown).

We congregated differential patterns of protein expression in unfractionated sera according to *m/z* area, using Eisen's hierarchical clustering [12] (Fig. 3), but limited the analysis to peaks whose *m/z* closely matched known serum proteins. Unsupervised clustering segregated individual samples into two groups according to the time of collection. Samples obtained before and after 1 dose (POST 1) of IL-2 (group I) clustered separately from samples obtained after 4 (POST 4) doses (group II). This is dramatically different from hierarchical clustering results that have been previously observed with protein arrays, where significant differences could already be detected after the administration of only one dose of IL-2. The levels of 11 out of 19 proteins (SAA, SAA-1R, TTR, truncated form of TTR, RBP, Apo A-I, Apo C-I, apo A-I, Apo C-II and 39,624 Da) were reduced in the POST 4 samples in all ten patients. In addition, hierarchical clustering highlighted differences in the expression of three proteins (25 476, 39 624 Da, truncated form of TTR) between samples obtained before and after IL-2 administration that would not have been otherwise detectable by visual inspection of individual SELDI spectra (Fig. 2).

3.4 Identification of up-regulated proteins by ProteinChip immunoaffinity capture

We confirmed the identity of the proteins up-regulated by IL-2 treatment by SELDI immunoaffinity capture. Using biologically active ProteinChip arrays, we captured SAA protein isoforms by applying an anti-SAA monoclonal antibody onto protein G-treated chips. The molecular masses of the fragments retained on the surface of the ProteinChip Arrays coincided to the masses of the putative SAA protein previously observed on SAX2. A cluster including three peaks (*m/z* ~11 700, 11 500 and 11 100 Da) was identified in sera obtained after four doses of IL-2 (Fig. 4A). The peaks at 11 700 and 11 500 Da closely matched the up-regulated peaks previously identified. The peak at 11,100 kDa was previously described as a des-1Arg/des-2Ser variant of SAA-1

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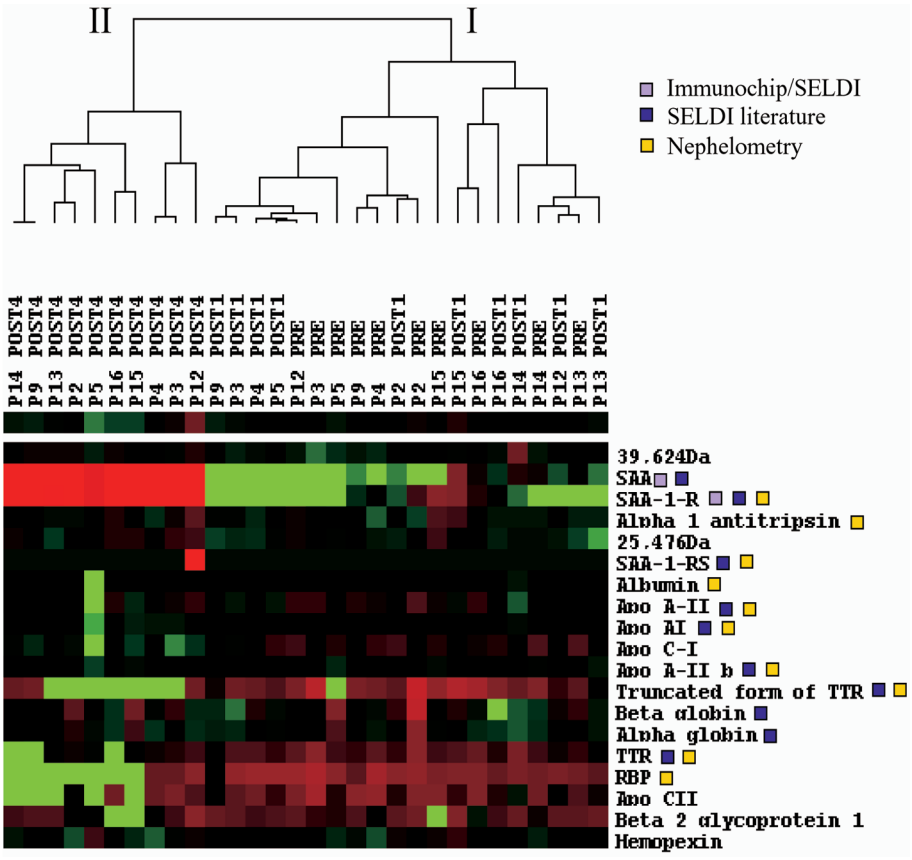

Figure 3. Unsupervised hierarchical clustering of serum samples from RCC patients obtained before and after one or four administrations of high-dose IL-2. Hierarchical clustering was applied to the data set encompassing the *m/z* area of 19 potentially identified peaks across all PRE and POST treatment samples. Patients' serum samples clustered according to time of collection in two main groups (I and II) with distinct soluble factor profiles.

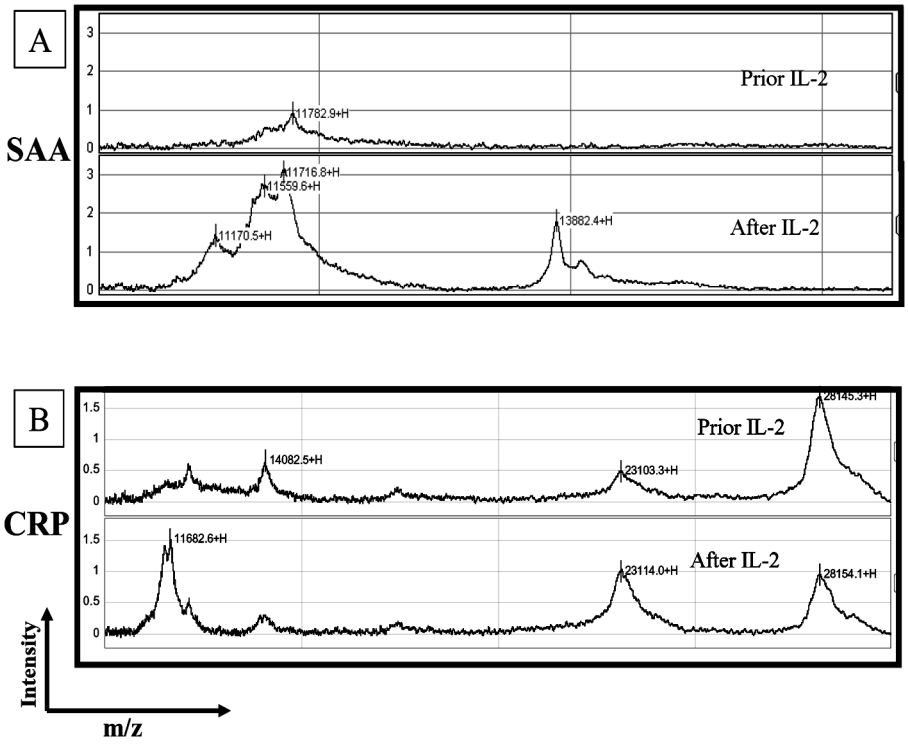

Figure 4. SELDI immunoaffinity capture of SAA and CRP. Monoclonal antibodies directed against putative proteins were immobilized on a protein G array and analyzed by SELDI. SELDI analysis of SAA and CRP in serum, obtained prior and after four doses of IL-2 treatment, captured on the protein G array chip surface are depicted in the spectra of one representative patient. The results of capture and analysis of SAA are shown in (A) and of CRP in (B).

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[16] that could not be detected by SAX2 chip analysis. A *m/z* 13 900-Da protein, potentially corresponding to TTR, co-purified with the three SAA isoforms. The concomitant occurrence of SAA and TTR as a two protein complex in serum has been previously described by Marhaug and Husby [17].

A similar procedure was followed for the identification of the peak at *m/z* 23 108 Da. A CRP-specific monoclonal antibody bound to protein G was able to detect the putative CRP protein previously observed by SAX2 (Fig. 2) at 23 100 Da (Fig. 4B). The intensity of this peak was greater in the POST 4 samples compared to the PRE samples. In addition, POST 4 samples displayed a potential double charge peak at 11 600 Da that was characteristic of CRP, and that was not present in PRE samples. A *m/z* 28 145-Da protein, potentially corresponding to Apo A-I, co-purified with CRP. The concomitant occurrence of CRP and Apolipoproteins has been previously described by Schwalbe *et al.* [18].

3.5 Quantification of protein and soluble factors present in serum

To further confirm the identity and verify the concentration of the 19 serum components identified by SELDI, we applied immunonephelometry and spectrophotometry to PRE, POST1 and POST4 samples. Only 4 out of 19 factors analyzed, SAA, CRP, Cys-C, and creatinine, were increased after four doses of IL-2. Large SDs reflect variations in absolute serum concentration of these proteins. Increases in CRP levels ranged from 7- to 160-fold, while increases in SAA levels ranged from 11- to 285-fold. Overall, the serum total protein levels were lower in the POST 4 IL-2 sera then in the PRE IL-2 sera. The down-regulation of total protein reflected the decreased concentration of negative acute-phase reactants (TTR, albumin, Apo A-II, RBP, Apo B, Apo A-I, TFN), cholesterol, and HDL-chol (Fig. 5).

A summary of the concentrations of serum components in all samples is portrayed by hierarchical clustering in Fig. 6. Unsupervised clustering segregated individual samples into two groups according to the time of collection. Samples obtained before and after one dose clustered in group I and those obtained after four doses clustered in group II with the exception of the patient 15 PRE and POST 1 samples which clustered in group II. Notably, this patient carried a diagnosis of arthritis, which may explain the relatively high levels of SAA and CRP in their PRE and POST 1 samples. Of interest was the close correlation of PRE and POST 1 samples in each patient, emphasizing the consistency of individual protein levels measured in each patient prior to, and in the early phases of, biological manipulation.

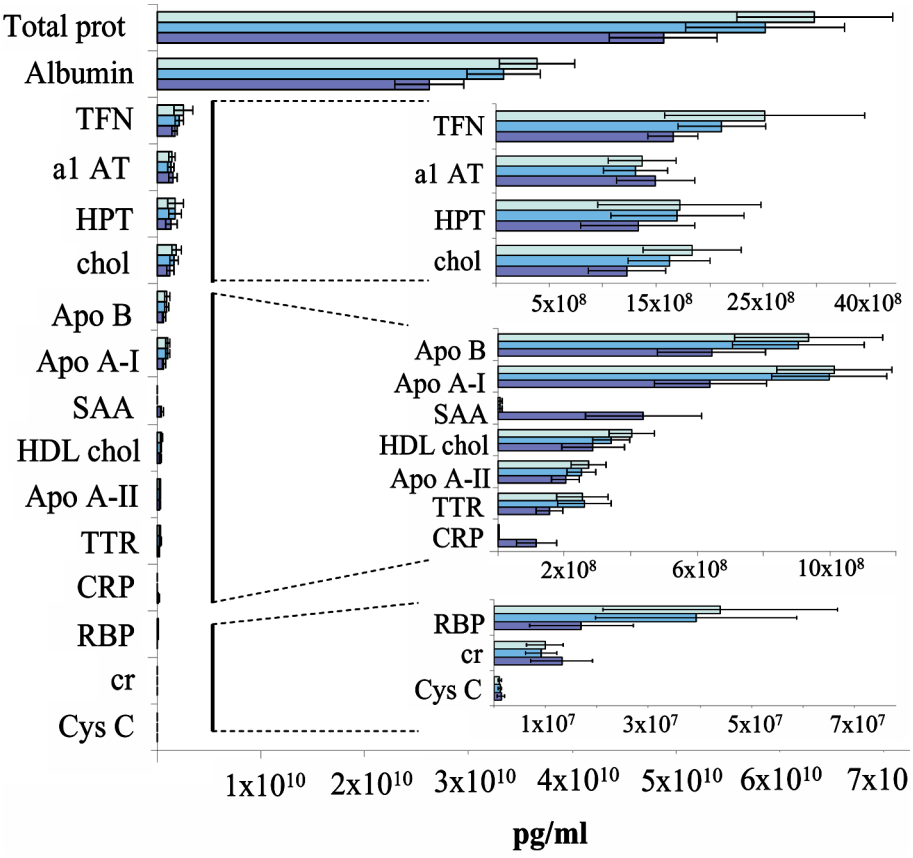

Figure 5. Quantification of serum components. Concentration of SAA, CRP, TTR, α1AT, Apo A-II, HPT, TFN, RBP, Cys-C, Apo A-I and Apo in PRE, POST 1 and POST 4 serum samples were measured by nephelometry. The concentration of several other serum components (chol, HDL-chol, creatinine, albumin and total protein) were measured spectrophotometrically. Values represent the average concentration of each factor in all patients (pg/mL) ± SD.

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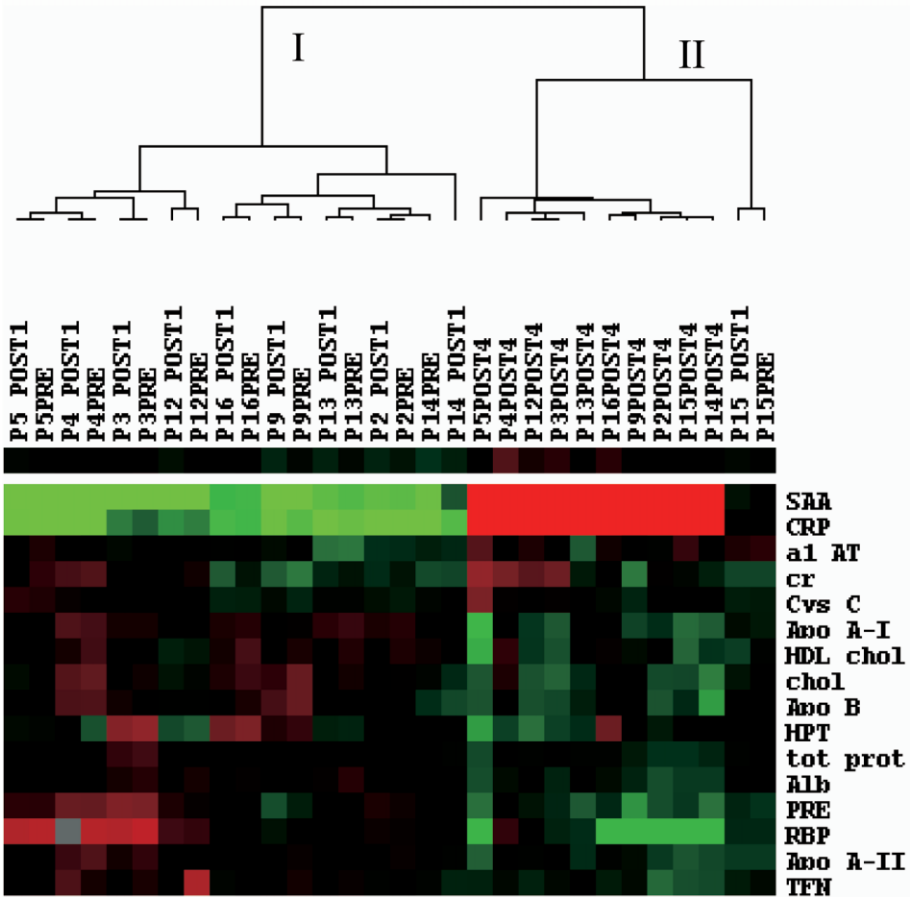

Figure 6. Hierarchical clustering of serum components before and after IL-2 treatment. Hierarchical clustering was applied to the data set encompassing the concentration of 19 serum components in all PRE and POST treatment samples. Patients' serum samples clustered according to time of collection in two main groups (I and II) with distinct soluble factors profiles.

3.6 Linear correspondence of *m/z* area obtained by SELDI and concentration of proteins in serum

We selected SAA to assess the correspondence of *m/z* area obtained by SELDI and quantitative assessment of protein concentration in serum. To assess the limit of detection of SAA in serum samples, we took advantage of the fact that SAA was undetectable by the SAX2 chip in samples obtained before IL-2 was given, but SAA levels were extremely high after four doses of IL-2. We serially diluted a serum sample obtained from one patient after IL-2 treatment (four doses) with the corresponding serum sample obtained prior to IL-2 treatment. The mixture was analyzed on SAX2 chips. Starting with the first dilution which contained SAA at a concentration of 22×10^7 pg/mL (0.3% of total protein concentration), approximately 4 μg total proteins (as assessed by nephelometry) was loaded on each spot. Analysis of the serial dilution revealed that SAA was still detected at a concentration of 6.7×10^7 pg/mL (corresponding to 3400 pg loaded on the chip surface), but was not automatically detected by the software at a serum concentration of 4.6×10^7 pg/mL (corresponding to 2300 pg loaded on the chip surface). This corresponds to a relative protein concentration of between 0.06% and 0.08% of the total serum protein (Fig. 7). The SAA concentration in the PRE IL-2 sera ranged from 1×10^7 to 3×10^7 pg/mL and was undetectable in our SELDI experimental conditions. To determine whether there was a linear correlation between *m/z* area obtained by SELDI and SAA relative abundance determined by nephelometry, we performed dilutions of sera from four patients (P3, P9, P2, P14) obtained after four doses of IL-2 with autologous serum obtained before treatment as described above. Proteins with marginal differences in expression between PRE and POST 4 samples maintained a constant concentration across dilutions. In contrast, SAA concentrations changed according to dilution and are indicated as a percentage of total serum protein. This relative measure was chosen to take into account changes in the amount of total proteins in different dilutions. A positive linear correlation was consistently found between SAA peak area and relative abundance in serum protein as shown by R^2 values >0.9. Furthermore, similar percentages of SAA corresponded to similar *m/z* area values in the four patients studied (Fig. 8). To determine whether a correlation between *m/z* area, obtained by SELDI, and concentration of proteins in serum measured by nephelometry could be established in all patients, we selected two differentially expressed proteins, SAA and TTR, for analysis. The concentration of SAA and TTR obtained by nephelometry in

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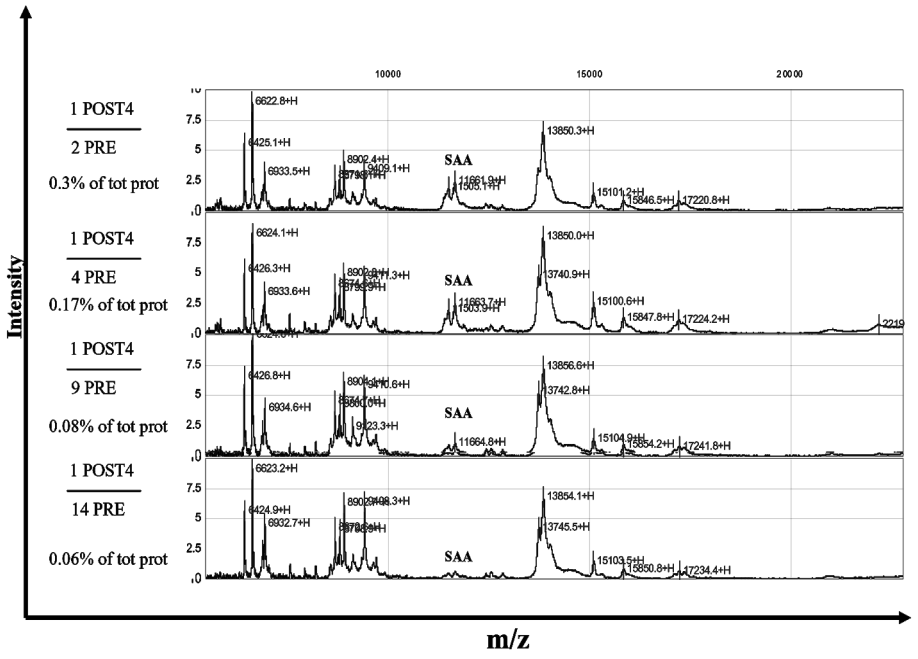

Figure 7. Limit of detection of SAA by SAX2 proteinchip in serum. Serum from patient 3 serum obtained after IL-2 treatment was analyzed by SELDI using the SAX2 chip. The samples analyzed were diluted at several ratios with autologous serum collected before IL-2 was given. The dilution ratios and relative percentage of SAA in each serum mixture are indicated on the left.

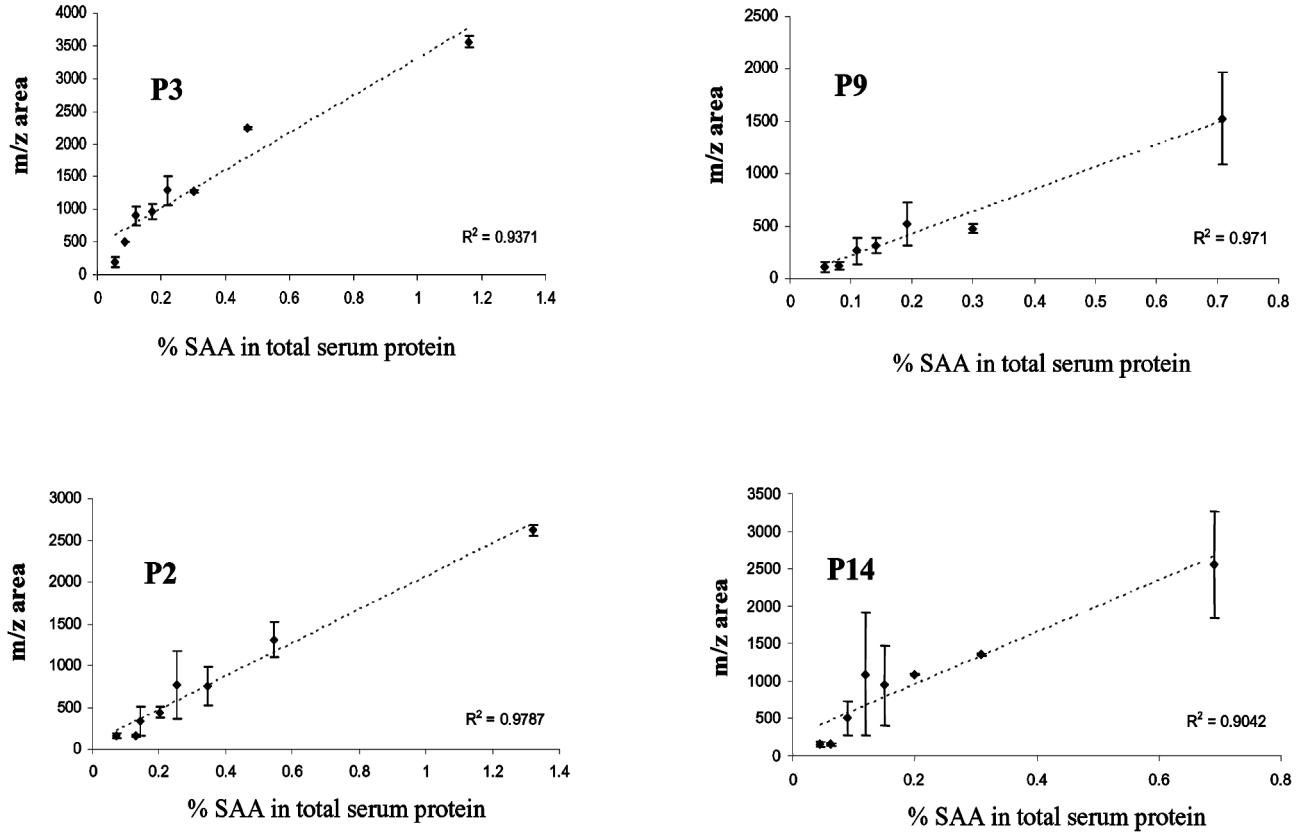

Figure 8. Linear correlation between SAA *m/z* area determined by SELDI and SAA relative concentration in serum. Serum from patients 3, 9, 2, and 14 obtained after IL-2 treatment was diluted (1:1, 1:2, 1:3, 1:4,1:6, 1:9,1:14) with corresponding autologous serum obtained before IL-2 treatment. The concentration of SAA in each mixture is expressed as a percentage of total serum protein, as assessed by nephelometry, and is correlated with the SELDI *m/z* area.

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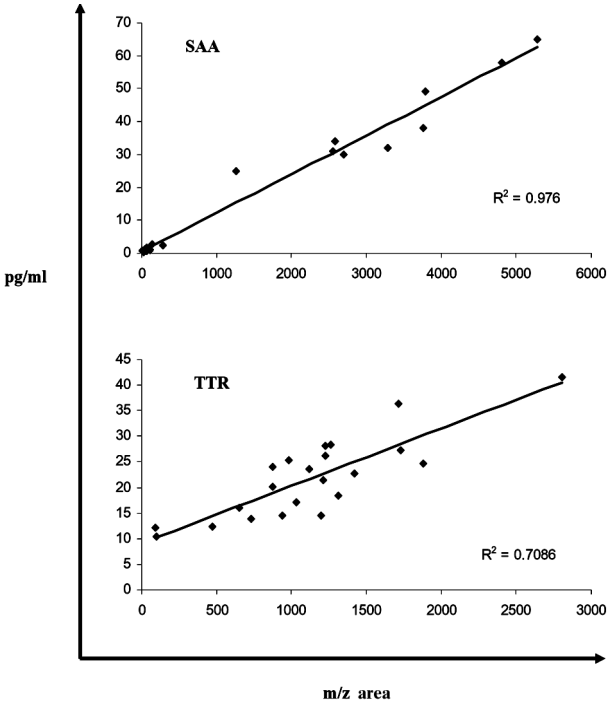

Figure 9. Correlation between serum SAA or TTR concentration and the relative *m/z* area in patient sera. SAA (A) and TTR (B) levels (pg/mL) detected by immunonephelometry were correlated to *m/z* area values in 22 serum samples. Linear correlation is expressed as R^2 values.

22 randomly selected samples (PRE, POST 1 and POST 4) positively correlated with SELDI *m/z* areas, as demonstrated in Fig. 9 (SAA-R^2 = 0.976, TTR-R^2 = 0.7086).

4 Discussion

We applied two methodologies to analyze serum components in samples collected from RCC patients undergoing systemic high-dose IL-2 therapy. SELDI was initially utilized to perform a parallel screening of protein profiles prior and after treatment and to complement pre-existing information reported by us and others on the cytokine and chemokine storm induced by IL-2 [12, 19–22]. Immunonephelometry, corroborated by clinical methods for blood chemistry, provided a quantitative validation of protein concentration changes detected by SELDI.

We established that the detection limit of SAA by SELDI in our experimental condition ranged between 4.6×10^7 and 6.7×10^7 pg/mL. Although this limit was evaluated for SAA, it could explain why cytokines and chemokines, previously detected in the same serum samples, could not be identified by SELDI. Indeed, they were an order of magnitude below the SELDI limit of detection even after four doses of IL-2 when most reached their peak abundance [12]. However, we could identify IL-2 induced-changes in the expression of several serum proteins that are relevant to acute-phase response and HDL metabolism. A preliminary analysis of SELDI spectra identified several peaks whose *m/z* values corresponded to the molecular weight of serum proteins reported in the GuessProt database. We were particularly interested in confirming the identity of two peaks at 11 666 and 23 108 Da that were consistently and exclusively expressed in samples obtained after four doses of IL-2. Database matching ascribed these *m/z* values to the acute-phase reactants SAA and CRP.

Confirmation of the identity of the serum components was facilitated by SELDI immunocapture platforms, which, in addition, allowed the identification of different isoforms of the same protein. The SAA immunocapture spectra included three different peaks at *m/z* ~11 700, 11 500, and 11 100 Da, which have previously been documented to be SAA variants [16]. Immunocapture also detected additional proteins associated/bound to the captured soluble factor, providing a practical and alternate methodology for co-immunoprecipitation. An *m/z* 13 900-Da protein, potentially corresponding to TTR, co-purified with the three SAA isoforms. The concomitant occurrence of SAA and TTR has been previously described in serum by Marhaug and Husby [17] as a two-protein complex.

SAA is the serum precursor of the amyloid A protein and proteolytic cleavage of SAA plays a central role in amyloid deposition [23, 24]. SAA concentrations were elevated more than a 1000-fold from resting levels during inflammation, and SAA levels can reach plasma concentrations exceeding 1 mg/mL [25–27]. Furthermore, SAA messenger RNA is a most abundant hepatic transcript during inflammation. Several cytokines may trigger SAA synthesis directly or indirectly [27]. The principal cytokines known to be involved in SAA induction are IL-1, TNF-α and IL-6 [28–35] and IL-2 is believed to play an indirect role in SAA induction by facilitating IL-1 expression [32]. Our data suggest that IL-2 induces increases in SAA levels indirectly through TNF-α and IL-6. In addition to SAA, high-dose IL-2 treatment induced in the same patients a sustained increment in the expression of 47 cytokines including IL-6 and sTNFR1 and 2 [12]. The levels of sTNFR1 and IL-6 were significantly upregulated 3 h after the first dose of IL-2, suggesting that IL-2 triggers an increase in serum levels of IL-6 and TNF-α that indirectly induces liver production of SAA, which can only be detected at a later stage (after the fourth dose in this study).

Several reports have described immunoregulatory properties of SAA that may contribute to the secondary effects of IL-2 administration, particularly in the later phases of treatment when toxicity becomes most significant. SAA can induce extra cellular matrix-degrading enzymes, such as matrix metalloproteinases (MMP) [36], which are involved in matrix destruction, regeneration and mononuclear phagocyte migration. In our previous study [12], we found that the concentration of MMP-2, -3, -8, -9, -10 and -13 were increased in sera collected after four doses of IL-2. This suggests that SAA may have a role as an intermediate factor in cytokine signaling pathways [37].

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SAA has also been demonstrated to be a chemoattractant for immune cells such as monocytes, polymorphonuclear leukocytes, mast cells and lymphocytes [38–40]. Preciado-Patt *et al.* [41] demonstrated that soluble SAA binds the extracellular matrix and promotes the adhesion of resting $CD4^+$ T cells. Thus, the extracellular matrix may provide an anchor for SAA, which allows it to become involved in regulating the recruitment and accumulation of immune cells in extravascular inflammatory compartments [42]. Furthermore, increases in SAA levels during IL-2 therapy may play a role in the enigmatic disappearance of lymphocytes from the peripheral circulation by facilitating their extravasation. SAA can also trigger the release of chemoattractants such as IL-8 by binding to the lipoxin A4 receptor [43]. This is consistent with the hypothesis that up-regulation of IL-8 expression observed during IL-2 therapy both at the mRNA and protein level [5, 12] may not be a direct effect of IL-2, but a consequence of SAA release.

Concomitantly to SAA, CRP was up-regulated following IL-2 therapy. This acute-phase reactant is a known marker of inflammation observed to increase up to 200-fold during an acute-phase response [44]. Several studies have reported the induction of CRP following low-intermediate dose IL-2 therapy [9, 20, 45–47].

Any type of inflammatory stimuli can, in most cases prompt the release of IL-1, IL-6 and TNF, which in turn stimulates hepatic production of CRP [48]. Thus, it could be speculated that, IL-2 treatment mimics an inflammatory stimulus capable of triggering the production of IL-1 and IL-6, both of which could induce the hepatic production of CRP [49]. CRP in turn can act as a pro-inflammatory agent inducing the release of ICAM, VCAM, E-selectin, MCP-1 and MMP-1 [48, 50–52], all which are significantly up-regulated during IL-2 therapy [12].

The application of hierarchical clustering to the analysis of protein data from high throughput platforms allowed the simultaneous extraction of information about the pattern of protein expression and the grouping of samples with similar profiles. Interestingly, hierarchical clustering of *m/z* area values obtained by SELDI analysis separated individual samples in two main groups. The group encompassing the POST 4 samples clustered separately from the PRE and POST 1 samples, indicating that dramatic variations in inflammatory protein profiles occur at a later stage of IL-2 administration. Other peaks identified as negative acute-phase reactants, such as Apo A-I, Apo C-I, Apo C-II, Apo A-II, TTR and RBP, were coordinately reduced in POST 4 samples as corroborated by hierarchical clustering of quantitative nephelometric data.

This pilot study demonstrates the advantages of a global approach to the analysis of cytokine therapy in cancer patients by demonstrating the limits and the advantages of distinct methodologies for high-throughput protein profiling. Given the wide range of concentrations in which distinct proteins are present in serum (a gap of 10 logs), we applied methodologies with different ranges of detection. Protein arrays [12] and nephelometry were applied to detect low and intermediate abundance proteins (chemokines, cytokines). SELDI, although limited by lower sensitivity, was able to capture novel information independent of pre-selected queries and could resolve isoforms of high-abundance serum proteins that could not have been detected otherwise. This may be of particular importance in assessing the biological activity of molecules that require biological activation through PTM.

We further compared the quantitative potential of different methods and observed a positive correlation between peak intensity by SELDI and protein relative abundance according to quantitative methods. Serial dilution of POST 4 with PRE IL-2 serum samples demonstrated that proteins other than SAA maintained a constant relative abundance. After adjustment of data according to total protein concentration which was found to change with treatment, the percentage of SAA correlated in several assays. These adjustments allowed (1) the representation of samples with different amount of SAA, and (2) the establishment of a linear correlation between SAA relative abundance (determined by nephelometry) and peak intensity. These results support a new quantitative role for SELDI analysis of protein expression in addition to pattern profiling.

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