RESEARCH ARTICLE

Proteomic analysis of inflammatory biomarkers in bronchoalveolar lavage

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To assess markers of lung inflammation, we used SELDI-TOF and 2-DE to study changes in bronchoalveolar lavage (BAL) protein in 33 subjects challenged with local bronchial lung endotoxin and saline and in 11 patients with acute respiratory distress syndrome (ARDS). Differences in the SELDI-TOF spectra were assessed by *t*-test after baseline subtraction, normalization to total ion current and alignment by *m/z* calibration. The temporal changes in acute inflammatory BAL (6, 24 and 48 h following endotoxin challenge) on hydrophobic binding chip surfaces revealed the differential presence of proteins of 9, 14, 18 and 28 kDa (all $p < 0.001$) in the inflammatory BAL. This differential pattern was also found in the ARDS BAL. Principal component analysis of the entire SELDI-TOF spectra separated normal BAL, experimental and clinical lung inflammation supporting the notion of a distinctive protein pattern associated with acute lung inflammation. An analysis of the hydrophobic fraction of the inflammatory BAL using 2-DE, identified increased levels of apolipoprotein A1, and S100 calcium-binding proteins A8 and A9 in the inflammatory BAL. This pattern was also found in ARDS BAL after immunoblot analysis. These approaches will be useful to improve current methods of monitoring lung inflammation and to identify new therapeutic targets.

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1 Introduction

Innate immunity to bacterial components is a common mechanism of lung inflammation [1]. While these responses provide a rapid defense against infection, they may also contribute to the development of severe diffuse lung injury or acute respiratory distress syndrome (ARDS). The resultant activation of the pulmonary epithelium, endothelium, and immune cells leads to an influx of inflammatory cells and alterations in the alveolar-capillary barrier. Alveolar flooding due to plasma protein exudation contributes to the hallmark of ARDS, hypoxemia, decreased lung compliance, and increased intrapulmonary shunt [2].

Endotoxin, a bacterial wall component, is a potent trigger of innate immunity. We have shown that direct instillation of endotoxin into a lung segment results in localized lung inflammation with a time-dependent profile of cell activation and inflammatory mediator release. At 6 h after instillation, the lavage has elevated levels of cytokines and chemokines and increased cellularity due to a neutrophil influx [3]. By 24 to 48 h, many secondary mediators in the

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Abbreviations: ApoA1, apolipoprotein A1; **ARDS**, acute respiratory distress syndrome; **ATIII**, anti-thrombin III; **BAL**, bronchoalveolar lavage fluid; **LPS**, lipopolysaccharide; **S100A8**, S100 calcium-binding protein A8; **S100A9**, S100 calcium-binding protein A9

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lavage return to baseline while the cellularity remains elevated due to increased numbers of macrophages, monocytes, lymphocytes and neutrophils [3]. This human model provides a unique means of evaluating the initiation and resolution of lung inflammation to a common bacterial component. These mechanisms are qualitatively similar to some of the inflammatory responses found in ARDS, *i.e.* activation of innate immunity, lung neutrophil infiltration, and elevated cytokine and chemokine levels [4, 5]. However, ARDS is a distinct entity because of the intensity, diffuse nature and prolonged duration of lung inflammation that results in tissue injury and life-threatening organ failure.

Recent studies highlight the interest in bronchoalveolar lavage (BAL) as a source of unique biomarkers in interstitial lung diseases [6, 7], acute lung injury [8] or in chronic lung diseases such as asthma [9]. We investigated potential markers of lung inflammation by using an unbiased proteomic approach to study BAL from healthy subjects challenged with lung endotoxin instillation and in patients with ARDS. Using complementary methodologies of SELDI-TOF, 2-D PAGE, MALDI-TOF, and a new spectral analysis approach, we identified biomarkers in acute inflammatory BAL. The use of samples from an experimental human setting allowed us to control for BAL protein expression variability and provided an impetus to confirm the importance of these inflammatory markers in a clinical disorder.

2 Materials and methods

2.1 Materials

BAL specimens from 33 healthy nonsmoking subjects (19 men, 14 women, 28.5 ± 1.2 years; mean ± SEM) were obtained as previously described [3]. Briefly, local lung inflammatory responses were evaluated using a bronchoscope and balloon catheter to instill saline (left lung subsegment, 10 mL) and endotoxin (right lung subsegment, 4 ng/kg *Escherichia coli* O:113 in a volume of 1–2 mL; Clinical Center Reference Endotoxin, NIH, Bethesda, MD). We have previously shown that this model is a reproducible means of eliciting local lung inflammatory responses isolated to the endotoxin-challenged segment [3]. BAL was performed with 180 mL of saline on each side (360 mL total) after 6 h (16 subjects), 24 h (9 subjects), or 48 h (8 subjects). The cellular and cytokine inflammatory responses of 20 of these subjects have been previously described [3]. Four additional healthy nonsmoking subjects (2 men and 2 women, ages 32 ± 2 years) underwent BAL alone (180 mL instilled in both left and right lung) without endotoxin challenge.

Eleven patients (5 men and 6 women, 46 ± 15 years) with ARDS had BAL performed (140 mL volume instilled each side with approximately 40–50% return of the instilled volumes) within 72 h of meeting the clinical definition [5, 6]. The underlying disorders were community-acquired pneumonia, acute pancreatitis, near drowning and peritonitis (Supplementary Table 1).

The BAL was placed immediately on ice and separated from the cellular component by centrifugation (300 × *g* for 10 min at 4°C). The supernatants were separated into aliquots and frozen at −80°C until thawed for analysis. The total BAL leukocyte number was determined with an automated cell counter (Coulter ZM, Miami, FL) in the endotoxin-challenged and healthy subjects. Differential cell counts were done by morphologic examination of stained cytospins (Diff Quick, Dade Behring, Newark, DE) and total protein was measured in non-concentrated BAL (BCA protein assay, Pierce, Rockford, IL) in the endotoxin study-participants, healthy subjects and patients with ARDS.

The institutional review boards of the National Institutes of Health, Bethesda, MD and the University of Tennessee at Memphis approved the studies of endotoxin challenge and ARDS, respectively. Written informed consent was obtained from all participants or their surrogates.

2.2 Analysis of BAL with SELDI-TOF

Due to the low protein concentration in the BAL, we concentrated each sample using either the YM3 Centriplus or Centricon (Millipore Corporation, Bedford, MA) filtration units at 4°C until a protein concentration of 2.3 mg/mL was achieved (BCA assay, Pierce). We diluted each sample 2:5 with a denaturing solution (8 M Urea/2% CHAPS). The samples were applied to the hydrophobic (H50) and metal-binding protein (IMAC30) ProteinChip arrays (Ciphergen Biosystems, Fremont, CA) according to the manufacturer's protocols. Briefly, the H50 arrays spots were activated for 10 min with 50% ACN and equilibrated for 10 min with the binding buffer (0.1% TFA, 25% ACN). For the IMAC30 ProteinChip, each spot was incubated twice with 100 mM $CuSO_4$ for 10 min followed by washing with HPLC water, 50 mM sodium acetate (pH 4.5), HPLC water and finally equilibrated with PBS.

We diluted the denatured BAL 1:10 in binding buffer and applied 9 μg of total protein/spot. Each sample was run in duplicate on different ProteinChip arrays and incubated for 2 h at room temperature in a shaking (900–1000 rpm) bioprocessor (Ciphergen Biosystems). The ProteinChip arrays were washed three times with the binding buffer and twice with HPLC water, and then air-dried. We then applied 1 μL (twice 0.5 μL) of a 100% saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (FLUKA, St Louis, MO) prepared in 0.5% TFA, 50% ACN.

The ProteinChip arrays were analyzed with the Ciphergen ProteinChip Reader (model PBSII). The mass spectra were generated using an average of 65 shots at a laser intensity of 240 arbitrary units. For data acquisition, the detection size range was between 3000–70 000 Da and the detector

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sensitivity was set at 7. The *m/z* of each protein captured on the array surface was determined according to externally calibrated standards (All-in-1 Protein Standard II. Ciphergen Biosystems).

2.3 Data analysis of BAL characteristics and SELDI-TOF BAL profiles

BAL characteristics in the endotoxin challenged subjects were compared using a one-way ANOVA and the Tukey-Kramer multiple comparison test (Prism, Graphpad Software, v3.06, San Diego, CA). The SELDI-TOF profiles were initially analyzed by application of the Ciphergen software baseline subtraction algorithm to all spectra. The peak intensities were normalized to the average of total ion current of all spectra between 3000 and 70 000 Da, the optimal size range for detection and *m/z* calibration. Each spectrum was then exported as raw data keeping their individual external calibration that was applied for each set of chips and aligned to a common set of *m/z* points using nearest-neighbor interpolation. A final normalization was applied to the logarithmic value of each spectrum by subtracting the median log intensity value. We applied a Student's *t*-test to data at each *m/z* value to determine where the spectra demonstrated significant differences ($p < 0.001$) comparing either lipopolysaccharide (LPS)-treated to saline-treated BAL (paired *t*-test) or ARDS to healthy BAL (unpaired *t*-test).

We calculated the variability of measurements from samples within the same treatment group, but arising from different individuals as an estimate of biological variability. We selected five intensity measurements (expressed as log base 10 spectral intensity, median normalized and rounded up to 0 to reduce the influence of values near baseline) from each sample corresponding to peaks of interest, with *m/z* at 9920.7906, 14157.199, 17784.288, 18294.631 and 28955.798. We studied the mean of duplicate samples at the same five peak locations by treatment group (6 h saline, 6 h LPS, 24 h saline, 24 h LPS, 48 h saline, 48 h LPS, ARDS, healthy). All analyses were performed using MATLAB version 6.5 (The MathWorks, Natick, MA). Principal component analysis of the entire transformed spectra was performed using JMP version 5.1.1 (SAS Institute, Cary, NC).

2.4 BAL fractionation using C8 spin columns

To simulate the binding conditions obtained in the SELDI experiments, we used the C-8 Vydac, 300-Å MacroSpin™ columns (AmiKa/Harvard Bioscience, The Nest Group, MA) and prepared 400 μg of BAL proteins using 0.1% TFA, 25% ACN, 480 mM urea and 0.12% CHAPS. Briefly, C-8 columns were activated with 50% ACN and equilibrated with 0.1% TFA/25% ACN. Each sample was applied ten times and the column washed three times with equilibration buffer. Elution of the sample was performed by applying 200 μL of 0.1% TFA/80% ACN.

The samples were dried in a Speed Vacuum (Heto-Holten A/S, Allerrød, Denmark) and then dissolved in 200 μL of 8 M urea, 2% CHAPS, 15 mM dithioerythritol and 0.5% IPG buffer 3–10 (Amersham Biosciences, Piscataway, NJ). Protein concentration was quantified using the 2D Quantum kit (Amersham Biosciences, Piscataway, NJ).

2.5 2-DE

The first dimension was performed in IPGphor unit (Amersham Biosciences), as previously described [10]. Immobiline DryStrips 24-cm length, pH 3–10 non-linear (Amersham Biosciences) were placed gel side-down on top of the fractionated BAL solution for in-gel rehydration for 12 h using low voltage 30 V at 20°C. The fractionated BAL contained 100 μg of protein in 450 μL of 8 M urea, 2% CHAPS, 0.5% IPG buffer 3–10, 65 mM dithioerythritol and a trace of bromophenol blue. The proteins were then focused for 36 500 Vh at a maximum voltage of 8000 V.

The second-dimensional separation (SDS-PAGE) was carried out with Ettan Dalt II System (Amersham Biosciences), using pre-cast 12.5% gels (dimensions 20.5 cm × 25.8 cm × 0.5 mm). In a first step, we used 2.5 W/gel for 30 min and then applied 170 W for 4 h 30 min. Staining with silver nitrate of the 2-D gels was performed as described by Schevchenko *et al.* [11].

2.6 Data analysis of 2-DE profiles of BAL

The analysis of protein spots on the gels was performed with Progenesis Workstation software (Nonlinear Dynamics, NC). The stained gels were digitized wet at 100 μm of resolution (FLA-5000 scanner, Fuji Film, Stamford, CT). The parameters for image digitizing was 532-nm laser, filter LPG 250-000/01 (Fuji Film) and the input voltage value applied to the photomultiplier tube was 250 V [12]. The protein spots of interest were punched out of gels manually and processed as described by Schevchenko *et al.* [11]. The recovered peptides were purified using Zip-Tip C18 (Millipore Corporation) following the manufacturer's instructions.

2.7 Analysis of tryptic digests with MALDI-TOF-TOF-MS

Mass spectra were acquired using a MALDI-TOF-TOF mass spectrometer (Bruker Daltonics, Billerica, MA). The mass spectrometer was used in the reflectron mode. The ions were accelerated using 25 kV and a time delay of 110 ns was used in the pulsed extraction ion source. Each spectrum was submitted with trypsin autodigestion peaks excluded to a PMF search using the search program MASCOT (http://www.matrixscience.com). The parameters for the searches were database NCBInr, species of origin *Homo sapiens*, the molecular range set to 5–3000 kDa, p*I* range 1–14, and digestion by trypsin allowing for no more than one missed cut. Monoisotopic peptide masses were used and the mass

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tolerance error was set to 0.3 Da. Cysteine was considered as carbamidomethylation cysteine and partial modification of methionine by oxidation was allowed. The samples not clearly identified by PMF were automatically submitted to LIFT TOF/TOF acquisition. Protein database searches, through MASCOT (http://www.matrixscience.com), using combined PMF and MS/MS datasets were performed via BioTools 2.2 (Bruker Daltonics).

2.8 Western blotting analysis for human apolipoprotein A-I, apolipoprotein E, S100 calcium-binding proteins A9 and A8, and anti-thrombin III

Briefly, 300 μg of each BAL protein sample was incubated with protein A-agarose 50% in PBS (Sigma-Aldrich, St Louis, MO), three times during 1 h at 4°C, to remove the cross-reactive human IgG. The protein concentration was measured by BCA Protein Assay kit (Pierce Biotechnology). The samples were normalized to 0.4 mg/mL and separated by SDS-PAGE on a 4–20% gradient pre-cast polyacrylamide gel (Invitrogen Life Technologies, Carlsbad, CA) and then stained with PhastGel BlueR (Amersham Biosciences).

For immunoblot analysis, gels were transferred to NC membranes (Invitrogen Life Technologies). The NC membranes were incubated with 1:10 000 dilution of a polyclonal goat anti-human apolipoprotein A-I (ApoAI), anti-human apolipoprotein E antibody, anti-human anti-thrombin III (ATIII) antibody (BioDesign International, ME) or with 1:200 dilution of a polyclonal rabbit anti-human S100 calcium-binding proteins A8 (S100A8) or anti-human S100A9 (Santa Cruz Biotech, Santa Cruz, CA) as primary antibodies. A 1:20000 dilution of a horseradish peroxidase conjugated anti-goat or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) were used as secondary antibodies and were visualized by the chromogenic substrate, BM Blue POD substrate (Roche Diagnostics, Indianapolis, IN).

3 Results

3.1 Identification of differential changes in BAL protein composition during lung inflammation

Segmental lung instillation of endotoxin is associated with an intense local pulmonary inflammatory response manifested by an increase in cellularity, proteins, and inflammatory mediators [3]. In the current study, the volume of lavage returned was comparable among the endotoxin-challenged subjects (n = 33, saline side 104 ± 4 and endotoxin side 115 ± 4 mL, p = 0.002). After endotoxin challenge, the BAL total cell number, percentage of macrophages and neutrophils and total protein content increased compared to the saline-challenged segments (all p <0.0001, Fig. 1). The BAL characteristics of the 4 healthy subjects were similar to the BAL of the saline-challenged segments (Fig. 1). Patients with

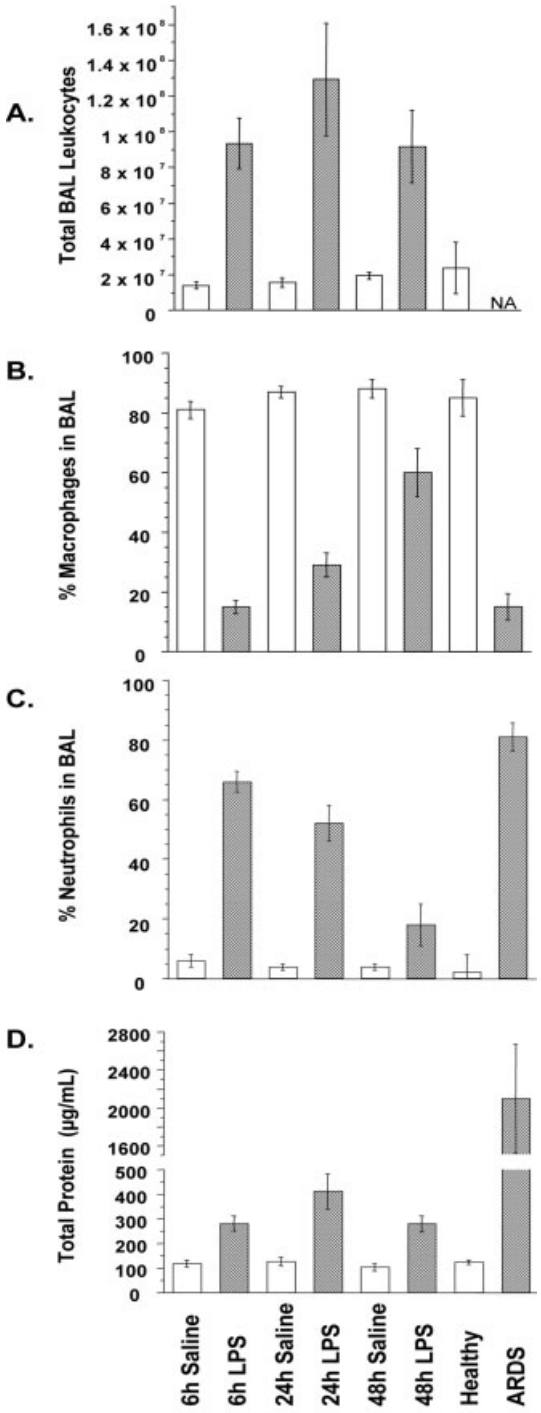

Figure 1. BAL characteristics of endotoxin-challenged subjects, healthy controls and patients with ARDS (mean ± SEM). Inflammatory BAL is shown by shaded bars and non-inflammatory BAL is shown by open bars. (A) Total BAL leukocytes after LPS challenge *vs.* saline (LPS effect p <0.001, 6 and 24 h – p < 0.01, 48 h – p >0.05). (B) BAL percent macrophages (LPS effect p <0.001, 6, 24 and 48 h, p <0.001), (C) BAL percent neutrophils (LPS effect p <0.001, 6 and 24 h – p <0.001, 48 h – p >0.05). (D) BAL total protein (LPS effect p <0.001, 6 and 24 h – p <0.01 and 48 h p <0.05), NA – not available.

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ARDS had increased neutrophils compared to the healthy subjects and total BAL protein was sixfold greater than levels observed after segmental endotoxin challenge (both $p < 0.0001$, Fig. 1).

Using SELDI-TOF proteomic analysis, we found several proteins were differentially present when comparing BAL from endotoxin- versus saline-challenged segments at 6, 24 and 48 h (33 duplicate arrays summarized in Fig. 2A–C). Analysis of the hydrophobic (H50) ProteinChip data revealed a significant increase in the endotoxin-challenged segment BAL spectra at 14-, 18-, and 28-kDa peaks while a 9-kDa peak was increased in the saline-challenged segment samples (Fig. 2A–D). Further, the intensity of the inflammatory protein expression persisted at 24 h and then diminished at 48 h when the inflammatory process was resolving with less cellularity and protein exudates [3].

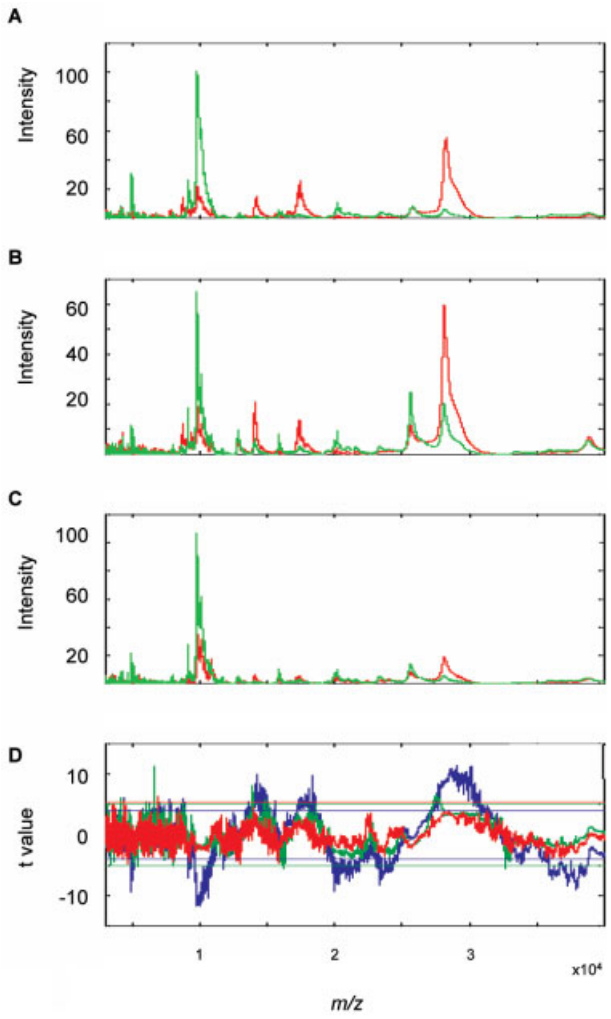

Figure 2. Average SELDI-TOF spectra obtained from LPS challenged BAL (red) and saline challenged BAL (green) at 6 h (A), 24 h (B) and 48 h (C) on H50 ProteinChip. In panel (D), t value versus *m/z* value at each time point is shown (6 h – blue, 24 h – green, 48 h – red). Horizontal lines represent the threshold t value of $p < 0.001$ for each respective time point.

To ascertain the precision of SELDI measurement, we compared values obtained from duplicates (same sample placed on distinct spots of the SELDI chip) obtaining a CV of 34% as the variance of duplicates. The average variability within group reached a CV of 0.75%.

The application of BAL to the metal-binding protein IMAC-Cu ProteinChips did not demonstrate significant differential expression with the exception of a 66-kDa peak (Supplementary Fig. 1), which likely represents an increase in albumin in the inflammatory lavage, as previously described [3].

BAL from patients with ARDS had a pattern of protein expression on the hydrophobic H50 ProteinChip qualitatively similar to the pattern observed at 6 h after endotoxin challenge (Fig. 3A and B). This profile showed a significant increase in the 14- and 28-kDa proteins that were absent from both the healthy volunteer BAL samples (no endotoxin) and the saline-challenged segments of the endotoxin-challenged volunteers. We performed principal component analysis on the complete log transformed spectra dataset. The first and second components accounted for 20 and 9% of the total variability, respectively (Fig. 4A and B).

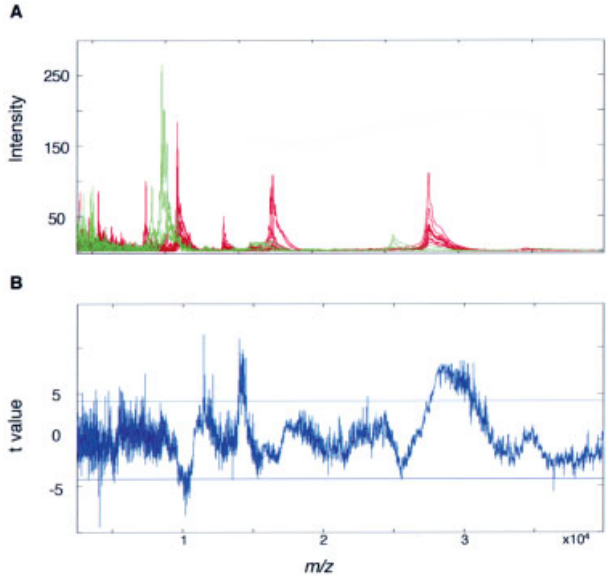

Figure 3. (A) Individual SELDI-TOF spectrum obtained from the BAL from healthy subjects (green) and patients with ARDS (red) on H50 ProteinChip. (B) The t value versus *m/z* with the horizontal lines representing the threshold t value of $p < 0.001$.

3.2 Analysis of inflammatory biomarkers by 2-DE

We isolated hydrophobic proteins from the BAL of six subjects obtained 6 h after endotoxin and saline challenge using C8 spin columns and conditions similar to those previously applied to the H50 ProteinChips. These hydrophobic proteins were applied to pH 3–10 non-Linear strips for first dimension separation and resolved in a 12% acrylamide gel in

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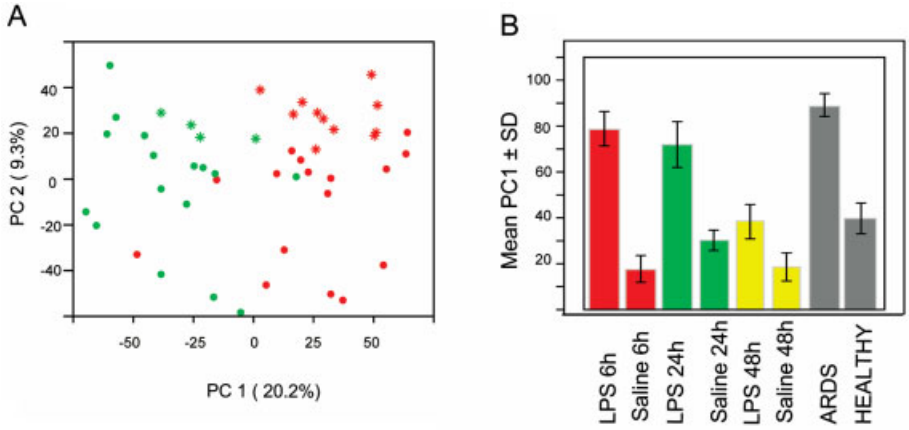

Figure 4. (A) Principal component analysis of the complete transformed SELDI-TOF H50 ProteinChip spectra data for 3000–70 000 Da; 6-h LPS challenged BAL (red circles), 6-h saline challenged BAL (green circles), ARDS BAL (red stars) and healthy BAL (green stars). (B) The mean of the first principal component value ± SD of each group studied.

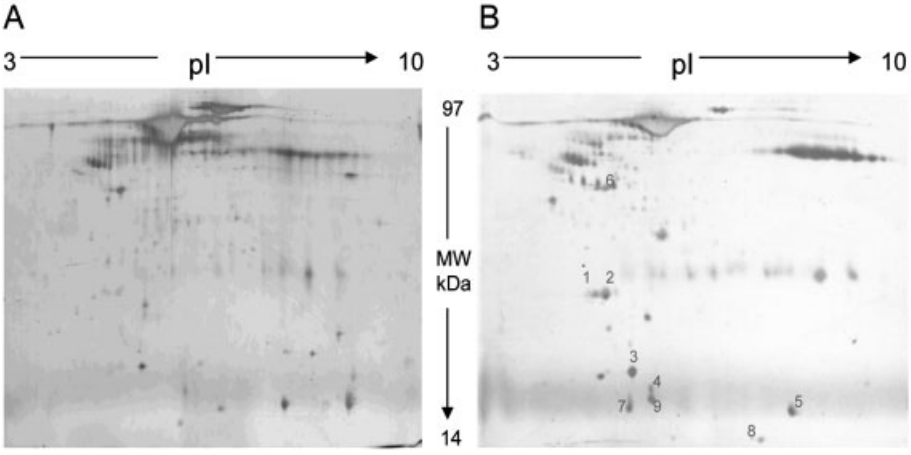

Figure 5. Differences in 2- DE protein patterns in paired BAL from lung segments challenged with (A) saline and (B) LPS. Each gel represents the summary of six subjects challenged with (A) saline and (B) LPS at 6 h. Sample preparation and 2-DE were performed as described in Section 2. Proteins (100 μg) were separated using nonlinear Immobiline gradient 3–10, followed by 12% SDS-PAGE and were detected by silver staining. Numbers (1–10) indicate the differentially expressed proteins listed in Table 1A and B.

the second dimension. The analysis of the BAL protein patterns was focused on the protein spots present in the endotoxin-BAL and absent or present in low amounts in the saline BAL. Ten spots met these criteria and were selected and identified by MALDI-TOF MS after in-gel trypsin digestion.

In the high molecular weight area of the gel, we detected a differential protein identified as ATIII. In the 30-kDa region, we detected two isoforms of ApoA1 and in the low molecular region of the gel we identified four proteins; transthyretin, hemoglobin A chain b, S100A8 and S100A9 (Fig. 5 and Table 1A and B). The plasma proteins, transthyretin and hemoglobin A chain b, were detected in both the endotoxin-BAL and the saline-BAL whereas S100A8, S100A9, ApoA1 and ATIII were detected in the endotoxin-BAL only.

To validate these proteins as inflammatory biomarkers, Western blots were performed on three samples of BAL from healthy volunteers and from patients with ARDS. The presence of ApoA1, ATIII, S100A8, and S100A9 in inflammatory BAL were increased compared to BAL from healthy subjects. Notably, apolipoprotein E, an inflammatory-related serum lipoprotein, was absent in all the BAL (Fig. 6) [13, 14].

4 Discussion

New biomarkers are needed to improve current methods of monitoring disease activity and to identify new therapeutic targets [15, 16]. One approach has been the high-throughput analysis of protein profiles in biological fluids using MS. Protein expression patterns based on SELDI-TOF have been used to discover early diagnostic markers, prediction of responses to therapy and prognosis of malignancies [17–19] as well as early diagnostic markers of infection [20, 21]. No information has been published using SELDI-TOF to assess changes in BAL in humans [22]. We demonstrate that the SELDI-TOF platform can semi-quantitatively assess the dynamic changes in protein expression that occur in BAL after the initiation of inflammation in humans challenged with local lung instillation of endotoxin. As noted in our previous work, segmental endotoxin instillation is associated with a neutrophil influx and increased protein content in the inflamed lung segment [3]. In the current study, unique hydrophobic proteins are detected within 6 h and resolve within 48 h of endotoxin challenge. Using SELDI-TOF, identical proteins were found in the BAL from patients with

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Table 1A. Analysis of tryptic digests with MALDI-TOF/TOF-MS (PMF)

Spot No.[a)]	Fragment[b)]	Match[c)]	Score value[d)]	Coverage[e)] (%)	Database i.d.[f)]	Protein name
1	87	36	300.0	83.1	gi4557321	Apolipoprotein AI precursor
2	126	34	221.0	88.0	gi4557321	Apolipoprotein AI precursor
3	65	10	99.20	73.5	gi30583217	Transthyretin
5	71	16	160.0	96.6	gi4504349	Beta globin

a) Spot No. related to the annotation in Fig. 5.
b) Total number of measured peptide masses obtained from the protein spots used in the database search.
c) Number of matched peptide masses related to the assigned protein.
d) MASCOT score value indicates the quality of database search results.
e) Sequence coverage, refers to the observed sequence coverage of the assigned protein
f) National Center for Biotechnology Information (NCBI)

Table 1B. Analysis of tryptic digests with MALDI-LIFT-TOF/TOF-MS (MS/MS)

Spot No.[a)]	Precursor ion	Fragment[b)]	Match[c)]	Score value[d)]	Sequence	Protein name
1	1400.7	26	17	101.00	DYVSQFEGSALGK	Apolipoprotein AI precursor
1	1815.9	28	21	82.30	DSGRDYVSQFEGSALGK	Apolipoprotein AI precursor
2	1400.7	21	16	101.0	DYVSQFEGSALGK	Apolipoprotein AI precursor
2	1815.9	31	17	74.70	DSGRDYVSQFEGSALGK	Apolipoprotein AI precursor
3	1366.8	57	28	131.00	GSPAINVAVHVFR	Transthyretin
3	1494.9	42	16	89.30	GSPAINVAVHVFRK	Transthyretin
4	1387.7	23	14	78.90	LPECEAVCGKPK	Haptoglobin
4	1857.9	48	26	109.0	AVGDKLPECEAVCGKPK	Haptoglobin
5	1274.7	59	16	89.20	LLVVYPWTQR	Beta globin
5	1449.8	42	28	120.00	VVAGVANALAHKYH	Beta globin
6	1232.8	48	16	65.20	ANRPFLVFIR	Antithrombin III
6	1389.8	29	14	96.00	EVPLNTIIFMGR	Antithrombin III
7	1455.7	21	18	116.00	LGHPDTLNQGEFK	S100 calcium-binding protein A9
7	1806.8	19	13	87.10	NIETIINTFHQYSVK	S100 calcium-binding protein A9
8	1421.7	28	13	79.50	LLETECPQYIR	S100 calcium-binding protein A8
8	963.4	34	17	73.90	GNFHAVYR	S100 calcium-binding protein A8
9	1857.8	57	30	152.00	AVGDKLPECEAVCGKPK	Haptoglobin
9	1439.6	11	10	71.80	TEGDGVYTLNNEK	Haptoglobin

a) Spot No. related to the annotation in Fig. 5. MS/MS spectra for each spot are shown in Supplementary Figs. 2–19.
b) Total number of measured peptide masses obtained from the protein spots used in the database search.
c) Number of matched peptide masses related to the assigned protein.
d) MASCOT score value indicates the quality of database search results.

severe clinical lung inflammation due to ARDS. These data suggest some common pathways in the development of this clinical syndrome.

The low protein content, high salt concentration and over representation of proteins such as albumin and immunoglobulins in lavage hamper the study of BAL [23]. To address these concerns, we concentrated the BAL using a 3-kDa cutoff and normalized the samples to the same concentration. The use of the hydrophobic ProteinChip (H50) and stringent buffer conditions helped obtaining protein profiles without albumin or immunoglobulins masking the binding of less abundant proteins to the chip. To reduce errors linked to noise or data acquisition, analyses of changes in the BAL protein profile were performed after subtracting baseline values, normalizing the spectra to the total ion current and aligning the spectra to a common set of m/z points [24–26]. Notably, the principal component analysis of the entire transformed spectra between 3 and 70 kDa separated the inflammatory from normal lavage, indicating the spectra contained information related to the treatment (*i.e.* endotoxin or saline) or to the condition (*i.e.* ARDS). Computing the principal component analysis only on selected portions

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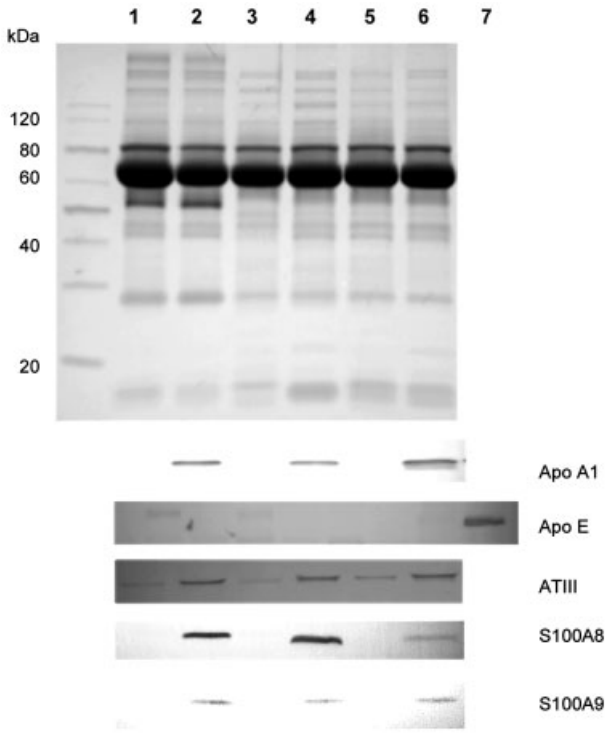

Figure 6. Electrophoretic and immunoblot analysis of 8 μg of BAL from ARDS patients (lanes 2, 4, 6) and healthy volunteers (lanes 1, 3, 5). Samples were prepared and subjected to 4–20% SDS-PAGE, as described in Section 2. (A) Gel stained with PhastGel BlueR. Lane 1 contains 5 μL of Magic Standards (Invitrogen life technologies, CA, USA). (B) Immunoblots obtained with anti-apoAI, anti-apoE, anti-ATIII, anti-S100A8 and anti-S100A9 antibodies as described in Section 2. Lane 7 contains 50 ng human apoE.

of the spectra where differences exist can give a misleading representation of the discriminating power of a sample set. When portions of a spectral dataset, which correlate with a specific sample grouping are selected, the principal component analysis of even random spectra can separate any set of samples. Further, we used a high level of stringency (t value with probability of $p < 0.001$) to assess changes between healthy and LPS BAL or ARDS samples.

The 2-DE analysis of the hydrophobic BAL fraction identified four inflammatory markers. Differences in the interactions of the BAL on C8 spin columns rather than a mixture of C6–C12 matrices, which is more representative of a protein chip surface, hampered our ability to identify SELDI differential peaks as ApoAI, S100A8, and S100A9. However, the use of a similar surface of interaction (C8) not only allowed identifying the inflammatory markers that enhanced our detection of differentially expressed proteins in the high molecular weight range. Transthyretin and hemoglobin A chain b could serve as inflammatory markers but the lack of a quantitative relation between spot intensity and protein amount with silver staining limited the analysis of these differences.

ApoA1 is as a major component of high-density lipoproteins and is synthesized primarily in the liver and the intestine [27]. In a recent study of gene expression distribution, ApoA1 was expressed in human trachea but is not described in other pulmonary tissue [28]. This lipoprotein both enables and neutralizes endotoxin bioactivity by its association with LPS-binding protein and high-density lipoprotein [29, 30]. Further, it may alter the binding and inhibition of the human antibacterial, cytotoxic and chemotactic LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin [31, 32]. In addition to these molecular interactions, ApoA1 has anti-inflammatory activity including inhibition of IL-1 and TNF production by blocking contact-mediated activation of monocytes by T lymphocytes [33], suppression of neutrophil degranulation and superoxide production in response to surface-bound IgG and formyl-methionyl-leucyl-phenylalanine [34], and inhibition of complement factor 9 incorporation into C5b-9 complexes on endothelial cells [35].

S100A8 (also called calgranulin A and myeloid-related protein 8, MRP8) and S100A9 (calgranulin B, MRP 14), are proinflammatory calcium-binding proteins that play a pivotal role in innate immunity [36] and were found differentially present in inflammatory BAL. Both proteins contribute to experimental and clinical lung inflammation. These molecules are present in high concentration in the neutrophil cytoplasm and are released upon cell activation, contributing to antibacterial activity [37, 38]. Murine S100A8 and S100A9 have chemotactic activity [39] but this is not reported for the human proteins [40]. Specific interactions of these proteins with the endothelial surface suggest a regulatory role in the adhesion of phagocytes to the endothelial surface [41]. Several sources of S100A8 and S100A9 may contribute to the increased amounts observed in inflammatory lavage; infiltrating inflammatory cells, tissue macrophages, or airway epithelium.

ApoA1, S100A8 and S100A9 have been detected in the BAL of patients with chronic interstitial lung disease (sarcoidosis, idiopathic pulmonary fibrosis, and hypersensitivity pneumonitis) using 2-DE [6, 8, 42–44]. Further research is needed to confirm the function and specificity of these molecules in acute lung inflammation using more rapid methods of analysis and applied to a larger number of clinical disorders. Other investigators have described a systematic evaluation of plasma and pulmonary edema fluid in patients with acute lung injury by directly aspirating edema fluid from the distal airways and analyzing the proteins with 2-D gels and MALDI-TOF [8]. Apo A1, S100A8 and S100A9 were not identified in the edema fluid of patients with acute lung injury [8]. This may be due to clinical differences in the patients. However, BAL examines a much larger number of distal airways and alveolar spaces than direct aspiration of edema fluid. As a result, BAL affords a wider sampling of lung tissue and inflammatory proteins. These observations suggest that different sampling methods will provide complementary details of proteins that are differentially expressed during acute lung inflammation.

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In the current investigation, we show that the SELDI-TOF platform can provide a useful screen to characterize the temporal sequence of biomarker protein expression in human experimental and acute clinical lung inflammation (ARDS). The nonproprietary analysis applied to the SELDI TOF data identified spectra that could be verified by complementary protein identification methods. Further, despite inter-individual variability in BAL protein expression, the entire transformed lavage spectra were separated with principal component analysis according to three different conditions (endotoxin-induced inflammation, saline or normal lavage, and ARDS). These results support the notion of a distinctive protein pattern that is present in acute lung inflammation. These data demonstrate the usefulness of this approach to monitor lung inflammation and to identify potential new biomarkers and therapeutic targets.

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