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Protein Expression Profiles Distinguish Between Experimental Invasive Pulmonary Aspergillosis and *Pseudomonas* Pneumonia

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Abstract

We hypothesized that invasive pulmonary aspergillosis (IPA) may generate a distinctive proteomic signature in plasma and bronchoalveolar lavage (BAL). Proteins in plasma and BAL from two neutropenic rabbit models of IPA and *Pseudomonas* pneumonia were analyzed by SELDI-TOF MS. Hierarchical clustering analysis of plasma time course spectra demonstrated two clusters of peaks that were differentially regulated between IPA and *Pseudomonas* pneumonia (57 and 34 peaks, respectively, $p < 0.001$). PCA of plasma proteins demonstrated a time-dependent separation of the two infections. A random forest analysis that ranked the top 30 spectral points distinguished between late *Aspergillus* and *Pseudomonas* pneumonias with 100% sensitivity and specificity. Based on spectral data analysis, three proteins were identified using SDS-PAGE and LC/MS and quantified using reverse phase arrays. Differences in the temporal sequence of plasma haptoglobin ($p < 0.001$), apolipoprotein A1 ($p < 0.001$) and transthyretin ($p < 0.038$) were observed between IPA and *Pseudomonas* pneumonia, as was C-reactive protein ($p < 0.001$). In summary, proteomic analysis of plasma and BAL proteins of experimental *Aspergillus* and *Pseudomonas* pneumonias demonstrates unique protein profiles with principal components and spectral regions that are shared in early infection and diverge at later stages of infection. Haptoglobin, apolipoprotein A1, transthyretin and C-reactive protein are differentially expressed in these infections suggesting important contributions to host defense against IPA.

Keywords

proteomics; *Aspergillus fumigatus*; *Pseudomonas aeruginosa*; pneumonia; neutropenia

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Conflicts of Interest

The authors have no financial or commercial conflicts of interest to declare regarding the content of this manuscript.

INTRODUCTION

Proteomic technologies carry the potential for a new era in molecular medicine, especially in the discovery and development of disease-related biomarkers [1–3]]. These technologies have been widely applied in cancer research for early detection and therapeutic monitoring of neoplastic disease [3–7]. Proteomic analysis of blood protein profiles reveals distinct patterns and potential biomarkers for ovarian cancer, colonic adenocarcinoma, and uroepithelial tumors. However, little is known about the proteomic profiles of invasive mycoses. Understanding the *in vivo* proteomics of invasive mycoses has important diagnostic, therapeutic and pathophysiological implications.

We hypothesized that pneumonia due to invasive pulmonary aspergillosis (IPA) may generate a distinctive proteomic signature in plasma and bronchoalveolar lavage (BAL) fluid compared to non-infected neutropenic controls and that this proteomic profile may have diagnostic and pathophysiological significance. As an additional control, the proteomic profiles of *Pseudomonas* pneumonia were compared with those of IPA. This study demonstrates a proof of principle that the proteins expressed in blood and BAL fluid distinguish IPA from non-infected neutropenic controls and from *Pseudomonas* pneumonia.

To our knowledge, this is the first report to describe the comparative proteomic profiles of invasive fungal and bacterial pneumonias and their kinetic proteomic profiles. These findings introduce the possibility of applying proteomic profiles and biomarkers as a new approach to advancing our understanding of the *in vivo* pathogenesis, molecular detection and host-pathogen interaction of IPA.

MATERIALS AND METHODS (Expanded methods section found in online supplement)

Immunosuppression and maintenance of neutropenia in the rabbit models

Healthy female New Zealand White rabbits (Covance Research Products, Inc., Denver, PA) were used in all experiments. All rabbits were monitored under humane care and use standards in facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International and according to the guidelines of the National Research Council for the care and use of laboratory animals and under approval by the Animal Care and Use Committee of the National Cancer Institute [8]. Immunosuppression and maintenance of neutropenia were established, as described previously [9]. The experimental plan and numbers of plasma and BAL samples analyzed are summarized in Table 1 A–C.

Rabbit model of invasive pulmonary aspergillosis

Pulmonary aspergillosis was established with *Aspergillus fumigatus* NIH isolate 4215 (ATCC no. MYA-1163) as described previously [9, 10]. The concentration was adjusted in order to give each rabbit a predetermined inoculum of $1.0\text{--}1.25 \times 10^8$ conidia of *A. fumigatus* in a volume of 250–350 μL on experiment day 1, while the rabbits were given general anesthesia. The model of IPA in persistently neutropenic rabbits recapitulates the histological, radiological, and microbiological features of IPA observed in patients [9, 11].

Rabbit model of *Pseudomonas* pneumonia

Pseudomonas aeruginosa is a normal constituent of the rabbit oral and upper respiratory bacterial flora. To establish *Pseudomonas* pneumonia, rabbits were immunosuppressed and maintained in the state of neutropenia as described above in the rabbit model of IPA, however, these rabbits did not receive prophylaxis against opportunistic bacterial infections

(*Pseudomonas aeruginosa*) with intravenous antibiotics nor did they receive vancomycin in their drinking water. This stands in contrast to the rabbit model of IPA that receives antibacterial agents for prevention of *P.aeruginosa* infections.

Specimen collection and processing

Blood—Blood was collected via the established vascular access into 3 mL heparinized syringes, transferred into 15 mL polypropylene conical Falcon tubes (Becton Dickinson Labware, Franklin Lakes, N.J.), separated via centrifugation at $400 \times g$ for ten min at 4°C and stored at -70°C in 2 mL Sarstedt microtubes until analyzed. Plasma was collected every other day for galactomannan antigen levels (Platelia™ *Aspergillus* EIA, Bio-Rad Laboratories, Redmond, Wash.), and processed according to manufacturer's recommendations as previously described [12]. Data are expressed as the plasma galactomannan index (GMI). All samples underwent one freeze-thaw cycle for GMI measurement prior to SELDI-TOF analysis.

Bronchoalveolar lavage—Rabbits were euthanized at the end of each experiment (typically day 14 for normal, neutropenic or *Pseudomonas* groups) or for humane indications (typically day 8–9 for the *Aspergillus* group). BAL was performed on each post-mortem excised tracheobronchial tree and lung preparation. Fungal infections were confirmed in post-mortem whole lung excisions as previously described [9, 13].

Analysis of plasma with SELDI-TOF

Plasma samples were denatured (8 M urea/2% CHAPS) and applied in duplicate to strong anion exchange (Q10) ProteinChip arrays (Ciphergen Biosystems, Fremont, Calif.) according to manufacturer's protocols for bioprocessor spotting. The specimens were randomized to three 96-well bioprocessors in a balanced fashion to control for bioprocessor assignment-bias upon infection type, experiment time, and experiment batch (Table 1). Each bioprocessor was run in duplicate. Robotic specimen processing was carried out with the Biomek 2000 (Beckman-Coulter, Thousand Oaks, Calif.). A 100% saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) (FLUKA, St Louis, Mo.) prepared in 0.5% TFA and 50% acetonitrile comprised the matrix that was applied robotically as two 0.5 μL aliquots. Proteins selectively retained on the chip surfaces were analyzed by SELDI-TOF with the Ciphergen ProteinChipReader PBS IIc (Ciphergen Biosystems).

Analysis of BAL fluid with SELDI-TOF

BAL fluid was concentrated, as previously described [14], using YM3 Centriplus or Centricon (Millipore Corporation, Bedford, Mass.) filtration units to a protein concentration of 2.3 mg/mL (BCA assay, Pierce Biotechnology Inc, Rockford, Ill.). Samples were denatured (8M Urea/2% CHAPS) and diluted 1:10 in binding buffer. A total of 9 μg of total protein was applied to each spot on Q10 ProteinChip arrays according to the manufacturer's protocols for direct spotting. Each sample was run in duplicate. An aliquot of 1 μL of matrix (0.5 μL two times), as described above, was applied manually. The ProteinChip arrays were analyzed with the PBS-II Ciphergen ProteinChip Reader as described above.

Data analysis of SELDI-TOF plasma profiles

Spectra were grouped together, baseline subtracted and normalized to total ion current in the range 5–200 kDa (CiphergenExpress Client 3.0). Supervised hierarchical clustering was performed with CiphergenExpress 3.0. Peaks were picked with criteria of greater than ten times the signal-to-noise (SNR) on the first pass and five times on the second pass for cluster completion. Cluster bins were limited 0.3% of m/z. Samples were analyzed according to infection type and experiment day. Replicates were analyzed as such. Peak

clusters with $p < 0.001$ were used to perform principal components analysis. One-way hierarchical clustering of m/z was performed relative to the *Aspergillus* infection time-course.

All baseline-subtracted and normalized spectra were exported as raw data, were downsampled to 1000 spectral points within the optimized m/z range, and were aligned to a common set of m/z values with nearest-neighbor interpolation. The spectra were then log-median normalized and replicates were averaged. ANOVA was performed versus experiment day (0, 2–9) for the *Aspergillus* group and day (0–14) for the *Pseudomonas* group. The significant m/z values were picked at level $p < 1e^{-10}$.

Principal component analysis (PCA) was performed for the *Aspergillus* group at all time points (day 0, 2–9) and *Pseudomonas* group at all time points (day 0–14). PCA reports a summary vector describing the mass spectral data in n-dimensional space. Typically the first three principal components account for the greatest separation of the data sets and results are projected in two-dimensional space for PC₁ versus PC₂ and PC₁ versus PC₃. The advantage is that n-groups can be compared in n-dimensional space. We performed a four-group PCA to address early changes in a group by analyzing *Aspergillus* day 0–2 (AspEarly) versus *Pseudomonas* day 0–2 (PseudoEarly) versus *Aspergillus* day 7–9 (AspLate) versus *Pseudomonas* day 7–14 (PseudoLate). All PCA were performed with JMP 5.1.1 (SAS Institute, Inc., Cary, N.C.).

A random forest (RF) analysis was used to classify the infectious etiologies of *Aspergillus* versus *Pseudomonas* pneumonia using the program R 2.1 [15]. RF is a classification method with internal cross-validation. This method takes a random subset of input variables (e.g. intensities at given m/z for mass spectroscopy data) from a subset of cases (e.g. plasma samples from rabbits) to determine to which class (e.g. *Aspergillus* versus *Pseudomonas*) each case belongs. Cases were defined by the average spectral response of each rabbit: *Aspergillus* early (day 0–2), *Pseudomonas* early (day 0–2), *Aspergillus* late (day 7–9), and *Pseudomonas* late (day 7–14). We explored the differences at early times and late times.

Data analysis of BAL with SELDI-TOF

All spectra underwent baseline subtraction and normalization to total ion current in the range 3–200 kDa. The spectra were exported as raw data and aligned with linear interpolation. Sample replicates were represented by the mean response. The averaged spectra were then log-transformed and re-normalized. PCA of the entire transformed spectra was performed at the group level comparing normal, neutropenic, *Pseudomonas* and *Aspergillus*. An un-paired Student's t-test was performed with MATLAB 6.5 (The MathWorks, Inc., Natick, Mass.) at every spectral point to determine significant differences ($p < 0.001$) between either *Aspergillus* and *Pseudomonas* groups, and *Aspergillus* and normal groups.

Protein identification and validation

1D SDS-Page gel and LC/MS—Rabbit plasma samples were run on a NuPage 4–12% Bis-Tris gel (Invitrogen, USA) in MES buffer for protein separation according to manufacture's protocol. The bands of interest were manually excised for protein digestion using modified trypsin (Promega, USA). Mass spectra were acquired using data dependent MS/MS with top three ions. After LC/MS/MS, the peptide ion list was generated with Biowork 3.0 and submitted to MASCOT (www.matrixscience.com) to search for matched proteins with S-PROT database with taxonomy set to "other mammalia". Only those hits matched to the rabbit sequence with significant Mowse Score were selected as possible protein identification.

Reverse Phase Array—Plasma samples were prepared for RPA and printed onto nitrocellulose-coated “Oncyte” slides (Grace Bio-Lab Inc, Bend, USA) using an Aushon 2470 solid-pin arrayer (Aushon Biosystems Inc, Billerica, MA). Primary antibodies; anti-rabbit haptoglobulin (HPT) (chicken pAb, ICLlab, Newburg, USA), anti-rabbit C-reactive protein (CRP) (chicken pAb, ICLlab), anti-transferrin (TTFY) (sheep anti-human TTFY pAb, Abcam, Cambridge, USA) and anti-rabbit apolipoprotein A1 (ApoA1) (chicken pAb, kindly provided by Lita Freeman NHLBI/NIH, Bethesda, USA) were prepared and incubated overnight on separate arrays at 4°C. A HRP-conjugated goat anti-chicken secondary antibody and an AlexaFluor 647 conjugated rabbit anti-HRP antibody solution was applied to the HPT and ApoA1 arrays. To detect the HRP on the CRP and TTFY arrays, fluorescein-conjugated tyramide, FITC-TSA (Perkin-Elmer, USA) and Cy5-conjugated mouse anti-FITC solution were applied followed by AlexaFluor 647 goat anti-mouse. Slides were scanned using a Fuji 8000 laser scanner and spots were quantified using MicroVigene (VigeneTech, Carlisle, USA). The final data were presented as relative concentrations with arbitrary units.

Statistical analysis of Galactomannan assay and Reverse Phase Array

Plasma GMI results were analyzed via a two-factor ANOVA for the interaction of infection type and experiment day. GMI in BAL fluid was analyzed by ANOVA for group (Normal, Neutropenic, *Pseudomonas*, *Aspergillus*). Least squares regression was used to characterize the time course of APOA1, HPT, TTFY and CRP.

RESULTS

Galactomannan assay in plasma and BAL fluid

Plasma and BAL GMI is a validated surrogate marker for organism burden and the course of IPA. Plasma GMI analyzed by two-factor ANOVA versus infection type and experiment day showed significant differences starting at experiment day 3 (*Aspergillus* baseline, 0.202 ± 0.055 vs day 3, 2.06 ± 1.371 GMI, $P < 0.05$, complete time course Figure 1 Supplement). The pairwise post hoc least squares mean was also significant from experiment day 3 ($p < 0.05$) through 9 ($p < 0.0001$). GMI in BAL fluid was not different among normal rabbits (0.25 ± 0.03 sem), neutropenic rabbits (0.29 ± 0.09) and rabbits with *Pseudomonas* 0.22 ± 0.09 , however GMI was significantly higher in rabbits with *Aspergillus* (6.21 ± 0.07) ($p < 0.001$).

Hierarchical clustering analysis of plasma response

Using hierarchical clustering analysis of protein peaks in the plasma samples, we explored the time course of *Aspergillus* and *Pseudomonas* infections (Figure 1). Hierarchical clustering analysis of *Aspergillus* showed two clusters of peaks that were differentially regulated. A set of 57 total peaks met our selection criteria ($S/N > 10$, m/z width 0.3%, $p < 0.001$). A cluster of 21 protein peaks were of high intensity at baseline and monotonically decreased by day 3. Protein peaks (14) of low intensity at baseline increased by day 3 and remained elevated. The remaining 22 peaks were variably regulated relative to time. Hierarchical cluster analysis of the *Pseudomonas* pneumonia time course revealed 34 total peaks with differential regulation ($S/N > 10$, m/z width 0.3%, $p < 0.001$). Of these, 10 peaks were elevated at baseline and decreased gradually by day 6. Fifteen peaks were of low intensity at baseline and increased between days 3 and 6 and nine peaks were variably regulated relative to time.

Principal component and random forest analysis of plasma

The PCA of *Aspergillus* and *Pseudomonas* groups at all time points compared to baseline responses showed separation of *Aspergillus* from *Pseudomonas* and baseline along PC₁ (Figure 2A). The *Aspergillus* group demonstrates a time-dependent response with separation along PC₁ between early and late responses (Figure 2B). Early and late protein responses in *Pseudomonas* pneumonia, however, were not well separated along PC₁ or PC₂ (Figure 2B).

Focusing on the late infection period, we performed a random forest analysis to rank the top 30 spectral points that can distinguish between *Aspergillus* and *Pseudomonas* groups and found absolute separation along dimension 1 (Figure 2C). This model has a 100% sensitivity and specificity for distinguishing the two groups (Figure 2D). The list of markers by decreasing importance is shown in Figure 2E.

Comparison of spectral regions in BAL

Protein markers were explored in the BAL fluid. Figure 3 demonstrates the heatmap response of the entire transformed spectra for all BAL fluid analyzed. The sources included normal rabbits, those with neutropenia, light *Pseudomonas* infection and *Pseudomonas* or *Aspergillus* pneumonia. There are qualitative differences in protein expression between the groups, with 10 and 11.7 kDa being relatively higher in the non-infected groups and elevation at 17 kDa in the *Pseudomonas* and *Aspergillus* groups. The PC₁ was uniformly -20 for the uninfected groups, intermediate at 0 for the *Pseudomonas* group and +15 for the *Aspergillus* group (Figure 3B). The entire transformed spectra from BAL fluid in the *Pseudomonas* and *Aspergillus* groups were averaged for each infection type. These averaged spectra were compared at every spectral point using a Student's t-test. Figure 3C shows 20 spectral regions (blue) with significant differences ($p < 0.001$ Student's t-test). A similar analysis examined the plasma response averaged over all animals in days 4 to 7 for each infection group (*Aspergillus* versus *Pseudomonas*) and compared at each spectral point. (Figure 2C, Supplement). Significant protein peaks that were differentially expressed in BAL and plasma of rabbits with invasive pulmonary aspergillosis are summarized in Table 1 Supplement.

Identification of major differentially expressed proteins (see on-line supplement results)

Analysis of the SELDI-TOF data by random forest comparing late *Aspergillus* and late *Pseudomonas* samples revealed the significant m/z values to be clustered around 28kDa and 13.6kDa. Heat map and PCA analysis of both plasma and BAL discovered qualitative differences in protein expression on 9.6, 11.7 and 17kDa peaks. To identify the proteins with 11.7kDa and 13.6kDa peaks, representative from *Aspergillus* and *Pseudomonas* plasma samples were run on 1D SDS Page selected for protein separation based on their SELDI-TOF data. The image of 1D gel of these two samples is shown in Figure 2, supplementary results with subsequent identification of tryptic digests by LCQ-LC/MS/MS (see Table 2 supplement). The results of this analysis suggest that the 11.7kDa peak is HPT, the 13.7kDa peak is TTHY and the 28kDa peak is apolipoprotein A1 (APOA1).

Trends noted in the degree of temporal change observed with the SELDI – based spectral analysis (Figures 4A–H) were quantified using antibody-based immunoassays for APOA1, HPT and TTHY. A complementary assay of another acute phase reactant, CRP (present within the 23 kDa band Figure 1 supplement) was additionally performed. Using a least squares regression of the changes in levels of the acute phase reactants, the rate of decline of APOA1 was significantly greater in rabbits with IPA vs *Pseudomonas* pneumonia (Figure 4A and B, $p < 0.001$). The rate of rise of HPT was significantly greater in rabbits with IPA compared to *Pseudomonas* pneumonia (Figure 4C and D, $p < 0.001$). Smaller differences were noted in the decrement of TTHY in animals with IPA vs *Pseudomonas* pneumonia

(Figure 9E and F, $p < 0.038$). The time course of rise in CRP was significantly greater in animals with IPA than *Pseudomonas* pneumonia (Figure 4G–H, $p < 0.001$).

DISCUSSION

The proteomic profiles of IPA and *Pseudomonas* pneumonia have potentially important diagnostic and pathophysiological implications for these life-threatening infections. We have shown that these proteomic profiles are distinct from the plasma proteomic background of a non-immunocompromised rabbit and the immunosuppression associated with a neutropenic animal. Our analytic approach was based on examining the spectra generated from neat plasma and concentrated BAL. SELDI-TOF mass spectroscopy methodology is based upon fractionation of proteins using different chip surface attractive properties [16]. SELDI-TOF profiles have been used to develop novel diagnostic protein signatures in cancer [17–20], graft-versus-host disease [21], infectious diseases [22–25], and pulmonary inflammation [14]. Our data shows that SELDI-TOF expression profiles can provide a semi-quantitative assessment of changes in protein expression over time following two distinct infections in an immunocompromised animal model. Using a principal component analysis of the entire transformed spectra, we were able to distinguish infected from non-infected animals and *Aspergillus* from *Pseudomonas* infection. These data analysis suggest that the spectrum contains information related to both the immune status (neutropenic versus non-neutropenic) and infectious etiology (*Aspergillus* versus *Pseudomonas*).

The proteomic profile for animals infected with IPA or *Pseudomonas* pneumonia reflects the expression of proteins in plasma and BAL fluid from both the host and pathogen. The early proteomic profile of IPA and *Pseudomonas* pneumonia did not distinguish between the two infections. As the infection evolved, the proteomic profile became distinctive for *Aspergillosis* and *Pseudomonas* infection. These profiles may reflect increasing host-specific responses to these pathogens or potentially pathogen-specific proteins. Our approach used strong anion exchange surface fractionation that biased the spectrum towards more abundant host proteins. The detection of galactomannan, a fungal wall constituent in plasma as early as day two- post inoculation of *Aspergillus* conidia (Figure 1 Supplement) as well as in the BAL, demonstrates that pathogen-related biomarkers are present in these fluids. However, the methods used in the current study did not succeed in identifying novel pathogen-associated proteins that have been described *in vitro* [26]. The use of additional fractionation methods may enhance the potential to detect some of these pathogen-associated molecules.

We analyzed some of the proteins that contributed to the unique SELDI-TOF spectra by fractionation of the plasma proteins with 1-D SDS Page gel. Three acute phase reactants; APOA1, HPT and TTHY were identified as being differentially expressed between the two infections.

APOA1 is a major component of high density lipoprotein that binds and neutralizes endotoxin and lipoteichoic acid [27–29]. Similarly, the hemolytic toxin, Asp-hemolysis isolated from *A. fumigatus* is inhibited by a related lipoprotein, APOB [30]. Lipid-free APOA1 has antioxidant, anti-inflammatory, and antithrombotic effects [31]. In models of sepsis, levels of APOA1 fall [32]. Administration of reconstituted high density lipoprotein which contains purified APOA1, neutralizes the effects of endotoxin in animals and humans and prevents lethality in models of sepsis [33]. In our immunosuppressed rabbit model, the decrements in plasma APOA1 were approximately two fold greater in IPA compared to *Pseudomonas* pneumonia (Figure 7). Supplementing APOA1 during IPA might serve as a novel means to ameliorate tissue injury from this invasive mycosis.

The virulence of *A. fumigatus* is related in part to the production of toxins such as Asp-hemolysin that produces hemolysis of erythrocytes and is cytotoxic to macrophages and endothelial cells [34]. The dramatic acute rise in HPT with IPA may relate in part to this hemolytic activity (Figure 8). While HPT has both anti-inflammatory and anti-bacterial properties [35–37], it also accelerates the clearance of free hemoglobin and thus prevents the use of hemoglobin-associated iron for microbial growth [35]. The HPT-hemoglobin complex undergoes macrophage endocytosis via CD 163 and the iron molecule catalytically released by heme oxygenase. Intracellular iron is either sequestered with ferritin or mobilized to an export protein (ferroprotein or MTP-1) [36]. Iron is a necessary nutrient for *Aspergillus* virulence factor production [34] and iron chelation strategies *in vitro* suggest a new means of treatment *Aspergillus* infections [38]. Thus, the acute rise in HPT during IPA may reflect both the scavenging of free hemoglobin and the sequestration of iron, an essential vital growth factor for *A. fumigatus*. Rabbits exposed to aerosols of *Aspergillus terreus* spores have acute increases in HPT levels and when given passive transfer of HPT rich plasma, hypoxemic responses were blunted suggesting a modulation of host pulmonary inflammatory response [39]. Further, HPT has immunomodulatory effects in part due to binding to CD11b and by inhibition of TH2 type cytokine responses. It shifts the balance of T cell cytokine responses in favor of TH1 immune responses suggesting a potential important role in the host response to fungal infections [37].

The rate of rise of CRP, a short pentraxin produced by the liver was strikingly greater in IPA compared to *Pseudomonas* pneumonia. The factors contributing to this rate of rise may be due in part to the acute organism burden from the inoculation of the fungal hyphae and magnitude of the host cytokine response inducing a greater hepatic acute phase response. The antimicrobial effects of CRP result from its activation of complement and its role as a bacterial opsonin. CRP promotes the phagocytosis of metabolically active conidia of *A. fumigatus* independent of complement activation [40]. Macrophages exposed to CRP increase production of reactive oxygen species, tissue factor, proinflammatory cytokines and adhesion molecules [41]. Endothelial cells exposed to CRP increase the release of chemokines (IL-8, MCP-1), upregulate adhesion molecule expression, and enhance monocyte adhesion [41, 42]. While elevated CRP levels were associated with specific IL-1 gene polymorphisms in patients with hematologic malignancies and IPA, CRP values were similar between patients with and without IPA [43].

The rate of decline of TTHY in the *Aspergillus* infected animals was less than that in the *Pseudomonas* infected rabbits. TTHY, a negative acute phase reactant made by the liver, transports retinol binding protein and thyroxine and has been associated with amyloid diseases [40]. While TTHY binds a variety of drugs and pollutants including plant flavonoids [40], significant interactions with fungal or bacterial elements have not been described.

Developing additional laboratory markers of IPA is essential as these markers could be useful for diagnosis, surveillance of at-risk patients, and for monitoring therapeutic efficacy [26]. The plasma galactomannan in this neutropenic model for IPA was significantly increased within the first three days, which is consistent with previous observations of the expression of galactomannan in IPA in neutropenic rabbit model systems and neutropenic patients with IPA [44]. Various PCR techniques for detecting *Aspergillus* species are being used on blood and BAL fluid [45, 46] however none of the PCR-based tests are yet standardized or validated for reference laboratory use. The SELDI platform is a semi-quantitative tool for discovery and for hypothesis generation. Its spectral patterns are rich with information that will vary with the method of fractionation used to simplify complex biologic fluids. However, as currently performed it is difficult to standardize sufficiently for routine clinical use. Future developments using new fractionation methods and capture

techniques may offer promise to improve the sensitivity, quantitation and standardization of this platform [47].

Proteomic profiles in patients with suspected IPA may provide important diagnostic information, as well as prognostic data based upon host response. In order to establish an experimental foundation for understanding the proteomic host-pathogen response in IPA, we analyzed differential protein expression with SELDI-TOF in plasma and BAL fluid from a well-characterized rabbit model of this medically important infection. Our results show that specific patterns of host protein expression distinguish *Aspergillus* pneumonia from *Pseudomonas* pneumonia in plasma as well as BAL fluid. Time-dependent patterns of protein expression were found in plasma that were associated with the development of IPA. The differential expression of the acute phase reactants APOA1, HPT, CRP and TTHY contribute to the differences between these two infectious etiologies. These data support the concept that patterns of proteins are expressed with different microbial infections and may provide a new approach to understanding host-pathogen interaction, detection, and prognostic stratification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BAL	bronchoalveolar lavage fluid
IPA	invasive pulmonary aspergillosis
APO1	apolipoprotein A1
TTHY	transthyretin
HPT	haptoglobin
CRP	C-reactive protein

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Figure 1. Hierarchical Clustering of Plasma Protein Peaks

SELDI-TOF spectra with automatic peak detection for peaks >10 times signal-to-noise ratio. Each column represents a single animal. Red denotes up-regulated or higher intensity peaks and green denotes down-regulated or lower intensity peaks. Only peaks with $p < 0.001$ are shown in each row. Rows are ordered by unsupervised hierarchical clustering. **A: Response to *Aspergillus fumigatus*.** The 21 peaks in the upper aspect are of high intensity at baseline and decrease by day 5. The 14 peaks in the lower aspect of the panel are of low intensity at baseline but peak by day 3. **B: Response to *Pseudomonas aeruginosa*.** The 10 peaks in the upper aspect are of high intensity at baseline and decrease by day 6. The 15 peaks in the lower aspect of the panel are of low intensity at baseline but peak between days 3 and 6.

Figure 2. PCA and Random Forest Analysis of Plasma

The PCA of plasma separates infection type and early from late infection. Each symbol represents the PCA of the entire spectrum for an animal with a distinct infection. **A:** *Aspergillus fumigatus* infection (red, mean across days 4–7) separates along PC1 (26.7%, $p < 0.001$) from *Pseudomonas aeruginosa* (blue, mean across day 4–7) and baseline (green, day 0) spectra. There is no significant separation along PC₁ or PC₂ of *Pseudomonas aeruginosa* from baseline. **B:** Late *Aspergillus fumigatus* infection (green, day 7–9) separates from early (red, day 0–2) *Aspergillus fumigatus* infection along PC₁. *Pseudomonas aeruginosa* early (blue, day 0–2) and late (grey, day 7–14) infection responses

do not separate from each other and appear similar to the early *Aspergillus fumigatus* response in PC₁–PC₂ space. Random forest analysis of late *Aspergillus* and *Pseudomonas* infections. **C.** Late *Aspergillus* (red, mean response of each animal days 7–9) and late *Pseudomonas* (blue, mean response of each animal days 7–14) groups are completely separated by random forest analysis along dimension 1. **D.** Sensitivity and specificity for predicting infectious type in this model are 100%. **E.** The protein peaks (m/z) are ranked in order of importance for separating *Aspergillus* from *Pseudomonas* late infections.

Figure 3. Heat Map and Principal Component Analysis of BAL Fluid

Entire transformed spectra for each animal ordered in columns by infection type. **A: Entire transformed spectra in BAL fluid.** Normal and neutropenic rabbit BAL fluid samples are compared to *Pseudomonas* and *Aspergillus*-infected BAL fluid. An additional group was discovered to have only light *Pseudomonas* growth in microbiologic evaluation and this correlated with a distinct proteomic profile. Qualitative differences in protein expression exist at 10, 11.7 and 17 kDa in the *Pseudomonas* and *Aspergillus* groups. **B:** The PC₁ was uniformly -20 for the uninfected groups, intermediate at 0 for *Pseudomonas* pneumonia and +15 for *Aspergillus* rabbits. **C:** Protein profiles were averaged across all animals with each

infection type. The averaged spectral responses were compared at every spectral point. Spectral regions with significant differences ($p < 0.001$ Student's t-test) are denoted in blue.

Figure 4. RPA validation of SELDI-TOF

The time course of the SELDI-TOF relative intensity compared to relative units of RPA are shown for the putative 28 kDa molecule, APOA1, 11.7 kDa molecule, HPT, and 13.7 kDa molecule TTHY as well as RPA measurement of C-reactive protein in *Aspergillus* infection (A, C, E, G) compared to *Pseudomonas* infection (B, D, F, H). Comparison of the time course of APOA1 (A and B), HPT (C and D), TTHY (E and F) and C-reactive protein expression (G and H) between the two infections were significantly different (APOA1, HPT and C-reactive protein all $p < 0.001$, TTHY, $p < 0.038$).

Table 1

Experimental design and number of samples evaluated

A.				
	Non-Neutropenic	Neutropenic		
Treatments	Non-Infected Controls N = 11	Non-Infected Controls N = 8	<i>Pseudomonas</i> Pneumonia N = 17	<i>Aspergillus</i> Pneumonia N = 30
Cytarabine ^a (days 1 to 5, 8, 9,13,14)		•	•	•
Methylprednisolone ^b (days 1 and 2)		•	•	•
Antibiotics ^c (start day 4, then daily)		•		•
Intratracheal <i>A.fumigatus</i> (instill on day 1)				•

B.			
	Neutropenic		
Plasma specimens	Non-infected Controls	<i>Pseudomonas</i> Pneumonia	<i>Aspergillus</i> Pneumonia
Analyzed for GMI		64 ^c	94 ^d
Analyzed by SELDI ^a	20 ^c	103 ^c	125 ^d
Analyzed by RPA ^b		100 ^c	122 ^d

c.				
	Non-Neutropenic	Neutropenic		
BAL specimens	Non-Infected Controls N = 11	Non-Infected Controls N = 8	<i>Pseudomonas</i> Pneumonia N = 17	<i>Aspergillus</i> Pneumonia N = 30
Analyzed for GMI	11	6	17	30
Analyzed by SELDI	11	7	10	21

^a Cytarabine (525 mg per m², days 1–5, 484 mg/m² days 8,9,13,14 IV, Pharmacia & Upjohn, Kalamazoo, Mich.),

^b Methylprednisolone (5 mg/kg IV, Abbott Laboratories, North Chicago, Ill.)

^c Ceftazidime (75 mg/kg IV twice daily; Glaxo, Inc., Research Triangle Park, N.C), gentamicin (5 mg/kg IV every other day; Elkins-Sinn, Inc., Cherry Hill, N.J) and vancomycin (15 mg/kg IV daily and 50 mg of vancomycin per liter of drinking water to prevent antibiotic-associated diarrhea due to *Clostridium spiroforme*; Abbott Laboratories, North Chicago, Ill.)

^a Samples obtained from all animals at baseline prior to treatments, then obtained during time course of neutropenia (n = 8), *Pseudomonas* (n = 17), and *Aspergillus* (n = 30) pneumonias

^b The number of samples analyzed at each time point varied: *Pseudomonas* – median 6 samples per time point (minimum 2, maximum 15) and *Aspergillus* – median 10 samples per time point (minimum 3, maximum 30)

^c time course: baseline, d1 – d14;

^d time course: baseline, d2 – d9

GMI – galactomannan index, RPA – reverse phase array