

Plasma Proteome Response to Severe Burn Injury Revealed by ¹⁸O-Labeled “Universal” Reference-Based Quantitative Proteomics

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Received May 19, 2010

A burn injury represents one of the most severe forms of human trauma and is responsible for significant mortality worldwide. Here, we present the first quantitative proteomics investigation of the blood plasma proteome response to severe burn injury by comparing the plasma protein concentrations of 10 healthy control subjects with those of 15 severe burn patients at two time-points following the injury. The overall analytical strategy for this work integrated immunoaffinity depletion of the 12 most abundant plasma proteins with cysteinyl-peptide enrichment-based fractionation prior to LC–MS analyses of individual patient samples. Incorporation of an ¹⁸O-labeled “universal” reference among the sample sets enabled precise relative quantification across samples. In total, 313 plasma proteins confidently identified with two or more unique peptides were quantified. Following statistical analysis, 110 proteins exhibited significant abundance changes in response to the burn injury. The observed changes in protein concentrations suggest significant inflammatory and hypermetabolic response to the injury, which is supported by the fact that many of the identified proteins are associated with acute phase response signaling, the complement system, and coagulation system pathways. The regulation of ~35 proteins observed in this study is in agreement with previous results reported for inflammatory or burn response, but approximately 50 potentially novel proteins previously not known to be associated with burn response or inflammation are also found. Elucidating proteins involved in the response to severe burn injury may reveal novel targets for therapeutic interventions as well as potential predictive biomarkers for patient outcomes such as multiple organ failure.

Keywords: human plasma • quantitative proteomics • ¹⁸O labeling • LC–MS • burn • inflammation • “universal” reference

Introduction

Second only to motor vehicle accidents as the leading cause of accidental deaths in the United States, burn injuries result in nearly half a million patients requiring medical treatments

and nearly 4000 deaths annually in the United States. Severe burn injury is one of the most devastating forms of trauma that affects the functions of nearly every organ system in the body by causing serious tissue damage, fluid loss, and overwhelming systemic metabolic and inflammatory responses.^{1–3} The pathophysiological response induced by severe burn injury has a marked inflammatory component stemming from the release of a wide range of inflammatory mediators that subsequently contribute to the development of a systemic inflammatory response syndrome (SIRS), immune dysfunction, and multiple organ failure (MOF).^{4,5} Despite recent advances in burn treatment and management, the complex interactions that occur during SIRS, as well as the mechanisms that lead to MOF have not been fully characterized.^{5–7} Moreover, current methods for

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[¶] Additional participating investigators in the Large Scale Collaborative Research Program entitled *Inflammation and the Host Response to Injury* are listed in the Acknowledgments.

predicting the likelihood of mortality are unreliable and nonindividualized.^{6–8}

Recently, there has been an increasing interest in applying high throughput genomics and proteomics approaches in large-scale studies of complex human diseases with the aims of elucidating the underlying signaling pathways of the diseases and discovering novel genes or proteins as predictors of disease outcomes and as new therapeutic targets.^{9–14} For example, several recent studies reported the application of genome-wide expression analyses to circulating blood leukocytes and tissue samples derived from trauma patients to gain some insight into the pathways that underlie systemic inflammation in humans.^{9,15,16} Proteomics technologies offer the advantages of directly measuring protein abundances, including cell-depleted biofluids such as blood plasma. In clinical research, plasma proteomics has become one of the most rapidly emerging fields because blood plasma is an easily accessible noninvasive source and a reservoir for circulating proteins throughout the body.^{17,18}

In this study, we report the first quantitative plasma proteome profiling in severe burn patients with the aim of identifying plasma proteins associated with the response to burn injury. Plasma samples were collected from 15 severely burned patients that had burns covering at least 20% of their body and 10 healthy subjects matched by ages and body weights. The application of a recently reported methodology that utilizes a stable isotope ¹⁸O-labeled “universal” reference¹⁹ in conjunction with immunoaffinity depletion, cysteinyl-peptide enrichment, and high resolution liquid chromatography–mass spectrometry (LC–MS) analyses enabled identification and relative quantification of 313 plasma proteins. The results reveal significant inflammatory and hypermetabolic responses to burn injury as many of the proteins that exhibited significant changes in abundance are associated with acute phase response signaling, complement, and coagulation system pathways. Moreover, nearly 50 proteins were revealed as novel burn-associated proteins after evaluating statistically significant protein abundance changes following injury. Importantly, this study provides a preliminary baseline of burn-responding plasma proteins for discovering novel biomarkers predictive of injury outcomes and targets for therapeutic interventions.

Experimental Section

Human Plasma Patient Samples. Human blood plasma samples from 10 healthy individuals and 15 burn patients were used in this study, which was approved by the Institutional Review Boards of the University of Texas Medical Branch (Galveston, TX), Loyola University Medical College (Chicago, IL), University of Texas Southwestern (Dallas, TX), University of Washington (Seattle, WA), Pacific Northwest National Laboratory (Richland, WA), Massachusetts General Hospital (Boston, MA), and University of Florida College of Medicine (Gainesville, FL) in accordance with federal regulations. All burn patients were admitted within 96 h after injury to the participating hospitals and had burns that covered more than 20% of their total body surface area that required at least one surgical intervention. Blood plasma samples collected at two different time-points after hospital admission consisted of early time-point plasma (T1), which was collected shortly after admission and late time-point plasma (T2), which was collected at the peak of the first MOF episode. A brief summary of patient demographics is provided in Table 1.

All plasma samples were supplied by the Department of Surgery at the University of Florida, College of Medicine, which

Table 1. Patient Demographics^a

	control	burn
	(n = 10)	(n = 15)
Age (yrs)	38 ± 4	45 ± 4
Weight (kg)	87 ± 17	80 ± 19
Gender (F/M)	3/7	2/13
Total body surface area burned (%)		54 ± 20
third degree burn (%)		30 ± 15
Time since injury (day)		T1, 4.1 ± 3.5; T2, 20 ± 11

^a Data are presented at average ± standard deviation. T1 indicates the early time-point and T2 the late time-point.

served as the sample collection and coordination site for this multicentered clinical study. A total of 300 μ L of plasma per healthy subject or per time-point for burn subjects was applied toward proteomics analysis. Initial protein concentrations in the plasma samples were determined by a BCA Protein Assay (Pierce, Rockford, IL). A reference sample was generated by pooling 100 μ L aliquots of plasma obtained from each of the 10 healthy controls and from each time for the 15 burn patients. Unless otherwise noted, protein sample processing was performed at 4 °C.

Immunoaffinity Depletion. Each patient plasma sample and the pooled reference sample were depleted individually of 12 abundant blood plasma proteins—albumin, IgG, α 1-antitrypsin, IgA, IgM, transferrin, haptoglobin, α 1-acid glycoprotein, α 2-macroglobulin, apolipoprotein A-I, apolipoprotein A-II, and fibrinogen—in a single step. Depletion was accomplished using a prepacked Seppro IgY12 LC-5 affinity column (GenWay Biotech, San Diego, CA) with loading capacity of 65 μ L human plasma and an Agilent 1200 series HPLC system (Agilent, Palo Alto, CA) per the manufacturer’s instructions.²⁰ For each patient, ~130 μ L of plasma was depleted, and only the flow-through fractions were collected and saved as samples. For the universal reference sample, ~3.0 mL of reference plasma sample was depleted (using the same procedure), but in this case, the flow-through fractions were pooled. Each flow-through portion was individually concentrated in iCON concentrators with 9 kDa molecular weight cutoffs (Pierce), followed by buffer exchange with 50 mM NH_4HCO_3 per the manufacturer’s instructions. Protein concentration was measured using a BCA protein assay (Pierce).

Plasma Protein Digestion. Protein samples were denatured and reduced in 50 mM NH_4HCO_3 buffer (pH 8.2), 8 M urea, and 10 mM dithiothreitol (DTT) for 1 h at 37 °C. The resulting protein mixture was diluted 10-fold with 50 mM NH_4HCO_3 before sequencing grade modified porcine trypsin (Promega, Madison, WI) was added at a trypsin:protein ratio of 1:50 (w/w). The sample was incubated for 5 h at 37 °C. Each digested sample was loaded onto a 1-mL SPE C18 column (Supelco, Bellefonte, PA) and washed with 4 mL of 0.1% trifluoroacetic acid (TFA)/5% acetonitrile (ACN). Peptides were eluted from the SPE column with 1 mL of 0.1% TFA/80% ACN and then lyophilized. Resulting peptide samples were reconstituted in 25 mM NH_4HCO_3 and the residual trypsin activity was quenched by boiling the samples for 10 min and immediately placing the samples on ice for 30 min. The resulting peptide concentration in each sample was measured using a BCA protein assay (Pierce).

Trypsin-Catalyzed ¹⁸O Labeling of the Reference Sample. ¹⁸O-labeling for preparing the reference sample was carried out using a previously described procedure.²¹ Briefly, the peptide

sample was lyophilized to dryness and initially reconstituted in 60 μL of acetonitrile, followed by the addition of 600 μL of 50 mM NH_4HCO_3 in ^{18}O -enriched water (95%; ISOTEC, Miamisburg, OH). Then, 6 μL of 1 M CaCl_2 and 30 μL of immobilized trypsin (Applied Biosystems, Foster City, CA) were added to the digest and the sample was mixed continuously for 5 h at 30 $^\circ\text{C}$. After labeling, the sample was acidified by adding 6 μL of formic acid, and the supernatant was collected after centrifuging the samples for 5 min at 15 000 $\times$ g. The labeled sample was lyophilized and reconstituted in 25 mM NH_4HCO_3 ; the peptide concentration was measured using a BCA Protein assay (Pierce). The labeled reference sample was divided into 40 identical aliquots and placed in 40 individual tubes, after which an equal amount of peptides from each patient sample was added to each tube to form 40 patient/reference mixed samples (i.e., 10 samples for healthy subjects, and 30 samples for the 15 burn patients with two time-points per patient).

Cysteinyl-Peptide Enrichment. The 40 patient/reference samples were fractionated into cysteinyl (Cys)- and non-Cys-peptide fractions by following a previously described procedure.^{21,22} Briefly, the tryptic digest was reduced with 5 mM DTT in Tris buffer, and Cys-peptides were captured by Thiopropyl Sepharose 6B thiol-affinity resin (4 $\times$ 100 μL ; Amersham Biosciences) following incubation of the reduced peptides with the resin. The non-Cys-peptide supernatant portion was collected. The resin was washed to remove any nonspecifically bound peptides. The captured Cys-peptides were released by incubation with 20 mM DTT for 30 min at room temperature, and the released peptides were then alkylated with 80 mM iodoacetamide. Both the eluted Cys-peptide and the unbound, non-Cys-peptide samples were desalted using a SPE C18 column and lyophilized. Both fractions were reconstituted in 25 mM NH_4HCO_3 and peptide concentrations were measured using a BCA Protein assay (Pierce).

Capillary LC–MS Analyses. Both Cys- and non-Cys-peptide samples from individual burn patients were analyzed using a fully automated custom-built two-column capillary LC system²³ that was coupled online via an in-house-manufactured electrospray ionization interface to an 11.5 T Fourier transform ion cyclotron resonance (FTICR) mass spectrometer. The capillary columns were prepared by slurry packing 5 μm Jupiter C₁₈ bonded particles (Phenomenex, Torrance, CA) into a 65-cm long, 150- μm i.d. fused-silica capillary (Polymicro Technologies, Phoenix, AZ). Mobile phases consisted of 0.2% acetic acid and 0.05% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in 90% acetonitrile/10% water (B). Ten μL aliquots of each peptide sample at concentrations of 0.1 $\mu\text{g}/\mu\text{L}$ were injected onto the reversed-phase column for LC–FTICR analysis. The mobile phase was maintained at 100% A for 20 min and then switched to an exponential gradient elution generated by increasing the mobile-phase composition to $\sim$ 70% B over 150 min. The LC–FTICR was configured and operated as described elsewhere.²⁴ Samples were analyzed in randomized order on the two columns. To assess analytical reproducibility, all samples were analyzed on the same instrument three months later.

LC–MS Data Analysis. LC–MS data analysis was similar to that previously described.¹⁹ Initially, LC–FTICR data sets were automatically analyzed using an in-house-developed software package that included Decon2LS and VIPER informatics software tools.^{25,26} Initial analysis of the raw LC–MS data involved the use of Decon2LS to perform a mass transformation or deisotoping step, which generated a text file report for each

LC–MS data set that for each mass spectrum included both the monoisotopic masses and the corresponding intensities for all detected species. Each data set was then processed by using the feature-matching tool VIPER to identify and quantify peptides, and then display the data in a two-dimensional mass and LC-elution time format. The feature matching process included “distinct feature” (i.e., a peak with unique mass and elution time) finding, searching for $^{16}\text{O}/^{18}\text{O}$ feature pairs, computing abundance ratios for pairs of features, an intensity report for all detected features, normalizing LC elution times via alignment to a database, and feature identification.

Feature identification was performed by matching the accurately measured masses and normalized LC elution time (NET) values of each detected feature to a pre-established human plasma proteome accurate mass and elution time (AMT) tag database. The plasma AMT tag databases were generated from the combined results of several comprehensive LC–MS/MS profiling investigations,²⁶ including a recent profiling of the human trauma patient plasma proteome.²² Note that the human IPI protein database (February 7, 2008, V3.39) was used for MS/MS peptide identification in these earlier investigations. The process of generating a AMT tag database and the criteria for peptide inclusion in the database have been described previously in detail²⁶ and the AMT tag database essentially serves as a “look-up” table for LC–MS feature identifications. The overall false discovery rate of the peptide identifications included in the AMT tag database is <5% based on a reversed database approach.²⁷ A dynamic modification of 4.0085 Da was applied during the peak (or feature) matching process to enable both unlabeled peptides and ^{18}O -labeled peptides to be identified simultaneously. The peptide sequences of a given feature or pair of features were assigned when the measured mass and NET for each given feature matched the calculated mass and NET of a peptide in the AMT tag database within a 4 ppm mass error and 2% NET error (the mass error tolerance was later refined to 2.5 ppm as the final cutoff). For each identified peptide, the relative $^{16}\text{O}/^{18}\text{O}$ abundance ratio was reported if the feature was paired. Details for computing the $^{16}\text{O}/^{18}\text{O}$ abundance ratios using VIPER are described elsewhere.²¹

The final identified peptide list was then used to generate a nonredundant protein group list using ProteinProphet²⁸ and all of the peptides were annotated based on protein group identification for downstream data normalization and for rolling-up peptide level abundance information to the protein level. An identified peptide was excluded for the purpose of calculating relative abundance ratios if the peptide was not observed in at least five data sets. Additionally, peptides were removed from downstream data analysis if they were shared by more than three protein groups.

Data Normalization and Statistical Analysis. Relative abundance $^{16}\text{O}/^{18}\text{O}$ data in each LC–MS data set were first transformed in Log_2 format and then normalized by centering the median Log_2Ratio to zero. Following data normalization, a rescaling procedure for peptide profiles across data sets was performed for each protein. In this procedure, all peptide profiles that originate from the same protein are scaled to the same level by using a simple scaling factor for each peptide obtained relative to a chosen reference peptide for the given protein. The peptide with the most observations serves as the reference peptide for a given protein and the scaling factor for every other peptide is calculated as the median ratio of the common data points between the peptide to be scaled and the

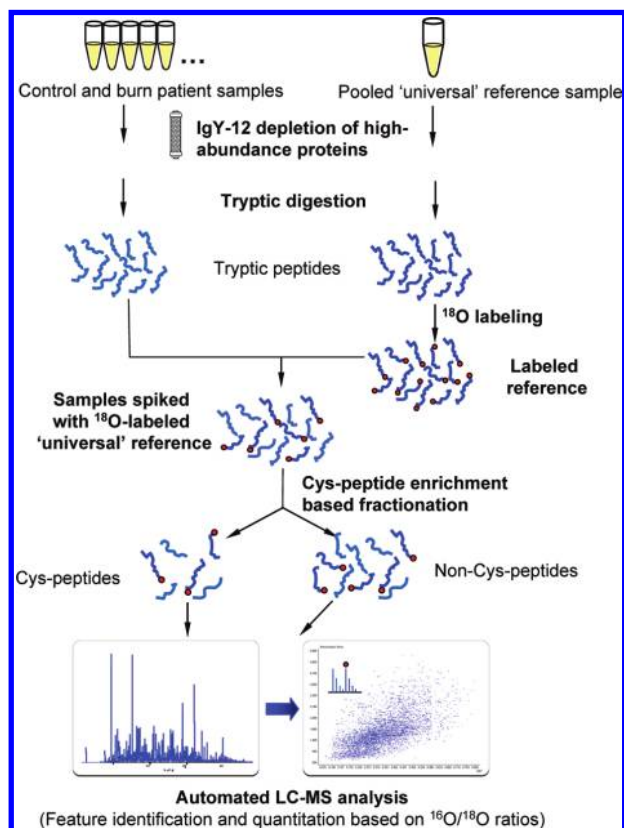


Figure 1. Flowchart depicting the overall experimental strategy. Control and burn patient plasma samples along with a pooled reference sample are subjected to initial IgY12 depletion followed by trypsin digestion. The reference digest is labeled with ^{18}O , and then an equal amount of labeled reference is mixed with each patient sample to generate individual samples spiked with the ^{18}O reference. The spiked samples are further fractionated using a cysteinyl-peptide enrichment strategy prior to LC–MS analysis.

reference peptide.¹⁹ Relative protein abundance ratios were calculated by averaging rescaled relative peptide abundance ratios for each data set. A statistical analysis of variance (ANOVA) test was applied to identify proteins with significant abundance differences across the control (healthy) subjects and the burn subjects at two different time-points postburn injury. Proteins with significant abundance changes across the three biological groups were identified by requiring an ANOVA *p*-value of <0.05 and a corresponding *q*-value of <0.1 . The *q*-value is an analog of the *p*-value and incorporates both a multiple testing correction and a posterior error probability, which corresponds to the local false discovery rate (FDR), that is, the probability that a given observation is drawn from the null distribution.²⁹ All data normalization, rescaling, and statistical analysis procedures were performed using DANte, a software tool developed for the quantitative analysis of “omics” data.³⁰

Results

Overall Analytical Strategy. Figure 1 depicts the overall analytical strategy for quantitative analysis of individual plasma samples. A recently reported ^{18}O -labeled universal reference-based quantitative methodology that employs inclusion of the same stable isotope labeled internal standards for all samples was applied to achieve robust quantitative measurements. As

a pooled sample, the ^{18}O -labeled universal reference sample contained all proteins that when spiked into individual samples, served as internal standards for quantification. We integrated immunoaffinity depletion with Cys-peptide enrichment-based fractionation to enhance single dimensional LC–MS detection²² of low abundance plasma proteins. The initial 40 plasma samples were fractionated into 80 final Cys- and non-Cys-peptide fractions that were individually analyzed using the AMT tag strategy.²¹ Peptides were identified by matching peak information to information in a pre-established human plasma peptide database.²² Relative peptide and protein abundances were quantified based on the $^{16}\text{O}/^{18}\text{O}$ ratios for all detected $^{16}\text{O}/^{18}\text{O}$ peptide pairs. Note that identifications and abundance data for the Cys- and non-Cys-fractions for each original patient plasma sample were subsequently combined to improve proteome coverage.

Reproducibility of Quantification. To assess the reproducibility or reliability of this quantification strategy, we analyzed the final 40 non-Cys-peptide samples two times each and at least three months apart. The two data sets allowed us to compare $^{16}\text{O}/^{18}\text{O}$ peptide abundance ratios for the same samples, as well as the relative abundance profiles across the 40 individual samples. Figure 2A shows the correlation of $^{16}\text{O}/^{18}\text{O}$ peptide abundance ratios between replicate analyses of the same biological sample. Although the analyses were performed more than three months apart and used different LC columns, the peptide abundance ratios appear reproducible based on the Pearson correlation coefficient of 0.92. The histogram in Figure 2B, which plotted the coefficient of variance (CV) for all peptides commonly quantified between the two replicated analyses of the 40 samples, provided another example of the reproducibility of peptide abundance ratio measurements. As shown, the majority of peptides are quantified with $<20\%$ CV, which is based on peptides detected in technical replicates in each of the two analyses. Figure 2C and D further illustrated the reproducibility of protein abundance profiles across the 40 different samples; in this case, using two proteins as examples. Collectively, these data illustrated that the use of an ^{18}O -labeled reference for relative quantification can effectively cancel out LC–MS platform performance variation in a large-scale study.

Plasma Protein Identifications. Following peak matching of each LC–MS data set against a pre-established plasma AMT tag database and data filtering, 5245 unique peptides were identified that had quantitative $^{16}\text{O}/^{18}\text{O}$ peptide abundance ratios measured across different data sets. The FDR at the unique peptide level was estimated to be 4.5%, which is comparable to those recently obtained using similar approaches.^{19,31} In total, 313 proteins were confidently identified with two or more unique peptides. The relative abundances for these quantified peptides and proteins in Log_2Ratio format against the reference across the 40 samples are listed in Supplemental Tables 1 and 2, respectively (Supporting Information). The observed coverage of 313 proteins with two or more peptide identifications is comparable to our previous results of 380 protein identifications obtained using SCX fractionation coupled to LC–MS/MS to analyze a single plasma sample.³² Although only single-dimensional LC–MS was applied in this study, the analyses of 40 different plasma samples improved the overall proteome coverage. Table 2 exemplifies low abundance proteins that were quantified with at least two unique peptides from different control and burn patient samples and that had concentrations in $\mu\text{g}/\text{mL}$ level based on previous literature reports under normal conditions.^{32–35} Most of these low

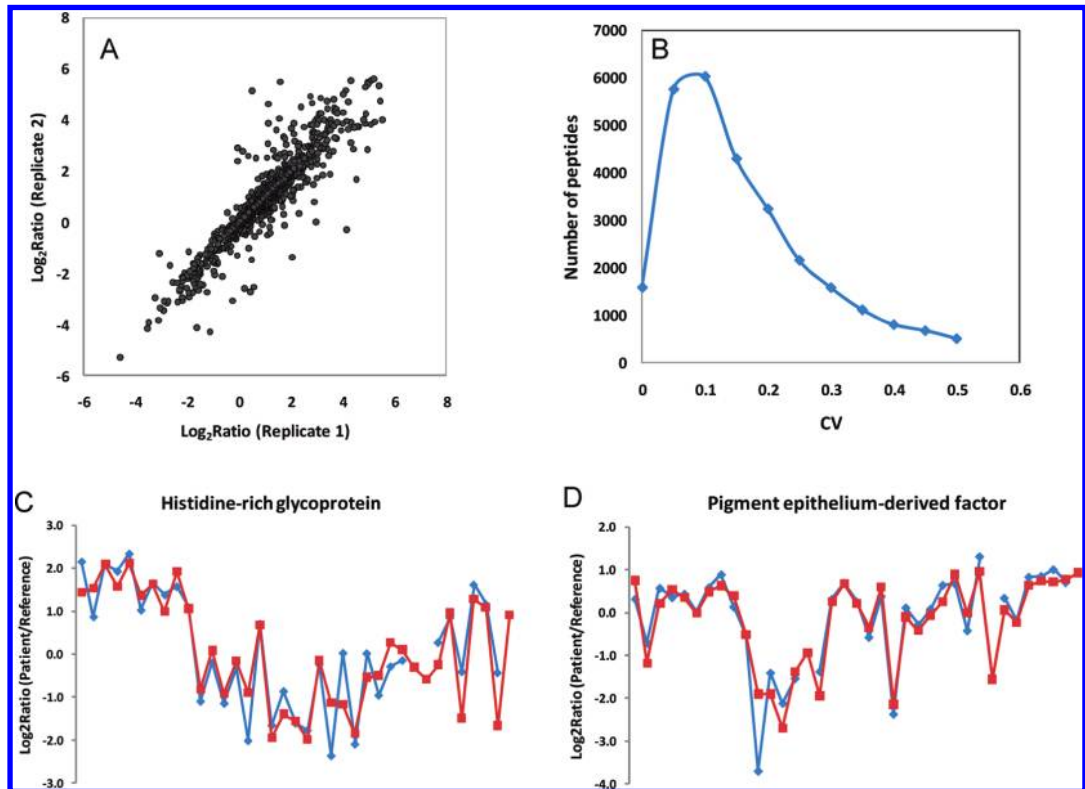


Figure 2. Reproducibility between replicate analyses performed more than three months apart. (A) Scatter plot for peptide Log₂Ratio data between the two replicates of a single sample. (B) Histogram of the distribution of coefficients of variation (CV) for all peptide Log₂Ratio data. CV values were calculated based on the standard deviations of the abundance ratio data between the two replicate analyses relative to the average abundance ratio for each peptide. (C and D) Protein abundance profile comparisons between the two replicate data for two selected proteins.

Table 2. Selected Low Abundance Plasma Proteins Obtained from Different Control and Burn Patient Samples and Quantified with at Least Two Unique Peptides

gene symbol	protein name	unique peptides	number of samples detected ^a	concentration (ng/mL) ^b
VCAM1	Vascular cell adhesion protein 1	7	35	483
MB	Myoglobin	6	40	90
IGF1	Insulin-like growth factor (IGF) 1	4	26	214
IGF2	IGF II	4	28	380
MMP9	Matrix metalloproteinase-9	2	26	189
CDH5	Cadherin-5	4	35	30
MST1	Hepatocyte growth factor-like protein	9	33	20
SELL	L-selectin	6	29	601
ALCAM	CD166 antigen	2	40	16
IGFBP2	IGF binding protein 2	5	32	305
MMP2	Matrix metalloproteinase-2	2	39	100
CTSD	Cathepsin D	3	28	9

^a Number indicates that the proteins are observed in the total number of samples among the original 40 plasma samples. ^b Protein concentrations were based previously literature reports from normal human subjects.^{32–35}

abundance proteins were detected in a portion of the original 40 samples, that suggested that their concentrations were below the limit of detection in some of the samples.

Plasma Proteome Response to Burn Injury. The quantitative protein abundance data for the 313 confidently identified proteins were subjected to ANOVA statistical analyses to reveal plasma proteins that exhibited significant abundance changes in response to severe burn injury. Figure 3 shows the *p*-value

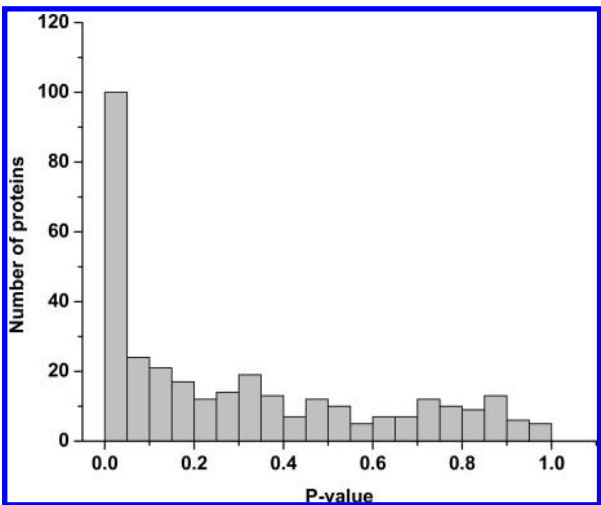


Figure 3. Histogram of *p*-value distribution. *p*-Values are based on the statistical ANOVA test for all quantified proteins across the three biological groups (control, burn time-point 1, burn time-point 2).

distributions for this set of proteins. Note that a significant proportion of the detected proteins were determined to have *p*-values of ≤ 0.05 . By requiring a *p*-value of < 0.05 and a corresponding *q*-value indicative of an FDR < 0.1 , 110 proteins were identified with statistically significant abundance changes across the three biological groups (control, burn time-point 1, and burn time-point 2). The quantitative data for these statistically significant proteins are listed in Supplemental Table 3 (Supporting Information). Among the 110 proteins, the

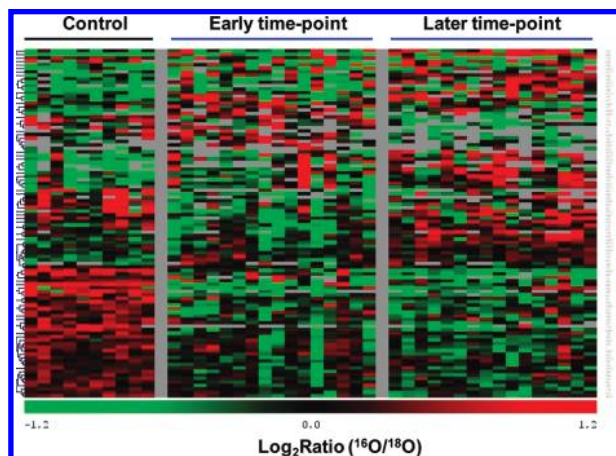


Figure 4. Heatmap of protein abundances across the 40 control and burn patient plasma samples. Only statistically significant proteins are displayed. Each row represents an individual protein, and each column represents a biological sample. All abundances are displayed in Log_2Ratio format relative to the labeled reference sample. Two blank columns were inserted to separate the three biological groups.

majority (~80%) of them are considered extracellular and plasma membrane proteins.

Figure 4 shows a heatmap of relative abundance profiles for the 110 proteins across the 40 plasma samples. All protein abundances are displayed in Log_2Ratio format, that is, protein abundances for each protein within a given sample are indicated as relative ratio values against the labeled reference. Note the large sample-to-sample variations. Also note that a good portion of proteins exhibit decreased concentrations in response to burn injury, which is apparent in the bottom set of proteins, while other proteins show increased abundances following injury. In Figure 5, the protein abundance changes in four example proteins further illustrate that abundance changes between burn and control samples are readily observed.

Inflammatory and Hypermetabolic Response Revealed by Burn-Responding Proteins. The 110 statistically different proteins were examined by applying the Ingenuity Pathway Analysis (IPA) tool to uncover biological pathways or functional processes associated with these burn-responding proteins. Figure 6A shows the top five canonical pathways mapped by these proteins. Activation of the acute phase response signaling pathway and the complement and coagulation systems suggest a significant postburn inflammatory and hypermetabolic response.³⁶ Figure 6B shows a partial representation of the acute phase response signaling pathway in which all quantified proteins mapped to the pathway exhibit decreased concentrations following burn injury. This finding is in good agreement with existing knowledge about these negative acute phase proteins during the acute phase response. Table 3 lists 36 proteins that are known to be associated with either the inflammatory or immune response; the majority of these proteins are known as acute phase proteins. The induction of the acute phase response³⁷ is supported by the increased concentrations for positive acute phase proteins and the decreased concentrations for negative acute phase proteins at both the early and later time-points following burn injury.

The quantitative changes for several proteins (i.e., C-reactive protein, plasma retinol binding protein, and transthyretin) are in good agreement with postburn changes of these proteins obtained using orthogonal nephelometry and ELISA tech-

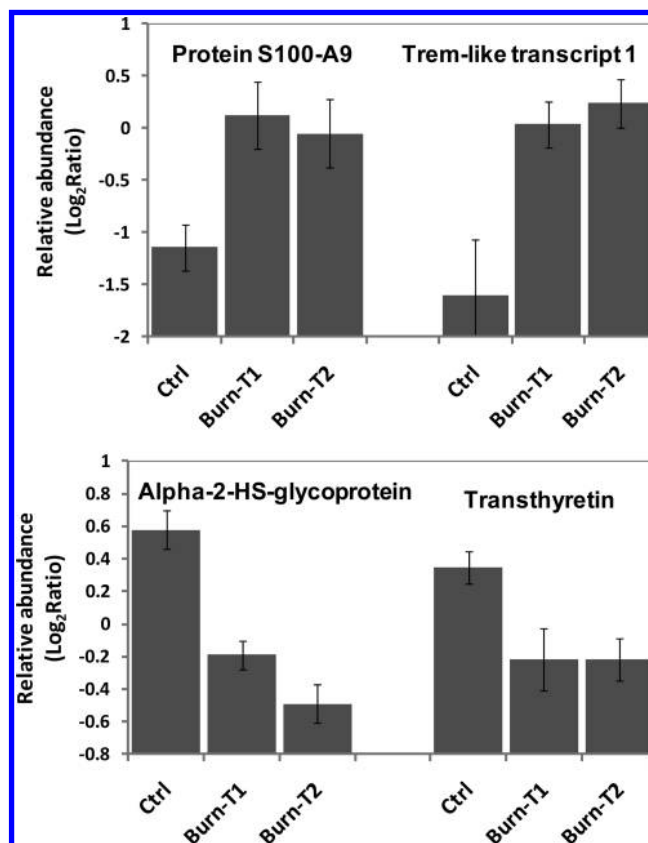


Figure 5. Protein abundance changes for selected proteins in bar graphs. Standard deviations were shown on each of the bars. Burn-T1 indicates sample collection at hospital admission and Burn-T2 indicates sample collection at a peak MOF episode. The differences between controls and both burn time-points are significant for all proteins ($p < 0.02$).

niques.³⁸ The overall good agreement between our quantitative proteomics data and the existing knowledge of the acute phase response confirms the robustness of our quantitative strategy for large-scale clinical studies and the confidence of this data set. Only complement C4-A shows an apparent disagreement of observed changes with reports that complement C4-A is a positive acute phase protein. The discrepancy could be due to the multiple isoforms that exist for complement proteins.

A number of proteins play a role in the immune response in addition to the acute phase response. For example, CD44, beta-2 microglobulin, HSPA5 protein, SPARC, and alpha-enolase have all been observed to be regulated by lipopolysaccharide (LPS) with directional changes in good agreement with the current data set.^{39–41} Additionally, vascular cell adhesion molecule 1 (VCAM1), protein S100-A9, TREML1, mannose-binding protein (MBL2), heat shock protein (HSPA5) have all been reported as pro-inflammatory mediators associated with sepsis and SIRS.⁴² The observed down-regulation of plasminogen, plasma prekallikrein, and kininogen-1 also supports increased proteolytic production of plasmin, kallikrein, and bradykinin, all of which are critical enzymes during inflammatory response to burn injury.^{37,43}

Among the 110 significant proteins are many proteins that have not been reported with clear implications in burn injury. These potentially novel proteins associated with burn injury are listed in Table 4. Some of these proteins have been implicated in immune response, inflammation, cell adhesion

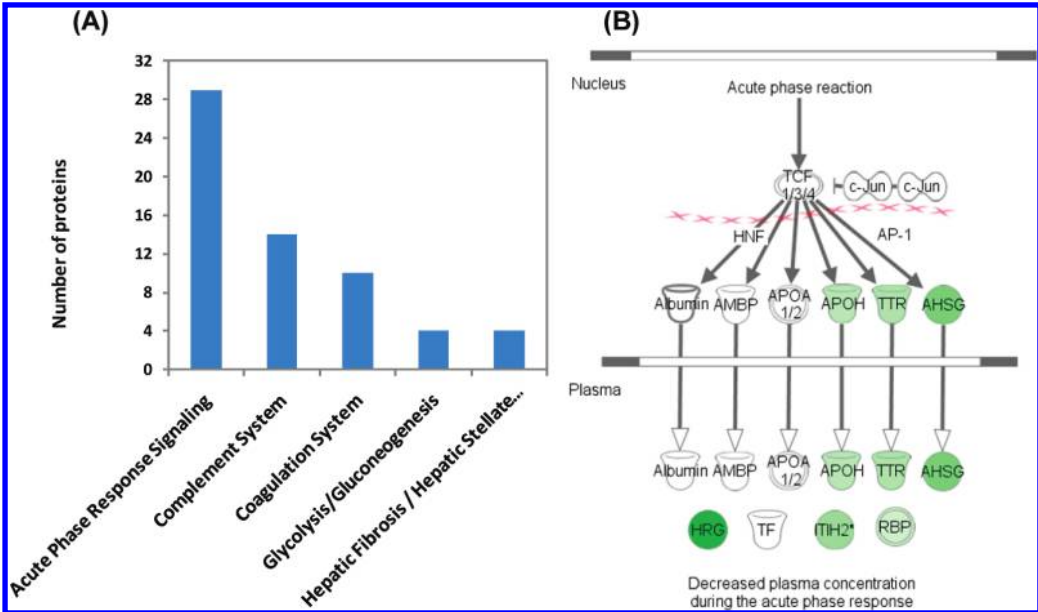


Figure 6. Pathways mapped by proteins with significant abundance changes. (A) Top 5 canonical pathways based on the number of proteins mapped. (B) Partial representation of acute phase signaling pathways. The green color of mapped proteins indicates a decreased concentration observed following burn injury.

Table 3. List of Burn-Responsive Proteins Previously Known to Be Associated with Either Inflammatory or Burn Responses

gene symbol	protein name	functions	burn-T1/control (Log ₂ Ratio)	burn-T2/control (Log ₂ Ratio)	up or down regulation
TREML1	Trem-like transcript 1 protein	Activated by platelet activation	1.6	1.9	UP
S100A9	Protein S100-A9	Immune response	1.3	1.0	UP
VCAM1	Vascular cell adhesion molecule 1	Inflammation marker	0.8	0.3	UP
SERPINA3	Alpha-1-antichymotrypsin	Positive acute phase protein	0.4	0.6	UP
SERPINA2	Alpha-1-antitrypsin-related protein	Positive acute phase protein	0.8	1.2	UP
MBL2	Mannose-binding protein C	Positive acute phase protein	0.6	-0.1	UP
VWF	von Willebrand factor	Positive acute phase protein	0.5	0.2	UP
CD44	CD44 antigen	Immune response	0.5	1.5	UP
B2M	Beta-2-microglobulin	Immune response	0.5	1.1	UP
FGG	Fibrinogen gamma chain	Positive acute phase protein,	0.5	1.0	UP
FGB	Fibrinogen beta chain	Positive acute phase protein	0.3	0.6	UP
CRP	C-reactive protein	Positive acute phase protein	0.4	1.0	UP ^a
MASP2	Mannan-binding lectin serine protease 2	Positive acute phase protein	0.3	1.3	UP
HSPA5	HSPA5 protein	Immune response	0.3	0.8	UP
ENO1	Alpha-enolase	Inflammatory mediator ⁵⁵	0.3	0.9	UP
ALCAM	CD166 antigen	Inflammatory disorder	0.3	-0.5	UP
MASP1	mannan-binding lectin serine protease 1	Positive acute phase protein	0.3	-0.5	UP
C9	Complement component C9	Positive acute phase protein	0.0	0.7	UP
SERPING1	Plasma protease C1 inhibitor	Positive acute phase protein	0.2	0.7	UP
ITIH3	Interalpha-trypsin inhibitor heavy chain H3	Positive acute phase protein	0.5	0.4	UP
RBP4	Plasma retinol-binding protein	Negative acute phase protein	0.0	-0.3	DOWN ^a
SERPINC1	Antithrombin III	Inflammatory marker	-0.3	-0.5	DOWN
ITIH1	Interalpha-trypsin inhibitor heavy chain H1 precursor	Negative acute phase protein	-0.4	-0.5	DOWN
ITIH2	Interalpha-trypsin inhibitor heavy chain H2	Negative acute phase protein	-0.6	-0.8	DOWN
SPARC	SPARC	Inflammatory marker	-0.5	0.8	DOWN
TTR	Transthyretin	Negative acute phase protein	-0.5	-0.6	DOWN ^a
APOH	Beta-2-glycoprotein 1	Negative acute phase protein	-0.6	-0.5	DOWN
C4A;C4B ^b	Complement C4-A	Positive acute phase protein	-0.6	-0.3	DOWN
FN1	Isoform 1 of Fibronectin precursor	Negative acute phase protein	-0.7	-0.6	DOWN
AHSG	Alpha-2-HS-glycoprotein	Negative acute phase protein	-0.8	-1.0	DOWN
PLG	Plasminogen	Acute phase protein	-0.8	-0.6	DOWN
PON1	Serum paraoxonase/arylesterase 1	Inflammatory marker	-0.9	-1.1	DOWN
HRG	Histidine-rich glycoprotein	Negative acute phase protein	-2.0	-1.7	DOWN
KLKB1	Plasma prekallikrein	Acute phase response	-1.3	-1.1	DOWN
KNG1	Isoform HMW of Kininogen-1	Acute phase response	-0.5	-0.5	DOWN

^a Postburn response of these proteins was reported by Jeschke et al.³⁸ ^b The observed abundance changes oppose previously reported literature knowledge.

and movement, metabolic response, and antioxidant response as annotated in the table. Additionally, two proteins—leucine-rich alpha-2-glycoprotein (LRG1) and cadherin-5 (CDH5)—were observed in a previous plasma proteome survey²¹ as up-regulated in human volunteers following lipopolysaccharide stimulation.

Discussion

The pathophysiology of severe burn injury manifests the full spectrum of the complexity of inflammation and the host response to injury. For example, severe burn patients often have increased susceptibility to infection, SIRS, adult respiratory

Table 4. List of Potentially Novel Burn-Responding Proteins

gene symbol	protein name	burn-T1/control (Log ₂ Ratio)	burn-T2/control (Log ₂ Ratio)	potential functional implications
SIGLEC14	Sialic acid-binding Ig-like lectin 14	1.3	0.1	Immune response ⁴⁷
CARD11	caspase recruitment domain family	0.7	1.0	Immune response ⁴⁵
PPBP	Platelet basic protein	0.6	0.9	Immune response
RNASE1	Pancreatic ribonuclease	0.6	0.5	Immune response ⁴⁶
RNASE1	Pancreatic ribonuclease	0.6	0.5	Immune response ⁴⁶
GPLD1	Phosphatidylinositol-glycan-specific phospholipase D	−0.4	−1.3	Immune response
DBH	Dopamine beta-hydroxylase	−0.9	−0.2	Immune response
GSN	Gelsolin	−0.8	−0.8	Immune response
PPIA	Peptidyl-prolyl cis–trans isomerase A	−0.9	0.1	Immune response
CHL1	Neural cell adhesion molecule L1-like protein	0.3	−2.4	Immune response
NCAM1	Neural cell adhesion molecule 1	−0.6	−1.3	Immune response
MYO9B	Myosin-IXb	−0.9	−0.6	Immune response
LRG1	Leucine-rich alpha-2-glycoprotein	0.9	1.6	Inflammation, observed in previous LPS study. ²¹
CA1	Carbonic anhydrase 1	0.9	0.3	Potential inflammation mediator ⁴⁸
CA2	Carbonic anhydrase 2	−1.0	−1.7	Inflammation
HGFAC	Hepatocyte growth factor activator	−0.1	−1.3	Linked with acute phase response ⁴⁹
APOB	Apolipoprotein B-100	−0.2	−0.5	Linked with inflammation ⁵⁰
APOA4	apolipoprotein A-IV precursor	−0.6	−0.7	Linked with inflammation
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	−0.8	0.0	Linked with inflammation
CFHR2	Complement factor H-related protein 2	−0.3	0.7	Complement system
CFH	Complement factor H	−0.9	−0.4	Complement system
AOC3	Membrane copper amine oxidase	0.5	1.4	Cell adhesion ⁵⁶
CDH5	Cadherin-5	0.9	1.0	Cell adhesion ⁵⁷
ANGPTL3	Angiopoietin-related protein 3	−1.0	−1.4	Cell adhesion
KRT10	Keratin, type I cytoskeletal 10	1.4	1.1	Cell movement
CAP1	Adenylyl cyclase-associated protein 1	1.2	1.4	Cell movement
IGF2	Insulin-like growth factor II	−0.4	−1.3	Metabolic response ⁵²
IGFALS	Acid labile chain	−0.8	−1.7	Metabolic response ⁵²
FBLN1	Fibulin-1	0.9	0.1	Hemostasis and thrombosis ⁵⁸
PGD	6-phosphogluconate dehydrogenase	0.9	1.6	Tissue injury marker
ALDOA	Fructose-bisphosphate aldolase A	0.4	0.7	Tissue injury marker
LDHAL6A	L-lactate dehydrogenase A-like 6A	0.8	0.9	Tissue injury marker
LCAT	Phosphatidylcholine-sterol acyltransferase	0.2	−0.6	Lipid metabolism ⁵⁹
GPX3	Glutathione peroxidase 3	−0.7	0.1	Antioxidant response ⁵⁴
SEPP1	Selenoprotein P	−1.0	−0.6	Antioxidant response ⁵³
GLUD1	Glutamate dehydrogenase 1	−1.1	−0.2	
SFN	14–3–3 protein sigma	0.4	1.0	
BCHE	Cholinesterase	−0.1	−1.2	
YWHAG	14–3–3 protein gamma	−0.1	−0.9	
TAGLN3	Transgelin-3	1.2	1.1	
CLEC3B	Tetranectin	−0.7	−0.7	
FETUB	Fetuin-B precursor	−0.7	−0.9	
SERPINF2	SERPINF2 protein	−0.9	−0.1	
COL18A1	Collagen alpha-1(XVIII) chain	−0.9	−0.5	
QSOX1	Sulfhydryl oxidase 1	−1.0	0.0	
AFM	Afamin	−1.2	−1.8	

distress syndrome, and multiple organ dysfunction syndrome, which may develop further into MOF and result in death.^{37,44} With no reliable biomarkers available at present for early assessment of patient outcomes following burn, trauma, and other critical illness, clinicians have been forced to rely on parameters such as heart rate, hypotension, and hyperglycemia to monitor patient trajectories. In the studies of the pathophysiological response to severe burn injury, a number of acute phase proteins and cytokines have been monitored in blood serum using traditional assays.^{36,38}

The present high throughput proteomics application to a relatively large set of healthy ($n = 10$) and severe burn subjects ($n = 15$) provides for the first time a relative broad picture of the human plasma proteome response to severe burn injury. Robust quantitative measurements for this relatively large

sample set were achieved by incorporating an ¹⁸O-labeled reference-based quantification strategy.¹⁹ Reproducibility is especially important for large-scale proteome studies where instrument performance variations typically challenge the ability to obtain reproducible measurements over extended time periods (e.g., months) when using label-free quantification approaches.¹⁷ In this work, the universal reference afforded relatively good reproducibility over several months for ¹⁶O/¹⁸O labeled pair data (Figure 2), although the reproducibility for label-free ¹⁶O intensities was poor, that is, a median CV >100% (data not shown).

Among the 313 proteins quantified with two or more peptides, 110 proteins exhibited statistically significant abundance changes in response to burn injury either at one or both time-points following the injury. Although we were unable to

detect subng/mL level proteins such as cytokines, a class of important inflammatory mediators, the results covered most of the relatively abundant and moderately abundant proteins with concentrations that range from $\mu\text{g/mL}$ to ng/mL , as evidenced by the coverage of acute phase proteins and some of the low abundance proteins listed in Table 2. Overall, the observed postburn quantitative changes appeared in good agreement with prior literature reports for the acute phase response, complement, coagulation, and the immune response (Table 3), including the postburn response of several acute phase proteins previously monitored in serum.³⁸ These changes support a significant postburn inflammatory and hypermetabolic response.

Even more interestingly, our results revealed ~50 potential novel burn-associated proteins (Table 4). Although most of these proteins are not known to be associated with burn injury, a number of these proteins have been implicated in immune response and inflammation. For example, CARD11, a protein containing a caspase recruitment domain (CARD) may play pivotal roles in signal transduction leading to apoptosis and NF-kappaB activation and inflammation.⁴⁵ Pancreatic ribonuclease (RNASE1) is reported to induce dendritic cell maturation and activation,⁴⁶ while sialic acid-binding immunoglobulin-like lectin (SIGLEC14) potentially regulates innate immune responses that modulate the life span of granulocytes.⁴⁷ Moreover, carbonic anhydrase 1, hepatocyte growth factor activator, and apolipoprotein B-100 link to inflammation.^{48–50}

Severe burn injury also causes significant metabolic disturbances and produces reactive oxygen species^{3,51} that are implicated in inflammation and systemic inflammatory response syndrome. Several of the novel burn-associated proteins support both the disruption of metabolic response and a reduced antioxidant capacity. For example, insulin growth factor II (IGF2) and IGF acid labile chain, two proteins known to modulate metabolic response in critical illness⁵² were observed with significant decreases in concentration following burn injury. Observation of down-regulation of glutathione peroxidase 3 and selenoprotein P, two proteins known to play a role in the antioxidant defense system^{53,54} support a potential decrease in antioxidant capacity.

In summary, the importance of the acute phase response, complement, coagulation, and related inflammatory responses in severe burn injury is supported by the plasma proteome changes observed in this study. The temporal changes of these known inflammatory proteins, as well as the nearly 50 novel burn-associated plasma proteins significantly expand our knowledge regarding the basic mechanisms of severe burn-induced inflammatory and metabolic responses. As such, this data set provides an initial basis for future studies of individual proteins as potential targets for therapeutic interventions, as well as for discovering novel protein biomarkers related to disease outcome.

Abbreviations: APP, acute phase proteins; APACHE, Acute Physiological and Chronic Health Evaluation; AMT, accurate mass and time; ARDS, acute respiratory distress syndrome; cAMP, cyclic adenosine monophosphate; BCA, bicinechonic acid; FTICR, Fourier transform ion cyclotron resonance; HLA, human leukocyte antigen; MOF, multiple organ failure; NET, normalized elution time; SIRS, systemic inflammatory response syndrome.

Acknowledgment. Portions of this research were supported by the National Institute of General Medical

Sciences (NIGMS; Large Scale Collaborative Research Grants U54 GM-62119-02 and T32 GM-008256), the NIH National Center for Research Resources (RR18522), and EMSL (Environmental Molecular Science Laboratory). EMSL is a national scientific user facility sponsored by the U.S. Department of Energy (DOE) Office of Biological and Environmental Research on the Pacific Northwest National Laboratory (PNNL) campus in Richland, Washington. PNNL is operated by Battelle for the DOE under contract DE-AC05-76RLO-1830. Additional participating investigators in the Large Scale Collaborative Research Program entitled *Inflammation and the Host Response to Injury*: Henry V. Baker, Ph.D., Ulysses Balis, M.D., Paul Bankey, M.D., Timothy R. Billiar, M.D., Bernard H. Brownstein, Ph.D., Steven E. Calvano, Ph.D., Irshad H. Chaudry, Ph.D., J. Perren Cobb, M.D., Joseph Cuschieri, M.D., Asit K. De, Ph.D., Bradley Freeman, M.D., Richard L. Gamelli, M.D., Nicole S. Gibran, M.D., Brian G. Harbrecht, M.D., Douglas L. Hayden, M.A., Laura Hennessy, R.N., Jureta W. Horton, Ph.D., Jeffrey Johnson, M.D., Matthew B. Klein, M.D., Stephen F. Lowry, M.D., Ronald V. Maier, M.D., John A. Mannick, M.D., Philip H. Mason, Ph.D., Grace P. McDonald-Smith, M.Ed., Carol L. Miller-Graziano, Ph.D., Michael N. Mindrinos, Ph.D., Joseph P. Minei, M.D., Ernest E. Moore, M.D., Avery B. Nathens, M.D., Ph.D., M.P.H., Grant E. O'Keefe, M.D., M.P.H., Laurence G. Rahme, Ph.D., Daniel G. Remick, Jr. M.D., David A. Schoenfeld, Ph.D., Michael B. Shapiro, M.D., Geoffrey M. Silver, M.D., John Storey, Ph.D., Robert Tibshirani, Ph.D., Mehmet Toner, Ph.D., H. Shaw Warren, M.D., Michael A. West, M.D.

Supporting Information Available: All identified peptides and proteins are listed along with their relative abundances in Supplementary Tables. The full pathway of Figure 6B is listed as a supplemental figure. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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PR1005026