Published in final edited form as:

J Proteome Res. 2020 March 06; 19(3): 1258–1266. doi:10.1021/acs.jproteome.9b00765.

TLR4-Independent Effects of LPS Identified Using Longitudinal Serum Proteomics

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Abstract

Sepsis remains one of the most lethal and costly conditions treated in U.S. hospitals, with approximately 50% of cases caused by Gram-negative bacterial infections. Septic shock is induced when lipopolysaccharide (LPS), a main component of Gram-negative outer bacterial membranes, signals through the Toll-like receptor 4 (TLR4) complex. Lethal endotoxemia, a model for septic shock, was induced in WT C57BL/6 and TLR4^{-/-} mice by administration of *Escherichia coli* LPS. WT LPS treated mice showed high morbidity while PBS treated LPS and treated TLR4^{-/-} mice did not. ANOVA analysis of label-free quantification (LFQ) of longitudinal serum proteome revealed 182 out of 324 proteins in LPS injected WT mice that were significantly changed across four time points (0, 6, 12, and 18h). No significant changes were identified in the two control groups. From the 182 identified proteins, examples of known sepsis biomarkers were validated by ELISA which showed similar trends as MS proteomics data. Longitudinal analysis within individual mice produced 3-fold more significantly changed proteins than pair-wise comparison. A

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Supporting Information

A MaxQuant analysis output summary file and a file containing the following supporting information is available free of charge at ACS website <http://pubs.acs.org>.

Data Availability

The sixty-four raw data files described in this manuscript have been deposited as a MassIVE data set number MSV000084416 (doi:10.25345/C5Z388). The URL to access this dataset is <ftp://massive.ucsd.edu/MSV000084416/>.

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subsequent global analysis of WT and TLR4^{-/-} mice identified pathways activated independent of TLR4. These pathways represent possible compensatory mechanisms that allow for control of Gram-negative bacterial infection regardless of host immune status.

Keywords

TLR4; LPS; mass spectrometry; serum proteomics; sepsis; endotoxemia

Introduction

Sepsis creates a large burden on human health with deaths outnumbering those from prostate cancer, breast cancer, and AIDS combined ¹. The high mortality is caused by dissemination of pathogens or pathogen associated molecules in the host's blood that activates innate immune receptors, which when overwhelmed produce a cytokine storm. Current treatment standards for a person presenting with sepsis include supportive intravenous fluids and antibiotics to eradicate the infecting bacteria. However, even with the use of antibiotics upon infection presentation, widespread dissemination of pathogen associated molecules can induce an unintended cytokine storm in the patient. During a cytokine storm, the host healthy immune system, normally required to intervene for resolution of a blood-borne infection, produces an uncontrolled pathogenic-driven inflammatory response. If the host immune response is not effectively controlled, the cytokine storm will lead to multiple organ dysfunction and eventually death ².

Septic shock and cytokine storm caused by Gram-negative bacteria is largely dependent on stimulation of Toll-like receptor 4 (TLR4) complex by the bacterial membrane component lipopolysaccharide (LPS) ³. The TLR4 receptor complex is comprised of several membrane bound/transmembrane proteins. Included in the receptor complex are lipopolysaccharide binding protein (LBP), cluster of differentiation-14 (CD14), lymphocyte antigen 96 (MD2), and a dimer of TLR4. Initially, LBP binds to LPS with high affinity after which, transfer of LPS to MD2 is facilitated by the CD14 protein. Once LPS binds MD-2 an active TLR4 dimer receptor complex is formed and the intracellular region of TLR4 recruits signaling molecules that initiate the production of a host immune response ⁴. Without a functional TLR4 receptor many of the pathogen downstream effects of Gram-negative sepsis are not initiated ⁵. Despite having an understanding of this signaling pathway, efforts devoted to developing effective therapies for sepsis over past decades have been unsuccessful ⁶. There is currently no FDA-approved drug for the treatment of sepsis, even though more than 40 clinical trials have been conducted⁷. It is estimated that effective early detection could save up to 80% of sepsis related deaths, which is in part due to the fact that the earlier sepsis is diagnosed and antibiotic therapy begun, the higher the survival rate ⁸. Additionally, diagnosis of sepsis proves to be extremely challenging due to the heterogeneity of responses and clinical symptoms among patients ⁹. Contributing to the difficulty of treating sepsis is the fact that more than 50% of patients show clinical symptoms but have negative culture results ¹⁰. Therefore, identifying reliable sepsis biomarkers could allow for early recognition and diagnosis, which would be a significant advance in the fight against this deadly condition. Nearly 180 proteins associated with sepsis have been reported and assessed

clinically, including the most widely used procalcitonin (PCT) and C-reactive protein (CRP). However, none exhibit sufficient specificity and sensitivity, which prohibit definitive use in clinical diagnostics ¹¹.

To identify the progression of sepsis in model systems, mass spectrometry based proteomics has been employed to improve understanding of molecular mechanisms of sepsis and in biomarker discovery efforts ^{6,12}. With the evolution of proteomics platforms, hundreds of proteins can be identified and quantified from a variety of biofluids, such as plasma, serum, urine, and cerebrospinal fluid. Serum is a particularly useful protein-rich biofluid as is readily obtained from patients and is capable of providing dynamic information about the circulatory system and disease state ¹³. However, proteomic analysis of serum is challenging due to the complex composition of proteins and their wide dynamic range of expression ¹⁴.

Although numerous protein putative biomarkers associated with sepsis have been reported, the failure of these to provide clinically significant sensitivity and specificity for predicting sepsis can be partially attributed to the poor understanding of the large differences in proteome variation between and within healthy individuals. This variation in healthy normal individuals contributes to underlying biological noise in the proteomics data that has made attempts to discover early markers of sepsis unsuccessful. To date, most proteomic studies have used simple pair-wise comparisons to try to extract biomarkers of significance, however, as shown by Nagaraj and Mann¹⁵, this analysis routinely cannot overcome the variations in normal proteomes that mask changes in abundance of potential new biomarkers. To circumvent the bias caused by pair-wise comparison, they analyzed the urinary proteome of seven healthy donors using a label-free quantification (LFQ) based proteomics strategy ¹⁵. Interpersonal variability was found and largely attributed to the total variability observed, which was 47.1%. In addition, technical and intra-individual variability were 7.5% and 45.5%, respectively. Thus, longitudinal sampling and analysis, which track a subjects' proteome change over time, may be considered a better approach to extract valid biomarkers from MS proteomic studies than simple pair-wise comparisons pre- and post-disease. In a second study from this group, samples collected longitudinally were used to identify two biomarkers with predictive value in lung cancer patients undergoing radical radiotherapy¹⁶. Finally, using longitudinal analysis Raju *et al.* identified dynamic serum proteome profiles between surviving and deceased patients from sepsis caused by *K. pneumoniae* ¹⁷.

Animal models of sepsis have long been used to investigate the dynamic host response in different phases of sepsis ^{16–18}. The three main categories of animal models of septic shock include lethal endotoxemia induced by injection of LPS, induction of an infection by administration of a single pathogenic organism, and polymicrobial sepsis induced by exposing an animal to a group of bacteria. Each of these categories of animal models of septic shock have value when investigating different facets of disease. Single- and polymicrobial infection models are most useful when investigating efficacy of therapeutics and when considering whole-host pathogenesis of particular infections. The lethal endotoxemia model is more suited for investigating molecular mechanisms activated during the innate immune sensing of Gram-negative bacteria or bacterially-derived molecules. Therefore, we have chosen this model for our studies which will allow for the elucidation of more

controlled insight(s) into how the host immune response is contributing to disease observed during active septic infections.

In this study, we use a controlled dose model of lethal endotoxemia to allow for the reduction of other biological variables that might be affected during an active infection. This allowed us to focus on the molecular consequences of TLR4/MD2 receptor complex activation in Gram-negative septic shock. To do so, small volume longitudinal serum samples (10 μ L) were collected from mice injected with a single lethal dose of *E. coli* LPS. Data dependent acquisition (DDA) during LC-MS/MS was used to acquire data and LFQ analysis used to quantify relative changes in protein abundance during the course of disease (Figure 1). By collecting longitudinal samples, changes in relative protein abundance could be followed over the course of infection in each animal, thus allowing us to overcome individual variability between mice that limited discovery of novel serum biomarkers of lethal endotoxemia. Here, we present data that further refines our understanding of lethal endotoxemia model with respect to septic shock. Specifically, we identified key serum proteins whose relative abundance changes during onset of lethal endotoxemia that were subsequently verified by ELISA analysis. By focusing on disease induced via TLR4/MD-2 complex activation and through the inclusion of TLR4^{-/-} mice as a control, we also identified pathways that are activated during lethal endotoxemia independent of TLR4.

Experimental Procedures

Mouse Model of Lethal Endotoxemia

Mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL6 WT (Strain: C57BL/6J) and TLR4^{-/-} (Strain: B6.B10ScN-*Tlr4*^{ps-del}/JthJ : *lr4*^{ps-del} spontaneous mutation exhibits a defective response to LPS stimulation, Stock # 007227) were used, all mice were female, age matched, and 8–10 weeks old at time of experiments. Intraperitoneal (i.p.) injection of 30 mg/kg LPS (*E. coli* BORT) in 500 μ L PBS was used to initiate lethal endotoxemia (LPS group, n =10) or TLR4^{-/-} mice (TLR4^{-/-} group, n =5). As a baseline comparator, 500 μ L of PBS was injected into WT control animals (PBS group, n =5). Five additional mice were included in the LPS injected WT group to confirm the disease timeline and were observed until a humane endpoint was reached. All animal studies were approved by the University of Maryland School of Medicine Institutional Animal Care and Use Committee (IACUC).

A small volume, approximately 25 μ L of blood was collected from the lateral saphenous vein immediately before the first injection was designated the 0 hr sample and then at 6, 12, and 18 hrs post-injection. Blood was mixed with 10.6 mM trisodium citrate in a pipette-tip-based centrifugal device¹⁰. After centrifugation at 10,000 $\times$ g for 5 minutes serum was transferred to a sterile tube and stored at -20°C until tryptic digestion for LC-MS/MS analysis. Internal temperature of individual mice was measured using a rectal thermometer (Kent Scientific, RET3, Torrington, CT) at each time point of blood collection.

LPS Extraction

LPS from *E. coli* BORT (O18ac:K1:H7) grown at 37°C in LB media supplemented with 1 mM MgCl₂ was isolated using an optimized hot phenol/water method¹⁹ that is based on the original protocol²⁰. Briefly, lyophilized bacterial pellets were resuspended in sterile water and mixed with 90% phenol for one hour incubation at 65°C. After incubation and centrifugation, the aqueous phase was collected. Water was added to phenol fraction, and then aqueous phases were combined for dialysis and lyophilization. The resultant pellet was purified by treating with DNase and Proteinase K. Water-saturated phenol extraction was performed on the digested solution, followed by dialysis, and freeze dried. The LPS was further purified by removing phospholipids and lipoproteins using chloroform/methanol mixtures (v/v 2:1) and water-saturated phenol extractions, and 75% ethanol precipitation consecutively.

Sample Preparation

Serum protein concentration was measured using a NanoDrop (Thermo Fisher Scientific, NanoDrop 1000 Spectrophotometer, Waltham, MA) at 280 nm. Sepsis samples were prepared using the protocol that was described by Geyer *et al.* with optimization for serum¹⁰. To 1 µL of murine serum, 24 µL of sodium deoxycholate (SDC) reduction and alkylation buffer was added, mixed briefly and incubated at 100°C for 10 minutes to allow protein denaturation. After incubation, samples were cooled to room temperature before treatment with 2 µL (0.5 µg/µL) mass spectrometric grade of Trypsin and Lys-C mix (Promega, Madison, WI), and incubation at 37°C for 2 hours. Trifluoroacetic acid (TFA) was added to reach a final concentration of 0.1% to quench the trypsin digestion. C18 StageTips (Thermo Scientific, SP301, West Palm Beach, FL) were used for downstream peptides mixture cleaning and desalting by following the manufacturer's protocol. Briefly, StageTips were initialized using 20 µL aqueous mixture of 80% acetonitrile (ACN) and 5% formic acid (FA) and re-equilibrated with 20 µL 5% FA. Peptide digests (10 µg) and 20 µL 5% FA were loaded on an equilibrated StageTip, consecutively. Samples were washed twice with 20 µL 5% FA and purified peptides were eluted out by 20 µL elution buffer (80% ACN, 5% FA) three times. The collected 60 µL purified samples were dried using a Speedvac at 37°C. Dried samples were suspended in buffer (97:3:0.1, H₂O/ACN/FA v/v/v) for LC-MS/MS analysis.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) Analysis

Triplicate biological samples from all groups at the 0, 6, and 12 hour post-injection time points were analyzed. Due to limited access to the instrument, only one sample at 18 hours was selected from the two control groups (WT-PBS and TLR4^{-/-}-PBS), while all three 18 hour samples from the WT-LPS injected group were included (Supp Table 1). A total of thirty-two samples were analyzed in duplicate (64 total injections) using an LC-MS system comprised of a nanoAcquity LC instrument (Waters nanoAcquity UPLC system, Milford, MA) connected to an Orbitrap Fusion Lumos tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Peptides were trapped on a nanoAcquity trap column (180 µm x 20 mm), washed with 99.5% mobile phase A (0.1% formic acid in water) and separated on a nanoAcquity C18 column (1.7µm BEH130Å, 100 µm x 100 mm) at 40°C. Peptides were

eluted with a linear gradient of 3% to 40% mobile phase B (0.1 % FA in acetonitrile) over 90 min. Gradient changes were followed at 91min to 85% B and then increased to 95% B at 95 min. The gradient was changed back to 3% of B to equilibrate for 20 minutes prior to the next injection. Each injection consisted of 1.5 µg of purified peptides and injection order was randomized to minimize bias.

Eluted peptides were ionized in positive ion polarity at a 2.1 kV of spraying voltage. MS¹ full scans were recorded in the range of m/z 380 to 1580 with a resolution of 120,000 at 200 m/z using the Orbitrap mass analyzer. Automatic gain control was set at 8×10^5 with 50 ms of maximum injection time. Top speed (3sec) data dependent acquisition mode was used to maximize the number of MS² spectra from each cycle. Higher-energy C-trap dissociation (HCD) was used to fragment selected precursor ions with a normalized collision energy of 30%. Tandem MS scans were performed in the ion trap mass analyzer with 35 ms maximum injection time, an ion target value set at 5×10^3 , and dynamic exclusion set to 30s.

Data Analysis

Sixty-four MS raw files were searched against the UniProt mouse database using MaxQuant (v1.6.0.1, Max Planck Institute, Munchen, Germany) for peptide and protein identification and relative LFQ. Cysteine carbamidomethylation was set as a fixed modification and N-terminal acetylation and methionine oxidations were defined in variable modifications. For Andromeda searches, peptide identification was analyzed with mass tolerance of 6 ppm for precursor ions and 0.5 Da for resulting fragment ions. Enzyme digestion was set as trypsin (cleavage at C-terminal arginine and lysine), and maximum of two missed cleavages were allowed for database search. For biomarker identification, false discovery rate (FDR) was set to 1% for both peptides and proteins, and only peptides with a minimum length of 7 amino acids were considered. LFQ was performed with a minimum ratio count of 2. Matches between run function was selected with a matching time window of 0.7 min and an alignment time window of 20 min. Default settings were applied for other parameters. A MaxQuant output file is provided as a supporting file.

Bioinformatics Analysis and Experimental Design and Statistical Analysis

MaxQuant search results were analyzed in Perseus (v 1.5.3.1, Max Planck Institute, Munchen, Germany) for bioinformatics analysis. MaxLFQ intensities for each identified protein were log 2 transformed and used for LFQ comparison between each time point and different treatment groups. UniProt annotations were added to classify all the identified proteins based on GOMF, GOCC and GOBP names. The 64 RAW files from 32 samples were categorically annotated based on experimental set up, technical replicates, biological replicates, mouse number, time points and conditions. ANOVA test was performed and p-values were adjusted with permutation-based FDR 5%. Z-score normalization was performed for ANOVA significantly changed proteins and followed by hierarchical cluster analysis with k-means clustering. Principle component analysis (PCA) was performed with the cutoff method of Benjamin-Hochberg FDR at 0.05. Presented graphics were generated using a combination of Perseus, Graphpad 5 (La Jolla, CA) and Power Point. To understand biological functions of the proteins with statistically significant changes in relative abundance, pathway analysis was performed using Ingenuity Pathway Analysis (IPA)

software (Qiagen, Redwood City, CA). Analyses of Canonical pathway, disease and function, and interaction network were performed.

Proteins identified during a global analysis of all treatment groups as being up- or down-regulated with over log 2 fold changes and ANOVA significant were entered into DAVID analysis software ²¹. Genes associated with each of the proteins were identified using the Gene Accession Conversion Tool and subjected to KEGG_PATHWAY analysis.

Enzyme-linked immunosorbent assay (ELISA)

An additional cohort of 8–10 week old female C57BL6 mice were purchased from Jackson Laboratory and lethal endotoxemia was initiated with the same procedures and dose described for proteomics samples. Blood was collected from three mice at each of the 6, 12, and 18 hour post-injection timepoints, as well as prior to the first injection designated 0 hr. Serum samples were stored at –80°C prior to ELISA analysis for serum amyloid A (R&D, DuoSet ELISA DY2948–05, Minneapolis, MN), CD14 (R&D, DuoSet ELISA DY982), Apoe (Aviva Systems Biology, OKEH04368, San Diego, CA), and CRP (Aviva Systems Biology, OKEH04495). ELISAs were run to measure serum protein amounts according to manufacturer's protocol. Values were determined using the average of technical duplicates and protein amounts were extrapolated from an exponential standard curve calculated by GraphPad Prism 7.00 (La Jolla, CA).

Results

Characterization of Murine Model of Lethal Endotoxemia

A murine model of lethal endotoxemia was used as a model to study the disease course of septic shock. Within 6 hours of LPS injection, WT mice became hypothermic, a hallmark of murine septic shock, while control PBS injected WT and LPS injected TLR4^{–/–} mice remained at a consistent basal temperature (Figure 2A). By 12 hours, the hypothermic response in the LPS treated WT mice decreased to a final core temperature to 25°C with lethality reached between 18 and 24 hours post LPS injection (Figure 2B). All animals were monitored for human endpoints, as approved by the University of Maryland – Baltimore IACUC. In contrast, neither the PBS nor TLR4^{–/–} treated mice showed any signs of hypothermia or lethality.

Proteome Analysis of Serum Samples

One µL of murine serum from each mouse, at each time point was used for LC-MS/MS sample preparation. Samples were processed using a bottom-up proteomics approach with trypsin digestion followed by a rapid StageTip based cleaning procedure. Purified peptides were analyzed using an optimized LC-MS/MS method and raw files were searched against the mouse UniProt database by the Andromeda search engine of MaxQuant (1.6.0.1) ²². The sepsis proteomics workflow overview is shown in Figure 1.

In this study, 32 unique biological samples were analyzed, in duplicate, by LC-MS/MS for a total of 64 injections (Supp Table 1). A total of 1161 proteins were identified from all injections after individual peptides and proteins were filtered using a 1% FDR cutoff.

MaxQuant results were imported into Perseus for bioinformatic analysis. Proteins were identified by site only, reverse sequence and contaminant databases defined in MaxQuant²² were excluded from the list. After these analyses, 672 proteins remained. To increase confidence of identification, proteins with more than one unique peptide identified were carried forward for subsequent analysis. This led to 572 proteins for whole proteome comparison between three groups: 1) WT C57BL6 injected with LPS, 2) WT C57BL6 injected with PBS, and 3) TLR4^{-/-} C57BL6 injected with LPS. After the described data filtering (*vide supra*), technical duplicates were combined leading to 200 – 350 proteins identified per sample (Supp Figure 1). These levels were within the expected range for serum proteomic studies run without depletion of abundant proteins. Consistent with previously reported results, the top ten most abundant serum proteins accounted for about 90% of the total protein abundance in serum, while other proteins had a very wide dynamic range²³.

To further characterize the dataset, a dynamic range analysis was carried out by ranking LFQ values for the identified 572 proteins. This analysis found that between the most and least abundant proteins, the dynamic range spanned about six orders of magnitude (Supp Figure 2). As expected, the most abundant protein was serum albumin followed by hemoglobin. Most protein log₁₀ LFQ values fell in the range of 6.5 – 8.5, with the 453 proteins in this 100-fold range accounting for nearly 80% of all identified proteins. The frequency distribution of all 572 proteins presents a ‘bell’ shape profile, indicating a comprehensive coverage of our LC-MS/MS analysis (Supp Figure 2). To evaluate the technical reproducibility of the proteomics workflow, the variability between duplicate injection of each unique serum sample was evaluated. The protein abundances of each technical replicate were correlated to generate a R² correlation value. The mean of all 32 samples’ R² value was 0.97, with the majority of duplicate LC-MS/MS runs (28 out of 32) being in the range of 0.95 to 0.99 (Supp Figure 3). These results indicate that the proteomics workflow had minimal technical variability.

Longitudinal comparison of the lethal endotoxemia murine serum proteome.

Despite clear clinical differences between treatment groups there were very few significant protein changes identified when using pair-wise comparison, this finding suggests that pair-wise comparisons might not be the most useful analysis for this data set/disease model. Next, to find inter-animal changes in protein abundance patterns over the course of disease, a longitudinal comparison was carried out for each condition separately by using each mouse 0 hour time point as its own control. The dataset was split based on treatment conditions (WT-LPS injected, WT-PBS injected, and TLR4^{-/-}-LPS injected) followed by z-score normalization within each grouping. For the WT-LPS injected group, one-way ANOVA was performed with permutation-based FDR correction (5%) among four time points (0, 6, 12, 18 hours) (Figure 3). Notably, 182 proteins were differentially modulated over time among all 324 proteins for the WT-LPS injected group. In addition, no ANOVA significant protein changes were found when this analysis method was applied to the WT-PBS injected and the TLR4^{-/-}-LPS injected groups. Lack of significant protein changes in the two control arms of this murine endotoxemia model indicate the role of TLR4 receptor in recognition of LPS

and the purity of the LPS as we would have potentially identified off target immune response associated pathways activated through non-LPS contaminants.

Hierarchical clustering was performed on the 182 ANOVA significant proteins found in the WT-LPS injected group with the resulting heat map is shown in Figure 3A. Three distinct clusters show either up- or down- patterns of difference. In cluster 1, 88 proteins decreased in abundance over time, mainly at 18 hours (Figure 3B). The majority of proteins in this group were associated with complement activation, immune responses, and regulation of multicellular organismal processes with high enrichment factors. Protein abundances of cluster 2 were increased over time with the transition state beginning early at 6 hours (Figure 3B). Some proteins in this cluster were associated with acute inflammatory response, such as literature reported sepsis biomarkers, serum amyloid A (Saa) 1 and 2²⁴, and S100A9²⁵. In cluster 3, abundance of 68 proteins gradually increased over time and majority of these were related to metabolic processes. A full list of the 182 ANOVA significant proteins is provided in Supplementary Appendix I.

Validation of observed protein changes by quantitative ELISA.

To validate the longitudinal MS proteomics results, protein specific ELISAs were run using serum collected from additional WT mice injected with the same lethal dose of LPS. Five proteins that are well known sepsis biomarkers, that are routinely used in clinical diagnosis, were selected for validation by quantitative ELISA and include: an acute phase inflammatory marker, serum amyloid A1 (Saa1); an LPS mediator/ macrophage activation marker, cluster of differentiation-14 (CD14); a protein involved in fat metabolism and checkpoint inhibition of the complement cascade, apolipoprotein E (ApoE); and a marker of inflammation, C-reactive protein (CRP). Concentrations of the proteins over time were well correlated with fold change values referenced to 0 hour from the proteomics study (Figure 4). Specifically, trends of Saa1 induction via ELISA analysis well correlated with fold change values referenced to 0 hour from proteomics results, with levels peaking at 12 hours post-induction of lethal endotoxemia. Changes in CD14 were elevated by 6 hours, and peaked at 12 or 18 hours, as measured by proteomics or ELISA, respectively, whereas ApoE levels steadily decrease over time in both the proteomics and ELISA assays. Interestingly, although CRP is one of the most characterized biomarkers of septic shock that is usually significantly elevated within 12 hours of disease induction ²⁵, it did not show a significant change in our serum proteomics study and is supported by ELISA analysis that showed CRP as being below the limit of detection in all samples tested.

TLR4-independent serum proteome changes identified by global analysis of data set

In addition to the first analysis, which was a within animal longitudinal analysis of the serum proteome, an ANOVA was run by grouping samples from all timepoints (6, 12, and 18 hours) for each of the experimental groups (PBS-WT, LPS-WT, and LPS-TLR4^{-/-}). The ANOVA was run comparing the three treated groups to the combined 0 hour PBS-WT and 0 hour LPS-WT samples. This analysis facilitates a broader look at overall serum protein changes between groups. From this global analysis, a set of proteins with significantly altered abundance, defined similarly to the previous analysis as log 2-fold changes and ANOVA significance, were identified and were graphed to show common proteins identified

among the three experimental groups (Figure 5). Of the 231 proteins identified as having a statistically significant change from this analysis, 97 are shared among all three experimental groups. These likely represent protein levels that are altered as part of a common stress response initiated by handling the mice (temperature reading and/or injection). As was found during the longitudinal analysis, there were no proteins that were significantly changed in the TLR4^{-/-}-LPS injected or the WT-PBS injected mice alone, further confirming the use of these control experimental arms. There were 69 proteins that were found to be altered in the WT-LPS injected group, these proteins represent a subset for which levels are altered during canonical TLR4 induced endotoxemia and include many of the previously identified markers of sepsis. There are also 51 significantly altered proteins that are shared between the TLR4^{-/-}-LPS injected and WT-LPS injected groups. Because they are also altered in the mice without TLR4, the proteins in this subset are altered by the presence of LPS but not specifically through the TLR4 signaling pathway. The lists of 69 and 51 proteins from Figure 5 were entered into DAVID analysis software²¹. Genes associated with each of the proteins were identified and DAVID was used to query the KEGG_PATHWAY database for significantly altered functional pathways associated with each of the two lists. Pathways associated with canonical TLR4-dependent endotoxemia are shown in Table 1, and pathways associated with TLR4-independent endotoxemia are shown in Table 2. Interestingly activation of the “biosynthesis of antibiotics” KEGG pathway, which provides a dynamic list of associated proteins, was shared among the WT LPS injected and TLR4^{-/-} LPS injected groups. This suggests that compensatory mechanisms to fight Gram-negative bacterial infection are activated by LPS without the associated canonical innate immune receptor.

Discussion

This study used a murine model of lethal endotoxemia to elucidate TLR4-dependent and independent pathways activated during septic shock. Longitudinal analysis of small volume serum samples allowed for increased identification of significantly altered proteins, as compared to pair-wise comparisons. This increase in disease-specific changes was observed without extensive processing of samples before analysis. During serum proteomics studies, enrichment or immuno-depletion approaches are commonly used to enhance detection of low abundance proteins by removing some portion of the most abundant proteins. While the top ten can account for more than 90% of total protein in serum and their depletion can extend detectable dynamic range, their depletion can also alter the quantitative results^{23, 25}. Samples in this study were not immuno-depleted of high abundance proteins to allow for detection of the full spectrum of serum proteins detectable by MS-based proteomics. Using affinity column based immunodepletion technology it is possible that non-targeted proteins may be nonspecifically removed during the interaction with the column, lower abundance proteins may also form complexes with high abundance proteins and be removed during the immunodepletion process. Additionally, reproducibility and consistency of immunodepletion may add artifactual protein variability over the disease time course.²⁶ During this initial query of serum proteins in our mouse model of lethal endotoxemia, we decided to test protein levels without any bias that may be introduced during immunodepletion. More targeted in-depth studies using this disease model and method may

benefit from removing high abundance proteins, this may change the list of significant proteins identified and is a possible future direction for this study.

Longitudinal analysis is beneficial when studying changes within large heterogeneous populations as the baseline for each individual is different. Interestingly, due to the fact that each mouse responded independently to the LPS stimulus with various initial protein abundances, no clear protein expression pattern was observed (Supp Figure 4). Even within this inbred genetically homogenous mouse strain, varied protein levels were observed. These differences cannot be attributed to variability or contamination of LPS inoculum, as we observed limited off target effects in the TLR4^{-/-} LPS-injected mice in the longitudinally collected samples. One concern about collecting longitudinal samples is the feasibility of repeated blood draws and blood volume loss. The small volume necessary for assays completed during this study would not require an additional blood draw from a patient and could be completed with serum collected to run other diagnostic tests or with venous blood collected from a simple finger stick. Advanced mass spectrometric analysis of just a few drops of blood is an exciting avenue to be pursued during the future of personalized medicine.

Protein abundances shown to be changed over time during proteomics studies were consistent with changes observed in the serum protein levels measured by ELISA from a different cohort of animals. Not only were the changes consistent between the two different assays, they were consistent among biological replicates analyses and suggests that these results will likely be able to be extrapolated across a wide range of Gram-negative sepsis models. Furthermore, the subtractive list of proteins from the TLR4^{-/-} mice during the global analysis provides insight into off target effects of LPS in the bloodstream. Activation of the TLR4 signaling pathway includes internalization of the receptor to allow for control of production of innate immune stimulated molecules. While controlling the host innate immune response is advantageous, it would also be helpful if the infected individual can continue to clear the infection without TLR4 signaling.

This study suggests that LPS is able to activate compensatory infection control mechanisms, including the synthesis of antibiotics, without the presence of TLR4. It is possible that the LPS is signaling through other more recently discovered intracellular innate immune receptors such as nucleotide oligomerization domain (NOD) proteins and caspase-11²⁷. The alteration of metabolic pathways and activation of coagulation and complement cascades also seem to be largely independent of TLR4 (Table 2). This is surprising considering purified LPS, not whole bacteria, was the only inoculum. For decades treatment of sepsis has focused on modulating the immune pathways and controlling the cytokine storm, but this study provides evidence that even without a functional immune response there are metabolic changes in the host²⁸. Further studies will be needed to confirm these observations and compare compensatory pathways activated with those from organisms that do not constitutively express the canonical LPS receptor. Novel therapeutic targets can be identified by subtracting components of disease. The observation of off target LPS effects would not be possible without the coordinate use of a genetically defined animal and advanced mass spectrometry techniques. This approach is not only useful in the advanced study of

pathogenic mechanisms of sepsis, but can also be applied to any defined animal model of disease to have a widespread impact in creating greater understanding of human disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank the Center for Food Safety and Applied Nutrition, FDA for instrumentation and bioinformatics support. Specifically, we thank Tim Croley of the FDA for access to the LC-MS instrument used in this work. DRG and RKE thank NIH R01 AI123820-01, PI: Ernst; EMH is supported by T32 AI095190, PI: Vogel. DRG thanks the International Centre for Cancer Vaccine Science project of the International Research Agendas program of the Foundation for Polish Science co-financed by the European Union under the European Regional Development Fund (MAB/2017/03) at the University of Gdansk.

Abbreviations

ACN	acetonitrile
ANOVA	analysis of variance
APOE	apolipoprotein E
CD14	cluster of differentiation-14
CRP	C-reactive protein
DAVID	database for annotation, visualization, and integrated discovery
DDA	data-dependent acquisition
ELISA	enzyme-linked immunosorbent assay
FA	formic acid
FDR	false discovery rate
H₂O	water
HCD	Higher-energy C-trap dissociation
IACUC	institutional animal care and use committee
IPA	Ingenuity pathway analysis
LC	liquid chromatography
LFQ	label-free quantification
LPS	lipopolysaccharide
MD-2	lymphocyte antigen 96
MS	mass spectrometry

NOD	nucleotide oligomerization domain proteins
PBS	phosphate buffered saline
PCT	procalcitonin
SAA	serum amyloid A
TFA	trifluoroacetic acid
TLR4	toll-like receptor 4
WT	wildtype

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Figure 1.
An overview of the proteomics workflow in serum sample analysis.

Figure 2. Characterization of murine model of lethal endotoxemia.

(a) WT C57BL6 mice (n=10) and TLR4^{-/-} mice on a C57BL6 background (n=5) were injected i.p. with 30 mg/kg of *E. coli* LPS (BORT strain), an additional control group of WT C57BL6 mice (n=5) were injected i.p. with PBS. Mean $\pm$ SD of mouse interal temperature over the course of the experiment is plotted. (b) Survival plot of WT C57BL6 mice (n=5) injected i.p. with 30 mg/kg of *E. coli* LPS (BORT strain) all of which reached a humane lethal endpoint within 24 hours post-injection.

Figure 3. Hierarchical Clustering Analysis.

(a) Heat map of all 182 proteins whose changes in abundance over time were of statistically significance by ANOVA test. Proteins whose abundance increased over time have z-scored normalized values colored in red while those whose abundance decreased are shown in blue. The grouping methods for z-score normalization was developed using longitudinal sample comparisons for each mouse. Color key displayed below heat map. Composite profile plots of changes in protein abundance from clusters 1–3 in panel A and shown in panel B 1–3, respectively.

Figure 4. ELISA measurement of protein abundance changes over time.

Tested proteins are (a) Saa1, (b) ApoE, and (c) CD14. Red line represented relative fold change data from LC-MS/MS proteomics experiments compared to 0 hour; mean $\pm$ SD of the six values associated with each timepoint are graphed. Blue bars represent the ELISA measured concentration (ng/mL) of proteins; mean $\pm$ SD of the three biological replicate serum is graphed.

Figure 5. Number of proteins with statistically significant changes in relative abundance identified in each of three groups.

The Venn diagram represents the number of proteins whose relative abundance changes were statistically significant that are both common to and unique to each experimental group. A list of the genes encoding proteins unique to the WT LPS injected group, and those that are shared among the WT LPS injected and TLR4^{-/-} LPS injected groups are shown in the dialog boxes next to the Venn diagram. Lists/tables of protein information from genes in dialog boxes are found in Appendix I.

Table 1.

KEGG_PATHWAY analysis of genes associated with significantly changed proteins unique to the LPS, WT group.

Pathway Term	Genes	% of Subset	Associated Gene List
Complement and Coagulation Cascades	22	32.4%	F2, F9, F10, F12, F13b, C1qa, C3, C8b, C8g, C9, Cfh, Cfi, Hc, Klkb1, Kng1, Mbl2, Plg, Serpina1d, Serpinc1, Serpind1, Serpinee1, Serpinf2
<i>Staphylococcus aureus</i> Infection	7	10.3%	C1qa, C3, Cfh, Cf1, Hc, Mbl2, Plg
Systemic Lupus Erythematosus	6	8.8%	C1qa, C3, C8b, C8g, C9, Hc
Prion Disease	5	7.4%	C1qa, C8b, C8g, C9, Hc
Pertussis	4	5.9%	Cd14, C1qa, C3, Hc
HIF-1 Signaling Pathway	4	5.9%	1300017J02Rik, Egfr, Serpine1, Trf
Amoebiasis	4	5.9%	Cd14, C8b, C8g, C9
Phagosome	4	5.9%	Cd14, C3, H2-Q10, Mb12
Proteasome	3	4.4%	Psm13, Psm2, Psm1
PPAR Signaling Pathway	3	4.4%	Apoa1, Apoc3, Pltp

Table 2.

KEGG_PATHWAY analysis of genes associated with significantly changed proteins shared by LPS, TLR4^{-/-} and LPS, WT groups.

Pathway Term	Genes	% of Subset	Associated Gene List
Metabolic Pathways	11	21.6%	Pgls, Adsl, Aldoa, Alad, Gclc, Mdh1, Pgd, Pgam2, Pnpo, Pklr, Pkm
Complement and Coagulation Cascades	9	17.6%	F5, F13a1, C1qb, Cfd, Fga, Fgb, Fgg, Masp1, Mbl1
Biosynthesis of Antibiotics	8	15.7%	Pgls, Adsl, Aldoa, Mdh1, Pgd, Pgam2, Pklr, Pkm
Carbon Metabolism	7	13.7%	Pgls, Aldoa, Mdh1, Pgd, Pgam2, Pklr, Pkm
Staphylococcus aureus Infection	5	9.8%	C1qb, Cfd, Fgg, Masp1, Mbl1
Pyruvate Metabolism	4	7.8%	Hagh, Mdhl, Pklr, Pkm
Glycolysis/Gluconeogenesis	4	7.8%	Aldoa, Pgam2, Pklr, Pkm
Biosynthesis of Amino Acids	4	7.8%	Aldoa, Pgam2, Pklr, Pkm
Pentose Phosphate Pathway	3	5.9%	Pgls, Aldoa, Pgd
Glucagon Signaling Pathway	3	5.9%	Calm1, Pgam2, Pkm
Platelet activation	3	5.9%	Fga, Fgb, Fgg