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Proteomic Profiling of Lipopolysaccharide-activated Macrophages by Isotope Coded Affinity Tagging

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Synopsis

Lipopolysaccharide initiates proinflammatory, proapoptotic, and anti-apoptotic pathways upon binding to macrophage TLR4. We performed ICAT, multi-dimensional liquid chromatography, and mass spectrometry to identify proteins that are differently expressed between naïve and LPS-activated cultured RAW 264.7 cultured mouse macrophages as well as C57BL/6 bone marrow derived mouse macrophages. We identified and obtained relative abundances for 1064 proteins, of which 36 had significantly different expression levels upon activation by LPS.

Lipopolysaccharide (LPS), a glycolipid component of the outer membranes of Gram-negative bacteria, initiates proinflammatory, proapoptotic, and anti-apoptotic pathways upon binding to macrophage TLR4. Macrophages that are exposed to LPS become activated and exhibit altered morphology and response to infection. We performed isotope coded affinity tagging (ICAT), multi-dimensional liquid chromatography, and mass spectrometry to identify proteins that are differently expressed between naïve and LPS-activated macrophages. We performed replicate ICAT analyses on RAW 264.7 cultured mouse macrophages as well as C57BL/6 bone marrow derived mouse macrophages. We identified and obtained relative abundances for 1064 proteins, of which we identified 36 as having significantly different expression levels upon activation by LPS. We also compared our results with a two color microarray gene expression assay performed by the Institute for Systems Biology and observed ~75% agreement between mRNA transcription and protein expression regarding up- or down-regulation of gene products. We used Western blot analysis to confirm the findings of ICAT and mRNA for one protein, sequestosome 1, the cellular concentration of which was observed to increase upon activation by LPS.

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INTRODUCTION

Lipopolysaccharide (LPS) is a glycolipid component of the outer membrane of Gram-negative bacteria that induces macrophages and monocytes to produce cytokines.¹ Recognition of LPS by Toll-like receptor (TLR) 4 induces MyD88-dependent activation of the proinflammatory transcription initiators nuclear factor-kappa B (NFkB) and mitogen-activated protein kinase (MAPK).² NFkB up-regulates expression of proteins that are anti-apoptotic,³ proinflammatory,⁴ and required for macrophage survival.⁵ Species of the genus *Yersinia* silence the proinflammatory response by inhibiting MAPK and NFkB with proteases introduced into the macrophages via a type III secretion system.^{6, 7} In the absence of these proinflammatory signals, TLR4-induced TRIF and other proapoptotic pathways cause the macrophage to die via caspase-3-dependent apoptosis without initiating an appropriate immune response.⁸ Macrophages that are exposed to LPS prior to infection by *Yersinia* instead undergo proinflammatory cell death characterized by the release of the proinflammatory cytokines interleukin- (IL) 1 β and IL-18.^{9, 10} This proinflammatory cell death, termed pyroptosis, is dependent on caspase-1, a cysteine protease that is not involved in apoptosis.¹¹ These dramatically different responses to the same stimulus suggest that activation of macrophages by LPS stimulates expression of proteins that play a key role in the life/death decision of a macrophage.

Numerous studies have been published detailing specific genes, proteins, and pathways affected by LPS activation, but there have been few attempts to characterize the phenomenon at the whole proteome level. Zhang et al¹² performed 2-dimensional gel electrophoresis (2DE) on naïve and LPS-treated RAW 264.7 cultured mouse macrophages. More than 400 proteins were separated and detected, but only the 11 most differently expressed gel spots were taken for identification by mass spectrometry, yielding only 7 unique identified proteins. Gadgil et al¹³ performed 2DE on LPS-treated human monocytes. Despite detecting more than 800 separated components, only 20 spots with significant changes in intensity were selected for identification by mass spectrometry, identifying 16 unique proteins. These two 2DE studies showed no overlap between the proteins identified as being expressed at significantly different levels. Gu et al¹⁴ used stable isotope labeling with amino acids in cell culture (SILAC) to investigate which proteins are differentially regulated by LPS-activation of GG2EE cultured macrophages from mice expressing LPS-hyposensitive TLR4. They identified 900 proteins of which 35 were differently regulated between wild-type and TLR4-mutant macrophages. Among the proteins whose up-regulation was attenuated by a hyposensitive TLR4 was IL-1 β . Patel et al¹⁵ used shotgun proteomics with quantification by spectral counting to investigate the effect of LPS and IFN γ activation of RAW 264.7 macrophages on microtubule associated proteins. They identified 409 proteins of which 94 were up- or down-regulated by a factor of 2 or more.

In this study we employed isotope coded affinity tagging (ICAT),¹⁶ a technique involving stable isotope labeling, multi-dimensional chromatography, and tandem mass spectrometry, to study at the proteome level the effect of LPS exposure on RAW 264.7 and C57BL/6 bone marrow-derived macrophages. We identified and quantified over 1000 proteins and employed statistical methods to identify 36 high-quality candidate proteins exhibiting significant up- or down-regulation due to LPS activation. Additionally, we compared our results with those of the above mentioned previous studies as well as with mRNA microarray data.

EXPERIMENTAL PROCEDURES

Specimens

Three replicate ICAT analyses were performed with RAW 264.7 macrophages and two replicate ICAT analyses were performed with C57BL/6 macrophages. The naïve cells used as the control sample in all three RAW 264.7 replicates were harvested at the same time. The LPS-activated RAW 264.7 macrophages used in replicate 1 were harvested from a different treatment than those used in replicates 2 and 3, which were harvested from the same treatment. The two C57BL/6 ICAT replicates were performed on different batches of macrophages. In each replicate, the naïve and LPS-treated macrophages were derived from the same culture. The RAW264.7 macrophage cell line was purchased from the American Type Culture Collection and cultured at 37 °C with 5% CO₂ in Dulbecco's minimal essential medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% FCS, 5 mM HEPES, 0.2 mg/ml L-glutamine, 0.05 mM β-mercaptoethanol, 50 mg/ml gentamicin sulfate and 10000 U/ml penicillin and streptomycin (DMEM-10). Bone marrow-derived macrophages were isolated from the femur exudates of C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and cultured for 7 days at 37 °C in 5% CO₂ in DMEM-10 supplemented with 30% L-cell-conditioned medium. The cells were analyzed by FACS and found to be 85%-94% positive for the macrophage marker CD11b. Macrophages were activated for 18-20 hours with ultra pure LPS from *Salmonella minnesota* (List Biologicals, Campbell, CA) at a final concentration of 100ng/ml, harvested using cell dissociation buffer (Invitrogen), pelleted, and stored at -80 °C until analysis.

Sample Preparation

Cellular homogenates were prepared by adding 100 µL 50mM tris(hydroxymethyl)aminomethane/3.5mM sodium dodecyl sulfate (Tris/SDS; Sigma Aldrich, St. Louis, MO) per 10⁶ cells to each tube and subjecting them to high-power sonication at 5 °C for 8 minutes at a duty cycle of 60. Tris(2-carboxyethyl)phosphine (TCEP; Sigma Aldrich) was added to a final concentration of 5mM in each of the tubes, which were then incubated 10 minutes in a 100 °C water bath. The sample tubes were centrifuged 10 minutes at 15000 rcf to settle the precipitate and the supernatant was transferred to fresh tubes. The total protein concentration of each sample was determined by triplicate analysis with a BCA assay (Pierce, Rockford, IL). Tris/SDS lysis buffer was added to a known volume of the more concentrated sample so that the naïve and LPS-treated samples had the same initial protein concentration.

ICAT Labeling

The ICAT protocols described below were based largely on modified protocols developed by the Goodlett group at the University of Washington.¹⁷ ICAT reagents were acquired from Applied Biosystems (Foster City, CA). For each of the three RAW 264.7 replicates and for the first C57BL/6 replicate, 6 tubes heavy label and 6 tubes light label were used, each tube containing 100µg (175nmol) label. The contents of each tube of label were dissolved in 20µL acetonitrile, then a volume of lysate containing 200µg of protein was added to each tube, naïve to the light ICAT label and LPS-treated to the heavy ICAT label. For the second C57BL/6 replicate, 10 tubes each of heavy and light ICAT label were used and lysate containing 150µg of protein was added to each tube. Assuming an average molecular weight of 50kDa and an average of 10 cysteines per protein, 200µg protein is approximated to contain 40nmol cysteine, giving a 4.4-fold molar excess of label to available cysteines in the labeling reaction (5.8-fold for 150µg protein). The tubes were incubated 2 hours at 37 °C, after which dithiothreitol (DTT; Gold Biotechnology, St. Louis, MO) was added to each tube to a final concentration of 12mM and allowed to incubate 10 minutes at room temperature. DTT acts as a scavenger, reacting with excess ICAT reagent to prevent cross-

contamination after combining the samples. The contents of all the tubes were combined and 0.1mg/mL sequencing grade modified trypsin (Promega, Madison, WI) in 5mM CaCl₂ (Sigma Aldrich) was added to the labeled sample to achieve a trypsin:protein ratio of 1:40 (w/w). The sample was then incubated 15 hours at 37 °C.

Strong Cation Exchange and Avidin Affinity Chromatography

Tryptic peptides were pre-fractionated by strong cation exchange using a PolySULFOETHYL A column (200mm × 4.6mm, 5μm beads, 300Å pores; PolyLC, Columbia, MD) on a Vision Workstation (Perseptive/Applied Biosystems). The mobile phase was 10mM KH₂PO₄ (Fisher Scientific, Pittsburgh, PA) in 25% acetonitrile (J.T. Baker, Phillipsburg, NJ) adjusted to pH 3.0 with phosphoric acid (Sigma Aldrich). Peptides were eluted at 0.8mL/min by an increasing gradient of KCl (EMD, San Diego, CA) according to the following program: 0-90mM KCl in 8 column volumes; 90-170mM KCl in 3 column volumes; 170-350mM KCl in 2 column volumes; 350-500mM KCl in 1.5 column volumes; and an additional 2 column volumes at 500mM KCl. The peptides were collected in 40 fractions of 1 mL each. Each fraction was collected into a tube containing 1mL 2X PBS (EMD) and 200μL 100mM Na₂HPO₄ (Fisher) pH 10.0, which brought the fraction to pH 7 in preparation for affinity chromatography. The ICAT-labeled peptides in each fraction were then isolated by avidin affinity chromatography with Applied Biosystems avidin cartridges and protocols developed for the Vision Workstation by Applied Biosystems. Each affinity-enriched fraction was then acid-cleaved according the ICAT protocol provided with the reagents.

Reverse Phase Liquid Chromatography and Mass Spectrometry

Each dried fraction was reconstituted in 2% acetonitrile (J.T.Baker)/5% acetic acid (EMD) and separated by reverse phase high performance liquid chromatography using an Agilent 1100 and an in-house packed column (75μm × 10.5cm, Jupiter Proteo C12; Phenomenex, Torrance, CA). The gradient profile from solvent A (0.6% acetic acid in H₂O) to 100% solvent B (0.6% acetic acid in CH₃CN) was as follows, at a flow rate of 250nL/min: 0-3 minutes 98% A; 3-6 minutes 98% A-94% A; 6-50 minutes 94% A-60% A; 50-60minutes 60% A-0% A; 60-70minutes 0% A. Eluent was introduced onto a Finnigan LCQ ion trap by positive mode electrospray ionization. The MS1 survey scan was limited to m/z 300-2000. The 4 most abundant ions of each scan were selected for collision-induced dissociation and subsequent MS2 scans. Dynamic exclusion was enabled such that a MS1 ion observed twice within a one-minute window (with a mass tolerance of -0.5/+1.1) was ignored for MS2 for the following 1.5 minutes, with a maximum exclusion list size of 30. Each LC-MS analysis was performed in triplicate.

Gas Phase Fractionation

For the second C57BL/6 ICAT replicate, the 5 most peptide-1 rich SCX fractions as determined from the first LC-MS replicate were analyzed by a gas-phase fractionation (GPF)¹⁸ protocol in which each sample was analyzed three separate times, limiting the MS1 to 300 - 670 Th the first time, 665-813 Th the second time, and 808-2000 Th the third time. The parameters for the gas phase fractions were determined by grouping all of the ~12,000 unique, high quality (Peptide Prophet probability > 0.9) peptides identified from all three RAW 264.7 experiments into three groups with equal numbers of peptides in each. Each GPF experiment was replicated three times per fraction analyzed.

Protein Identification and Quantification

Peptide and protein identification and quantification were done through the Trans Proteomic Pipeline (TPP) v.4.2, developed by the Institute for Systems Biology.¹⁹ Mass spectra were

searched through Sequest version 27 (executable provided by Jimmy Eng, University of Washington) against ipi.MOUSE.v3.48.fasta (published 2 September 2008 by IPI) appended to add human keratin and bovine serum albumin. The following Sequest search parameters were used: Fully tryptic cleavages (K and R) with up to two missed cleavages; fixed modification of 227.13 on all cysteines with a variable modification of 9 for acid-cleavable light and heavy ICAT labels; variable modification of 16 for oxidation of methionine; mass tolerance of 1.0 for parent ions and 0.0 (default) for fragment ions. Peptides were identified by Peptide Prophet²⁰ and proteins were identified by Protein Prophet.²¹ In the case where identified peptides corresponded to multiple proteins, the protein assigned the highest probability by Protein Prophet's Occam's razor function was accepted from among such protein groups. The ratios of light:heavy-labeled proteins were quantified by XPRESS²², which calculates a mean protein ratio and standard deviation for each protein from the ratios of all the quantified peptides used to make the protein identification. Protein IPI numbers were used to obtain Entrez Gene numbers and gene symbols through ISB's Protein Information and Property Explorer (PIPE).²³ A literature search of identified proteins in PubMed was aided by iHOP.²⁴ Protein Prophet results were manually validated. The only ratios that were altered were those in which one or more peptide XPRESS ratios were incorrectly set to 0, as was the case when the m/z of a peptide was near the limit of the GPF fraction and only the light- or heavy-labeled peptide was quantified while its corollary heavy- or light-labeled partner was excluded. In those cases, the incorrect ratio was removed and the ratio mean and standard deviation protein ratio was recalculated.

Data Analysis

The default protein identification parameters in the TPP use nearly all identified peptides, filtering out only the poorest identifications (peptide probability < 0.05). While expanding the peptide pool in this manner increases the likelihood of finding multiple identifications of multiple tryptic peptides per protein, thus improving identification and quantification statistics, it also increases the likelihood of skewing the final protein ratio by including peptide ratios of incorrectly identified peptides. We chose to include only peptides identified with a Peptide Prophet probability greater than 0.9 (error rate 2% in all replicates) for protein identification and quantification. It is typical to report only proteins with Protein Prophet probabilities greater than 0.9. We accepted all non-zero Protein Prophet probabilities since our identifications were made with only high-quality peptides. The number of single hit proteins (proteins identified by only one tryptic peptide) was greater and the Protein Prophet probabilities were lower than if we had included lower quality peptide identifications, but this was seen as an acceptable trade-off for higher accuracy quantification. Confidence in identification was increased by only accepting proteins identified in multiple ICAT replicates.

Of the two quantification programs bundled with the TPP, ASAP is by far the more sophisticated, employing base-line subtraction, quantification of the peptide at multiple charge states, grouping of multiple spectra from the same peptide, outlier tests to reject seemingly incorrect peptide ratios, and an algorithm to correct bias and provide a p value for protein ratios.²⁵ XPRESS is much simpler: it quantifies peptide ratios without background subtraction and reports the mean and standard deviation of all peptide ratios associated with a certain protein.²² We chose to use the XPRESS ratios and perform our own statistical analysis because we observed that while ASAP's advanced algorithms perform quite well for high quality, high abundance mass spectra, the background subtraction algorithms and multiple charge state detection begin to introduce gross errors when the detected ion has a low signal-to-noise ratio. Since many of the proteins that are significantly up- or down-regulated have low abundance peptides in either the light- or heavy- labeled sample,

ASAP's quantification errors disproportionately affect the protein ratios of the greatest interest.

The three replicate LC-MS analyses for each ICAT replicate were combined and analyzed together. Only peptides identified by Peptide Prophet with probabilities ≥ 0.9 were accepted for protein identification and quantification. The Peptide Prophet models for the three RAW 264.7 ICAT replicates had the following sensitivities and errors at a minimum probability to accept threshold (MPT) of 0.9: replicate #1 sensitivity 0.673, error 0.016; replicate #2 sensitivity 0.643, error 0.017; replicate #3 sensitivity 0.658, error 0.016. The two C57BL/6 ICAT replicates had the following sensitivities and errors at a MPT of 0.9: replicate #1 sensitivity 0.644, error 0.018; replicate #2 sensitivity 0.638, error 0.020. Since only high quality peptides were taken for protein identification, all proteins identified by Protein Prophet with non-zero probabilities were accepted (model sensitivity = 1.00). The Protein Prophet models gave the following minimum non-zero probabilities and errors: RAW #1 lowest probability 0.4622, error 0.097; RAW #2 lowest probability 0.5990, error 0.060; RAW #3 lowest probability 0.4805, error 0.095; C57BL/6 #1 lowest probability 0.3702, error 0.148; C57BL/6 #2 lowest probability 0.4510, error 0.118.

The protein ratios were normalized based on the method employed in the program ASAPRatio.²⁵ For each ICAT replicate, a Gaussian curve was fit to the plot of the frequencies of the $\log_{10}$ of all the accepted ratios of light-labeled:heavy-labeled (naïve:LPS-treated) proteins. The mean ratio extracted from this curve was used to normalize the data to center around a mean Naïve:LPS-treated ratio of 1:1 ($\log_{10}[\text{ratio}] = 0$) by setting $r_n = r_p/r_0$, where r_n is the normalized protein ratio, r_p is the initial XPRESS protein ratio, and r_0 is the mean ratio extracted from the curve. The standard deviation Δr_n of each normalized protein ratio r_n was calculated as $\Delta r_n = r_n [(\Delta r_p/r_p)^2 + (\Delta r_0/r_0)^2]^{0.5}$ where Δr_p is the standard deviation of the unadjusted protein ratio and Δr_0 is the fitting error extracted from the curve. The normalized protein ratios from the three RAW ICAT replicates were combined, as were the normalized protein ratios from the two C57BL/6 ICAT replicates (figure 1). For proteins found in two or more replicates, the mean ratio r_m was taken and the error calculated as $\Delta r_m = r_m [(\Delta r_1/r_1)^2 + (\Delta r_2/r_2)^2 + (\Delta r_3/r_3)^2]^{0.5}$. A Gaussian curve was fit to the $\log_{10}$ of the ratios of this new population comprised of all the proteins identified for the cell type (C57BL/6 or RAW 264.7). The ratios were again normalized, though the mean for each population was already very close to zero ($\log[\text{naïve:LPS-treated}]$ was -5.15×10^{-3} for the combined C57BL/6 and 5.94×10^{-3} for the combined RAW 264.7). A p value was calculated for each protein ratio r_m by the equation $p = \text{erfc}[|\log(r_m/r_0)|/(2((\Delta \log r_m)^2 + (\Delta \log r_0)^2 + \sigma^2)^{0.5})]$ where $\Delta \log r = 0.4343 \Delta r/r$.

Western Blot of Sqstm1

The cellular homogenates used to perform ICAT analysis were also used for Western blot analysis to confirm that expression of sequestosome-1 (Sqstm1) is increased following treatment with LPS. 60µg of total protein from each sample (RAW +/- LPS and C57BL/6 +/- LPS) was separated by 15% SDS-PAGE and transferred to a nitrocellulose membrane. Sqstm1 expression was assessed by Western blotting using a mouse anti-Sqstm1 antibody (clone 2C11, Sigma) followed by a peroxidase-conjugated secondary antibody (Amersham Biosciences, Buckinghamshire, UK). The membrane was then stripped and reprobed with a peroxidase-labeled anti-beta-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) to confirm equal loading. Antibody binding was detected with an enhanced chemiluminescence system (Amersham Biosciences). Quantification of immunoblots was performed by densitometry using NIH Image, version 1.63.

RESULTS

Identification and Quantification of Proteins

Isotope Coded Affinity Tagging (ICAT) was employed to identify and quantify the differential expression of proteins in macrophages activated by lipopolysaccharide (LPS). A total of five ICAT analyses were performed, three replicates with RAW 264.7 cultured mouse macrophages and two replicates with C57BL/6 bone marrow derived mouse macrophages. RAW 264.7 macrophages are derived from virally-induced leukemia in Balb/c mice. We chose to include RAW 264.7 cells in our study because they are widely used in investigations of innate immunity and are easy to culture. However, caution is warranted when using RAW 264.7 macrophages as a model system. It stands to reason that an immortalized cell line derived from cancerous tissue will be in some ways fundamentally different than primary cells. For this reason we also included in our study macrophages derived directly from C57BL/6 mice.

Macrophages were treated with LPS for 18-20 hours to promote activation of the cells. The protein concentration was determined and equalized between cellular homogenates of naïve and LPS-activated macrophages. The naïve and LPS-activated homogenates were labeled with light and heavy ICAT tags, respectively, then combined and digested with trypsin. The tryptic digests were separated into 40 fractions by strong cation exchange chromatography (SCX) and the labeled peptides of each fraction were isolated by avidin affinity chromatography. The isolated labeled peptides from each SCX fraction were then separated by reverse phase high performance liquid chromatography (RP-HPLC) and introduced onto an ion trap mass spectrometer by electrospray ionization. Each fraction was subjected to at least three replicate LC-MS analyses.

Sequest and the Trans Proteomic Pipeline were used to identify proteins by searching the MS2 fragmentation spectra of the tryptic peptides against a FASTA database of the mouse genome. Pooling the results of the 5 ICAT replicates of both cell types, peptides corresponding to a total of 1064 unique proteins were identified at high confidence and quantified with XPRESS ratios. 860 unique proteins with naïve:LPS-activated ratios were identified from the three replicate RAW 264.7 ICAT analyses; 274 were identified in all three replicates, 178 were identified in only two replicates, and 408 were identified in only one replicate (Supplemental Table 1). 549 unique proteins with naïve:LPS-activated ratios were identified from the two C57BL/6 ICAT analyses; 247 proteins were identified in both replicates and 302 were identified in only one replicate (Supplemental Table 2). Of the 1064 proteins identified in at least 1 ICAT replicate, 345 proteins were found in both cell types.

As would be expected, we observed some overlap in the proteins identified and agreement in the levels of protein expression between the two cell types. However, there were also many proteins identified in one cell type and not the other, as well as proteins identified in both cell types but with markedly different and sometimes contradictory expression levels. Some of the difference in protein coverage is attributable to the ICAT technique: only the most abundant proteins are reliably identified and quantified, and the stochastic sampling of the ion stream in the mass spectrometer means that no two replicates will identify exactly the same peptides. Differences in expression, on the other hand, likely reflect real differences between the cell types. Conversely, for proteins that are identified in both cell types as having significant differential regulation and for which there is agreement concerning the magnitude and direction of change, the case is made even stronger for a significant role in innate immunity that is not unique to the model organism.

The program XPRESS was used to obtain ratios of light-labeled:heavy-labeled (naïve:LPS-activated) proteins. The normal distribution of protein ratios was used to correct

experimental bias and to assign p values for determining the significance of the ratios. Proteins were considered likely to be positive indicators of macrophage activation if they were identified in at least two RAW 264.7 ICAT replicates or in both C57BL/6 ICAT replicates and if the ratio of light-labeled:heavy-labeled protein had a p value < 0.05. 24 such proteins were identified from RAW 264.7 macrophages and 15 were identified from C57BL/6 bone marrow derived macrophages (Tables 1 and 2, Supplemental Table 3). Two proteins, Sequestosome 1 (Sqstm1) and predicted gene product RIKEN cDNA 9230020A06, were found among the significant proteins of both RAW 264.7 and C57BL/6. In many cases, the significant proteins identified in one cell type were also identified in the other cell type but did not meet the above criteria for a significant result. Tubulin beta 4 (Tubb4, IPI00109073.5) was identified as significantly down-regulated by LPS activation in RAW 264.7 according to the above criteria, but this result was discarded because the peptides used to make the identification were also associated with several other isoforms of tubulin and it could not be confirmed that Tubb4 alone was the source of the quantified peptides.

Western Blot of Sqstm1

Sqstm1 was selected for validation by Western blot because it was one of the highest quality candidates, (identified as significantly up-regulated by LPS in all 5 ICAT replicates) and because it has a commercially available monoclonal antibody. Figure 2 shows the results of the Western blot confirming that Sqstm1 is up-regulated by LPS activation in both RAW 264.7 and C57BL/6 macrophages. The level of Sqstm1 protein was observed to increase 36-fold in LPS-treated RAW 264.7 cultured mouse macrophages and 17-fold in C57BL/6 bone marrow derived macrophages.

Comparison with Microarray Data

The Institute for Systems Biology has published on their website ²⁶ the results of two-color microarray expression experiments with RAW 264.7 cultured mouse macrophages treated with LPS for various lengths of time. Tables 1 and 2 compare the ratios of the significant proteins identified here by ICAT with those determined by the ISB with microarray. Of the 35 proteins we identified as having significant differential regulation, 30 were also identified in the microarray study. Comparing our protein expression results after 18-20 hours of exposure of RAW 264.7 macrophages to LPS with mRNA expression levels after 24-hour exposure, there is a ~75% agreement with respect to up-regulation, down-regulation, or no significant change.

DISCUSSION

Rationale for the ICAT Technique

The authors are aware of the limitations of ICAT compared to other currently available stable isotope labeling techniques such as iTRAQ, most notably that labeling and selecting for analysis only cysteinyl peptides greatly reduces the total number of peptides available for protein identification. ICAT was selected for two reasons: first, the biological system in question, activated vs. naïve macrophages, is a test vs. control experiment ideally suited for testing by ICAT; second, and more importantly, ICAT is a well-established technique which was well-suited to our capabilities at the time the experiment was designed and undertaken. Sample preparation has been automated by the manufacturers of the Vision Workstation, data analysis software (the Trans Proteomic Pipeline from the Institute for Systems Biology) is freely available with excellent technical support, and the samples can be analyzed on widely available ion trap mass spectrometers which are unable to detect the low mass range reporter ions of iTRAQ. While ICAT has its limitations, we make the case here that, with the appropriate data analysis, the technique can still be used to obtain meaningful proteomic information.

ICAT for Identification of Significant Proteins

Extracting biologically significant information from ICAT experiments is not a simple, straight-forward procedure. Rather, at each step in the identification and quantification of peptides and proteins the researcher must decide on threshold probabilities and confidence intervals. SEQUEST, Peptide Prophet, and Protein Prophet provide statistical tools that can be used to predict the quality of protein identifications by modeling sensitivity (the percentage of total correct identifications reported as correct) and error (the percentage of reported identifications that are incorrect). The protein ratios calculated by the Trans Proteomic Pipeline provide further metrics for error analysis.

The method we used for correcting and assigning a p value to protein ratios is similar to the method described in the ASAP publication, the only difference being that we applied the method to the population of observed protein ratios rather than peptide ratios. Using this method was very important for two reasons: first, fitting the population of observed protein ratios to a normal distribution allowed us to observe and correct a non-reproducible and often significant bias in the observed protein ratios; second, the p value gave us an objective and statistically defensible way to determine which proteins were significantly differently expressed. Many of the proteins ratios we report as being significant may not appear to be so at first glance, differing less than two-fold from control, but emerge as being significant when seen in context of the population as a whole. It is quite likely that the protein ratios observed do not actually reflect the true concentrations. For example, the ICAT ratios we report for Sqstm1 show a roughly 3-fold higher concentration in LPS-activated C57BL/6 and RAW 264.7 macrophages, but a Western blot of the same sample shows Sqstm1 to be present in LPS-activated macrophages at a concentration 17-fold greater than control in C57BL/6 and 36-fold greater than control in RAW. As the signal-to-noise level of the mass spectra of the peptide ions decrease, the magnitude of the protein ratios is masked. Examining the protein ratios in the context of the whole population of observed ratios provides a means of identifying significant protein ratios in spite of the masking effect of poor spectra.

Sequestosome 1

Sequestosome 1 (Sqstm1) was one of only two proteins identified in both RAW 264.7 and C57BL/6 as a significant indicator of LPS-activation. One published study did not observe any increase in Sqstm1 after treatment of murine macrophages with epidermal growth factor, LPS, or TNF α , and proposed that Sqstm1 plays a role in oxidative stress response signal transduction.²⁷ Our results are more consistent with the observation that Sqstm1 is involved with regulation of NF κ B as seen in response to IL-1 and TNF α signaling by its formation of a complex with aPKC.²⁸ Like recognition of LPS by TLR4, IL-1 initiates a MyD88-dependent activation of NF κ B.² In this pathway it has been seen that a complex formed by Sqstm1, aPKC, and TRAF6 is required for IL-1 activation of NF κ B. Furthermore, depletion of Sqstm1 has been observed to significantly decrease activation of NF κ B by both IL-1 and TNF α .^{29, 30} Our ICAT experiments and Western blot analysis showed LPS activation to cause significant up-regulation of Sqstm1. Additionally, the ISB's mRNA experiment showed a 3-fold increase in expression of the Sqstm1 gene after 1 hour of LPS treatment, reaching a maximum 7-fold up-regulation after 2 hours and remaining at least 4-fold up-regulated for the duration of the 24 hour exposure, and Patel et al found Sqstm1 to increase 4-fold upon activation of RAW 264.7 with LPS and IFN γ .¹⁵

Comparison with Literature

Based on previous studies we would expect treatment of macrophages with LPS to activate factors downstream of TLR signaling, such as proteins involved in the MAPK cascade, NF κ B activation, antiviral and interferon responses, regulation of apoptosis, and expression

of pro-inflammatory cytokines. NFkB is known to be one of the primary transcription factors involved in the proinflammatory response to LPS activation. We observed increased levels of the NFkB subunit Nfkb2 after LPS treatment of C57BL/6 macrophages. No significant change in Nfkb2 was observed in RAW 264.7 macrophages, though the ISB's mRNA study showed transcription of the Nfkb2 in RAW 264.7 macrophages to increase significantly upon treatment with LPS, reaching a maximum 13-fold greater than control after 8 hours. Vasodilator-stimulated phosphoprotein (VASP) is phosphorylated by Protein Kinase A (PKA) in a mitogen-activated protein kinase kinase kinase (MEKK1)-dependent fashion that involves stimulation of the NFkB pathway.³¹ We observed LPS activation to cause Vasp levels to increase in both C57BL/6 and RAW 264.7. The ISB's mRNA study showed expression of the Vasp gene in RAW 264.7 reaching a maximum (3-fold greater than control) after 4 hours of treatment with LPS and decreasing nearly to baseline after 24 hours. Oddly, Patel et al observed Vasp levels to decrease nearly 10-fold in RAW 264.7 macrophages after 12 hours of exposure to LPS and IFN γ . Inducible nitric oxide synthase (iNOS) is involved in inflammation and has been shown to be produced in response to activation of NFkB by LPS.³² Catalase (Cat), expression has been seen to be increased by LPS activation of human monocytes¹³, and has been shown to induce NFkB-dependent expression of iNOS in RAW 264.7 macrophages,³³ as well as to inhibit TNF α -induced apoptosis in murine cerebral microvascular endothelial cells.³⁴ We detected Cat but not iNOS. Our results and those of the ISB's mRNA study showed levels of Cat to increase in LPS-treated RAW 264.7, although Cat levels were seen to decrease in C57BL/6.

A somewhat unexpected result was found with aldo-keto reductase family 1, member B3 (Akr1b3), which has been shown to modulate NF-kB-dependent activation of inflammatory cytokines and chemokines. Inhibiting Akr1b3 has been seen to prevent biosynthesis of TNF α and IL-1 β in LPS-treated RAW 264.7 macrophages, significantly attenuating the LPS-induced activation of PKC and nuclear translocation of NFkB, and ultimately attenuating the cytotoxicity of LPS-activation.³⁵ SILAC studies by Gu et al have shown Akr1b3 to be up-regulated in a TLR4-dependent fashion in LPS-treated GG2EE murine macrophages.¹⁴ Patel et al observed a slight, though not significant, decrease (1.6-fold, p value of 0.21) in LPS/IFN γ -activated RAW 264.7 cells.¹⁵ We observed no significant change in levels of Akr1b3 in RAW 264.7 macrophages, and we observed a decrease in Akr1b3 levels in C57BL/6 macrophages. Furthermore, the ISB's mRNA experiment showed a steady decrease in mRNA transcription of Akr1b3 in RAW 264.7 beginning at 1 hour of LPS-treatment and reaching a 4-fold decrease after 24 hours of exposure. It is curious that a gene that has been shown to be essential to the NFkB-dependent response to LPS appears to be down-regulated by this same stimulus. It is possible that this gene product is only required during the initial stages of LPS activation and is subsequently turned down by a negative control feedback loop.

Some of the significant proteins we identified have previously been implicated in response to viral infection. DEAD (Asp-Glu-Ala-Asp) box polypeptide 58 (Ddx58), a.k.a. Retinoic acid-inducible gene (Rig-1), has been shown to initiate production of type I interferon as an innate antiviral response³⁶ via a pathway that is independent of toll-like receptor pathways.³⁷ We observed LPS activation to cause down-regulation of Ddx58/Rig-1 in both C57BL/6 and RAW 264.7 macrophages, though it was not observed in the ISB's mRNA experiment. The 2'-5'-oligoadenylate synthetase-like (Oasl) proteins are homologues of human 2'-5'-oligoadenylate synthetases, interferon-induced proteins with roles in antiviral response and apoptosis.³⁸ We observed up-regulation of Oasl1 by LPS-treated RAW 264.7. Furthermore, the mRNA study showed expression of the Oasl1 gene increasing with LPS activation of RAW 264.7, reaching a maximum 68-fold increase compared to control after 24 hours. Interestingly, however, one study has suggested that murine Oasl1 is not active as an OAS (a role filled by Oasl2), and has evolved some as-yet unidentified function.³⁹

CD40 antigen, a member of the tumor necrosis factor receptor superfamily, is a B-cell surface antigen that is a marker of cell maturation. Monocyte-derived dendritic cells (DC) infected with paramyxovirus Simian Virus 5 that were treated with pancaspase inhibitor have been observed to have decreased expression of CD40. In the same study, treatment of infected DC with either LPS or IL-1 β was seen to cause enhanced surface expression of CD40 and other surface antigen maturation markers in a NF κ B-dependent fashion while reducing cytopathic effect and apoptosis.⁴⁰ We observed LPS treatment to cause CD40 antigen levels to increase in C57BL/6 macrophages activated by LPS. The ISB mRNA study showed the CD40 gene reaching maximum expression in RAW 264.7 cells (8-fold up-regulation compared to control) after 8 hours of treatment with LPS.

Some of the significant indicators of LPS activation we observed have previously been seen to be inducible by LPS and interferon (IFN). Interferon-induced protein with tetratricopeptide repeats 3 (Ifit3), a.k.a. glucocorticoid-attenuated response gene 49 protein (Garg49), has previously been shown to be induced in RAW 264.7 macrophages by LPS and interferons alpha, beta, and gamma.⁴¹ We observed LPS treatment to cause up-regulation of Ifit3/Garg49 in both C57BL/6 and RAW 264.7 macrophages, an effect consistent with the ISB's mRNA experiment. We observed Interferon-induced guanylate-binding protein 5 (Gbp5) to be up-regulated by LPS treatment in C57BL/6, though it was not observed in either ICAT or mRNA RAW 264.7 experiments. Macrophage myristoylated alanine-rich C kinase substrate (Marcks11 or MacMarcks), a substrate of protein kinase C (PKC), has been seen to be induced by LPS and plasmid DNA in RAW 264.7 and bone-marrow derived macrophages⁴². We observed large (3-fold) increases in Marcks11 in both C57BL/6 and RAW 264.7 macrophages in response to LPS activation. The ISB mRNA study showed the Marcks11 gene reaching maximum expression in RAW 264.7 (22-fold up-regulation compared to control) after 4 hours of treatment with LPS and decreasing to roughly 6-fold increased transcription after 24 hours of LPS exposure, and Patel et al observed a nearly 5-fold increase after activation of RAW 264.7 cells with LPS and IFN γ .¹⁵ Another PKC substrate, Pleckstrin (Plek), has also been shown to be induced by LPS and IFN-gamma and is thought to be involved in phagocytosis.⁴³ We observed LPS activation to increase levels of Plek in both C57BL/6 and RAW 264.7. The ISB mRNA experiment showed expression of the Plek gene reaching a maximum in RAW 264.7 macrophages (4-fold increase compared to control) after 8 hours of treatment with LPS, decreasing to roughly 2-fold greater than control after 24 hours of treatment. Patel et al observed a slight, though not significant, increase (1.4-fold, p value of 2.74) in LPS/IFN γ -activated RAW 264.7.¹⁵

CONCLUSION

We have employed ICAT to study differential protein expression resulting from LPS activation of RAW 264.7 cultured macrophages and C57BL/6 bone marrow derived macrophages. We used statistical methods to correct for experimental bias and identify proteins with significant differential expression. Many of the 36 proteins identified as markers of LPS activation are consistent with current models of pro-inflammatory pathways in innate immunity and were consistent with findings of other studies employing different methods to study activated macrophages. Still, more than half of the significant proteins we identified here have not previously been shown be directly involved with pro-inflammatory pathways, including two hypothetical gene products whose identity has yet to be disambiguated. Further studies will be needed to validate and elucidate their role in pro-inflammatory cell death.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

2DE	2-dimensional gel electrophoresis
BCA	bicinchoninic acid
C57BL/6	C57 black 6
DMEM	Dulbecco's minimal essential medium
DTT	dithiothreitol
FACS	fluorescence activated cell sorting
GPF	gas-phase fractionation
HPLC	high performance liquid chromatography
ICAT	isotope coded affinity tagging
iHOP	information hyper-linked over proteins
IL	interleukin
ISB	The Institute for Systems Biology
LPS	lipopolysaccharide
LC-MS	liquid chromatography-mass spectrometry
MAPK	mitogen-activated protein kinase
NFκB	nuclear factor-kappa B
PAGE	polyacrylamide
PIPE	Protein Information and Property Explorer
RAW	mouse leukaemic monocyte macrophage cell line
RCF	relative centrifugal force
SCX	strong cation exchange
SDS	sodium dodecyl sulfate
SILAC	stable isotope labeling with amino acids in cell culture
TCEP	tris(2-carboxyethyl)phosphine
TLR	toll-like receptor
TNFα	tumor necrosis factor alpha
TPP	Trans Proteomic Pipeline
Tris	tris(hydroxymethyl)aminomethane

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Figure 1. Distribution of protein ratios obtained from the combined ICAT replicates

The histograms show the distribution of corrected log₁₀ of the ratios of light:heavy ICAT labeled proteins (naïve:LPS-treated). **A)** The normal curve fit to the distribution of the combined C57BL/6 experiments has a mean value of 0.005 ± 0.005 with $\sigma = 0.094 \pm 0.005$ and $\chi^2 = 1275$. **B)** The normal curve fit to the distribution of the combined RAW 264.7 experiments has a mean value of -0.006 ± 0.003 with $\sigma = 0.028 \pm 0.003$ and $\chi^2 = 1291$.

Figure 2. Sequestosome-1 expression is increased in LPS-treated RAW 264.7 and bone marrow-derived C57BL/6 macrophages

Protein lysates were generated from RAW264.7 and C57BL/6 macrophages that had been incubated overnight with 100ng/ml LPS or left untreated. Equal amounts of macrophage cell lysate were separated by SDS-PAGE and analyzed by Western blotting with an anti-Sqstm1 antibody. The level of Sqstm1 protein increased 36-fold in RAW264.7 cells and 17-fold in C57BL/6 macrophages, as determined by NIH Image analytical software. The membrane was then reprobbed with an anti-β-actin antibody to confirm equivalent protein loading.

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Proteins significantly up-regulated by LPS-activation

Table 1

IPI	Entrez Gene	Gene Symbol	Description	RAW 264.7 LPS:Naïve ^a	p	coverage	peptides ^b	spectra ^c	ISB mRNA LPS:Naïve ^d	C57BL/6 LPS:Naïve ^a	p	coverage	peptides ^b	spectra ^c
IP100652070.1	619299	9230020A06Rik	RIKEN cDNA 9230020A06 gene	3.99 ± 0.06	4.40E-09	4.7%	1	2	-	6.26 ± 1.23	3.9E-10	4.7%	1	4
IP100133374.5	18412	Sqstm1	sequestosome 1	3.66 ± 2.81	0.018	20.1%	4	35	5.79	2.80 ± 0.10	2.9E-06	5.9%	1	4
IP100281011.7	17357	Mareks1l	MARCKS-like 1	2.81 ± 0.39	1.50E-04	17.5%	1	12	5.57	2.57 ± 0.46	8.1E-04	17.0%	1	5
IP100331612.3	15364	Hmga2	high mobility group AT-hook 2	2.51 ± 0.40	1.30E-03	12.0%	1	5	1.42	-	-	-	-	-
IP100280233.1	219132	D14Erd668e	DNA segment, Chr 14, ERATO Doi 668, expressed	2.26 ± 0.40	5.70E-03	9.3%	1	27	-	2.39 ± 0.29	4.4E-04	9.3%	1	6
IP100119551.1	56193	Plek	pleckstrin	1.97 ± 0.44	0.038	9.4%	2	17	1.91	1.52 ± 0.12	0.071	4.0%	1	8
IP100128953.3	231655	Oasl1	2'-5' oligoadenylate synthetase-like 1	1.89 ± 0.33	0.031	5.9%	2	11	67.58	-	-	-	-	-
IP100123570.3	15953	Ifi47	interferon gamma inducible protein 47	1.88 ± 0.19	0.014 19	5.0%	1	8	0.67	2.33 ± 0.03	9.8E-05	5.0%	1	1
IP100312058.5	12359	Cat	catalase	1.76 ± 0.02	0.016	8.2%	2	3	2.26	0.62 ± 0.09	0.067	10.1%	3	7
IP100133417.1	15959	Ifi3	interferon-induced protein with tetra-ricopeptide repeats 3	1.64 ± 0.08	0.040	6.7%	2	2	2.73	3.01 ± 0.84	1.9E-03	17.4%	6	43
IP100226205.3	21356	Tapbp	TAP binding protein	1.64 ± 0.02	0.036	2.3%	1	2	1.05	2.23 ± 0.25	0.001	5.7%	2	5
IP100624876.3	22323	Vasp	vasodilator-stimulated phosphoprotein	1.46 ± 0.27	0.21	6.7%	1	45	1.55	2.11 ± 0.28	3.3E-03	6.7%	1	23
IP100663627.1	286940	Flnb	filamin, beta	1.18 ± 0.23	0.59	7.2%	8	25	2.20	2.32 ± 0.04	1.1E-04	0.5%	1	3
IP100123474.2	18034	Nfk62	nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100	1.05 ± 0.02	0.85	2.0%	1	3	10.66	1.71 ± 0.03	0.014	2.0%	1	3
IP100128410.3	22040	Trex1	three prime repair exonuclease 1	1.03 ± 0.08	0.91	8.6%	2	8	5.13	1.78 ± 0.25	0.026	4.5%	1	29
IP100132950.1	66481 37	Rps21	ribosomal protein S21	0.89 ± 0.11	0.67	10.8%	1	43	-	1.69 ± 0.15	0.025	10.8%	1	14

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IP1	Entrez Gene	Gene Symbol	Description	RAW 264.7 LPS:Naïve ^a	p	coverage	peptides ^b	spectra ^c	ISB mRNA LPS:Naïve ^d	C57BL/6 LPS:Naïve ^a	p	coverage	peptides ^b	spectra ^c
IP100120113.5	22169	Cmpk2	cytidine monophosphate (UMP-CMP) kinase 2, mitochondrial	-	-	-	-	-	82.66	2.51 ± 0.74	0.011	9.2%	2	64
IP100117572.4	21939	Cd40	CD40 antigen	-	-	-	-	-	2.40	31.90 ± 0.03	3.3E-03	4.2%	1	3
IP100229277.1	229898	Gbp5	guanylate binding protein 5	-	-	-	-	-	-	1.73 ± 0.18	0.023	2.0%	1	12
IP100112549.1	14081	Acs1l	acyl-CoA synthetase long-chain family member 1	-	-	-	-	-	0.78	1.72 ± 0.04	0.013	4.9%	2	3

^aICAT ratios as quantified by XPRESS. **Bold** ratios indicate proteins with p values <0.05 that were identified in both C57BL/6 ICAT replicates and/or at least two RAW 264.7 ICAT replicates.

^bNumber of unique peptides used to identify the protein and calculate a protein ratio.

^cNumber of MS/MS spectra in which peptides corresponding to the protein were identified.

^dGene expression ratio in RAW 264.7 after 24-hour exposure to LPS as determined by two color mRNA microarray experiments performed at the Institute for Systems Biology. MS/MS spectra identifying the peptides of single-hit proteins are provided in Supplemental Figure 1.

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Table 2

Proteins significantly down-regulated by LPS-activation.

IPI	Entrez Gene	Gene Symbol	Description	RAW 264.7 Naïve:LPS ^a	p	coverage	peptides ^b	spectra ^c	ISB mRNA Naïve:LPS ^d	C57BL/6 LPS:Naïve:LPS ^a	p	coverage	peptides ^b	spectra ^c
IP100273914.2	230073	Ddx58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	4.49 ± 1.92	2.0E-03	2.2%	2	5	-	1.36 ± 0.02	0.16	1.4%	1	1
IP100551078.8	338523	Jhdm1d	jumonji C domain-containing histone demethylase 1 homolog D (S. cerevisiae)	2.73 ± 0.05	2.1E-05	3.9%	2	2	0.93	-	-	-	-	-
IP100263313.1	13494	Drg1	developmentally regulated GTP binding protein 1	2.55 ± 0.03	7.2E-05	6.0%	1	2	1.52	-	-	-	-	-
IP100115653.1	17970	Ncf2	neutrophil cytosolic factor 2	2.47 ± 0.04	1.2E-04	2.7%	1	3	0.39	-	-	-	-	-
IP100129250.1	76905	Lrg1	leucine-rich alpha-2-glycoprotein 1	2.33 ± 0.04	3.4E-04	2.0%	1	3	1.19	1.38 ± 0.02	0.14	2.0%	1	1
IP100420185.1	13859	Eps15l1	epidermal growth factor receptor pathway substrate 15-like 1	-	-	-	-	-	0.97	1.91 ± 0.07	3.3E-03	1.1%	1	6
IP100119114.2	11363	Acadl	acyl-Coenzyme A dehydrogenase, long-chain	1.82 ± 0.13	0.015	4.7%	1	4	1.21	-	-	-	-	-
IP100320905.7	70497	Arhgap17	Rho GTPase activating protein 17	1.73 ± 0.08	0.022	2.7%	1	4	0.99	-	-	-	-	-
IP100136134.1	29811	Ndrp2	N-myc downstream regulated gene 2	1.72 ± 0.25	0.048	8.4%	2	6	1.82	-	-	-	-	-
IP100117611.3	108150	Galnt7	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetyl-galactosaminyl transferase 7	1.69 ± 0.02	0.027	4.4%	2	2	1.59	-	-	-	-	-
IP100130246.2	72462	Rpl1b	ribosomal RNA processing 1 homolog B (S. cerevisiae)	1.66 ± 0.02	0.032	2.8%	1	2	1.69	-	-	-	-	-
IP100229859.1	27979	Eif3b	eukaryotic translation initiation factor 3, subunit B	1.65 ± 0.16	0.05	2.6%	2	8	1.94	-	-	-	-	-

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IPI	Entrez Gene	Gene Symbol	Description	RAW 264.7 Naïve:LPS ^a	p	coverage	peptides ^b	spectra ^c	ISB mRNA Naïve:LPS ^d	C57BL/6 LPS;Naïve:LPS ^a	p	coverage	peptides ^b	spectra ^c
IP100313672.3	16985	Lsp1	lymphocyte specific I	1.64 ± 0.02	0.037	4.2%	1	2	73.09	1.05 ± 0.09	0.83	4.2%	1	4
IP100380280.3	69719	Cad	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	1.62 ± 0.03	0.041	2.5%	3	3	3.22	-	-	-	-	-
IP1002223757.4	11677	Akr1b3	aldo-keto reductase family 1, member B3 (aldose reductase)	0.89 ± 0.13	0.68	8.2%	2	7	4.32	1.69 ± 0.03	0.016	3.8%	1	2
IP100222035.3	70719	Hmha1	histocompatibility (minor) HA-1	-	-	-	-	-	1.84	1.53 ± 0.03	0.050	1.2%	1	2

^aICAT ratios as quantified by XPRESS. Bold ratios indicate proteins p values <0.05 that were identified in both C57BL/6 ICAT replicates and/or at least two RAW 264.7 ICAT replicates.

^bNumber of unique peptides used to identify the protein and calculate a protein ratio.

^cNumber of MS/MS spectra in which peptides corresponding to the protein were identified.

^dGene expression ratio in RAW 264.7 after 24-hour exposure to LPS as determined by two color mRNA microarray experiments performed at the Institute for Systems Biology. MS/MS spectra identifying the peptides of single-hit proteins are provided in Supplemental Figure 1.