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A Methodology for Comprehensive Analysis of Toll-Like Receptor Signaling in Macrophages

Marijke Koppenol-Raab¹ and Aleksandra Nita-Lazar¹

¹Cellular Networks Proteomics Unit, Laboratory of Systems Biology, National Institute of Allergy and Infectious Diseases National Institutes of Health Bethesda USA

Abstract

A combination of high-throughput, multiplexed, quantitative methods with computational modeling and statistical approaches is required to obtain system-level understanding of biological function. Mass spectrometry (MS)-based proteomics has emerged as a preferred tool for the analysis of changes in protein abundance and their post-translational modification (PTM) levels at a global scale, comparable with genomic experiments and generating data suitable for use in mathematical modeling of signaling pathways. Here we describe a set of parallel bottom-up proteomic approaches to detect and quantify the global protein changes in total intracellular proteins, their phosphorylation, and the proteins released by active and passive secretion or shedding mechanisms (referred to as the secretome as reviewed in Makridakis and Vlahou, *J Proteome* 73:2291–2305, 2010) in response to the stimulation of Toll-like receptors (TLRs) with specific ligands in cultured macrophages. The method includes protocols for metabolic labeling of cells (SILAC: stable isotope labeling by amino acids in cell culture; Ong et al., *Mol Cell Proteomics* 1:376–386, 2002), ligand stimulation, cell lysis and media collection, in-gel and in-solution modification and digestion of proteins, phosphopeptide enrichment for phosphoproteomics, and LC-MS/MS analysis. With these methods, we can not only reliably quantify the relative changes in the TLR signaling components (Sjoelund et al., *J Proteome Res* 13:5185–5197, 2014) but also use the data as constraints for mathematical modeling.

Keywords

Toll-like receptor; Phosphoproteomics; Proteomics; Secretome; Macrophage; Innate immunity; SILAC; TLR2; TLR4; TLR7

1 Introduction

Toll-like receptors (TLRs) are a group of pattern recognition receptors and among the first sentinels of the innate immune system, acting as detectors of invading pathogens and playing a crucial role in initiating the immune response of the host organism [1]. These transmembrane sensors are located at the cell surface or in the endosome [2], and upon binding to pathogen-derived ligands (such as bacterial cell wall components or viral nucleic acids), they undergo ligation and rapidly generate a cascade of intracellular signals leading to changes in gene expression and, ultimately, to the production of inflammatory mediators that trigger host defense mechanisms [3].

There are multiple TLRs that recognize various specific microbial compounds [4, 5], and their signaling is mediated by the association of their intracellular domains with combinations of five adaptor molecules (reviewed in detail in [6]). The stepwise activation of the signaling pathway components is tightly regulated. Any imbalance of the TLR signaling may lead to aberrant cytokine expression and, in turn, dysregulated inflammation; chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease; or an acute immune reaction such as septic shock, a leading cause of death due to a lack of treatment options [7].

The tight control of TLR signaling is achieved by changes in expression of proteins involved in the signaling cascade and their post-translational modifications (PTMs). Phosphorylation is the best understood regulatory PTM, and many other PTM types are emerging as the complexity of the system is revealed, and the measurement accuracy improves (for an excellent review of PTM analysis by mass spectrometry, see [8]). Many components of the signaling network are shared between all TLRs; thus the regulation has to be executed quantitatively in a spatially and temporally controlled manner rather than qualitatively.

The complexity of TLR signaling and its clinical relevance make it an ideal system for mathematical modeling. Accurate measurement of absolute protein copy numbers with the use of targeted proteomics is the basis for the models we create [9, 10], and they are described elsewhere (for example [10]), but discovery-based proteomic studies of temporal changes in the protein and PTM levels are important as a basis for the design of targeted proteomics experiments as well as for obtaining additional constraints for model refinement. The multiparameter nature of the studies requires strict control and can be technically challenging. This protocol describes the design and execution of parallel mass spectrometry-based standardized [11] assays to analyze the changes in the proteome, phosphoproteome [3], and secretome [1] of cultured macrophages stimulated with TLR ligands (Fig. 1; [12]) and can be modified for any complex signaling network.

2 Materials

2.1 Cell Culture Reagents

1. RAW264.7 murine monocyte/macrophage cell line: American Type Culture Collection (ATCC) or Sigma-Aldrich.
2. RAW cell growth medium: Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 20 mM HEPES, and 2 mM L-glutamine. Combine 435 mL DMEM containing high glucose without L-glutamine with 50 mL FBS, 10 mL HEPES buffer, and 5 mL L-glutamine in the top chamber of a 500 mL filter system with 0.22 μ m filter. Filter the media and swirl briefly to mix. Store at 4 °C when not in use.
3. Growth medium for stable isotope labeling with amino acids in cell culture (SILAC [13]): DMEM with stable glutamine minus L-lysine and L-arginine supplemented with 10% FBS, 20 mM HEPES, and labeled or unlabeled amino acids: 0.398 mM L-arginine HCl and 0.798 mM L-lysine 2HCl. For light SILAC medium, unlabeled L-arginine (Arg0) and L-lysine (Lys0) are added. For

medium-labeled SILAC medium, add $^{13}\text{C}_6$ -L-arginine (Arg6) and $^2\text{D}_4$ -L-lysine (Lys4). For heavy-labeled medium, add ^{13}C $^{15}\text{N}_4$ -L-arginine (Arg10) and $^{13}\text{C}_6$ $^{15}\text{N}_2$ -L-lysine (Lys8). Unlabeled amino acids and stable isotope-labeled amino acids are available from various suppliers. Prepared media should be stored at 4 °C when not in use.

2.2 Toll-Like Receptor Ligands

1. Lipopolysaccharide (LPS) from *Salmonella Minnesota* R595, a TLR4 ligand.
2. Pam3CSK4 (P3C), a synthetic triacylated lipoprotein and TLR2/1 agonist.
3. Resiquimod (R848); a TLR7 ligand.

2.3 Lysate and Conditioned Media Preparation Reagents

1. Modified RIPA lysis buffer (for the proteome analysis): 50 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% Nadeoxycholate, 1% IGEPAL. Can be prepared and stored at 4 °C.
2. Urea lysis buffer (for the phosphoproteome analysis): 8 M urea. Prepare fresh just before use.
3. Protease inhibitor cocktail: Complete Mini EDTA-free Protease Inhibitor Cocktail tablets. Add one tablet per 10 mL of lysis buffer immediately before use.
4. Phosphatase inhibitor cocktail: PhosSTOP tablets. Add one tablet per 10 mL of lysis buffer immediately before use.
5. Spin filters to remove dead cells from conditioned media: Ultrafree-MC 0.22 µm spin filters.
6. Protein concentration determination: Bicinchoninic acid (BCA) Protein Assay Kit.

2.4 Protein Fractionation Reagents

1. Lithium dodecyl sulfate (LDS) (Life Technologies) sample loading buffer with reducing agent (50 mM dithiotreitol, DTT).
2. SDS-PAGE gel system: NuPAGE Bis-Tris gels. *See* Note 1.
3. Coomassie protein stain: SimplyBlue SafeStain.

2.5 Chemical Modification and Digestion Reagents

1. H_2O : LC/MS grade.
2. Acetonitrile: LC/MS grade.
3. Formic acid: LC/MS grade.

4. Dithiothreitol (DTT): Prepare a fresh stock of 1 M DTT in 100 mM ammonium acetate pH 8.9 for in-solution modification or 10 mM DTT in 100 mM ammonium bicarbonate for in-gel modification.
5. Iodoacetamide (IAA): Prepare a fresh stock of 0.8 M IAA in 100 mM ammonium acetate pH 8.9 for in-solution modification or 55 mM IAA in 100 mM ammonium bicarbonate for in-gel modification.
6. Sequencing grade modified trypsin. For in-gel trypsin digestion, dissolve one 20 µg vial of trypsin in 1.5 mL of 10 mM ammonium bicarbonate buffer containing 10% acetonitrile. Prepare just before use.
7. Peptide extraction buffer for in-gel digest: Dilute formic acid to 5% (v/v) in ultrapure water. Combine one part 5% formic acid with two parts acetonitrile for a 1:2 (v/v) mixture of 5% formic acid/acetonitrile to extract peptides from gel pieces.
8. HPLC fractionation solvent A: 5 mM KH₂PO₄ pH 2.7, 25% acetonitrile.
9. HPLC fractionation solvent B: 5 mM KH₂PO₄ pH 2.7, 500 mM KCl, 25% acetonitrile.

2.6 Reagents for Enrichment of Phosphopeptides

1. Oasis HLB column for desalting of in-solution digests.
2. Enrichment of phosphopeptides: TiO₂ Phosphopeptide Enrichment and Clean-up Kit.

2.7 Buffers for LC-MS/MS

1. Solvent A: 0.1% (v/v) formic acid.
2. Solvent B: 0.1% formic acid in acetonitrile.

2.8 Equipment

1. Benchtop microcentrifuge for spins at room temperature and 4 °C.
2. Vacuum evaporator centrifuge such as Savant SpeedVac Concentrator SPD111V.
3. Liquid chromatography system for SCX fractionation such as AKTAdesign.
4. Nano-flow HPLC system such as Eksigent nano-HPLC.
5. Electrospray ionization (ESI) capable ion-trap tandem mass spectrometer such as LTQ-Orbitrap Velos.

3 Methods

3.1 Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC)

1. RAW264.7 cells are grown in RAW growth medium on sterile tissue-culture treated plates in a humidified incubator at 37 °C, 5% CO₂ and passaged every 2–

3 days using phosphate-buffered saline (PBS) supplemented with 2 mM EDTA to detach cells.

2. To transition cells to SILAC media, harvest cells as usual with PBS + EDTA. Seed $1\text{--}1.5 \times 10^5$ cells per labeling condition in a 6-well plate with 3 mL of the appropriate SILAC medium. For a triplex experiment, you need cells adapted to light, medium, and heavy SILAC conditions.
3. Passage the cells in SILAC media every 4–5 days (*see* Note 1) by pipetting in the well to detach the cells and diluting them 1:10 into 3 mL of fresh SILAC media in a new well of a 6-well plate.
4. After five passages, evaluate the stable isotope incorporation in whole cell lysates by mass spectrometry (Fig. 2).
5. Once label incorporation of >95% is achieved, expand the cells in each SILAC condition in 10–15 cm dishes to ensure sufficient cells for stimulation.
6. Seed SILAC-labeled cells for treatment 1 day prior to stimulation. For global proteomic and secretome analyses, 10^6 cells seeded in 12-well plates are sufficient per treatment and time point. For phosphoproteomic analysis, seed 2.5×10^7 cells in a 10 cm dish for each treatment and time point.

3.2 Stimulation with Toll-Like Receptor Ligands

1. Prior to stimulation, prepare TLR ligands in SILAC media: 100 ng/mL LPS, 1 μ M P3C, or 1 μ M R848. IMPORTANT: For secretome analysis, prepare ligands in SILAC media without FBS.

Sample treatment scheme:

SILAC medium	Proteome	Secretome
Light – TLR ligand	0 h (untreated)	0 h (untreated)
Medium + TLR ligand	6 h	6 h
Heavy + TLR ligand	12 h	24 h

2. Remove media from the SILAC-labeled cells. For proteome and phosphoproteome analysis, add SILAC media containing TLR ligands to the cells: 1 mL per well for cells in 12-well plates or 10 mL per 10 cm dish of cells. For the untreated control, add the appropriate SILAC media without TLR ligand. For secretome analysis, wash cells once with SILAC media lacking FBS to remove residual serum proteins. After the wash, add SILAC media lacking FBS containing TLR ligands, 1 mL per well of cells in 12-well plates.
3. Incubate cells in a humidified incubator at 37 °C for the desired treatment time.

3.3 Preparation of Cell Lysates for Global Proteomic Analysis

1. After stimulation, wash cells three times with ice-cold phosphate-buffered saline (PBS).

2. Keep dishes on ice and lyse the cells in 50 μ L ice-cold modified RIPA buffer containing protease and phosphatase inhibitors.
3. Transfer the lysates to cold microcentrifuge tubes, and incubate on ice with occasional vortexing for 20 min.
4. Clear lysates by centrifugation at $12,000 \times g$ at 4 °C for 10 min.
5. Transfer the supernatant to a fresh microcentrifuge tube for proteomic analysis. Cleared lysates can be stored at –80°C.
6. Determine the total protein concentration using a BCA assay according to the manufacturer's protocol.
7. For proteomic analysis, combine the light, medium, and heavy-labeled time points in a microcentrifuge tube at a 1:1:1 ratio by weight using 10 μ g total protein from each time point.
8. Proceed to Subheading 3.6 for protein fractionation.

3.4 Preparation of Cell Lysates for Phosphoproteomic Analysis

1. After stimulation, wash cells three times with ice-cold phosphate-buffered saline (PBS).
2. Keep dishes on ice and lyse cells in 3 mL ice-cold 8 M urea buffer containing protease and phosphatase inhibitors.
3. Transfer lysates to cold 15 mL conical tubes and incubate on ice with occasional vortexing for 20 min.
4. Clear lysates by centrifugation at $14,000 \times g$ at 4 °C for 20 min.
5. Transfer the supernatant to a fresh tube. Cleared lysates can be stored at –80 °C.
6. Determine the total protein concentration using a BCA assay according to the manufacturer's protocol.
7. For phosphoproteomic analysis, combine the light, medium, and heavy-labeled time points in a microcentrifuge tube at a 1:1:1 ratio by weight using 1.3 mg total protein from each time point.
8. Proceed to Subheading 3.8 for in-solution modification and digestion.

3.5 Preparation of Conditioned Media for Secretome Analysis

1. After stimulation, collect 900 μ L conditioned media.
2. Filter media using spin filters with 0.22 μ m pore size, 450 μ L at a time, for 2 min at $12,000 \times g$ and 4 °C to remove any floating, dead cells.
3. Transfer the filtered media to a fresh microcentrifuge tube and flash freeze in liquid nitrogen. Store samples at –80 °C until ready for processing.

4. For secretome analysis, combine the light, medium, and heavy-labeled time points in a microcentrifuge tube at a 1:1:1 ratio by volume using 250 μ L from each sample for a total sample volume of 750 μ L.
5. Reduce the sample volume to approximately 25–30 μ L by vacuum centrifugation. The reduced sample will be viscous.
6. Proceed to Subheading 3.6 for protein fractionation.

3.6 Protein Fractionation by One-Dimensional Gel Electrophoresis (Proteome and Secretome)

1. Add LDS sample loading buffer to 1 $\times$ concentration, and load the sample on a NuPAGE Bis-Tris gel for protein separation using NuPAGE SDS running buffer (see Note 2).
2. Wash the gel three times with ultrapure water before staining with SimplyBlue SafeStain. After staining, destain the gel with ultrapure water to remove background stain.
3. Using a light box and clean scalpel, excise the gel lanes, and cut each lane into 20 equal pieces. Transfer each piece to a clean microcentrifuge tube and store at -80°C .
4. Proceed to Subheading 3.7 for in-gel modification and digestion.

3.7 In-Gel Chemical Modification and Trypsin Digestion of Extracted Proteins (Proteome and Secretome)

1. Thaw gel bands and mince into cubes of ca. 1 mm^3 using ultra-fine tip straight forceps.
2. Add 500 μ L acetonitrile per tube, and incubate at room temperature for 10 min to dehydrate the gel pieces. The gel pieces will shrink and become opaque when dehydrated.
3. Spin the gel pieces down using a benchtop microcentrifuge and remove all liquid (see Note 3).
4. Add 50 μ L 10 mM DTT in 100 mM ammonium bicarbonate per tube and incubate at 56°C for 30 min to reduce the cysteines. Ensure the gel pieces are covered with liquid.
5. Chill tubes to room temperature, and dehydrate the gel pieces with 500 μ L acetonitrile per tube. Incubate at room temperature for 10 min.
6. Spin the gel pieces down using a benchtop microcentrifuge and remove all liquid.
7. Add 50 μ L 55 mM iodoacetamide in 100 mM ammonium bicarbonate per tube, and incubate at room temperature in the dark for 30 min to alkylate the cysteines. Ensure that the gel pieces are covered with liquid.

8. Add 500 μ L acetonitrile per tube and incubate at room temperature for 10 min to dehydrate the gel pieces.
9. Spin the gel pieces down using a benchtop microcentrifuge and remove all liquid.
10. Add 50 μ L trypsin solution and ensure that the gel pieces are covered by liquid. Incubate on ice for 30 min.
11. After 30 min, check that the trypsin solution has been absorbed, adding more as needed to cover the gel pieces.
12. Allow the trypsin digestion to proceed overnight at 37 °C.
13. Chill tubes to room temperature and spin down gel pieces in a benchtop microcentrifuge. Transfer the supernatant to a fresh microcentrifuge tube. Do not discard as this supernatant contains peptides.
14. Add 100 μ L peptide extraction buffer and incubate the samples in a sonication water bath at room temperature for 15 min.
15. Use a gel-loading tip to transfer the supernatant with extracted peptides to the tube with supernatant from step 13. Extracted peptides can be stored at –20 °C. Gel pieces can be stored at –80 °C, and peptides can be re-extracted if necessary.
16. Concentrate the extracted peptides by vacuum centrifugation to a minimal volume (1–2 μ L). Dried extracts can be stored at –20 °C.
17. Resuspend dried peptides in 10–15 μ L 0.1% (v/v) formic acid, and incubate in a sonication water bath at room temperature for 10 min (*see* Note 4).
18. Centrifuge the peptides for 15 min at full speed to pellet insoluble particulates. Carefully transfer the supernatant with peptides to a new tube without disturbing the pellet. Peptides are now ready for LC-MS/MS analysis.

3.8 In-Solution Modification and Digestion of Extracted Proteins (Phosphoproteome)

1. Add DTT buffer to the combined urea lysate samples to a final concentration of 2 mM DTT. Incubate the samples at 56 °C for 1 h to reduce cysteines.
2. Cool the samples to room temperature before adding iodoacetamide buffer to a final concentration of 4 mM IAA. Incubate the samples in the dark at room temperature for 1 h to alkylate cysteines.
3. Dilute the samples with 100 mM ammonium acetate buffer pH 8.9 to 2 M urea.
4. Add trypsin at a ratio of 1:100 (w/w) and incubate the samples overnight at 37 °C for digestion.
5. Quench the digestion reaction with glacial acetic acid to reduce the pH to below 2.

6. Prepare the Oasis HLB columns for sample desalting by washing with 1 mL of acetonitrile, followed by 0.1% acetic acid. Load the trypsin reactions on the column for desalting and wash with 3 mL 0.1% acetic acid.
7. Elute the peptides from the column with 1 mL of 0.1% (v/v) acetic acid, 75% (v/v) acetonitrile.
8. Dry the eluted peptides by vacuum centrifugation.
9. Proceed to Subheading 3.9 for fractionation and enrichment of phosphopeptides.

3.9 Fractionation and Enrichment of Phosphopeptides

1. Dissolve the dried peptides in 500 μ L of HPLC Fractionation solvent A.
2. Inject the peptides onto a PolySULFOETHYL A SCX column (4.6 mm i.d. $\times$ 20 cm length, 5 μ m particle size, 200 Å pore size) for fractionation.
3. Elute peptides with a gradient of 0% HPLC Fractionation solvent B for 2 mL, 0–14% B for 33 mL, 14–100% B for 1 mL, and 100% B held for 4 mL at a flow rate of 0.3 mL/min. Collect 2 mL fractions and desalt as above using Oasis HLB columns.
4. Dry the desalted peptide fractions by vacuum centrifugation.
5. Pierce TiO₂ phosphopeptide enrichment kit: Resuspend the dried SCX peptide fractions in 100 μ L binding buffer and apply to a TiO₂ tip. Wash the spin tip and elute the peptides with ammonium hydroxide and then pyrrolidine in a final volume of 200 μ L according to the manufacturer's protocol.
6. Acidify the phosphopeptide eluates with 100 μ L of 2.5% (v/v) TFA and desalt using the graphite columns supplied with the Pierce TiO₂ kit.
7. Dry the desalted phosphopeptides by vacuum centrifugation.
8. Resuspend the dried peptides in 10–15 μ L 0.1% (v/v) formic acid and incubate in a sonication water bath at room temperature for 10 min.
9. Centrifuge the peptides for 15 min at full speed to pellet insoluble particulates. Carefully transfer the supernatant with peptides to a new tube without disturbing the pellet. Peptides are now ready for LC-MS/MS analysis.

3.10 LC-MS/MS Analysis of Peptides (See Note 5)

1. Inject the (phospho-) peptides for analysis onto an Eksigent nano-LC system coupled to an Orbitrap Velos mass spectrometer. For the proteome and secretome analyses, a 60-min gradient at a flow rate of 200 nL/min is sufficient to separate peptides: 5% B to 37% B in 50 min, followed by 37% B to 90% B in 2 min and holding at 90% B for 3 min. For the phosphoproteome analysis, use a longer gradient of 3% B to 40% B over 120 min with a 200 nL/min flow rate.
2. Use a top ten instrument method to perform data-dependent acquisition to automatically cycle between Orbitrap full scan MS and LTQ MS/MS.

4 Notes

1. The doubling time of cells in SILAC media is longer than in rich media; thus cells do not need to be passaged as often in SILAC media.
2. We achieve good protein separation on both 10% and 4–12% NuPAGE Bis-Tris gels run in either NuPAGE MOPS or MES running buffer (Life Technologies).
3. Pipette carefully as dehydrated gel pieces may stick to the pipette tip. Use a gel-loading tip when removing liquid to help prevent clogs in the tip and loss of gel pieces during sample preparation.
4. 2% (v/v) acetonitrile can be added to the 0.1% formic acid to help dissolve peptides without affecting LC-MS/MS.
5. Adjust parameters as needed based on the available LC-MS/MS instrumentation.

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Fig. 1.

Experimental workflow for the mass spectrometry-based analysis of changes in the proteome, secretome, and phosphoproteome of cultured macrophages stimulated with TLR ligands. SILAC-labeled cells are treated with TLR ligands over a time course. Lysates are prepared for the proteome and phosphoproteome analyses, or conditioned media is collected for the secretome analysis. Protein fractionation by gel electrophoresis and in-gel trypsin digestion are used to generate peptides for the proteome and secretome analyses. Proteins for phosphoproteome analysis are trypsinized in solution. The resulting peptides are fractionated by SCX chromatography, and the phosphopeptides are enriched using TiO₂. Once peptides for each type of analysis have been obtained; they are analyzed by LC-MS/MS.

Fig. 2.

Example of labeling efficiency. A small aliquot of lysate can be prepared and analyzed by LC-MS/MS. This peptide contains a heavy-labeled Arg10 residue. There is no detectable peak at the corresponding m/z of the unlabeled peptide, indicating label incorporation of near 100%