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Host-pathogen dynamics through targeted secretome analysis of stimulated macrophages

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Abstract

The pattern recognition receptors (PRRs) facilitate an organism's first line of defense against interlopers and shape the overall innate immune response through sensing and sampling pathogen-associated molecular patterns (PAMPs). The Toll-like receptor (TLR) family is the prototypic PRR family. Upon recognition of PAMPs, TLRs promote MyD88 dependent and independent responses. Understanding how different PAMPs are recognized by their specific TLRs and how pathogen recognition initiates immune activation is an intense area of research. Previously, we have reported the discovery of the temporal changes in signaling cascades of macrophage proteome and secretome post-stimulation with three different PAMPs. To extend our global proteomics approach to targeted protein abundance quantification, we describe the macrophage secretome targeted proteomics assay. We chose three different pathogens that specifically stimulate diverse TLRs (TLR2, TLR4, and TLR7). Using a simple targeted proteomics approach, combining data-dependent acquisition with an inclusion list, an array of cytokines, chemokines, and transcription factors can be profiled for their secretome abundance. This strategy facilitates the profiling and validation of pathogen-specific temporal changes in the macrophage secretome.

The innate immune system not only provides the host with a first line of defense against invading pathogens but also facilitates the adaptive immunity [1]. The host surveys pathogens via their archetypical pathogen recognition receptors (PRRs) that recognize pathogen-associated molecular patterns (PAMPs), for instance Toll-like receptor-4 (TLR4) recognizes Gram-negative bacterial lipopolysaccharide [2, 3]. To sense different microbes and their products, which include from bacteria, fungi, and viruses, humans and mice express eleven and thirteen TLRs, respectively. Although different TLRs recognize distinct

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PAMPs, they all lead to activation of the NF- κ B signaling pathway through either the myeloid differentiation primary response gene 88- (MyD88-) dependent mechanisms and/or via the TIR-domain-containing adapter-inducing interferon- β - (TRIF-) dependent routes [4]. TLR4 recruits both the MyD88-dependent and independent routes whereas TLR2, which recognizes lipopeptides, and TLR7, which senses single stranded nucleic acids through late endosomes, signal through only the MyD88- dependent pathway [4]. Post-TLR activation, many of the proteins that shape immune responses such as chemokines and cytokines are secreted, and facilitate intercellular communication and cell-to-cell coordination between immune cells in order to mount an inflammatory response and help clear the invading pathogen. Recently, secretome analyses have improved, both conceptually, taking into consideration the secretion machineries and technically, through improvement of mass spectrometry tools [5, 6]. However, only a few proteomic studies of immune activation, particularly comparing and contrasting TLR-mediated immune responses, have been reported [5–9]. In our recent work, we reported a systems biology study spanning quantitative transcriptomics, proteomics (intracellular), and secretomics of macrophages stimulated with different toll-like receptor ligands [5], which elucidated the differences and similarities of signaling between individual TLRs. In the present work, we describe a targeted proteomics based strategy using the inclusion list and an LTQ-Orbitrap Velos to quickly assay the differences in host-pathogen dynamics mediated through different TLR receptors following stimulation with whole pathogens.

In our earlier study, we analyzed the intracellular proteome and secretome of macrophages stimulated with a TLR4 ligand (lipopolysaccharide (LPS)), a TLR2 ligand (Pam3CSK4 (P3C)), and a TLR7 ligand (resiquimod (R848)). Here we have expanded the targeted proteins repertoire by profiling the changes in secreted proteins when macrophages are exposed to complex ligands. Heat-killed *Escherichia coli* (a Gram-negative bacterium), heat-killed *Staphylococcus aureus* (a Gram-positive bacterium), and live intracellular *Burkholderia cenocepacia* bacteria were used as exemplar pathogens signaling through TLR4, TLR2, and endosomal TLR7 respectively (Figure 1A). A secretome subset of 28 significantly regulated proteins from single ligand stimulations (LPS, P3C, and R848) were selected (Table S1, [5]). For the whole pathogen stimulation, the light (Arg0, Lys0)-labeled cells were left untreated for 24 hours while the medium (Arg6, Lys4) and heavy (Arg10, Lys8)-labeled cells were treated with pathogens for 6 and 24 hours, respectively, in serum-free media. To examine the secretome changes and the TLR-mediated signaling pathway activation, samples were SDS-PAGE fractionated and SILAC quantification [10] was achieved using high-resolution mass spectrometry data-dependent acquisition (DDA) with an inclusion list (Figure 1B, Supplementary Methods).

The proteins exhibited different temporal changes in response to stimulations as evidenced by the perturbations being distinctive to the TLR4 *versus* the TLR2 and TLR7 stimulations (Figure 2). Although the magnitude of the secretome perturbations varied, for most of the targeted proteins the trend of changes maintained directional similarity. The majority of the proteins showed roughly similar protein levels for whole-pathogen stimulations to those observed for the individual ligand stimulations (App, CD14, Ctsb, Hexa, Man2b1, Pf4, Lyz2; Figure 2). However, for some proteins, stimulation with the whole pathogens resulted in temporal changes, which are different from those observed for single-ligand stimulations.

For example, C3 (Figure 2B), CCL9 (Figure 2C), and H2-K1 (Figure 2F) demonstrated relatively weak responses to the whole pathogen stimulations. Also, for the whole pathogen treatment using intracellular *B. cenocepacia*, a decrease in protein abundance was observed at 24 hours post-stimulation compared to at 6 hours. Therefore, -omics analyses using individual purified ligands can provide insight into TLR-mediated responses to whole pathogens. The present targeted proteomics work profiling macrophages stimulated using whole pathogens is consistent with our earlier single ligand stimulation results. The majority of the measurements are displaying the same trends and patterns (with the exception of Lyz2 under one condition, the *S. aureus* stimulation), although the work was performed on a different batch of cells, by a different scientist and more than a year later. However, differences in the magnitude of response (single ligand experiments *versus* whole pathogen stimulations) may be explained in terms of ligand concentrations and availability of distinct PAMPs in the macrophage microenvironment. For example, the global proteomics study employing single purified ligands may have used higher or even saturating concentrations of single ligands compared to the whole bacteria stimulations. This may explain the observation that the whole pathogen experiments produced comparatively weaker responses, which are prone to more variability than single, purified ligand stimulations. In addition, complex combinations of ligands, presented by whole pathogens, can facilitate synergistic immune responses [5], and the intracellular pathogen *B. cenocepacia* may induce host cell death in response to infection, and this may explain the observed consistent decrease in protein abundance in the *B. cenocepacia* stimulated macrophages (at 24 hours stimulation). On the other hand, the difference may be a result of the undersampling in DDA acquisition mode even when using inclusion list. Thus, some proteins of interest are not measured in one or several replicates.

A source of difference between this work and our MCP paper [5], however surprisingly little, may also be the use of *E. coli* and not *P. aeruginosa* as the Gram-negative pathogen. We chose to use *E. coli* because its LPS structure is closer to the LPS we used in the pure ligand stimulations than the *P. aeruginosa* dominant LPS structure [11].

In summary, the dynamics of the target proteins, discovered using single ligand experiments, can be measured after much more complex stimulations using the targeted proteomics assay, and the results provide insight into the temporal profile of macrophage stimulation *in vivo*.

In the present work, we used mass spectrometry-based targeted immunoproteomics to decipher TLR-mediated innate immune signaling in response to whole pathogen stimulation. The differing response by the TLR4-induced cells, compared to the TLR2 and TLR7-mediated responses, is likely because TLR4 is known to activate both the MyD88-dependent and independent pathways. The systems-level analysis of TLR4-mediated signaling reveals an early-phase up-regulation of proteins (App, Cd14, Ctsb, Man2b1, Lyz2, Hexa) that are rapidly down-regulated during the late-phase (6 hours *versus* 24 hours; Figure 2). Tight control of signaling is required for mounting an effective immune response. However, down-regulation of some of the proteins (IL6; Table S2) might be due to proteasomal activities in the secretome [12]. We have demonstrated the robustness of this method and its feasibility in the laboratories whose main focus is not necessarily mass spectrometry method development and the instrument of choice is the versatile LTQ-Orbitrap. The present systems immunology

workflow highlights the importance of correlating global immunoproteomics data with targeted systems-level analyses for different pathogens and demonstrates the utility of the targeted proteomics assay as an alternative to the antibody-based cytokine measurements.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SIGNIFICANCE

Strategies to profile the secretome of TLR2-, TLR4, and TLR7- stimulated macrophages using whole pathogens were developed. Stable isotope labeling with amino acids in cell culture (SILAC) of macrophages was integrated with whole pathogen macrophage stimulation and subsequent targeted proteomics to quantify cytokines, chemokines, and transcription factors.

Single Ligand Stimulations (Global)

Whole Pathogen Stimulations (Targeted)

Single Ligand Stimulations (Global)

Whole Pathogen Stimulations (Targeted)

FIGURE 2.

Quantitative macrophage secretomics of shotgun LC-MS of single ligand stimulations [5] alongside targeted LC-MS of whole pathogen stimulations (present work). Values are the mean $\pm$ standard deviation calculated across three biological replicates.