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Protein networks and activation of lymphocytes

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The signal transduction pathways initiated by lymphocyte activation play a critical role in regulating host immunity. High-resolution mass spectrometry has accelerated the investigation of these complex and dynamic pathways by enabling the qualitative and quantitative investigation of thousands of proteins and phosphoproteins simultaneously. In addition, the unbiased and wide-scale identification of protein–protein interaction networks and protein kinase substrates in lymphocyte signaling pathways can be achieved by mass spectrometry-based approaches. Critically, the integration of these discovery-driven strategies with single-cell analysis using mass cytometry can facilitate the understanding of complex signaling phenotypes in distinct immunophenotypes.

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Introduction

High-resolution mass spectrometry (MS) has emerged as an attractive wide-scale approach to studying intracellular and extracellular signaling events in lymphocytes. Identification and quantitation of thousands of proteins, as well as their modifications and interaction partners can now be achieved with this approach. Furthermore, the development of novel technologies utilizing MS, such as mass cytometry, has opened up a new age of performing highly parallel single-cell analysis. In this review, we will discuss recent applications of MS technology to unraveling complex signaling networks in lymphocytes.

Shotgun proteomics to interrogate lymphocyte signaling

A comprehensive investigation of protein signaling networks requires the ability to evaluate the dynamic composition of molecular components in time and space. Shotgun proteomics using high-resolution MS provides an unbiased, quantitative, and wide-scale analysis of complex protein mixtures, delivering snapshots of proteome compositions during cellular processes. In a typical workflow, proteins extracted from biological samples are enzymatically digested and analyzed using reversed-phase liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). Peptides identified using MS are fragmented and sequenced using tandem mass spectrometry (MS/MS), and resulting spectra are analyzed through database search engines to identify the corresponding peptide sequences and proteins. Finally, statistical validation of the data is performed, often through decoy search strategies in which the MS/MS spectra are competitively matched against databases containing both normal and reversed protein sequences to estimate the false discovery rate (FDR) for sequence assignment.

Shotgun proteomics is a powerful approach for quantitation of relative changes in peptide and protein abundance across different cellular states or treatments (Table 1). Both label-free and label-based strategies can be employed for quantitation and have been reviewed in detail elsewhere [1,2]. In LC–MS/MS experiments, MS data is collected for quantitation by the sequential determination of the m/z values and intensities of all peptides eluted into the instrument. Although selection of peptides for fragmentation and MS/MS generation is stochastic and based on the most abundant signals, MS-based quantitation using both label-free and SILAC strategies allows for quantitation in the absence of MS/MS spectra by retention time alignment and accurate mass analysis of MS spectra using information from replicate samples. Importantly, our group has demonstrated the power of this approach by showing that calculating phosphopeptide replicate selected ion chromatogram peak areas using retention time alignment increases the number of replicate peptides quantified fivefold [3]. Conversely, quantitative information in isobaric mass tag experiments is generated from MS/MS spectra and thus can suffer from 'missing data' that may undermine the application of statistical tests.

Shotgun proteomic approaches have benefited investigations of critical signaling pathways in lymphocytes as they have enabled the identification and quantitation of thousands of global alterations in the proteomes of lymphocytes across a diverse range of cellular processes. These MS-based proteomic studies have revealed: insights into proteomic changes occurring in response

Table 1

Overview of quantitative approaches in MS-based proteomics

	Method	Sample application	Number of comparisons	Quantitation level	Sequencing depth	Quantitative accuracy	Cost
Label-free	Spectral counting	Primary and cultured cells	Many	MS	+++	+	$
	Ion intensity	Primary and cultured cells	Many	MS	+++	++	$
Label-based	SILAC	Cultured cells	2–3	MS	++	+++	$$
	Isobaric mass tag	Primary and cultured cells	2–8	MS/MS	++	++	$$$

to various cellular treatments [4–8]; unique proteomic signatures across the maturation states of lymphocytes [9,10]; and proteins with clinical significance in lymphocyte disease states [11–13]. Another powerful utility of quantitative MS-based proteomics is the interrogation of protein networks within isolated subcellular locations in lymphocytes. Already, the membrane proteomes of primary B cells, NK cells, and T cells have been characterized utilizing this technique on plasma membrane enriched samples [14–16]. Studies have also performed proteomic characterizations of enriched secretory lysosomes from cytotoxic T cells and NK cells [17–19].

Emerging 'omics' fields from MS-based proteomics

Harnessing the high-throughput power of MS, investigators have begun the characterization of the secretome and immunopeptidome from signaling lymphocytes. Secreted proteins, which include cytokines, interleukins, and growth factors, are critical intercellular messengers in the immune system, mediating both the communication between effector cells and the orchestration of the immune response. Recently, a quantitative, high-resolution MS workflow was described to detect and quantify the time-resolved release of proteins from immune cells, the secretome, upon receptor ligation. From this investigation, the authors detected secreted proteins whose abundance increased by a factor of greater than 10,000, illustrating the significant sensitivity and dynamic range of this label-free quantitative approach [20•]. Although this particular workflow was applied to macrophages, it will have great utility in interrogating lymphocyte responses to various stimuli. Another recent study implemented quantitative MS along with next-generation sequencing technology to profile the serum antibody repertoire elicited by vaccination, detailing its molecular composition and characteristics [21]. Utilizing similar approaches will be invaluable to defining the serum antibody repertoire and how its diversity and specificity changes in response to diseases.

Another area of research that is enabled by MS is the comprehensive analysis of both the membrane-bound and plasma soluble human immunopeptidome [22]. The immunopeptidome is the collection of thousands of peptides displayed by MHC molecules. Presentation of MHC molecules with their bound immunopeptidomes facilitates the scrutiny of the health-state of cells by circulating T cells. To perform this analysis, peptides bound to immunoaffinity purified MHC are extracted and analyzed by LC–MS/MS. Already, MS-based investigations have revealed the complexity and plasticity of the immunopeptidome and have highlighted critical information on the internal state of the cell that can be gleaned from a system-levels analysis of its composition [23–25,26•,27].

MS-based investigations of protein–protein interaction networks

Protein–protein interaction (PPI) networks play a critical role in signal transduction in lymphocytes. For example, in T cell receptor (TCR) signaling, scaffolding molecules such as LAT nucleate the assembly of multiprotein complexes that are critical for amplification and diversification of signals that mediate responses resulting from TCR engagement [28]. The study of PPIs has been greatly facilitated by MS technologies, in particular, affinity purification mass spectrometry (AP-MS), which requires the ectopic expression of epitope tags on target 'bait' proteins of interest as affinity capture probes. Using this approach, a broad scope of PPI networks can be obtained with high sensitivity, accuracy, versatility and speed. A collection of approaches to define PPIs using AP-MS has been reviewed elsewhere [29••,30–36]. Many of these approaches have been implemented to interrogate PPI networks in lymphocyte signaling [37,38,39••,40]. Recently, mice bearing a genetic tag that permit AP-MS of signaling complexes containing ZAP-70, LAT, and SLP-76 was described using label-free quantitation. From this system, a membrane-proximal TCR signaling network was identified along with quantitative insights into the temporal regulation of these complexes [39••]. A concern for AP-MS experiments is the potential loss of weak interactors. To address this issue, Li *et al.* implemented an enzyme-generated proximity labeling strategy combined with AP-MS to investigate the molecular composition of B cell receptor (BCR) clusters. In this approach, B cells were treated with HRP-conjugated IgM followed by tyramide-biotin, resulting in biotinylation of proximal proteins. These biotinylated