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APPLICATION OF PROTEOMICS TO NEUTROPHIL BIOLOGY

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Abstract

Polymorphonuclear leukocytes or neutrophils are a primary effector cell of the innate immune system and contribute to the development of adaptive immunity. Neutrophils participate in both the initiation and resolution of inflammatory responses through a series of highly coordinated molecular and phenotypic changes. To accomplish these changes, neutrophils express numerous receptors and use multiple overlapping and redundant signal transduction pathways. Dysregulation of the activation or resolution pathways plays a role in a number of human diseases. A comprehensive understanding of the regulation of neutrophil responses can be provided by high throughput proteomic technologies and sophisticated computational analysis. The first steps in the application of proteomics to understanding neutrophil biology have been taken. Here we review the application of expression, structural, and functional proteomic studies to neutrophils. Although defining the complex molecular events associated with neutrophil activation is in the early stages, the data generated to date suggest that proteomic technologies will dramatically enhance our understanding of neutrophil biology.

1. Introduction

As the primary effector cell of the innate immune system, polymorphonuclear leukocytes (neutrophils) provide the first line of defense against bacterial and fungal infections. The importance of this role is underscored by the high risk of infection in individuals with leukopenia. On the other hand, inappropriate or prolonged activation of neutrophils leads to tissue injury associated with a number of autoimmune and inflammatory diseases, including rheumatoid arthritis, Wegener's granulomatosus, systemic lupus erythematosus, multisystem organ failure associated with sepsis, and ischemia-reperfusion injury. In healthy individuals circulating neutrophils are poorly responsive to pro-inflammatory stimuli. Neutrophil activation results from a highly regulated series of phenotypic changes. A complex and interactive array of extracellular stimuli and intracellular signal transduction pathways regulate the transition of resting neutrophils to cells primed for enhanced responses and, finally, to fully activated cells. Resolution of inflammation is also an active process resulting in interruption of neutrophil activation, followed by neutrophil apoptosis and their engulfment by monocytes. As an introduction to the uses of proteomic technologies to study neutrophil biology, the processes leading to neutrophil participation in inflammation and to resolution of inflammation will be briefly described.

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2. Neutrophil Participation in Inflammation

Neutrophils continually "sense" vascular endothelial cells for changes induced by underlying inflammation through a selectin-dependent process of loose adhesion called rolling. Release of pro-inflammatory cytokines from cells at a site of infection or tissue injury triggers vascular endothelial cells to increase expression E-selectins, P-selectins, and intercellular adhesion molecules (ICAMs) and to generate another set of cytokines and chemokines. The increased selectin expression results in slowing of neutrophil rolling, allowing neutrophils to be activated by bacterial byproducts (eg. formylated peptides and lipopolysaccharides), complement fragments (e.g., C5a), or products released from endothelial cells (e.g., leukotriene B_4, TNF-α, and interleukin (IL)-8. These agents increase neutrophil plasma membrane expression and activation of integrins (CD11a-c/CD18) and shedding of selectins [1–3]. Integrin interaction with its counter-receptor, ICAM-1, results in firm adhesion and spreading of neutrophils on endothelial cells [2–4].

Transmigration of neutrophils across the vascular wall is an active process for both migrating neutrophils and the activated endothelium. Proteins that hinder paracellular migration are degraded or escorted from endothelial junctions, while those required for the transmigration process are mobilized on endothelial cells to the junction interface [5]. Release of extracellular matrix-degrading enzymes from neutrophil storage granules clears a path through the vessel wall and produces binding sites for integrin adhesion and chemotactic fragments of matrix proteins [6].

Once neutrophils cross the vessel wall, they move along a gradient of substrate-bound (haptotaxis) and/or soluble (chemotaxis) chemoattractants to seek out their targets. Chemotactic signals are released from bacteria, necrotic cells, or other inflammatory cells; including IL-8, leukotriene B_4, epithelial cell-derived neutrophil attractant (CXCL5), thrombospondin, formylated peptides, and complement C5a [7,8]. Each of these chemotactic signals interacts with specific receptors to direct neutrophil migration via rearrangement of the actin cytoskeleton.

Phagocytosis results in the engulfment of foreign pathogens and cell debris. Complement receptors and immunoglobulin Fcγ receptors (FcγR) induce reorganization of the cytoskeleton leading to pathogen ingestion by either pseudopod-like extensions which surround the pathogen or membrane zippering and sinking of the pathogen into the neutrophil [9]. Upon pathogen ingestion, the nascent phagosome acquires microbicidal capability by a process termed maturation. Fusion of phagosomes with intracellular secretory granules is required to supply the components necessary for maturation, including transporters that generate an acidic lumen, proteases, components of the NADPH oxidase, and microbicidal peptides.

Four classes of neutrophil granules have been identified based on morphology, separation by density gradient fractionation, and contents. Sengeløv *et al.* [10,11] established a hierarchy of granule mobilization by examining granule content release during in vivo exudation and upon fMLP stimulation. Stimulation of human neutrophils in vitro by fMLF resulted in exocytosis of greater than 60% of secretory vesicles, 30% of gelatinase (tertiary) granules, 15% of specific (secondary) granules, and less than 5% of azurophil (primary) granules. Exudative neutrophils in skin windows in human volunteers demonstrated 100% release of secretory vesicles, 38% of gelatinase granules, 21% of specific granules, and 7% of azurophil granules. Exocytosis of these granule subsets leads to incorporation of receptors, adhesion molecules, and signaling molecules into plasma or phagosomal membranes and release of proteases and other host-defense proteins into phagosomes or the surrounding tissue.

In addition to granule microbicidal contents, generation of oxygen radicals is necessary for neutrophil microbicidal activity. The NADPH oxidase is a multicomponent enzyme that

transfers electrons from cytosolic NADPH to molecular oxygen to produce reactive oxygen species. Flavocytochrome b_{558}, comprised of $gp91^{phox}$ and $p22^{phox}$, is the membrane-bound core of the complex. The major cytoplasmic components required for activation of the complex include $p67^{phox}$, $p47^{phox}$, $p40^{phox}$, and rac2. The combination of the azurophil granule protein myeloperoxidase with hydrogen peroxide forms the highly toxic hypochlorous acid. Toxic oxygen and nitrate species act synergistically with neutral proteases and bactericidal peptides to kill bacteria [12,13].

Once the offending agents are removed, an inflammatory reaction must be terminated to prevent excessive damage to normal tissue. Resolution of inflammation was previously considered to be a passive process in which inflammation would simply 'burn out' with time. Resolution of inflammation, however, is now known to be an active process involving synthesis of anti-inflammatory cytokines and pro-resolution lipid mediators and induction of neutrophil apoptosis. Resolution is initiated by release of anti-inflammatory cytokines by macrophages, including IL-10, IL-13, and transforming growth factor (TGF)-β, and lipid-derived mediators of resolution (lipoxins, protectins, and resolvins) by vascular endothelial cells and macrophages [14].The anti-inflammatory cytokines suppress the production of TNF-α and inhibit the upregulation of endothelial cell adhesion molecules [15]. Lipoxins are a group of lipid mediators that reduce vascular permeability, preventing further recruitment of neutrophils [16]. Resolvins and protectins are two recently described families of lipid mediators with anti-inflammatory properties. These endogenous compounds derived from the omega-3-fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), block the production of pro-inflammatory mediators and stop neutrophil infiltration and migration in vivo [17].

The final stage in resolution is apoptosis of infiltrating neutrophils and their removal by tissue macrophages. During active inflammation, production of pro-inflammatory cytokines and bacterial products delay constitutive neutrophil apoptosis [18]. With the onset of resolution, apoptosis is induced by a number of anti-inflammatory cytokines. Cell surface molecular changes trigger phagocytosis of the apoptotic neutrophils by macrophages, release of anti-inflammatory mediators such as TGF-β1, IL-10, and leukocyte protease inhibitors, and induce a switch of the macrophage phenotype from an activated to a reparative cell [19,20,21].

3. Neutrophil Receptors and Signal Transduction Pathways in Activation and Resolution of Inflammation

As indicated above, the ability of neutrophils to respond to inflammatory stimuli in a coordinated manner requires plasma membrane expression of a large number of receptors. Toll-like receptors (TLRs) are a subfamily of pattern recognition receptors (PRRs) that initiate neutrophil responses by recognizing microbial products and damage-associated molecular pattern (DAMP) molecules released from injured cells [22]. Seven transmembrane-spanning G-protein coupled receptors (GPCRs) recognize chemoattractants, including formylated peptides, C5a, platelet activating factor, leukotriene B_4, and IL-8. Specific cytokine receptors recognize TNF-α, interleukin-1α and β, colony stimulating factors for granulocyte-macrophages (GM-CSF) and granulocytes (G-CSF), and interferons. Integrin receptors and selectins not only mediate adhesion, but participate in "inside-out" and "outside-in" signal transduction [23]. Complement receptors and FcγRs link engagement of opsonized particles with actin-mediated phagocytosis, as discussed above.

Ligand binding to the various receptors results in an overlapping array of signal transduction pathways which link receptors with functional responses. These receptors induce assembly of multi-component signaling complexes on the cytoplasmic side of the plasma membrane which contain kinases that transmit signals through a series of protein and lipid phosphorylation events. For example, Fcγ receptor ligation results in the Src tyrosine kinase-dependent

phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) located in the cytoplasmic tail of FcγRs [24]. Syk tyrosine kinases bind to phosphorylated ITAMs through their SH2 domains inducing activation of the Rho family of GTPases (Rac, Cdc42, Rho) via Rho family guanine nucleotide exchange factors (GEFs). This leads to reorganization of the actin cytoskeleton [25]. Chemoattractant GPCRs are coupled by heterotrimeric G proteins to signal transduction pathways containing Src tyrosine kinases, phospholipase $C\beta_2$, protein kinase C, phospholipase D, Akt, phospholipase A_2, Erk, p38 MAPK, and PI 3-K. TLRs initiate sequential phosphorylation reactions leading to activation of NF-κB and two members of the mitogen-activated protein kinase (MAPK) family, p38 and JNK [26]. A number of common tyrosine kinases and serine/threonine kinases are activated by different receptors resulting in different phenotypic changes. For example, TNFα and bacterial lipopolysaccharide do not stimulate respiratory burst activity, but prime neutrophils for an enhanced respiratory burst upon stimulation by a second agent. This priming effect is dependent on activation of p38 MAPK [27–29]. On the other hand, the chemoattractant f-Met-Leu-Phe stimulation of respiratory burst activity is dependent on the p38 MAPK pathway [30], and activation of p38 MAPK was shown to be involved in neutrophil apoptosis [31]. As opposed to promoting cell death pathways, p38 MAPK activation has also been shown to promote Akt activation and cell survival in neutrophils [32–34]. Our understanding of the interconnections among the many signal transduction pathways that regulate neutrophil activation is incomplete. Elucidating the molecular details of interactions among these various signal transduction pathways through proteomic approaches is likely to greatly expand our understanding of the initiation and resolution of inflammation.

4. Application of Proteomics to Understanding Neutrophil Biology

Proteomic approaches can be grouped into three broad categories: expression, structural, and functional. The goal of expression proteomics is to define and quantify all proteins within a cell or subcellular organelle under normal, diseased, or treated conditions. Structural proteomics aims to determine the composition of protein complexes and to define protein-protein interactions. Functional proteomics aims to define the post-translational modifications of proteins that regulate their activation or molecular interactions. Table 1 summarizes the proteomic studies reviewed in the subsequent sections.

5. Application of Expression Proteomics to Neutrophils

Not all expressed genes are translated into proteins, highlighting the need to identify the protein composition of cells and subcellular organelles. The inconsistent correlation between mRNA and protein expression has proven true for neutrophils undergoing differentiation or during response to inflammatory stimuli [35,36]. Piubelli *et al*. [37] performed a global analysis of rat neutrophil proteins using 2-dimensiontal gel electrophoresis (2DE) to separate proteins from neutrophils isolated from blood. A wide immobilized pH 3–10 gradient and a narrower IPG pH 4.5–8.5 gradient in the first dimension were used to create a 2D protein map. Three different MS approaches, MALDI-TOF MS in both linear and reflectron modes and LC-ESI MS/MS, were used to identify 52 proteins from 2DE gel spots. Lippolis and Reinhardt [38] used a combination of one-dimensional electrophoresis followed by reversed phase chromatography in line with ESI-MS/MS to identify 250 proteins from bovine neutrophils. Proteins were classified into functional categories of cell metabolism, cell motility, immune response, protein synthesis, cell signaling, and membrane trafficking. These studies established the ability to define the neutrophil proteome.

To better understand the consequences of neutrophil activation, a number of subsequent expression proteomic studies focused on subcellular organelles. Redistribution of intracellular storage granules to the plasma membrane or to phagosomes is an important mechanism by

which neutrophils rapidly respond to inflammatory stimuli. To define the consequences of granule mobilization, Lominadze *et al*. [39] used a combination of proteomic techniques to perform a comprehensive analysis of gelatinase, specific, and azurophil granules isolated by three-layer Percoll density gradient centrifugation. 2DE combined with MALDI-TOF MS identified 87 proteins, only one of which was a membrane-spanning protein. The resolving capacity of two-dimensional gels was limited due to large amounts of basic lumenal proteins. However, 2DE revealed differential amounts of actin associated with each granule subset. Application of two-dimensional chromatographic separation with reversed phase followed by strong cation exchange in line with ESI-MS/MS identified 247 proteins in the granule subsets. Of these, 86 were found only in gelatinase granules, 28 only in specific granules, and 26 only in azurophil granules. A number of these proteins, including several GTPases, had not previously been identified on neutrophil granules.

Feuk-Lagerstedt *et al*. [40] isolated lipid rafts from human neutrophil azurophil granules, separated proteins by 10% SDS-PAGE, and identified excised bands by LC-MS/MS. This study identified 106 proteins, including cytoskeletal, structural, and membrane fusion proteins. A similar study used gradient gel electrophoresis and MALDI-MS or MALDI-MS/MS to identify 23 proteins from plasma membrane lipid rafts [41]. Nine of these 23 proteins, primarily cytoskeletal proteins, were common to those identified from azurophil granule lipid rafts. These studies represent initial attempts to define the function of lipid rafts in neutrophils.

Two studies have compared the components of secretory vesicle membranes with those of plasma membranes. Jethwaney *et al.* [42] analyzed multiple slices from one-dimensional gels of separated proteins from secretory vesicle membranes and plasma membranes isolated by free-flow electrophoresis. By MALDI-TOF-MS only 27 proteins were identified, while analysis by HPLC-MS/MS identified 43 and 37 proteins from vesicle and plasma membrane fractions, respectively. Two proteins unique to secretory vesicles, desferlin and 5-lipoxygenase-activating protein, were validated by immunoblot analysis, and desferlin was demonstrated to translocate to the plasma membrane following stimulation with the formylated peptide, f-Met-Leu-Phe. Uriarte *et al*. [43] identified proteins from secretory vesicles and plasma membranes by 2D-LC-MS/MS following extraction of membrane proteins using 60% methanol in ammonium bicarbonate [44]. Of the 1118 proteins identified, 337 were categorized as integral membrane proteins by Gene Ontology. A total of 573 proteins were present only in plasma membrane fractions, 418 only in secretory vesicles, and 127 in both membrane fractions. Differences in biologic functions and canonical pathways of these proteins were identified using a proprietary bioinformatics platform, Ingenuity Pathways Knowledge Base. For example, components of the PI 3-K/Akt pathway were only found in the secretory vesicle fractions, while components of the TLR pathway were localized to the plasma membrane fraction. Up to 25% of identified proteins in both of these studies were predicted to be from contaminating mitochondria, endoplasmic reticulum, or Golgi. This finding indicates that the sensitivity of current mass spectrometry techniques makes the ability to obtain highly purified intracellular organelles the limiting factor in obtaining an accurate organelle proteome.

The studies described above examined the proteome of neutrophils or subcellular organelles in unstimulated neutrophils. Application of expression proteomic approaches to neutrophil activation has also been reported. Burlak *et al.* performed the first large scale proteomic analysis of proteins comprising human neutrophil phagosomes [45]. This study found a number of proteins previously identified with macrophage phagosomes, including proteins involved in cytoskeletal reorganization and proteins typically found in the ER [46,47]. Maturation of neutrophil phagosomes is thought to be dependent, in part, on fusion of intracellular granules with the phagosomal membrane. This hypothesis was supported by their finding that many phagosomal proteins were previously identified on neutrophil granules by Lominadze *et al*. [39]. On the other hand significant differences in the proteins identified on macrophage and

neutrophil phagosomes was found, indicating that the mechanisms of phagosomal maturation differ between the two cell types [48,49].

2D DIGE allows the comparison of multiple sample sets within the same gel, thus eliminating gel-to-gel variability. The data are not only visually quantitative, but can also be accompanied by mass spectrometric analysis. Xu *et al.* [50] used protein separation by 2DE followed by MALDI-TOF-MS analysis of tryptic digests to compare the proteins associated with subcellular cytoskeletal compartments associated with the cytosol, phagosomes, and plasma membrane. Although the majority of proteins were cytoskeletal proteins, a number of metabolic enzymes were found to be associated with the cytoskeleton. Shimada *et al.* [51] used 2DE and MALDI-TOF-MS to determine that a combination of crosslinking CD69 and stimulation with GM-CSF induced increased expression of the calcium binding protein S100A9.

Fessler *et al.* [52] compared the changes in neutrophil gene transcription and protein expression following exposure to bacterial lipopolysaccharide (LPS). Exposure to LPS for 4 hr resulted in upregulation of 100 genes and downregulation of 56 genes contained on an Affimetrix gene array. Protein expression was determined by 2DE, which identified about 1200 well resolved spots. In each of six independent experiments about 100 proteins were found to have increased expression by 50% and another 100 proteins had a 50% reduction in expression. A poor correlation was found between the changes in expression of proteins and mRNA.

Mass spectrometry approaches have been used to identify agents that act on neutrophils leading to resolution of inflammation. Serhan and colleagues used LC-UV-MS/MS with low-energy ionization electrospray to identify two groups of lipid mediators that are generated from fatty acids during resolution of inflammation. These two groups of mediators, termed resolvins and protectins, are derived from eicosapentenoic acid and docosahexaenoic acid and possess potent anti-inflammatory properties [53,17,54]. Using LC-MS/MS, Haas-Stapleton *et al.* [55] identified one of these resolvins, Resolvin E1 (RvE1), as a biosynthetic product of *Candida albicans* produced in the presence of eicosapentaneioc acid. This product blocked IL-8-stimulated neutrophil chemotaxis, while enhancing neutrophil-mediated phagocytosis, killing, and clearance. The authors concluded that RvE1 production is a method used to deter migration of neutrophils to sites of inflammation. Ohira *et al.* [56] investigated the kinase signaling pathways regulated by the pro-resolving mediator 15-epi-16-(*p*-fluoro)-phenoxy-lipoxin A4 methyl ester (ATLa). Using mass spectrometry, they determined that ATLa blocked phosphorylation of leukocyte-specific protein 1, a downstream component of the p38 MAPK cascade, which is known to regulate neutrophil chemotaxis. Greenlee *et al.* [57] used 2DE followed by MALDI-TOF-MS to identify substrates of matrix metalloproteinases 2 and 9 from the bronchoalveolar lavage fluid of MMP2−/− and MMP9−/− transgenic mice. Three substrates, Ym1, S100A8, and S100A9, demonstrated chemotactic activity that was reduced following cleavage with MMP2 and 9.

6. Application of Structural Proteomics to Neutrophils

Although transfection of cDNAs into neutrophils has been documented [58,59], genetic manipulation of these terminally differentiated cells leading to high efficiency introduction of tagged proteins remains a challenge. Consequently, techniques to immunoprecipitate endogenous proteins have been developed to define the composition of protein complexes without altering the genetic composition of neutrophils. The applicability of immunoprecipitation of specific proteins from neutrophils to allow protein complex structures to be defined was demonstrated by Rane *et al.* [60]. This study examined the molecular mechanisms by which Akt regulated neutrophil functional responses. Proteins contained in the Akt complex immunoprecipitated from neutrophil lysates were resolved by 2DE, and unique protein spots were identified by in-gel trypsin digestion and peptide mass fingerprint analysis

by MALDI-MS. One of the proteins identified was the C-terminal tail of the γ-amino butyric acid receptor 2 ($GABA_BR2$). The presence of Akt and $GABA_BR2$ in a protein complex was confirmed by reciprocal immunoprecipitation followed by immunoblot analysis. $GAGA_BR2$ agonists stimulated Akt phosphorylation and activation, stimulated neutrophil chemotaxis *in vitro* in an Akt dependent manner, and enhanced neutrophil influx into areas of cerebral ischemia-reperfusion injury in mice. Bioinformatic analysis of other proteins contained in the Akt-$GAGA_BR2$ complex identified a number of proteins that play a role in microtubule organization; including microtubule associated regulatory kinase 4, the dynein H chains, merlin, and focal adhesion kinase. $GABA_BR2$ ligation was shown to stimulate neutrophil chemotaxis through microtubule cytoskeleton reorganization in a PI-3K/Akt-dependent manner. Thus, a proteomic analysis of Akt immunoprecipitates led to the identification of a novel receptor signaling pathway that regulates neutrophil migration into areas of ischemia.

A similar study was performed to define the role of the Akt scaffolding protein, heat shock protein (Hsp) 27, in neutrophil apoptosis. Proteins were identified from Akt immunoprecipitates before and after disruption of the Akt-Hsp27 complex by introduction of anti-Hsp27 antibodies by three proteomic approaches. First, immunoprecipitated proteins were separated by 2DE and unique protein spots were subjected to in-gel digestion and identified by MALDI-MS. Second, immunoprecipitated proteins were directly eluted from protein A sepharose beads, digested with trypsin, separated by reversed phase capillary HPLC, and subjected to MALDI-MS/MS. Third, following direct elution and trypsin digestion, peptides were analyzed by HPLC-ESI-MS/MS. From these three analyses about 100 proteins were identified (Rane *et al.* unpublished data). Disruption of Akt-Hsp27 resulted in dissociation of positive regulators of Akt activity, such as p38 MAPK, mitogen-activated protein kinase-activated protein kinase-2 (MAPKAPK2), and 3-phosphoinositide-dependent protein kinase-1 (PDK1), from the Akt signal complex. In contrast, Akt-Hsp27 disruption resulted in recruitment of a negative regulator of Akt activation, PKC-δ, to the Akt signaling complex [61]. In addition to PKC-δ, apoptosis-related proteins PARP-2, caspase-3, and caspase-8 associated with Akt after disruption of the Akt-Hsp27 interaction, suggesting that disruption of the Akt signaling complex may promote caspase-3 activation, leading to neutrophil apoptosis.

7. Application of Functional Proteomics to Neutrophils

Post-translational modifications are important regulators of individual protein functions. Given the sensitivity of current technology, most biologically relevant post-translational modifications, including phosphorylation, glycosylation, ubiquitination, methylation, acetylation, isoprenylation, can be detected by mass spectrometry techniques. However, some post-translational modifications, including acetylation and citrullination, can impair detection of peptides by mass spectrometry due to changes in charge leading to alterations in the peptide ionization state.

Taylor *et al.* [62] employed a combination of MALDI MS and LC-MS/MS to analyze the structure of the flavocytochrome b_{558} (Cyt b) purified from both human neutrophil membrane fractions and PLB-985 cells expressing recombinant $gp91^{phox}$. This study confirmed $gp91^{phox}$ glycosylation sites by identifying post-translational modifications from mass spectra. The ability of glycosylation to regulate three-dimensional protein structure and the interactions with other oxidase components was required for NADPH oxidase activation.

Protein kinases are the most common mediators of signal transduction in eukaryotic cells. Manning *et al.* [63] determined that the kinome of the human genome contained 518 kinases, representing 1.7% of the genome. Identification of targets of these kinases remains a challenge due to the expression of target proteins at low levels compared to the most abundant cell

proteins, the low stoichiometry of phosphorylation, and the transient nature of phosphorylation. Advances in enrichment techniques of organelles and phosphoproteins and in proteomic technology now permit large-scale, global identification of phosphorylated proteins and targeted identifications of specific phosphorylated residues. These advances have stimulated a focused approach to tracking the functional effects of phosphorylation as they relate to neutrophil biology.

Using a combination of *in vitro* phosphorylation of [^{32}P]-orthophosphate-loaded neutrophillysates, one-dimensional SDS-PAGE, and peptide identification by MADLI-TOF-MS, our laboratory identified eight substrates of MAPKAPK2, a downstream kinase in the p38 MAPK pathway [64]. One of these substrates, 14-3-3ζ was found to be phosphorylated at Ser-58, and this phosphorylation regulated 14-3-3ζ dimerization and binding to Raf-1. In this proteomic analysis, a number of abundant neutrophil proteins were identified, but these were considered contaminating artifacts stemming from the inherent limitations of one-dimensional gel analysis. 2DE and immunoblot analysis was used to detect pI shifts in 14-3-3ζ upon fMLF stimulation of intact neutrophils. This pI shift was reversed by PP2A phosphatase treatment of cell lysates and prevented by preincubation of intact neutrophils with the p38 MAPK inhibitor, SB203580. These findings indicate that the pI shift was due to phosphorylation mediated by the p38 MAPK pathway. Singh *et al.* [65] used a combination of 2DE and MALDI-MS to identify MAPKAPK2 substrates in [^{32}P]orthophosphate-loaded neutrophil lysates. This analysis identified the p16-Arc subunit of the Arp2/3 complex as a MAPKAPK2 substrate. The functional consequences of its phosphorylation are not yet known, but may explain the participation of the p38 MAPK pathway in actin-dependent cellular functions. Lominadze et al. [66] used a similar approach to identify the calcium-binding protein myeloid-related protein-14 (MRP-14) as a target of p38 MAPK phosphorylation in neutrophils stimulated with fMLF. MRP-14 translocation to neutrophil granules following fMLF stimulation was blocked by inhibition of p38 MAPK activity, suggesting a role for MRP-14 in stimulated exocytosis.

Kettritz *et al.* [67] used 2DE and immunoblot analysis with an anti-phosphotyrosine antibody to investigate intracellular signaling events involved in fibronectin-accelerated TNF-α-mediated neutrophil apoptosis. The RhoGTPase regulator, LyGDI, was identified by MS/MS and was found to be tyrosine phosphorylated. The authors determined that LyGDI phosphorylation was followed by increased caspase-3-mediated LyGDI cleavage, and this cleavage was a signaling event in TNF-α-mediated apoptosis.

Pacquelet *et al.* [26] used LC-MS/MS analysis of neutrophil lysates separated by SDS-PAGE to demonstrate that interleukin-1 receptor-associated kinase-4 (IRAK-4) is downstream of toll-like receptor 4 (TLR-4) -dependent activation of the NADPH oxidase component p47^{phox}. They determined that multiple sites on p47^{phox} were directly phosphorylated by IRAK-4. 2DE was used to demonstrate that IRAK-4 phosphorylated p47^{phox} at serine and threonine residues, and phosphorylation of these sites led to enhanced NADPH oxidase activity.

Receptor ligation leads to the activation of many intracellular signaling cascades regulating neutrophil functional responses. Boldt *et al*. [68] used 2D-DIGE to identify protein patterns that differed between basal neutrophils and neutrophils activated through the formyl peptide receptor. They identified a number of proteins involved in cytoskeletal reorganization with differential expression, including L-plastin, meosin, cofilin, and strathmin. Using phosphoprotein-specific gel staining (Pro-Q Diamond), they determined that the changes in expression of each of these proteins were due to phosphorylation or dephosphorylation. Dang *et al.* [69] employed MS/MS to confirm that a component of the NADPH oxidase, p47^{phox}, is phosphorylated on Ser345 by extracellular signal-regulated protein kinase-1/2 (ERK1/2) in response to GM-CSF, and by p38 MAPK in response to TNF-α. This selective phosphorylation is a point of convergence for MAPK signaling that primes the respiratory burst.

Despite advances in mass spectrometry techniques that allow identification of post-translational modifications, detection of protein phosphorylation on a proteome scale remains difficult due to issues of abundance and poor ionization characteristics of phosphoproteins. Ions of low-abundance phosphoproteins are suppressed in complex samples, making post-translational modification detection difficult. Enrichment of phosphoproteins and phosphopeptides is, therefore, a necessity for their identification. Immobilized metal affinity chromatography (IMAC) takes advantage of the affinity of phosphopeptides for divalent and trivalent cations (e.g Ni^{2+}, Mg^{2+}, Fe^{3+}, Ga^{3+}). IMAC is not without its limitations, including contamination by peptides containing acidic amino acids (Asp, Glu, His, Cys) and the preference of multiply phosphorylated peptides. The use of off-line sources for IMAC enrichment (spin column, zip-tip) may also contribute to sample loss. Metal oxide affinity chromatography (MOAC) employing TiO_2 or related oxide compounds demonstrated increased phosphopeptide affinity and coverage over other IMAC media [70,71]. Online microcolumns are commonly used just upstream of other HPLC separation techniques (reverse phase, strong cation exchange chromatography) [72].

In an attempt to establish a connection between kinase signal transduction pathways and functional responses, our laboratory used a combination of IMAC with ESI-MS/MS to identify phosphorylated proteins that are targets of kinases known to regulate exocytosis. To address problems with dynamic range, intact specific granules isolated from unstimulated or fMLF-stimulated human neutrophils were subjected to trypsin digestion. Thus, peptides were generated only from portions of proteins on the cytoplasmic surface of these granules. Following methyl esterification to reduce non-specific binding by carboxylate anions, the peptides were subjected to immobilized Ga^{3+} affinity chromatography to enrich for phosphopeptides. HPLC-separated peptides were eluted directly into a LTQ linear ion trap mass spectrometer for ESI-MS/MS. Acquired spectra were monitored for 80 Dalton shifts consistent with phosphorylated amino acid residues. A total of 31 phosphopeptides were identified from specific granules from unstimulated cells, while 49 were identified from granules isolated from fMLF-stimulated neutrophils. Table 2 lists the identified phosphopeptides from proteins likely to participate in exocytosis. One peptide that contained two phosphoserines was identified as Slp homolog lacking C2 domains b (Slac2-b). The Slac2 family member, Slac2-a, mediates the transfer of melanosomes from microtubules to the actin cytoskeleton in melanocytes and couples the vesicles to the myosin molecular motors required for actin-dependent transport to the plasma membrane [73]. The presence of Slac2-b in neutrophils was confirmed by tandem mass spectrometry analysis of secretory vesicle membranes [43] and by RT-PCR (Luerman et al. unpublished observations). The Slac2-b peptide which contained the two phosphoserines comprised a known p38 MAPK phosphorylation motif [74], as identified by GPS (Group-based Prediction System) 2.1 [75]. We and others previously reported that p38 MAPK regulates stimulated exocytosis in human neutrophils [28,76,77]. We are currently evaluating the role of Slac2-b as a candidate to link p38 MAPK activation to granule exocytosis in human neutrophils.

8. Limitations

A number of limitations to the application of proteomics to neutrophils exist. A large number of proteases are contained in neutrophil granules. Release of some granules contents accompanies disruption of neutrophils for subcellular organelle isolation, making protease inactivation necessary to achieve accurate analysis by mass spectrometry. Studies of neutrophil subcellular organelles indicate that these organelles are complex and, thus, present problems related to the dynamic range of proteins. The current sensitivity of mass spectrometers can lead to identification of proteins from contaminating organelles, making enrichment techniques of critical importance. For this reason, advances in technologies that will identify the subcellular location of individual proteins are required to allow confirmation of proteomic data [78,79].

Identification of membrane associated and transmembrane proteins may be limited due to lack of available trypsin cleavage sites outside the membrane. Thus, other proteolytic enzymes (e.g. chymotrypsin, Glu C, cyanogen bromide) may improve generation of peptides and coverage of these proteins. Comprehensive identification of phosphopeptides is affected by the different approaches for phosphopeptide enrichment. The immobilized metals comprising IMAC columns demosntrate different affinities for singly- and multiply-phosphorylated peptides [80]. HPLC titanium dioxide affinity columns showed increased binding specificity for phosphopeptides, but decreased binding of multiply-phosphorylated peptides [70]. Thus, the characteristics of enriched phosphopeptides will depend on the type of column used. Taken together, the studies reviewed reinforce previous observations that a combination of proteomic approaches, including different methods of protein extraction and separation, is necessary to optimally identify any proteome.

9. Conclusions

Neutrophils play a major role in the initiation and resolution of the inflammatory response, and they clearly perform tasks in addition to killing microorganisms. Although once considered as terminally differentiated, static cells, neutrophils demonstrate significant transcriptional and translational activity. Understanding these changes at the protein level will advance understanding of neutrophil participation in health and disease. The promise of proteomics is that identifying all proteins expressed in a cell, determining their localization within a cell, defining their protein-protein interactions, and identifying their post-translational modifications will dramatically enhance our understanding of how cells respond to various physiologic and pathophysiologic conditions. The application of proteomic technologies to neutrophils is clearly feasible, but subject to the same limitations seen in other biologic systems. These studies are in their infancy, and significantly more data on how neutrophils respond to various combinations of stimuli will be required. The data will require computational analysis, using systems biology methodologies, to define the dynamic regulatory networks that control neutrophil responses. The studies reviewed herein provide the initial steps in achieving the goal of a global understanding of neutrophil biology at the molecular level.

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Table 1

A selected list of proteomic studies contributing to the understanding of neutrophil biology

Sample/Organelle	Protein Separation	MS Analysis	Major Findings
Expression			
Rat neutrophil lysate	2DE, HPLC	MALDI-TOF, LC-ESI-MS/MS	52 proteins identified
Bovine neutrophil lysate	1DE, HPLC(RP)	LC-ESI-MS/MS	250 proteins identified
Storage granules	2DE, gel-free HPLC (RP/SCX)	2DE/MALDI-TOF, LC-ESI-MS/MS	2DE/MALDI identified 87 proteins ESI-MS/MS identified 247 proteins
Lipid rafts	1DE	LC-FT-ICR MS/MS	106 proteins identified
Lipid raft cytoskeleton	1DE	MALDI-TOF, MS/MS	23 proteins identified
Secretory vesicles and plasma membrane fractions	1DE, free-flow electrophoresis	MALDI-TOF, HPLC-MS/MS	MALDI identified 27 proteins HPLC-MS/MS identified 80 proteins
Secretory vesicles and plasma membrane fractions	2D HPLC (SCX/RP)	LC-MS/MS	1118 total proteins identified, including 337 integral membrane proteins
Phagosomes	2DE	MALDI-TOF, LC-MS/MS	198 proteins identified, including ER machinery
Phagosome and plasma membrane skeletons	2DE	MALDI-TOF	138 proteins identified, metabolic enzymes associate with the cytoskeleton
Neutrophil lysate	2D-DIGE	LC-MS/MS	FPRLR-1 ligation (de)phosphorylates cytoskeletal proteins
Neutrophil lysate	2D-DIGE	MALDI-TOF/TOF	Cross-linking CD69 enhanced expression of S100A9
Neutrophil lysate	2DE	MALDI-TOF	Pharmacologic inhibition of p38 attenuated 18% of LPS-regulated proteins by ≥ 40%
Brochoalveolar lavage fuid	2D-DIGE	MALDI-TOF/TOF	Identified 3 proteins having MMP2 and 9 cleavage sites: Ym1, S100A8, S100A9
Structural			
Neutrophil lysate	IP, 2DE	MADLI-TOF	Identified $GABA_BR2$ as an AKT substrate
Functional			
Immunoaffinity-purified cytochrome *b*	1DE	MALDI, LC-MS/MS	Reported extensive sequence coverage and PTMs for Cyt *b*
Neutrophil lysate	1DE	MALDI	Identified substrates of MAPKAPK2, including 14-3-3ζ
Neutrophil lysate	2DE	MALDI	Identified substrates of MAPKAPK2, including p16-Arc of Arp2/3
Neutrophil lysate	2DE	MALDI	Identified substrates of p38 MAPK, including MRP-14

Table 2

IMAC-enriched specific granule-associated proteins identified by LC-MS/MS.

Protein Description	Peptide	Role in Neutrophil Function	Potential Kinase Motif[a]
Unstimulated			
Proline-rich synapse-associated protein 2(proSAP1)	GGS*LLSIGE#GGF	Clusters vesicle membrane proteins to actin	Rho kinase (ROCK)
Gamma-aminobutyric acid (GABA) A receptor	GFSQMPTSSVKDET*ND#NIT*IF	GPCR, PMN chemoattractant receptor	GPCR kinase, PAK/MLK
Stimulated			
Complement component 1	VD#VPCS*HFCNNF	Serine protease	TAK1
RAB3 interacting protein 3	RST*E#QRPLLE#RTTTRSRST	Rab-specific effector	Protein Kinase C
RAB3 interacting protein 3	KVVGGKMTES*GRLCAF	Rab-specific effector	Raf kinase
Paracingulin	VLE#TE#GS*QE#STVIR	RhoGTPase regulator	Casein kinase 1
RAB21	DT*AGQE#RFHALGPIY	Vesicle targeting	Casein kinase 1a
Protein kinase C, alpha	TRPTICQY*	Kinase	Csk (Src)
G protein-coupled receptor 126	GS*NQNEIVSL	GPCR	Casein kinase 2b
G Protein receptor kinase 5	KVNSQFVVNLAY*AY*ETKD	Ser/Thr GPCR Kinase	Tec kinase, JAK2
Muted	GT*AGSAHLIIKD#L	Biogenesis of granules	MAPK11
Dynein	AAHMADYS*VF	Vesicular motor protein	TAK1
RAN binding protein 3	KLNEVSSD#AN RENAAAES*GS* E-SS*SQEATPE	Essential to nucleocytoplasmic transport	Casein kinases(for all S*)
Gamma-taxilin	RT*DPPD GQQDSECNRNKE#KT	Syntaxin-binding protein, Ca^{2+}-dependent exocytosis	MAPK9
PKC-delta-interacting protein kinase	VKE#TAHD#LDVKS*PPEPRS	Kinase, PKC inhibition blocks exocytosis of MMP-9	Multiple MAPKs
Slac2-b	H KTSCDSLD#LSSAALPDS*S*PS K	Rab27-specific effector	Multiple MAPKs (for all S*)
Dedicator of cytokinesis 3(DOCK3)	KLDSMVSE#GKGD#E#S*YRE	Regulates RhoGTPase activation	Casein kinase 2
Cdc42 effector protein 4	RDS*SSLSSCTS*GILE#ERS	Control of actin polymerization	PKG2, Dyrk1

Peptide modification searches identified residues modified by methyl esterification of D and E (#) and STY phosphorylation (*)

[a] for each phosphorylated residue, as identified by Group Prediction System 2.1 (GPS) under high stringency [74]