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Analysis of Detergent-Insoluble and Whole Cell Lysate Fractions of Resting Neutrophils Using High-Resolution Mass Spectrometry

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Neutrophilic granulocytes play a major role in the initiation and resolution of the inflammatory response, and demonstrate significant transcriptional and translational activity. Although much was known about neutrophils prior to the introduction of proteomics, the use of MS-based methodologies has provided an unprecedented tool to confirm and extend previous findings. In the present study, we performed a Gel-LC-MS/MS analysis of neutrophil detergent insoluble and whole cell lysate fractions of resting neutrophils. We achieved a set of identifications through the use of high-resolution mass spectrometry and validation of its data. We identified a total of 1249 proteins with a wide range of intensities from both detergent-insoluble and whole cell lysate fractions, allowing a mapping of proteins such as those involved in intracellular transport (Rab and Sec family proteins) and cell signaling (S100 proteins). These results represent the most comprehensive proteomic characterization of resting human neutrophils to date, and provide important information relevant for further studies of the immune system in health and disease. The methods applied here can be employed to help us understand how neutrophils respond to various physiologic and pathophysiologic conditions and could be extended to protein quantitation after cell activation.

Keywords: proteomic profiling • neutrophils • cell fractionation • whole cell lysate • high resolution mass spectrometry • shotgun proteomics.

Introduction

Neutrophilic polymorphonuclear cells (PMNs) are the most abundant leukocytes in human peripheral blood and have a specially tailored cell biology to mediate the first responses that characterize the innate immune system early in inflammation.[1] Once at the target site, they quickly attack microorganisms and debris by phagocytosis, which stimulates the assembly and activation of the NADPH oxidase enzyme complex, undergoing a burst of oxygen consumption and generation of reactive oxygen species (ROS) inside the PMNs.[2] Furthermore, PMNs are rich in intracellular granules that contain proteolytic enzymes, in addition to proteins that are directly toxic to microbes, such as bactericidal permeability increasing proteins, and proteins that convert H_2O_2 into more potent antimicrobial species.[3] In addition to their granules, PMNs also contain secretory vesicles, a unique compartment that cosediments with the plasma membrane in the light membrane fraction of resting neutrophils. Whereas the lumen of secretory vesicles houses plasma proteins, the membranes of this intracellular compartment contain a variety of functionally important membrane proteins.

PMNs rely on the redistribution of functionally important proteins, from intracellular compartments to the plasma membrane, as the means to respond quickly.[5] The application of proteomics for the characterization of human neutrophils is steadily increasing, and its potential to achieve a better understanding of neutrophil biology has recently been addressed.[6] Subcellular proteomic studies have focused on the identification of human neutrophil proteins in the cytoskeleton,[7] membrane and secretory vesicles[8,9] and granule fractions.[5] We recently published a comprehensive analysis of total cellular proteins,[10] using gel-LC-MS/MS and a low resolution Q-TOF mass spectrometer. At that time we were able to identify 251 proteins, of which 22% had not been found previously in human neutrophils.

Mass spectrometry-based proteomics is a powerful approach for analyzing highly complex samples,[11] and has taken great strides in development, mostly with respect to the implementation of instruments with high speed and resolution that allow the acquisition of very accurate mass data.[12] This is particularly true regarding hybrid mass spectrometers such as the LTQ-

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Orbitrap,[13,14] which combines the high-resolution features of a Fourier transformer electrode with a low-resolution, but highly sensitive, linear ion trap (LTQ). The improvement in determining accurate peptide masses resulted in more reliable protein identifications. Consequently, new software packages were developed specifically to exploit this type of data in order to improve the workflow without compromising false-discovery rates.[15] Consequently, the technical improvements are expected to have an impact on the current knowledge about neutrophilic cells.

The current study was undertaken with the objective to generate a high quality identification repertoire and to improve the current knowledge about the neutrophil proteome by applying the high resolution LTQ-Orbitrap. To enhance the identification of proteins, we performed a prefractionation of the PMNs by analyzing detergent-insoluble and whole cell lysate fractions. We identified a total of 1249 different proteins with a false-positive identification rate of 1%. These identifications confirmed previous findings and provided new information about neutrophil biology, such as the identification of new proteins involved in intracellular transport (Rab and Sec family proteins) or cell signaling (S100 proteins). By correlating the PMNs proteomic data with the protein literature on PMNs, we try to move beyond identification-based proteomics to obtain an overall picture of human neutrophils.

Experimental Section

PMNs were isolated from healthy donors from the Centro de Química de Proteínas or donated by the Centro Regional de Hemoterapia, Brazil. This study was approved by the Research Ethics Committee of the Federal University of São Paulo/São Paulo Hospital (# 1706/05).

Neutrophil Isolation. Human neutrophils (~8×10^7 cells) were obtained from venous EDTA blood (64 mL) of healthy donors and isolated by Percoll gradient centrifugation.[16] The granulocyte pellet was washed in an erythrocyte lysis buffer containing 150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA and then in Hanks' solution to eliminate mononuclear cells and debris. The viability of neutrophils (>95% purity and >80% nonactivated cells) was determined by flow cytometry on the basis of their size and internal complexity and the expression of CD62L (L-selectin).

Extraction of the Proteins from Detergent-Insoluble and Whole Cell Lysate Fractions. The detergent-insoluble membrane domains were isolated as described previously.[17] Briefly, approximately 4×10^7 neutrophils were washed with cold phosphate-buffered saline (PBS), lysed in 1.5 mL of hypotonic buffer containing 10 mM Tris-HCl, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$, 1% NP-40, and 10% (v/v) protease inhibitors (P8340, Sigma-Aldrich, MO) and broken by being passed through a 25-gauge needle 20 times.

For whole cell lysate preparations, the remaining 4×10^7 neutrophils were resuspended in a lysis buffer containing 1% SDS, 1% Triton X-100, 50 mM Tris, 150 mM NaCl, pH 7.5, and 10% (v/v) protease inhibitors (P8340, Sigma-Aldrich, MO). To extract the proteins, both the detergent-insoluble fraction and the whole cell lysate were resuspended in a ratio of 1×10^7 cells to 500 μL of extraction buffer and the procedures were done at 4 °C. The protein content was quantified by Bradford's method.[18]

SDS-PAGE and Protein Digestion. Fifty micrograms of the whole cell lysate and of the detergent-insoluble fraction was mixed with electrophoretic sample buffer (NuPAGE kit, Invitrogen, CA) containing 1 μL of 100 mM dithiothreitol (DTT), and boiled for 5 min at 56 °C prior to the electrophoretic run. Proteins were separated in duplicate for each fraction using a NuPage 4−12% Bis-Tris Gel in MES (2(*N*-morpholino)ethane sulfonic acid) buffer (Invitrogen) at 200 V constant voltage for 30 min. The proteins were visualized using a Colloidal Coomassie Novex kit (Invitrogen). After staining, each lane was sliced into 18 pieces and submitted to in-gel digestion with trypsin, as described by de Souza et al.[19]

In short, the proteins in the gel pieces were reduced using 10 mM DTT for 1 h at 56 °C and alkylated with 55 mM iodoacetamide for 45 min at room temperature. The reduced and alkylated proteins were digested with 0.125 μg of trypsin (Sequence grade modified, Promega, WI) for 16 h at 37 °C in 50 mM NH_4HCO_3, pH 8.0. The reaction was stopped by acidification with 2% trifluoracetic acid (Fluka, Buchs, Germany). The resulting peptide mixture was eluted from the gel slices, and further desalted using RP-C18 STAGE tips.[20] The peptide mixture was dissolved in 0.1% formic acid for nano-HPLC-MS analysis.

Mass Spectrometry Analysis. The tryptic peptide digests were submitted to a Dionex Ultimate 3000 nano-LC system (Sunnyvale CA) connected to an LTQ-Orbitrap mass spectrometer (ThermoElectron, Bremen, Germany) equipped with a nanoelectrospray ion source. For LC separation, we used an Acclaim PepMap 100 capillary column (C18, 3 mm, 100 Å) (Dionex) of 12 cm bed length. The flow rate used was 0.3 mL/min for the nanocolumn, and the solvent gradient used was 7−40% B in 87 min and then 40−80% B in 8 min. Solvent A was aqueous 0.1% formic acid, whereas solvent B was aqueous 90% acetonitrile (ACN) in 0.1% formic acid.

The mass spectrometer was operated in the data-dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. Surveys of full scan MS spectra (from m/z 300 to 2000) were acquired in the Orbitrap with resolution of 60 000 at m/z 400 (after accumulation to a target of 1 000 000 charges in the LTQ). The method used allowed sequential isolation of the most intense ions, up to six, depending on the signal intensity, for fragmentation on the linear ion trap using collisionally induced dissociation at a target value of 100 000 charges.

For accurate mass measurements, the lock mass option[21] was enabled in the MS mode and the polydimethylcyclosiloxane ions generated in the electrospray process from ambient air were used for internal recalibration during the analysis. Target ions already selected for MS/MS were dynamically excluded for 60 s. General MS conditions were: electrospray voltage, 1.5 kV; no sheath and auxiliary gas flow. Ion selection threshold was 500 counts for MS/MS, and an activation Q-value of 0.25 and activation time of 30 ms were also applied for MS/MS. This proteomic analysis was performed twice with neutrophils from two different donors.

Database Searches and Peptide/Protein Validation. All acquired data were processed and analyzed using MaxQuant (version 1.0.13.13), a software script specifically developed for data acquired using high-resolution instrumentation.[15] MS/MS peak lists from 138 individual RAW files were generated using the Quant.exe tool from the MaxQuant package. Protein identification was performed by searching combined data from each neutrophil fraction against the International Protein Index (IPI) human database, version 3.55, available at the European Bioinformatics Institute Web site (75 554 protein sequences). The database was also modified to contain reversed sequences

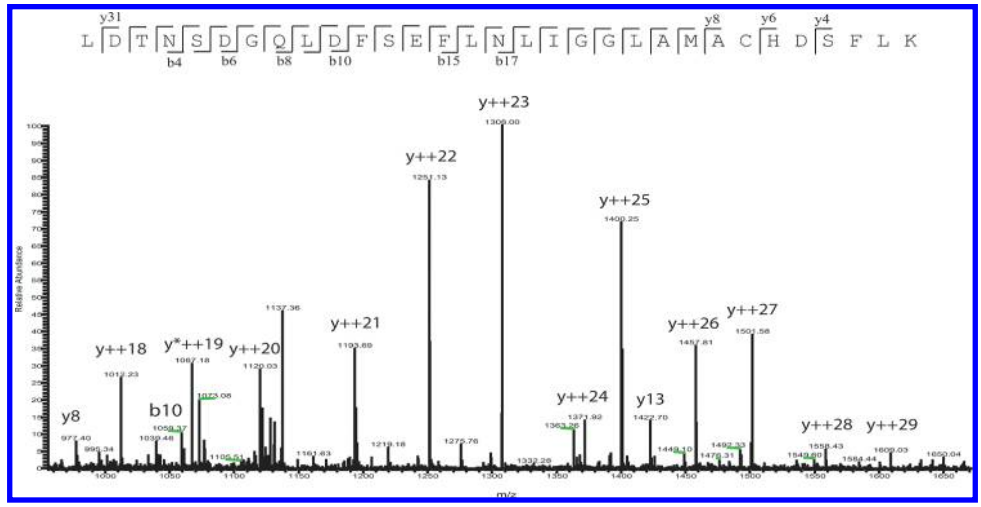

Figure 1. MS/MS profile of ion $m/z = 1149.21$ (triple charged). The peptide was randomly fragmented at each peptide bond, resulting in carboxy-terminal y-ions or amino-terminal b-ions. When the fragment masses were submitted to Mascot, the peptide was identified as LDTNSDGKLDFSEFLNLIGGLAMACHDSFLK from protein S100-A11 (inset, with detected y and b ions represented), with a score of 134.

of all entries as a control of false-positive identifications during analysis. In addition, common contaminants such as keratins, bovine serum albumin and trypsin were also added to the database (database final size of 151 137 protein sequences). We used MASCOT Deamon for submission of multiple searches to a local Mascot server v2.2 (Matrix Science). The search parameters were: Enzyme, trypsin with no proline restriction; Maximum missed cleavages, 3; Carbamidomethyl (C) as fixed modification; *N*-acetyl (Protein), oxidation (M), Pyr-Q (Gln to 2-pyrrolidone-*5*-carboxylic acid-Glu) and Pyr-E (Glu to 2-pyrrolidone-*5*-carboxylic acid-Glu) as variable modifications; Peptide mass tolerance of ± 15 ppm; MS/MS mass tolerance of 0.5 Da.

Protein identification and validation was performed with Identify.exe from MaxQuant using the following parameters: peptide and protein false discovery rate, 0.01 (1%); minimal peptide length was 7; and to guarantee a high confidence identification rate, the maximal posterior error probability was set to 0.1 (from a range of 0 to 1, where 1 represents very high error chance); minimal number of unique peptides per protein, 1. The average mass accuracy for the identified peptides was 400 ppb.

The MS/MS fragments assignments for all identified peptide sequences (including for single peptide-based protein identifications) are freely available at the Tranche network (http://proteomecommons .org) (see Data Availability section for more details).

Gene Ontology (GO) Analysis and Database Mining. Database mining of the identified proteins was performed using the DAVID annotation tool,[22] available at the address http://david.abcc.ncifcrf.gov/. This online resource includes different types of analysis and predictions. The GO annotation analysis grouped the proteins according to their role in biological processes and their main cell locations in order to visualize the identified proteins in main biological processes in which neutrophils participate. In addition, we also used the DAVID tool to perform molecular pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway maps for human species.[23]

Data Availability. MS/MS information for all identified peptides is public available at Tranche network (http://proteomecommons.org) under the following Hash code: LpcXHDEum1rlgSAGcO1hC4TGzWAHfscbCNpMIIQx4qtNl7fRl+xOqRY+WFNiZ7iozQE rimKMfYx7xgTdG92fMu4QXNkAAAAAAAAADag== (hyphens are not part of the hash code).

Results

Purity Assessment of PMN Samples. Preparations of PMNs for MS were analyzed for purity and activation state using flow cytometry on the basis of their size, internal complexity and the expression of CD62L, which gave us at least 95% viability and 80% nonactivated cells (data not shown). In any case, there were <5% contaminating cells, and most likely only highly abundant proteins from those cells might be identified in conjunct with neutrophil extracts.

Proteomic Analysis by Gel LC-MS/MS. Since the use of different strategies to reduce complexity can increase the number of low-abundance proteins that can be subsequently analyzed, we also performed an enrichment step to identify detergent-insoluble proteins, in addition to the analysis of whole cell lysate proteins of resting human neutrophils. PMNs isolated from healthy donors were subjected to cell fractionation, and both samples were analyzed separately by mass spectrometry.

Fifty micrograms of each protein fraction was separated by SDS-PAGE in duplicate, the gel was cut into 18 slices and proteins were in-gel digested with trypsin. Peptides resulting from the protein digestion were analyzed by automated reversed-phase nanoscale liquid chromatography (LC) coupled to tandem mass spectrometry (MS/MS). Using the MaxQuant software set with stringent criteria, and using an IPI human database containing both forward and reverse sequences, we were able to identify a total of 1249 proteins by merging the data from the duplicate fractions. These results were obtained with a false-positive discovery rate of 1% and represent a high quality set of identifications. Of those, 240 were present only in the detergent-insoluble fraction (DIF), 430 only in whole cell lysate (WCL), and 579 in both fractions.

Figure 1 shows a mass spectrum of a selected peptide in the MS mode. The Mascot engine identified this spectrum as the peptide LDTNSDGKLDLSEFLNLIGGLAMACHDSFLK, present

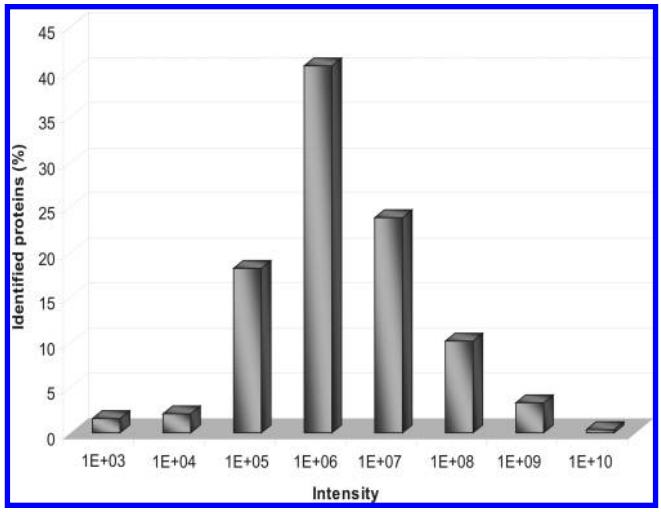

Figure 2. Protein abundance in the resting human neutrophil proteome. The bars represent the individual amounts of 1249 proteins (in percent) identified with an intensity range, as calculated by MaxQuant software based on peptide XIC values.

in the protein S100-A11. Figure 1 also illustrates the fragmentation pattern and identification of y/b series of the sequence (sequence input). In this example, Mascot correlated the majority of y fragments for this sequence. The comprehensive analysis of the 1249 identified proteins from the whole data set, with information about the peptide sequences, length, mass and charge of the peptides, protein group to which a peptide belongs, modification state, calibrated and uncalibrated mass errors and MS/MS counts are available in Supplementary files S1 and S2. Both data sets can be found in Supplementary files S1 and S2 in different sheets: (A) DIF and (B) WCL.

Relative Protein Abundance. Among the identified proteins, we observed ions with different intensities. Very abundant ions derived from proteins were detected, such as plastin-2 and matrix metalloproteinase-9, with intensities around 3×10^9, and protein S100-A11, beta-actin, and nucleophosmin, with intensities between 2 and 5×10^7. The identification of proteins with high ion intensities could mask the identification of proteins with low ion intensities. The least abundant identified proteins, such as cathepsin B, had ion intensities around 10^3-10^4. Figure 2 shows the ion intensities observed for the 1249 proteins identified in the present study. Following a bell-shaped curve, 83% of the proteins had ion intensities of the order of 10^5 to 10^7, while 13% had ion intensities of up to 10^8 and only 4% were of low abundance (intensities $\leq 10^4$). This is in agreement with the limitation of current MS instrumentation, with dynamic ranges of 4−6 orders of magnitude. This correlates with the good number of identifications achieved, but also demonstrates that a better dynamic range is needed to reach further identifications of the neutrophils proteome. Therefore, the use of extensive fractionation for further research might be desirable. Our data appear to be the first to report protein abundance levels in neutrophilic extracts.

Gene Ontology Analysis. The GO distribution pattern of the identified proteins is shown in Figure 3. Biological process annotation analysis revealed that 29% and 13% of the identified proteins are involved in metabolic processes and localization, respectively. Cell organization and biogenesis and transport represent 11% and response to stimulus represents 10% of the identified proteins. Moreover, 4% of the proteins are responsible for cell development and immune system processes and 3% for cell death and cell proliferation. Furthermore, the cell location annotation analysis revealed that 44% of the identified proteins are cytoplasmic proteins, while 16% are membrane proteins and 9% are proteins located at endosomes, lysosomes and peroxisomes. The GO analysis allowed us to make an overview of the expressed proteins, and also to sort them according to main relevant processes in the biology of neutrophil granulocytes.

Building a Catalogue of the Resting Human Neutrophil Proteome. The data collected in the present study, representing detergent-insoluble fractions and whole cell lysates of PMNs, were compared with previous data obtained from plasma and vesicle membrane fractions.[9] We merged 1249 proteins from the present study with 1118 proteins identified in those cell structures. Supplementary file S3 shows the merged result, with previous data in red and our data in black. More than 266 proteins were observed in both studies, with 983 proteins specific to the current work and 850 specific to previous data (Figure 4). This indicates that an effective fractionation method was used to separate plasma and vesicular proteins. However, it is important to note that differences in methodology employed by the two studies, such as the use of different databases (NCBInr and IPIhuman) may result in different reported entries. Some of the discrepant identifications may actually represent the same gene. This can especially be true for proteins classified as chromosomal open reading frames (C6orf1, for example), DKFZ entries and others. We manually checked both lists to add any further validation made on those entries in posterior database versions in order to decrease redundancy in the final list. For example, the entry DKFZp434J1015 is now named Zink finger protein 853 (ZNF853), while the DKFZp434J1015 entry was discontinued in NCBInr. Discontinued names are shown in parentheses in Supplementary file S3 appearing after the updated entry name. Two such cases were observed in the vesicular/plasma membrane data. Those entries were deleted, resulting in a total of 1116 proteins from these extracts. The combined proteome catalogue for resting human neutrophil reported here contains 2099 unique protein groups. Supplementary file S4 illustrates the most characterized pathways in human neutrophils with the 2099 proteins uploaded to KEGG maps. Proteins marked in red at the KEGG pathways represent genes present in our neutrophil catalogue.

Discussion

Neutrophils play a major role in the initiation and resolution of the inflammatory response, and demonstrate significant transcriptional and translational activity.[6] Although much was known about the PMNs before the introduction of proteomics, the use of MS-based methodologies has provided an unprecedented depth of analysis to confirm and extend previous findings. Actually, many proteome profiling studies have been performed on blood constituents,[24] which can be directly compared to the present results. As many proteins are accessible via shotgun proteomics, the number of specifically expressed proteins identified by this methodology is much larger compared to the other approaches.[5,8,9] Our group has previously described a catalog of human neutrophil proteins, where we showed the expression of 55 proteins for the first time in human neutrophils, such as centaurin beta-1, a cytoplasmic protein that interacts with NOD-1 and −2 to down-regulate NF-κB activity.[10]

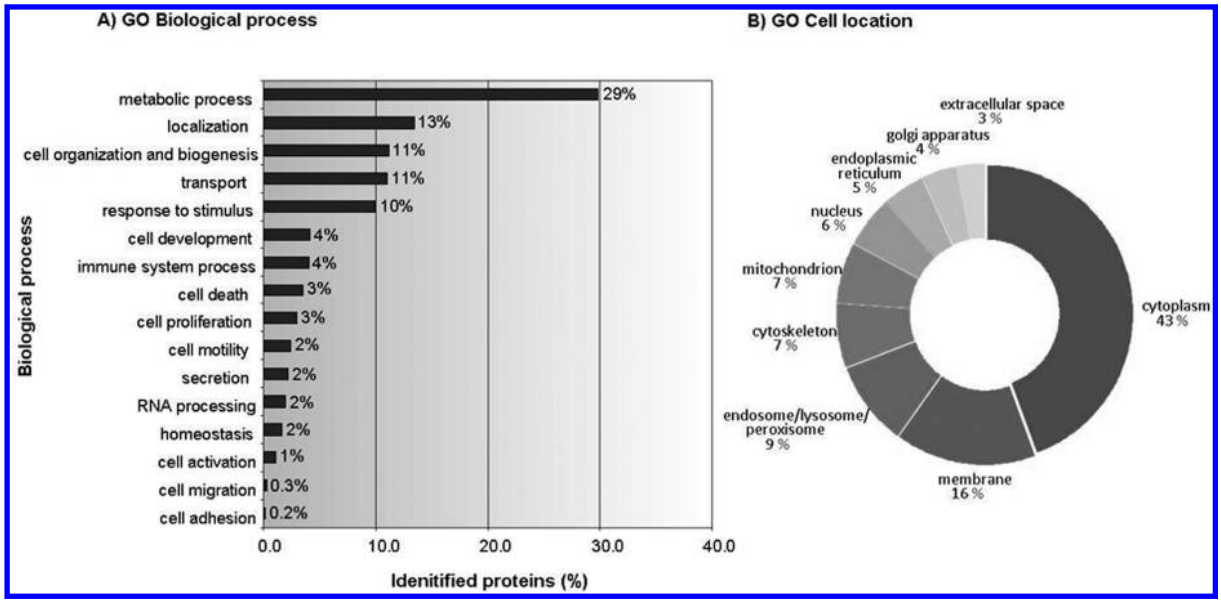

Figure 3. Protein ontology. Proteins identified from human neutrophils were classified according to the biological process and cell location GO annotation using the DAVID functional annotation analysis.

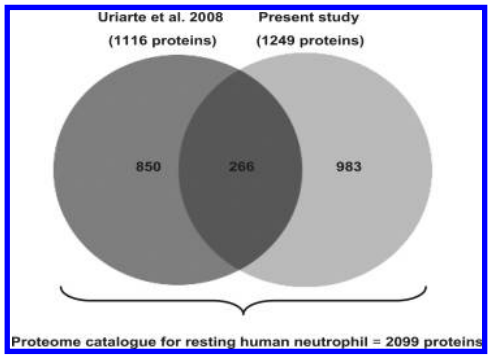

Figure 4. Proteome catalogue for resting human neutrophils. We merged 1249 proteins from the present study with 1118 proteins (two entries had to be deleted due to database discontinuation) identified by Uriarte et al. Overall, 266 proteins were detected in both studies, with 983 proteins specific to the present study and 850 specific to the report of Uriarte et al.,[9] resulting in a proteome catalogue for resting human neutrophil containing 2099 proteins.

In the present study, using methods for high confidence identification with a LTQ-Orbitrap and a software (MaxQuant) specifically designed for high resolution MS data, we identified a total of 1249 proteins in the resting human neutrophil with a 1% false discovery rate. Furthermore, the present study is the most comprehensive human neutrophil proteome analysis reported. However, it is highly probable that we failed to identify some membrane-associated and transmembrane proteins, because we used trypsin as a single protease to enzymatically digest the proteins. In some cases, the identification of membrane-associated and transmembrane proteins may be low due to a lack of available trypsin cleavage sites outside the membrane.[6] Uriarte et al.[9] reported a well validated plasma membrane and vesicular membrane analysis using similar instrumentation (a low resolution but highly sensitive LTQ instrument) with a false positive discovery rate of 10%. If the data reported here are used together with the 1116 proteins identified by Uriarte et al.[9] in this cell (two entries had to be deleted because they were discontinued from the database), one can assume that these data represent an important effort to characterize human neutrophils at the molecular level, resulting in a catalogue of 2099 proteins.

The analysis of the GO annotation of the identified proteins showed that almost 29% of the proteins are involved in metabolic processes, which was expected because housekeeping proteins are important in all cells. Furthermore, the GO analysis allowed us to sort the proteins according to the biological processes of neutrophils. A series of 33 proteins from the Rab family, which are involved in intracellular transport, were detected, such as Rab2A, Rab3A, Rab13 and Rab18. These identifications extend previous studies which also identified 10 Rab proteins known to regulate membrane trafficking,[9] but only two known Rab proteins (Rab15 and Rab3GAP2) were not identified in our data set. These proteins could contribute to the elucidation of mechanisms of secretory vesicle exocytosis. Regarding proteins involved in intracellular transport, we also identified 3 components of the exocyst complex: Sec1 family domain containing 1, SEC31 homologue A (*S. cerevisiae*) and Sec61 beta subunit. The exocyst complex is a group of eight proteins involved in vesicle targeting and docking at the plasma membrane.[25]

The present study also detected a number of S100 proteins, which is the largest subfamily of the EF-hand Ca^{2+}-binding proteins.[26] Some S100 proteins have been investigated and associated with inflammatory processes. The S100A8/S100A9 heterodimer might have a role in the propagation of inflammation by inducing neutrophil chemotaxis and adhesion,[27] and recruiting monocytes to inflammatory sites.[28] S100A12 interacts with RAGE, resulting in the secretion of proinflammatory mediators, IL-1β and TNF-α,[29] and may contribute to leukocyte migration in chronic inflammatory responses.[30] S100B, through RAGE signaling in astroglial cells, mediates pro-inflammatory cytokine production,[31] and represents an endogenous factor implicated in neuroinflammation.[32] RAGE also plays a crucial role in the inflammatory response, involved in recruiting inflammatory cells and interacting with their ligands to activate cellular signals.[33] S100A11 is expressed by diverse tissues at different levels, high in placenta, intermediate in heart, lung, and kidney, low in skeletal muscle and liver and brain,[34] but

has not been correlated to human neutrophils so far. It has been proposed that S100A11 has biological functions in the processes of endo- and exocytosis,[35] regulation of enzyme activity,[36] cell growth regulation,[37] apoptosis,[38] and inflammation.[39] However, since S100 proteins appear to lack enzymatic activity, it is believed that they bind to other proteins and modifies their activity or cell location. Recently, some proteins have been demonstrated to be able to associate with S100A11, and thus, the biological functions of S100A11 would be gradually revealed. S100A11 signaling through RAGE activates p38 MAP kinase, which then increases the expression of type X collagen and the size of the chondrocyte. On the basis of these studies it is for example concluded that S100A11 and its receptor, RAGE, could be involved in the process of low-grade inflammation which contributes to progression of osteoarthritis.[40]

Conclusion

The present report represents an update of the resting human neutrophil proteome. With the help of cell fractionation, we identified a total of 1249 proteins with a wide range of intensities from both detergent-insoluble and whole cell lysate fractions, revealing proteins involved in intracellular transport (Rab and Sec family proteins) and cell signaling (S100 proteins). The present study is the most comprehensive proteomic analysis of resting human neutrophils to date and provides important information for future structural and functional proteomic studies. Together with previous characterizations of the plasma and vesicular membrane proteomes, we constructed a high confidence data set of neutrophil proteins which contains the identification of a total of 2099 proteins. This catalog can be used as a reference for future studies in order to understand how neutrophils respond to various physiologic and pathophysiologic conditions.

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Supporting Information Available: Supplementary File S1, list of all identified proteins. This table represents the total list of identified proteins in (A) DIF (detergent-insoluble fraction) and (B) WCL (whole cell lysate) of human neutrophils. Highlighted columns represent the minimal information required by the journal for each protein sequence (Protein ID; Total number of Unique peptides; Sequence coverage; Posterior Error Probability (PEP) Score). Supplementary File S2, list of all sequenced peptides. This table represents the total list of sequenced peptides in (A) DIF (detergent-insoluble fraction) and (B) WCL (whole cell lysate) of human neutrophils. This file also include information about length, mass and charge of the peptides, protein groups to which a peptide belongs, modification state, mass error (calibrated and un-calibrated) and MS/MS count. Highlighted columns represent the minimal information required by the journal for each peptide sequence identification (Peptide Sequence; Modifications; precursor mass, charge and observed mass error; Scores as PEP and Mascot Score). Supplementary File S3, high confidence protein list from detergent-insoluble and whole cell lysate fraction and plasma membrane and secretory fractions. The list reports a merged list of identifications obtained in this work (black) and obtained by Uriarte et al.[9] (red). Supplementary File S4, schematic representation of the most characterized pathways in human neutrophils, using our final repertoire of 2099 proteins submitted to the KEGG pathway map. Proteins described in Supplementary File S3 are shown in red inside the KEGG pathways. This material is available free of charge via the Internet at http://pubs.acs.org.

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