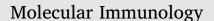
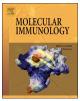
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Proteomic analysis of lipopolysaccharide activated human monocytes

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ARTICLEINFO	A B S T R A C T
<i>Keywords:</i> Monocyte Lipopolysaccharide Proteome Mass spectrometry	Monocytes are key mediators of innate immunity and comprise an important cellular defence against invading pathogens. However, exaggerated or dysregulated monocyte activation can lead to severe immune-mediated pathology such as sepsis or chronic inflammatory diseases. Thus, detailed insight into the molecular mechanisms of monocyte activation is essential to understand monocyte-driven inflammatory pathologies. We therefore investigated the global protein changes in human monocytes during lipopolysaccharide (LPS) activation to mimic bacterial activation. Purified human monocytes were stimulated with LPS for 17 h and analyzed by <i>state-of-the-art</i> liquid chromatography tandem mass spectrometry (LC–MS/MS). The label-free quantitative proteome analysis identified 2746 quantifiable proteins of which 101 had a statistically significantly different abundance between LPS-stimulated cells and unstimulated controls. Additionally, 143 proteins were exclusively identified in either LPS stimulated cells or unstimulated controls. Functional annotation clustering demonstrated that LPS, most significantly, regulates proteasomal- and lysosomal proteins but in opposite directions. Thus, seven proteasome subunits were upregulated by LPS while 11 lysosomal proteins were downregulated. Both systems are critically involved in processing of proteins for antigen-presentation and together with LPS-induced regulation of CD74 and tapasin, our data suggest that LPS can skew monocytic antigen-presentation towards MHC class I rather than MHC class II. In summary, this study provides a sensitive high throughput protein analysis of LPS-induced monocyte activation and identifies several LPS-regulated proteins not previously described in the literature which can be used as a source for future studies.

1. Introduction

Monocytes are circulating myeloid cells comprising 3–10% of leucocytes in peripheral blood. They are important innate effector cells participating in pathogen recognition and elimination. During infection, monocytes sense invading pathogens through a variety of conserved innate immune receptors known as pattern recognition receptors (PRR). These receptors recognize different microbial-restricted structures collectively called pathogen associated molecular patterns (PAMPs). Among PRRs the Toll-like receptor (TLR) family plays important roles in microbial sensing and initiation of innate immunity. An archetypical PAMP-PRR interaction is the recognition of lipopolysaccharide (LPS) by TLR4. LPS is a highly immunogenic glycolipid found in the outer membrane of Gram-negative bacteria. Recognition of LPS by TLR4 is facilitated by the co-receptors CD14 and MD-2 (Lu et al., 2008). Recognition leads to receptor dimerization and initiates an intracellular signaling cascade which ultimately leads to activation and nuclear translocation of several transcription factors, which lead to transcriptional activation of a variety of functionally different genes.

LPS-induced TLR4 activation is a key process in the immunological response to gram-negative bacterial pathogens, but the interaction may also be harmful to the host as seen during bacterial sepsis. In sepsis caused by gram-negative bacteria, LPS activates TLR4 in the bloodstream causing an exaggerated systemic inflammatory response that may lead to multiple organ failure and ultimately death. TLR4 signaling is not only activated during bacterial infections, but can also be activated during chronic inflammatory and autoimmune conditions. TLR4 is targeted by several endogenous ligands known as danger associated molecular patterns (DAMPs) such as S100 proteins or high mobility group box 1 (HMGB1), which are released during various inflammatory

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https://doi.org/10.1016/j.molimm.2018.09.016

Abbreviations: DAMPs, danger associated molecular patterns; ER, endoplasmic reticulum; HMGB1, high mobility group box 1; IL-1Ra, IL-1 receptor antagonist; LPS, lipopolysaccharide; MS, mass spectrometry; PAMPs, pathogen associated molecular patterns; SDC, sodium deoxycholate; TAP, transporter associated with antigen processing; TLR, toll-like receptor; UPLC-MS/MS, ultra performance liquid chromatography tandem mass; V-ATPase, vacuolar H + - ATPase

Received 2 May 2016; Received in revised form 20 August 2018; Accepted 25 September 2018 0161-5890/ © 2018 Elsevier Ltd. All rights reserved.

pathologies such as rheumatoid arthritis and inflammatory bowel disease. Thus, detailed knowledge on the cellular and molecular mechanisms acting downstream of TLR4 activation is important to understand pathophysiological events taking place during bacterial infection and other inflammatory conditions mediated by TLR4 activation. For this reason, *in vitro* stimulation of various cell types by LPS has been widely used to mimic immune cell activation under different pathological conditions. However, common for these studies is that only a single or a few cellular systems are investigated and thus potentially important interconnected pathways may be overlooked. To get the full understanding of the effects of TLR4 activation the global cellular response should be investigated and this can be achieved using tandem mass spectrometry (MS).

Quantitative proteomics offers an opportunity to identify and quantify more than thousand proteins within a single cell population and by MS analysis it is possible to identify relatively small changes in protein expression between differently conditioned samples (Kruse Meyer and Andersen, 2015). Thus, small, but biological important protein alterations taking place in multiple protein systems can be studied simultaneously. Two studies have previously investigated the proteome of LPS activated primary monocytes. However, these studies were based on two dimensional (2D)-gel based proteomics, which has several limitations in terms of sensitivity and only studies protein size and pI range (Bennike et al., 2014, 2015). The studies identified 23 different proteins with a statistically significant change of abundance, collectively (Gadgil et al., 2003; Pabst et al., 2008). One study has analyzed the LPS-induced activation of the human monocytic THP1 cells by high throughput MS (Tarasova et al., 2015). However, LPS activation of THP1 monocytic cells poorly resembles the LPS activation of freshly isolated monocytes ex vivo (Bosshart and Heinzelmann, 2016). Therefore, we applied a state-of-the-art gel-free ultra performance liquid chromatography tandem MS (UPLC-MS/MS) analytical method to identify novel regulated proteins in human monocytes following LPS activation.

2. Methods

2.1. Antibodies

The following antibodies were used for flow cytometry in the study: PE Mouse Anti-Human CD14 (clone M5E2, 2.5 µl/test, BD Bioscience, NJ, USA), FITC Mouse Anti-Human CD18 (clone 6.7, 10 µl/test BD Bioscience), APC anti-human CD68 (clone Y1/82 A, 2.5 µl/test, Biolegend, CA, USA), anti-human CD74 (clone LN2, 2 µg/ml, Biolegend), anti-human HLA-ABC (clone W6/32, 2 µg/ml ImmunoTools, Germany), Anti-HLA-DR APC-Cy[™]7 (clone L243, 2.5 µl/ test, BD Bioscience) and FITC Mab 1.3.2 against interleukin-1 α (IL-1 α) propiece (Loke Diagnostics, Risskov, Denmark). All isotype controls were purchased from BD Bioscience. Anti-interleukin-1 α (IL-1 α) propiece (clone 1.3.2, 5 µg/ml, Loke Diagnostics, Risskov, Denmark) and Anti-Human CD68 (clone KP1, 4.29 µg/ml Dako, Glostrup, Denmark) were used for immunofluorescence staining and microscopy.

2.2. Monocyte isolation and culturing

Blood samples were obtained from one healthy donor by approval from the ethics committee of Region Nordjylland, case number N-20150073. Blood samples were collected in S-Monovette[®] (Sarstedt, Nümbrecht, Germany) and diluted (1:2) in 0.9% NaCl solution with heparin (7 IE/ml). PBMCs were isolated using density gradient centrifugation on LymphoPrep[™] (STEMCELL Technologies[™], Vancouver, Canada) at 300 x g for 30 min at 20 °C. Following centrifugation, PBMCs were harvested and washed twice in wash medium consisting of PBS with 2% FCS and 1 mM EDTA with centrifugation at 120 x g for 10 min at 20 °C between each step. PBMCs were resuspended in wash medium and adjusted to 1×10^8 cells/ml. Monocytes were purified

from PBMCs by immunomagnetic positive separation using EasySepTM Human CD14 Positive Selection Kit (STEMCELL TechnologiesTM) essentially according to manufacturer's instructions. During magnetic separation we used Hank's Balanced Salt Solution in all washing steps instead of the recommended medium (PBS + 2% FCS, + 1 mM EDTA) to avoid serum albumin that may interfere with the MS analysis.

Purified monocytes were suspended in ProDoma-1 (Lonza, Basel, Switzerland) serum-free medium with 1.65 mM L-glutamine and cultured with and without LPS (2 µg/ml) in two separate Greiner CELLS-TAR® 96 well plates (Sigma-Aldrich, MO, USA) with 1 × 10⁶ cells per well. Cells were cultured at 37 °C in 5% CO₂ for 17 h. All samples were cultured in duplicates.

2.3. Flow cytometry

Monocyte purity was assessed by flow cytometry using PE Mouse Anti-Human CD14 or PE Mouse IgG2a Isotype Control as described (Carlsen et al., 2015). CD14 antibody and matched isotype control was added during the monocyte purification procedure at a concentration of $0.4 \,\mu$ g/ml to avoid epitope blocking by CD14 antibodies in the monocyte purification kit. Following purification, monocytes were suspended in PBS and analyzed immediately. Monocytes were identified based on forward/side scatter characteristics and CD14 expression using a matched isotype control.

To analyze expression of CD18, CD74, HLA-ABC, HLA-DR, and IL-1 α stimulated and unstimulated PBMCs were incubated with primary antibodies or matched isotype controls in PBS + 0.1% BSA and 0.05% sodium azide for 60 min at room temperature. To stain intracellular antigens cells were fixed in 1% formaldehyde for 15 min and permeabilized and blocked in PBS + 0.5% saponin + 0.5% BSA for 30 min at room temperature and subsequently stained as described above in PBS + 0.1% saponin + 0.1% BSA.

Stained cells were analyzed immediately on a CytoFLEX S flow cytometer (Beckman Coulter, IN, USA). Data were acquired using CytExpert Software v. 1.2 (Beckman Coulter) and analyzed using Kaluza Analysis Software (Beckman Coulter). The monocyte population was gated based forward/side scatter characteristics and CD14/CD68 expression.

2.4. Immunofluorescence staining and confocal microscopy

PBMCs were seeded in 8 well Lab-Tek[®] Chamber Slide[™] Permanox slides (Thermo Scientific) at a concentration of 3×10^5 cells/well in medium containing RPMI 1640 (Biowest, Nuaillé, France), 16.5% FCS, 1.65 mM L-glutamine, and 0.01 mg/ml gentamicin. The cells were incubated at 37 °C for 90 min. Following incubation, media was removed and the wells were washed twice in PBS to remove non-adherent cells. Prodoma-1 serum-free medium (Lonza) was added to the remaining cells and the cells were incubated and stimulated with LPS (2 µg/ml) for 17 h. Control cells were cultured for 17 h without LPS.

Cells were processed for immunofluorescence staining according to Carlsen et al (Carlsen et al., 2015). Cells were fixed in 3.7% formalin in PBS for 20 min at 4 °C and subsequently permeabilized for 7 min in 0.2% Triton X-100 at room temperature. Next, the cells were blocked for 15 min in 0.1% BSA in PBS at 37 °C and subsequently stained for 30 min using anti-IL-1 α propiece (Loke Diagnostics) and Anti-Human CD68 (Dako, Glostrup, Denmark). FITC-conjugated affinity purified Goat Anti Mouse IgG (Jackson immunoResearch, PA, USA) was used as secondary antibody and nuclei were visualized using 1 μ M TO-PRO-3 iodide (Life Technologies, CA, USA).

The cells were inspected and imaged using a Leica SP5 confocal microscope with a CX PL Apo 100x/1.47 objective (Leica Microsystems, Wetzler, Germany).

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