Study of monocyte membrane proteome perturbation during lipopolysaccharide-induced tolerance using iTRAQ-based quantitative proteomic approach

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Abstract

Human monocytes exposure to low-level lipopolysaccharide (LPS) induces temporary monocytic insensitivity to subsequent LPS challenge. The underlying mechanism of this phenomenon could have important clinical utilities in preventing and/or treating severe infections. In this study, we used an iTRAQ-based quantitative proteomic approach to comprehensively characterize the membrane proteomes of monocytes before and after LPS exposure. We identified a total of 1651 proteins of which 53.6% were membrane proteins. 94% of the proteins were quantified and 255 proteins were shown to be tightly regulated by LPS. Subcellular location analysis revealed organelle-specific response to LPS exposure: more than 90% of identified mitochondrial membrane proteins were significant down-regulated, whereas the majority of proteins from other organelles such as ER, Golgi, Ribosome were up-regulated. Moreover, we found that the expression of most receptors potentially involved in LPS signal pathway (CD14, TLR4, CD11/CD18 complex) were substantially decreased, while the expression of molecules involved in LPS neutralization were enhanced after LPS challenge. Together, these findings could be of significance in understanding the mechanism of LPS tolerance and provide values for designing new approaches for regulating monocytic responses in sepsis patients.

Keywords: Endotoxin tolerance (ET), iTRAQ, Lipopolysaccharide (LPS), Membrane proteome, Monocyte, Quantitative proteomics, Sepsis

Abbreviations: ET, endotoxin tolerance (ET); LPS, lipopolysaccharide; PMNC, peripheral blood mononuclear cells; iTRAQ, isobaric tag for relative and absolute quantitation.
1. Introduction

Human monocytes play a vital role in an innate immune response to infections initiated by the invasion of pathogens, bacteria and inflammatory agents [1]. Among various infectious species, lipopolysaccharide (LPS) is one of the most potent endotoxins well-characterized in gram-negative bacteria [2]. Sensitive detection of LPS induces strong immune response cascades and serves as an early warning of bacterial invasion in many individuals. Over-response to LPS exposure, however, frequently leads to severe sepsis, septic shock or systemic inflammatory response syndrome with which high motility rate is associated [3]. Thus, understanding the ways to regulate monocytic responses to LPS is of great clinical interest.

Monocytes exposure to low level of LPS could result in temporary monocytic insensitivity to a secondary LPS challenge, a phenomenon termed as endotoxin tolerance (ET) [4]. The underlying mechanism of the dampening monocyte response in the ET could thus have important clinical utility in preventing and/or treating severe infections such as sepsis. As such, many attempts have been made to understand this phenomenon. In vitro experiments showed that LPS-induced tolerance was associated with an increased expression of the anti-inflammatory cytokines, IL-10, IL-1 receptor antagonist (IL-1ra) or transforming growth factor (TGF) [5], but a reduction in the expression of chaperonic invariant chain [6], antigen presentation molecules (HLA-DQ, CD86) [7], toll-like receptor 4 (TLR4) [8, 9], and monocytic scavenger receptor B1 [10]. Furthermore, some signaling pathways and key regulators have also been identified to be involved in the LPS tolerance. For example, Sly et al [11] demonstrated that LPS tolerance is mediated by the up-regulation of cytosolic phosphatase SHIP through LPS-induced autocrine-acting TGF-β via a SMAD-dependent pathway. These findings had led to clinical trials for target
therapy of sepsis patients [12] but the outcome was either poor or yet to be determined [13]. This could be probably explained by the fact that an immune response is regulated by a complex system of receptors and cytokines, and thus targeting a single or a few molecules provides poor or no benefit. Furthermore, most of these targets were intracellular proteins [12], which are less accessible compared to membrane receptors. Systematic analysis of the membrane proteome perturbation after LPS-treatment may thus provide new insight into the adaptation of innate immunity and aid to the discovery of novel therapeutic targets for the sepsis patients.

Rapid development of proteomics technologies including difference gel electrophoresis (DIGE) [14], isotope labeling reagents (e.g. ICAT [15] and iTRAQ [16]) and the data management [17] are making it possible to perform global protein expression analysis and accurately detect quantitative changes. In particular, the iTRAQ technology has gained great popularity in quantitative proteomics applications due to its accurate quantitation and relatively high sensitivity and reproducibility [18-20]. In this study, we used the iTRAQ-based quantitative proteomic approach to comprehensively characterize the membrane proteome of human monocytes before and after LPS challenge. We showed that LPS induced organelle-specific proteome expression changes: more than 90% of identified mitochondrial proteins were significant down-regulated, whereas the majority of proteins from other organelles such as ER, Golgi, Ribosome were up-regulated. Furthermore, we found that the expression of most receptors potentially involved in LPS signal pathway (CD14, TLR4, CD11/CD18 complex) was substantially decreased, while the expression of molecules involved in LPS neutralization was significantly enhanced after LPS challenge. These findings could be of significance in understanding the mechanism of LPS tolerance, and provide values for designing new approaches for regulating monocytic responses in sepsis patients.
2. Materials and Methods

2.1 Antibodies

The antibodies (Abs) used for flow cytometry are FITC-conjugated CD11a (HI111) and CD14 (61D3), PE-conjugated CD11b (ICRF44), CD11c (3.9) and CD18 (CLB-LFA-1/1), PE-Cy7 conjugated HLA-Dr (LN3), CD33 (WM53) (eBioscience, San Diego, CA, USA); PE-conjugated CD97 (VIM3b) (BD Pharmingen, San Diego, CA, USA). The Abs used for Western blot are ATP synthase beta (10/ATP) (BD Pharmingen), NADH dehydrogenase (17D95) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and Actin (LV1435643) (Millipore, Billerica, MA, USA).

2.2 Preparation of monocytes from human peripheral blood

Blood samples of 12 healthy donors were collected from Health Sciences Authority, Singapore. Peripheral blood mononuclear cells (PMNC) were separated from buffy coats using Ficoll-Hypaque (Sigma-Aldrich, St. Louis, MO, USA) density gradient centrifugation. To minimize perturbation, monocytes were negatively isolated from the PMNC by magnetic-activated cell sorting using monocytes isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). Aliquots of the monocytes were then immediately cultured in tissue culture petri-dishes either in the medium (IMDM supplemented with 10% serum) alone or in the presence of 100 ng/mL E. coli LPS (Sigma-Aldrich) for 24 hrs to induce LPS tolerance as described [21].

2.3 Membrane protein preparation

The monocytes were harvested and washed three times with cold PBS (4 °C). They were then resuspended in 1 mL HES buffer (20 mM HEPES, pH 7.4, 1 mM EDTA and 250 mM sucrose) supplemented with protease inhibitor (Roche diagnostics, Mannheim,
Germany). The cells were lysed by sonication of three times on ice using a Vibra Cell™
high intensity ultrasonic processor (Jencon, Leighton Buzzard, Bedfordshire, UK). The
remaining unbroken cells and debris were removed by centrifugation at 1000 × g at 4 °C
for 10 min. The supernatant was transferred to a new tube and centrifuged at 100,000 ×
g at 4 °C for 45 min. The pellet containing membrane fraction was washed once with
Na₂CO₃ (0.1 M, pH11) and twice with Milli-Q water sequentially by centrifugation at
100,000 × g at 4 °C. The membrane pellet was then dissolved in 8 M urea solution, and
protein content was determined using 2-D Quant Kit (GE healthcare, Milwaukee, WI,
USA).

2.4 Protein digestion and iTRAQ labeling

Approximately 200 µg of proteins from both LPS-treated and control monocytes were
equally divided into two tubes respectively. The proteins were reduced with 5 mM tris-
(carboxyethyl phosphine hydrochloride (TCEP) for 60 min at 37 °C, and alkylated with 10
mM methylethanethiosulfonate (MMTS) for 20 min at room temperature (RT). They were
then diluted 7-fold with 50 mM triethylammonium bicarbonate (TEAB) prior to digestion
with trypsin (Promega, Madison, WI, USA) overnight at 37 °C in a 1:50 trypsin-to-protein
mass-ratio. The protein digests were desalted using Sep-Pak C18 cartridges (Waters,
Milford, MA, USA) and dried in a SpeedVac (Thermo Electron, Waltham, MA, USA). The
digests were labeled with iTRAQ reagents according to the manufacturer’s protocol
(Applied Biosystems, Framingham, MA, USA). Briefly, desalted digests were reconstituted
in 30 µL of dissolution buffer (0.5 M TEAB) and mixed with 70 µL of ethanol-suspended
iTRAQ reagents (one iTRAQ reporter tag per protein sample). The samples were
labeled with the respective tags as follows: two control (un-treated) monocyte samples
were labeled with reporter tags 114 and 116 respectively; and the two LPS-treated
samples with reporter tags 115 and 117. Labeling reactions were carried out at RT for 60 min before all the samples were mixed into a single tube and dried in a SpeedVac.

2.5 Strong cation exchange fractionation of peptide mixture

The dried iTRAQ labeled samples were reconstituted with 200 µL buffer A (10 mM KH$_2$PO$_4$, pH 3.0, ACN/H$_2$O 25/75 (v/v)) and loaded into a PolySULFOETHYL A column (200 mm length x 4.6 mm ID, 200-Å pore size, 5 µm particle size) (PolyLC, Columbia, MD, USA) on a prominence HPLC unit (Shimadzu, Kyoto, Japan). The sample was fractionated using a gradient of 100% buffer A for 5 min, 5 - 30% buffer B (10 mM KH$_2$PO$_4$, pH 3.0, 500 mM KCl, ACN/H$_2$O 25/75 (v/v)) for 40 min, 30 - 100% buffer B for 5 min, and finally 100% buffer B for 5 min, at a constant flow rate of 1 mL/min for a total of 60 min. The eluted fractions were monitored through a UV detector at 214 nm wavelength. Fractions were collected at 1-min intervals and consecutive fractions with low peak intensity were combined. Finally a total of 20 fractions were obtained and dried in a SpeedVac. Each fraction was reconstituted in 0.1% trifluoroacetic acid and desalted using a Sep-Pak C18 cartridge. Desalted samples were dried and stored at -20 ºC until mass spectrometric analysis.

2.6 Mass spectrometric analysis using Q-TOF mass spectrometer

Each dried fraction was reconstituted in 100 µL of 0.1% formic acid, injected using an auto-sampler (Shimadzu) and concentrated in a Zorbax peptide trap (Agilent, Palo Alto, CA, USA). The peptide separation was performed in a home-packed nanobored C18 column with a picofrit nanospray tip (75 µm ID x 15 cm, 5 µm particles) (New Objectives, Wubrun, MA, USA). Mobile phase A (0.1% formic acid in H$_2$O) and mobile phase B (0.1% formic acid in ACN) were used to establish a 90-min gradient comprised of 5 min of 0-5% B; then 55 min of 5-25% B; followed by 12 min of 25-60% B; maintained at 80%
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B for 8 min; finally re-equilibrated at 5% B for 10 min. The HPLC (prominence™, Shimadzu) was operated at a constant flow rate of 30 µL/min and a splitter was used to create an approximate 300 nL/min flow rate at the electrospray tip. The mass spectrometer (QSTAR elite, Applied Biosystems) was set to perform data acquisition in the positive ion mode, with a selected mass range of 300-2000 m/z. Peptides with +2 to +4 charge states were selected for MS/MS and the time of summation of MS/MS events was set to 2 sec. The three most abundantly charged peptides above a 5 count threshold were selected for MS/MS and dynamically excluded for 30 s with ±30 mmu mass tolerance.

Protein identification and quantification were performed using ProteinPilot v2.0.1 (Applied Biosystems) by searching the combined data from each sample against the International Protein Index (IPI) human database (IPI_human version 3.34.fasta, including 69164 sequences and 29064824 residues) and its reversed complement. Trypsin was selected as the digestion enzyme and cysteine modification was set as methylethanethiosulfonate. A minimum unused score of 2 (equivalent to 99% confidence) was required for all reported proteins. For quantitative analysis, a protein must have minimum two unique peptide matches with iTRAQ ratios, and at least one of them with an expectation less than 0.05.

2.7 Western blot

Monocyte lysate was prepared using the mammalian cell lysis kit (Promega) according to the manufacturer’s instructions. The Western blot was performed as described [22]. Briefly, 15-30 µg of proteins in the lysate were separated with 10-15% SDS-Tris-glycine gels and transferred onto PVDF membrane using Tran-Blot ® semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA, USA) at a voltage of 15 V for 30 min. After blocking
with 5% non-fat milk for 1 hr at RT, the membrane was incubated with primary antibody for 1 hr at RT, followed by secondary antibody conjugated with horseradish peroxidase (HRP) for 1 hr at RT. After each incubation, the membrane was washing three times with PBST buffer (1 × PBS with 0.1% Tween-20). The immune-reaction was visualized using chemiluminescence HRP substrate (Thermo Fisher Scientific, Rockford, IL, USA).

2.8 Flow cytometric analysis

For cell surface staining, fluorochrome-conjugated antibodies were incubated with the monocytes for 15 min at 4 °C and the staining intensity was measured on a FACSCalibur flow cytometer (BD Bioscience). Appropriate isotype antibodies were used as negative controls. The data were analyzed on the FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.9 Measurement of reactive oxygen species (ROS)

The monocytes were cultured either in the presence of 100 ng/mL LPS for 2, 4, and 24 hrs, or in the absence of LPS for 24 hrs. Their intracellular ROS levels were assessed by incubation with 10 M of either dichlorofluorescin diacetate (DCFH-DA) or dihydroethidine (DHE) (Molecular probes, Carlsbad, CA, USA) followed by analysis using flow cytometry.

2.10 In-silico biological and functional analyses

Subcellular and functional categories were based on the annotations of Gene Ontology using Mouse Genome Informatics (MGI). The number of trans-membrane helices of identified proteins was predicted using TMHMM 2.0. Pathway analysis was preformed based on Kyoto Encyclopedia of Genes and Genomes pathway collection (KEGG).
3. Results and Discussion

3.1 Experiment design.

Monocytes account for about 10-20% of PMNC in human blood [23]. To obtain sufficient material for a triplicate experiment, we isolated monocytes from the peripheral bloods of 12 healthy donors using Miltenyi's monocyte isolation kit II, which routinely provides a purity of >98%. Purified monocytes from every four samples were pooled and equally divided into two cultures: control and LPS treatment. Membrane proteins were purified from each culture according to our previous protocol [24, 25]. As there are four tags available in the 4-plex iTRAQ, we labeled each sample with two tags to set up technical replicates, i.e. control samples were labeled with tags 114 and 116, whereas LPS-treated samples were labeled with tags 115 and 117. The iTRAQ labeled samples were analyzed using a well-established platform comprising of QSTAR mass spectrometer and ProteinPilot software (Supplemental Fig. 1). This workflow could ensure statistical consistency and better coverage for identification and quantitation.

3.2 Protein identification and quantification

A similar number of proteins (1129, 1187 and 1245) were identified from each of the three replicates respectively. The overall false positive assignment was estimated around 1% (with 0.8%, 1.2%, and 1.2% each) by a target-decoy search strategy [26], suggesting the high quality of our dataset. In total, 1651 proteins including 881 membrane proteins were identified (Supplemental Table 1). Of these, 94% proteins were quantified with iTRAQ ratios, and 762 proteins were common in all three biological replicates.
To set an optimal threshold for measuring the significant changes of protein expression of monocytes upon challenge by LPS across three biological replicates, the experimental variations were evaluated not only between the technical replicates within each biological replicate, but also among the biological replicates. **Fig. 1** showed the good consistency in quantitative measurement of the technical replicates within a biological replicate. Theoretically, the ratios of technical replicates 116/114 and 117/115 should both give a value of 1. However, this ideal situation is not practically achievable due to pipetting error, differential efficiency in sample labeling and instrumental error. In this study, of the 762 common identifications, 99% proteins have <1.30 fold changes for technical replicates (116/114 and 117/115), and 95% protein have <1.2 fold changes (**Table 1**). Hence, a 1.3-fold variation was determined as the cut-off value for measuring the significant changes of protein expression to achieve 99% confidence (p<0.01). The inter-experimental variations between the biological replicates were evaluated by their correlation coefficients of the common identified proteins. All ratios (115/114 and117/114) of the common identified proteins from the biological replicates were log-transformed and plotted to estimate the similarity of protein expression as well as reproducibility of the different biological replicates. As shown in **Fig. 2**, all correlation coefficients between any of the two replicates were about 0.85, indicating the good technical and biological reproducibility of the LPS induced expression changes.

Using the ±1.3 fold cut-off value, we identified that a large set of proteins were significantly (p<0.01) and consistently up- or down-regulated in all three biological replicates. Of these, 112 proteins were found to be up-regulated whereas 143 proteins were down-regulated in response to LPS challenge (**Supplemental Table 2**). Earlier, Pabst et al. [27] identified 19 LPS-regulated proteins including one membrane proteins from human monocytes using 2-DE based method, while a report using SILAC-based
approach identified more than 1000 cytosolic and nuclear proteins in response to LPS exposure from murine macrophage cell line [28]. In contrast, we analyzed proteins from human monocyte membrane extracts using iTRAQ-based quantitative proteomics. Expectedly, we identified many known LPS-regulated membrane proteins such as CD14, CD44 and CD74. In addition, we identified some membrane proteins that have not previously been reported to respond to LPS challenge. For example, CD33, CD300LF were identified to be >2.5-fold down-regulated. CD33 is an inhibiting receptor and it could efficiently prevent the maturation of dendritic cell from either CD34+ cell precursors or peripheral monocytes [29]. It has also been shown that treatment of human monocytes with anti-CD33 mAb or RNA interference induces the production of the proinflammatory cytokines [30]. Thus down-regulation of CD33 may be necessary for LPS triggered cytokine release.

3.3 LPS down-regulates mitochondrial proteins, but up-regulates proteins of Golgi, ER, and Ribosome

Based on their subcellular locations, we categorized the 255 proteins with significant expression changes using gene ontology tool from MGI. The two most abundant groups were mitochondria and plasma membrane, which together accounted for about 60% of the analyzed proteins. The others were from nucleus (10%), Golgi/ER/lysosome (9%), cytoskeleton/cytoplasm (7%), ribosome (3%) and unclassified proteins (12%) (Fig. 3A). Interestingly, 14 of the 16 cytosolic proteins were significantly up-regulated (Fig. 3B), suggesting that there was possible protein translocation to membrane or proteins tightly bound to membrane complex after LPS treatment. For example, we observed 2.1 and 1.6 fold increase in the expression of cytosolic proteins NCF1 and NCF2. This is consistent with an early report that showed p47-phox (NCF) to be enriched in the plasma membrane preparation due to the translocation [31]. Similarly, cytosolic heat shock
protein 70 (HSP70) has been shown to be enriched in lipid raft preparation after LPS challenge [32].

Of the identified mitochondrial proteins, 94% of them were down-regulated. In contrast, more than 87% of the nuclear proteins, Golgi/ER proteins, and ribosomal proteins were significantly up-regulated (Fig. 3B). Expression of two selected mitochondrial proteins ATP5B and NDUFS3 was further confirmed using Western blotting (Fig. 4). The reciprocal regulation between mitochondria and other organelles indicates their coordinating response to LPS induced infection. The down-regulation of mitochondrial proteins suggests that mitochondria were the major targets of bacterial infection. Nuclear, ribosomal and Golgi/ER proteins are mainly involved in protein synthesis, folding and post-translational modification. The significant up-regulation of these proteins suggests active transcription and translation involved at this state, possibly to compensate the mitochondrial damage. Similar phenomenon was reported at DNA in an in vivo study: LPS administration led to the decrease of the copy number of mitochondrial DNA and the reduction of the intactness of the mitochondria in rat heart, while nuclear DNA encoded mitochondrial genes were significantly increased [33].

The mitochondrion is not only a major organelle contributing to energy metabolism but also a main site of ROS production [34]. Upon LPS infection, TNF-alpha production was induced, which then stimulates ROS and reactive nitrogen species (RNS) production [35]. We next examined the overall ROS release of monocytes under LPS stimulation. As expected, ROS level was dramatically increased at the initial stage of the LPS treatment, but it dropped to a slightly lower level after 24 hr treatment compared to the control (Fig. 5). The TNF-induced ROS production occurs mainly in mitochondria in most of cells [34]. As mitochondrial constituents are susceptible to oxidation, large amount of
ROS produced at the early LPS treatment stage would cause the damage of mitochondrial DNA thus leading to altered mitochondrial gene expression. This is evidenced by the observation of down-regulation of most mitochondrial proteins.

There were a total of 48 plasma membrane proteins with approximately equal number of proteins in down-regulation and up-regulation groups respectively (Fig. 3B). Interestingly, most of the integral membrane proteins were down-regulated after LPS treatment for 24 hrs. These include CD14, CD33, CD97, CD74, CD300lf, ITGAX, ICAM3 and SORL. Fig. 6 showed the flow cytometric confirmation of the down-regulation of the selected surface antigens. In contrast, membrane-associated and membrane-anchored proteins were observed to be up-regulated such as Ras-related protein Rab-3D, IL1B Interleukin-1 beta precursor, Serum amyloid P-component precursor, Apolipoprotein A-I precursor, Ras-related protein Rab-14 and Annexin A5. Up-regulation of some of the proteins such as Apo protein is well documented in maturation of murine monocytic precursor [36] and LPS infection of monocytic cell lines [37].

3.4 Down-regulation of proteins in LPS signaling pathway

In this study, several potential LPS receptors were found significantly down-regulated after 24 hr LPS exposure. These include CD14, Toll-like receptor 4 (TLR4) and CD11b/CD18. The CD14 and TLR4 are well recognized as key receptors in the LPS signaling pathway in human monocytes [38]. Monocytes respond to LPS infection by firstly coupling the endotoxin with CD14 and then transferring it to TLR4 [39], which activates downstream signaling pathway and subsequently initiates immune response by release of large amount of reactive species and cytokines [39]. CD11/CD18 complex receptor has also been demonstrated to be potential LPS receptor [40]. This complex could probably utilize the same downstream signaling regulators of TLR4 to initiate the
LPS-induced signal transduction although the efficiency is relatively low compared to CD14 [41]. On the other hand, many lipoproteins and LPS binding proteins were found to be significantly up-regulated 1.6-3.9 fold. These include Apolipoprotein B-100; Apolipoprotein D; Apolipoprotein A-I, Apolipoprotein E and Serum amyloid P-component. The binding of LPS to the individual lipoproteins attenuates LPS-induced activation of macrophages and endothelial cells [42]. Similarly, the binding of Serum amyloid P (SAP) to LPS inhibits the LPS-induced activation of PMNC in vitro and cell activation in whole blood [43]. Together, the up-regulation of these “neutralized” proteins could be in concert with the down-regulation of LPS receptors for the dampening monocyctic response to LPS infection and thus inhibit the activation of LPS signaling pathway, leading to an LPS tolerance state.

3.5 Alteration of proteins involving in TLR-associated signaling pathways

Despite the down-regulation of TLR receptor after LPS treatment for 24 hrs, some of TLR-induced proteins were observed to be significantly up-regulated. For example, several proteins (SYK, HCK, LYN and VAV1) involved in Fcγ receptor-mediated phagocytosis in monocytes were increased 1.3-2.3 fold; Proteins involved in JAK/Stat Signaling (STAT6, PTPN6 and PTPN1) and p38 MAPK signaling (MAPK14, IL1B and TNFRSF1B) were also up-regulated. Fcγ receptor-mediated phagocytosis has been reported to be up-regulated upon LPS exposure [44]. This increase in phagocytosis could occur despite the being in LPS tolerance state [45]. The poor proinflammatory activity in ET together with high phagocytic ability might avoid development of septic shock, while allowing bacterial clearance [46]. Up-regulation of both p38 and phagocytosis pathway in our data is consistent with an early report that the activation of phagocytosis by Toll-like receptor was likely mediated via p38 MAPK signaling pathway [47]. JAK/Stat signaling molecules were reported to negatively regulate TLR-induced
production of pro-inflammatory cytokines [48]. The up-regulation of these molecules in JAK/Stat signaling pathway indicates an LPS tolerance state of monocytes after 24 hr LPS exposure.

3.6 Differential expression of antigen presentation molecules

We observed that two types of antigen presentation molecules, i.e. major histocompatibility complex I (MHC I) and MHC II, responded differently to LPS treatment. MHC II molecules CD74 and HLA-DR were both significantly down-regulated. However, of the identified MHC I molecules, HLA-A, B, C were up-regulated but B2M was substantially decreased in the expression. Similar expression of HLA-A, B, C and HLA-DR has been reported in human endothelial cells [7, 49]. During bacterial infection, monocytes not only phagocytose and kill pathogens but also process bacterial endotoxins and present them to T lymphocytes via MHC I and MHC II antigen presentation molecules [50]. It has been reported that exposure of monocytes to LPS led to impaired antigen presentation [7, 46]. Our data not only confirmed that LPS challenge could induce impaired antigen presentation in monocytes, but also suggested that this impairment is likely specific to MHC II-mediated activation of CD4+ T lymphocytes, rather than the activation of CD8+ T lymphocytes by MHC I molecules.

4. Concluding remarks

In this study, we used iTRAQ quantitative approach to obtain the proteomics survey of monocyte membrane proteins before and after LPS treatment. We reported a total of 1651 proteins of which 53.6% were membrane proteins and 94% proteins have been quantified (Supplemental Table 1), representing the most comprehensive membrane dataset in the study of monocyte response to bacterial infection. We identified 255
proteins that were tightly regulated by LPS (Supplemental Table 2). Subcellular analysis of these proteins revealed organelle-specific response of monocytes to LPS challenge (Fig. 3), that is, majority of the monocyte mitochondrial membrane proteins was down-regulated after LPS exposure. It has been shown that mitochondrial intactness in rat heart was reduced after LPS administration [33]. Our data provide strong molecular evidence showing that more than 90% of the identified mitochondrial membrane proteins required for maintaining its structure were significantly down-regulated. In addition, mitochondria are the major organelle for energy production and also central executor of programmed cell death [51]. Hence, the large scale of down-regulation of mitochondrion-specific proteins in the LPS infection would adversely affect monocyte function, leading to immune disorders as in sepsis or septic shock. This suggests that mitochondria alone might be an effective organelle therapy target during endotoxin induced infection. Indeed, protective treatment of mitochondria increased the survival rate in sepsis patients [52]. Furthermore, we found that the LPS exposure altered the monocyte surface proteins reciprocally. Many potential LPS receptors including CD14, TLR4 and CD11/CD18 complex, and scavenger receptor CD36 were suppressed, whereas negative regulators of LPS pathway were significantly up-regulated. Collectively, these dynamic protein expressions could be at least partly responsible for the LPS induced monocytic insensitivity, suggesting that an effective therapy may need to target a combination of key molecules.

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5. Reference


[35] Corda, S., Laplace, C., Vicaut, E., Duranteau, J., Rapid reactive oxygen species production by mitochondria in endothelial cells exposed to tumor necrosis factor-


Table 1. Experimental variation across the technical replicates. 99% proteins were found to have a variation ≤1.3 fold within technique replicates.

<table>
<thead>
<tr>
<th>Percentage of proteins</th>
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Figures and figure legends.

Fig. 1. Experimental variation across the technical replicates
Fig. 2. Experimental variation across three biological replicates
Fig. 3. Subcellular categories of the 255 proteins identified with significant expression change across three replicates. (A) Subcellular locations. (B) Comparison of the numbers of the up- and down-regulation of proteins from each group.
Fig. 4. Immunoblotting confirmation of the down-regulation of mitochondrial proteins. Consistent down-regulations of APT5B and NDUFS3 in the monocyte lysate were observed from three healthy donors after LPS challenge. Actin was used as loading control.

![Immunoblotting Confirmation](image)

Fig. 5. Temporal analysis of ROS using both dichlorofluorescin diacetate (DCFH-DA) and dihydroethidine (DHE) staining. ROS level was dramatically increased at the initial stage of the LPS treatment (2 hr), but it dropped to a slightly lower level after 24 hr treatment compared to the control in the two different staining.

![Temporal Analysis of ROS](image)
Fig. 6. Flow cytometric analysis confirmed the monocyte surface antigen expression changes over LPS exposure. Significant down-regulation of CD11/CD18 complex, CD14, CD33, CD97 and HLA-Dr were observed after LPS exposure. The number in the figure indicates the mean fluorescence intensity (MFI).
Supplemental Fig. 1. Experimental flow chart for comparative analysis of LPS-treated and control monocyte membrane proteins.

Supplemental Table 1. Total proteins identified and quantified from this study.

Supplemental Table 2. Proteins were significantly (p<0.01) up-regulated or down-regulated in response to LPS challenge, and reproducibly identified from three biological replicates.