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Selenoproteome Identification in Inflamed Murine Primary Bone Marrow-derived Macrophages by nano-LC Orbitrap Fusion Tribrid Mass Spectrometry

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Abstract

Selenium (Se) functions as a cellular redox gate keeper through its incorporation into proteins as the 21st amino acid, selenocysteine (Sec). Supplementation of macrophages with exogenous Se (as sodium selenite) downregulates inflammation and intracellular oxidative stress by effectively restoring redox homeostasis upon challenge with bacterial endotoxin lipopolysaccharide (LPS). Here we examined the use of a standard Tandem Mass Tag (TMT)-labeling mass spectrometry-based proteomic workflow to quantitate and examine temporal regulation of selenoproteins in such inflamed cells. Se-deficient murine primary bone marrow-derived macrophages (BMDMs) exposed to LPS in the presence or absence of selenite treatment for various time periods (0–20 hours) were used to analyze the selenoproteome expression using isobaric labeling and shotgun proteomic workflow. To overcome the challenge of identification of Sec-peptides, we used the identification of non-Sec containing peptides downstream of Sec as a reliable evidence of ribosome readthrough indicating efficient decoding of Sec codon. Results indicated a temporal regulation of the selenoproteome with a general increase in their expression in inflamed cells in a Se-dependent manner. Selenow, Gpx1, Msrb1, and Selenom were highly upregulated upon stimulation with LPS when compared to other selenoproteins. Interestingly, Selenow appeared to be one amongst the highly regulated selenoproteins in macrophages that was previously thought to be mainly restricted to myocytes. Collectively, TMT-labeling method of non-Sec peptides offers a reliable method to quantitate and study temporal regulation of selenoproteins; however, further optimization to include Sec-peptides could make this strategy more robust and sensitive compared to other semi-quantitative or qualitative methods.

Graphical Abstract

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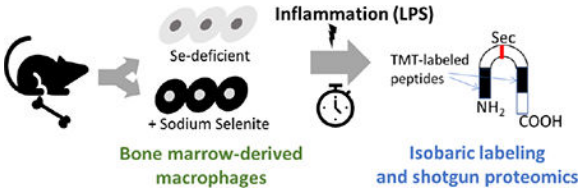

Keywords

Lipopolysaccharide; inflammation; resolution; proteomics; redox

INTRODUCTION

The essential trace element selenium (Se) functions through its incorporation as the 21st amino acid, selenocysteine (Sec; U), in proteins. Sec has a unique redox chemistry that endows selenoproteins with high redox activity in many biological systems [1, 2]. Decoding of the UGA stop codon by Sec $tRNA^{[Ser]Sec}$ leads to the specific incorporation of Sec within 25 (24 in mice) selenoproteins in humans [3]. The expression of these selenoproteins in macrophages is not only regulated by the bioavailability of Se, but also by pathways related to stress and inflammation that helps to reduce free radicals and efficiently restore redox homeostasis [4, 5]. Inflammation is a cellular response to diverse extracellular stimuli leading to the release of plethora of mediators, which is sequentially followed by resolution [6], where macrophages play a dual role. During an inflammatory response, macrophages that are activated by pathogen-associated molecular patterns (PAMPs), such as bacterial endotoxin lipopolysaccharide (LPS), cause collateral damage to the surrounding tissues via the oxidative burst that is associated with the production of free radicals or reactive oxygen and nitrogen species (RONS) eventually leading to cancer [7]. Thus, efficient control of intracellular oxidative stress and redox imbalances in macrophages along with initiation of beneficial functions like phagocytosis and antigen presentation are necessary to facilitate resolution of inflammation [8, 9]. We have previously shown that macrophage selenoproteins play an important role in phenotypic switching in inflammatory models from a classically activated phenotype (M1) to a pro-resolving phenotype (M2) that promotes resolution of inflammation [10]. The ability to downregulate inflammatory pathways while activating pro-resolution pathways was attributed to differential regulation of two transcription factors, nuclear factor-kB (NF-κB) and peroxisome proliferator-activated receptor-gamma (PPARγ). Our studies characterized the central role of cyclopentenone prostaglandins, Δ^{12}-PGJ_2 and 15d-PGJ_2, produced in the presence of selenoproteins, which inhibit NF-κB, while activating PPARγ in a ligand-dependent manner [11, 12]. In addition, selenoproteins also downregulated inflammatory gene expression through epigenetic mechanisms [13]. Despite this data, it is not clear which selenoprotein(s) are key regulators of such a mechanism.

Literature is replete with reports of selenoproteins such as methionine-R-sulfoxide reductase B1 (Msrb1; Selenor; Selenox), ethanolaminephosphotransferase 1 (Selenoi), selenoprotein F (Selenof), selenoprotein H (Selenoh), selenoprotein K (Selenok), selenoprotein M (Selenom), selenoprotein N (Selenon), selenoprotein O (Selenoo), selenoprotein S (Selenos),

selenoprotein W (Selenow), thioredoxin reductase-like selenoprotein T (Selenot) that are regulated by stress-related pathways; while, glutathione peroxidase 1 (Gpx1), phospholipid hydroperoxide glutathione peroxidase (Gpx4), thioredoxin reductase 1 (Txnrd1), and thioredoxin reductase 2 (Txnrd1), perform a housekeeping role [14]. While some of these proteins have been characterized, the biological role of a few selenoproteins, such as Selenow, have not been fully characterized. Selenow is a 10 kDa selenoprotein with a conserved redox CXXU motif like the CXXC motif in thioredoxin (Trx) [15, 16]. Selenow displays glutathione-dependent antioxidant activity. Selenow was reported to reduce disulfides in Cdc25B, which prompts its dissociation from 14–3-3β/ζ, and promotes cells cycle progression [17–20]. Selenow enhances skeletal muscle differentiation by inhibiting the interaction of Taz (transcriptional coactivator with PDZ binding motif) with 14–3-3 [18]. Recently, our laboratory reported the expression of Selenow in splenic erythroid cells to support stress erythropoiesis [21], but its expression and regulation during inflammation has not been described in macrophages. In addition, previous studies using macrophage-specific deletion of *Trsp* (encoding the Sec $tRNA^{[Ser]Sec}$) have demonstrated selenoprotein expression to be key in efficiently resolving inflammation [22–23]. While selenoproteins are important macrophage function, the identity and modulation of the selenoproteome under such inflammatory conditions is still not well understood.

To better understand the role of selenoproteins under various pathophysiological conditions, it is essential to accurately quantify their expression at the proteome level given the post-transcriptional, co-translational, and post-translational regulation of these proteins which is seldom captured at the level of the transcriptome or even by ^{75}Se labeling technique [24]. Even the current proteomic approaches are not without challenges, such as the identification and characterization of the Sec-containing peptides of selenoproteins. In the recent past, semi-quantitative and non-targeted mass spectrometry (MS)-based workflows have proven to be popular without the use of radioactive isotopes. The most widely used techniques to analyze selenoprotein include Laser Ablation Inductively Coupled Plasma Mass Spectrometry (LA-ICP-MS) and laser ablation of isoelectric focused immobilized pH gradient strips coupled to ICP MS detection (IEF-LA-ICP MS) [25–27]. Each of these methods have advantages, limitations, and challenges in selenoprotein research (reviewed in detail [28]). A combination of shotgun proteomic workflow and LA-ICP-MS method identified eight non-Sec containing peptides for Gpx3 and seven peptides for selenoprotein P (Selenop) with a good MS/MS spectrum that matched well with the National Institute of Standards and Technology peptide mass spectral library in depleted human plasma, where human Standard Reference Material 1950 was used [29, 30]. In a recent study, Se-encoded isotopic signature targeted profiling (SESTAR) computational method was developed to detect selenoproteins by utilizing the distinct natural isotopic distribution of Se that demonstrated the natural distribution across different cell lines and tissues [31].

Here we report the use of standard Tandem Mass Tag (TMT)-labeling [32] based proteomic workflow for selenoprotein quantification in murine primary BMD coupled to orbitrap fusion tribrid MS. We report for the first time a snapshot of the temporal regulation of selenoprotein expression at the proteome level following LPS-stimulation of macrophages. The work entailed the identification of peptides downstream of Sec, particularly in selenoproteins where Sec residue is present in the N-termini suggesting ribosomal read

through the Sec codon. Our studies indicate high levels of expression of a subset of selenoproteins (Selenow, Gpx1, Msrb1, and Selenom) upon exposure to LPS challenge. In addition, our studies also complement the existing methods to analyze the expression of these proteins in order to delineate their function in immune mechanisms associated with the efficient control of inflammation.

EXPERIMENTAL

Materials

Dulbecco's Modified Eagle Medium (DMEM) and TMT labeling reagent kit were purchased from Thermo Scientific (Gaithersburg, MD). Fetal bovine serum (FBS) was purchased from Atlanta biologicals (Atlanta, GA), and lipopolysaccharide from *E. coli* (Serotype 0111:B4) and sodium selenite were purchased from Sigma Aldrich (St. Louis, MO). L929 murine fibroblasts were purchased from the American Type Culture Collection (Manassas, VA). Basal levels of Se in the culture media was 7 nM. Unless otherwise mentioned, all chemicals and reagents were of MS grade.

Mice and Bone Marrow-derived Macrophage Culture and LPS Stimulation

Three-week-old C57Bl/6 male mice were purchased from Taconic Biosciences, Inc. (Hudson, NY) and maintained on an AIN-76-based semi-purified Se-deficient diet (<0.01 ppm) from Harlan-Teklad (Madison, WI) for at least four weeks before being used in experiments as previously described [22]. Femoral bone marrow was harvested from 7 to 12 weeks old mice. Bone marrow was prepared into a single cell suspension and plated in DMEM with 5% (v/v) fetal bovine serum, 100 lU/ml of penicillin (Corning), 100 μg/ml of streptomycin (Corning, NY), 1mM L-glutamine (Corning, NY), and 10% (v/v) L929 fibroblast-conditioned DMEM as a source of macrophage-colony stimulating factor (M-CSF). Cells were cultured in biological triplicates for seven days with or without Se (as sodium selenite; 250 nM) as described [22]. On the seventh day, cells were stimulated with LPS (100 ng/ml) and were harvested at zero, four, eight and twenty-hours post stimulation, and stored at −80 °C until further processing. Cell viability analysis suggested no signs of toxicity with 250 nM Se treatment with or without LPS stimulation; viability decreased only upon treatment with > 2 μM of Se (data not shown). All animal studies were preapproved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee at The Pennsylvania State University, University Park, PA.

Sample Preparation for MS Analysis

Harvested cell pellets were washed with phosphate-buffered saline (PBS) and the proteome was extracted in RIPA buffer (Thermo Fisher Scientific, MA). Protein concentration was determined by BCA assay (Thermo Fisher Scientific, MA). Equal amounts of protein (100 μg) diluted with 100 mM triethyl ammonium bicarbonate (Sigma Aldrich, St. Louis, MO) buffer was subjected to in-solution digestion. Proteins were reduced with dithiothreitol (10 mM) at 45 °C for 30 min, followed by alkylation with 2-chloroacetamide (65 mM) at room temperature in the dark for 30 min. The proteome was digested with proteomics-grade trypsin at 1:40 (enzyme to substrate) ratio overnight at 37 °C. The peptide digest was labelled with TMT isobaric labeling kit (Lot number SH253273), and channels for each of

the samples are provided in Table S1. An overview of the complete study design is shown in Figure 1. The MS analysis at all time points were done in biological triplicate.

Liquid Chromatography-High Resolution/Accurate MS Orbitrap Fusion

To increase the accuracy and confidence in measurements of protein abundance, a multinotch-MS3 method was employed for analyzing MS data [33]. Raw data were acquired by using RSLC Ultimate 3000 nano-UPLC (Dionex) coupled to Orbitrap Fusion MS (Thermo Fisher Scientific, MA). Two microliters from each fraction were resolved on an Acclaim PepMap C18 reverse phase column (2 microns, 75 μm i.d. × 50 cm) using a 0.1% formic/acetonitrile gradient at 300 nl/min. The mass spectrometer was set to collect one MS1 scan (Orbitrap; 120 K resolution; AGC target 2×10^5; max IT 100 ms) followed by data-dependent, “Top Speed” (3 s) MS2 scans (collision induced dissociation; ion trap; NCD 35; AGC 5×10^3; max IT 100 ms). For multinotch-MS3, the top ten precursors from each MS2 scan were fragmented by high-energy collisional dissociation (HCD) followed by Orbitrap analysis (NCE 55; 60,000 resolution; AGC 5×10^4; max IT 120 ms, 100–500 m/z scan range).

Data Analysis

Mass-spectral data-set was analyzed with Proteome Discoverer™ (v2.2, Thermo Fisher Scientific, MA) and queried against the SwissProt *Mus Musculus* database (Reviewed 17,424 proteins) using the following parameters: peptide and fragment mass tolerance were 10ppm and 0.5Da, respectively, with two miscleavages. The oxidation of methionine (15.995Da) and deamidation of asparagine and glutamine (0.984Da) were considered as variable modifications. TMT labeling of the N-termini of peptides, as well as lysine (229.163Da) and cysteine carbamidomethylation (57.021Da) were considered as static modifications. Relative quantitation using TMT reporter ions was performed using high-quality MS3 spectra (with an average reporter signal-to-noise ratio of 7 and 40% as co-isolation threshold). A percolator algorithm was used to determine the false discovery rate (FDR) and only proteins/peptides with an FDR < 0.01 were retained for further analysis. To be more inclusive, we replaced ‘U’ to ‘C’ in data-base FASTA sequence, since Proteome Discoverer™ does not assign mass-value on U. This method worked well for unlabeled peptides (not part of this manuscript). Possible loss of Sec has been reported during sample processing by conversion to dehydroalanine [34], which was also used as a variable modification. In this study, the protein abundance values were normalized to beta-actin and was made relative to zero hour, zero-selenium sample, which would behave as naïve cells.

Statistical Analysis

All statistical analyses were performed by using GraphPad Prism v7.04 for Windows (San Diego, CA). Heat maps were generated by using GraphPad Prism. Regular two-way ANOVA with uncorrected Fisher’s LSD test was used to compare means over time in the presence or absence of Se and stimulation with LPS.

RESULTS AND DISCUSSION

Identification and Quantitation of Selenoproteins by nano-LC- Orbitrap Fusion MS

MS-based proteomic workflows render relative quantification of proteins across biological samples with great ease. Isotope labeling of proteins in samples prior to MS acquisition is a popular method to quantify the proteome. Furthermore, MS/MS based analysis strategies using TMT labelling allows simultaneous identification and quantification of peptides and serves as a robust method in multiplex quantitative proteomics [32]. Using this method, 6197 proteins were identified with at least one unique peptide, including 13 selenoproteins, in the murine primary BMDMs. A total of 67 peptides were identified belonging to 13 selenoproteins (Table S2). Of these, 22 peptides were identified for Txnrdl, followed by 10 and 9 peptides each for Gpx4 and Gpx1, Txnrd2 respectively. Four and three peptides each for Selenof and Selenos, respectively, were identified. Furthermore, two and one peptide each for Selenon, Selenot, Selenok and Selenom, Selenow, Msrb1, Selenoi, respectively, were identified (Table 1). Details of the identified peptides are provided in Table S2, while details of the TMT-labeled relevant unique peptides and peptide spectral matches (PSMs) are listed in Table 1 and protein sequence coverage by peptides are shown in Figure 2 and Figure S2. Corresponding MS/MS spectra of TMT labelled peptides identified are provided in Figure S1 and a representative MS/MS of Gpx4 peptide TDVNYTQLVDLHAR (625.3375 Da) is shown in Figure 3.

Use of classical shotgun proteomic workflows cannot be undermined as it serves as an unambiguous method to identify both Sec containing and non-Sec containing peptides. However, given that we were unable to identify Sec containing peptides using the TMT-labeling technique, we reasoned that identification of non-Sec containing peptides downstream of Sec could serve as a reliable evidence of ribosome readthrough indicating an efficient decoding of Sec codon. While this strategy holds good for selenoproteins that possess Sec in the N-termini, identification of Sec containing peptides still poses an anal ytical ch allenge, particularly in those selenoproteins where Sec is present at the C-terminus, making one merely rely on upstream peptides. Recently, the development of a novel Sec-specific MS-based technique (SecMS) was reported, where Sec-containing peptides were preferentially enriched by selective alkylation of Sec at an acidic pH allowing systematic profiling of selenoproteome [35].

Differential Modulation of Selenoproteins in Inflamed Macrophages Treated with LPS

Using the TMT-labeling method, we analyzed the expression of a select few LPS-responsive proteins in macrophages treated with LPS. Prototypical inflammatory markers such as Il1b, Nos2, Tlr4, Stat1, and Icam1 were upregulated in these cells indicating that our cell culture system, treatments, and proteomic analysis were in close agreement with that reported in the literature (Figure 4A) [36]. The TMT-labeled grouped abundance raw values of selenoproteins were normalized to cytoplasmic 3-actin (Uniprot accession P60710). The normalized values were made relative to base line sample that corresponded to zero hour zero Se (considered as “naïve” cells). A total of 13 selenoproteins were identified and their relative abundance values are depicted as heat map in Figure 4B. Selenoprotein abundances were found to be higher in macrophages cultured with 250 nM Se supplementation,

suggesting their expression is dependent on the bioavailability of Se in comparison to their deficient counterparts (Figure 4B). A two-way ANOVA analysis showed statistically significant differences in relative abundances across different time points post LPS. Orthogonal validation was performed using immunoblotting with antibodies for Selenow, Gpxl and Txnrdl (Figure 4C). The lack of selenoproteins in cells cultured under Se-deficient conditions were clearly in agreement with previous studies [13], including those performed with BMDMs from $Trsp^{fl/fl}LysM^{Cre}$ mice (data not shown) that lacked the entire selenoproteome. Based on our previous experiments, lack of a selenoproteome would further exacerbate inflammatory pathways leading to experimental artifacts and complexities, and therefore, $Trsp^{fl/fl}LysM^{Cre}$ macrophages were not used in this investigation. Uncorrected Fisher's LSD multiple comparisons test suggested that out of total 13 selenoproteins, the relative abundance of four selenoproteins, Selenow, Gpxl, Msrb1 and Selenom, were significantly different at either one time point as shown in Figure 4B, suggesting differential regulation of selenoproteins is not just limited to bioavailability of Se, but also to other factors prevailing in the *in-vitro* culture system. The fold change in the expression of Gpxl, Selenow, and Txnrdl between western immunoblotting (Figure 4D) and MS methods at various time points post LPS challenge showed an approximately similar patterns in these selenoproteins with the fold-differences reasonably closer, though not identical, between two different platforms (Table S3).

More importantly, proteomic analysis indicated differential modulation of selenoproteins in response to LPS. Se-dependent upregulation of Selenow, Gpxl, Msrbl, and Selenom was observed (Figure 4B). Both Selenow and Msrbl were detected with low PSMs; however, increased expression at 24-hour post LPS stimulation was observed. This agrees with the previous report where elevated MsrBl expression regulates actin polymerization in macrophages 18-hour post LPS stimulation [37]. Expression of Msrbl upon LPS treatment in the presence of Se is likely required to reduce methionine oxidation suggesting a new paradigm in selective selenoprotein regulation under Th2 conditions that prevail during asthma or helminth infections [38]. However, further studies are required to validate this observation. Expression of Selenow in macrophages in the presence of bioavailable Se and its regulation under inflammatory conditions was unexpected given that most of the literature related to this selenoprotein is restricted to myocytes [16-18], and more recently, reported in erythroid progenitors [21]. This also raises an important question regarding its role in macrophage biology, particularly in pathways related to downregulation of inflammatory responses in response to changes in the cytokine environment, which could also have an impact on the expression of the selenoproteome and subsequent resolution of inflammation.

CONCLUSIONS

Here we report the isobaric labeling and shotgun proteomic workflow to identify and quantitate selenoproteins in mammalian cells. Using the TMT-labeled selenoproteome identification and quantification, we report an interesting pattern of regulation of the selenoproteome in macrophages treated with pro-inflammatory bacterial endotoxin LPS that warrants further characterization of their identification as well as regulation. Our studies found the expression of Selenow in macrophages that was previously thought to be mainly

restricted to myocytes. While this method simplifies the process of quantitation of selenoproteins, the ability to identify Sec containing peptides is of utmost importance for at least those selenoproteins where Sec is at C-terminus.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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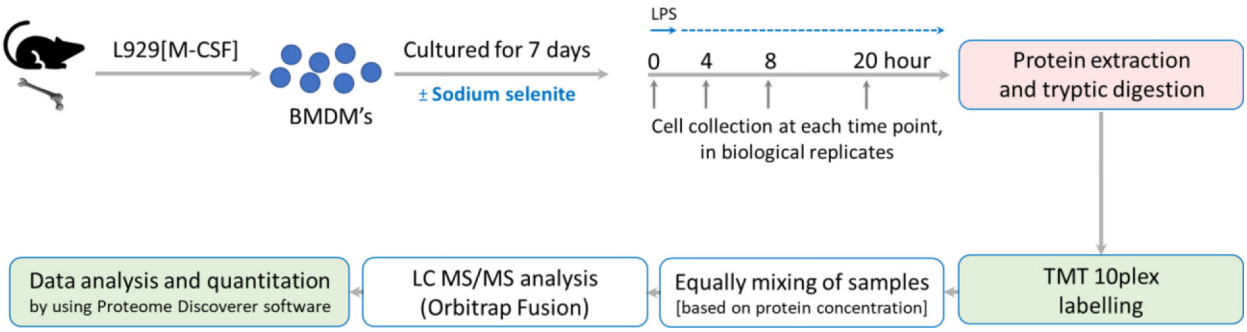

Figure 1.

Overview of the complete study design. BMDMs were cultured with or without 250 nM sodium selenite (Na_2SeO_3), stimulated with LPS and harvested at various time points. The lysates were hydrolyzed with trypsin and labelled with TMT, proteomic data-set was acquired on nano-LC coupled to orbitrap tribrid fusion MS and analyzed with Proteome Discoverer software.

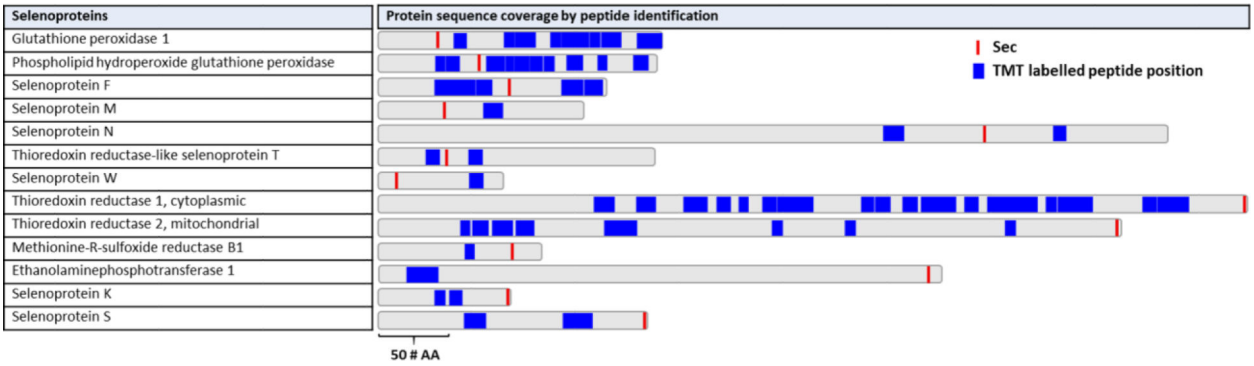

Figure 2.

TMT labelled 67 peptide positions and coverage for selenoprotein sequence. Sequence length of selenoproteins were scaled, the red bar represents Sec and the blue boxes represent the TMT labeled peptides that were identified in our assay.

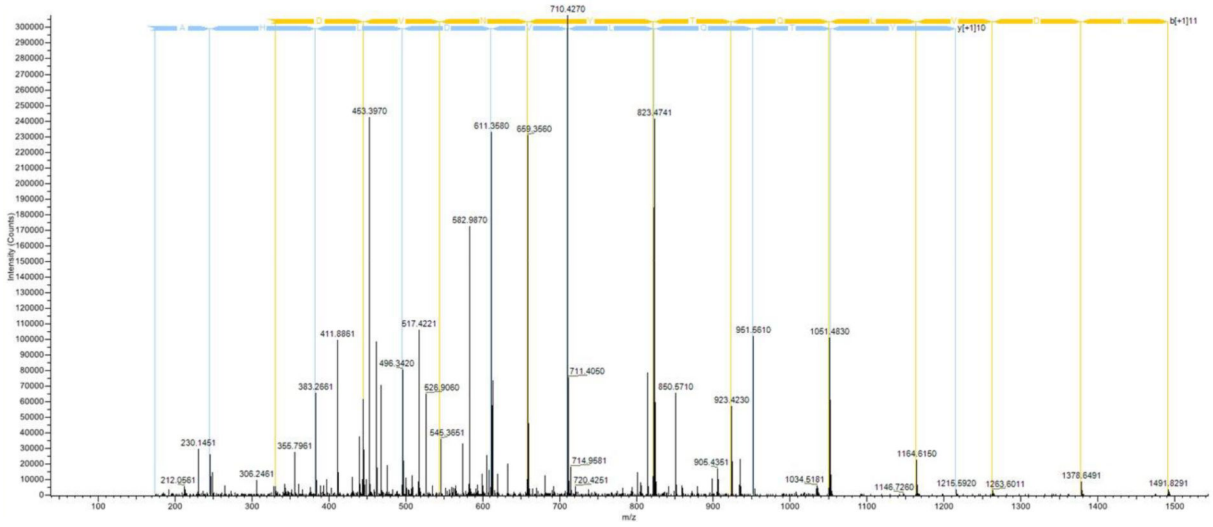

Figure 3.

A representative selenoprotein peptide MS/MS. Protein- Phospholipid hydroperoxide glutathione peroxidase (Gpx4). Sequence- TDVNYTQLVDLHAR, T1-TMT6plex (229.16293 Da), Charge: +3, Monoisotopic m/z: 625.33752 Da (+0.58 ppm error), MH+ - 1873.99802 Da. XCorr-6.13, Percolator q-Value- 0.0e0, Percolator PEP-5.3e-8. Fragment match tolerance used for search- 0.6 Da. Fragments used for search- b; b-H_2O; b-NH_3; y; y-H_2O; y-NH_3.

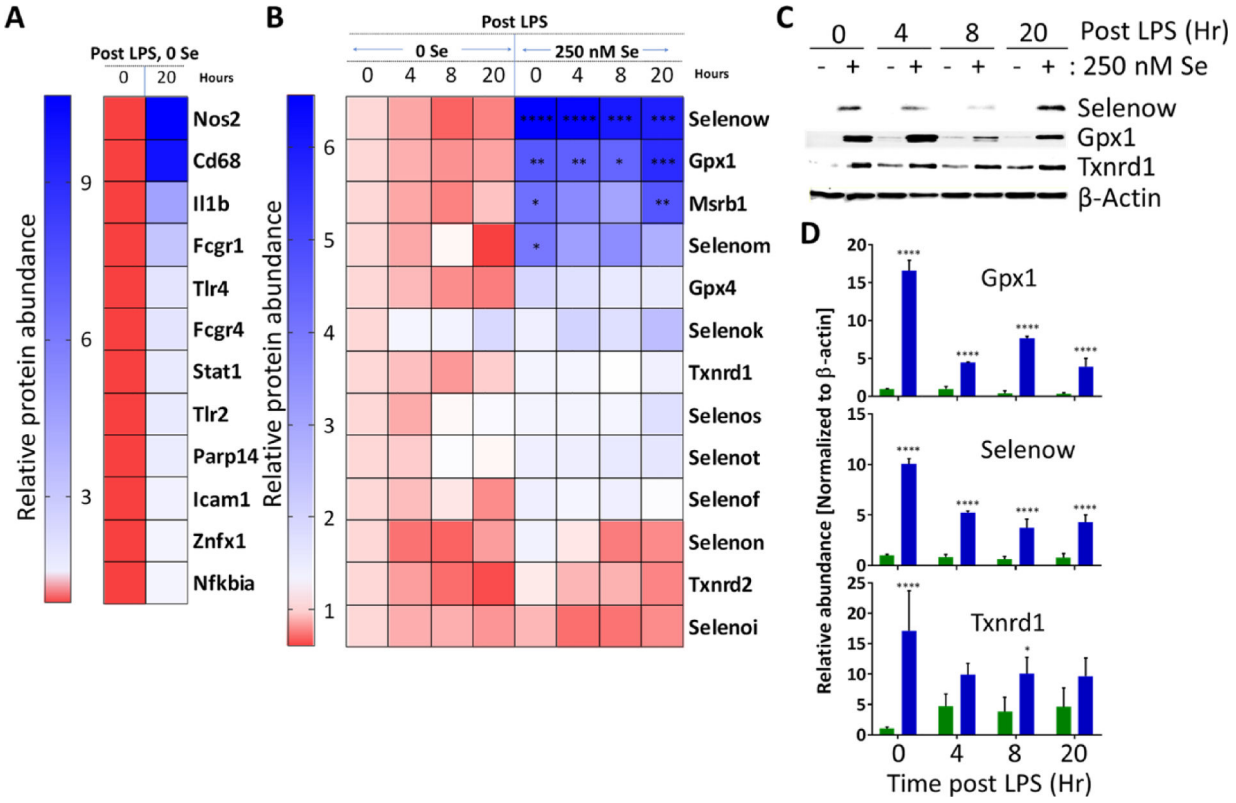

Figure 4.

Heat map for differentially regulated selenoproteins in macrophages post LPS treatment. Briefly, BMDMs were cultured with 100 ng/ml LPS for 20 hours. The samples were processed as described under “Methods”. Panel A shows upregulation of proto l proteins upregulated in response to LPS. Panel B shows temporal modulation of selenoproteins in macrophages. The color scale bar represents protein relative abundance, red (low) to blue (high). Panel C & D show representative (of n= 3) Western immunoblot validation of Selenow, Gpxl, and Txnrdl and densitometric values in bar graphs. NS- Nonspecific immunoreactive band. Two-way ANOVA multiple comparisons *, $p<0.05$; **, $p<0.009$; ***, $p<0.0009$; ****, $p<0.0001$. All experimental data are expressed as mean ± S.E.

Table 1.

Selenoproteins identified by TMT labeling and analyzed on Orbitrap Tribrid Fusion MS. Selenoproteins can be divided into two groups depending on the Se position in amino-acid sequence. In first group, the Sec position is towards N-terminal or in the middle region, the other group have towards C-terminal end. Selenoproteins identified in macrophages by TMT 10plex labelling and corresponding TMT labelled peptide position is provided in Figure 2 and Figure S2.

Sl	Uniprot Accession	Protein name	Gene Symbol	#AAs	Sec position	TMT-LBUP	#PSMs
Sec in the N-terminal or middle region							
1	P11352	Glutathione peroxidase 1	Gpx1	201	47	9	91
2	O70325	Phospholipid hydroperoxide glutathione peroxidase	Gpx4	197	73	10	72
3	Q9ERR7	Selenoprotein F	Selenof	162	93	4	56
4	Q8VHC3	Selenoprotein M	Selenom	145	48	1	2
5	D3Z2R5	Selenoprotein N	Selenon	557	428	2	3
6	P62342	Thioredoxin reductase-like selenoprotein T	Selenot	195	49	2	19
7	P63300	Selenoprotein W	Selenow	88	13	1	2
Sec in the C-terminal region							
8	Q9JMH6	Thioredoxin reductase 1, cytoplasmic	Txnrd1	613	612	22	199
9	Q9JLT4	Thioredoxin reductase 2, mitochondrial	Txnrd2	524	523	9	23
10	Q9JLC3	Methionine-R-sulfoxide reductase B1	Msrb1	116	95	1	1
11	Q80TA1	Ethanolaminephosphotransferase 1	Selenoi	398	388	1	2
12	Q9JLJ1	Selenoprotein K	Selenok	94	92	2	3
13	Q9BCZ4	Selenoprotein S	Selenos	190	189	3	6