Osteopontin depletion in macrophages perturbs proteostasis and leads to UCHL1 deficiency and mitochondria-mediated apoptosis

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Short title: Role of OPN in UPS-mediated macrophage homeostasis
ABSTRACT

Osteopontin (OPN; also known as SPP1), an immunomodulatory cytokine highly expressed in bone marrow-derived macrophages (BMMΦ), is known to regulate diverse cellular and molecular immune responses. We previously revealed that glatiramer acetate (GA) stimulation of BMMΦ upregulates OPN expression, promoting an anti-inflammatory, phagocytic, pro-healing phenotype, whereas OPN inhibition triggered a pro-inflammatory phenotype. Here, we applied a global proteome profiling via mass spectrometry analysis to gain a mechanistic understanding of OPN suppression versus induction in macrophages. We identified over 630 differentially expressed proteins (DEPs) in OPN knockout (OPN\(^{\text{KO}}\)) or GA-stimulated versus wild type (WT) macrophages. Two topmost downregulated DEPs in OPN-deficient macrophages were ubiquitin C-terminal hydrolase L1 (UCHL1), a crucial component of ubiquitin-proteasome system (UPS), and the anti-inflammatory Heme oxygenase 1 (HMOX-1), whereas GA stimulation upregulated their expression. We confirmed UCHL1 expression in BMMΦ, which was previously recognized as a neuronal-specific protein. Further, immunoprecipitation assays suggest a direct interaction of OPN and UCHL1 proteins. Functional pathway analyses revealed two inversely regulated pathways in OPN-deficient macrophages: activated oxidative stress and lysosome-mitochondria-mediated apoptosis (ROS, Lamp1/2, ATP-synthase subunits, cathepsins, and cytochrome C and B subunits) and inhibited translation and proteolytic pathways (60S and 40S ribosomal subunits and UPS proteins). In agreement with the proteome-bioinformatics data, Western blot and immunocytochemical analyses revealed that OPN deficiency perturbs protein homeostasis (proteostasis) in macrophages—inducing apoptosis and inhibiting translation—whereas GA-stimulated OPN induction restores cellular proteostasis. Taken together, OPN is essential for macrophage homeostasis via regulation of cell viability, UCHL1-UPS, and protein synthesis, indicating its potential application in immunotherapy.

Keywords: secreted phosphoprotein 1, early T-lymphocyte activation (ETA-1), bone/sialoprotein I (BSP-1 or BNSP), monocytes, innate immunity, mitochondrial dysfunction, endocytosis.
INTRODUCTION

Osteopontin (OPN), encoded by the secreted phosphorylated protein 1 (Spp1) gene, is a multifaceted matricellular glycoprotein secreted by various immune cells, such as macrophages and T cells. OPN is an immunomodulatory cytokine, which is highly expressed by bone marrow-derived macrophages (BMMΦ) and regulates diverse cellular immune responses, including migration, communication, and immunological responses. Notably, OPN has dual roles in peripheral immune cells influencing both inflammatory and anti-inflammatory responses depending on the acute or chronic inflammatory microenvironment. Although, previous studies have reported that OPN is associated with type 1 pro-inflammatory macrophage and T cell polarization, other reports demonstrate that OPN is associated with macrophage and T cell polarization towards anti-inflammatory phenotypes. In addition, macrophage-derived OPN induces an anti-inflammatory immune response during Cryptococcus neoformans infection. Furthermore, a recent report revealed that OPN can reduce inflammation, tissue injury, and bacterial loads during concurrent pneumococcal infection in a murine model. These studies suggest dual roles for OPN in regulating peripheral immune functions, and a strict modulation of OPN function is crucial to accomplish optimal immune responses.

In the context of the central nervous system (CNS), OPN was shown to be a critical regulator of neuroprotection in several neurological disorders, such as brain ischemia, stroke, and traumatic brain injury. Notably, macrophages are the key regulators of immune responses due to their rapid and diverse activities. Macrophages exhibit high plasticity and adaptability to various adverse conditions, which promote repair and restore tissue homeostasis. Hence, regulating macrophage OPN could have a significant impact on various peripheral and CNS inflammatory conditions.
We recently reported that stimulating BMMΦ with glatiramer acetate (GA; generic name Copaxone®), an FDA-approved drug for the treatment of the neurological autoimmune disease, relapsing-remitting multiple sclerosis, upregulates OPN expression, promoting an anti-inflammatory response. Our in vivo and in vitro studies indicated that GA-stimulation of macrophages substantially induces their ability to clear pathogenic forms of amyloid β-protein (Aβ), including Aβ40 and Aβ42 fibrils. Moreover, GA treatment protected both synapses and the cognitive function in transgenic murine models of Alzheimer’s disease (AD). We found that GA immunomodulation can lead to increased cerebral infiltration of monocytes, which are directly involved in Aβ plaque clearance. Further, in BMMΦ cultures, GA exposure improved phagocytosis of fibrillar Aβ via increased expression of surface scavenger receptors (i.e., CD36, Scara-1). These receptors recognize and bind Aβ fibrils to facilitate their uptake. Importantly, GA promoted a shift into anti-inflammatory microglia and monocyte/macrophage phenotypes, as evidenced by marked increases in anti-inflammatory interleukin (IL)-10, insulin-like growth factor-1 (IGF-1), matrix metalloproteinase-9 (MMP-9), and OPN production, as well as decreases in pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and IL-12 release.

Recent proteomic studies on microglia and macrophages identified distinct protein expression profiles of pro-inflammatory versus anti-inflammatory-type macrophages related to cell metabolism and signaling. Additionally, a few whole proteome analyses were carried out to decipher GA’s effects and mechanisms of action. In the current study, we applied quantitative mass spectrometry (MS)-based proteomics profiling to analyze the effects of OPN deficiency vs. GA immune modulation on macrophage protein networks and functional pathways. Top up and down-regulated proteins were defined as differentially expressed proteins (DEPs), whereas protein
expression networks were examined using functional clustering analysis. Then proteins were enriched for canonical pathways to identify specific signaling pathways. Upstream regulator analysis was performed to identify molecules that can influence the changes in downstream signaling. We comprehensively identified and quantified proteins from OPN-deficient and GA-stimulated BMMΦ relative to untreated macrophages through proteomics. In addition, key identified DEPs were validated \textit{in vitro} by employing immunocytochemistry, immunoprecipitation, Western blot, and mitochondrial tracing analyses. Findings from this study provide new insights into the immune mechanisms of OPN deficiency in macrophages, affecting cellular homeostasis and survival via reduced ubiquitin-proteosome system (UPS) functions.

**MATERIALS AND METHODS**

**Mice**

OPN knock-out (OPN$^{\text{KO}}$) mice from the B6.129S6 (Cg)-\textit{Spp1}$^{\text{im1Blh/J}}$ strain (Jackson laboratories stock #004936|OPN$^{\text{KO}}$) and aged-match wild type controls (Jackson laboratories) were used for in vitro experiments. Animal experiments were performed in accordance with Cedars-Sinai Medical Center Institutional Animal Care and Use Committee (IACUC) guidelines under an approved protocol.

**Primary cultures of bone marrow-derived macrophages**

Bone marrow-derived macrophages (BMMΦ) were collected from 8 to 16 weeks old WT or OPN$^{\text{KO}}$ mice and cultured as previously described $^6$. 
On the sixth day of culture, cells were either plated in 24-well plate on glass coverslips for immunocytochemistry (1x10^5 cells per well; 3-5 wells per condition) or 6-well plate for protein assays (3x10^5 cells per well; 3 wells per condition). Next, some wells were treated with 30 µg/mL GA overnight. The control group was not treated. Then cells were washed three times with 1X PBS before fixation with 4% paraformaldehyde (PFA) (15 min at room temperature). Cells were rinsed 3 times with 1X PBS and stored at 4 °C until immunocytochemistry.

**Immunocytochemistry (ICC)**

Cells plated on glass coverslips were blocked with serum-free protein blocking (Dako Cytomation) for 30 min at room temperature, then hybridized with primary antibodies overnight at 4 °C (see Supplementary table 1). On the following day, cells were incubated with appropriate secondary polyclonal antibodies for 1 h at room temperature. Coverslips were then mounted using ProLong Gold with DAPI (Molecular Probes, Life Technologies). In cases where cells were stained with Dihydroethidium (DHE; Sigma-Aldrich #D7008), fluorescent dye was added after secondary antibody for 30 min at 37 °C (10 µM final concentration in PBS) before mounting.

**Mitotracker**

Cells treated with Mitotracker™ (Thermofisher #M7512) were incubated for 15-30 min with 250 µl of the fluorescent dye at 37 °C, then washed three times with 1X PBS before fixation with 4% PFA (15 min at room temperature). Cells were rinsed 3 times with 1X PBS and mounted for microscopy analysis.

**Microscopy and quantification**
Representative images were captured with a Carl Zeiss Axio Imager Z1 fluorescence microscope equipped with ApoTome, AxioCam MRm, and AxioCam HRc cameras (Carl Zeiss MicroImaging, Inc.). Images were analyzed using ImageJ software (NIH).

**Western blot**

Cells plated on 6-well plates were lifted with 2 mM EDTA-PBS, collected in tubes, and pelleted (1000 rpm for 5 min). Cell pellets were lysed and re-suspended in a cocktail of RIPA lysis buffer (Thermofisher Scientific, #89900) supplemented with 1% protease inhibitor (Calbiochem #539131) and stored at -80 °C until further analysis. Protein concentration was determined using a BCA Protein Assay Kit (Thermofisher Scientific, #23225). Equal amounts of total protein samples were electrophoretically separated onto 4% to 20% Tris-glycine gels (Invitrogen, #XP04205BOX), then transferred to nitrocellulose membranes, blocked for 1 h at room temperature in Tris-buffered saline with Tween 20 (TBST; 10 mmol/L Tris-HCl buffer, pH 8.0, 150 mmol/L NaCl, and 0.1% Tween 20) containing 5% BSA, and hybridized with appropriate primary antibodies overnight at 4 °C. After 4 washes in TBST, membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature then washed again 4 times in TBST prior to development with chemiluminescence substrate kit (Thermofisher Scientific, #34580). Images were taken on iBright imaging system (iBright imaging system; Thermofisher Scientific). Protein expressions were analyzed using ImageJ software and normalized to β-Actin.

**Immunoprecipitation (IP)**

Cells were lysed in lysis buffer (Pierce™ IP Lysis Buffer, Cat. 87787) on ice for 20 min, and lysates were centrifuged at 8000 rpm for 10 min. The supernatant was collected and incubated
with 50 ml of IP beads (TrueBlot Anti-Rabbit) for 30 min on a rocking platform at 4 °C. After incubation, the supernatant was collected by centrifugation and added 5 mg of the primary antibody and incubated on a rocking platform overnight at 4 °C. After overnight incubation, 50 ml of IP beads was added into the supernatant, and 1 h later the beads were collected by centrifugation and washed with lysis buffer. Beads were then loaded with 2X loading buffer and run on the sodium dodecyl-sulfate polyacrylamide gel electrophoresis.

**LC-MS/MS Analysis**

*Sample preparation for mass spectrometry (MS).* Cell pellet processing for MS as outlined in Parker *et al.*

Briefly, proteins were reduced with 5 mm TCEP, alkylated with iodoacetamide, digested with sequencing grade modified trypsin (Promega, Madison, WI) at a 1:100 ratio of enzyme/substrate, and the digestion was stopped by the addition of formic acid. Tryptic peptides were desalted using reverse phase cartridges Sep-Pak C18 (Waters, Milford, MA) according to the following procedure. Tryptic peptides were dried using a vacuum centrifuge and resolubilized in 0.1% formic acid with synthetic iRT calibration peptides (Biognosys, Schlieren, Switzerland) at a 1:20 v/v ratio.

*MS including Assay Library Generation and individual sample analysis by data independent acquisition-MS (DIA-MS).* Tryptic peptides were separated on a ChromXP column (3 um particle size, 150 um x 10 cm) Eksigent technologies) from 3–35% acetonitrile over 60 min gradient by an Eksigent NanoLC Ultra 2D Plus HPLC system coupled to a 5600 TripleTOF mass spectrometer (AB Sciex, Framingham, MA). To build the sample-specific peptide library, the tryptic peptides data was generated using data-dependent acquisition as outlined in Parker *et al.*

Briefly, the top 20 most intense MS1 precursors (collected between 360 and 1460 m/z for 250 msec) with charge
states between 2 and 5 were selected for MS2 fragmentation, with a 15 sec exclusion window. Fragment MS2 ions were collected for 100 ms across a 50–2000 \( m/z \) range. Raw MS spectra were converted to mzML using AB Sciex converter (v1.3) and subsequently converted to mzXML using msconvert (ProteoWizard, v3.04.238). They were then searched parallelly using OMSSA and X!Tandem algorithms against UniProt database of human appended with decoys (Human database, March 2016). Search engine results were then converted to pepXML format using omssa2pepXML (v2.1.9) and Tandem2XML (v4.6.0). Peptide spectral match probability scoring was modeled in PeptideProphet (v4.6.0), and the resulting interact.pepXML files of the two search engines were combined in iProphet (v4.6.0). A Peptide assay library was generated with SpectraST (v4.0) from the identified peptides with a Peptide Prophet probability > 0.95. The resulting spectrast .splib file was submitted as input to the custom spectrast2spectrast_irt.py converter script that was used to align RT using iRT peptides. Spectral libraries were formatted for OpenSWATH first using the custom script spectrast2tsv.py followed by the OpenSWATH tool ConvertTSVtoTraML. Finally, OpenSwathDecoyGenerator was used to append shuffled decoys to the full assay library.

Each individual sample was analyzed by DIA-MS on the tripleTOF 5600 mass spectrometer in data-independent mode as outlined in Parker et al.\textsuperscript{32}, and Howlenski et al.\textsuperscript{33}. Within DIA-MS, MS1 was collected with 100 \( m/z \) windows across a 400–1200 \( m/z \) range with 0.5 \( m/z \) overlap at either end of a given window. Data were analyzed using OpenSWATH workflow (REF). Briefly, raw intensity data for peptide fragments were extracted from DIA files using the open source openSWATH workflow against the custom-generated library. Target and decoy peptides were then extracted, scored, and analyzed using the mProphet algorithm to determine scoring cut-offs consistent with 1\% FDR. Peak group extraction data from each DIA file was combined using
the “featurealignment” script, which performs data alignment and modeling analysis across an experimental data set. Normalized transition-level data was then processed using the mapDIA software to perform pairwise comparisons between groups at the peptide level.

Functional Network and Computational Analysis.

Detectable protein hierarchies displayed as heatmaps, and principal component analysis (PCA) were created by using ClustVis (https://biit.cs.ut.ee/clustvis/). Volcano plots were created using Prizm 9. Pie chart of Protein annotation through evolutionary relationship (PANTHER) protein classification analysis was created using http://pantherdb.org/geneListAnalysis.do. Data were analyzed using IPA (Ingenuity Pathway Analysis, Qiagen (https://digitalinsights.qiagen.com). Differentially expressed genes (with corresponding fold-changes and p values) were incorporated in canonical pathways and upstream regulators analyses and were used to generate diagrams.

Statistical analysis

Experimental data were analyzed using GraphPad Prism (GraphPad Software). One-way ANOVA with Tukey’s multiple comparison test was performed in case of 3 groups comparisons. Two-tailed unpaired Student’s t-tests were used in two-group comparisons. Results are shown as means ± standard errors of the mean (SEM). Degree of significance between groups is represented as follows: *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. A p-value lower than 0.05 was considered significant.

RESULTS

Proteome signatures of OPN-deficient and GA-stimulated macrophages
We previously found that GA stimulation upregulates OPN expression in BMMΦ, promoting an anti-inflammatory macrophage phenotype, whereas OPN deletion in BMMΦs results in pro-inflammatory phenotypes \(^6\). We explored \textit{in vitro} molecular network effects of OPN deficiency versus GA-mediated OPN induction in BMMΦ using applied MS-based quantitative proteomics profiling on protein extracts from OPN\(^{KO}\) versus GA-stimulated BMMΦ compared to untreated wild type (WT) control BMMΦ. The scheme of the experimental procedure, which encompasses biochemical and MS analysis, data curation, and profiling followed by data validation, is outlined in Figure 1A. In brief, bone marrow was isolated from WT or OPN\(^{KO}\) mice and cultured for 7 days in MCSF-enriched media to differentiate into macrophages. On day 6, one group of WT cells underwent overnight treatment with GA. On day 7, cells were collected and pelleted, processed for quantitative proteomic and bioinformatics analyses, and followed by immunocytochemical (ICC) and Western blot validation experiments.

Mass spectrometry analysis identified 2505 peptides corresponding to 631 DEPs amongst the three BMMΦ cell groups (Supplemental Table S2). Of those, 249 proteins were significantly altered exceeding our 1.2-fold change cutoff (Table 1). In OPN-deficient (OPN\(^{KO}\)) versus WT BMMΦ, 66 proteins were upregulated while 16 were downregulated. In GA-treated compared to WT BMMΦ, 56 proteins were upregulated and only 2 proteins were significantly downregulated. DEPs numbers were further enriched in comparing between GA-stimulated and OPN\(^{KO}\) BMMΦ groups, in which 84 proteins were upregulated and 25 were downregulated (Table 1). A heatmap with hierarchical clustering analysis of the most abundant up (red)- and down (blue)-regulated DEPs across three experimental groups demonstrated a distinct cluster profile for each group (Figure 1B). Prediction ellipses by principal component and Venn diagram analyses showed that each
group has a discrete protein expression profile, and notably the OPN\textsuperscript{KO} group had the least overlapping profile compared to the GA-treated BMMΦ group (Figure 1C-D).

**Top DEPs in OPN-deficient macrophages**

Among the top 10 DEPs in OPN\textsuperscript{KO} vs. WT BMMΦ, we focused on the 2 most significantly downregulated proteins (Figure 1E). These include the ubiquitin carboxyl-terminal hydrolase isozyme 1 (UCHL1; 2.86-fold downregulation), a deubiquitinating enzyme of the UPS system with an important role in maintaining ubiquitin homeostasis and protein synthesis turnover \textsuperscript{34}; Heme oxygenase 1 (HMOX1; 1.53-fold downregulation), a heme degrading enzyme that produces antioxidant and anti-inflammatory compounds \textsuperscript{35}. The Volcano plot in (Figure 1E) highlights additional downregulated proteins that include the 60S ribosomal proteins RL3, cellular nucleic acid-binding protein (CNBP), and Farnesyl pyrophosphate synthase (FPPS). These data indicate that macrophages lacking OPN exhibit deficiencies in proteins involved in ubiquitin-proteosome system, oxidative stress, inflammation, and translation.

Conversely, among the top 10 DEPs in OPN\textsuperscript{KO} vs. WT BMMΦ, we detected 7 upregulated proteins: including signaling lymphocytic activation molecule-associated protein (SAP), fatty acid binding protein 5 (FABP5), DExD-Box Helicase 39B (DX39B), gamma-interferon-inducible lysosomal thiol reductase (GILT), palmitoyl-protein thioesterase 1 (PPT1), proteoglycan 2 (PRG2), and cyclooxygenase (COX2) (Figure 1E). These findings implicate the involvement of OPN in lysosomal organization, mitochondrial functions, oxidative stress, as well as metabolic and proteolytic responses in BMMΦ.

**Top DEPs in GA-stimulated versus WT and OPN\textsuperscript{KO} macrophages**
In GA-treated macrophages, the top 10 DEPs highlight the histone H3.2 variant as markedly downregulated by 2.64- and 2.41-folds in GA-stimulated macrophages compared to WT and OPN$^{KO}$ macrophages, respectively (Figure 1F-G heatmaps). This histone protein is a DNA replication-dependent nucleosome assembly protein, associated with replication and/or cell division $^{36, 37}$. Volcano plot analysis further show that low-density lipoprotein receptor-related protein 1 (LRP1), which is involved in the positive regulation of lysosomal protein catabolic process and cell death $^{38, 39}$, was downregulated by GA treatment vs. WT controls (1.32-folds; Figure 1F). These results suggest that GA can reverse detrimental lysosomal catabolism and cell death-mediated signaling. Additionally, two other proteins, Lyz2 (involved in cytolysis) and SDHA (a mitochondrial electron transport protein) were downregulated following GA stimulation compared to OPN$^{KO}$ BMMΦ (Figure 1G, J).

Notably, as described in Table 1 and Figure 1F and 1G volcano plots, other important proteins were upregulated in macrophages by GA stimulation, including the scavenger receptors CD36 and CD68, which are involved in facilitating uptake and clearance of Aβ and were also previously shown to increase expression following GA stimulation $^{6, 19, 20}$. Further, several anti-inflammatory and antioxidant proteins were upregulated by GA treatments. These include UCHL1, superoxide dismutase C (SODC), HMOX1, troponin I3 (TNNI3), Y-box binding protein 1 (YBox1), which negatively regulates of apoptotic processes and promotes cell proliferation, and ribosomal proteins like RL36 (Figure 1F-G, Table 1). Importantly, the GA-influenced topmost upregulated proteins were anti-inflammatory and antioxidant-related proteins (Table 1). Taken together, GA-immunomodulation downregulated lysosomal and apoptotic cell death-related proteins while upregulating anti-inflammatory and antioxidant proteins, at least in part, via increased OPN production in macrophages.
**Functional and canonical pathways in OPN-deficient and GA-stimulated macrophages**

What are the leading biological pathways that OPN deficiency triggers in macrophages? To address this question, we studied the relative distribution of functionally classified DEPs in OPN-deficient and GA-immunomodulated BMMΦ. Pie charts based on the PANTHER functional classification, display the proportions between the 19 PANTHER clusters for each OPN KO vs. WT as well as the GA vs. OPN KO BMMΦ (Figure 2A). When we compared these 2 pie charts, the most substantial difference occurred in the ‘translational protein’ class with 6.6-fold differences, upregulated from 2.7% in OPN KO to 18% in GA-treated BMMΦ. While the most notable protein cluster changes in OPN KO BMMΦ were related to metabolic and protein modifying enzymes in GA-treated BMMΦ, the prominent clusters were related to metabolites, translational and cytoskeletal proteins. Further comparison between the pie clusters of GA vs. OPN KO BMMΦ reveal more clusters related to defense immunity, chromatin binding, and chaperone and cell adhesion class proteins; while fewer clusters related to intercellular signaling, extracellular matrix, and transmembrane signal receptor following GA treatment in macrophages (Figure 2A; Figure S1A).

Next, ingenuity pathway analysis (IPA) identified the principal canonical pathways and downstream signaling in OPN-depleted and GA-stimulated macrophages (Figure 2B). A top altered pathway that was inhibited in OPN KO BMMΦ while activated when GA-stimulated was the EIF2 signaling (Figure 2B and S1B), which is essential for most forms of translation initiation and protein synthesis. Another top altered pathway that was activated in OPN KO and conversely inhibited in GA-stimulated BMMΦ was related to oxidative phosphorylation (Figure 2B, S1C). Oxidative phosphorylation is a metabolic pathway in mitochondria that is associated
with cellular proliferation. Protein Interaction Network Extractor (PINE) analysis (Figure S1A) displays the top 5 canonical ontology networks that were identified across the experimental macrophage groups. In alignment with our aforementioned results, the most significantly altered signaling pathways were related to the innate immune system, translation, and translation initiation.

To gain a deeper understanding of the biological pathways that are altered following GA stimulation in macrophages, we conducted IPA for GA vs. OPN\textsuperscript{KO} BMMΦ (Figure 2C-E). Top activated biological pathways were related to cell survival, cell viability (Z scores = 3.54–4.08) as well as microglia/macrophage activation, and protein synthesis (Z scores = 2.21–1.40). In contrast, top inhibited functions were necrosis (Z-score = -3.637), apoptosis (Z-score = -1.655), and synthesis of reactive oxygen species (ROS; Z score = -1.538) (Figure 2C). The top interconnecting molecular networks are displayed in Figure 2D. Opposite IPA functional directions of inhibited cell survival, viability, and macrophage activation and activation of necrotic, apoptotic, and ROS markers were detected for OPN\textsuperscript{KO} vs. GA-treated BMMΦ (not shown). These results were further validated by gene ontology (GO) analysis (Tables 2 and Table 3). Next, we identified the specific upstream regulators across all three comparison groups (Figure 2E-G). Previous literature supports IL-4 as one of the top upstream activated regulators in GA-treated BMMΦ (Z-score = 1.472), promoting an anti-inflammatory phenotype in macrophages. IL-10 is another anti-inflammatory cytokine that was a top inhibited upstream regulator of OPN\textsuperscript{KO} BMMΦ (Z-score = -1.595), which supports a pro-inflammatory phenotype for OPN-deficient macrophages.

Overall, the combined canonical pathway and network analyses suggest that proteins associated with translation, protein synthesis, metabolism, and apoptosis were oppositely regulated in OPN-deficient and GA-stimulated macrophage, emphasizing their role in cellular homeostasis.
UCHL1 protein is expressed in BMMΦ and downregulated in OPN$^{KO}$ macrophages.

Our proteomics data identified UCHL1 as the most downregulated protein in OPN-deficient BMMΦ (Figure 1E and Table 1) and most upregulated protein upon GA stimulation compared to OPN$^{KO}$ macrophages (Figure 1G and Table 1). We confirmed these findings by immunocytochemistry and Western blot analyses. These studies revealed that UCHL1 was expressed by CD36-positive BMMΦ cells (Figure 3A), and concomitant with enhanced OPN expression, UCHL1 was 2.4-fold upregulated following GA stimulation (Figure 3A-D and Figure 3E-G). Furthermore, along with OPN deficiency in macrophages, UCHL1 expression was absent in OPN$^{KO}$ BMMΦ cultures. To explore a possible interaction between OPN and UCHL1 proteins, we performed a co-immunoprecipitation that pulled down both OPN and UCHL1 (Figure 3H). These results suggest that OPN and UCHL1 may interact with each other in a protein complex. In support of this, we identified UCHL1 expression in BMMΦ and found that UCHL1 expression is OPN-dependent.

OPN deficiency upregulates lysosomal and mitochondrial markers in macrophages

Given that the proteomic analyses indicate marked changes in translation, lysosomal, and mitochondrial proteins in OPN$^{KO}$ and in GA-stimulated BMMΦ (Figure 4A-F), we aimed to further verify this data by immunolabeling macrophages with antibodies against late lysosomal marker (Lamp1) and mitochondrial markers (cytochrome C—CytC and ATP-β; Figure 4G). ICC quantification in OPN$^{KO}$ BMMΦ showed significant increases in the expression of CytC (1.5-fold), ATP-β (2.2-fold), and Lamp1 (1.8-fold; Figure 4H-J). In contrary, Lamp1, mitochondrial CytC, and ATP-β proteins were downregulated in GA-treated BMMΦ. These data were further
confirmed via Western blot analysis (Figure 4K-N). Application with an oxidative phosphorylation (OXPHOS) mitochondrial-targeted cocktail resulted in the presence of three protein bands from BMMΦ cell lysates: the ATP synthase complex V (ATP5A), ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), and the mitochondrial encoded cytochrome c oxidase I (MTCO1) (Figure 4K). The OXPHOS mitochondrial-targeted cocktail contained antibodies against 5 different subunits of mitochondrial chain (CI-CV) and added CytC antibody (with no overlap with the molecular weight of OXPHOS proteins). Quantification of the two additional mitochondrial OXPHOS proteins, ATP5A and MTCO1, indeed showed increased expression in the OPN$^{KO}$ and reductions in GA-stimulated macrophages (Figure 4L-N).

Collectively, OPN deficiency was associated with lysosomal and mitochondrial imbalance in BMMΦ, suggesting a role for OPN-primed UCHL1 in regulating mitochondrial functions and cellular homeostasis. Combined with our bioinformatics analysis, we revealed that this dual OPN-UCHL1 deficiency is associated with oxidative phosphorylation and mitochondrial dysfunctions in macrophages (Figure S1C).

**Anti-inflammatory and antioxidant profile was upregulated with GA and downregulated in OPN-deficient BMMΦ**

Given that OPN-primed UCHL1 expression was linked to mitochondrial homeostasis and protection, we assessed whether such protective effects in mitochondria were, in part, associated with the expression of heme oxygenase 1 (HMOX1) and the superoxide dismutase (SODC, encoded by the SOD1 gene). We found that HMOX1 and SOD1 are two top upregulated DEPs in GA-stimulated vs. OPN$^{KO}$ BMMΦ, which were downregulated in OPN$^{KO}$ BMMΦ (Figure 1G and Table 1). HMOX1 is a heme degrading enzyme that catalyzes the production of antioxidant and
anti-inflammatory compounds and provides beneficial effects for cellular homeostasis and switching macrophages to an anti-inflammatory phenotype \(^{35,44-46}\). HMOX1 deficiency renders macrophages sensitive to cell death. Our quantitative ICC analysis showed a substantial 2.3-fold reduction of HMOX1 in OPN\(^{KO}\) vs. GA-stimulated BMM\(\Phi\) (Figure 5A-B). SOD1 is another potent antioxidant that has pleiotropic biological functions to counter oxidative stress in mitochondria \(^{47,48}\). We further reaffirmed the proteomics findings of SOD1 in OPN\(^{KO}\) BMM\(\Phi\) by employing ICC analysis (Figure 5A, C). Our Western blot analysis indicated that the expression of both of these antioxidant enzymes (HMOX1 and SOD1) were upregulated upon GA stimulation and decreased in OPN\(^{KO}\) BMM\(\Phi\) (Figure 5D-G).

**OPN deficiency in macrophages induces ROS production, mitochondrial membrane damage, and subsequent apoptosis**

We sought to determine whether GA stimulation vs. OPN deficiency in macrophages leads to decrease oxidative stress. Indeed, IPA revealed that GA-stimulated vs. OPN-deficient BMM\(\Phi\) had inhibited the synthesis and production of ROS as well as reduced necrosis and apoptosis pathways (Figure 2C, D). To directly evaluate ROS production, we further employed fluorescence dye-based detection method using dihydroethidium (DHE) staining. As shown in Figure 6A, ROS accumulation was much stronger in OPN\(^{KO}\) than in WT BMM\(\Phi\). This effect was reversed upon GA treatment. Quantitative analysis of ROS (DHE)-immunoreactive area (Figure 6B) showed 2-fold increase in ROS levels in OPN\(^{KO}\) BMM\(\Phi\), whereas GA treatment reduced it by 1.3-fold. Additionally, ICC labeling with annexin-V antibody (an apoptotic marker), demonstrated 2.4-fold increase in OPN-deficient BMM\(\Phi\) that was decreased following GA stimulation (Figure 6C-D).
Finally, to determine levels of mitochondrial membrane potential (MMP) in BMMΦ, we employed Mitotracker-red assay on live macrophage cells. The MMP of healthy cells is relatively high and show red fluorescence, while MMP of apoptotic cells is decreased and show diminished fluorescence (Figure 6E-F). The red fluorescence of GA-treated cells did not significantly change, implying preserved mitochondrial membranes after GA treatment. By contrast, in OPN\textsuperscript{KO} vs. WT macrophages, the ratio of red fluorescence was diminished by 2.4-fold, indicative of impaired mitochondrial membranes in cells that undergo apoptosis. In conclusion, OPN deficiency in macrophages induces ROS production, mitochondrial membrane damage, and subsequent apoptosis. In contrary GA treatment decreased ROS, promoted MMP integrity.

Figure 7A-B illustration summarizes the effects of OPN-deficiency as compared with GA-stimulation on phenotypes of BMMΦ. We found that OPN deficiency perturbs cellular homeostasis in macrophages through increased ROS production, and lysosome-mitochondria-mediated apoptosis, whereas GA immunomodulation restores cellular homeostasis by markedly increasing OPN and UCHL1 expression, inducing anti-inflammatory phenotype and cell survival, reducing ROS production and mitochondrial dysfunction.

Discussion

In this study, we identified a novel role for OPN in macrophages related to UPS and proteostasis, employing both OPN-deletion loss-of-function and GA stimulation gain-of-function in vitro paradigms. Proteomics profiling by mass spectrometry analysis detected 630 DEPs when comparing between OPN\textsuperscript{KO}, GA drug-stimulated, and WT macrophages. OPN deletion in macrophages induced activation of pro-inflammatory, mitochondrial dysfunction, apoptotic, and
lysosomal signaling-related pathways and inhibition of UPS, translation, and antioxidant-related processes, leading to disrupted cellular homeostasis. Notably, GA stimulation reversed most of these adverse pathways through induction of OPN expression in macrophages, restoring an anti-inflammatory phenotype and macrophage proteostasis. These data were further confirmed via Western blot and immunocytochemistry analyses, as well as immunoprecipitation and mitochondrial tracing assays. Overall, this study provides substantial evidence for the essential role of OPN in maintaining macrophage homeostasis.

One of the most notable findings revealed a direct connection between OPN and UCHL1 in macrophages. UCHL1 was the most significantly downregulated protein in OPN-deficient macrophages. While UCHL1 is recognized as a neuronal-specific protein, here, we demonstrate UCHL1 expression also in peripheral macrophages. Importantly, our finding that UCHL1 was downregulated upon OPN deficiency and upregulated by OPN enhancement through GA stimulation indicates that UCHL1 expression is OPN-dependent. Moreover, we show that OPN interacts with UCHL1 in protein complex by co-immunoprecipitation, further affirming that UCHL1 expression is tightly regulated by OPN. Two recent studies indicating that OPN and UCHL1 were intracellularly localized to mitochondria, align with our results, suggesting a potential interaction in the mitochondria to regulate oxidative reaction and maintain mitochondrial function.

UCHL1 is a deubiquitinating enzyme (DUB), a key cellular proteolytic enzyme responsible for the removal of oxidized and/or damaged proteins, and a prominent component of the UPS. The UPS plays a key role in preserving cellular homeostasis through the regulation of key cellular processes such as transcription, protein quality control and degradation, cell stress responses, cell cycle progression, and apoptosis; such pleotropic cellular functions make UPS
as a central regulator of diverse cellular processes. A recent study showed that DUBs inhibition in macrophages resulted in acute perturbation of cellular ubiquitin homeostasis and induction of oxidative stress through ROS \(^{57}\), indicating that DUBs are responsible for mitigating oxidative damage and maintaining cellular homeostasis in macrophage. Furthermore, crosstalk between the UPS and mitochondrial proteins is reported to play a major role in cellular homeostasis \(^{58-60}\).

Indeed, a lower level of UCHL1 has been detected in apoptotic cells with severe loss of the mitochondrial membrane potential \(^{61}\). Another report found that UCHL1 inhibition increased neuronal and apoptotic cell death \(^{62}\) and UCHL1 deficiency in skeletal muscle cells resulted in altered mitochondrial oxidative phosphorylation \(^{59}\). Accordingly, in the current study, we found that UCHL1 deficiency in OPN\(^{\text{KO}}\) macrophages was associated with harmful cellular processes. These processes include lower expression of potent antioxidant enzymes, such as SOD1 and HMOX1 \(^{47,63}\), along with increased ROS production as well as elevated lysosomal, mitochondrial, and apoptotic proteins as lysozyme C-2, ATP-synthase subunits, cathepsins, and cytochrome C and B subunits, and annexin-V. Moreover, there was also a severe loss of mitochondrial membrane potential measured in UCHL1-deficient OPN\(^{\text{KO}}\) macrophages. Therefore, our data support a role for the UCHL1-OPN axis in regulation of mitochondrial oxidative stress in macrophages.

A summary of our hypothesis on OPN-deficiency and OPN-induction effects in macrophages is illustrated in Figure 7. Following lysosomal membrane permeabilization (LMP), lysosomal proteases, such as cathepsins, are released into the cytosol and mediate cytochrome-C release and caspase activation; this ultimately triggers mitochondria-mediated apoptotic cascade \(^{64}\). During apoptosis, caspase-dependent cleavage of translation-initiation factors leads to the inhibition of protein synthesis and translational shutdown \(^{65}\). OPN also influenced lysosomal protein degradation and protease activation as it triggers lamp1 production. The current study
reveals that loss of OPN resulted in reduced expression of UCHL1 that ultimately triggered apoptotic markers. As mentioned above, lower UCHL1 levels were detected in apoptotic cells with severe loss of mitochondrial membrane potential. The collective data clearly indicate that OPN-UCHL1 signaling can maintain cellular homeostasis by controlling apoptotic processes. We also found that OPN was involved in the regulation of protein synthesis and translation. Since UCHL1 expression in macrophages is found to be regulated by OPN, among other UPS proteins, OPN has crucial role in protein translation and turnover, and cellular homeostasis. This result is supported by a previous report that found OPN to regulate the homeostasis and function of natural killer cells.

Our data also revealed that OPN deficient macrophages were exhibiting a pro-inflammatory phenotype. In fact, our data indicate that OPN may inhibit inducible nitric oxide synthase, thus inhibiting nitric oxide production in macrophages leading to suppressed oxidative stress and inflammation. Indeed, we recently reported the potential anti-inflammatory effects of OPN as it can resolve inflammatory cascades through promoting macrophages polarization towards their anti-inflammatory and highly phagocytic phenotypes. Similarly, anti-apoptotic functions of OPN have been previously reported as inhibiting OPN by monoclonal antibody dramatically promoted the apoptosis of activated T cells. These independent studies suggest the antioxidant, anti-inflammatory, and anti-apoptotic effects of OPN in different immune cells.

Importantly, our current study also revealed the key OPN-associated functions of GA, as GA treatment not only increased the OPN production in macrophages, but it also reversed the expression of almost all proteins influenced by OPN deficiency. As an immunomodulator, GA has been reported to influence pleiotropic cellular functions in multiple immune cells such as macrophages, microglia, and T cells both in vivo and in vitro disease models of neurodegenerative...
disorders including AD. We previously reported that administration of GA to transgenic murine models of AD enhanced brain recruitment of blood-borne monocytes, which were involved in the clearance and degradation of Aβ plaques, reduced neuroinflammation, and induced production of neurotrophic factors, resulting in significant neuroprotection and cognitive improvement \(^{19, 21, 69-71}\). In addition, GA-influenced neuroprotection against AD-related pathology in transgenic mice model was associated with the induction of a phenotypic shift in microglia, which typically expressing IGF-1. Such microglia were involved in uptaking cerebral Aβ plaques and regulating detrimental chronic inflammation \(^{24}\). These effects of GA on immune cells leading to neuroprotection against AD pathogenesis is, at least in part, associated with increased production of OPN in immune cells influencing their anti-inflammatory and neuroprotective functions. In fact, a recent report demonstrated that OPN promoted neuroprotection by inhibiting NLRP3 inflammasome and inflammatory microglial activation following focal ischemic brain injury in mice as well as LPS-stimulated rat primary microglia \(^{72}\), further affirming the strong anti-inflammatory potential of OPN.

**CONCLUSIONS**

This study reveals that OPN deficiency in macrophages is associated with homeostatic imbalance by affecting multiple downstream signaling pathways such as ROS production, oxidative stress, mitochondrial-related dysfunctions, and subsequent apoptosis. Reciprocally, the induction of apoptosis leads to a substantial inhibition of protein synthesis and translation, which was a feature of OPN\(^{\text{KO}}\) macrophages. On the other hand, GA appears to maintain cellular homeostasis through the induction of OPN production, which in turn regulates UPS, protein recycling, and protein synthesis. Our proteomics data in conjunction with bioinformatics analysis offer the molecular
insights into phenotypic changes that occur in OPN-deficient and GA-stimulated macrophages.

We revealed the importance of OPN modulation in cellular proteostasis and ROS production. On aggregate, this study indicates that targeting of UPS and lysosome-mitochondrial pathways may hold therapeutic potential for enhanced macrophage function and immunomodulation therapies.
ACKNOWLEDGEMENTS

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

AUTHOR’S CONTRIBUTIONS


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COMPETING INTERESTS

None.
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Figure legends

**Figure 1. Proteome signatures of OPN-deficient and GA-stimulated macrophages.** (A) Schematic illustration of experimental workflow for the proteomics profiling and validation studies. (B) Heatmap with hierarchical clustering analysis of the most abundant up (red)- and down (blue)-regulated DEPs across three experimental groups. (C) Hierarchical clustering of the top up and downregulated 225 DEPs by principal component analysis (PCA performed with ClustVis). Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. Prediction ellipses are such that with probability of 0.95, a new observation from the same group will fall inside the ellipse. (D) Venn diagram showing the number and percentage (%) of overlapping DEPs according to statistical significance ($p<0.05$) and 1.2-fold change threshold criteria in the 3 analyzed groups. The number of common DEPs between pair groups is also shown. (E-G) Heatmaps and volcano plots of the top 10 most differentially expressed proteins (FDR < 0.05, ranked by FC) generated by differential analysis of the proteome profiles between (E) OPN$^{KO}$ vs WT, (F) WT vs GA and (G) GA vs OPN$^{KO}$. Significantly upregulated proteins are shown as red dots and down-regulated proteins as green dots. The proteins with fold change $>$1.2 and p value $<$0.05 were considered significantly differentially expressed.

**Figure 2. Functional and canonical pathways in OPN-deficient and GA-stimulated macrophages.** (A) PANTHER analysis Pie chart by Protein Class distribution in percentages showing fraction and percentage of significant DEPs (up- or downregulated proteins) grouped by protein class category in the OPN$^{KO}$ vs WT and GA vs OPN$^{KO}$. (B) IPA was performed to identify canonical pathways, most differentially regulated pathways in all 3 groups. (C) IPA of top up and down-regulated biological functions in GA vs OPN$^{KO}$ group and (D) network displaying 5
important biological functions and their interactions with each other. (E) IPA of upstream regulator analysis in all 3 groups and network displays of upstream regulators for (F) IL-10 in OPN\textsuperscript{KO} vs WT and (G) IL4 in GA vs OPN\textsuperscript{KO} (G).

**Figure 3. UCHL1 protein is expressed in BMMΦ and downregulated in OPN\textsuperscript{KO} macrophages.** (A) Representative fluorescent micrographs of BMMΦ cells immunostained for OPN (red), UCHL1 (green), CD36 (white) and DAPI (blue for nuclei of cells). Quantitative analysis of immunoreactive (IR) area of (B) OPN, (C) UCHL1 and (D) CD36 expression in BMMΦs. (E) Western blot analysis of cell lysates and quantitative analysis of (F) OPN and (G) UCHL1 bands. (H) Co-immunoprecipitation of OPN and UCHL1 and analysis by western blots. Immunoprecipitation demonstrates that OPN and UCHL1 interact with each other in protein complex. Individual data points, group means, and SEMs are shown. * p < 0.05, *** p < 0.001, **** p < 0.0001 by one-way ANOVA with Tukey’s post-hoc multiple comparison test.

**Figure 4. OPN deficiency upregulates lysosomal and mitochondrial markers in macrophages.** (A-F) Heatmaps of (A) UPS proteins, (B) 60S ribosomal proteins, (C) 40S ribosomal proteins in GA/WT vs OPN\textsuperscript{KO}/WT, (D) ATP-synthase subunits, (E) cathepsins and (F) cytochrome C and B subunits in OPN\textsuperscript{KO}/WT vs GA/WT. (G) Representative fluorescent images of BMMΦ cells immunostained for Cytochrome C\textsuperscript{+} (green), ATP-β\textsuperscript{+} (white) and Lamp1\textsuperscript{+} (green). (H-J) Quantitative analysis of IR of (H) CytC, (I) ATP-β and (J) Lamp1 expression in BMMΦs. (K) Western blot analysis of cell lysates and quantitative analysis of Oxphos proteins: (L) CytC, (M) ATP5A and (N) MTCO1 bands. Individual data points, group means, and SEMs are shown.
* p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 by one-way ANOVA with Tukey’s post-hoc multiple comparison test.

**Figure 5.** Anti-inflammatory and antioxidant enzymes were downregulated in OPN-deficient BMMΦ. (A) Representative fluorescent micrographs of BMMΦ cells immunostained for HMOX1+ (upper panel), and SOD1+ (lower panel) either untreated or pretreated with GA, or OPNKO. Quantitative analysis of ICC reveals increased level of (B) HMOX1 and (C) SOD1 following GA treatment, but reduced expression in OPNKO cells. (D) Western blot and (E) quantitative analysis of HMOX1 bands from cell pellets. (F) Western blot and (G) quantitative analysis of SOD1 bands from cell pellets. Individual data points, group means, and SEMs are shown. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 by one-way ANOVA with Tukey’s post-hoc multiple comparison test.

**Figure 6.** OPN deficiency induces ROS production and subsequent apoptosis in BMMΦ. (A) Representative fluorescent micrographs of BMMΦ cells stained for DHE and (B) quantitative analysis of ROS/DHE IR area in BMMΦ. (C) Representative fluorescent micrographs of BMMΦ cells immune-stained for Annexin-V and (D) quantitative analysis of Annexin-V IR area in BMMΦ. (E) Representative fluorescent micrographs of BMMΦ cells immune-stained for Mitotracker-red and (F) quantitative analysis of Mitotracker-red IR area in BMMΦ. Individual data points, group means and SEMs are shown. ** p < 0.01, and **** p < 0.0001 by one-way ANOVA with Tukey’s post-hoc multiple comparison test.
Figure 7. OPN/Spp1 deficiency in BMMΦ triggers lysosome-mitochondria-mediated apoptosis accompanied by reduced translation and recycling (A) Illustration summarizing lysosome-mitochondria-mediated apoptosis accompanied by reduced translation and UPS recycling in OPN-deficient macrophages. (B) Illustration of macrophage proteostasis emphasizing up- and downregulated proteins/groups and functions in OPN\textsuperscript{KO} vs GA-stimulated macrophages in vitro. Illustration made with Biorender.com.
Figure 1. Proteome signatures of OPN-deficient and GA-stimulated macrophages.

Figure 1

See above image for figure legend.
Figure 2

See above image for figure legend.
Figure 3. UCHL1 protein is expressed in BMMΦ and downregulated in OPNko macrophages.
Figure 4. OPN deficiency upregulates lysosomal and mitochondrial markers in macrophages.
Figure 5. Anti-inflammatory and antioxidant enzymes were downregulated in OPN-deficient BMMΦ

See above image for figure legend.
Figure 6. OPN deficiency induces ROS production and subsequent apoptosis in BMMΦ

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Figure 7

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Supplementary Files

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