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The role of selenoproteins in neutrophils during inflammation

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Abstract

Polymorphonuclear neutrophils (PMNs)-derived ROS are involved in the regulation of multiple functions of PMNs critical in both inflammation and its timely resolution. Selenium is an essential trace element that functions as a gatekeeper of cellular redox homeostasis in the form of selenoproteins. Despite their well-studied involvement in regulating functions of various immune cells, limited studies have focused on the regulation of selenoproteins in PMN and their associated functions. Ex-vivo treatment of murine primary bone marrow derived PMNs with bacterial endotoxin lipopolysaccharide (LPS) indicated temporal regulation of several selenoprotein genes at the mRNA level. However, only glutathione peroxidase 4 (Gpx4) was significantly upregulated, while Selenof, Selenow, and Gpx1 were significantly downregulated in a temporal manner at the protein level. Exposure of PMNs isolated from $tRNA^{Sec}$ (*Trsp*) $^{fl/fl}$S100A8^{Cre} ($Trsp^{N}$) PMN-specific selenoprotein knockout mice, to the Gram-negative bacterium, *Citrobacter rodentium*, showed decreased bacterial growth, reduced phagocytosis, as well as impaired neutrophil extracellular trap (NET) formation ability, when compared to the wild-type PMNs. Increased extracellular ROS production upon LPS stimulation was also observed in $Trsp^{N}$ PMNs that was associated with upregulation of redox-related genes, including Alox12, Cox2, and iNOS, as well as proinflammatory cytokines such as TNFα and IL-1β. Our data indicate that the inhibition of selenoproteome expression results in alteration of PMN proinflammatory functions, suggesting a potential role of selenoproteins in the continuum of inflammation and resolution.

Graphical Abstract

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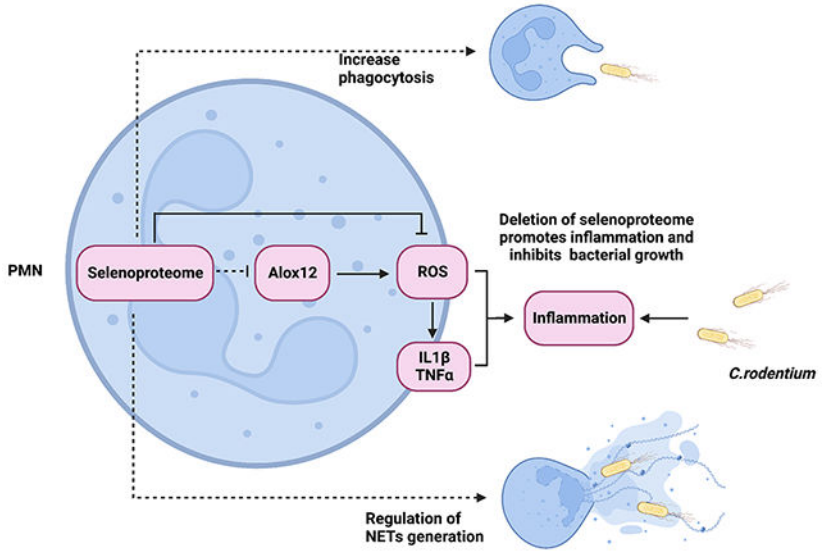

Keywords

PMNs; reactive oxygen species; Gpx4; Alox12; NET

Introduction

Polymorphonuclear neutrophils (PMNs) are innate immune cells that are among the first responders recruited to the site of inflammation[1]. Being an important effector in limiting bacterial infection, their strong bactericidal ability is due, in part, to their ability to generate significant amounts of intracellular and extracellular reactive oxygen species (ROS) through the activation of the membrane-associated nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2)[2,3]. PMN-released ROS directly contribute to the extracellular and intraphagosomal pathogen killing, as well as the activation of redox-dependent signaling pathways, which facilitate the pro-inflammatory functions of PMNs, such as phagocytosis[4], generation of inflammatory cytokines[5], and the release of neutrophil extracellular traps (NETs)[2]. Despite these antimicrobial functions, ROS are also highly reactive and cytotoxic. Excessive ROS production can result in PMN cell death and host tissue damage, which further results in immune cell recruitment and activation in the affected tissue, leading to prolonged inflammation[2,3]. Thus, regulation of ROS concentration in PMNs is of paramount importance to maintain functional antimicrobial functions, while preventing and/or minimizing collateral tissue damage.

Selenium is an essential trace element that exhibits its antioxidant role in the form of the 21st amino acid, selenocysteine, in selenoproteins[6]. In mice, 24 selenoproteins have been identified through bioinformatic approaches following genome sequencing. Some of the most well-studied selenoproteins are known to possess reductase activities against disulfides, oxidized methionine, and hydroperoxides to protect cells from oxidative stress[7,8]. Considering the significant amount of ROS generated in PMNs, it is likely that selenium in the form of selenoproteins could be involved in regulating the function of PMNs. Indeed, a

few studies have shown that selenium deficiency results in abnormal PMN pro-inflammatory functions, including alteration in ROS generation[6], phagocytosis[9], bacterial killing[10–12], and Netosis[13–15]. Several selenoproteins are also found to participate in regulating the function of PMNs. For example, the lipid peroxidation regulator glutathione peroxidase 4 (GPX4) has been found to regulate PMN ferroptosis in lupus patients[16]. Selenoprotein K deletion in mouse PMNs shows a reduction in Ca^{2+} influx and PMN migration ability upon stimulation with KC (also known as CXCL1)[17]. In female animals, PMNs lacking the expression of the thyroid hormone deiodinase 3 (DIO3) also show impaired NOX enzymatic ability[18]. Altogether, these findings implicate the importance of selenoproteome in the regulation of PMN antimicrobial functions. However, many of these studies have utilized selenium diets or whole-body deletion of specific selenoproteins. Since PMNs are tightly regulated by other cell types in the microenvironment, as seen during bone marrow hematopoietic maturation[1,19], steady state surveillance[1], or inflammation[1], utilizing nonspecific selenium or selenoprotein deprivation models could also contribute to non-PMN-specific effects.

In this study, we generated PMN-specific selenoproteome knockout mice ($Trsp^{N}$), by crossing the $tRNA^{Sec}$ (*Trsp*)$^{fl/fl}$ mice with mice carrying *S100a8*^{Cre}. PMNs isolated from the $Trsp^{N}$ mice exhibited increased inhibition of bacterial growth, reduction in phagocytosis, and impaired NET generation against *Citrobacter rodentium* (*C. rodentium*). Diogene assay showed that the $Trsp^{N}$ PMNs have an earlier onset and higher generation of extracellular ROS production upon bacterial endotoxin lipopolysaccharide (LPS) stimulation, associated with significant upregulation of redox-sensitive signaling pathways. Together, the present study suggests that the inhibition of selenoproteome expression results in the alteration of PMN proinflammatory functions, demonstrating a potential role of the neutrophilic selenoproteome in the continuum of inflammation and resolution.

Method

Mice

C57BL/6 mice carrying floxed $tRNA^{Sec}$ (*Trsp*) allele (*Trsp*$^{fl/fl}$), obtained from Dr. Dolph Hatfield's laboratory (National Institutes of Health, USA)[20], were crossed to mice carrying the S100A8-Cre-GFP (*S100A8*^{Cre}) from Jackson Laboratory (Bar Harbor, ME). Sex and age-matched mice were genotyped for $Trsp^{fl/fl}$ [20] and Cre[21], and TrspWT and $Trsp^{N}$ mice were used for all experiments, with the average age being 3-4 months. All procedures were preapproved by the Institutional Animal Care and Use Committee at Penn State University.

Complete blood count (CBC)

Peripheral blood was collected by penetrating the retro-orbital sinus with a sterile hematocrit capillary tube. Samples were analyzed using Hemavet 950 (Drew Scientific) to enumerate white blood cell (WBC) and neutrophil (NE) counts.

Mouse bone marrow PMNs isolation

Mouse femurs were collected in sterile D-PBS (Corning). After the removal of muscle tissue, the bone marrow cells were than flushed with RPMI 1640 (Corning) containing 10% FBS, 25mM HEPES, 2mM EDTA, and 100nM sodium selenite (Na_2SeO_3), pelleted down

and followed by red blood cell lysis with ACK buffer (0.15M NH_4Cl, 0.01M $KHCO_3$, 0.1mM Na_2EDTA, pH 7.2-7.4). Cells were washed with D-PBS containing 0.5% bovine serum albumin and 2mM EDTA, counted, and PMNs were purified using the MojoSort™ Mouse neutrophil isolation kit as per manufacturer's instructions (Biolegend). Following isolation, PMNs were rested in $CaCl_2$ containing media (RPMI media containing 10% FBS, 2.5mM HEPES, 1mM $CaCl_2$ and 100nM sodium selenite) at 37 °C for 30 min before any further treatment. Purity of isolated PMNs were measured by flow cytometry, with an average of 98 % of Gr-1^+ cells and 95 % of Ly6G^+ cells.

LPS treatment

Following culture in $CaCl_2$ containing media, PMNs were treated with 100ng/ml of LPS from *E.coli* (*Escherichia coli* serotype 0128:B12; MilliporeSigma). At predetermined timepoints post-LPS stimulation, PMNs were spun down at 800*g* to pellet down the suspended PMNs, and further treated with 0.25% Trypsin-EDTA (GIBCO) for 3 min at 37°C to harvest the PMNs. Following a quick incubation in the complete culture media, PMNs were again centrifuged, as above, washed once in PBS, and used in experiments.

mRNA expression analysis

Total RNA from the above prepared PMNs were isolated using Tri-Reagent (Sigma), followed by cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosynthesis). Sybr green qPCR analysis was performed for selenoprotein expression (primers sequences obtained from Hoffmann et. al[22]), 18*S* (forward – 5-CGTTCAGCCACCCGAGATT-3; reverse 5-CGGACATCTAAGGGCATCACA-3), and *Alox12* (forward – 5-CGCGGGGCAAGGAGGAGGAGT-3; reverse – 5-GGGGTTGGCGCCATTGAGGA-3). qRT-PCR was used with commercially available Taqman probes purchased from ThermoFisher, including 18*S* (Hs99999901_s1), *Ptgs2* (Mm004478374_m1), *Nos2* (Mm00440502_m1), *Sod2* (Mm00690588_m1), *Tnf* (Mm00443258_m1), *Il1b* (Mm00434228_m1), and *Gls2* (Mm01164862_m1). The relative expression of selenoproteins was normalized to *Gapdh* according to the $\Delta\Delta CT$ calculation, and further normalized to 18*S* expression. Relative expression of *Alox12* and above mentioned Taqman probe targeting genes were normalized to *Gapdh* and 18*S*, respectively.

Protein isolation and immunoblotting analysis with PMNs

PMNs were lysed with Pierce™ RIPA buffer (ThermoFisher) containing 1% proteinase inhibitor cocktail (Sigma) and 25mM sodium vanadate. Protein samples were isolated as per manufacturer's instructions, followed by protein estimation with Pierce™ BCA Protein Assay (ThermoFisher). Samples were subjected to standard sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western immunoblot. Antibodies for immunoblotting were used as following – rabbit anti-mouse Msrb1 (1:1000, Abclonal), rabbit anti-mouse Sps2 (GeneTex, 1:1000), rabbit anti-mouse Gpx4 (1:1000, abclonal), rabbit anti-mouse Gpx1 (1:1000, abcam), rabbit anti-mouse Gpx1 (1:1000, GeneTex), rabbit anti-mouse Sep15 (Selenof) (1:1000, abcam), rabbit anti-mouse Selenok (1:1000, GeneTex), rabbit anti-mouse Selenon (1:500, Proteintech), rabbit anti-mouse Txnrd1 (1:2000, Proteintech), rabbit anti-mouse Txnrd2 (1:500, Proteintech), rabbit anti-mouse Selenoh (1:1000, abcam), rabbit anti-mouse Selenom (1:1000, Epigenetex), rabbit

anti-mouse Gpx2 (1:1000, Abclonal), rabbit anti-mouse Selenow (1:1000, Rockland), mouse anti-mouse β-actin (1:25000, Fitzgerald), rabbit anti-mouse TNFα (1:1000, Bioss), and rabbit anti-mouse Cox2 (Ptgs2; 1:1000, Cayman). Densitometry analysis was performed using Image J (National Institutes of Health), and target protein expression were normalized to β-actin.

C. rodentium growth assay

Five hundred thousand of the isolated PMNs rested for 30 mins were cultured with $5x10^6$ CFU of *C. rodentium* for 1 h at 37 °C. Samples were cultured in the 5ml polypropylene round-bottom tubes on a 3-D Mini Rocker during incubation to ensure proper mixing of PMNs and bacteria. At the end of the incubation, 10 μl of samples were mixed in 90 μl of sterile Milli-Q water for 10 min for PMN lysis, followed by plating upon serial dilution on the MacConkey Agar plate for 18 h for CFU count. Bacterial numbers in PMN-free culture media were designated as 100 % that was used to calculate relative bacterial growth.

Phagocytosis assay

Five hundred thousand of the isolated and rested PMNs were cultured with $5x10^6$ CFU of PKH26-labeled *C. rodentium* in 12 well plate for 30 min at 37°C, 50 rpm. Following culture, supernatant containing suspension of PMNs were collected, and attached PMNs were treated with 0.25% Trypsin-EDTA for 3 mins at 37°C to facilitate the detachment of PMNs. Samples were incubated with penicillin-streptomycin and 2% FBS containing PBS to remove the extracellular bacteria. After washing, samples were resuspended in 3% FBS containing PBS, treated with Fc block (1:100, BD Bioscience) for 10 min, followed by staining with APC-conjugated Gr-1 (1:100, Biolegend) for PMNs labeling. Samples were washed, fixed in 4% paraformaldehyde (PFA), and analyzed on BD LSR-Fortessa followed by data analysis with BD FlowJo software program (Ashland, OR).

NETosis assay

NETosis measurement by flow cytometry was following the protocol from Gavillet M et.al.[23] Five hundred thousand of the isolated PMNs after resting for 30 min were cultured with or without $5x10^6$ CFU of *C. rodentium* in 12 well plate at 37 °C, 50 rpm for 4 hours. After culturing, PMNs were collected and treated with 0.25% Trypsin-EDTA to collect the bottom-attached PMNs. Following washing with 3% BSA, samples were blocked with 3% BSA for 30 min at 37 °C, washed and incubated with Fc block (1:100, BD Bioscience) for 10 min. Samples were then stained with anti-mouse citrullinated histone-3 antibody (1:100, Cayman) extracellularly for 30 mins, washed once with 3% BSA, and again incubated with Fc block for 10 min, followed by extracellularly stained with Alexa Fluor® 568 conjugated anti-mouse antibody (1:300, Invitrogen), PeCy7-conjugated Ly6G (1:100,BD Bioscience), and Alexa Fluor® 647 Anti-Myeloperoxidase antibody (1:50, Abcam) for 30 mins. After wash, samples were fixed with 4% PFA and analyzed using on BD- LSR-Fortessa followed by data analysis with BD FlowJo Software Program (Ashland, OR).

Diogenes assay

Extracellular superoxide of WT and $Trsp^N$ PMNs were detected using the chemiluminescence-based Diogenes assay (National Diagnostics) as per manufacturer's instructions. Briefly, $5x10^5$ of PMNs in 100 μl was added to the 96 well plate, rested for 30 min in $CaCl_2$ containing media at 37 °C. After 30 min of culture, 100 μl of the Diogenes complete enhancer solution was added to each well, followed by LPS. Luminescence was detected every 5 mins for 5 h at 37 °C in the plate reader equipped with shaking function.

Intracellular lipid ROS measurement

Lipid ROS accumulation in PMNs was measured with BODIPY™ 581/591 C11 (Invitrogen), a dye that shifts from red fluorescence to green fluorescence upon oxidation. Isolated PMNs (500,000) with and without LPS stimulation were washed and stained with 5μM BODIPY in PBS for 30 min. Cells were then trypsinized and washed once with PBS, followed by flow cytometry analysis on BD- LSR-Fortessa. Data were analyzed with BD FlowJo Software Program for the mean fluorescence intensity (MFI) of the green fluorescence (510 nm).

Statistical analysis

Graph and statistical analysis were performed using GraphPad-Prism9. Detail specific statistical analysis for each experiment is provided in the figure legends. Generally, p-values were analyzed with one-way or two-way ANOVA using multiple comparison, paired (Wilcoxon test) or unpaired-t test (Mann-Whitney). Significance was set as $p<0.05$. Error bars represent mean ±SEM of independent PMN isolations from mice.

Results

Selenoprotein mRNA and protein expression are differentially regulated in PMNs upon LPS stimulation.

Early LPS stimulation primes PMN activation of multiple proinflammatory functions.[24] We thus investigated the transcription of selenoproteome in response to short term LPS stimulation. The transcript levels of 24 selenoproteins were measured in unstimulated cells and compared to those PMNs that were stimulated with LPS for 30 min. These cells were cultured in a selenium-replete media of 100 nM. As shown in Figure 1A, *Selenor* (*Msrb1*) appeared to be the most highly expressed selenoprotein mRNA in naive PMNs followed by *Sps2* and *Gpx4*. Stimulation of these cells with LPS resulted in significant upregulation of multiple selenoproteins, including *Selenor*, *Selenof*, *Txnrd1*, *Selenot*, *Selenoo*, *Txnrd2*, *Txnrd3*, and *Dio2*, while *Dio3* and *Gpx2* were significantly downregulated in activated PMNs (Fig. 1B). *Selenov* and *Dio1* were below the limit of detection. In addition, expression of *Sbp2* in PMNs was also significantly upregulated upon LPS stimulation, suggesting the need of selenoprotein synthesis during PMN activation. We also examined the temporal expression of multiple selenoproteins at the protein level in PMNs post-LPS stimulation (Fig. 1C, 1D). 1h and 4h post-LPS stimulation timepoints are chosen as we observed ROS generation in PMNs initiated at 1h and peak at 4h, as shown in later figure. Compared to all the other selenoproteins, Gpx4 showed sustained increased expression in

PMNs upon LPS stimulation. Gpx4 is known inhibitor for ferroptosis, thus, the increased in Gpx4 expression was likely in response to the increased lipid peroxidation in LPS-activated PMNs. Indeed, we observed significantly increased lipid ROS accumulation in PMNs under 30 min and 1h post-LPS stimulation (Fig. 1E), consistence to the upregulation of Gpx4. Protein expression of Selenof and Selenow showed a transient increase initially at 1 h post-LPS stimulation; while their expression was significantly downregulated at 4 h, suggesting a potential role in the early activation of PMNs. Interestingly, Gpx1 expression was significantly downregulated throughout the inflammatory phase. These results suggest that selenoprotein expression is differentially regulated in PMNs subjected to activation by LPS.

Generation of PMN-specific selenoprotein knockout mice.

To further investigate the role of selenoproteome in PMNs, we generated PMN-specific selenoprotein knockout mice ($Trsp^N$), produced by genetic deletion of the floxed *Trsp* gene [25], in granulocytic cells, predominantly PMNs, expressing Cre recombinase driven by S100A8 promoter. As shown in Fig. 2A, PMNs isolated from the $Trsp^N$ mice showed significant reduction of Gpx1 and Txnrd1 protein expression. Due to the heterozygous Cre insertion, residual selenoproteins could still be detected in the $Trsp^N$ PMNs. As a control for cell-specific deletion within other innate immune cells, expression of Gpx1 and Txnrd1 in the bone marrow-derived macrophages (BMDMs) isolated from $Trsp^N$ mice showed no changes in the expression pattern mimicking those in the WT control cells, suggesting greater level of PMN-specific deletion of selenoproteins in the $Trsp^N$ mice (Fig. 2B). Of note, the $Trsp^N$ mice were healthy, with comparable circulating white blood cells (WBC) and PMN numbers, despite a slight reduction in the body weight of the male mice compared to the WT counterparts (Fig. 2C).

Anti-bacterial activity of $Trsp^N$ PMNs against *C. rodentium*.

Selenium deficiency is known to affect PMN functions during inflammation[12,26,27]. Multiple selenoproteins have also been found to be associated with PMN proinflammatory functions[4,12,15,17]. Therefore, we examined if the deletion of entire selenoproteome would result in the alteration of PMN proinflammatory functions. We first looked at the inhibition of bacteria growth ability of WT and $Trsp^N$ PMNs. Using the murine enteropathogenic bacteria, *C. rodentium* that causes gastrointestinal inflammation[28], we observed $Trsp^N$ PMNs has a greater ability in suppressing *C. rodentium* growth at 1h compared to bacteria cultured with WT PMNs (Fig. 3A). Interestingly, the phagocytosis ability of $Trsp^N$ PMNs was also significantly reduced (Fig. 3B), suggesting existence of additional mechanisms for bacterial growth inhibition in $Trsp^N$ PMNs. Furthermore, given that host selenium status is known to affect NETs production[14,15], we investigated NETosis events in $Trsp^N$ PMNs. Flow cytometric analysis, Ly6G staining, and levels of extracellular citrullinated histone 3 (H3Cit) and myeloperoxidase (MPO) were used to evaluate NETs. WT PMNs showed a significant increase in the NET production when exposed to *C. rodentium* for 4 h. However, co-culture with *C. rodentium* was not able to induce an increase in NET generation in the $Trsp^N$ PMNs when compared to WT PMNs (Fig. 3C). Together, these results suggested that the lack of selenoprotein expression in PMNs results in the alteration of antibacterial activity against *C. rodentium* that was independent of phagocytosis and NETosis.

Increased ROS generation and upregulation of proinflammatory genes.

The role of selenoproteins in PMNs following activation with an emphasis on ROS that is reported to regulate multiple PMN functions was further probed. Since we observed alteration of antibacterial functions of $Trsp^N$ PMNs, and given the antioxidant function of selenoproteins, we examined if extracellular ROS generation was affected in $Trsp^N$ PMNs using the Diogenes chemiluminescence assay. As shown in Figure 4A, $Trsp^N$ PMN stimulated with LPS showed varied temporal kinetics of ROS production along with increased magnitude in $Trsp^N$ PMN compared to TrspWT PMNs. The ROS generated from $Trsp^N$ PMNs peaked at around 3 h post LPS stimulation, while the WT PMNs reached the highest ROS production at 4 h (Fig. 4A). At the peak, ROS production was also significantly higher in the $Trsp^N$ PMNs compared to the WT control. These results suggested that selenoproteins may help in buffering the ROS production in PMNs. Respiratory burst results from the activation of NOX and multiple ROS-producing enzymes such as the iron-containing enzyme, arachidonate acid 12-lipoxygenase (Alox12) that catalyzes the dioxygenation of polyunsaturated fatty acids to produce lipid hydroperoxides.[29,30] We observed a significant upregulation of *alox12* mRNA expression in $Trsp^N$ PMNs at 30 min of LPS stimulation, but not at 3 h, suggesting Alox12 may contribute to the early ROS generation of the $Trsp^N$ PMNs, to initiate the respiratory burst (Fig.4B). We also examined downstream proinflammatory targets and found that *Pghs2* (*Cox2*) and *Inos* (*Nos2*) were upregulated in $Trsp^N$ PMNs at 3 h post LPS stimulation, which may also contribute to the respiratory burst in the $Trsp^N$ PMNs (Fig. 4B, 4C). We further tested the expression of proinflammatory cytokines *Il1b* and TNFα and observed a significant upregulation of both proinflammatory cytokines (Fig. 4C, 4D). Additionally, the expression of superoxide dismutase 2 (*Sod2*) and Nrf2 downstream target glutaminase 2 (*Gls2*) were also upregulated[31], suggesting increased oxidative stress in the $Trsp^N$ PMNs following LPS stimulation (Fig. 4C). These results suggest that deletion of the selenoproteome in PMNs results in increased ROS production, through the upregulation of multiple oxidative stress-related genes, potentially leading to increased inflammation.

Discussion

Respiratory burst in PMNs plays a major role during the inflammatory process while also contributing to host tissue damage if dysregulated. Selenium and selenoproteins are involved in regulating immunity through the modulation of the redox status in various immune cells[32–35]. However, limited studies have focused on selenoprotein regulation in PMNs, due to the lack of animal models for *in vivo* and *in vitro* studies involving PMN-specific selenoproteins and their role in inflammation and resolution. Our study utilized PMN-specific selenoproteome knockout mice produced by deletion of the Trsp gene in the S100A8 expressing granulocytes, predominantly PMNs, thereby providing a model for selenoproteome investigation in these cells.

We observed a hierarchy within selenoprotein expression in PMNs stimulated with LPS. The differential expression of selenoproteins indicates their role in the regulation of ROS-related functions during the PMNs activation. Temporal increase in the expression of Gpx4 suggested that it was likely involved in regulating ferroptosis in these cells. It is

known that during PMN activation, the generation of ROS results from NOX enzymes and pathways involving lipid peroxidation[2]. We observed significantly increased in lipid ROS accumulation in PMNs post-LPS stimulation, consistent with the upregulation of Gpx4. The upregulation of Gpx4 is, therefore, likely to assist in the response to the accumulation of lipid peroxides upon PMN activation[16]. On the contrary, the downregulation of Gpx1 during PMN activation may suggest an increase in the proinflammatory signaling pathway in PMNs, as Gpx1 has been found to play an inhibitory role of proinflammatory responses[36–38]. We also observed a transient increase in Selenof and Selenow followed by a decrease in 4h LPS-treated cells, suggesting the differential expression of selenoproteins may be a consequence and/or cause of the fine-tuning of redox status for temporal control of proinflammatory functions of PMN. Of note, the observed discordance of our transcriptional regulation with the protein expression data suggests that these selenoproteins are subject to post-transcriptional and/or translational control, even when selenium is not limiting.

We observed increased inhibitory activity on *C. rodentium* growth of the selenoproteome-depleted PMNs. Interestingly, PMN phagocytosis ability was also inhibited in $Trsp^{N}$ PMNs. Multiple studies have reported the impact of selenium deficiency on bactericidal and phagocytosis abilities of PMN; however, these results suggest considerable variation between them. PMNs isolated from cattle and rodents upon selenium supplementation showed increased killing ability against *Candida albicans*, with either increasing or unaffected phagocytosis ability[10,11,26,27]. In human, PMNs isolated from individuals with high serum selenium concentration showed no effect on their phagocytosis ability compared to the low serum selenium cohort[39]. The study with PMNs isolated from *Dichelobacter nodosus* infected sheep also showed no effect on bacteria-killing ability [40]. The large variation in the results may be due to a wide range of selenium used in these studies from 0.1mg/kg to 5mg/kg. Along these lines, the effect of selenium supplementation on phagocytosis has also been shown to be dosage dependent[6], which may explain the variability in the above studies. Another basis of variability may be contributed by the microenvironment. For instance, deletion of Selenos expression in arterial endothelial cells was found to promote NET generation upon co-culture with PMNs in the arthritis model[15]. A previous study from our group using the macrophage-specific selenoproteome deletion in mice showed increased MPO activity in the jejunum upon *Nippostrongylus brasiliensis* infection, suggesting the increased migration of PMNs along with macrophages that were devoid of selenoproteins [41]. In addition, the selenium-dependent thyroid hormone metabolism may also affect the function of PMNs[10,18].

Selenium has been found to regulate NETs generation in different disease models[13–15]. As ROS regulates the generation of NETs, selenoproteins may be involved in the regulation of ROS-dependent NETs through regulating cytoplasmic and mitochondrial ROS accumulation[2,42]. We did not observe a difference in NETs abundance in the $Trsp^{N}$ PMNs; however, the ability of increased NETs generated in the presence of *C. rodentium* was only seen in the control, but not the $Trsp^{N}$ group. NETs are involved in bridging the inflammation and resolution process during an inflammatory response, which occurs via priming the recruitment and activation of proinflammatory macrophages as damage-associated molecular patterns, while also promoting the pro-resolving phenotypic transition of macrophages[43–45]. The lack of response in terms of NET generation in *C.rodentium*

infected Trsp^{N} PMNs may suggest a potential role of selenoproteins in this process. Further studies involving *in vivo* and co-culture experiments with other immune cell types are needed to confirm the effect of selenoproteome-deletion on NETs formation and its impact on proinflammatory and resolution processes.

As expected, we observed increased extracellular ROS production in the Trsp^{N} PMNs earlier than in TrspWT PMNs correlated with the expression of Alox12 and Cox2. Both iron-containing enzymes are involved in arachidonic acid metabolism leading to the generation of proinflammatory lipid mediators, 12-hydroperoxy eicosatetraenonic acid (12-HPETE) and prostaglandins, respectively[29,30,46]. 12-HPETE promotes multiple proinflammatory functions of PMNs, including degranulation and chemotaxis[47,48]. Thus, the upregulation of Alox12 in Trsp^{N} PMNs may result in the alteration of PMN degranulation and migration activities. Alox12 is also involved in the regulation of ferroptosis through the modulation of lipid peroxidation[49]. Since Trsp^{N} PMNs lacked the expression of the endogenous ferroptosis inhibitor Gpx4, while also upregulating Alox12, the Trsp^{N} PMNs may be prone to increased ferroptosis. Current studies are being carried out to test this hypothesis. On the other hand, the upregulation of Cox2 and iNOS in Trsp^{N} PMNs suggests an upregulation of the proinflammatory signaling pathways, e.g., NF-κB (not shown) that could be the result of such Fe-containing enzymes, which produce ROS as a byproduct of their catalytic activities. This is further confirmed by the increased expression of proinflammatory cytokines, TNFα and IL1β. In addition, the upregulation of Sod2 further confirmed that the Trsp^{N} PMNs were under oxidative stress, and such alternative antioxidant systems were likely upregulated to compensate for the lack of selenoproteins.

In conclusion, here we report the role of selenoproteins in PMNs upon activation with LPS. PMNs lacking the selenoproteome isolated from mice displayed altered proinflammatory functions. Deletion of the selenoproteome resulted in increased PMN extracellular ROS production, associated with the upregulation of proinflammatory signaling pathways, resulting in the increase in proinflammatory cytokines expression that was also associated with increased inhibition of *C. rodentium* growth. Studies are currently underway in our laboratory to understand how selenoproteome regulates PMN functions during inflammation and its timely resolution in an *in-vivo* model of infection.

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Highlight

- Expression of selenoproteins was differentially regulated in neutrophils upon LPS stimulation.
- Neutrophils lacking the selenoproteome expression displayed altered antibacterial activities.
- Increased ROS production and upregulation of proinflammatory genes were seen in neutrophils lacking the selenoproteome.

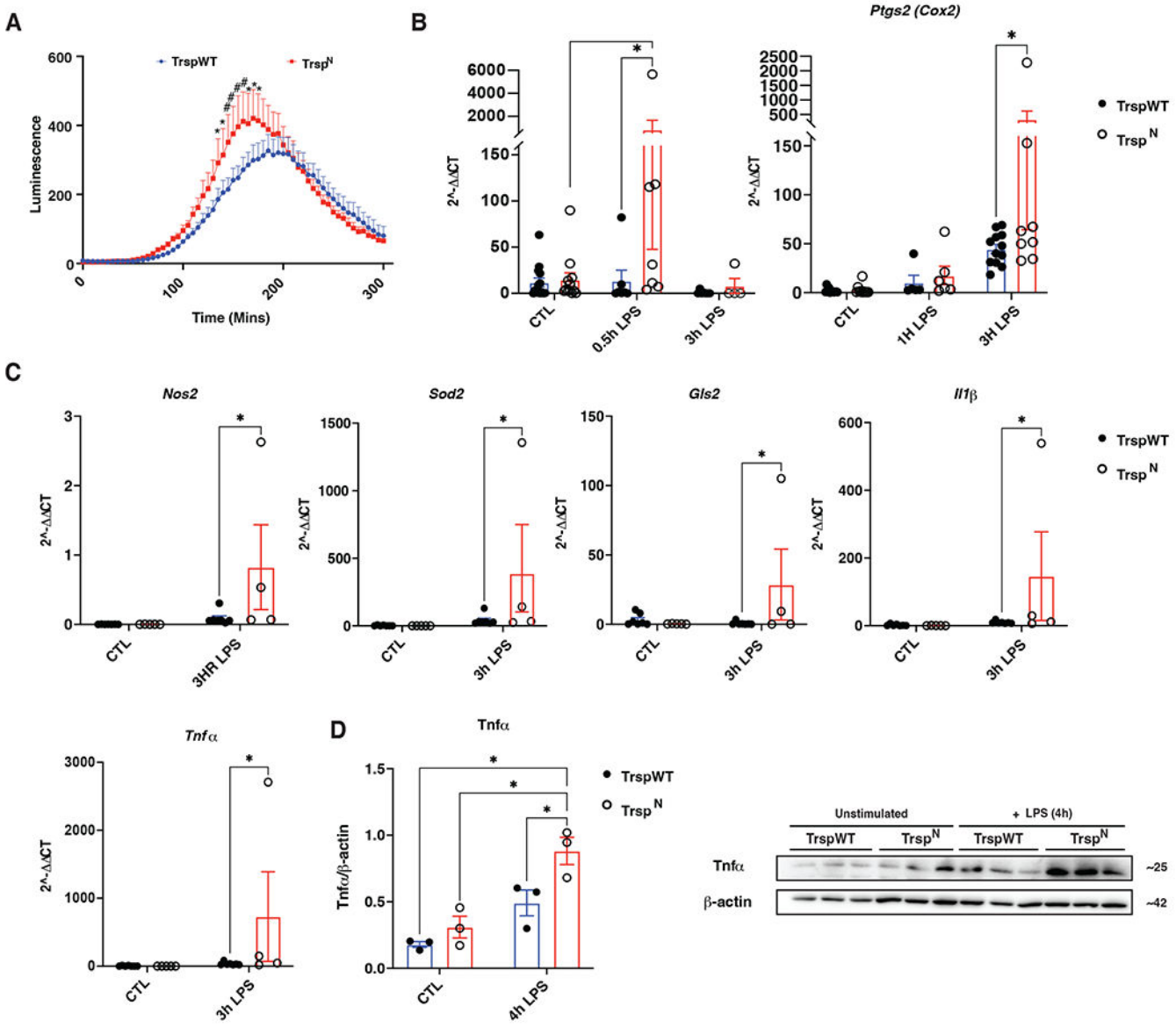

Figure 1. mRNA and protein expression of selenoproteins in PMNs upon LPS stimulation.

(A) Selenoprotein mRNA expression in PMNs under naive conditions (n=4 independent isolations). The relative expression of selenoproteins was first normalized to *Gapdh* according to the $\Delta\Delta C_T$ calculation, and further normalized to 18S expression. **(B)** 23 selenoproteins and *Sbp2* mRNA expression in PMNs following 100 ng/ml LPS treatment for 30 min (n=4). Multiple Mann-Whitney tests were applied. Data are shown as mean ± SEM. * $p < 0.05$. **(C)** Representative images of western blot, and **(D)** densitometric analysis of selenoprotein expression in PMNs treated with or without 100ng/ml LPS for 1 and 4 h (n≥3 independent PMN isolation from mice). One-way ANOVA Kruskal-Wallis test were applied. Data are shown as mean ± SEM. * $p < 0.05$, **$p<0.01$, ***$p<0.001$. (**E**) Lipid ROS accumulation measured as MFI of green fluorescence (510 nm) in post-LPS stimulated PMNs at 0, 15, 30, and 60 min. One-way ANOVA Friedman test was applied. Data are shown as mean ± SEM. * $p < 0.05$, **$p<0.01$.

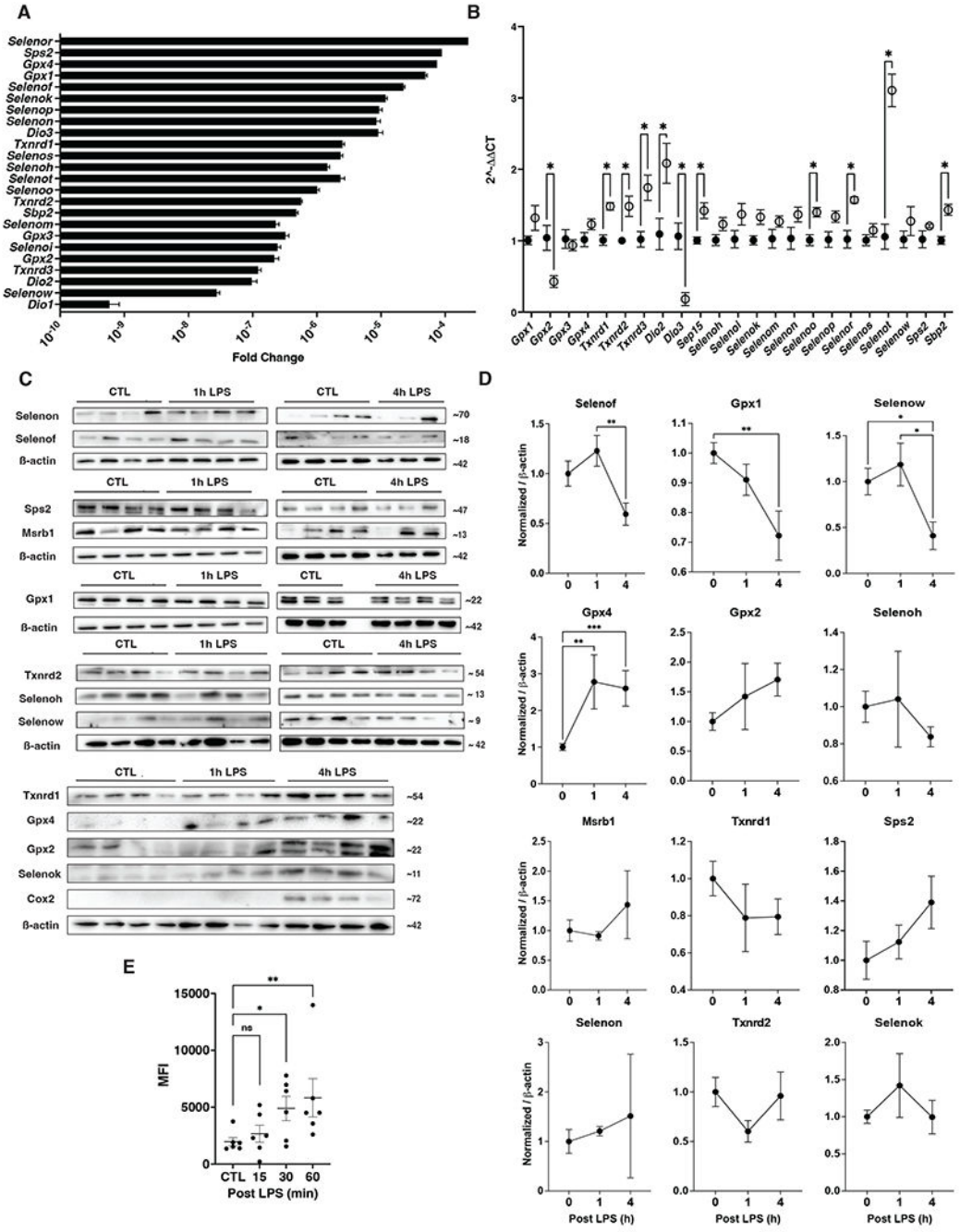

Figure 2. Generation of PMN – specific selenoprotein knockout mice.

Representative western blot images and densitometric analysis of Gpx1 and Txnrd1 expression in **(A)** bone marrow isolated TrspWT and $Trsp^N$ PMNs and **(B)** bone marrow-derived macrophages. PC – positive control (WT bone marrow-derived macrophages). Multiple Mann-Whitney tests were applied. Data are shown as mean ± SEM. ** $p < 0.01$, ***$p<0.005$.**(C)** Weight and CBC cell count comparison between 14-week-old TrspWT and $Trsp^N$ mice. WBC—white blood cell; NE – neutrophil. Multiple Mann-Whitney tests were applied. Data are shown as mean ± SEM. ** $p < 0.01$.

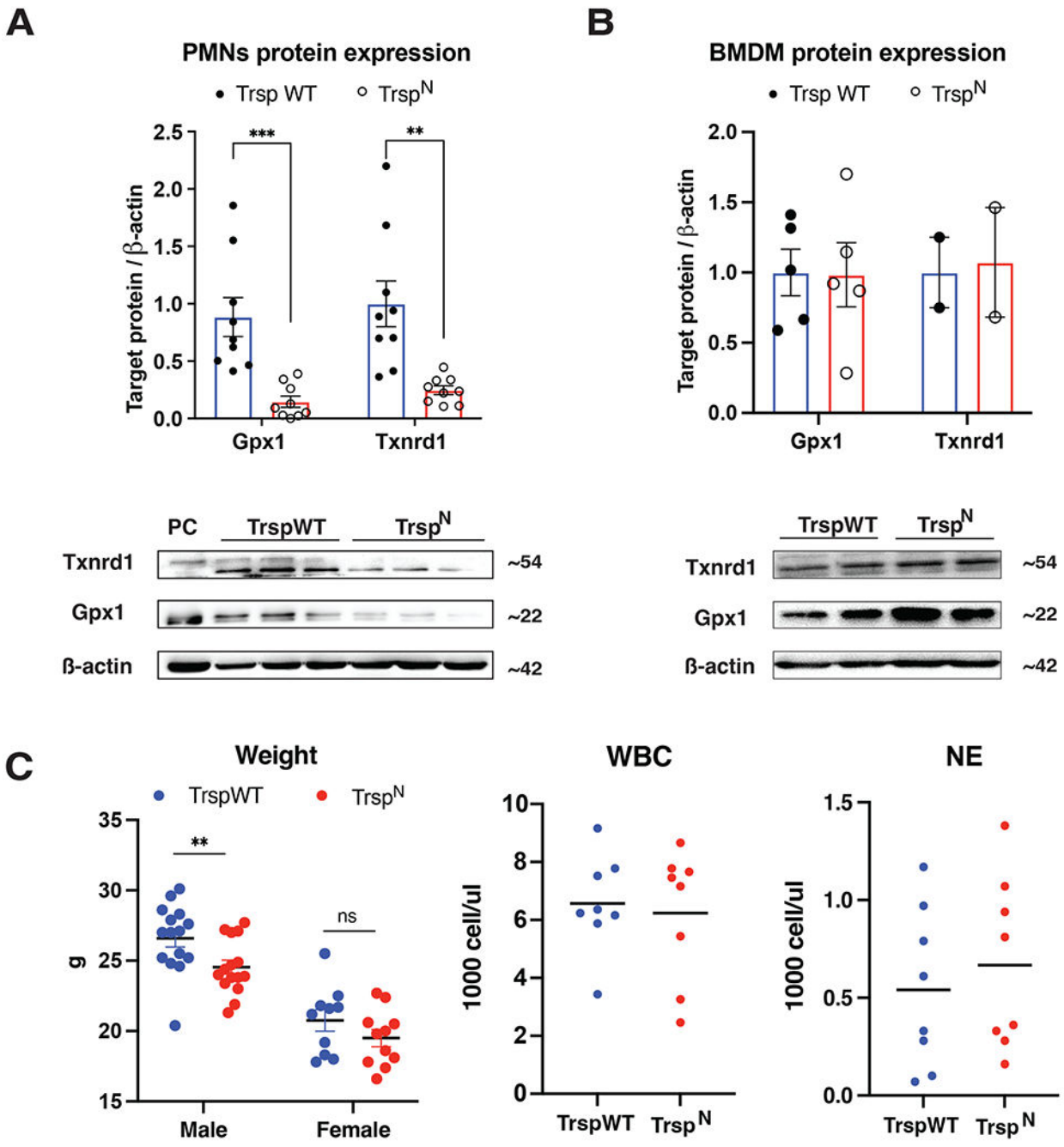

Figure 3. Anti-bacterial activity of Trsp^N PMNs against *C. rodentium*.

(A) PMNs inhibition of *C. rodentium* growth following co-culture for 1h. Bacterial numbers in PMN-free culture media were designated as 100 %, used to calculate relative bacterial growth. Wilcoxon test was applied. Data are shown as mean ± SEM. * $p < 0.05$ **(B)** Phagocytosis ability of PMNs against PKH26 dye-labeled *C. rodentium* following co-culturing for 30 min are represented as the percentage of Gr-1$^+$ PKH26$^+$ population. Mann-Whitney test was applied. Data are shown as mean ± SEM. * $p < 0.05$. **(C)** PMNs NET formation ability with and without *C. rodentium* co-culturing for 4 h. NETosis positive

population is represented as $Ly6G^{+}H3Cit^{+}MPO^{+}$ population. Representative flow cytometry images are presented showing NETosis population of $H3Cit^{+}MPO^{+}$ in the $Ly6G^{+}$ population of TrspWT and $Trsp^{N}$ PMNs co-cultured with or without *C. rodetnium*. Two-way ANOVA with multiple comparisons was applied. Data are shown as mean ± SEM. * $p < 0.05$.

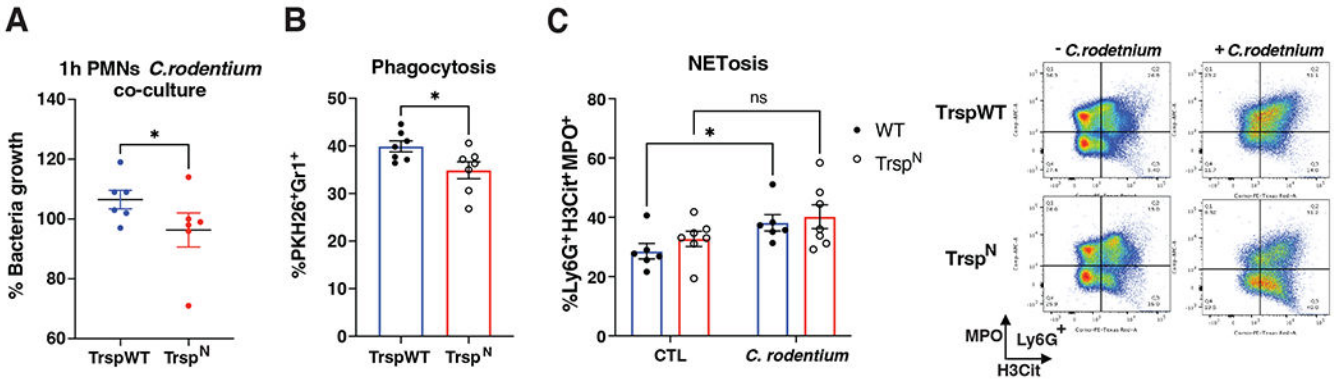

Figure 4. Trsp^N PMNs ROS generation and expression of ROS-related signaling pathways upon LPS stimulation.

(A) Diogenes assay measurement of the extracellular ROS generation of PMNs upon 100ng/ml LPS stimulation (n=6 independent experiments). Two-way ANOVA with multiple comparisons was applied. Data are shown as mean ± SEM. * $p < 0.05$, # $p<0.01$ **(B)** mRNA expression of *Alox12* and *Ptgs2* (*Cox2*) of PMNs treated with or without 100ng/ml LPS for 0.5 h and 3 h. Mix-effect analysis with multiple comparison were applied. Data are shown as mean ± SEM. * $p < 0.05$ **(C)** mRNA expression of *Nos2, Sod2, Gls2, Il1β, Tnf* of PMNs treated with or with 100ng/ml for 3h. Mix-effect analysis with multiple comparisons were applied. Data are shown as mean ± SEM. * $p < 0.05$ **(D)** Western immunoblot and densitometric analysis of TNFα protein expression in PMNs treated with or without 100ng/ml LPS for 4 h. Two-way ANOVA with multiple comparisons was applied. Data are shown as mean ± SEM. * $p < 0.05$.