RESEARCH ARTICLE

Transcriptome profiling reveals new insights into the roles of neuronal nitric oxide synthase on macrophage polarization towards classically activated phenotype

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Abstract

In response to various stimuli, naïve macrophages usually polarize to M1 (classically activated) or M2 (alternatively activated) cells with distinct biological functions. Neuronal nitric oxide synthase (NOS1) is involved in M1 macrophage polarization at an early stage. Here, we show for the first time that NOS1 is dispensable for M2 macrophage polarization for the first time. Further, differentially expressed genes (DEGs) regulated by NOS1 signaling in M1-polarized macrophages stimulated with lipopolysaccharide (LPS) were characterized by transcriptome analysis of wild-type (WT) and *NOS1* knockout mouse macrophages. Thousands of affected genes were detected 2 h post LPS challenge, and this wide-ranging effect became greater with a longer stimulation time (8 h post LPS). *NOS1* deficiency caused dysregulated expression of hundreds of LPS-responsive genes. Most DEGs were enriched in biological processes related to transcription and regulation of the immune and inflammatory response. At 2 h post-LPS, the toll-like receptor (TLR) signaling pathway, cytokine–cytokine receptor interaction, and NOD-like receptor signaling pathway were the major pathways affected, whereas the main pathways affected at 8 h post-LPS were Th1 and Th2 cell differentiation, FoxO, and AMPK signaling pathway. Identified DEGs were validated by real-time quantitative PCR and interacted in a complicated signaling pathway network. Collectively, our data show that NOS1 is dispensable for M2 macrophage polarization and reveal novel insights in the role of NOS1 signaling at different stages of M1 macrophage polarization through distinct TLR4 plasma membrane-localized and endosome-internalized signaling pathways.

Introduction

As an essential player in the innate immune system, macrophages function in the inflammation response and host defense against different pathogens [1]. Traditionally, two different activation states of macrophages have been defined, namely, classically activated (M1) and

alternatively activated (M2), which mirror the polarization of T cells toward T-helper type 1 (Th1) and type 2 (Th2) cells, respectively [2,3]. Lipopolysaccharide (LPS), interferon-γ (IFN-γ), and tumor necrosis factor α (TNFα) often stimulate macrophages toward the M1 phenotype, whereas M2 macrophages are induced with interleukin (IL) 4 (IL4) or IL13 [4]. M1 macrophages produce proinflammatory mediators, including IL1β, IL6, inducible nitric oxide (NO) synthase (iNOS), and TNFα, with strong tumoricidal and microbicidal activity [5]. In contrast, M2 macrophages upregulate the expression of arginase 1 (Arg1) and IL10, exhibiting anti-inflammatory functions [2]. To date, the molecular mechanisms underlying macrophage polarization are poorly understood.

As a short-lived gaseous and free radical, NO not only functions as an antibacterial and tumoricidal effector molecule in the innate immune system but also acts as a cytoprotective effector strongly influencing the adaptive immune response [6]. NO is synthesized from L-arginine by three distinct isoforms of NO synthase (NOS), specifically neuronal NOS (nNOS), iNOS (also known as NOS2) and endothelial NOS (eNOS) [6]. iNOS is induced by M1 polarization stimuli as a central signaling molecule for M1 macrophages [6,7]. In addition, nNOS and eNOS (alternatively termed NOS1 and NOS3 respectively) also play roles in the immune system, although they are constitutively and primarily expressed in neurons and endothelial cells, respectively [8]. Given the neuronal expression of NOS1, it is involved in viral encephalitis [9]. Further, NOS1 also regulates dendritic cell maturation in humans and is required for iNOS expression in allergen-induced asthma in mice [10,11]. In oxidized low-density lipoprotein (oxLDL)-induced bone marrow-derived macrophages (BMDMs), the levels of inflammatory cytokines IL6 and TNFα were associated with NOS1 expression [12]. In addition, inflammation can be promoted by NOS1-synthesized NO at lower concentrations [13,14]. However, the mechanism underlying NOS1 function is not well known.

Recently, it has been demonstrated that NOS1 is required for inflammation-mediated changes in transcription in macrophages and host-tissue injury in sepsis mice [15]. Knockout of NOS1 protects mice from septic injury and mortality and inhibits production of proinflammatory mediators, such as IL6, IL1β, and TNFα, in BMDMs stimulated with LPS [15]. In LPS-challenged $NOS1^{-/-}$ macrophages, suppressor of cytokine signaling 1 (SOCS1) mediates degradation of the NFκB p65 subunit via the proteasomal pathway instead of the NFκB p50 subunit early (2 h) after LPS stimulation, thereby attenuating the production of proinflammatory cytokines [15]. In addition, NOS1 affects macrophage polarization toward the M1 state through the nuclear translocation and subunit assembly of activator protein 1 (AP1) [16,17]. Moreover, macrophage NOS1 modulates the inflammatory response and foam cell formation in atherosclerosis via the uptake of low-density lipoprotein (LDL) [18,19]. Thus, NOS1 participates in the regulation of macrophage polarization toward the M1 phenotype; however, whether NOS1 is involved in M2 macrophage polarization is not known.

Irrespective of the state of LPS activation, NOS1 mainly localizes in macrophage nucleus [15], and thus, it may have a regulatory role in gene expression. NOS1 is constitutively expressed in macrophages during polarization toward the M1 state, whereas NOS2 is induced and can be detected 4 h after LPS stimulation and is highly expressed 7 h post-LPS [15]. As a result, it was demonstrated that synthesis of NO by NOS1 is required for macrophage function early (within 2 h) after LPS stimulation [15–17]. However, whether NOS1 acts for a longer period in macrophages is not known. Here, we first investigated the role of NOS1 in M2 macrophage polarization. Then, to comprehensively reveal the functions of NOS1 in M1 macrophage polarization, we compared the expression of genes between wild-type (WT) and NOS1 knockout ($NOS1^{-/-}$) macrophages stimulated with LPS at an early time (2 h) and that at a later time (8 h) through transcriptome analysis.

Materials and methods

Materials

6 to 8 weeks old WT and $NOS1^{-/-}$ C57BL/6 J mice were obtained from the Jackson Laboratory. The study was approved by the ethics board of Chongqing University of Posts and Telecommunications (CQUPT) (No. CA2019-01). Animal handling and experiments were conducted in accordance with the policies of the Animal Care Facility of CQUPT. LPS from *Escherichia coli* O111:B4 was obtained from Sigma. IL4 was purchased from PeproTech. The RNeasy Mini Kit was purchased from Qiagen, and the High Capacity cDNA Reverse Transcription Kit and Fast SYBR® Green Master Mix were obtained from Applied Biosystems. The STAT6 (C9) mouse monoclonal antibody (mAb) was purchased from Santa Cruz Biotechnology. β-Actin (8H10D10) mouse mAb, NF-κB p65 (D14E12) XP® rabbit mAb, iNOS (D6B6S) rabbit mAb, PPARγ (C26H12) rabbit mAb, and Phospho-Stat6 (Tyr641) (D8S9Y) rabbit mAb were obtained from Cell Signaling Technology. The SOCS1 rabbit antibody (ab62584) was purchased from Abcam.

BMDM culture and treatment

Mice were kept on a 12 h/12 h light/dark cycle with standard chow. Mice were sacrificed by ether anesthesia and bone marrow was collected immediately. Macrophages derived from the bone marrow of WT and $NOS1^{-/-}$ littermates were cultured according to an established protocol [15]. Briefly, bone marrow was flushed from femur and tibia with culture media under aseptic conditions. Single-cell suspensions of bone marrow were then cultured in DMEM containing 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20% L929 cell-conditioned complete medium to prepare macrophages. Macrophages were collected using an 18-gage needle to spray cells with PBS containing 1 mM EDTA. Macrophages were plated for 24–48 h and stimulated with either 100 ng/mL LPS or 20 ng/mL IL4 for the indicated duration toward an M1 or M2 state, respectively. Cells were treated with vehicle alone as naïve macrophages.

RNA extraction and sequencing

Macrophages from four (two males and two females) littermate WT and $NOS1^{-/-}$ mice were stimulated with LPS for 2 h, for 8 h, or with vehicle as control (0 h), performing four replicates for each stimulus duration per group. Macrophage total RNA was extracted using the RNeasy Mini Kit, and genomic DNA was digested with DNase I (Ambion) during the process. RNA concentration was measured using a NanoDrop™ One spectrophotometer, and RNA quality was verified with a 260/280 ratio of ~2. Samples with RNA integrity number values of 6.3 and higher were used for RNA library construction and then sequencing on an Illumina HiSeq2500.

Analysis of RNA sequencing data and differentially expressed genes (DEGs)

The overall quality of sequencing data was evaluated by FastQC software, and low-quality reads were removed [20]. The clean reads were then mapped to the mouse reference genome with Tophat2 and Bowtie, and gene expression levels were computed with HTSeq (https://pypi.python.org/pypi/HTSeq) [21]. Gene expression was normalized using the reads per kilobase per million reads method [22]. Threshold limits were applied for the identification of low-expression genes that have at least 1 count per million reads (CPM $\geq$ 1) in a minimum of three samples. DEGs were screened with the R package EdgeR with a threshold false discovery

rate of <0.01 and fold change (FC) of ≥2. DEGs of WT and $NOS1^{-/-}$ macrophages at 2 and 8 h post-LPS stimulation relative to vehicle control (0 h) were screened based on the differentially expressed analyses (DEAnalyses_pairwise_WT and DEAnalyses_pairwise_NOS files in the Supporting information section, files removed as a result of retraction), and underwent integrated analysis including Venn diagram preparation using the open source software TBtools [23] (Chen et al., 2020). Heat maps were prepared using Biocloud (https://international.biocloud.net/zh/software/tools).

Functional annotation of DEGs

Gene Ontology (GO) enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID) Knowledgebase (https://david.ncifcrf.gov/). The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database (https://www.kegg.jp/kegg/tool/map_pathway2.html) and the KEGG Orthology Based Annotation System (KABOS) webserver (http://kobas.cbi.pku.edu.cn/kobas3) were used for pathway annotation and mapping. Significance level was evaluated by Fisher's exact test. Gene interaction network analysis was performed using the STRING database (https://string-db.org/) [24].

Real-time-quantitative PCR

Total RNA was extracted and purified as described in Section 2.3 and then reverse transcribed to cDNA with random primers. Synthesized cDNA was diluted at a ratio of 1:10 as template. Real-time quantitative PCR (qPCR) was performed with Fast SYBR® Green Master Mix on a QuantStudio 6 Flex Real-Time PCR System, using the primers shown in Table 1. To account for differences in cell numbers, all cycle threshold (Ct) values of sample replicates were

Table 1. Primers used to quantify mRNA levels.

Gene	Primer Sequence (5'-3')
Arg1	TTGGGTGGATGCTCACACTG
	TTGCCCATGCAGATTCCC
FIZZ1	CCAATCCAGCTAACTATCCCTCC
	ACCCAGTAGCAGTCATCCCA
GAPDH	AACGACCCCTTCATTGAC
	TCCACGACATACTCAGCAC
IL1β	TCCCATTAGACAGCTGCACTG
	GTCGTTGCTTGT CTCTCCTTG
IL12b	CAGCACCAGCTTCTTCATCA
	ACACCCCTCCTCTGTCTCCT
MARCO	GATGCTAGAGATGTGCTGTGGC
	GTGCTCTAGGTACCAGGTGTG
NOS2	GTTCTCAGCCCAACAATACAAGA
	GTGGACGGGTCGATGTCAC
Rpl39l	AGAGTCAGGCGCTCAAATCC
	TTGGCCAGGAATCGCTTGAT
TNFα	AGTTCTATGGCCCAGACCCT
	CACTTGGTGGTTTGCTACGA
Xlr4a	AAGTGCCGCCTACAGGATTC
	AGCTGCTGTGAATCGTCA GA
YM1	GGGCATACCTTTATCCTGAG
	CCACTGAAGTCATCCATGTC

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normalized to that of *GAPDH*. Relative mRNA levels are expressed as the FC relative to controls with the ΔΔCt method [25].

Immunoblotting analysis

Macrophages were harvested, briefly sonicated, and then lysed using RIPA buffer with protease and phosphatase inhibitors, followed by centrifugation at $15,000 \times g$ for 15 min at 4˚C. Total protein of the supernatant was determined by protein concentration assay with a Pierce BCA Protein Assay Kit, followed by immunoblotting. Samples were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. Membranes were incubated with blocking buffer (TBST containing 5% BSA) for 1 h, followed by incubation with primary antibodies at 4˚C overnight. Antibodies were diluted in blocking buffer at a ratio of 1:1000, with the exception of anti-NOS2 (1:500) and anti-β-actin (1:5000). After secondary HRP staining, protein signal was detected by enhanced chemiluminescence. The blots were quantitated by ImageJet (1.43) software.

Statistical analysis

Data are presented as mean ± standard deviation (SD). Groups were compared by one-way ANOVA and by post hoc analysis using the Student–Keuls method. A significant difference between means was determined as $P < 0.05$.

Results

NOS1 knockout does not affect M2 macrophage polarization

Whether NOS1 expression influences M2 macrophage polarization is not known. Here, we stimulated macrophages with IL4 for different durations and analyzed the expression of mouse M2 macrophage gene markers by qPCR. The widely used M2 markers included arginase 1 (*Arg1*), resistin like α (*Retnla*, also known as *Fizz1*), and chitinase-like 3 (*YM1*) [26]. As shown in Fig 1, under naïve conditions, *Arg1*, *Fizz1*, and *YM1* were expressed at very low levels in WT and *NOS1*$^{-/-}$ macrophages. Under M2 polarizing conditions, expression of all three genes was markedly increased at the indicated times, but induction of *Arg1*, *Fizz1*, and *YM1* was not significantly affected in *NOS1*$^{-/-}$ macrophages relative to the WT group at any time. These results indicate that NOS1 deficiency does not affect the polarization of macrophages toward the M2 phenotype.

The phosphorylation of signal transducer and activator of transcription 6 (STAT6) at Tyr641 activates transcription of M2 macrophage-specific genes, such as *Arg1*, *Fizz1*, and *Ym1* [26]. As expected, we found that STAT6 was not phosphorylated in both WT and *NOS1*$^{-/-}$ macrophages under naïve conditions (0 h) (Fig 2A). In contrast, STAT6 was phosphorylated in IL4-stimulated macrophages (Fig 2A). However, there were no significant differences in the levels of phosphorylated STAT6 normalized to total STAT6 in *NOS1*$^{-/-}$ macrophages compared with those in WT macrophages at the three indicated times (Fig 2B). In addition, IL4 stimulation strongly induces expression of peroxisome proliferator activated receptor-γ (PPARγ) [27], and activation of PPARγ is essential for M2 macrophage polarization [28]. As shown in Fig 2A and 2C, PPARγ expression was not significantly affected by NOS1 deficiency under naïve conditions or after IL4 activation. Thus, NOS1 does not regulate key transcriptional factors, such as STAT6 and PPARγ, related to M2 polarization and is not required for M2 macrophage polarization.
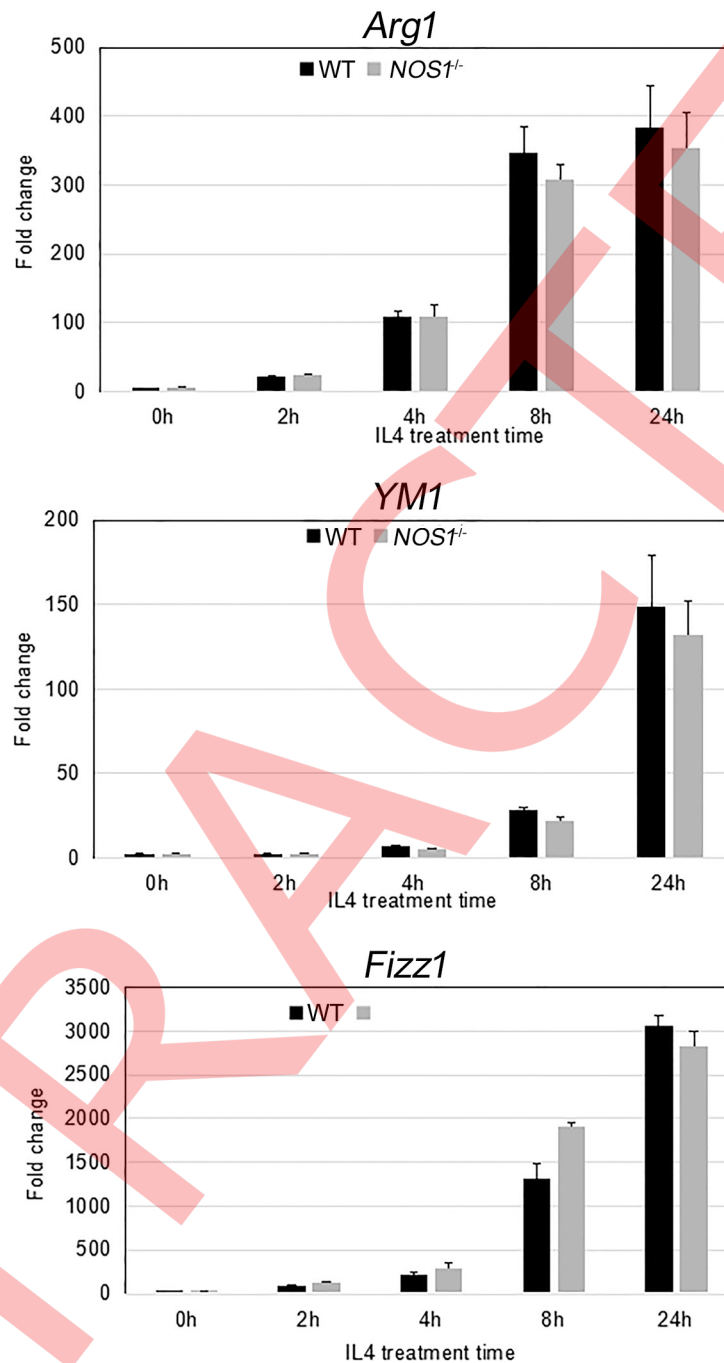
Fig 1. M2 markers in BMDM from WT and *NOS1*$^{-/-}$ mice. RNA was isolated from macrophages stimulated with IL4 for 2, 4, 8 and 24 h or not stimulated (0 h), and cDNA was prepared for RT-qPCR analyses of M2 markers gene expression, including *Arg1*, *YM1* and *Fizz1* genes. Fold expression of each marker relative to that of IL4-untreatd WT cells is presented. Data are presented as mean ± SD (n = 5 independent measurements). *, $p < 0.05$, *NOS1*$^{-/-}$ significantly different from WT.

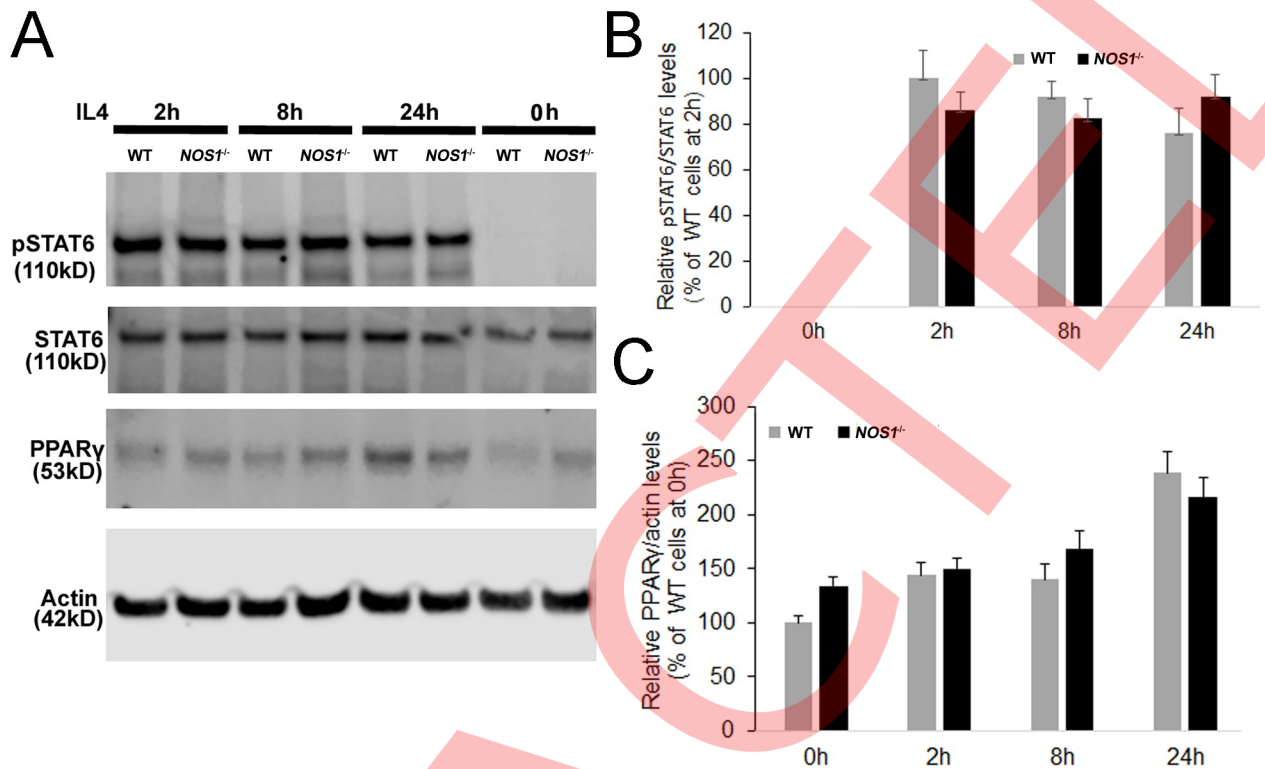https://doi.org/10.1371/journal.pone.0257908.g001

Fig 2. Neither the phosphorylation of STAT6 nor the levels of PPARγ were affected in IL4-stimulated NOS1$^{-/-}$ macrophages. Total protein was extracted from WT and NOS1$^{-/-}$ BMDMs treated with IL4 for 2, 8 and 24 h or not treated (0 h), and then the expression of total STAT6, phosphorylated STAT6 (pSTAT6), PPARγ and β-actin was detected by immunoblotting assay. Migration of molecular mass standard proteins is indicated on the left of the figure. Figures are representative of three separate experiments (A). The levels of pSTAT6 and PPARγ were quantified with normalization to total STAT6 and β-actin respectively (B and C), n = 3.

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Overview of RNA-seq data

RNA-seq data quality was evaluated using FastQC, followed by data filtering. As shown in Table 2, there were between 47 million and 77 million total reads for all samples. The GC content was normally distributed and approximately 50%. The used reads of samples were about 99.7% of total reads. There were between 35 and 63 million mapped reads (75%–81% of total reads), of which 22–38 million were genome-mapped reads and 13–25 million were junction-mapped reads (46%–51% and 27%–33% of total reads, respectively). Our mapping results showed that there were approximately 6.4×10^7 transcript exon-mapped reads and an average of 4.2×10^7 reads were mapped to gene counts.

Table 2. Summary of mRNA-seq data.

	WT 0h	WT LPS 2h	WT LPS 8h	NOS1$^{-/-}$ 0h	NOS1$^{-/-}$ LPS 2h	NOS1$^{-/-}$ LPS 8h
Total reads	53790269	60401256	50847867	77227944	47143722	65567226
Used reads	53674496 (99.78%)	60270477 (99.78%)	50737239 (99.78%)	77061271 (99.78%)	47042792 (99.79%)	65423714 (99.78%)
Mapped reads	43612406 (81.08%)	49400859 (81.79%)	38204449 (75.13%)	63565872 (82.31%)	35662114 (75.65%)	50408974 (76.88%)
Mapped reads (Genome)	25842214 (48.04%)	30745906 (50.9%)	23411455 (46.04%)	38586163 (49.96%)	22131310 (46.94%)	32296186 (49.26%)
Mapped reads (Junction)	17770192 (33.04%)	18654952 (30.89%)	14792994 (29.09%)	24979710 (32.35%)	13530804 (28.7%)	18112787 (27.62%)
Gene count	39388592 (73.23%)	44177601 (73.14%)	34771013 (68.38%)	57054584 (73.88%)	32157625 (68.21%)	45223524 (68.97%)
Exon count	62123021 (115.49%)	67850526 (112.33%)	52887936 (104.01%)	88853447 (115.05%)	49239878 (104.45%)	67759842 (103.34%)

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Baseline DEG expression analysis

First, DEGs between WT and *NOS1*⁻/⁻ macrophages in the absence of external stimulus were identified. We identified 37 upregulated genes in *NOS1*⁻/⁻ macrophages with expression at least twofold higher than that found in WT cells (S1 Table, removed as result of retraction). Among this set of prominent genes, we found that there were three X-linked lymphocyte-regulated (Xlr) genes, *Xlr3b*, *Xlr4a*, and *Xlr4b*. In contrast, we found only seven genes with expression below 50% of WT macrophages at baseline in *NOS1*-deficient cells. On the basis of these findings, *NOS1* deficiency does not influence the basal mRNA level of a number of genes in macrophages, suggesting that NOS1 may play a more important role under stimulating conditions.

NOS1 deficiency dysregulates select genes 2 h post-LPS stimulation

To characterize the regulatory role of NOS1 in M1 macrophage polarization, DEGs were first screened following LPS treatment relative to untreated macrophage baseline level (0 h). All the According to cutoffs of more than or equal to twofold increase or <50% baseline expression, we found that the expression of over 2200 genes was rapidly affected 2 h post LPS treatment (Fig 3A). Most of these dysregulated genes (n = 1732 genes) overlapped in both WT and *NOS1*⁻/⁻ macrophages, whereas 307 and 177 genes were specifically expressed in WT and *NOS1*⁻/⁻ macrophages, respectively (Fig 3A). Of the 307 uniquely expressed genes in WT macrophages, 111 were upregulated and 196 were downregulated in response to LPS (Fig 3B). It should be noted that the expression changes of some of these genes, such as *Il12a* and *Il33*, was over a hundred fold (S2 and S3 Tables, removed as result of retraction). Similarly, we found 51 upregulated and 126 downregulated genes within the 177 uniquely dysregulated DEGs in *NOS1*⁻/⁻ macrophages (Fig 3C, S4 and S5 Tables, tables removed as result of retraction). Of the 1732 DEGs common to both WT and *NOS1*⁻/⁻ macrophages at 2 h post-LPS stimulation, we found that the extent of the expression change can be either attenuated or strengthened in *NOS1*⁻/⁻ macrophages compared with that in WT macrophages. We identified only 49 upregulated and 30 downregulated genes in NOS1 knockout macrophages compared with those in WT macrophages, even if the threshold for FC was lowered from 2 to 1.5 (Fig 3B, S6 and S7 Tables, tables removed as result of retraction). The divergence between gene expression of WT and *NOS1*⁻/⁻ macrophages is further shown by heat map analysis (Fig 3E). On the basis of row changes shown in the heat map, the expression of each gene was affected by NOS1 deficiency under 2 h LPS treatment conditions.

To further characterize our DEGs, functional classification was performed by GO enrichment analysis. We found that screened DEGs were enriched in 80 GO terms under the three independent categories of the GO database: "Biological Process" (36 terms), "Cellular Component" (16 terms), and "Molecular Function" (28 terms) (Fig 4A and S8 Table, table removed as result of retraction). The most enriched terms were "regulation of transcription, DNA-templated" and "transcription, DNA-templated" in "Biological Process"; "cytoplasm" and "nucleus" in "Cellular Component"; and "protein binding" and "metal ion binding" in "Molecular Function" (Fig 4A and S8 Table, table removed as result of retraction). Moreover, "positive and negative regulation of transcription from RNA polymerase II promoter" (GO term IDs: 0045944 and 0000122) were highly enriched in "Biological Process," indicating that the regulation of transcription was significantly affected by NOS1 deficiency. Furthermore, other terms in "Biological Process," such as "immune system process," "immune response," "negative regulation of inflammatory response," and "positive regulation of inflammatory response," confirmed that NOS1 deficiency in macrophages influences immune and inflammatory response. In the "Molecular Function" category, several terms related to transcription, including "transcription factor activity," "transcriptional activator activity," "transcription factor
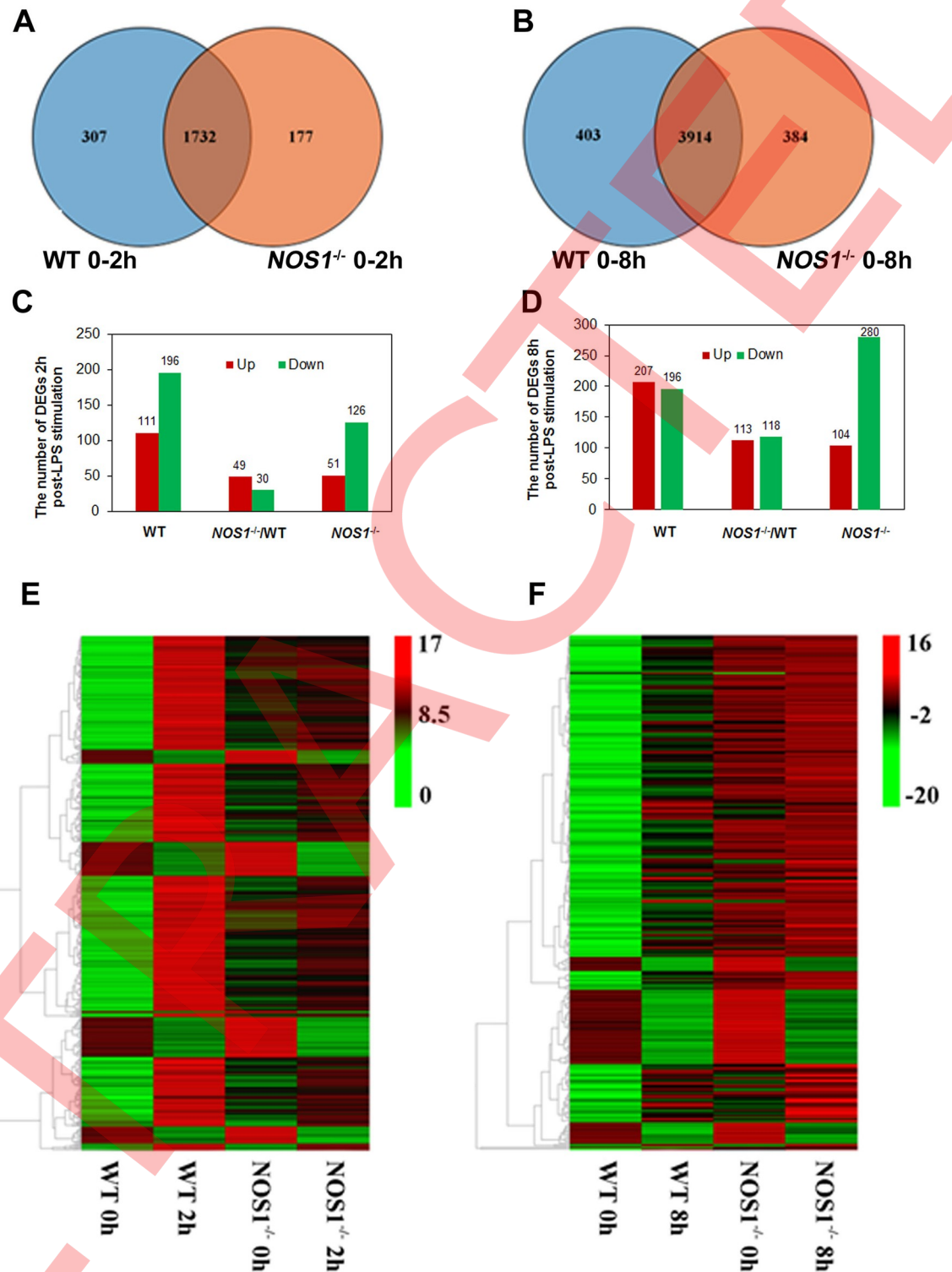
Fig 3. Integrated analysis of DEGs in WT and *NOS1*^{−/−} macrophages post-LPS stimulation. A and B, Venn diagram showing the total number of genes upregulated (more than 2-fold) and downregulated (less than 0.5-fold) in WT and *NOS1*^{−/−} BMDMs treated with LPS for 2 (A) and 8 h (B) compared with untreated cells (0h). Each portion of a Venn diagram displays the number of DEGs in *NOS1*^{−/−} BMDMs compared to *WT* BMDMs. **C and D**, Histogram showing the number of genes upregulated (red) and downregulated (green) in *NOS1*^{−/−} compared with WT BMDMs post 2 and 8 h of LPS stimulation. **E and F**, Heat map of DEGs in macrophages with 2 and 8 h of LPS stimulation or without LPS stimulation. Each row represents a gene, and each column represents a time point.
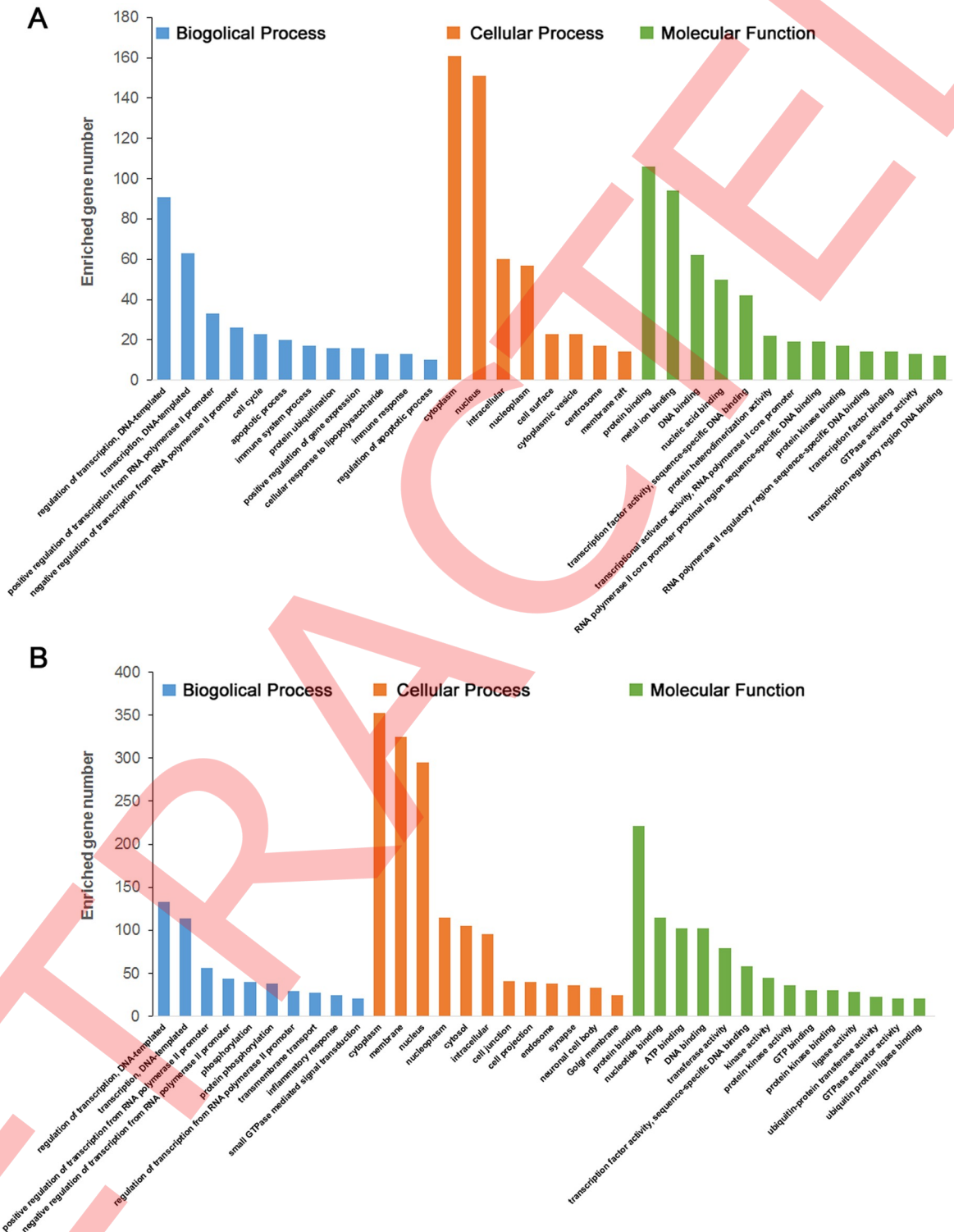
https://doi.org/10.1371/journal.pone.0257908.g003

Fig 4. Functional classification of the DEGs obtained from the comparison of WT and *NOS1*$^{-/-}$ macrophages stimulated with LPS for 2 (A) and 8 h (B). According to a GO analysis, unigenes with BLAST hits were classified into three categories: "Biological Process," "Molecular Function," and "Cellular Component." The Y-axis represents the number of enriched DEGs in each subcategory. The X-axis indicates the most enriched GO terms with the number of enriched genes above 10 and 20 in A and B respectively.

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binding," and "transcription regulatory region DNA binding," were also highly enriched, showing that NOS1 was involved in the regulation of LPS-stimulated genes through the regulation of transcription.

Next, we elucidated the pathways of DEGs uniquely found in WT and $NOS1^{-/-}$ macrophages. On the basis of our analysis, we found that the unique DEGs in WT macrophages were involved in few pathways, most of which were affected by gene downregulation (Fig 5A). Among the three pathways affected by downregulated genes, "inositol phosphate metabolism"

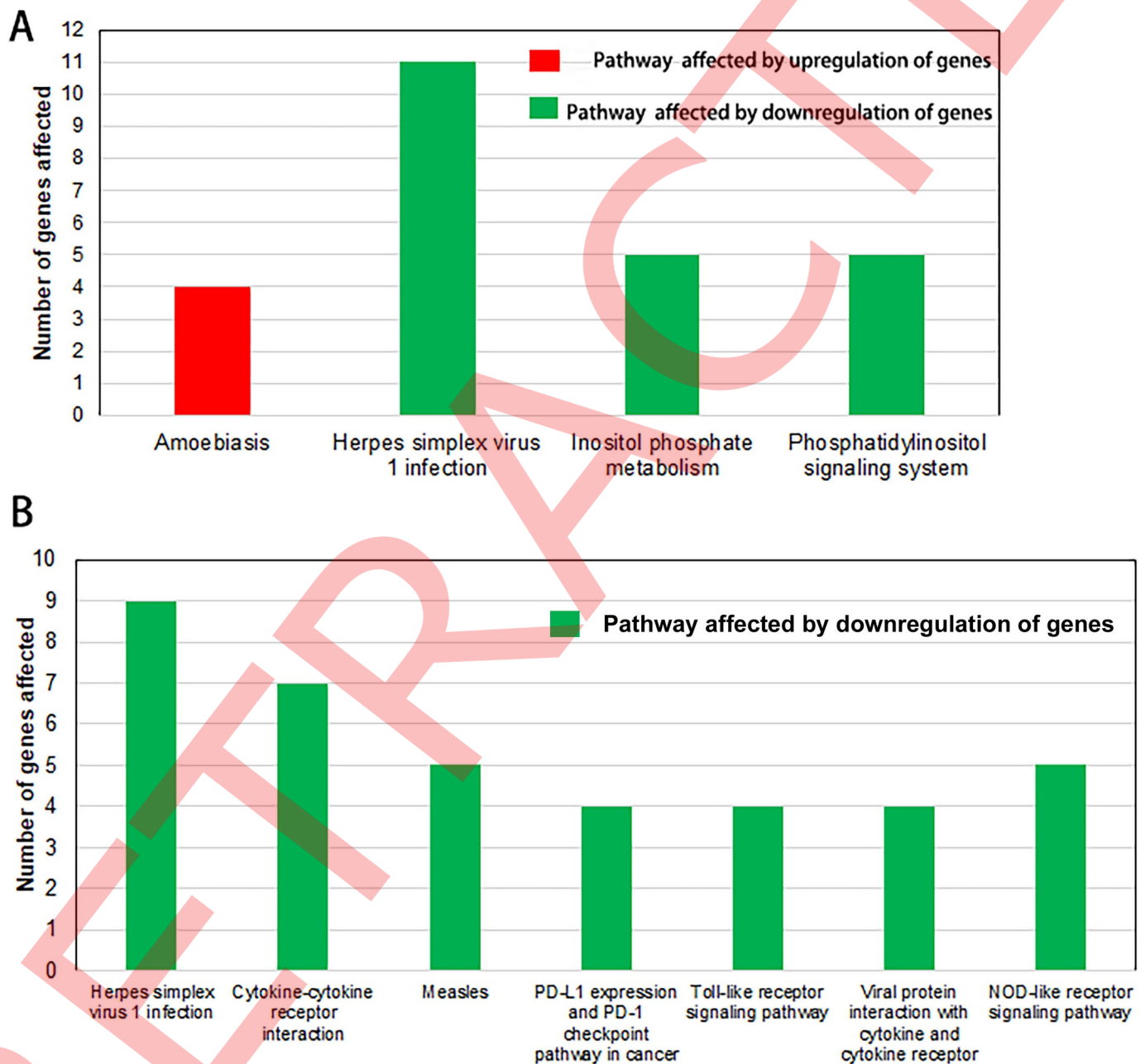**Fig 5. NOS1 deficiency affects several pathways related to macrophage polarization towards M1 after 2 h of LPS stimulation.** Pathway analysis showing the major pathways affected by the downregulation (green) or upregulation (red) of genes by NOS1 knockout in WT macrophages alone (A) and $NOS1^{-/-}$ macrophages (B) in response to 2 h of LPS treatment. Y-axis displays the number of genes implicated in each pathway.

and "phosphatidylinositol signaling system" are of note, indirectly suggesting that NOS1 deficiency may affect phosphatidylinositol metabolism and signaling. The sole pathway affected by upregulated DEGs was "amoebiasis," involving *Il12a* and the genes encoding interleukin 1 receptor-like 1 and 2 (*Il1r1* and *Il1r2*). For *NOS1*$^{-/-}$ macrophages, we identified several pathways that were affected by downregulated DEGs (Fig 5B), of which the "toll-like receptor signaling pathway," "cytokine-cytokine receptor interaction," and "NOD-like receptor signaling pathway" are related to M1 macrophage polarization. Chemokine (C-X-C motif) ligand 9 (*Cxcl9*), interleukin 2 receptor, alpha chain (*Il2ra*), *Il19*, *Il16*, C-C motif chemokine receptor 7 (*CCR7*), and TLR 4 were all downregulated in NOS1 knockout macrophages than in WT macrophages. Thus, NOS1 signaling may play a novel role through these genes in the early stage of M1 macrophage polarization.

DEGs affected by NOS1 deficiency 8 h post-LPS challenge

A longer duration of LPS stimulation resulted in more DEGs in WT and *NOS1*$^{-/-}$ macrophages 8 h post-LPS compared with those in macrophage baseline level (0 h). Over 4300 DEGs in both WT and *NOS1*$^{-/-}$ macrophages were screened, of which 3914 genes were overlapped, whereas 403 and 384 genes were unique in WT and *NOS1*$^{-/-}$ macrophages, respectively (Fig 3B). The unique 403 DEGs in WT macrophages consisted of 207 upregulated and 196 downregulated genes (Fig 3D), including 5′-nucleotidase, cytosolic IA (*Nt5c1a*), SRY (sex determining region Y)-box 5 (*Sox5*), and cholinergic receptor, nicotinic, alpha polypeptide 5 (*Chrna5*), which were increased hundredfold (S9 Table, removed as result of retraction), and resistin like gamma (*Retnlg*), which was downregulated hundredfold (S10 Table, removed as result of retraction). In *NOS1*$^{-/-}$ macrophages, we found that the unique set of 384 DEGs was composed of 104 upregulated and 280 downregulated genes (Fig 3D, S11 and S12 Tables, tables removed as result of retraction). Within the overlapped set of 3914 DEGs, we found that 113 genes were upregulated and 118 genes were downregulated in NOS1-deficient macrophages compared with those in WT macrophages, using a lower FC threshold from 2 to 1.5 (Fig 3D, S13 and S14 Tables, tables removed as result of retraction). Several ILs and their receptors, such as IL12b (*Il12b*), IL12 receptor subunit beta 1 (*Il12rb1*), and IL2 receptor, alpha chain (*Il2ra*) were downregulated (S14 Table, removed as result of retraction). The expression pattern of each gene affected by NOS1 deficiency at 8 h post-LPS stimulation is also shown by heat map analysis (Fig 3F).

Next, screened DEGs were further functionally classified by GO enrichment analysis in the categories of "Biological Process," "Cellular Component," and "Molecular Function," identifying 56, 20, and 28 terms, respectively (Fig 4B and S15 Table, table removed as result of retraction). In the "Biological Process" category, the most enriched GO terms were "regulation of transcription, DNA-templated," and "transcription, DNA-templated" in most DEGs similar to that found at 2 h post-LPS (Fig 4). In addition with the terms "positive and negative regulation of transcription from RNA polymerase II promoter" (GO term IDs: 0045944 and 0000122), these terms showed that transcription and its regulation were the most important biological processes affected by NOS1 deficiency. Moreover, other terms in "Biological Process," such as "inflammatory response," "response to lipopolysaccharide," and "positive regulation of T-helper 1 type immune response," further confirmed that NOS1 deficiency in macrophages influences M1 polarization and the proinflammatory response. In addition, the terms "cytoplasm," "membrane," and "nucleus" in "Cellular Component," as well as "protein binding" and "nucleotide binding" in "Molecular Function," were significantly enriched (Fig 4B and S15 Table, table removed as result of retraction). In the "Molecular Function" category, terms related to transcription, namely, "transcription factor activity," "histone deacetylase binding,"

and "DNA binding," were also highly enriched, showing that NOS1 participates in the regulation of gene expression response to LPS stimulation via transcriptional control.

To further understand the functions of DEGs identified 8 h after LPS stimulation, KEGG pathway mapping was performed on upregulated and downregulated DEGs uniquely found in WT and $NOS1^{-/-}$ macrophages. As shown in Fig 6A, the unique DEGs in WT macrophages largely had roles in pathways affected by upregulated genes. Among these pathways, signal transducing adaptor molecule (SH3 domain and ITAM motif) 2 (*Stam2*), Janus kinase 1 (*Jak1*), *Jak3*, SOS Ras/Rac guanine nucleotide exchange factor 1(*Sos1*), and protein tyrosine phosphatase, non-receptor type 6 (*Ptpn6*) were upregulated in the Jak-STAT signaling pathway. This finding conversely suggests that NOS1 deficiency does not affect Jak-STAT signaling. Further, two pathways affected by downregulated DEGs were RNA degradation and RNA polymerase, which suggest that RNA stability may be attenuated in $NOS1^{-/-}$ macrophages. Most of the pathways affected by dysregulated DEGs in $NOS1^{-/-}$ macrophages were associated with downregulated genes (Fig 6B). Of these pathways, we found that metabolic pathways contained the most DEGs, indicating that metabolism is distinctly different in WT and $NOS1^{-/-}$ macrophages 8 h after LPS stimulation. The pathway of Th1 and Th2 cell differentiation was also downregulated, involving *Il12rb1*, *Il2ra*, mastermind-like transcriptional coactivator 3 (*Maml3*), mitogen-activated protein kinase 12 (*Mapk12*), and *Il12b*. In addition, the FoxO and AMPK signaling pathway, both of which are related to the regulation of M1 macrophage polarization, were affected. In contrast, the IL17 signaling pathway was the sole pathway affected by the upregulation of DEGs, in which the genes *Cxcl1*, *Cxcl3*, colony stimulating factor 3 (*CSF3*), and FosB proto-oncogene, AP-1 transcription factor subunit (*FosB*) were upregulated in $NOS1^{-/-}$ macrophages compared to WT macrophages. The changes in these pathways suggest that NOS1 signaling still functions at a later stage of M1 macrophage polarization.

DEGs interaction network by NOS1 deficiency in response to LPS treatment

Although we identified several important pathways related to M1 macrophage polarization that are affected by NOS1 knockout, these pathways may also crosstalk through common DEGs. Thus, the interaction network was further detailed on the basis of relationships among DEGs to identify the key connected genes under LPS stimulation. As shown in Fig 7A, among the first shell of interactors in response to 2 h LPS stimulation, *Il12a*, *Il2ra*, *Tlr4*, *Ccr7*, *Cxcl9*, *Plcg1*, and proto-oncogene tyrosine-protein kinase LCK (*Lck*), which are involved in several pathways, were identified as central nodes interacting with other genes. Through the proteins encoded by these genes, we identified Th1 and Th2 cell differentiation, inositol phosphate metabolism, NFκB, and TLR signaling pathway as the strongest pathways mediating several biological processes, such as regulation of macrophage cytokine production and the IL1-mediated signaling pathway. Among the first shell of interactors in response to 8 h LPS stimulation, a number of genes, including *Il2ra*, *Il12b*, *Il12rb1*, *Cxcl1*, *Jak1*, *Jak3*, *Foxo1*, and *Foxo3*, were identified as central nodes connecting to additional genes (Fig 7B). We constructed a more complex signaling pathway network with these genes (Fig 7B), and found that the interleukin-2-mediated signaling pathway was the strongest. On the basis of these findings, we conclude that NOS1 mediates different signaling pathway networks to regulate macrophage polarization response to LPS for different durations.

Validation of screened DEGs by real-time qPCR

Next, mRNA expression of several screened DEGs was further validated by real-time qPCR. We found that the mRNA levels of M1 cytokine genes, such as *Il1β*, *Il6*, *Il12b*, and *TNFα*, and
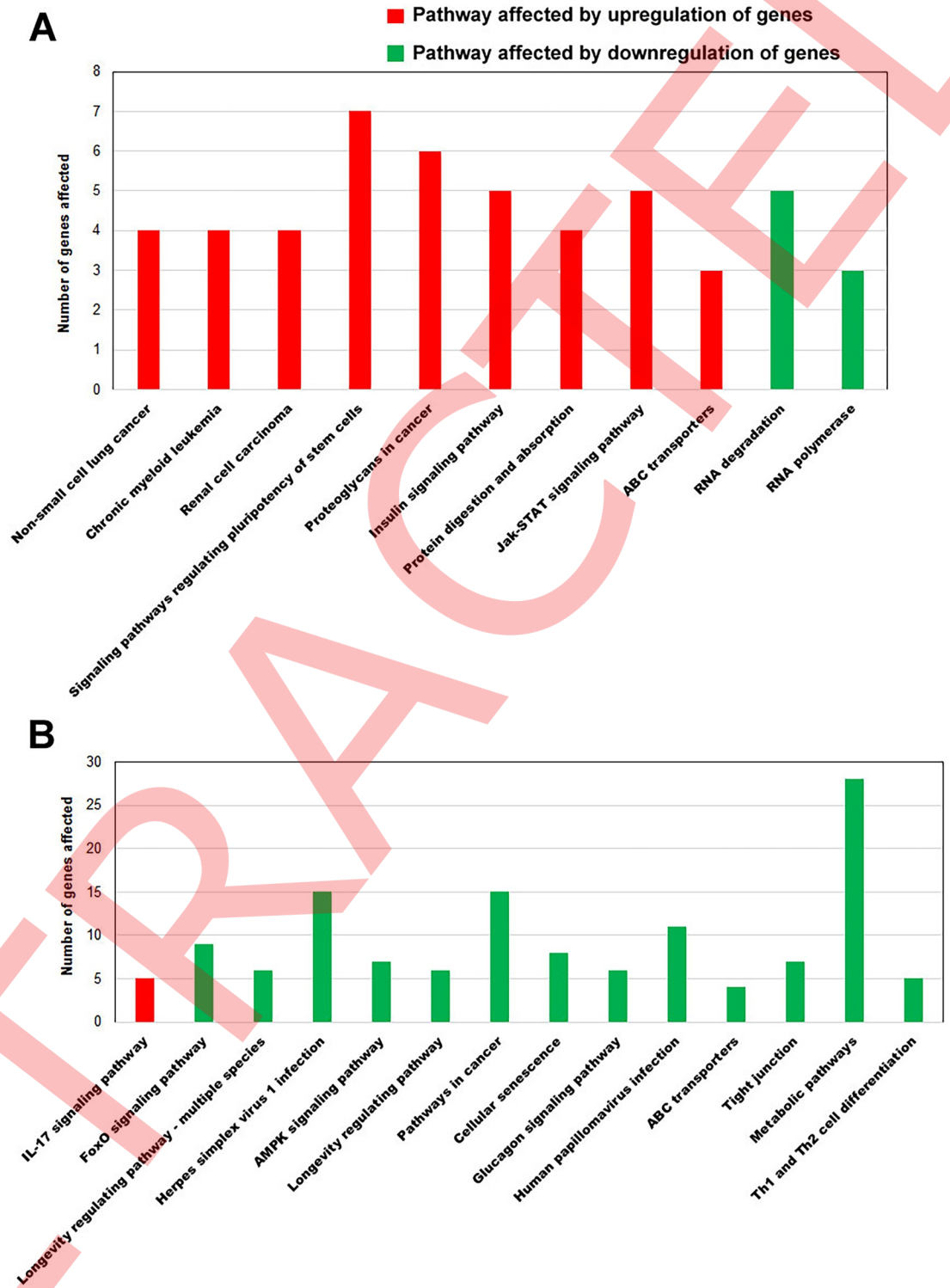
Fig 6. Analysis of pathways affected by NOS1 knockout post 8h LPS stimulation. The major pathways affected by the downregulation (green) or upregulation (red) of genes in WT macrophages alone compared with $NOS1^{-/-}$ macrophages (A), in $NOS1^{-/-}$ macrophages (B) compared with WT macrophages in response to 8 h of LPS stimulation. Y-axis displays the number of genes involved in each pathway.

https://doi.org/10.1371/journal.pone.0257908.g006

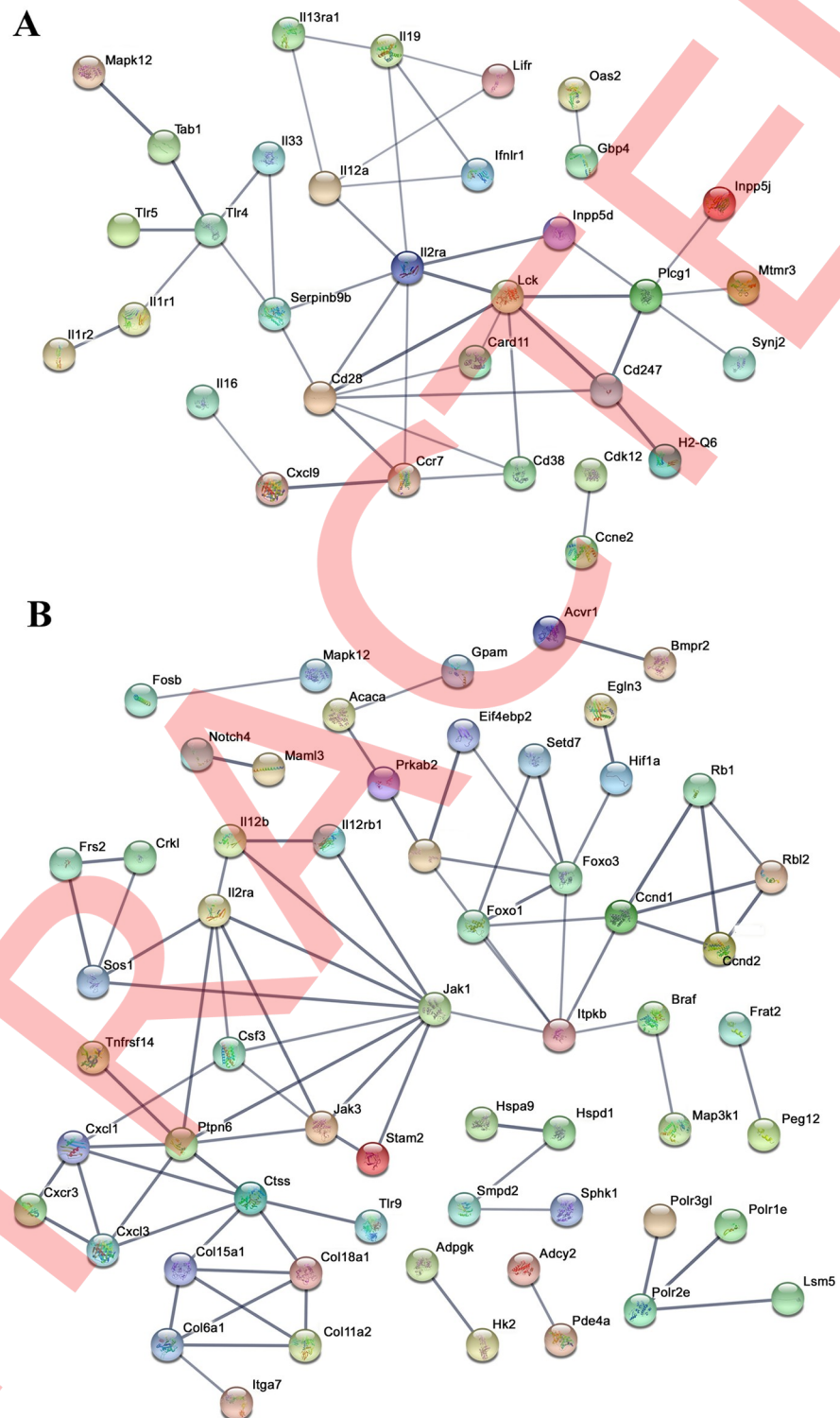**Fig 7. Interaction network analysis for DEGs post 2 h (A) and 8 h (B) LPS stimulation.** Colored nodes are proteins in the first shell of interactors. The edges indicate both functional and physical protein associations. Line thickness indicates the strength of data support. Empty and filled nodes are proteins of unknown and known or predicted 3D structures respectively.

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the M1 marker gene *NOS2*, were significantly increased in WT macrophages in response to LPS stimulation, whereas their expression was downregulated in *NOS1*$^{-/-}$ macrophages 2 and 8 h post-LPS treatment (Fig 8). These findings confirmed that NOS1 deficiency attenuates the inflammatory response to LPS in macrophages to some degree. Furthermore, we verified the upregulated DEGs, macrophage receptor with collagenous structure (*MARCO*), ribosomal protein L39-like (*Rpl39l*), and *Xlr4a*. Compared with that in WT macrophages, the expression of these genes was significantly increased in *NOS1*$^{-/-}$ macrophages stimulated with LPS for 2 and 8 h (Fig 8).

SOCS1-mediated degradation of p65 in *NOS1*$^{-/-}$ macrophages

A previous study demonstrated that the proteasomal degradation of SOCS1 promoted by NOS1-synthesized NO maintained stability of the NFκB p65 subunit in macrophages during the early stage of LPS stimulation (0-2h) [15]. In *NOS1*$^{-/-}$ macrophages, stable SOCS1 led to proteasomal degradation of the NFκB p65 subunit, resulting in the decreased production of proinflammatory cytokines early during 2 h exposure [15]. However, it was not known whether this SOCS1-p65 signaling pathway remains involved post 8 h of LPS treatment, thus the expression of SOCS1and p65 was further investigated in the current study (Fig 9). Over the course of the first 2 h of LPS treatment, SOCS1 levels were stable in *NOS1*$^{-/-}$ macrophages and decreased in WT macrophages (Fig 9A and 9C). In contrast p65 levels were decreased in *NOS1*$^{-/-}$ macrophages but not in WT macrophages (Fig 9A and 9D), which is consistent with previous results [15]. Furthermore, these changes to SOCS1 and p65 were maintained until 8 h post-LPS stimulation (Fig 9). In addition, we found that NOS2 expression was downregulated in *NOS1*$^{-/-}$ macrophages compared with WT macrophages at 8 h post-LPS, whereas the expression of NOS2 could not be detected early (2 h) after LPS stimulation (Fig 9A and 9B). Our findings show stable SOCS1-mediated proteasomal degradation of p65 even for 8 h of LPS stimulation in *NOS1*$^{-/-}$ macrophages.

Discussion

Macrophages can polarize toward M1 and M2 cells to promote and attenuate inflammation, respectively. As a primarily expressed NOS in neurons, NOS1 is constitutively expressed in macrophages stimulated by LPS [8]. Although the expression of NOS1 in macrophages was found to be lower than that of NOS2 4 h after LPS stimulation, the essential roles of NOS1 in macrophage polarization toward an M1 phenotype and host-tissue injury in sepsis mice have been demonstrated [15]. In this study, we demonstrate for the first time that NOS1 is not involved in M2 macrophage polarization for the first time (Fig 10), a conclusion that was demonstrated by expression changes of M2 marker genes, such as *Arg1*, *Fizz1*, and *YM1*, as well as essential signaling factors including phosphorylated-STAT6 and PPARγ (Figs 1 and 2).

The next most important effort is to explore and discover new functional mechanisms of NOS1 in M1 macrophage polarization through transcriptome sequencing analysis (Fig 10). For expression profiling we performed RNA-seq on WT and *NOS1*$^{-/-}$ macrophages at three times: 0 h (treatment with vehicle) and 2 h and 8 h post-LPS stimulation (Fig 10). Although NOS1 is expressed in macrophages independent of LPS stimulation, our analysis identified only dozens of DEGs between WT and *NOS1*$^{-/-}$ macrophages at baseline (0 h) [15]. Thus, NOS1 may function in macrophages under stimulated conditions.

At 2 h post-LPS stimulation, we identified over 2200 DEGs compared with those at baseline, a finding that shows the wide-ranging effects of LPS in both WT and *NOS1*$^{-/-}$ macrophages. The number of DEGs increased after 8 h of LPS exposure (Fig 3). Over 85% of DEGs common in WT and *NOS1*$^{-/-}$ macrophages were expressed at both 2 and 8 h post-LPS

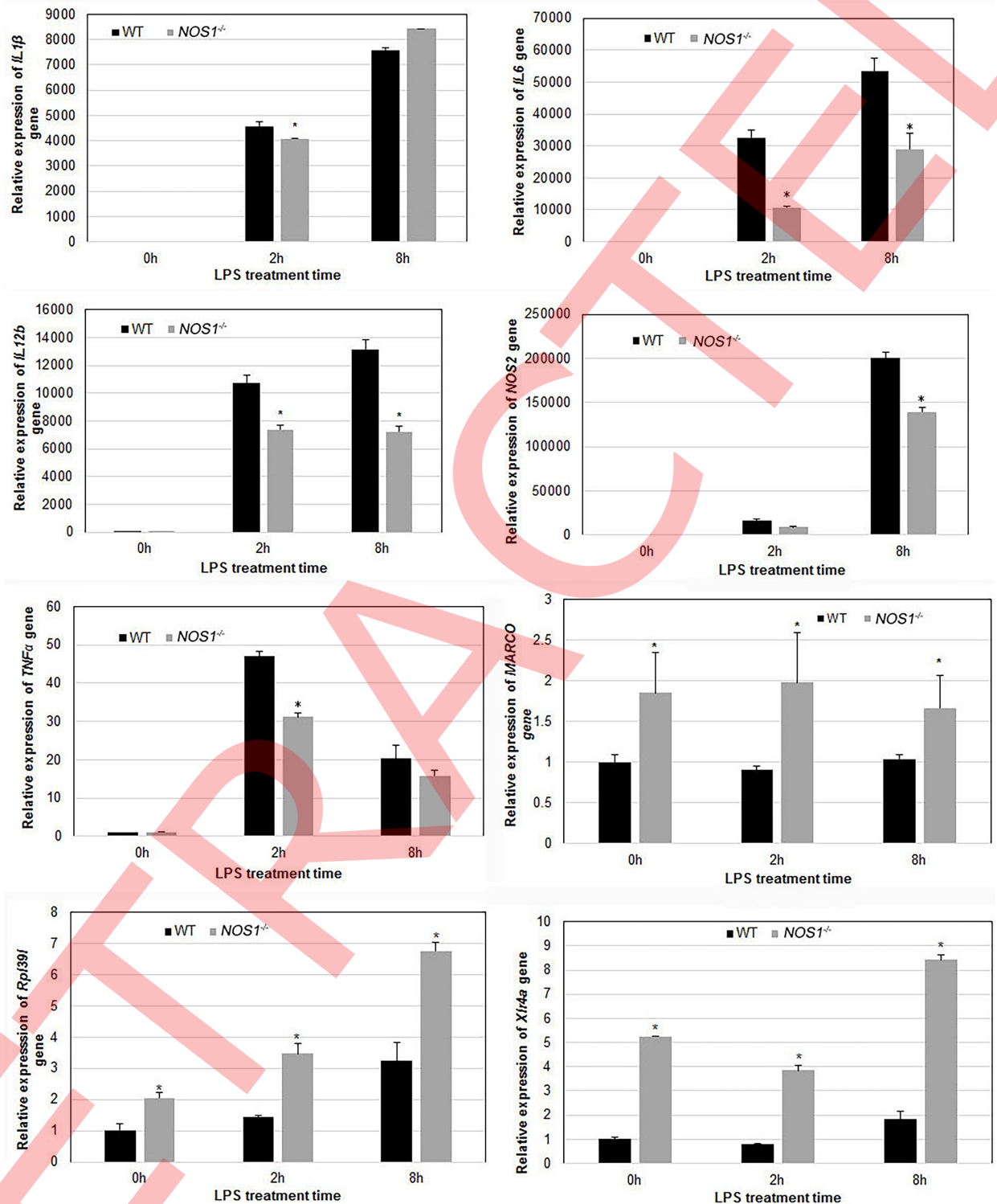**Fig 8. RT-qPCR validation of selected DEGs in WT and *NOS1*$^{-/-}$ macrophages stimulated with LPS for 2, 8 h or not stimulated (0 h).**
Relative expression of genes is presented as fold change compared with WT macrophages without LPS stimulation normalized to the expression of *GAPDH* gene according to the ΔΔCt method. n = 3, * $p < 0.05$ compared to WT macrophages.
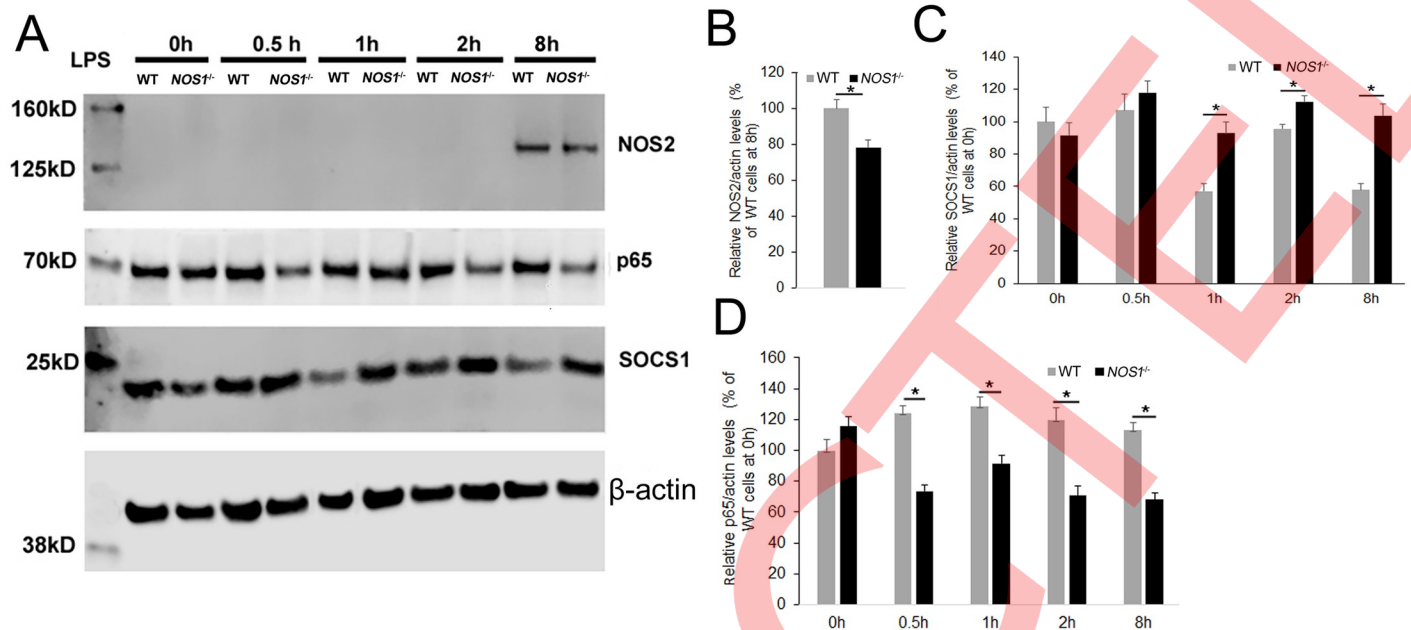
https://doi.org/10.1371/journal.pone.0257908.g008

Fig 9. Immunoblotting analysis of NOS2, p65 and SOCS1 in WT and *NOS1*⁻/⁻ macrophages stimulated with LPS or not. Total protein was extracted from WT and *NOS1*⁻/⁻ BMDMs treated with LPS for 0.5, 1, 2 and 8 h or stimulated (0 h). Then the expression of NOS2, p65, SOCS1, and β-actin was detected by immunoblotting assay. Migration of molecular mass standard proteins is indicated on the left of the figure. Figures are representative of three separate experiments (A). The relative levels of NOS2 (B), SOCS1 (C), and p65 (D) were normalized to β-actin and quantified. n = 3, * $p < 0.05$ compared to WT macrophages.

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stimulation, which is in contrast to those unique DEGs found in WT and *NOS1*⁻/⁻ macrophages (Fig 3). In addition, GO enrichment analysis showed that DEGs at 2 and 8 h post-LPS were enriched in "Biological Process" terms related to transcription and regulation, immune, and inflammatory response (Fig 4). DEGs were also frequently associated with terms related to transcription and regulation in "Molecule Function," such as "transcription factor activity," "transcriptional activator activity," and "transcription factor binding." Thus, the most pronounced effects of NOS1 deficiency on M1 macrophage polarization response to LPS is on transcription and regulation of the immune and inflammatory response.
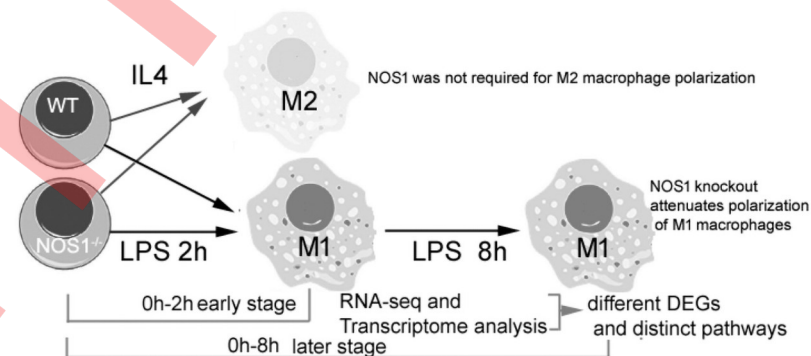
Fig 10. A model of the role of NOS1 in macrophage polarization. Naïve macrophages polarize to M2 and M1 cells in response to IL4 and LPS respectively. NOS1 is not required for M2 macrophage polarization. In contrast, NOS1 deficiency inhibits macrophage polarization toward M1 cells. Potential mechanisms were investigated by comparable transcriptome analysis at the early and the later stages of LPS challenge in the current study.

https://doi.org/10.1371/journal.pone.0257908.g010

Compared with those in WT macrophages, there were about 150 downregulated and 100 upregulated DEGs including unique and overlapped genes in *NOS1*$^{-/-}$ macrophages in response to LPS for 2 h. Among these DEGs, several cytokines, such as *Il16* and *Il19*, and interleukin receptors including *Il2r*a and *Il28ra*, were downregulated. Further, the expression of *Cxcl9* and *CCR7* was decreased, suggesting that NOS1 deficiency also affects chemokine secretion in addition to cytokine secretion. The most notable DEG was *TLR4* as it was downregulated in *NOS1*$^{-/-}$ macrophages. Most LPS signaling pathways are mediated through TLR4 [29]. Thus, downregulation of *TLR4* may trigger downstream cascades that attenuate the proinflammatory response to LPS in *NOS1*$^{-/-}$ macrophages. Other downregulated DEGs in *NOS1*$^{-/-}$ macrophages participated in signaling pathways related to M1 macrophage polarization, such as TLR signaling pathway, cytokine–cytokine receptor interaction, and NOD-like receptor signaling pathway (Fig 5).

We also characterized a set of unique DEGs in WT macrophages at 2 h post-LPS, in which interleukins, *Il12a* and *Il33*, and interleukin receptors, such as *Il1r1*, *Il1r2*, and *Il13ra1*, were upregulated. The identified pathways affected by DEGs unique to WT macrophages were related to inositol phosphate metabolism and the phosphatidylinositol signaling system, and included the downregulated genes, inositol polyphosphate 5-phosphatase (*Inpp5*) J, *Inpp5d*, and phospholipase C, gamma 1 (*Plcg1*). TIR domain-containing adaptor protein (TIRAP) and myeloid differentiation primary response gene 88 (MyD88) in macrophage plasma membrane are a pair of TLR4 adaptors [30,31]. TIRAP binds phosphatidylinositol 4,5-bisphosphate [PI(4,5)P2] through a domain enriched in aromatic and basic residues, and this interaction is required for TLR4 signaling [32,33]. Activation of type I PI3-kinase by the TLR4–TIRAP–MyD88 signaling complex can phosphorylate PI(4,5)P2 to phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P3], which induces internalization of TLR4 and activation of Akt [34,35]. In turn, the activation of Akt mediates inhibition of ERK1/2 and NFκB signaling cascades and leads to limited production of IL10 as an anti-inflammatory cytokine [36]. Inositol polyphosphate 5-phosphatase is a novel regulator of PI3-kinase and Akt1 [37,38]. We found that LPS stimulation downregulated the expression of *Inpp5j* and *Inpp5d* specifically in WT macrophages, which may attenuate dephosphorylation of PI(3,4,5)P3 and reversely strengthen the effect of PI3-kinase on phosphorylation of PI(4,5)P2 and Akt activation. In addition, Plcg2 catalyzes PI(4,5)P2 to diacylglycerol and PI(1,4,5)P3, and inhibition/knockdown of Plcg2 abolishes the internalization of TLR4 in murine macrophages stimulated with LPS and dendritic cells [36,39]. In the current study, we found that *Plcg1* was downregulated in WT macrophages at 2 h post-LPS stimulation and speculate that this change may affect endocytosis of TLR4. Thus, the unique DEGs in WT macrophages identified in our study may reflect a regulatory mechanism of NOS1 knockout in M1 macrophage polarization through opposing changes in plasma membrane localization and endosome internalization of TLR4.

In the context of longer duration LPS stimulation, we identified more DEGs in WT and *NOS1*$^{-/-}$ macrophages at 8 h than at 2 h LPS exposure. Most of the pathways regulated solely in WT macrophages were characterized by upregulated DEGs at 8 h exposure and were different from the pathways characterized by downregulated DEGs at 2 h LPS exposure (Figs 5A and 6A). Among these pathways, Jak-STAT signaling attracted attention as both *Jak1* and *Jak3* were upregulated. Jak1 and Jak3 are involved in inflammation and naïve T cell differentiation through activation of the proinflammatory cytokine Il6 and the common cytokine INFγ [40]. In contrast to those pathways only affected in WT macrophages, altered pathways in *NOS1*$^{-/-}$ macrophages were mainly characterized by the downregulation of DEGs (Fig 6B). In addition to *Mapk12* and *Maml3*, *Il12* and its receptor encoding gene *Il12rb1* participated in Th1 and Th2 cell differentiation. Moreover, we found that *FoxO1* and *FoxO3* were downregulated. Both are involved in the FoxO signaling pathway that mediates proinflammatory cytokine regulation

in macrophages [41]. Further, the AMPK signaling pathway was also affected that has a known role in M1 macrophage polarization [31]. In addition, the AMPK signaling pathway not only suppresses mammalian target of rapamycin (mTOR) signaling, but also regulates phospholipase D (PLD) in a reciprocal manner [42,43]. Therefore, AMPK-mTOR-PLD signaling loops may play a role in NOS1 deficient macrophages 8 h post LPS. It is well known that AMPK acts as a critical signaling node that responds to cellular energy levels and alters cellular metabolism [42]. The pathway containing the most affected DEGs was the metabolic pathway (Fig 6B). Thus, in contrast to those pathways affected by DEGs at 2 h post-LPS, NOS1 deficiency affected more distinct pathways involved in M1 macrophage polarization after 8 h of LPS exposure.

To validate the screened DEGs identified by transcriptome analysis, the expression of several DEGs was measured by qPCR. First, we confirmed that the expression of M1 cytokine genes, such as *Il1β*, *Il6*, *Il12b*, and *TNFα*, and the M1 marker gene *NOS2*, was significantly downregulated in *NOS1*$^{-/-}$ macrophages compared with WT macrophages after LPS stimulation (Fig 8). These results also match previous results in *NOS1*$^{-/-}$ and NOS1 inhibitor 1-(2-trifluo-romethylphenyl) imidazole (TRIM)-treated macrophages [15–17]. In addition, the qPCR results further confirm that NOS1 deficiency attenuates the inflammatory response to LPS in macrophages to some extent. Similarly, the significantly increased expression of three upregulated DEGs, *MARCO*, *Rpl39l*, and *Xlr4a*, was validated by qPCR in *NOS1*$^{-/-}$ macrophages compared with WT macrophages (Fig 8). MARCO, RPL39, and XLR are implicated in inflammation, NOS signaling, and immunodeficiency disorder [44–48]. On a final note, interactions among the identified DEGs between WT and *NOS1*$^{-/-}$ macrophages constitute a complicated signaling pathway network (Fig 7).

In recent years, macrophage polarization has been attracted attention for its translational implication [49]. The induction of NOS1 was associated with oxidative stress and the activation of nuclear factor (erythroid-derived 2)-like 2 (NRF2) signaling in CXCL14-expressing cancer-associated fibroblasts (CAF), which stimulated tumor growth and metastasis [50]. As a master regulator of the antioxidant network, NRF2 not only contributed to chemoresistance to gemcitabine, but was also associated with a poor prognosis in pancreatic cancer patients [51]. These results suggested that NOS1 may be involved in chemoresistance. Indeed, NOS1 expression enhanced chemoresistance to cis-diamminedichloroplatinum (cisplatin/DDP) in ovarian cancer and inhibited the interferon response of lung cancer cells [52–54]. Thus, NOS1 plays a role in therapy resistance and might be a modulator of therapeutic improvements through the regulation of cancer-associated macrophage polarization.

Conclusions

NOS1 is not required for M2 macrophage polarization; in contrast NOS1 knockout attenuates polarization of M1 macrophages (Fig 10). The results from transcriptome analysis of WT and *NOS1*$^{-/-}$ macrophages provide evidence that NOS1 signaling is involved in M1 macrophage polarization through TLR4 plasma membrane localization and endosome internalization pathways. Our findings demonstrate that NOS1 plays a fundamental role in transcriptional regulation of inflammation-related genes affecting different dysregulated genes and distinct pathways not only at an early stage but also at a later stage of the inflammation response (Fig 10).

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