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< Supplementary Materials >

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Anti-inflammatory mechanisms of suppressors of cytokine signaling target ROS via NRF-2/thioredoxin induction and inflammasome activation in macrophages

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Gene Transduction: SOCS1, SOCS3 or thioredoxin1 (Trx1) cDNAs previously described (15, 26) were subcloned into a retroviral vector, pMSCV. The constructs were transfected into HEK 293T cells using Lipofectamine 2000 (Invitrogen). Recombinant viruses were purified from the HEK 293T culture supernatants and used for infection into THP1 monocytic cells. For SOCS1 or thioredoxin (Trx1) knock-down, the sh-negative control (sh), shSOCS1, or shTrx were cloned into pLKO.1 lentiviral vectors (These were from Sigma Aldrich). The transduced cells with viral vectors were selected with puromycin to establish stable cell lines over-expressing or deficient for specific gene product and maintained in RPMI media.

Primary Cell Culture: BMDM preparation was performed based on the reported procedures (27) using rM-CSF (recombinant M-CSF: R&D Systems, Mineannapolis). Briefly, the femurs and tibia of 8 week-old C57BL/6 mice were flushed using a 10 cc needle and syringe in cold MEM alpha media. After gentle disruption, bone marrow cells were washed and cultured in the growth media containing 10% FBS. M-CSF was added at 30 μg/ml from day 3 to induce differentiation to macrophages. On day 5 and day 7, adherent cells were scraped and cultured at $1x10^6$/ml in the media containing M-CSF to obtain F4/80 and CD11b positive cells. Cells were then subjected to transduction of mouse shSOCS1 using the lentiviral system as described above.

Antibodies: Antibodies to SOCS1, SOCS3, catalase, superoxide dismutase(SOD1), ASC, procaspase-1 and cleaved caspase-1 (Santa Cruz Biotechnology); antibodies to p-Erk, p-Jnk, p-p38, STAT1, STAT3, STAT5, pS-STAT1, pY-STAT1, pY-STAT3, and pY-STAT5 (Cell Signaling Technologies); antibodies to p65 and thioredoxin (Upstate Biochemicals); antibodies to peroxiredoxin (Abcam); antibodies to Nrf-2, Flag M2 , and beta-actin (Sigma-Aldrich). Densitometric analysis of immunoblots was performed using MCID software version 7.0 (Imaging Research). Fluorochrome-labelled antibodies to TNFR, IL-1R, and IL-6R alpha were purchased from Biorbyt, Santa Cruz, and Biolegend, respectively.

Inflammasome Assay: For NLRP3 inflammasome activation, the medium was replaced with OPTI-MEM medium supplemented with 10 μM nigericin. After 15 to 30 min of treatment, supernatants and cell lysates were collected for immunoblot analysis. For the apoptosis-associated speck-like protein containing a CARD (ASC) pyroptosome analysis, pellets from whole-cell lysates were cross-linked with disuccinimidyl suberate and subjected to immunoblotting.

Chromatin immunoprecipitation (CHIP) Assay : PMA-differentiated cells were stimulated with LPS and fixed by formaldehyde. The lysates were incubated at 4°C with anti-NRF2 antibodies for overnight after which protein A/G agarose beads were added to obtain immunoprecipitates. PCR amplification was performed using primers to detect 163 bp product containing the anti-oxidant response element (ARE)-related sequence (-564 GTGAAAGGGCT -555) of the thioredoxin1 promoter. Left primer: ATGGGCAAAACTTTCGGTTA, Right primer:TACCCTGCACATCACTGCTC.

Proposed model for inhibitory mechanism of SOCS1 on TLR4 signaling for inflammatory cytokine production via down-regulation of ROS: Fig 4F

LPS signal through TLR4 induces intracellular ROS which activates MAPKs(Erk/Jnk), NF-κB/p65 and STATs 1/3/5 leading to the production of pro-inflammatory cytokines. ROS is also participating in the NRLP3 inflammasome activation required for caspase1 activity and IL-1β maturation. Over-expression of SOCS1 results in the induction of Nrf-2 /Trx1, which down-regulates LPS-induced ROS levels, thereby suppressing the downstream signals for inflammatory cytokine production.

< Supplementary Fig 1 >

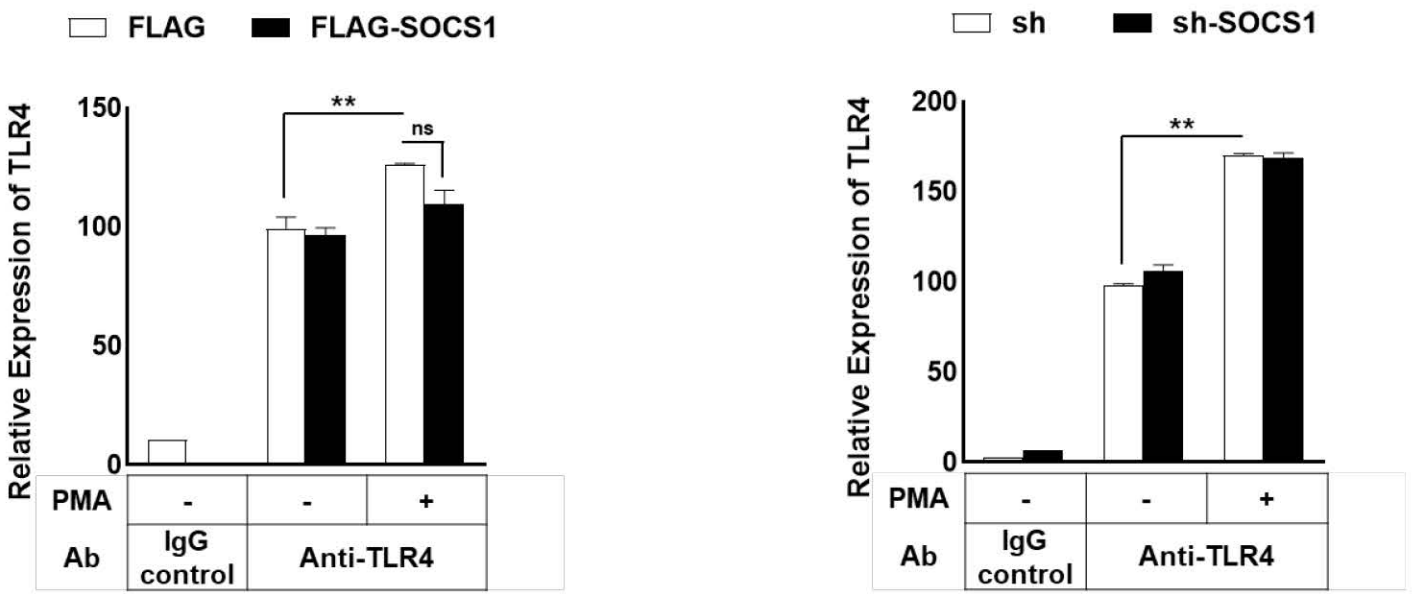

Fig S1. Analysis of TLR4 expression levels in PMA-treated THP1 cells with SOCS1 or sh SOCS1 transduction. SOCS1 over-expression and knock-down in THP1 cells by transduction with Flag-SOCS1 or shSOCS1 was performed as described. Cells gated for CD11b were analyzed for surface TLR4 levels by flow cytometry. While PMA treatment (16 h) up-regulated TLR4 by 125~160 %, cells expressed similar TLR4 levels regardless of SOCS1 transduction. IgG control: stained with isotype control Ab. PMA-treated (+) or untreated (-) cells were stained with anti-TLR4 Ab conjugated with PE.

< Supplementary Fig 2 >

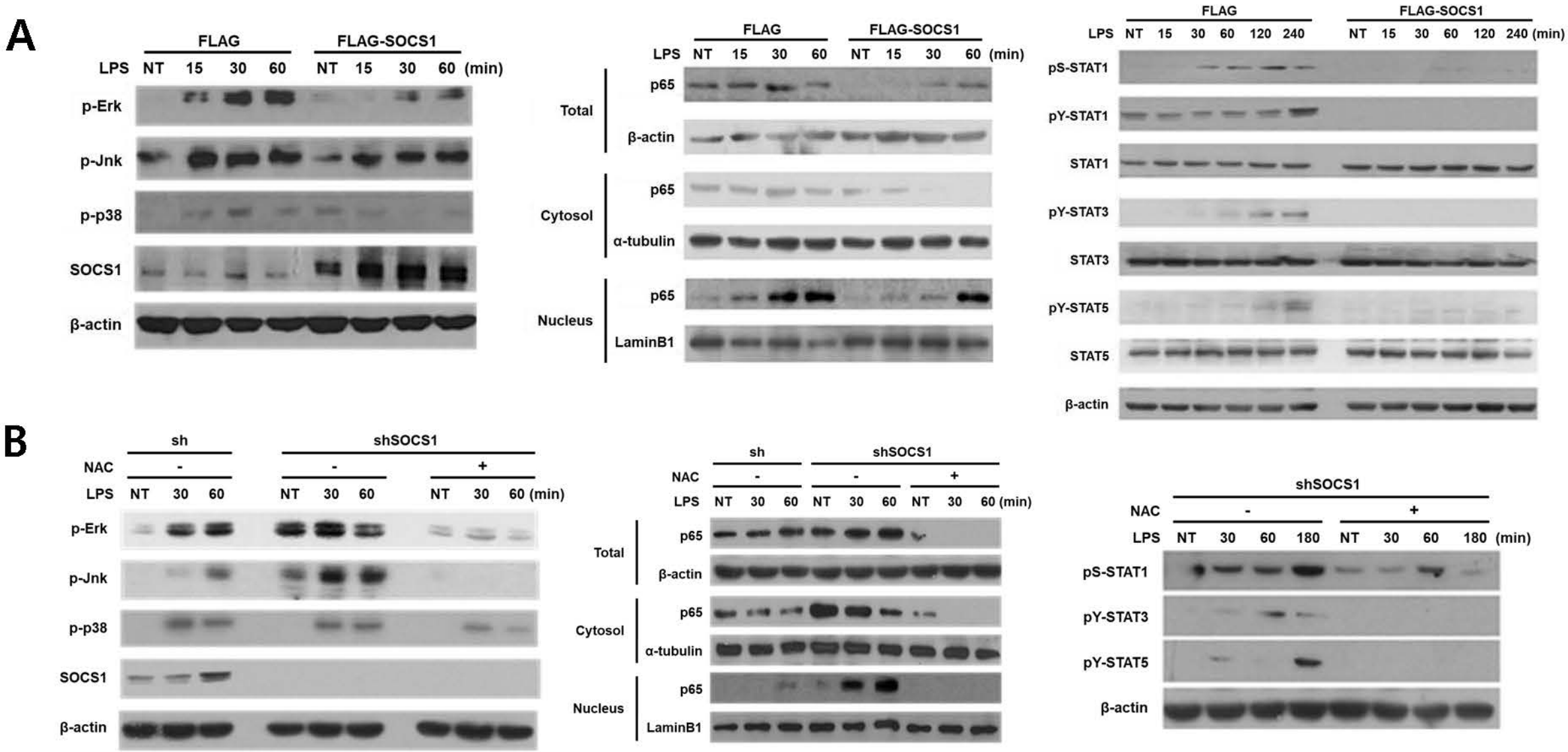

Fig S2. (A) Suppression of LPS-induced MAPK, NF-κB and STAT activation by SOCS1. Flag or Flag-SOCS1 THP1 cells were stimulated with LPS (1 μg/ml) and lysed. MAPKs, NF-κB/p65 levels, and STAT activations were analyzed by immunoblotting.
(B) Role of ROS in MAPK, NF-κB, and STAT activities up-regulated in shSOCS1 cells. sh(mock) and shSOCS1 THP1 cells were stimulated with LPS with or without NAC pretreatment. The cells lysates were subjected to immunoblotting for detection of MAPKs. NF-κB/p65 levels, and STAT activations.

< Supplementary Fig 3 >

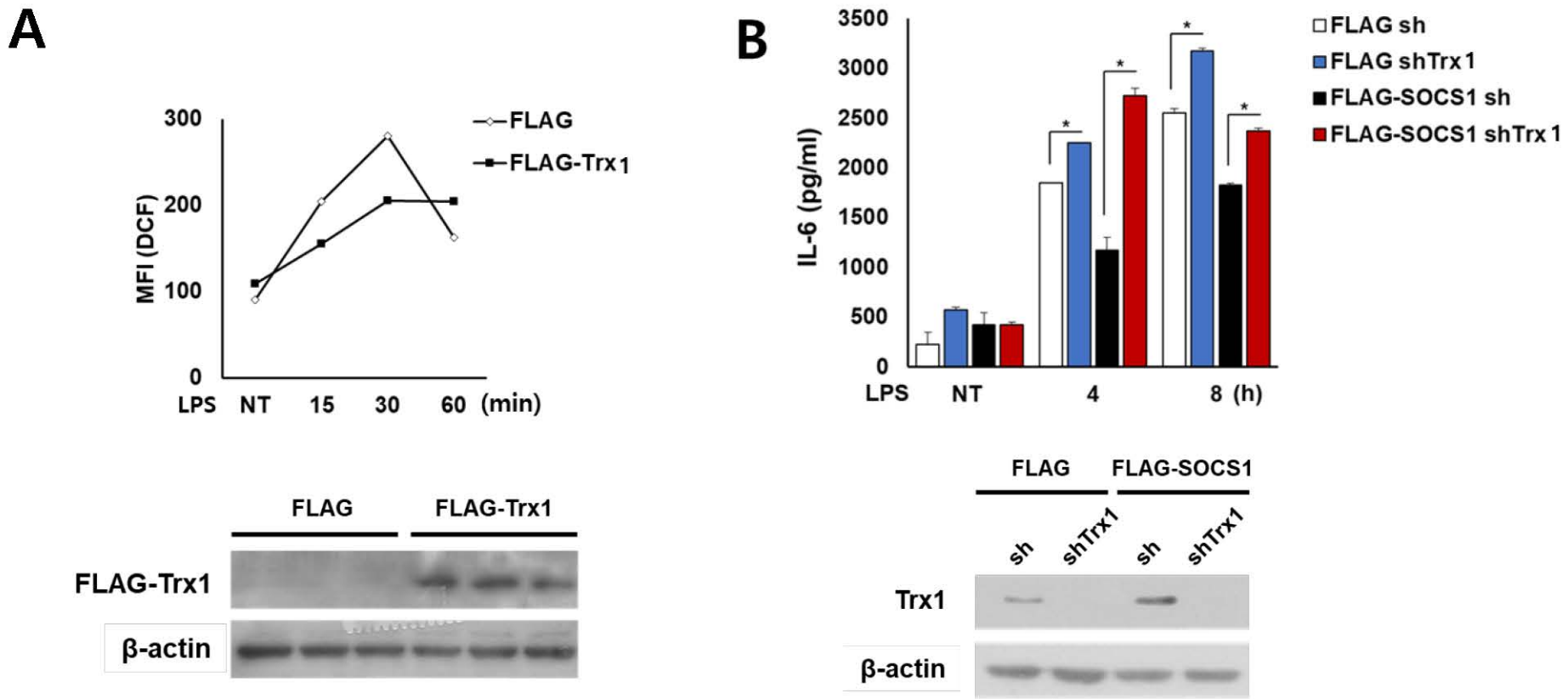

Fig S3. Role of thioredoxin in SOCS1-mediated suppression of cytokine production induced by LPS.
(A) THP1 cells tranduced with Flag or Flag-Trx1 were analyzed for LPS-induced ROS generation. The early ROS generation was significantly reduced upon Trx1 transduction. **(B)** Flag or Flag-SOCS1 THP1 cells were further transfected with sh/shTrx . These cells were stimulated with LPS then analyzed for IL-6 production by Elisa.

< Supplementary Fig 4 >

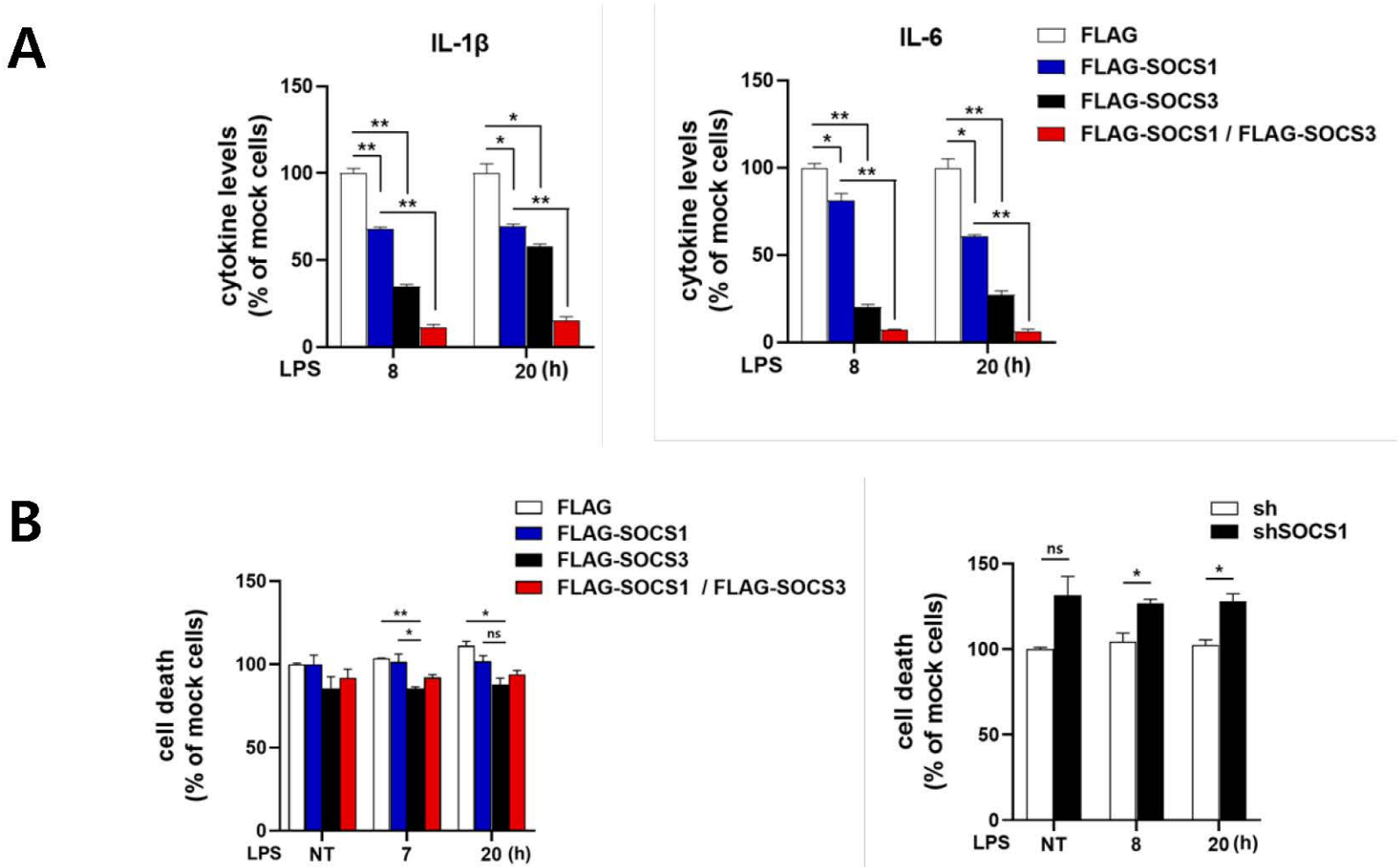

Fig S4. (A) Synergistic inhibition of LPS-induced cytokine production by SOCS1 and SOCS3 in THP1 cells. THP1 cells transduced with both SOCS1 and SOCS3 were compared with cells transduced with SOCS1 or SOCS3 alone for cytokine production (IL-1β and IL-6) at 8 h and 20 h after LPS stimulation.
(B) SOCS1 or SOCS3 did not affect cell death during the LPS treatment : THP1 cells transduced with SOCS1, SOCS3, SOCS1 and SOCS3, or shSOCS1 were subject to cell death analysis after LPS stimulation using AnnexinV and PI staining. Cells positive for either Annexin V or PI were included for cell death measurement. The PMA-treated mock/sh cells typically show 10~20 % spontaneous cell death during the 20 h of culture time employed in the study.

< Supplementary Fig 5 >

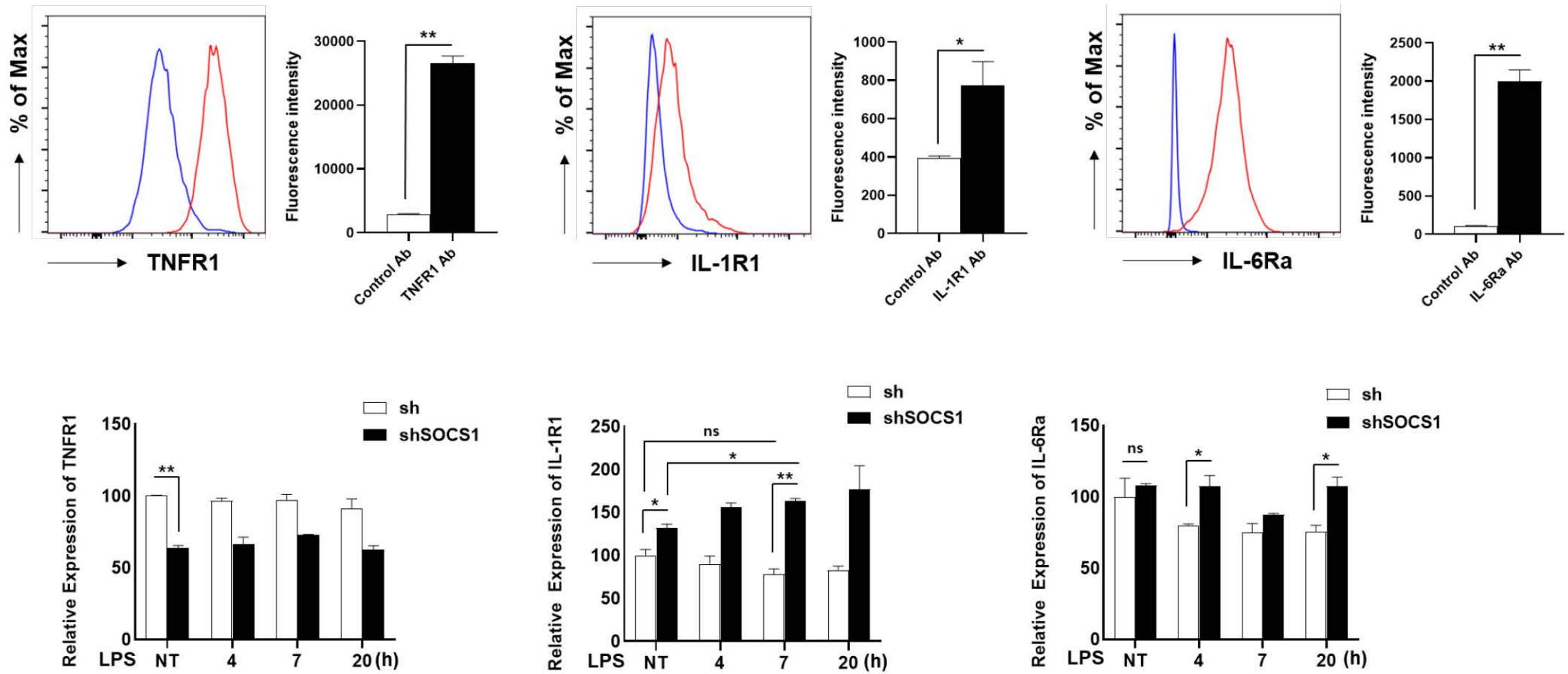

Fig S5. (A) Expression of receptors for inflammatory cytokines on THP1 cells. THP1 cells were analyzed for inflammatory cytokine receptor expression using antibodies against TNFR1, IL-1R1, and IL-6R alpha labelled with FITC, PE, and APC, respectively. Each isotype control Abs labeled with respective fluorochrome was used to stain cells as control.

(B) Analysis of cytokine receptor levels during LPS treatment for cytokine production in sh and sh SOCS1-transduced THP1 cells. Modulations of cytokine receptors are not observed during LPS treatment except for IL-1R in shSOCS1 cells.