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SOCS3 Attenuates Dexamethasone-Induced M2 Polarization by Down-Regulation of GILZ via ROS- and p38 MAPK-Dependent Pathways

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Conflict of Interest
The authors declare no potential conflicts of interest.

Abbreviations
mM-CSF, mouse macrophage-colony stimulating factor; NAC, N-acetylcysteine; Arg1, arginase1; BMDM, bone-marrow-derived macrophage; Dex, dexamethasone; GC, glucocorticoid; GILZ, glucocorticoid-

ABSTRACT

Suppressors of cytokine signaling (SOCS) have emerged as potential regulators of macrophage function. We have investigated mechanisms of SOCS3 action on type 2 macrophage (M2) differentiation induced by glucocorticoid using human monocytic cell lines and mouse bone marrow-derived macrophages. Treatment of THP1 monocytic cells with dexamethasone (Dex) induced ROS generation and M2 polarization promoting IL-10 and TGF-β production, while suppressing IL-1β, TNF-α and IL-6 production. SOCS3 over-expression reduced, whereas SOCS3 ablation enhanced IL-10 and TGF-β induction with concomitant regulation of ROS. As a mediator of M2 differentiation, glucocorticoid-induced leucine zipper (GILZ) was down-regulated by SOCS3 and up-regulated by shSOCS3. The induction of GILZ and IL-10 by Dex was dependent on ROS and p38 MAPK activity. Importantly, GILZ ablation led to the inhibition of ROS generation and anti-inflammatory cytokine induction by Dex. Moreover, GILZ knock-down negated the up-regulation of IL-10 production induced by shSOCS3 transduction. Our data suggest that SOCS3 targets ROS- and p38-dependent GILZ expression to suppress Dex-induced M2 polarization.

Keywords: Suppressors of cytokine signaling (SOCS)3; Type 2 macrophage (M2) polarization; Reactive oxygen species; Glucocorticoid-induced leucine zipper (GILZ); p38 MAPK

INTRODUCTION

Macrophages are key players of the immune-inflammation response. They perform pathogen recognition and elimination by producing pro-inflammatory mediators. They also participate in wound healing and tissue repair process by releasing anti-inflammatory cytokines. The pro-inflammatory (classically activated: M1) macrophages dominant in the early infection phase needs to be converted to anti-inflammatory (alternatively activated: M2) macrophages to reduce the inflammation-induced damage and to restore tissue homeostasis (1). While specific triggers inducing distinct types of macrophage differentiation have not been clearly defined, certain factors in the microenvironment of infected tissues have been suggested to lead to M1 vs. M2 activation (1,2). LPS in combination with IFN-γ and/or TNF-α acts as a strong inducer of M1 differentiation, whereas glucocorticoids (GCs), TGF-β, IL-4 and IL-13 can induce M2 differentiation (2,3).

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GCs, well-known for the anti-inflammatory and immune-suppressive effects, act as a potent inducer of M2s (4). In the absence of inflammatory stimuli *in vitro*, GC can trigger macrophage polarization toward M2, leading to the production of anti-inflammatory cytokines and markers of the M2 phenotype such as Arg1, CD206, and CD163. However, molecular mechanisms of GC action inducing M2 differentiation have not been clearly elucidated (4,5).

The role of MAPKs in GC-induced response has been widely recognized. While MAPKs modulate GC receptor expression and transcription, GC-induced proteins via GC receptor activation in turn regulate MAPKs. The GC-induced factors negatively regulating MAPK signal pathways include mitogen-activated protein kinase phosphatase-1 (MKP-1) (6) and glucocorticoids-induced leucine zipper (GILZ) (7,8). MKP-1 inhibits activated MAPKs Erk and p38 involved in the inflammatory signaling and it is considered an important mediator of anti-inflammatory function of GC (9,10). In activated T cells, GILZ is reported to antagonize the inflammatory gene expression through the inhibition of AP-1 (11) and NF-κB (8). In macrophages, the GC-induced GILZ expression attenuates the pro-inflammatory response as well (12,13). It is shown that over-expression of GILZ suppresses the activation of NF-κB by IL-1β and TLR ligands both *in vivo* and *in vitro* (14,15). On the other hand, GILZ deficiency is associated with enhanced production of inflammatory mediators (16,17). These observations support anti-inflammatory function of GC-induced GILZ by counter-acting pro-inflammatory signaling in immune cells. Yet a direct role of GILZ in the induction of anti-inflammatory cytokines during the GC-induced macrophage differentiation has not been demonstrated.

Suppressors of cytokine signaling (SOCS) were initially identified as inhibitors of the Jak/STAT pathway activated by cytokines (18). Among 7 members of SOCS proteins, SOCS1 and SOCS3 were shown to inhibit inflammatory cytokine signaling mediated by IFN-γ and IL-6, respectively (19). In fact, SOCS1 has been widely reported to exhibit anti-inflammatory effects in various cell systems (19-22). SOCS3, however, has been implicated for both anti-inflammatory and pro-inflammatory functions. Indeed, signaling pathways evoked by pro-inflammatory cytokines IFN-γ, IL-1β, IL-6 as well as anti-inflammatory cytokines IL-10, TGF-β, are both subject to regulation by SOCS3 (23-25). While SOCS3 has emerged as an important modulator of inflammation in diverse immune environments, its role during M1 vs. M2 differentiation remains to be established.

In our recent study, SOCS3 was found to attenuate LPS-induced M1 differentiation of monocytic cells. SOCS3 was induced during LPS/TLR4 signaling and its over-expression up-regulated the Nrf2-Trx1 axis to attenuate the LPS-induced ROS signal involved in the production of TNF-α, IL-1β and IL-6 (26). We now have examined modulatory effects of SOCS3 on M2 differentiation using a GC-induced polarization system and investigated the signaling mechanisms regulated by SOCS3.

It is observed that treatment of PMA-differentiated THP1 cells with a synthetic GC dexamethasone (Dex) promoted the generation of ROS, which participates in the production of IL-10 and TGF-β for M2 polarization. SOCS3 over-expression reduced, whereas SOCS3 ablation enhanced IL-10 and TGF-β induction with corresponding regulation of ROS. SOCS3 over-expression also inhibited p38 MAPK activation and GILZ expression leading to the suppression of Dex-induced M2 response. The induction of GILZ by Dex is both ROS- and p38-dependent, and GILZ depletion blocked ROS generation and M2 cytokine induction.

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Collectively our data suggest that SOCS3 induced in a feed-back loop during the GC-mediated M2 polarization, attenuates GC signaling by targeting ROS- and p38-dependent GILZ expression required for M2 activation.

MATERIALS AND METHODS

Cell culture and generation of SOCS over-expressing or knock-down cell systems

Human acute monocytic leukemia cell lines, THP1 were maintained in RPMI media containing 10% FBS (Invitrogen, Carlsbad, CA, USA) and cultured in a humidified 5% CO_2 incubator. The viral transduction of SOCS3 genes were performed as described (26). Briefly, Flag-tagged SOCS3 was subcloned into pMSCV retroviral vectors, and transfected into complementing HEK 293T cells using Lipofectamine 2000 (Invitrogen). Recombinant viruses were purified from the HEK 293T culture supernatants and used for infection of THP-1 cells. For SOCS3 and/or GILZ knock-down cells, shSOCS3 and shGILZ vectors along with respective negative controls were cloned into pLKO.1 lentiviral vectors. The transduced cells with viral vectors were then selected with puromycin to establish stable cell lines over-expressing or deficient for the specific gene product and maintained in RPMI media.

Mouse bone-marrow-derived macrophage (BMDM) preparation and shSOCS3 transduction were performed as described (26,27). Briefly, the femurs and tibia of 8-week-old C57BL/6 mice were flushed in cold MEM media. After gentle disruption, bone marrow cells were washed and cultured in the growth media containing 10% FBS. Mouse M-CSF (mM-CSF) was then added at 30 µg/ml from day 3 to induce differentiation to macrophages. On day 5, adherent cells were scraped and cultured with mM-CSF to obtain F4/80 and CD11b positive cells. Cells were then subjected to transduction of mouse shSOCS3 using the lentiviral system.

M2 polarization by Dex and inhibitor treatments

THP1 cells over-expressing or knock-down for SOCS as above, were induced to differentiate to acquire macrophage phenotypes by treating with PMA (20 ng/ml) for 16 h. The cells were then treated with 10–40 ng/ml Dex (Sigma-Aldrich, St. Louis, MO, USA) or 10 ng/ml IL-4 for M2 induction for 24 to 48 h. The following inhibitors of MAPK and MKP-1 were used at the final concentrations: PD98059(PD), SP600125(SP), SB203580(SB) at 30 µM each and BCI at 1 µM. As an anti-oxidant N-acetylcysteine (NAC) was treated at 1 to 2 mM 1 h prior to stimulation by Dex.

Determination of intracellular ROS by FACS

Intracellular ROS was measured using a cell-permeable dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), which becomes fluorescent upon oxidation by intracellular peroxide/hydroperoxides. Cells ($4×10^5$/well) were treated with Dex for the indicated times. Freshly collected cells were then loaded with 1 mM H_2DCF-DA at 37°C for 15 min. This was then exchanged with pre-warmed, dye-free bath solution for 15 min to wash out the extracellular dye and allow the conversion of intracellular dye into its non-ester form. 2',7'-dichlorofluorescein fluorescence was measured with excitation at 480 nm and emission at 530 nm to assess intracellular ROS using a FACS Calibur (Becton Dickinson, Franklin Lakes, NJ, USA).

Measurement of cytokines by ELISA

Cytokine levels in the culture supernatants were assessed by using TNF-α, IL-1β, IL-6, IL-10 and TGF-β ELISA kits (eBioscience, San Diego, CA, USA).

Cell fractionation and Western blots analysis

Cells were lysed to prepare total, cytosolic and nuclear extracts in the respective lysis buffer as described (28). Proteins were separated by SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Burlington, MA, USA). The membranes were probed with the respective antibodies and developed using an ECL system. Antibodies to SOCS3, SOCS1, SOD1 were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Antibodies to MKP1, p38, Erk, Jnk, p-p38, p-Erk, p-Jnk, pY-STAT1, pY-STAT3 and pY-STAT6 were from Cell Signaling Technologies (Danvers, MA, USA). Antibodies to p65 and Trx1 are from Upstate Biochemicals (Lake Placid, NY, USA). Anti-GILZ, anti-Flag M2, anti-GAPDH, anti-β-actin antibodies were from Sigma-Aldrich.

Quantitative RT-PCR analysis

After stimulation with Dex, total cellular RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Gene expression levels of the macrophage surface markers and cytokines were detected by qRT-PCR analysis. qRT-PCR amplification with POWER SYBR® Green (Applied Biosystems, Warrington, UK) was performed using a Mastercycler realplex thermalcylcer (Eppendorf AG, Hamburg, Germany). The qRT-PCR primers used are described (**Supplementary Table 1**).

Statistical analysis

Experiments were performed at least in 3 independent sets, and the values are shown as means ± SE. For statistical analysis, data were analyzed using GraphPad Prism 6 software (GraphPad Software Inc., San Diego, CA, USA).

Differences between 2 means were assessed using a Student's t-test. A value of $p < 0.05$, $p < 0.01$ and $p < 0.001$ was considered statistically significant.

RESULTS

Induction of M2 polarization by Dex and analysis of signaling pathways

To study the regulatory effects of SOCS3 in M2 differentiation we have utilized human monocytic THP1 cells. Initially THP1 cells were pretreated with PMA to induce differentiation and acquire macrophage phenotype (M0) including TLR up-regulation and adhesive properties as described (26). Then as an inducer of M2 polarization, a synthetic GC, Dex (1–100 ng/ml) or IL-4 (5–40 ng/ml) was tested for the production of anti-inflammatory cytokines and the expression of M2 phenotype markers. Though the preliminary experiments, the optimal concentration for M2 induction was determined for Dex (40 ng/ml) and IL-4 (10 ng/ml). The production of IL-10 a representative anti-inflammatory M2 cytokine peaked at 24–48 h upon stimulation by Dex or IL-4 (**Fig. 1A**). The gene expression level of IL-10, arginase1 (Arg1) and a scavenger receptor CD163, analyzed by qRT-PCR peaked at 24–40 h (**Fig. 1B**). Dex treatment also resulted in a noticeable reduction of pro-inflammatory cytokines including IL-1β, TNF-α and IL-6 (**Fig. 1C**), without significantly affecting cell viability as assesses by apoptosis and MTT assays (**Supplementary Fig. 1**). The result indicates that Dex induces M2 polarization of monocytic cells by promoting anti-inflammatory mediators, while suppressing pro-inflammatory cytokines.

According to prior studies, ROS and MAPKs are considered as critical signaling mediators for macrophage polarization by LPS (26). Thus, the activation and expression levels of these
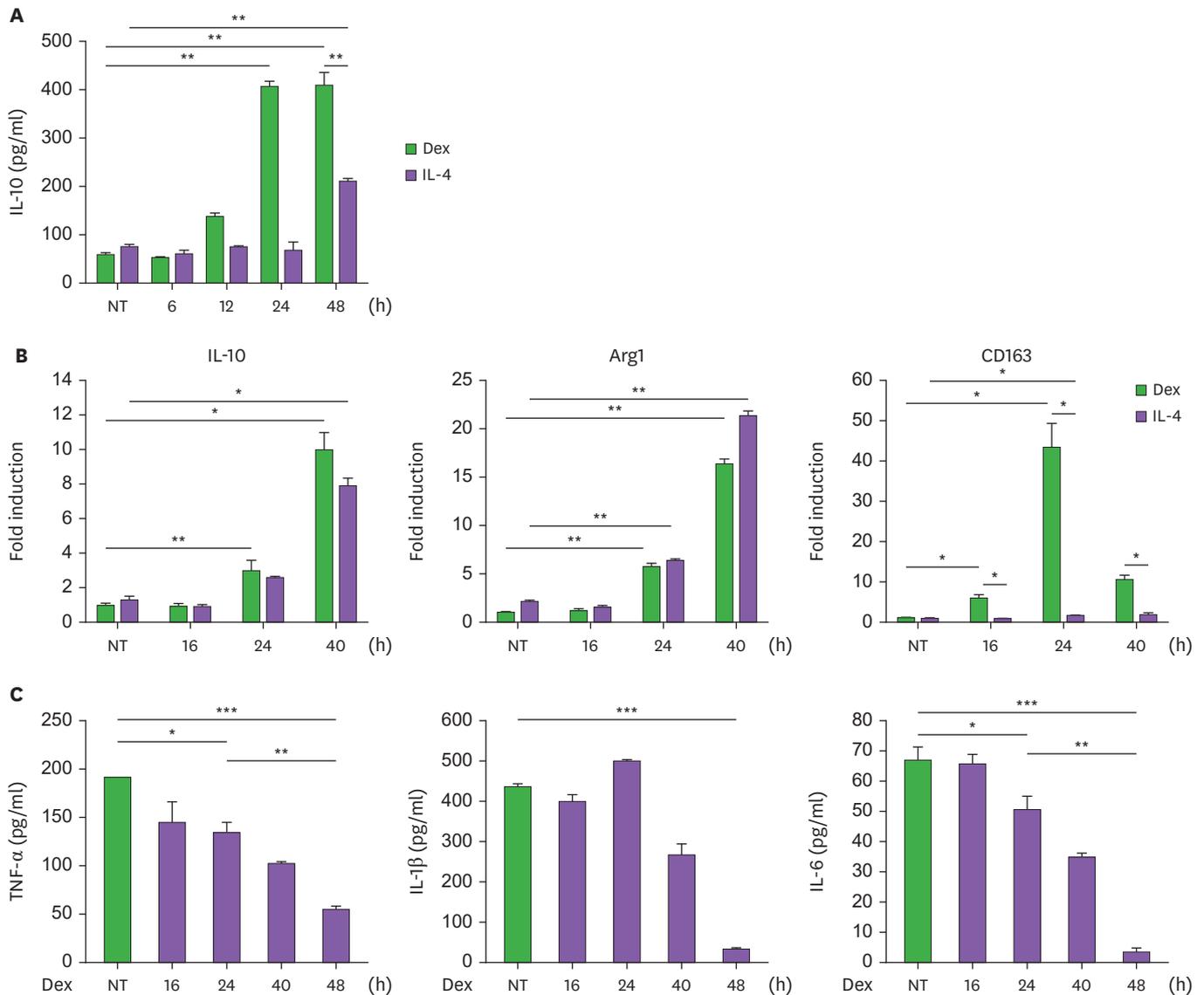
Figure 1. Kinetics of M2 polarization of monocytic cells by Dex. THP1 promonocytic cells cultured with PMA for 16 h to acquire macrophage (M0) phenotype were NT or treated with Dex (40 ng/ml) or IL-4 (10 ng/ml) for the indicated durations up to 48 h. The production of IL-10 was analyzed by cytokine ELISA (A) and the induction of M2 markers were by qRT-PCR (B). The levels of inflammatory cytokines (TNF-α, IL-1β and IL-6) were measured after Dex treatment by ELISA (C). Data are obtained from 3 independent experiments and shown as mean ± SE. Statistical significance was determined by a Student's *t*-test. NT, not treated.
*p<0.05; **p<0.01; ***p<0.001.

signaling molecules were analyzed during M2 differentiation by Dex. There was a gradual increase in ROS levels which starts at 4 h after Dex treatment. Among MAPKs, Erk and p38 activation becomes evident by 5 to 16 h (**Fig. 2A and C**). In addition, the GC-induced factor, GILZ expression was prominent in a similar kinetic window, suggesting its potential function in the Dex-induced M2 differentiation. Induction of both SOCS1 and SOCS3 was also noted, implying their regulatory roles in this process. Notably, the induction of MAPK activation, SOCS and IL-10 was suppressed upon NAC treatment without affecting cell viability (**Supplementary Fig. 1B**), indicating the role of ROS signal during M2 polarization by Dex (**Fig. 2B and C**).
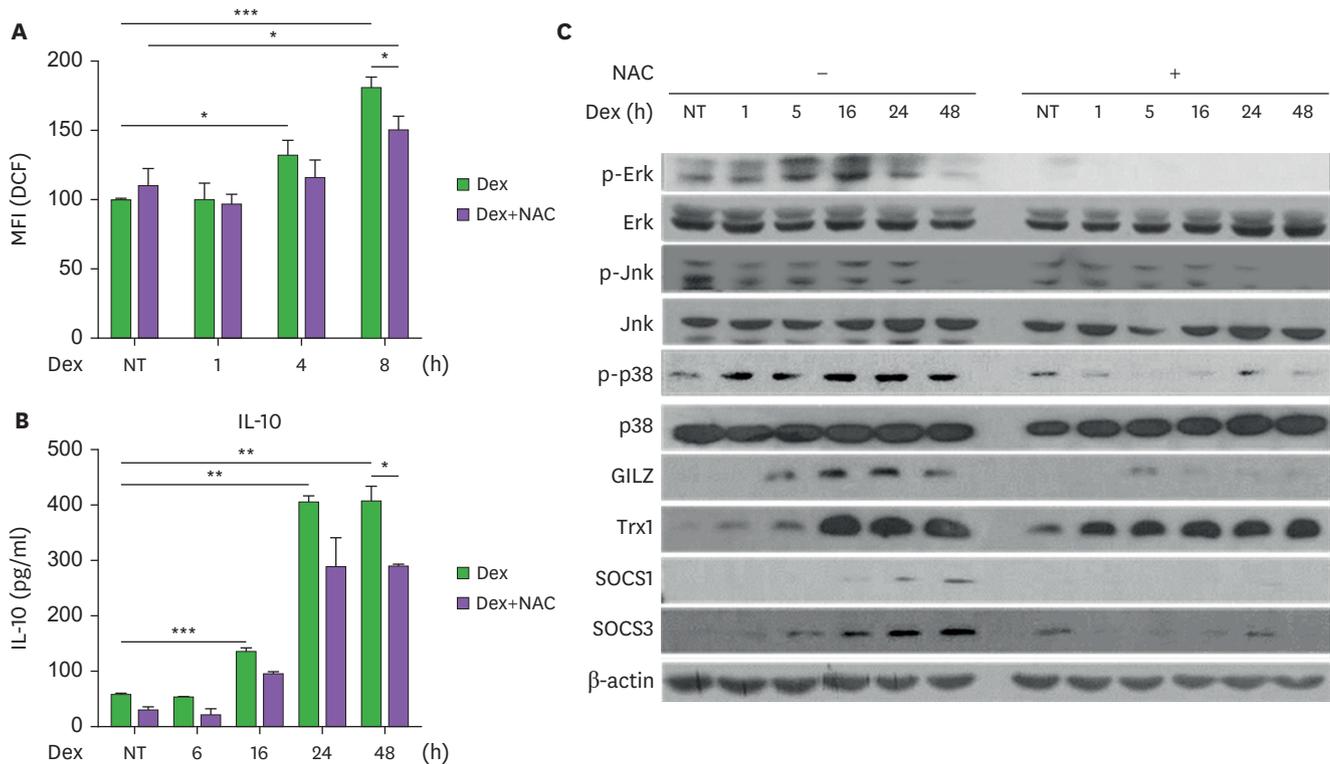
Figure 2. Induction of ROS and MAPKs during Dex-induced M2 polarization of monocytic cells. PMA-differentiated THP1 cells were NT or treated with Dex (40 ng/ml) for the indicated periods with or without NAC (1 mM) pretreatment for 1 h. Cells were harvested and analyzed for ROS generation (A) and IL-10 production (B), where data are shown as mean ± SE. The expression levels of MAPKs, GILZ, Trx1 and SOCS proteins were examined by Western blotting. A representative blot is shown (C).
NT, not treated; MFI, mean fluorescence intensity; DCF, 2′,7′-dichlorofluorescein.

SOCS3 suppresses whereas shSOCS3 promotes Dex-induced M2 polarization of THP1 cells

The regulatory effects of SOCS3 on the Dex-induced M2 polarization were examined using THP1 cells transduced with Flag-SOCS3 or shSOCS3. As compared with mock cells, the induction of IL-10 by Dex was inhibited by 65% in SOCS3-transduced cells, while it was promoted by 240% at 24 h in cells introduced with shSOCS3 (**Fig. 3A and B**). Likewise TGF-β in Dex-treated cells peaking at 12 to 24 h was reduced by 50%–52% in SOCS3-transduced cells, while it was increased by 170%–265% upon shSOCS3 transduction (**Fig. 3A and B**). The data demonstrate that SOCS3 negatively regulates the Dex-induced M2 polarization of THP1 cells.

Role of ROS in Dex-induced IL-10 production and its regulation by SOCS3

Having observed inhibitory effects of anti-oxidant NAC and SOCS3 on the Dex-induced M2 cytokine induction (**Fig. 2**, **Fig. 3**), the ROS-regulating effect of SOCS3 during M2 differentiation was of interest. In SOCS3-transduced cells, the Dex-induced ROS generation was impaired as compared to mock cells (**Fig. 4A**). On the contrary, ROS levels were upregulated in SOCS3-ablated cells (**Fig. 4B**). In fact, the regulation profile of ROS in SOCS3 vs. shSOCS3 cells under M2 polarization by Dex, resembled that of IL-10 and TGF-β levels observed in **Fig. 3**, which suggests ROS as a target of SOCS3-mediated suppression of M2 cytokines induced by Dex. Indeed the increased IL-10 production in shSOCS3 cells was attenuated by NAC (**Fig. 4C**). As reported for the LPS-induced inflammatory signaling (26), SOCS3 over-expressing cells exhibited increased Trx1 levels, while SOCS3-deficient cells expressed significantly reduced Trx1 levels during Dex-induced M2 polarization (**Fig. 4D and E**).
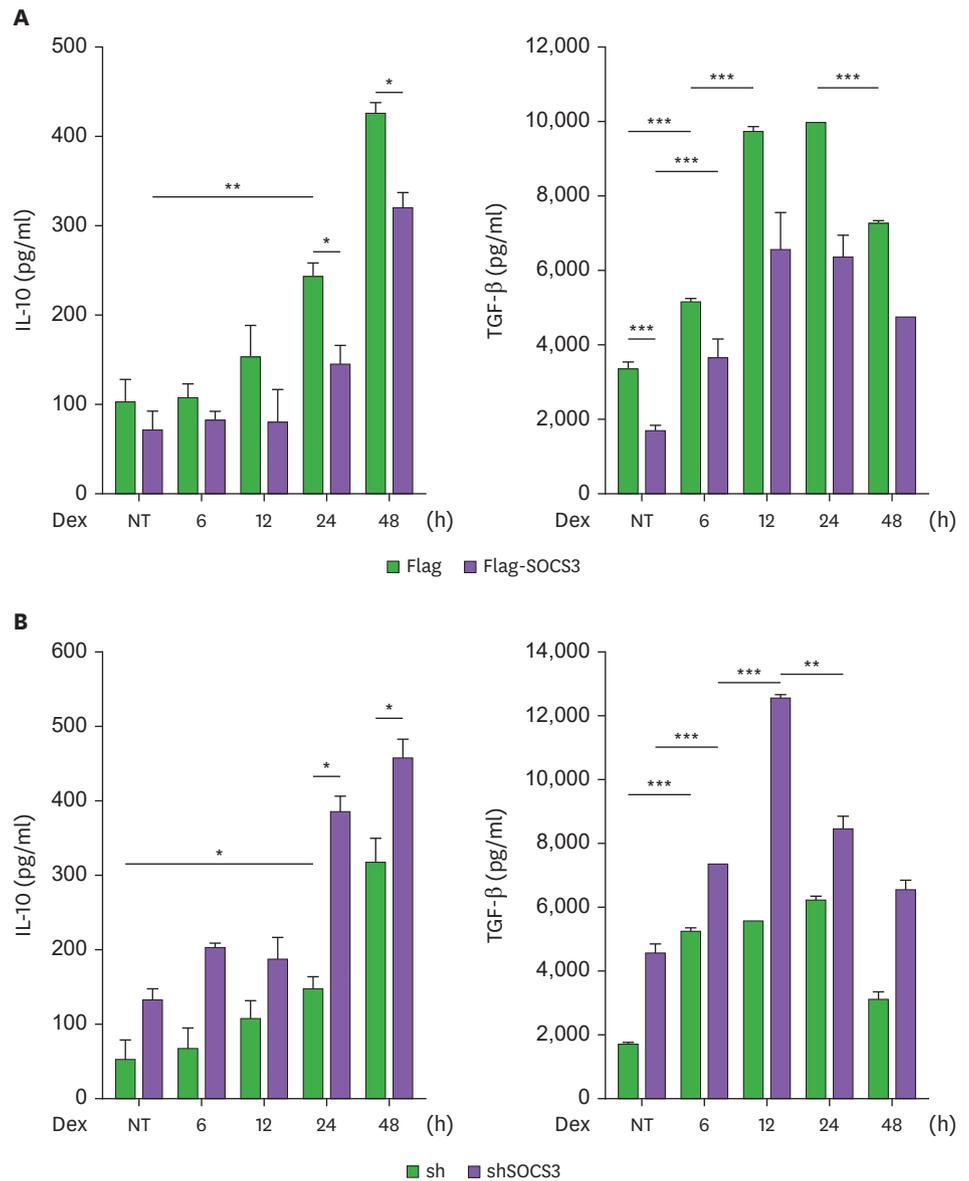
Figure 3. SOCS3 negatively regulates M2 polarization of monocytic cells. THP1 cells transduced with SOCS3 for over-expression (Flag-SOCS3 vs. Flag) and shSOCS3 for knock-down (shSOCS3 vs. sh) were NT or stimulated with Dex for indicated durations. The levels of IL-10 and TGF-β were analyzed by ELISA to assess the effect of SOCS3 on M2 polarization by Dex. ELISA data are are shown as mean ± SE. Statistical significance was determined by a Student's *t*-test.
NT, not treated.
*p<0.05; **p<0.01; ***p<0.001.

The difference in Trx1 expression may reflect the differential ROS scavenging ability of Flag-SOCS3 vs. shSOCS3 cells. For example, the elevated ROS level in shSOCS3 cells is likely due to the reduced Trx1, a key anti-oxidant enzyme in macrophages.

Down-regulation of p38MAPK and GILZ by SOCS3 during M2 induction by Dex

In order to identify molecular targets regulated by SOCS3 involved in ROS-mediated anti-inflammatory cytokine production by Dex in THP1 cells, the signaling pathways activated by Dex were analyzed in mock vs. SOCS3-transduced or shSOCS3-transduced cells. Of note,
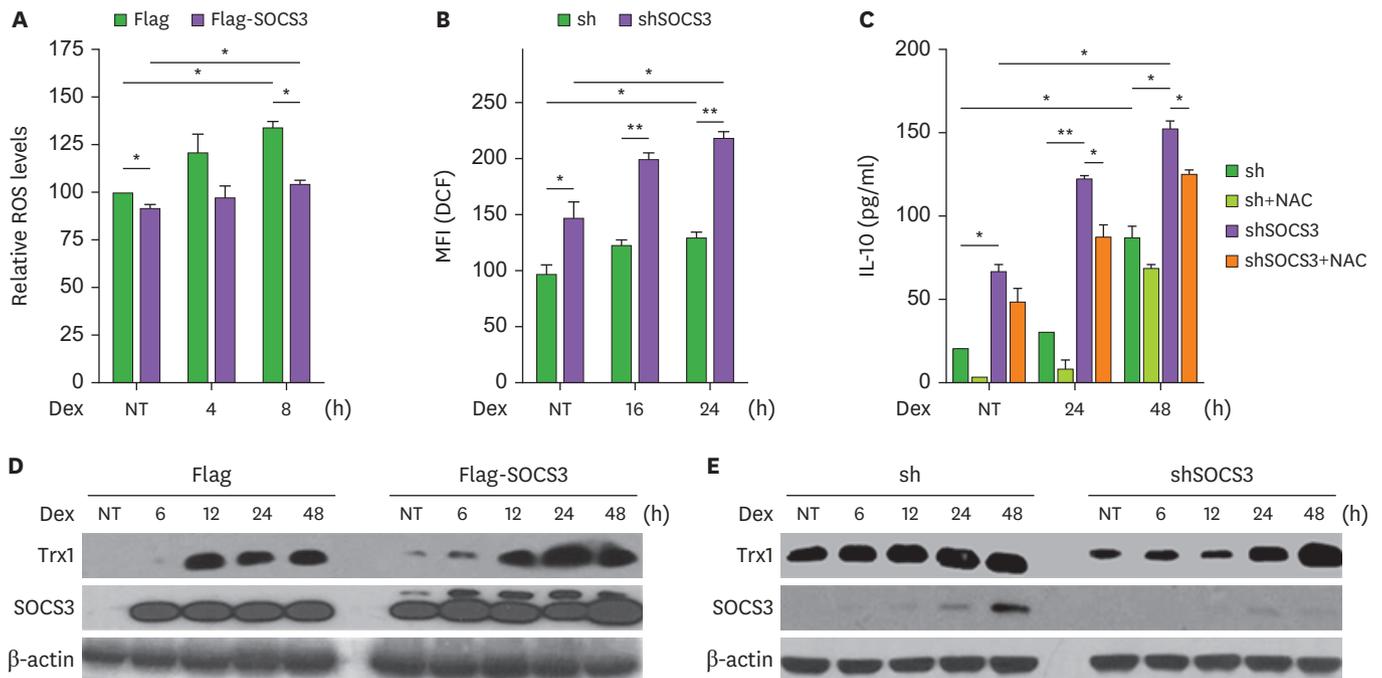
Figure 4. SOCS3 down-regulates ROS which mediates IL-10 production during M2 polarization. Flag-SOCS3 vs. Flag and shSOCS1 vs. sh mock THP1 cells were analyzed for ROS levels NT or upon Dex treatment (A, B). shSOCS3 vs. sh mock cells were pretreated with 1mM NAC and incubated with Dex for indicated durations. IL-10 production was analyzed by ELISA (C). SOCS3- or shSOCS3-transduced THP1 cells were analyzed for Trx1 and SOCS3 expression levels by Western blot under Dex treatment for M2 induction (D, E). Both ROS determination and ELISA data represent mean ± SE obtained from 3 independent experiments. NT, not treated; MFI, mean fluorescence intensity; DCF, 2',7'-dichlorofluorescein.
*p<0.05; **p<0.01.

the p38 activation was reduced with an enhanced MKP-1 expression by Dex upon SOCS3 transduction (**Fig. 5A**). By contrast, SOCS3-depleted cells exhibited high basal p38 activities, which were maintained throughout Dex treatment with significantly suppressed MKP-1 levels (**Fig. 5B**). Hence, there is a reciprocal regulation of p38 activation and MKP-1 levels in Flag-SOCS3 vs. shSOCS3 cells. In addition, GILZ induction was reduced by SOCS3 and promoted by shSOCS3 transduction, which coincided with regulation of M2 cytokine induction and p38 activities in these cells.

As we have observed MKP-1 up-regulation and p38 suppression in SOCS3-transduced cells and the opposite in SOCS3-depleted cells, we have then examined the counter-regulatory role of p38 and MKP-1 by SOCS3 vs. shSOCS3 in the Dex-induced M2 differentiation by conducting inhibitor studies. The results demonstrate that treatment of an MKP-1 inhibitor, not only promoted IL-10 production by Dex, but also restored reduced IL-10 levels caused by SOCS3 over-expression (**Fig. 5C**). On the other hand, the enhanced IL-10 production in shSOCS3 cells was significantly reduced by a p38 inhibitor (**Fig. 5D**), suggesting that the p38 pathway is an important target of SOCS3 action during M2 differentiation by Dex.

Dex-induced M2 polarization in mouse BMDMs and its regulation by SOCS3

To investigate the SOCS3-regulated M2 polarization in primary immune cells, BMDMs from C57BL/6 mice were employed. Upon Dex treatment of BMDMs, IL-10 production was obtained by 16 to 24 h (**Supplementary Fig. 2A**). In addition, there noted a time-dependent increase in ROS levels along with up-regulation of GILZ and CD206 as observed for human monocytic THP1 cells during the M2 polarization response induced by Dex (**Supplementary Fig. 2B and C**). Importantly, the induction of ROS and M2 markers were upregulated in
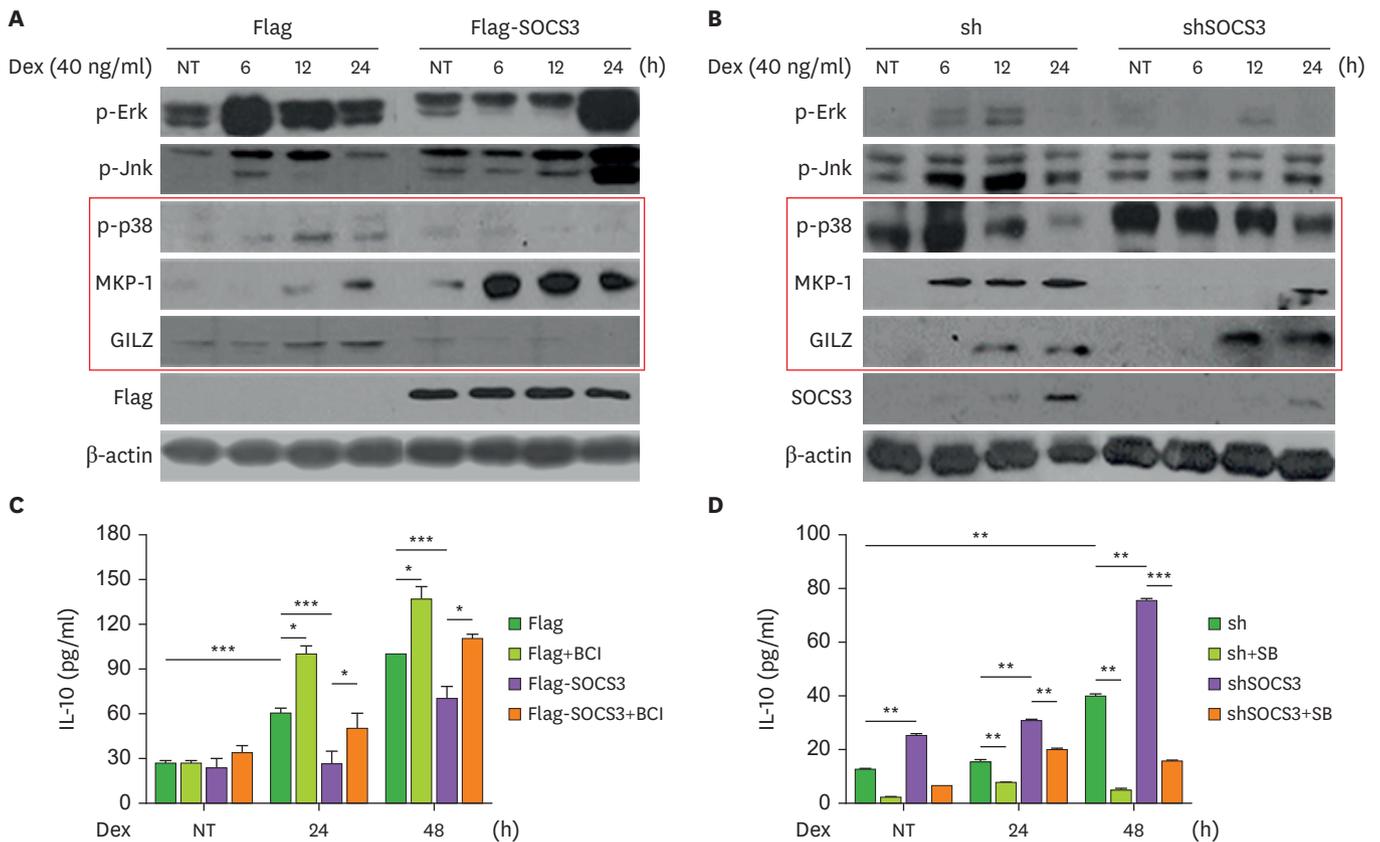
Figure 5. SOCS3 inhibits p38 MAPK and GILZ induction by Dex to suppress M2 polarization. Flag-SOCS3 vs. Flag and shSOCS1 vs. sh mock cells were induced for M2 polarization by Dex treatment. At each time point, cells were harvested and analyzed for pMAPKs, MKP-1 and GILZ expression levels by Western blotting (A and B). Flag-SOCS3 or shSOCS3 cells were treated with inhibitors for MKP-1(BCI) or p38 MAPK(SB), respectively. These cells were induced for M2 by Dex treatment and IL-10 production was analyzed by ELISA (C and D). Data are shown as mean ± SE.
NT, not treated.
*p<0.05; **p<0.01; ***p<0.001.

shSOCS3-introduced BMDM cultures over mock cultures. Not only the Dex-induced IL-10 production in control BMDMs was sensitive to both NAC and SB treatment, the shSOCS3-induced up-regulation of IL-10 were abolished by NAC or SB (**Supplementary Fig. 2D**). These observations indicate that as in the case for THP1 monocytic cell-derived macrophages, Dex induced ROS/p38-mediated M2 polarization and SOCS3 attenuates M2 phenotype acquisition likely through the regulation of ROS/p38-dependent pathways in primary macrophages as well.

SOCS3 targets GILZ which mediates anti-inflammatory cytokine production for M2 polarization by Dex

GILZ is recognized as an inducible protein which participates in the GC-triggered anti-inflammatory response (12). Based on the observation that both of the GILZ expression and p38 activation are attenuated in Flag-SOCS3 cells with M2 suppression, we have postulated that p38-dependent GILZ expression is a target of SOCS3. To test this possibility, effects of p38 inhibition on the expression of GILZ and phenotypic markers of M2 were examined first. Treatment of SB, a representative p38 inhibitor caused a substantial reduction in GILZ induction and a drastic suppression of IL-10 as well as markers of M2 such as Arg1 and CD163 as analyzed by qRT-PCR (**Supplementary Fig. 3A**). Although other MAPK inhibitors such as PD and SP reduced the induction of M2 markers, the Dex-induced GILZ expression was specifically attenuated by the p38 inhibitor in a dose-dependent manner (**Supplementary Fig. 3B and C**).

The result suggests that the p38 pathway is playing a key role in Dex-induced GILZ expression and the overall acquisition of M2 phenotype.

Next, the role of GILZ in the Dex-induced M2 polarization was investigated employing GILZ-ablated cells obtained by transduction of shGILZ into THP1 cells (**Fig. 6**). In GILZ-deficient cells, Dex-induced IL-10 expression was completely blocked as compared to mock cells, which
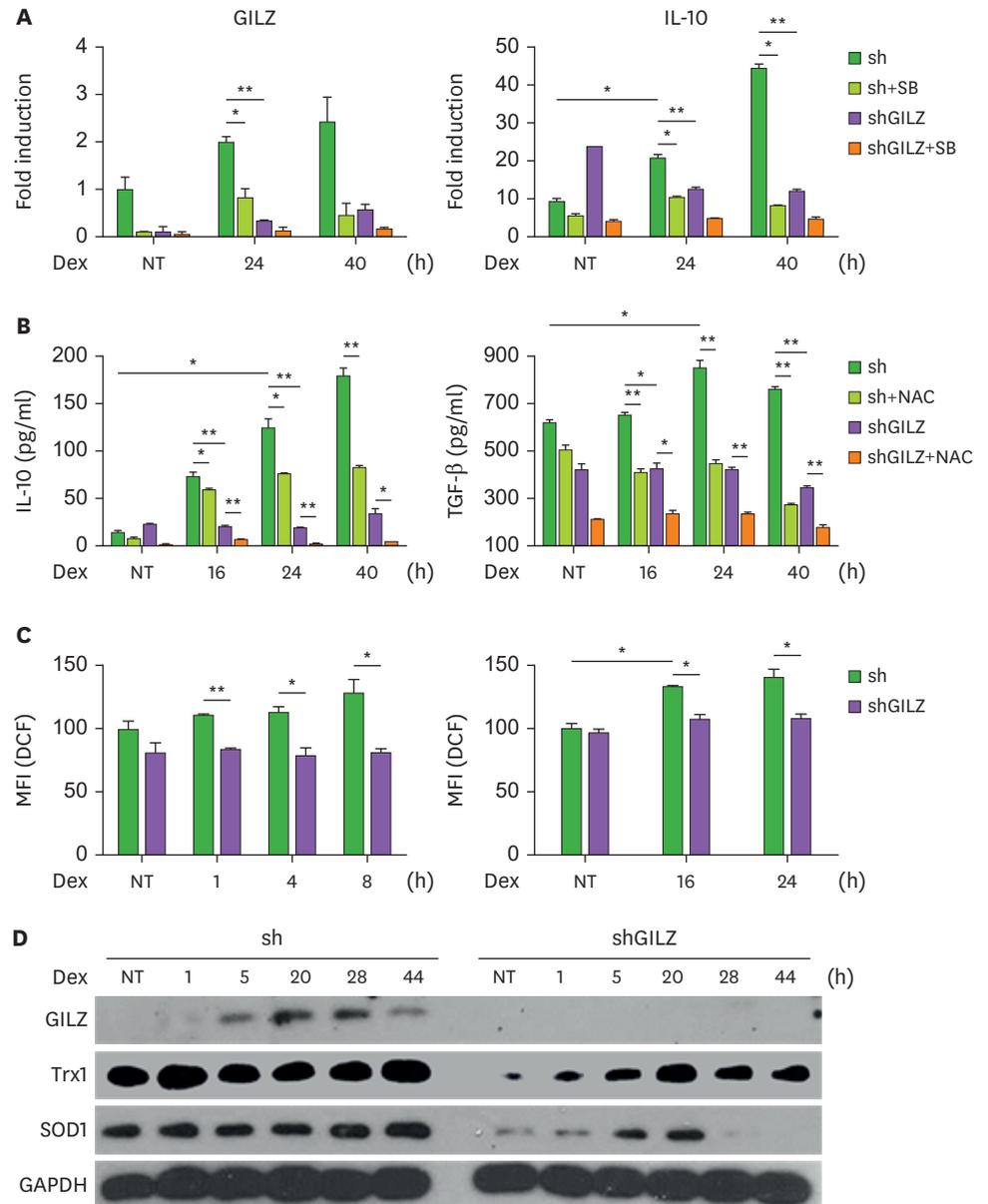

Figure 6. GILZ mediates Dex-induced M2 polarization via ROS- and p38-dependent pathways. GILZ-deficient THP1 cells were generated by viral transduction of shGILZ as described in the text. shGILZ vs. sh mock cells were analyzed for M2-inducing ability to examine the role of GILZ in M2 differentiation. Cells were treated with SB or NAC to compare the role of GILZ with those of p38 and ROS during M2 induction by Dex as shown by qRT-PCR analysis for GILZ and IL-10 (A), and ELISA for IL-10 and TGF-β (B). ROS was measured by 2',7'-dichlorofluorescein staining (C) and anti-oxidant factors were analyzed by Western blotting (D). shGILZ transduction did not adversely affect the cell viability. Both qRT-PCR and ELISA data are shown as mean ± SE.
NT, not treated; MFI, mean fluorescence intensity; DCF, 2',7'-dichlorofluorescein.
$^*p < 0.05$; $^{**}p < 0.01$.

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indicates GILZ-dependency of IL-10 induction (**Fig. 6A**). In fact, the effect of GILZ ablation on IL-10 induced by Dex resembled that of the p38 inhibitor. GILZ knock-down also blocked the production of TGF-β, similar to the effect of NAC treatment (**Fig. 6B**).

Interestingly, it is noted that the GILZ-ablated cells maintain low ROS levels and fail to generate intracellular ROS upon Dex treatment, suggesting that the reduced production of M2 cytokines in shGILZ cells is partly due to the attenuation of Dex-induced ROS signal (**Fig. 6C**). The immunoblot analysis of anti-oxidant proteins indicate that low expression levels of Trx1 and SOD1 in GILZ-deficient cells, which are likely due to the defect in ROS generation required to trigger the induction of the anti-oxidant enzymes (**Fig. 6D**). GILZ ablation not only blocked the M2 differentiation, but also impaired the reduction in pro-inflammatory cytokine IL-6 (**Fig. 7A and B**), suggesting its role during the Dex-induced M2 polarization.
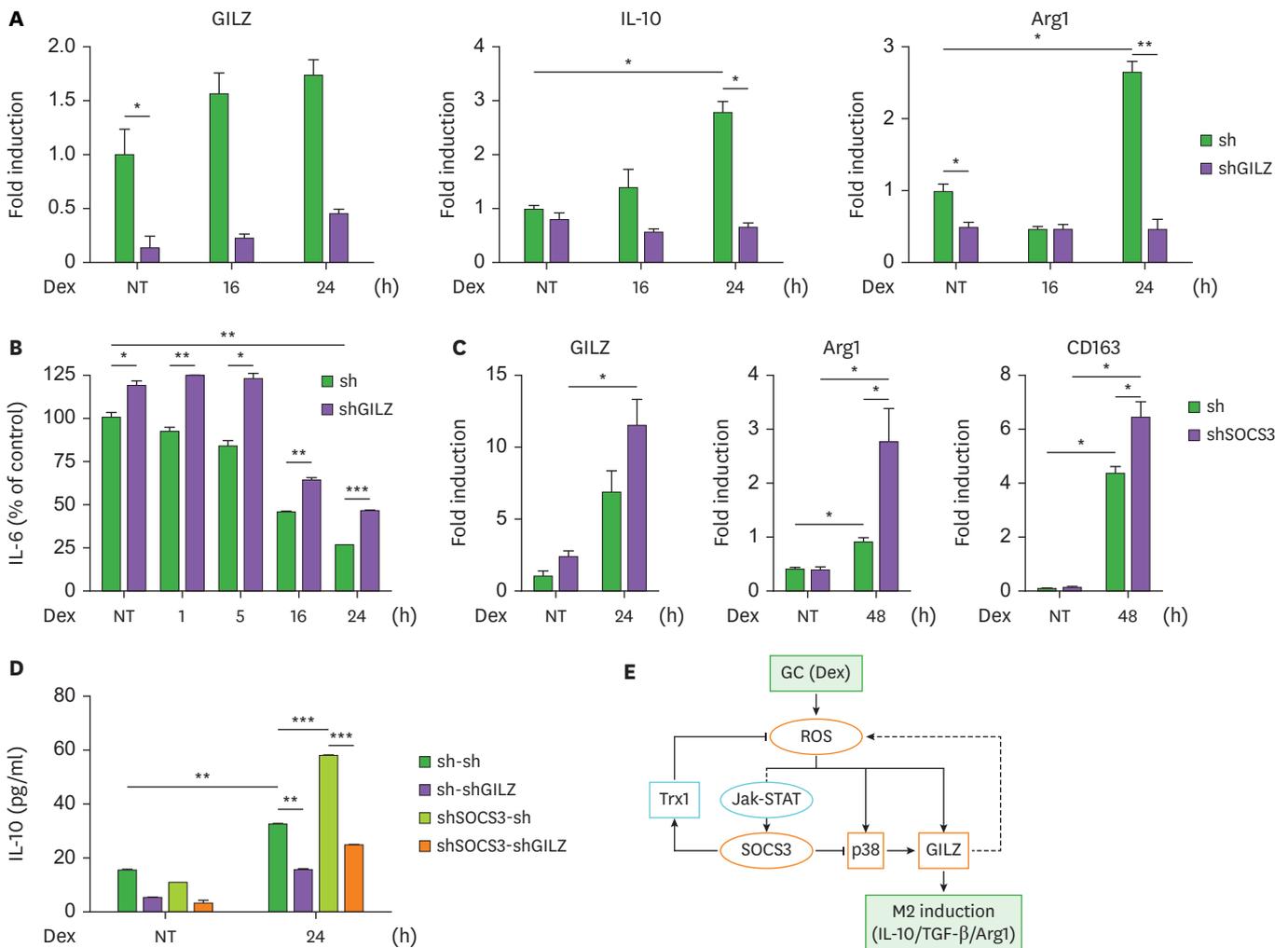

Figure 7. Role of GILZ in the IL-10 up-regulation in shSOCS3 cells. shGILZ vs. sh mock cells were NT or treated with Dex under M2 polarization condition. At indicated time points, the gene expression levels of GILZ, IL-10 and Arg1 were analyzed by qRT-PCR (A) and IL-6 production by ELISA (B). shSOCS3 cells exhibited elevated expression of GILZ, Arg1 and CD163 (C). The introduction of shGILZ into the shSOCS3 cells negated the increase in IL-10 production (D). Both qRT-PCR and ELISA data were shown as mean ± SE. The proposed scheme for the mechanism of SOCS3 action to attenuate the GC-induced M2 induction through the inhibition of GILZ expression via ROS- and p38-dependent pathways (E).
NT, not treated.
*p<0.05; **p<0.01; ***p<0.001.

Finally, while shSOCS3 transduction promoted M2 polarization by Dex, as shown by increased levels of IL-10, Arg1 and CD163, GILZ ablation in these cells negated the shSOCS3-induced increase of IL-10 production (**Fig. 7C and D**). Together, these data indicate that GILZ is a crucial factor participating in the shSOCS3-mediated promotion of M2 induction through p38- and ROS-dependent pathways.

DISCUSSION

GCs are known as strong anti-inflammatory agents and potent inducers of M2. The anti-inflammatory actions of GC have been revealed through a number of *in vitro* and *in vivo* studies. Most of these studies have been conducted focusing on the counter-acting mechanisms of GC action against the inflammatory triggers such as infection, cytokines, and TLR agonists (8,9,13,14,16). On the other hand, the signaling mechanisms of GC directing to M2 polarization are not fully understood (15,29). Therefore, in order to identify the molecular mechanism of SOCS3 action on macrophage polarization by GC, we have first analyzed Dex-induced M2 differentiation of monocytic cells. We have observed an optimal M2 polarization capacity of Dex at 40 ng/ml within 24 to 48 h as shown by effective induction of anti-inflammatory cytokines (IL-10 and TGF-β), a key M2 metabolic enzyme (Arg1), and a surface marker of M2 (CD163), as well as a significant reduction in the production of pro-inflammatory cytokines (**Fig. 1**).

During M2 polarization of monocytic cells, Dex induced a time-dependent ROS generation and MAPK (Erk and p38) activation, which are both sensitive to NAC treatment (**Fig. 2**). In addition, the suppression of Dex-induced IL-10 production by NAC suggests a positive role of ROS signal in M2 differentiation. This is in line with a recent finding that ROS is required during M2 polarization of monocytes induced by IL-4, and supports a notion that ROS generation and its downstream signals are critical in mediating not only M1 but also M2 polarization of macrophages (30).

As in the case for the LPS-induced M1 differentiation (26), the induction of SOCS1 and SOCS3 under the Dex-induced M2 polarization may have incurred by ROS-dependent Jak/STAT activation, which implies the potential role of SOCS as feed-back regulators of Dex-induced M2 polarization. Indeed the negative regulatory effect of SOCS3 on the M2 induction by Dex is clearly demonstrated by a significant reduction of anti-inflammatory cytokines IL-10 and TGF-β upon SOCS3 transduction and substantial increase upon SOCS3 ablation. Upon shSOCS3 transduction, the Dex-induced Arg1 and CD163 expression levels were also promoted, suggesting potent inhibitory effects of SOCS3 on M2 polarization (**Fig. 7C**).

Changes in ROS levels observed in SOCS3-transduced vs. SOCS3-deficient cells correlated well with the M2 cytokine-inducing capacity of Dex. Importantly, the increase in IL-10 levels in shSOCS3 cells was negated by NAC treatment, suggesting that the up-regulation of ROS is responsible for the shSOCS3-mediated promotion of M2 polarization. As a down-stream target of ROS regulated by SOCS3, the potential role of MAPKs and MKP-1 was examined. All 3 MAPKs appear to be involved in the Dex-induced M2 differentiation (**Supplementary Fig. 3A**). Among them, p38 MAPK seems to play a key role in the SOCS3-mediated inhibition of M2 response, as it is reciprocally regulated by SOCS3 vs. shSOCS3 transduction (**Fig. 5A and B**). Moreover, the counter-regulatory effect of the inhibitor of p38 and MKP-1 on the SOCS3-mediated changes in IL-10 production, strongly suggests that SOCS3 targets p38/MKP-1 pathways to down-regulate the Dex-induced M2 polarization (**Fig. 5C and D**).

At present, the mechanism by which Dex induces MKP-1 expression in macrophages is not known. While ROS has been proposed as a signal mediator for the MAPK/MKP-1 induction and activity regulation in human endothelial cells (6,31), the role of ROS in MKP-1 gene expression to control inflammatory activities during M2 polarization is unclear. In addition, the mechanism by which SOCS3 upregulates MKP-1 to reduce p38 activity needs to be investigated in future studies.

In addition to the PMA-differentiated THP1 monocytic cell lines, the suppressive function of SOCS3 on the Dex-induced M2 differentiation is noted in primary macrophages derived from mouse bone marrow. The BMDMs transduced with shSOCS3 displayed increased levels of IL-10 as well as GILZ and CD206 as compared to mock cells. Similar to THP1 cells, Dex-induced M2 polarization of BMDMs appears to involve ROS/p38-medited pathways which are regulated by SOCS3 (**Supplementary Fig. 2**).

An important finding in the present study is the critical role of GILZ in M2 polarization. GILZ was induced during M2 differentiation and its ablation resulted in a striking impairment in Dex-induced M2 cytokine production. Although GILZ is known to mediate anti-inflammatory action through the inhibition of NF-κB, AP-1, and Ras/Raf activated under inflammatory stimuli, no direct roles of GILZ in the GC-induced M2 polarization via regulation of anti-inflammatory molecules have been documented (11,12,32). In the present work, we have demonstrated that GILZ is required for the Dex-induced IL-10, TGF-β, and Arg1 expression, which strongly suggests a crucial role of GILZ in M2 function. Moreover, GILZ is found as a molecular target of SOCS3 which attenuates the production of GC-induced anti-inflammatory cytokines and M2 markers. It is noted that the induction of GILZ by Dex was both ROS- and p38-dependent as the inhibitory effects of SOCS3 on M2 differentiation were. These data indicate that SOCS3 suppresses Dex-induced M2 polarization by targeting GILZ expression via ROS- and p38-dependent pathways.

Through recent years we have reported that SOCS proteins are induced by ROS signal in diverse cell systems including lymphocytes, monocytes/macrophages and tumor cells, mediated by Jak/STAT pathways (26,33-36). We now have shown that Dex treatment also induces ROS generation and activation of STATs, leading to the up-regulation of pY-STAT1, pY-STAT3, and pY-STAT6, which are followed by increase in SOCS3 expression (**Supplementary Fig. 4A-C**). The Dex-induced M2 polarization appears to be dependent on Jak activation and the shSOCS3-mediated promotion of IL-10 production is also sensitive to Jak inhibition (**Supplementary Fig. 4D**). However, which Jak/STAT members are playing a key role in Dex-induced M2 polarization and the role of ROS signal in Dex-induced Jak/STAT activation leading to M2 differentiation are not clear at present.

Still, ROS appears critical in Dex-induced signaling pathways leading to M2 polarization through p38 activation and GILZ expression (**Fig. 2B, Fig. 6A and B**). Thus, the induction of SOCS3, which then targets ROS/p38 and GILZ expression by Dex likely represents a natural negative feed-back loop to attenuate the M2 polarizing function of Dex (**Fig. 7E**).

Using GILZ-ablated cells, we have demonstrated that GILZ is critical in the GC-induced M2 polarization including the induction of IL-10, TGF-β, and Arg1 as well as the reduction of IL-6. We have also shown that the SOCS3-induced regulation of IL-10 by GC is mediated by GILZ. However, the mechanism by which GILZ mediates GC-induced M2 polarization remains unclear. An interesting observation is a lack of ROS generation in GILZ-deficient

cells, which suggests that GILZ, once induced by GC, acts in a positive feed-back loop to promote or maintain ROS levels necessary for the M2 polarization process. As ROS signal is required for M2 differentiation by IL-4 (30), GILZ may serve as a crucial factor in the IL-4-induced M2 function as well.

The regulatory function of SOCS3 on inflammation has been reported to be diverse ranging from pro-inflammatory to anti-inflammatory actions (23-26). SOCS3 is normally kept at low levels in the basal state, while it is induced by infection and inflammatory cytokines in tissues with immune-inflammatory conditions (23,37). We have demonstrated that SOCS3 expression is induced not only by a strong M1 inducer (LPS), but also by a potent M2 inducer (Dex). Under these conditions, SOCS3 exhibited negative regulatory effects to attenuate pro-inflammatory and anti-inflammatory response by the inhibition of M1 and M2 cytokines, respectively. Taken together these findings suggest that SOCS3 can be utilized as a tool for fine-tuning multiple signals to change macrophage phenotypes upon complex environmental cues generated during chronic inflammation and disease progression in diverse model systems.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1
Primers used for qRT-PCR analysis of M2 markers

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Supplementary Figure 1
Determination of cell viability during the Dex-induced M2 polarization. The PMA-differentiated THP1 monocytic cells were not treated (NT) or cultured with Dex under M2-inducing conditions. At indicated time points cell viability was analyzed by performing apoptosis assay using Annexin-V staining (A) and MTT assays (B, C). Flag vs. Flag-SOCS3 cells (D) as well as sh vs. shSOCS3 cells in the presence or absence of NAC treatment (E) were also analyzed for cell viability during Dex-induced M2 polarization. No significant changes in cell viability were noted. Treatment of NAC (2 mM) or SB (30 μM) only slightly affected cell viability during M2 induction with less than 10% reduction in MTT-stained cells.

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Supplementary Figure 2
Inhibitory effect of SOCS3 on the Dex-induced M2 polarization on mouse BMDMs. Mouse BMDMs were prepared from C57BL/6 mice as described. Cells were not treated (NT) or treated with Dex at 40 ng/ml to induce M2 differentiation in the presence of NAC or SB, after which IL-10 production was analyzed using the mouse cytokine ELISA kit (A). SB or NAC

did not affected cell viability under this condition. BMDMs transduced with shSOCS3 were treated with Dex and analyzed for ROS generation (B) and the expression levels of SOCS3, GILZ, and CD206 by qRT-PCR (C). Inhibitory effects of NAC and SB on the shSOCS3-induced increase of IL-10 in BMDM cultures were determined (D).

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Supplementary Figure 3
Role of p38 MAPK in GILZ induction and M2 polarization by Dex. The PMA-differentiated THP1 monocytic cells were not treated (NT) or cultured with Dex under M2-inducing conditions in the presence of MAPK inhibitors and harvested at indicated times. The expression levels of IL-10, Arg1 and CD163 were analyzed by qRT-PCR (A). Cell extracts were prepared to analyze the effect of the MAPK inhibitors on the expression levels of GILZ, and the effect of SB on MAPKs activity by Western blotting (B and C). PD, SP, and SB were used at 30 µM unless otherwise indicated.

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Supplementary Figure 4
Role of Jak/STAT pathways and SOCS3 during M2 polarization by Dex. THP1 cells transduced with shSOCS3 (A) or Flag-SOCS3 (B, C) were not treated (NT) or treated with Dex under M2 polarizing condition. At indicated time points, cells were harvested and analyzed for STAT activation status and SOCS3 expression levels. THP1 cells (sh vs. shSOCS3-transduced) were treated with Jak inhibitor AG490 (10 µM) to examine the role of Jak/STAT activation on the IL-10 induction promoted by shSOCS3 (D).

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