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SOCS3 ablation enhances DC-derived Th17 immune response against *Candida albicans* by activating *IL-6/STAT3 in vitro*

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ABSTRACT

Aims: Enhancing the potency of dendritic cells (DCs) by downregulating negative immunoregulatory pathways may provide immunotherapeutic possibilities against candidiasis.
Main methods: In this study, a si-RNA method is used to repress expression of the cytokine signaling-3 suppressor (SOCS3) in murine bone marrow-DCs, and then the maturation of DCs and the subsequent T-cell response after exposure to *C. albicans* are monitored *in vitro*.
Key findings: Along with a higher expression of the DC maturation markers CD40, CD86 and MHC-II, IL-6/STAT3 is markedly upregulated in the SOCS3 siRNA-treated DCs after exposure to *C. albicans* as compared with control DCs. In response to DCs maturation, $CD4^+$ T cells have an increased expression of Th17 cell markers — including the retinoic acid-related orphan nuclear hormone receptors γt (RORγt), IL-17A and IL-23R — and increased release of IL-17. We note that this enhanced Th17 cell differentiation induced by siSOCS3-treated DCs in presence of *C. albicans* can be partly offset when anti-IL-6 antibody is added into the co-culture.
Significance: As with SOCS1 in our previous report, suppression of SOCS3 alone also has the potential to fully activate DCs maturation. However, while SOCS1 knockdown in DCs during *C. albicans* infection specifically augments Th1 differentiation, SOCS3 silencing particularly increases Th17 differentiation.

1. Introduction

The fungus *Candida albicans* (*C. albicans*), being a commensal organism, can cause invasive infections and even death in patients with critical conditions. > 400,000 life-threatening cases of invasive *C. albicans* infections have been reported each year worldwide in the last three decades [1], due in part to the widespread use of corticosteroids, antibacterial agents, immunosuppressive agents, radiotherapy, and anti-tumor chemotherapies [2]. Despite the availability of several antifungal drugs, failure of such therapies due to acquired resistance is not uncommon. Since T helper type 17 (Th17) cells have been identified as the third T-cell subset after Th1 and Th2 [3], this T-cell subset has received considerable attention for its effects on mediating inflammation in autoimmune diseases [4] and against microbes, and especially against extracellular bacterial and fungal pathogens [5–7].

The host Th17 cell response to fungal infection has therefore been closely investigated to uncover its specific antifungal mechanism. As with Th1 and Th2 immune responses, three signals are required for the differentiation and immune response of Th17: i) TCR-MHC-peptide complex; ii) co-stimulatory molecules; and iii) pro-inflammatory cytokines. Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) that deliver all three signals and thereby determine the character (activity/energy) of naive T cells. Furthermore, DCs are the central architects of the part of the immune response that affect inflammatory or tolerogenic immunity, depending on their developmental stages [8]. For example, mature DCs can efficiently drive the activation and clonal expansion of T cells, thereby prolonging the immune response. In essence, maturation and activation of DCs are the fundamental and necessary steps for an effector T cell response. As such, DCs are highly attractive therapeutic targets and may hold the potential to improve immune response in critical cases.

Suppressors of cytokine signaling (SOCSs) operate in negative

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feedback loops to terminate cytokine signaling through janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway [9,10], which regulates neuronal growth and immune cell differentiation (*e.g.*, neuronal cells, T cells and DCs) [11]. Also, SOCS3 has been assumed to decrease the antigen-presenting function of DCs. While SOCS3 binds to gp130, a common receptor for signal transduction *via* interleukin-6 (IL-6), or JAK1 and JAK2, the signal transduction for DCs activation are then subsequently inhibited [12,13]. Therefore, knockdown of SOCS3 can dramatically enhance the activation of DCs; on the other hand, up-regulation of SOCS3 expression induced by some drugs can inversely inhibit the activation of DCs to decrease Th17-cell differentiation [14,15]. Because the SOCS3-mediated IL-6/STAT3-signaling pathway regulates multiple cytokine signaling pathways in DCs, suppression of SOCS3 has been widely investigated as a potential therapeutic target for autoimmune and infectious diseases [16].

Although several studies have been explored the roles of SOCS3on DC maturation in autoimmune disease, the question of whether silencing of SOCS3 can induce the maturation of DCs in response to fungal infection remains open. In this study, we seek to further understand the specificity of immune response mechanisms of SOCS3-suppression in DCs isolated from murine bone marrow. Using siRNA method, we discovered that the suppression of SOCS3 in murine bone marrow-DCs enhances DC maturation and phagocytosis by promoting the IL-6/STAT3 pathway. The cytokine profile reveals that the immune response favors Th17-cell differentiation in presence of fungi, which is an important host response to against *C. albicans* cells.

2. Materials and methods

2.1. Mice and C. albicans strain preparation

For all animal experiments, we used female C57 mice that were purchased from the Experimental Center of Yangzhou University, Jiangsu Province. The mice were bred in the Animal Experimental Center of the Institute of Dermatology, Chinese Academy of Medical Sciences (CAMS) in Nanjing. At the beginning of the experiments, the mice had an age of 8 weeks and the body weight ranged from 16 to 18 g. The mice were kept in standard laboratory cages at a temperature of 22 ± 4 °C and a photoperiod with a light-dark cycle (12:12 h) and supplied with standard rodent chow and water available *ad libitum.* The Animal Study Committee of the Institute of Dermatology at CAMS reviewed and approved all animal care and experimental procedures used in this study; all methods were performed in accordance with the relevant guidelines and regulations. Wild type of *C. albicans* (SC5314) strain was cultivated on YPD plates. Prior to the experiment, the fungal cells were cultivated overnight in liquid YPD on a shaker at 25 °C.

2.2. Generate dendritic cells

We cultured and prepared the bone marrow-derived DCs following the methods as fully described elsewhere [32], with minor modifications. In brief, bone marrow-derived cells were flushed aseptically from femurs and tibiae of female C57 mice, and were washed and cultured in completed RPMI 1640 (Invitrogen, Life Technologies, Eugene, OR) containing 10% FCS (GIBCO, Grand Island, NY), 100 U/ml penicillin, 100 mg/ml streptomycin, and 100 mM L-glutamine and, supplemented with 2 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF). At day 2, we added 5 ml of RPMI 1640 medium containing rmGM-CSF (200 U/ml) to the culture. At days 4, 6, and 8, we harvested and centrifuged halves of the culture supernatants. Then, each cell plate as re-suspended using 5 ml of fresh RPMI 1640 medium containing rmGM-CSF (200 U/ml), and were returned to the original culture plate. At day 8, we collected DCs for the experiments. Flow cytometric analysis confirmed that > 95% of the cells in the culture plate were $CD11c^+$ cells.

2.3. siSOCS3 design and transfection

We designed the siRNAs targeting the murine SOCS3 gene by using the methods described elsewhere [25]. The three sense strand sequences of siRNA target sites that are designed to silence murine SOCS3 gene are: siSOCS3 #1 (5′- AGAGCTTACTACATCTATT-3′), siSOCS3 #2 (5′-GCTTCGACTGTGTACTCAA-3′), siSOCS3 #3 (5′-GATTCTACTGGAG CGCCGT-3′), and the control siRNA (5′- CAGCCTTCCTTCTTGGG TAT-3′). We used the recombinant lentivirus expression vector GV248 as the vector for SOCS3 siRNA, and the non-targeting siRNA as negative control. We conducted transient transfection of siRNA with the lipofectamine 2000 reagent (Invitrogen) following the manufacturer's instructions.

2.4. Cytotoxicity assay

The tolerance of DCs to SOCS3 silencing was tested by measuring the staining capacity of an FITC-coupled Annexin V (BD Bioscience, USA) [25]. Briefly, DCs were seeded at 1×10^5 cells/well in 24-well plates. DCs were incubated for 24 h and treated with siSOCS3 or with non-targeting control siRNA. They were incubated for 20 min in Cytofix/Cytoperm solution on ice, with the concentration of 2×10^6/ml. After subsequent re-suspension in 100 μl Perm/Wash buffer (BD Biosciences, San Jose, CA, USA), the cells were incubated with 20 μl Annexin-v-FITC (BD Biosciences, San Jose, CA, USA) for 15 min in the dark at room temperature. Cells were stained with PI for 3 min and were analyzed using flow cytometry. Fractions of the culture were labeled with trypan blue (Sigma, USA) to determine cell viability.

2.5. DCs phagocytosis

DCs with and without SOCS3 silencing were harvested, washed, and plated in 96-well plates at a density of 5×10^5 in 100 μl per well for 2 h. Cells of *C. albicans* (1×10^8) labeled with FITC (Sigma, 1.25 mM in 0.1 mM sodium bicarbonate buffer with 0.5% DMSO, pH 9.0) were cultured overnight at 4 °C, and then collected. Labeled yeast cells were washed 3 times with PBS to remove unbound dye, and re-suspended in 100 μl of DMEM with 10% FBS and co-cultivated with a DCs:yeast ratio of 1:1 at 4 °C for 15, 30, 45, 60 and 75 min. DCs were labeled with PKH-26 (Sigma-Aldrich). At each time point, we added 100 μl of trypan blue (250 mg/ml in PBS) to quench the fluorescence of yeast that was bound but not internalized, and then incubated at room temperature for 1 min. The cells were collected and analyzed using flow cytometry. In parallel, we used a laser scanning confocal microscope to visualize the internalized and fluorescent of yeast.

2.6. Assay of DC killing capacity

DCs were prepared and seeded in a 96-well plate of RPMI 1640 at the concentration of 5×10^4 in 150 μl for 12 h at room temperature (37 °C), as previously reported [33]. We diluted the overnight cultures of the fungal strain with DMEM to a concentration of 2×10^6/ml. We added 50 μl aliquot to the first column of the plate, mixed with DCs, then serially diluted 1:4 for six times. Strains incubated without DCs were diluted in parallel. We incubated all plates at 37 °C for 24 h in an atmosphere with 5% CO_2. Colonies of fungus were collected and diluted in DMEM, and the number of fungal cells was counted using a microscope. We calculated the killing rate of each strain as (1 - ((numbers of fungal colonies with DCs)/(number of fungal colonies in the absence of DCs))) × 100%. Each experiment was repeated seven times.

2.7. Measurement of surface antigen expression in DCs infected with C. albicans

To observe the mature state of DCs, immature DCs were co-cultured with *C. albicans*, as described above. Then DCs were collected, and the

Fig. 1. SOCS3 enhances DC phagocytosis as well as the killing of *C. albicans in vitro*. (a) Here untreated DCs served as the negative control. DCs were exposed to FITC-labeled *C. albicans* at a DC: yeast ratio of 1:1 for 3 h, then washed with PBS three times. Cells were collected and stained with PKH-26 to detect phagocytosis of *C. albicans*; (a1 and a5) Laser scanning confocal microscope (LSCM) images of FITC-labeled *C. albicans*; (a2 and a6) LSCM images of PKH-26-labeled DCs; (a3 and a7) Merged images; (a4) Magnified merged image of a3, a8 magnified merged image of a7. (b) DCs were exposed to FITC-labeled *C. albicans* at a DC: yeast ratio of 1:1 for 75 min, then were washed with PBS and examined using flow cytometry. (c) DCs were exposed to FITC-labeled *C. albicans* at a DC: yeast ratio of 1:1 or 1:2 for 75 min. A serial PBS-diluted mixing of each DCs-*C. albicans* co-culture was inoculated on the SDA plates and cultured overnight at 30 °C. The fungal colonies were counted. The killing rate of each strain is calculated as ((100 - [number of fungal colonies with DCs]/number of fungal colonies that are DC-free) × 100%). Data are represented as mean ± SD of seven independent experiments. $^{**}p < 0.01$.

expression of surface molecules of DCs was assessed using flow cytometry with FITC-coupled anti-CD11c, PE-coupled anti-I-A/anti-I-A/I-E, PE-coupled anti-CD40 (Clone 3/23), PE-coupled anti-CD80 (Clone 16-10A1) and PE-coupled anti-CD86 (Clone GL1) (eBioscience Inc., San Diego, CA, USA). We used FITC Mouse IgG2aκ Isotype as isotype control for CD11c, and PE Mouse IgG2aκ for MHC-II, CD40, CD40 and CD86. We analyzed the samples using flow cytometry and CellQuest software (FACSCalibur, Becton Dickinson, USA).

2.8. Migration of DCs

We used the 24-well transwell plates from Millicell (5.0 μm; Millipore, Bedford, MA, USA) in the experiments [34]. The suspensions of DCs prior to the treatment with or without SOCS3 siRNA were co-cultured with or without *C. albicans* in the upper well. Complete medium containing 100 nM CCL19 were added to the lower chamber. We incubated the cultures at 37 °C for 3 h. Then, the cells in the lower wells were collected and counted to calculate the percentage of the cells that had been transfused to the lower well. The procedure was repeated seven times.

Meanwhile, DCs prior to the treatment with SOCS3 siRNA were co-cultured with *C. albicans,* or without *C. albicans* as a control. Then, CCR7 expression of DCs were measured using flow cytometry with PE-conjugated rat anti-mouse CCR7 (Clone 4B12, BD Biosciences, San Jose, CA, USA). PE Mouse IgG2aκ was used as isotype control.

2.9. Analysis of cytokine secretion by DCs

At day 8, immature DCs were treated with SOCS3 siRNA at 37 °C for 24 h. Then, DCs were either stimulated with *C. albicans* for 48 h or kept as a control group. The levels of IL-4, IL-6, IL-12, IL-23, IL-1β, TNF-α and TFG-β in the supernatants were measured using ProcartaPlex Mulplex (eBioscience Inc., San Diego, USA) with Luminex 200.

2.10. RNA isolation and qRT-PCR

We purified total RNA by using TRIzol reagent (Invitrogen) following the manufacturer's instructions. cDNA was synthesized with 1 μg

Fig. 2. DCs treated with SOCS3 siRNA show a maturation phenotype. (a) DCs are treated with or without siSOCS3 for 2 h, and co-cultured with *C. albicans* for 24 h. Then, cells were collected and the expression of co-stimulatory molecules was detected using flow cytometry. Data from one of seven independent experiments are shown. The black lines represent unstained negative control cells; the red lines represent control DCs stimulated with or without *C. albicans*, SOCS3 siRNA-treated DCs co-cultured with or without *C. albicans,* respectively. The upper right numbers represent the mean fluorescent intensity (MFI). (b) Data represent mean ± SD of seven independent experiments. Y-axis represents the mean fluorescent intensity (MFI). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. NS: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

of total RNA by reverse transcriptase (Takara). Supplemental Table 1 lists the primers used for measuring the transcript levels for each gene. Primers for each tested gene and for the reference gene were designed using Primer Express software (Applied Biosystems). Data were analyzed using the comparative cycle threshold method to obtain quantitative measurements.

2.11. Western blotting

We used western blot to determine the protein levels of total STAT3, p-STAT3 and SOCS3. The lysates were separated on 10% SDS-PAGE (Bio-Rad) and transferred to polyvinylidene difluoride membrane. The membranes were blocked for 2 h at room temperature with 5% non-fat milk in TBS (20 mM Tris and 500 mM NaCl) supplied with 0.1% Tween 20. The membranes were then incubated with primary Abs against p-STAT3 (Tyr705), total STAT3 (124H6), and SOCS3 overnight at 4 °C; all Abs were rabbit polyclonal Ab and purchased from Cell Signaling Technology, Inc. Secondary HRP-conjugated Ab was added at a ratio of 1:2000 for 1 h at room temperature. After three additional washes, the signal detection was carried out with an ECL Detection System (Santa Cruz Biotechnology, USA), and the protein bands were quantified by using ImageJ.

2.12. Assays of T cell proliferation

$CD4^+$ T lymphocytes were separated from spleen of C57 mice using a $CD4^+$ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany). We used flow cytometry with PE-conjugated anti-CD4mAbs (eBioscience Inc., San Diego, CA, USA) to confirm that the cells purity reached > 95% in the cell population.

Fig. 3. SOCS3 silencing enhances the migration of DCs and CCR7 expression. (a) Transwell experiments were performed using 24-well plates (Millicell, 5.0 μm). The suspensions of SOCS3 siRNA-treated *vs.* non-treated DCs were placed in the upper well; then *C. albicans* were added in the assigned panels. Complete medium containing CCL19 was added to the lower chamber. The cells in the lower wells were collected and counted to calculate the percentage of the cells migrated to the upper wells. Y-axis represents the percentage of DC migration. (b) Immature DCs were exposed to medium alone, or treated with SOCS3 siRNA, and then *C. albicans* was added to induce maturation for 24 h. CCR7 expression on DCs was analyzed by rat anti-mouse CCR7 antibody. The cells were collected for detecting the expression of CCR7 protein on DCs using flow cytometry. Representative data from one of the seven experiments are shown. The black lines represent rabbit IgG isotype control antibody, and the red lines represent FITC-CCR7 positive cells. The upper right numbers represent the mean fluorescent intensity (MFI). (c) Y-axis represents the percentage of CCR7 positive DCs. The figure shows the mean ± SD of data from seven independent experiments. *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$. NS: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We assessed T cell proliferation by CFSE-labeled T cells, as previously described [34], with minor modifications. Briefly, $CD4^+$ T cells isolated from the spleen of C57 mice were incubated with 10 μM CFSE (Invitrogen, Carlsbad, CA, USA) at 37 °C for 8 min. Parallel DCs were incubated with *C. albicans* at 37 °C for 24 h, then washed and co-cultured with the CFSE-labeled allogeneic T cells at the DCs: T cell ratios of 0, 1:10, 1:20, 1:40 and 1:80 in RPMI containing 5% FCS for 2 days at 37 °C in triplicate. After 2 days, we examined T-cell proliferation by formation of T cell clusters under a microscope, and the proliferation of $CD4^+$ T cells were determined using flow cytometry.

2.13. Determination of cytokine production by $CD4^+$ T cells

Purified $CD4^+$ T cells (1×10^5) were co-cultured with irradiated DCs (1×10^4, 30 Gy). Recombinant murine anti-IL-6 (R&D Systems, USA) at 10 ng/ml was added to T cell cultures that had been stimulated with DCs following incubation with SOCS3 siRNA. After two days, cells were washed with PBS containing 1% FBS, and stained with the monoclonal antibody anti-CD4-APC (Clone RPA-T4) (BD Biosciences, San Jose, CA, USA). Then, cells were washed using PBS containing 1% FBS and re-suspended in Fixation/Permeabilization Solution (BD Biosciences, San Diego, CA, USA). After permeabilization, cells were washed with Perm/Wash/Buffer (BD Biosciences, San Diego, CA, USA) and stained with the monoclonal antibodies: anti-IFN-γ-FITC (Clone B27), anti-IL-17A–PE (Clone N49–653), anti-TNF–FITC (Clone MAb11) or anti-IL-4-PE (Clone 8D4–8). IgG1κ-FITC (Clone MOPC-21) for IFN-γ and TNF, IgG1κ-PE (Clone MOPC-21) for IL-17A and IL-4 (BD Biosciences, San Jose, CA, USA) were used as isotype controls. All staining and permeabilization were performed for 20 min in the dark at 4 °C. After intracellular staining, cells were washed with Perm/Wash

Fig. 4. SOCS3 silencing enhances the migration of DCs and CCR7 expression. (a) Transwell experiments were performed using 24-well plates (Millicell, 5.0 μm). The suspensions of SOCS3 siRNA-treated *vs.* non-treated DCs were placed in the upper well; then *C. albicans* were added in the assigned panels. Complete medium containing CCL19 was added to the lower chamber. The cells in the lower wells were collected and counted to calculate the percentage of the cells migrated to the upper wells. Y-axis represents the percentage of DC migration. (b) Immature DCs were exposed to medium alone, or treated with SOCS3 siRNA, and then *C. albicans* was added to induce maturation for 24 h. CCR7 expression on DCs was analyzed by rat anti-mouse CCR7 antibody. The cells were collected for detecting the expression of CCR7 protein on DCs using flow cytometry. Representative data from one of the seven experiments are shown. The black lines represent rabbit IgG isotype control antibody, and the red lines represent FITC-CCR7 positive cells. The upper right numbers represent the mean fluorescent intensity (MFI). (c) Y-axis represents the percentage of CCR7 positive DCs. The figure shows the mean ± SD of data from seven independent experiments. *$p < 0.05$; **$p < 0.01$. ***$p < 0.001$. NS: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Buffer, suspended in PBS containing 1% FBS and collected using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA).

Furthermore, the gene expression of the transcription factors for each T-cell subset (*e.g.*, Tbet for Th1, GATA3 for Th2, and RORγt for Th17) were measured by qRT-PCR. The primers and probe sequences and concentrations (Supplemental Table 1) were optimized following the manufacturer's instructions for TaqMan Universal PCR Master Mix Protocol part number 4304449 revision C. The expression of mouse MMP-3 mRNA was measured using TagMan Gene Expression Assay (Mm00440295 m1, Applied Biosystems).

2.14. Statistical analyses

All results are presented as mean ± SD. Statistical significance ($p < 0.05$) between groups were tested using one-way ANOVA (GraphPad Prism 5.01, GraphPad, San Diego, CA, USA), followed by Student–Newman–Keuls analyses. Data analyses were performed in a blinded fashion.

3. Results

3.1. SOCS3 small interfering RNA (siRNA) has no effect on DC viability

The down-regulation of SOCS3 expression in murine bone marrow-DCs has been achieved by using siRNA technology. To ensure that this silencing gene has minor effects on DCs viability, we measured the viability of SOCS3 siRNA-treated DCs by using Annexin-V staining, a marker for early apoptosis. We observed that < 4% of DC cells are stained positively with FITC-Annexin-V mAb following treatment with either SOCS3 siRNA or non-target siRNA (Supplemental Fig. 1a–c), indicating that both SOCS3 siRNA and the non-target siRNA are not unacceptably toxic to DCs. We also confirm these results with a trypan blue exclusion test, in which few cells are positively stained regardless of their treatments (Supplemental Fig. 1d).

3.2. SOCS3-siRNA-transfection effectively suppresses SOCS3 at both gene and protein levels

To verify the efficiency of SOCS3 siRNA, the expression levels of SOCS3 gene and protein were measured *in vitro* using qRT-PCR and western blot analysis, respectively. The levels of SOCS3 mRNAs in different groups of DCs were quantified by qRT-PCR, and normalized to the β-actin levels (see Supplemental Table 1 for the primers). Since SOCS3-siRNA #2 was more stable and more effective in inhibiting SOCS3 expression at gene and protein levels when compared with SOCS3-siRNA #1 and SOCS3-siRNA #3, the SOCS3-siRNA #2-infected DCs were used in all subsequent experiments of this study. Supplemental Fig. 2(a, b and c) shows that the expression of SOCS3 at

Fig. 5. Ablation of SOCS3 promotes Stat3 expression. At day 8, iDCs were treated with and without siSOCS3 for 2 h prior to being co-cultured with or without *C. albicans* for 6 h, then cells were collected for detecting Stat3 and p-Stat3 protein by western blotting. (a) Mean ± SD of data from seven independent experiments are shown. Y-axis represents the protein expression of Stat3 with β-actin serving as a reference gene. (b) Mean ± SD of data from seven independent experiments are shown. Y-axis represents the protein expression of pStat3 with β-actin serving as a reference gene. (c) Stat3 and pStat3 protein expression. Representative data from two of the seven experiments are shown. In the panel c1 and c2, lanes 1, 2, 3, 4, 5 and 6 represent untreated control DCs, non-target siRNA DCs, siSOCS3-treated DCs, control DCs co-cultured with *C. albicans,* non-target siRNA DCs co-cultured with *C. albicans*, and siSOCS3-treated DCs co-cultured with *C. albicans*, respectively. $^{**}p < 0.01$; $^{***}p < 0.001$. NS: not significant.

the gene and protein levels are significantly decreased in SOCS3-siRNA #2-transfected DCs.

Next, we measured the durational time of silencing of SOCS3 in this model with siRNA method. We find that the inhibition of SOCS3 at gene levels is in a time-dependent manner. The inhibitory effect reaches its peak at 24 h after siRNA treatment in the DCs and starts to reduce after 48 h. After 72 h, the gene expression of SOCS3 relative to control DCs is about 82% (Supplemental Fig. 2d), and SOCS3 gene expression shows no significant difference between siRNA treated and untreated DCs after that time.

3.3. SOCS3 silencing in DCs increases fungal phagocytosis and killing capacity

An important function of DCs is to capture and present antigens to effector immune cells. To explore whether SOCS3 silencing can affect phagocytosis capacity, *C. albicans* cells were co-cultured with DCs. We use laser scanning confocal microscopy (LSCM) to observe the antigen (*C. albicans* cell) uptake both in the SOCS3 siRNA-treated DCs (Fig. 1a) and untreated DCs. DCs were exposed to FITC-labeled *C. albicans* at a ratio of DC: yeast = 1:1 or 1:2 for 3 h, then washed with PBS, and examined by flow cytometry. The phagocytosis of fungal cells in all DCs groups reaches a peak at 60 min post *C. albicans* infection (Fig. 1b) and then declines as the co-cultured time extends. The SOCS3 siRNA-treated DCs have a higher phagocytotic activity from 15 to 75 min with *C. albicans.* We next compare the fungal killings between SOCS3 siRNA-treated and untreated groups. The results show that after 24 h of co-culture with *C. albicans*, the killing rate is higher in the SOCS3 siRNA-treated DCs than for the DCs in the control group (Fig. 1c). These data suggest that SOCS3 siRNA-treated DCs have a greater uptake rate and killing capacity for *C. albicans* than do untreated DCs.

3.4. SOCS3 silencing promotes DC maturation in response to C. albicans

Maturation of DCs requires upregulation of the expressions of MHC-II and other co-stimulatory molecules [8]. To determine whether SOCS3 siRNA modulates the maturation of DCs in response to *C. albicans* infection, we incubated SOCS3 siRNA-treated DCs with and without exposure to *C. albicans.* The DC surface markers MHC-II, CD86, CD80 and CD40 were used in flow cytometry analysis to measure the response to *C. albicans* co-inoculation. There is no significant difference between SOCS3 siRNA-treated DCs and control DCs without *C. albicans* stimulation. All DC groups without *C. albicans* showed a typical immature phenotype at the beginning of the experiments with a low level of MHC-II, CD86, CD80 or CD40. The DCs undergo maturation when co-inoculated with *C. albicans,* as indicated by the significantly increased expression levels of MHC-II, CD86, CD80 and CD40. The maturation is enhanced by reducing SOCS3, indicated by increased expression levels of MHC-II, CD86 and CD40 in the SOCS3 siRNA-treated DCs compared to control DCs co-cultured with *C. albicans* (Fig. 2). However, the expression level of CD80 remains the same in siSOCS3-treated and

Fig. 6. DCs treated with siSOCS3 RNA in response to *C. albicans* enhance the activation of T cells. Purified $CD4^+$ T cells were counted and seeded into 96-well plates at 10^5 cells per well at room temperature with an equal volume of 2.5 μm CFSE. DC cells exposed to medium alone, to non-target siRNA, or to SOCS3 siRNA were treated with *C. albicans* for 24 h to stimulate T cells. We added 500, 2000 or 10,000 treated DCs to triplicate wells. After 5 days, the cells were collected and stained with PE-conjugated anti-CD4 mAbs for flow cytometry analysis. Y-axis represents the percent of $CD4^+$ T cells that are proliferating. X-axis represents different ratios of DC-to-T cells. Mean ± SD of data from seven experiments are shown. $^{**}p < 0.01$; $^{***}p < 0.001$. NS: not significant.

-untreated DCs in the presence of *C. albicans* (data not shown). These results suggest that suppression of SOCS3 can accelerate DC maturation in response to *C. albicans* infection.

3.5. SOCS3 silencing promotes DC migration or CCR7 expression

After activation, DCs migrate to the T cell zone of the secondary lymphoid organs and present antigens to initiate the immune response against fungal infections. To test whether SOCS3 silencing affects the migration of DCs, we measured the migration in an *in vitro* transwell system and the CCR7 expression on DCs was detected by flow cytometry. Compared to DC groups without *C. albicans*, the migration of DCs pre-incubated with *C. albicans* is slightly increased in control DC groups or non-target siRNA-treated DCs, but is significantly enhanced in SOCS3 siRNA-treated DCs (Fig. 3a). Homing chemokines (*e.g.*, CCL19 and CCL21) and chemokine receptor CCR7 have been associated with DC migration. Here the CCR7 expression on DCs was analyzed by flow cytometry. We find that the expression level of CCR7 in each DC group corresponds to the DCs migration phenotypes. As shown in Fig. 3b and c, the basal levels of CCR7 expression among control DCs, non-target siRNA-treated DCs, and SOCS3 siRNA-treated DCs are barely detectable without *C. albicans* stimulation. However, after inoculation with *C. albicans,* the CCR7 expressions in all DC groups are greatly increased with the highest being in SOCS3-silenced DCs. By suppressing SOCS3, the expression of CCR7 and migration of DCs are enhanced in the response to *C. albicans* infection.

3.6. SOCS3 silencing activates IL-6/STAT3 at the gene and protein levels in response to C. albicans

The initiation of adaptive immune response depends upon cytokines released by DCs. For example, IL-6 binding to the gp130 receptor results in SOCS3 activation, which is a transcriptional target gene of the STAT3 signaling pathway. IL-6, as a pivotal cytokine promoting Th17-cell differentiation [17], plays an important role in host response to fungal infection. IL-12 and IL-4 are essential cytokines as well that respectively induce Th1 and Th2 differentiation from naïve (Th0) cells. To clarify the effects of SOCS3 silencing on T-cell polarization, we measured the activity of these cytokines. Our results show that the basal levels of IL-6, IL-4 and IL-12p70/IL-12p35 expression in DCs without *C. albicans* stimulation are very low regardless of trans-infection of SOCS3 siRNA. However, the gene and protein expression levels of IL-6 and IL-12p70/IL-12p35 in DCs are significantly increased upon *C. albicans* stimulation (Fig. 4). Moreover, with *C. albicans* stimulation, protein level of IL-6 in SOCS3 siRNA-treated DCs is higher than non-target siRNA and untreated DC controls. In contrast with IL-6, there are no significant differences in IL-12p35 mRNA abundance and IL-12p70 protein among control DCs, non-target siRNA-treated DCs and SOCS3 siRNA-treated DCs upon *C. albicans* stimulation. Finally, the mRNA and protein levels of IL-4 are not significantly affected by *C. albicans* infection even if SOCS3 silencing has been induced into the DCs (data not shown).

Since IL-6 expression is altered by SOCS3 silencing, we next evaluate the STAT3 pathway activation by measuring p-STAT3 and total STAT3 proteins by western blot. As shown in Fig. 5, without *C. albicans* stimulation there are no significant differences in protein expression of p-STAT3 and total STAT3 among control DCs, non-target siRNA-treated DCs and SOCS3 siRNA-treated DCs. However, the total STAT3 and p-STAT3 protein levels in each of the DC groups are significantly increased with *C. albicans* stimulation compared to their corresponding non-stimulated DCs. In presence of *C. albicans,* the SOCS3 siRNA-treated DCs have greater expression levels of p-STAT3 and total STAT3 protein when compared with untreated DCs and non-target siRNA-treated DCs. These results suggest that suppression of SOCS3 can activate IL-6/STAT3 pathway.

3.7. SOCS3-silenced DCs stimulated with C. albicans enhance $CD4^+$ T lymphocyte proliferation

To analyze the functions of SOCS3 in the priming of $CD4^+$ T cells, DCs were co-cultivated with $CD4^+$ T cells prior to being challenged with and without *C. albicans*. Without *C. albicans* stimulation, there is no significant difference in $CD4^+$ T lymphocyte proliferation among all DC groups. However, DCs stimulated with *C. albicans* enhance the proliferation of $CD4^+$ T cells compared to DCs without *C. albicans*

Fig. 7. SOCS3 siRNA-treated DCs co-cultured with *C. albicans* induce a strong Th17 response. Proliferative capacity of allogeneic T cells from C57 mice co-cultured with DCs. (a, c and e) Cells were collected and stained with anti-IL-4-PE, anti-IFN-γ-FITC and IL-17-FITC to detect intracellular cytokine production by $CD4^+$ T cells. Meanwhile, IFN- γ, IL-4 and IL-17 concentrations were measured by flow cytometry. Mean ± SD of data from seven independent experiments are shown. Y-axis represents the percentage of IFN-γ^+ $CD4^+$ T cells, IL-4^+ $CD4^+$ T cells and IL-17^+ $CD4^+$ T cells, respectively. (b, d and f) The cells were collected for detecting gene expression by qRT-PCR. β-actin served as a reference gene. mRNA level in uninfected control cells is set as 100%. Mean ± SD of data from seven independent experiments are shown. $^{**}p < 0.01$; $^{***}p < 0.001$. NS: not significant.

stimulation.

In response to *C. albicans,* SOCS3-silenced DCs further increase the proliferation of T cells at a DC-to-T cell ratio of 1:50 as well at a ratio of 1:200 when compared to control DCs and non-target siRNA-treated DCs (Fig. 6a and b). These data suggest that downregulation of SOCS3 expression by siRNA transfection in DCs can effectively activate T cells in response to stimulus by *C. albicans*.

Upon increasing the DC-to-T cell ratio to 1:10, allogeneic T cell proliferation is no longer increased in SOCS3 siRNA-treated DCs and remains at the level achieved by the DC-to-T cell ratio of 1:50 in the presence of *C. albicans*. Also, at this ratio the difference in T cell proliferation between SOCS3-silenced and non-silenced DCs could not be detected. The plateau effect at the ratio of 1:10 may be due in part to an excess of co-stimulatory molecules on the DC's surface (Fig. 6c). As

Fig. 8. Exogenous anti-IL-6 mAbs partly reverse the enhancement of IL-17$^+$ CD4$^+$ T cells proliferation. (a, c and e) DCs were exposed to SOCS3 siRNA and were treated with *C. albicans* for 24 h. At day 10, T cells were stimulated with PMA and ionomycin for 5 h in the presence of brefeldin A. Cells were collected to detect protein expression of RORγt, IL-17A and IL-23R by western blotting. β-actin served as a reference gene. Y-axis represents the gene expression of RORγt, IL-17A and IL-23R, respectively. Data were mean ± SD of seven independent experiments. (b, d and f) Two representative examples from the seven experiments are shown. In the panel b1, b2, d1, d2, f1 and f2, lane 1, 2, 3, and 4 represents control DCs, control DCs treated with *C. albicans*, siSOCS3-treated DCs co-cultured with *C. albicans,* and siSOCS3-treated DCs co-cultured with *C. albicans* and anti-IL-6, respectively. *$p < 0.05$; ***$p < 0.001$. NS: not significant.

shown above, siSOCS3 increases the expressions of MHC-II and co-stimulatory molecules. Once the secretion of these surface molecules reaches saturation, T-cell proliferation capacity is not expected to increase further.

3.8. SOCS3-silenced DCs in response to C. albicans promote Th17 cell differentiation

Th1 and Th17 cells are the principal effector cells responsible for protective immunity against fungi, while Th2 responses are thought to be associated with variant deleterious effects [18]. Along with the increase in T cell proliferation in SOCS3 siRNA-treated DCs, we also measure T cell polarization under SOCS3 siRNA treatment. After being co-cultured with *C. albicans*, SOCS3 siRNA-silenced and untreated DCs were used to stimulate the purified CD4$^+$ T cells.

The gene markers of Tbet for the Th1, GATA3 for Th2 and RORγt for Th17 are quantified by qRT-PCR [19]. In the presence of *C. albicans,* Tbet, GATA3 and RORγt are all significantly increased in DCs in comparison with DCs without *C. albicans*. However, the increased expression of Tbet and GATA3 by *C. albicans* stimulation is similarly independent of whether control DCs or silenced DCs have been applied. By contrast, the increased RORγt expression is greater in silenced SOCS3 DCs than in control DCs or non- SOCS3 targeted DCs in presence of *C. albicans*. As shown in Fig. 7b, d and f, silencing SOCS3 can only enhance RORγt expression of Th17 cells, but not the Tbet and GATA3 of Th1 and Th2 cells.

These results are further supported by the percentage distribution of CD4$^+$ T cells that produce IFN-γ, IL-4 or IL-17 as measured by

intracellular cytokine staining. The protein level of IFN-γ, IL-4 or IL-17 produced by $CD4^+$ T cells after being co-cultured with DCs is markedly enhanced when stimulated with *C. albicans*, but there is no noticeable difference among control DCs, non-target treated DCs and siSOCS3-treated DCs. However, compared with control DCs, the siSOCS3-treated DCs co-cultured with *C. albicans* generate a greater population of IL-17-producing $CD4^+$ T cells (Fig. 7a, b and c). These results suggest that *C. albicans* can induce the proliferation of Th1, Th2 and Th17 cells, and SOCS3 suppression in DCs can increase the IL-17 producing $CD4^+$ T cells (Th17 cell) but not Th1 (IFN-γ^+CD4^+ T cell) or Th2 (Il-4^+CD4^+ T cell) proliferation (Fig. 7).

3.9. Exogenous anti-IL-6 mAbs partly reduces the enhancement of Th17 differentiation in SOCS3 siRNA-treated DCs

Like Th1- and Th2-cells, the differentiation of Th17 cells is orchestrated by cytokines secreted from APCs. The differentiation of Th17 cells can occur in the presence of the polarizing cytokines IL-6 [20]. We hypothesize that enhancement of IL-6 production of DCs by pretreatment with SOCS3 siRNA may be responsible for the increased Th17 cell differentiation. To test this hypothesis, we added anti-IL-6 mAbs to the co-culture experiments. We find that this anti-IL-6 treatment could partly reduce the Th17 polarization found in SOCS siRNA DCs exposed to *C. albicans* (Fig. 8). These results suggest that pro-inflammatory cytokine IL-6 in SOCS3-silenced DCs only partly mediates the enhancement effect of Th17 cells. Other cytokines, such as IL-1β and IL-23 may also contribute to the development and differentiation of Th17 cells during the fungal infection.

4. Discussion

This is one of the first studies to address the effect of SOCS3 on DCs in counteracting the fungal pathogen *C. albicans*. In this study, we investigated the anti-fungal effects of down-regulation of SOCS3 in DCs at the molecular level. We find that decreased expression of SOCS3 promotes DCs' ability to drive Th17 immune response against *C. albicans* by increasing the levels of the key mediators IL-6 and STAT3. Furthermore, SOCS3-silenced DCs mediate innate immunity leading to an increased *C. albicans* phagocytosis and killing rate. Thus, lowering the expression of SOCS3 in DCs could possibly be a novel medical treatment strategy to improve immune responses against fungal infection.

C. albicans is a leading cause of hospital-acquired infections, accounting for 15% of all sepsis cases, and 40% of all bloodstream infections in clinical settings [21]. T cells play a central role in mediating protection against systemic fungal pathogens. DCs are a major player in the initiation and augmentation of T cells in response to fungal infection [22]. Once the fungal pathogen is taken into DCs phagosomes, the digested fungal peptides are then presented to the T cells [23] which in turn are primed. Therefore, elimination of fungal pathogens requires both DCs and intact T cell immunity [24]. The host immune system includes negative feedback mechanisms to modulate immune response, of which DCs use SOCS to regulate the strength of immune response by a variety of cytokine activations and antigen-presenting processes. We previously showed that inhibiting SOCS1 could induce the maturation of DCs and subsequently stimulate Th1 cell differentiation in response to *C. albicans* [25]. In this study, we demonstrate that the suppression of SOCS3 results in an overall antifungal DC response by increasing *C. albicans* phagocytosis, killing capacity and DC maturation. Furthermore, cytokine responses indicate that SOCS3 silencing can increase Th17 differentiation from naïve T cells at least in part by IL-6 activation. Our two studies in SOCS1 and SOCS3 suggest that there may be a specificity of SOCS in T cell response against fungal infection. In the current study, we also demonstrate that silencing of SOCS3 can enhance the phagocytic capacity of DCs with a peak at 60 min after *C. albicans* exposure. As the co-cultivation time increases, phagocytosis declines slightly, possibly due to saturation and maturation of DCs. In addition, silencing of SOCS3 improves the diffusion capacity of DCs. DC diffusion to the lymph nodes is an indispensable step in immune surveillance. In the lymph nodes, DCs make contact with a vast repertoire of naïve Ag-specific T cells and mediate T cells through an increased expression of CCR7 and decreased expression of CCR6 [26]. Clearly, *C. albicans* can stimulate CCR7 expression in both siRNA-treated and untreated DCs. However, the SOCS3 siRNA-transfected DCs have higher expressions of CCR7 than untreated or non-targeted siRNA-treated DCs. The elevated CCR7 level after *C. albicans* stimulation indicates that the mechanisms underlying the promotion of DC migration by SOCS3 silencing might be due in part to the induced expression of CCR7.

As we mentioned above, co-stimulatory molecules are the key for the activation of T cells. For example, expression of CD40 by polyI:C stimulation can induce a Th17 immune response [15]. In support of this, knockdown of SOCS3 promotes the up-regulation of co-stimulatory molecules including MCH-II, CD40 and CD86 following the stimulation with *C. albicans*. All three molecules are commonly used to predicate the maturation of DCs. However, there is no significant change at the expression of CD80 in both control DCs and SOCS3-silenced DCs with or without *C. albicans* stimulation.

Like Th1 and Th2 cells, the differentiation of Th17 cells is mediated by cytokines secreted by APCs such as IL-6. Despite regulating T cell differentiation, IL-6 balances pro-inflammatory T cell subsets and immunosuppressive regulatory T cells [27,28]. IL-17 mediates the severity of autoimmune and inflammatory disease and confers to some protection against bacterial and fungal infections [29]. In this study, we demonstrate that SOCS3 silencing promotes the secretion of IL-6, IL-17 in DCs that leads to an IL-6-mediated Th17 cell proliferation. This is consistent with the parallel experiment using anti-IL-6 mAbs to suppress cytokines upon Th17 cells activation. By contrast, we find no effect on IL-4-driven Th2 differentiation or on IFN-γ-driven Th1 differentiation responses to *C. albicans*.

Cytokine signaling mediated by the JAK/STAT pathway plays an important role in the specialization, proliferation, and apoptosis of various types of cells including DCs. Once SOCS3 is bound to JAK, the activity of JAK2 kinase is inhibited, which in turn blocks the activation of STAT3 and the IL-6/STAT3 signaling pathway through a classic negative feedback loop [30]. Our results show that suppression of SOCS3 in DCs enhances IL-6-induced tyrosine phosphorylation of STAT3 that may explain the increased maturation of DCs and Th17 cell proliferation in response to *C. albicans*.

The siRNA-mediated silencing of gene expression has shown promise for the development of novel therapeutic strategies to treat autoimmune and inflammatory diseases [31]. In the present study, by using SOCS3 siRNA we can effectively downregulate the SOCS3 expression at both the gene and protein levels in 24–48 h. After 72 h post siRNA treatment, treated DCs restore their original function and response to SOCS3 and inhibitory effectiveness decreases dramatically. In consideration of the possibility that the continuous activation of DCs may induce a hyper-inflammatory response, which may affect the overall survival and health of the host, delivery of siRNA targeting SOCS3 can promptly and effectively activate DCs and yet avoid an overlong activation of those DCs.

5. Conclusion

In summary of this *in vitro* experiment, we demonstrate that the suppression of SOCS3 in DCs leads to [1] enhanced activation and specialization of DCs; [2] augmented phagocytosis and killing of the fungal pathogen by DCs; [3] increased production of IL-6; and [4] improved capability of DCs to prime naïve T cells and initiate Th17 proliferation. As a result, SOCS3 silencing promotes IL-6-induced tyrosine phosphorylation of STAT3 in DCs, which favors the differentiation of naive CD4 T cells to the Th17 subset and protects against intracellular fungal pathogens (Supplemental Fig. 3). Further studies in a mouse model or in human cells would help us to clarify whether SOCS3

down-regulation can be used in mammalian environments and be applied eventually in clinical practice.

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Author contributions

W. L. and D. S. conceived and designed the experiments; J. Y., Q. L. Z. and W. Huan Mei performed the experiments; D. S. and D. L. analyzed the data; D. S. and D. L. wrote the paper.

Conflicts of interest

The authors declare no conflict of interest.

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