Upregulation of SOCS3 in lung CD4⁺ T cells in a mouse model of chronic PA lung infection and suppression of Th17-mediated neutrophil recruitment in exogenous SOCS3 transfer *in vitro*

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Abstract. Neutrophilic airway inflammation in chronic lung infections caused by *Pseudomonas aeruginosa* (PA) is associated with T helper (Th)17 responses. Suppressor of cytokine signaling 3 (SOCS3) is the major negative modulator of Th17 function through the suppression of signal transducer and activator of transcription (STAT)3 activation. The aim of the present study was to investigate the expression of SOCS3 in lung CD4⁺ T cells in a mouse model of chronic PA lung infection and the effect of exogenous SOCS3 on Th17-mediated neutrophil recruitment *in vitro*. A mouse model of chronic PA lung infection was established and the activation of STAT3 and Th17 response in lung tissues and lung CD4⁺ T cells was assessed. The protein and mRNA expression of SOCS3 in lung CD4⁺ T cells was analyzed by western blotting and reverse transcription-quantitative polymerase chain reaction. The authors constructed a recombinant lentivirus carrying the SOCS3 gene and transferred it into lung CD4⁺ T cells isolated from a mouse model. These transfected cells were stimulated with interleukin (IL)-23 *in vitro* and the protein level of p-STAT3 and retinoid-related orphan receptor (ROR)γt was determined by western blotting. The expression of IL-17A⁺ cells was analyzed by flow cytometry and the level of IL-17A in cell culture supernatant was measured by ELISA. The mouse lung epithelial cell line, MLE-12, was cocultured with lung CD4⁺ T cells that overexpressed the SOCS3 gene and the culture supernatant was harvested and used for a chemotaxis assay. Compared with control mice, mice with chronic PA lung infection had significantly higher level of p-STAT3 and Th17 response in both lung tissues and lung CD4⁺ T cells. The protein and mRNA level of SOCS3 in lung CD4⁺ T cells increased as the chronic PA lung infection developed. Exogenous SOCS3 gene transfer in PA-infected lung CD4⁺ T cells decreased p-STAT3 and RORγt expression and suppressed the level of IL-17A⁺ cells *in vitro*. MLE-12 cells cocultured with SOCS3-overexpressing lung CD4+ T cells expressed a significantly lower level of neutrophil chemoattractants chemokine (C-X-C motif) ligand (CXCL) 1 and CXCL5, and recruited significantly smaller numbers of migrating neutrophils than those cocultured with control cells. SOCS3 was upregulated in lung CD4⁺ T cells following the activation of STAT3/Th17 axis in a mouse model of chronic PA lung infection. Exogenous SOCS3 transfer in PA-infected lung CD4⁺ T cells suppresses Th17-mediated neutrophil recruitment *in vitro*.

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Introduction

Chronic airway infection with *pseudomonas aeruginosa* (PA) represents a therapeutic challenge. Host immune responses to PA often result in persistent airway inflammation and immuno-pathological lung injury, characterized by polymorphonuclear leukocyte infiltration (1). Although the cause of PA-related airway inflammation remains incompletely explored, it has been demonstratedthat Th17 responses are associated with the neutrophil recruitment and activity in lung defense against the infection. Significantly elevated levels of interleukin (IL)-17A are reported in the sputum of patients with cystic fibrosis who were colonized with PA at the time of pulmonary exacerbation, and the levels declined with therapy directed against PA (2,3). IL-23 mediates inflammatory responses to mucoid PA lung infection, which induces IL-17 production and the subsequent local production of cytokines and chemokines that are critical to airway inflammation (4). IL-23 and the downstream cytokine IL-17A are important molecules for proinflammatory gene expression and are likely involved with the immunopathological injury in chronic PA lung infection.

   Th17 cells are a subset characterized by a unique transcriptional program dependent on signal transducer and activator of transcription 3 (STAT3) transduction pathways (5). The Th17

transcription factor RORγt induces the expression of IL-23 receptor through STAT3-dependent mechanisms, rendering the differentiating cells responsive to IL-23, which is an innate immune cell cytokine essential for stabilization of the Th17 phenotype (6). When STAT3 is genetically ablated in CD4+ cells, neither naturally occurring Th17 cells nor Th17-dependent autoimmunity occurs (7). In PA lung infections, STAT3 activation has been demonstrated to be essential for the translocation of nuclear factor-κB into the nucleus, which induced elevated inflammatory cytokines (IL-6, tumor necrosis factor-α, and IL-12) and increased superoxide release in the lung (8). These studies suggest that targeting STAT3/Th17 pathway may be a potential therapeutic strategy for controlling immunopathological injury during chronic PA lung infection.

Suppressor of cytokine signaling (SOCS) proteins are feedback inhibitors of the JAK/STAT pathway. The major function of SOCS3 is inhibition of signaling by the IL-6 family of cytokines, causing inhibition of STAT3 activation and Th17 generation (9). Furthermore, SOCS3 expression in T cells inhibits IL-23 signaling, which constrains Th17 cell differentiation (10). In the central nervous system, the STAT3/SOCS3 axis influences the T-cell repertoire, with SOCS3 providing protection against autoimmune diseases by blocking Th17 development (11). So far, in the field of chronic lung infection, data regarding the effect of SOCS3 on STAT3/Th17 signal pathway remains scarce.

In the present study, the authors investigated the activation of the STAT3/Th17 signal pathway and the expression of SOCS3 in the lung CD4+ T cells in a mouse model of chronic PA lung infection. Following this, the SOCS3 gene was lentivirally delivered into the CD4+ T cells isolated from lung tissues of the mouse model and the effect of exogenous SOCS3 on Th17-mediated neutrophil recruitment was investigated *in vitro*.

Materials and methods

Animal model of chronic PA lung infection. Specific pathogen-free female C57/BL6 mice (8-12 weeks of age, 18-22 g) were obtained from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China), and were kept under environmentally controlled conditions with standard food and water. Animal experimentations were approved by the Institutional Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine (Shanghai, China).

PA (ATCC© 27853) was purchased from China General Microbiological Culture Collection Center (Beijing, China) and loaded on agarose beads at a final concentration of $2x10^6$ colony-forming units/50 μl phosphate-buffered saline (PBS). The beads were measured to be 70 to 150 μm in diameter by microscopic quantification. Mice were anesthetized with intraperitoneal injection of 20 μl/g body weight of a stock solution of 0.5 ml ketamine (100 mg/ml), 0.5 ml 1% xylazine hydrochloride (equivalent to 10 mg/kg body weight) and 9 ml sterile 0.9% NaCl.

Mice were placed in the dorsal recumbent position. The trachea was cannulated, and 50 μl PA-laden agarose beads were introduced into the right lung. Mice were monitored, and allowed to recover until euthanized by CO_2 asphyxiation on d3, 5 and 7. Bacterial load was evaluated by quantitatively

culturing lung homogenates on sheep blood agar plates overnight at 37˚C. Mice in the control group were inoculated with sterile agarose beads. A total of 72 C57BL/6 mice were randomly divided into a control group (n=36) and a PA group (n=36). In each group, 12 mice were euthanized at each time point. For PA group, the percentage of mice positive for PA growth in homogenate cultures was 100% with the bacterial load more than $1x10^5$ colony forming units (CFU)/g lung tissue at each time point.

Bronchoalveolar lavage cell counts. The lungs were lavaged with 1.8 ml PBS. Bronchoalveolar lavage (BAL) fluid was centrifuged at 300 x g. The cell pellet from the BAL fluid was resuspended in 1 ml PBS, and the cells were counted using a microscope counting chamber. The percentage of neutrophils was determined by counting a minimum of 100 cells following staining with haematoxylin and eosin. A pathologist, blinded to the experimental protocols, checked the total and differential cell counts.

Histological examination and immunohistochemistry. For histologic analysis, the right low lobe was removed, fixed with 4% paraformaldehyde in PBS and embedded in paraffin. The paraffin was sectioned and stained with haematoxylin and eosin. The expression of phospho-STAT3 (p-STAT3) in the lung tissues was analyzed by immunohistochemistry. Lung sections were deparaffinized and stained with rabbit anti-mouse p-STAT3 antibody (1:400; catalog no. 9145; Cell Signaling Technology, Inc., Danvers, MA, USA) at 4˚C overnight. The sections were incubated with horseradish peroxidase-conjugated secondary antibody (1:1,000; catalog no. 6721; Abcam, Cambridge, UK) at 37˚C for 30 min. Then the DAB substrate solution was applied to reveal the color of antibody staining. Finally, sections were counterstained with haematoxylin to visualize the nuclei.

Measurement of IL-17A and p-STAT3 concentrations in lung tissues by ELISA. The right middle lobes were weighed and homogenized in 1 ml normal saline. Concentrations of IL-17A and p-STAT3 were measured using commercially available ELISA kits (catalog nos. M1700 and DYC4607B-2; R&D Systems, Inc., Minneapolis, MN, USA). The samples were assayed in duplicate and values were expressed as pg/mg lung tissue.

Mouse lung CD4+ T cells isolation. The lungs were excised aseptically and homogenized in 0.5 ml normal saline. Minced lungs were enzymatically digested, using a mixture containing 6 Udispase II (catalog no. D4693; Sigma-Aldrich, Merck KGaA, Darmstadt, Germany), 20 μg collagenase I (catalog no. 1148089; United States Pharmacopeia, Rockville, MD, USA). Following digestion, lung tissues were passed through a 40 μm nylon mesh to obtain single-cell suspensions. CD4+ T cells were isolated using a CD4+ T cell isolation kit and Mini-MACS columns (MiltenyiBiotec, Inc., Auburn, CA, USA). Following centrifugation at 300 x g, 4˚C for 10 min and washing with cold PBS, collected CD4+ T cells were dissolved in culture medium consisting of RPMI 1640 (catalog no. 11875093; Gibco; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 25 mM

HEPES, 2 mM glutamine, 10% fetal bovine serum, penicillin (100 IU/ml) and streptomycin (100 IU/ml) at 37°C in a 5% CO_2 incubator.

Western blotting analysis of RORγt, p-STAT3 and SOCS3 expression in lung CD4+ T cells. Proteins were extracted from lung CD4+ T cells using a commercially available Mammalian Protein Extraction Reagent (Pierce; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The protein concentration was calculated using the bicinchoninic acid protein assay (catalog no. 23227; Thermo Fisher Scientific, Inc.). The extracts were diluted, mixed with protein loading buffer and boiled for 5 min before loading onto an SDS-PAGE gel (with a 10% separating gel and a 5% stacking gel). Following electrophoresis, proteins were transferred onto polyvinylidene fluoride membranes, which were blocked with 5% skim milk for 1 h, and blotted against rat anti-mouse RORγt monoclonal antibody (1:1,000; catalog no. 14-6981-82; eBioscience, Inc., San Diego, CA, USA), rabbit anti-mouse SOCS3 polyclonal antibody (1:500; catalog no. sc-9023, Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-mouse p-STAT3 monoclonal antibody (1:2,000; catalog no. 9145, Cell Signaling Technology, Inc.), and rabbit anti-mouse Histone H3 polyclonal antibody (1:1,000; catalog no. sc-8654-R;Santa Cruz Biotechnology, Inc.) at 4°C overnight. Membranes were washed and incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000; catalog no. 111-035-144; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) or goat anti-rat IgG (1:5,000; catalog no. 97057;Abcam) at room temperature for 1 h. Protein bands were visualized by enhanced chemiluminescence substrate (catalog no. 32106; Thermo Fisher Scientific, Inc.) and quantification was performed by densitometry analysis in duplicate using Quantity One software version 4.6.3 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The data were normalized to Histone H3 expression in samples.

Reverse transcription-polymerase chain reaction (RT-PCR) and RT-quantitative polymerase chain reaction (RT-qPCR) analysis of SOCS3 mRNA in lung CD4+ T cells. Lung CD4+ T cells were lysed in TRIzol (catalog no. 15596026; Invitrogen, Thermo Fisher Scientific, Inc.) and total RNA was extracted. The RT-PCR reaction for murine SOCS3 was carried out by standard methods using RNA LA PCR Kit (Takara Bio, Inc., Otsu, Japan). The RT-qPCR reaction was conducted using the GMR-Super™ Universal Master Mix Kit (Shanghai GenePharma Co., Ltd., Shanghai, China). The reaction was performed using the Mx3000P qPCR system (Stratagene; Agilent Technologies, Inc., Santa Clara, CA, USA). β-actin was used as an internal positive control for normalization of data. The comparative threshold cycle method was used to assess the relative mRNA levels of target genes (12). The sequences of the primers were as follows: Mouse SOCS3 sense, 5'-ACC TTC AGC TCC AAA AGC GAG TAC-3' and anti-sense, 5'-CGC TCC AGT AGA ATC CGC TCT C-3'; mouse β-actin sense, 5'-AAG ATC AAG ATC ATT GCT CCT CC-3' and anti-sense, 5'-GAC TCA TCG TAC TCC TGC TTG C-3'.

Production of SOCS3 recombinant lentiviral vectors. A fifth generation of the self-inactivating lentiviral vector reporter

was purchased from Shanghai GenePharma Co., Ltd. The lentiviral vector system had four sections, including the pGLV-EF1a-GFP plasmid, the pLV/helper-SL3 (gag/pol element) plasmid, the pLV/helper-SL4 (pRev element) plasmid, and the pLV/helper-SL5 (pVSV-G element) plasmid. The pGLV-EF1a-GFP plasmid was constructed to encode the full length of the mouse SOCS3 gene (NCBI reference sequence ID, NM_007707.3). PCR and DNA sequencing confirmed the accurate insertion of the SOCS3 cDNA. The transient transfection method was used to prepare the recombinant lentiviral vectors. The plasmids were co-transfected into subconfluent 293T cells in serum-free medium using the cationic liposome based transfection reagent (Lipofectamine 2000; Invitrogen; Thermo Fisher Scientific, Inc.). Following 8 h incubation, the medium was completely exchanged. High-titer recombinant lentiviral vectors with SOCS3 were harvested 48 h later. The viruses were precipitated at 1,500 x g and resuspended with Opti-MEM (catalog no. 31985062; Thermo Fisher Scientific, Inc.) medium.

SOCS3 overexpression in lung CD4+ T cells by lentiviral infection in vitro. CD4+ T cells were isolated from mouse lung tissues 3 d following inoculation of PA-laden agarose beads using methods described above. The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc.) containing 10% horse serum, 1.5 g/l NaHCO$_3$ and 30 ng/ml IL-2 at 37°C in a 5% CO_2 incubator. Cells were seeded on six-well plates at a density of $1x10^6$ cells/well and incubated with SOCS3 recombinant lentiviral vectors at a titer of $1x10^9$ TU/ml for 72 and 96 h. ~90% of cells were infected as assessed by GFP staining. The overexpression of SOCS3 gene expression in lung CD4+ T cells was confirmed by RT-qPCR and western blotting. Cells incubated with PBS were used as blank controls. Cells transfected with the empty vector, which was not inserted with SOCS3, were used as negative controls.

Western blotting and RT-qPCR analysis in lung CD4+ T cells stimulated by IL-23. Following lentiviral infection, cells were seeded at a concentration of $5x10^6$ cells/well into a 96-well culture plate and incubated with recombinant mouse IL-23 (10 ng/ml, eBioscience, Inc.) for 24 h. The protein levels of p-STAT3 and RORγt were analyzed by western blotting following the method described above. The mRNA levels of STAT3 and RORγt in lung CD4+ T cells were analyzed by RT-qPCR. The sequences of primers were as follows: Mouse STAT3 sense, 5'-TGT CTC CAC TTG TCT ACCT-3' and anti-sense, 5'-CAG CAC CTT CAC CGT TAT-3'; mouse RORγt sense, 5'-TCT CTG CAA GAC TCA TCG ACA AG-3' and anti-sense, 5'-GCA CAG GCT CCG GAG TTTT-3'. β-actin was used as an internal positive control for normalization of data. The results were compared between SOCS3 overexpressing group and control groups.

Measurement of IL-17A expression in cell supernatant and flow cytometry analysis of IL-17A+ cells. Following IL-23 stimulation, protein levels of IL-17A in supernatant of SOCS3 overexpressing cells were measured by ELISA using commercially available kits (catalog no. M1700; Quantikine ELISA; R&D Systems, Inc., Minneapolis, MN, USA). For

flow cytometry analysis, these cells were washed and incubated with fixation/permeabilization solution for 1 h in the dark. Following a blocking step with anti-mouse CD16/CD32 (1:1,000; catalog no. 14-0161-81; eBioscience, Inc.), allophycocyanin-conjugated IL-17A antibody (1:200;catalog no. 17-7177; eBioscience, Inc.) was incubated with prepared cells in a final volume of 100 μl flow staining buffer at 4°C for 90 min. The percentage of IL-17A+ cells within the CD4+ T-cell population was determined using FACScan flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) in the FL4 channel. Background fluorescence was assessed using appropriate isotype- and fluorochrome-matched control antibodies (eBioscience, Inc.).

Neutrophil isolation. Neutrophils were isolated from the peripheral blood of healthy mice according to the manufacturer's instructions. In brief, venous blood was drawn and neutrophils were isolated immediately by mouse peripheral neutrophils separation medium (Sangon Biotech Co., Ltd., Shanghai, China). Following lysis of the erythrocytes, the neutrophils were harvested, washed twice and resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum at a cell concentration of $1x10^6$/ml. The percentage of viable neutrophils was assessed by morphology and the trypan blue exclusion test. A population of >95% of viable neutrophils was confirmed in the purified cells.

Chemotaxis analysis. Boyden chambers of 3 μm pore size (Corning Incorporated, Corning, NY, USA) was used to assess chemotaxis for neutrophils. MLE-12 cells (mouse lung epithelial cell lines, ATCC CRL-2110; American Type Culture Collection, Manassas, VA, USA) were plated in 24-well plates ($4x10^5$ per well) and incubated with the three groups of lung CD4+ T cells (blank control group, negative control group and SOCS3 overexpressing group) for 48 h. The culture supernatant was harvested. Then, the treated supernatant was added to the lower chambers, while neutrophils were added to the top chambers for incubation for another 90 min at 37°C in a humidified atmosphere with 5% CO_2. The filters were fixed with ethanol and stained with DAPI to visualize the nuclei. The number of cells that had migrated through the entire thickness of the filter was evaluated, and the results were expressed as the chemotactic index, being the number of cells that migrated towards the sample divided by the number of cells that migrated towards the control medium. Triplicate chambers were used in each experiment and five fields were examined in each filter. CXCL1 and CXCL5 were measured in the culture supernatant using commercially available ELISA kits (catalog nos. MKC00B and MX000; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistical analysis. Data were summarized as means ± standard deviation. Comparisons between groups were made with one-way analysis of variance followed by the Tukey post hoc test, and a two-tailed unpaired Student's t-test. Mann Whitney U test was used to analyze the non-normal distribution data. $P<0.05$ was considered to indicate a statistically significant difference. SAS version 8.1 statistical software (SAS Institute, Cary, NC, USA) was used for data analysis.

Results

SOCS3 is upregulated in lung CD4+ T cells following the activation of the STAT3/Th17 pathway during chronic PA lung infection. Lung tissue histology indicated that mice with chronic PA lung infection had an intense neutrophilic infiltration within and around the small- and medium-sized bronchi, and had significantly higher expression of p-STAT3 in the lung tissues compared with control mice (Fig. 1A). Both the number and percentage of recruited BAL neutrophils were significantly higher in mice with chronic PA lung infection ($P<0.01$; Fig. 1B). The concentrations of IL-17A and p-STAT3 in lung tissues were also significantly higher in mice with chronic PA infection ($P<0.01$; Fig. 1C).

The authors examined RORγt, p-STAT3 and SOCS3 expression in the lung CD4+ T cells. The RORγt and p-STAT3 protein were expressed more strongly in mice with chronic PA infection than control mice on d5 and 7 ($P<0.01$). The SOCS3 protein was at low levels in control mice, while in mice with PA infection, it gradually increased as chronic lung infection developed (Fig. 2A). In addition SOCS3 mRNA level in lung CD4+ T cells was examined using RT-PCR and RT-qPCR analysis. SOCS3 mRNA level was demonstrated to increase significantly in mice with chronic PA infection on d5 and d7 ($P<0.01$; Fig. 2B).

In vitro SOCS3 gene transfer suppresses p-STAT3 expression and Th17 response in lung CD4+ T cells. To investigate the significance of SOCS3 expression in the regulation of STAT3/Th17 activation in lung CD4+ T cells during chronic PA infection, the authors overexpressed the SOCS3 gene in lung CD4+ T cells isolated from the chronic PA-infection mouse model. These cells were stimulated with IL-23 and the p-STAT3 expression and Th17 response were compared between SOCS3-overexpressing cells and control cells. The overexpression of SOCS3 strongly suppressed the protein level of p-STAT3 and RORγt in the lung CD4+ T cells ($P<0.01$; Fig. 3A). The effect is more obvious when the SOCS3 expression is higher after 96 h of lentiviral infection compared with that of 72 h of infection ($P<0.05$). The mRNA level of RORγt was significantly lower in the SOCS3 overexpressing cells ($P<0.01$), but no significant change of STAT3 mRNA level was observed between these groups (Fig. 3B).

Flow cytometry analysis indicated that the percentage of IL-17A+ cells was significantly lower in SOCS3-overexpressing cells compared with that of control cells ($P<0.01$; Fig. 4A and B). The level of IL-17A was analyzed in the cell supernatant by ELISA. IL-23 induced IL-17A production was significantly decreased in the supernatant of SOCS3-overexpressing cells, but not in that of control cells ($P<0.01$; Fig. 4C).

In vitro SOCS3 gene overexpression in lung CD4+ T cells suppresses neutrophil recruitment. The mouse lung epithelial cell line, MLE-12, was incubated with lung CD4+ T cells that overexpressed the SOCS3 gene. Using a chemotaxis assay it was demonstrated that, compared with those incubated with control cells, the MLE-12 cells incubated with SOCS3-overexpressing lung CD4+ T cells induced significantly smaller number of migrating neutrophils isolated from peripheral blood of healthy mice ($P<0.01$, Fig. 5A). The expression of
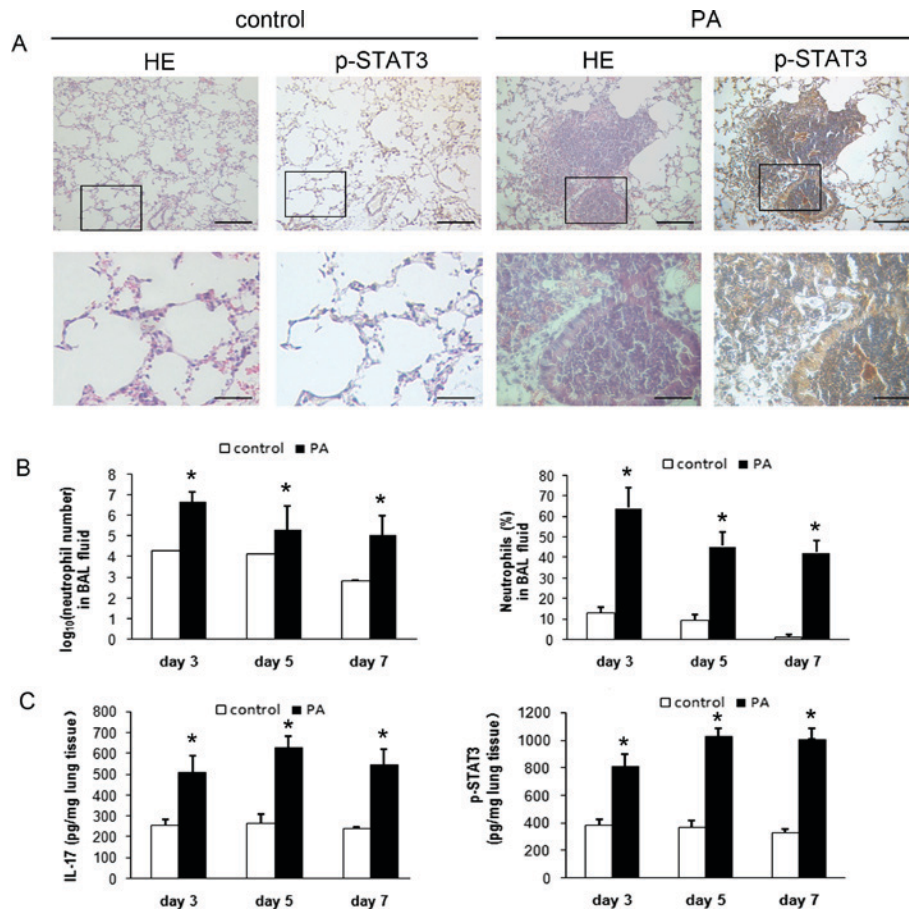
Figure 1. Neutrophillic airway inflammation and high expression of p-STAT3 and IL-17A in a mouse model of chronic *Pseudomonas aeruginosa* lung infection. (A) Histological examination of airway inflammation and immunohistochemical detection of p-STAT3 in lung tissues. Slides were stained with hematoxylin-eosin (left) and immunostained with anti-p-STAT3-specific antibodies (right). Boxes in the upper photomicrographs (x100 magnification, internal scale bar=200 μm) specify sections of lungs that are presented in lower photomicrographs (x400 magnification, internal scale bar=50 μm). (B) Absolute numbers of neutrophils and % neutrophils in BAL fluid. (C) Concentrations of IL-17A and p-STAT3 in lung tissues by ELISA analysis. Values are presented as the means ± standard deviation for 12 mice per group. *P<0.01 vs. control. HE, hematoxylin-eosin; STAT3, signal transducer and activator of transcription 3; p-STAT3, phospho-STAT3; BAL fluid, bronchoalveolar lavage fluid; IL, interleukin.
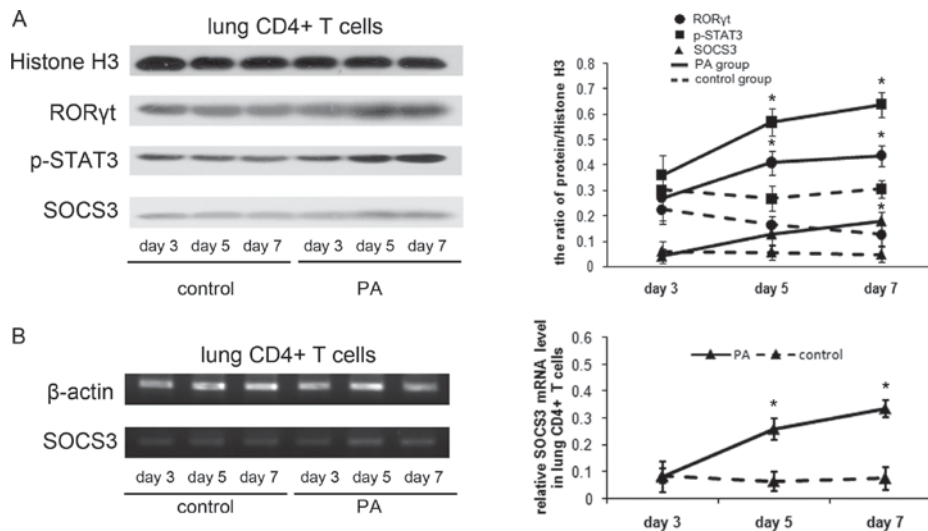

Figure 2. Induction of SOCS3 following the activation of STAT3/Th17 in a mouse model of chronic PA lung infection. (A) Protein expression of RORγt, p-STAT3 and SOCS3 in the lung CD4+ T cells by western blot analysis. Total cell proteins were extracted from lung CD4+ T cells on days 3, 5 and 7, then were immunoblotted with anti-RORγt, anti-p-STAT3 or anti-SOCS3 antibodies. Protein quantification was performed by densitometry analysis using Quantity One software. (B) Reverse transcription-PCR analysis of SOCS3 mRNA in the lung CD4+ T cells on days 3, 5 and 7. The relative expression of SOCS3 mRNA was reverse transcription-quantitative PCR analysis. Values are presented as the means ± standard deviation for 12 mice per group. *P<0.01 vs. control. B, blank control group; NC, negative control group; OE, SOCS3 overexpression group. PA, *Pseudomonas aeruginosa*; RORγt, retinoid-related orphan receptor γt; STAT3, signal transducer and activator of transcription 3; p-STAT3, phospho-STAT3; SOCS3, suppressor of cytokine signaling 3; Th17, T helper 17; PCR, polymerase chain reaction.
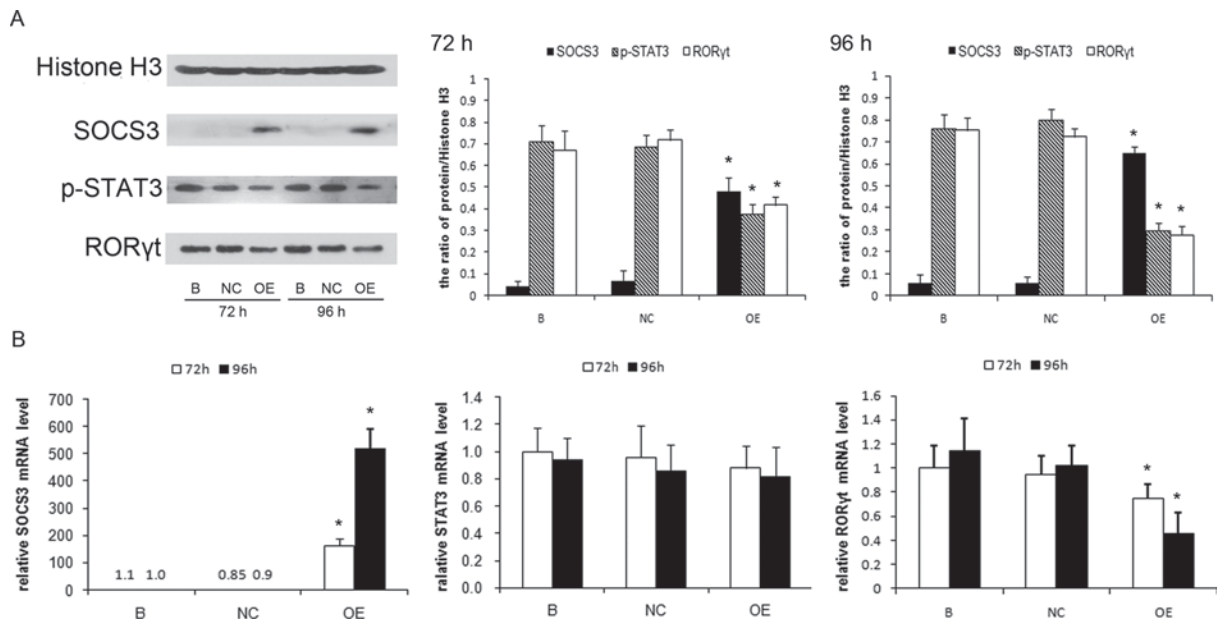
Figure 3. Decreased expression of p-STAT3 and RORγt in *Pseudomonas aeruginosa*-infected lung CD4+ T cells when stimulated by IL-23 following 72 h and 96 h of lentivirus-mediated SOCS3 gene transfection *in vitro*. (A) The protein expression of SOCS3, p-STAT3 and RORγt was determined by western blotting analysis. Histone H3 was used as an internal control. Protein quantification was performed by densitometry analysis using Quantity One software. (B) The mRNA level of SOCS3, STAT3 and RORγt was determined by reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as mean ± standard deviation of three separate experiments. *P<0.01 vs. control. RORγt, retinoid-related orphan receptor γt; STAT3, signal transducer and activator of transcription 3; p-STAT3, phospho-STAT3; SOCS3, suppressor of cytokine signaling 3; IL, interleukin.
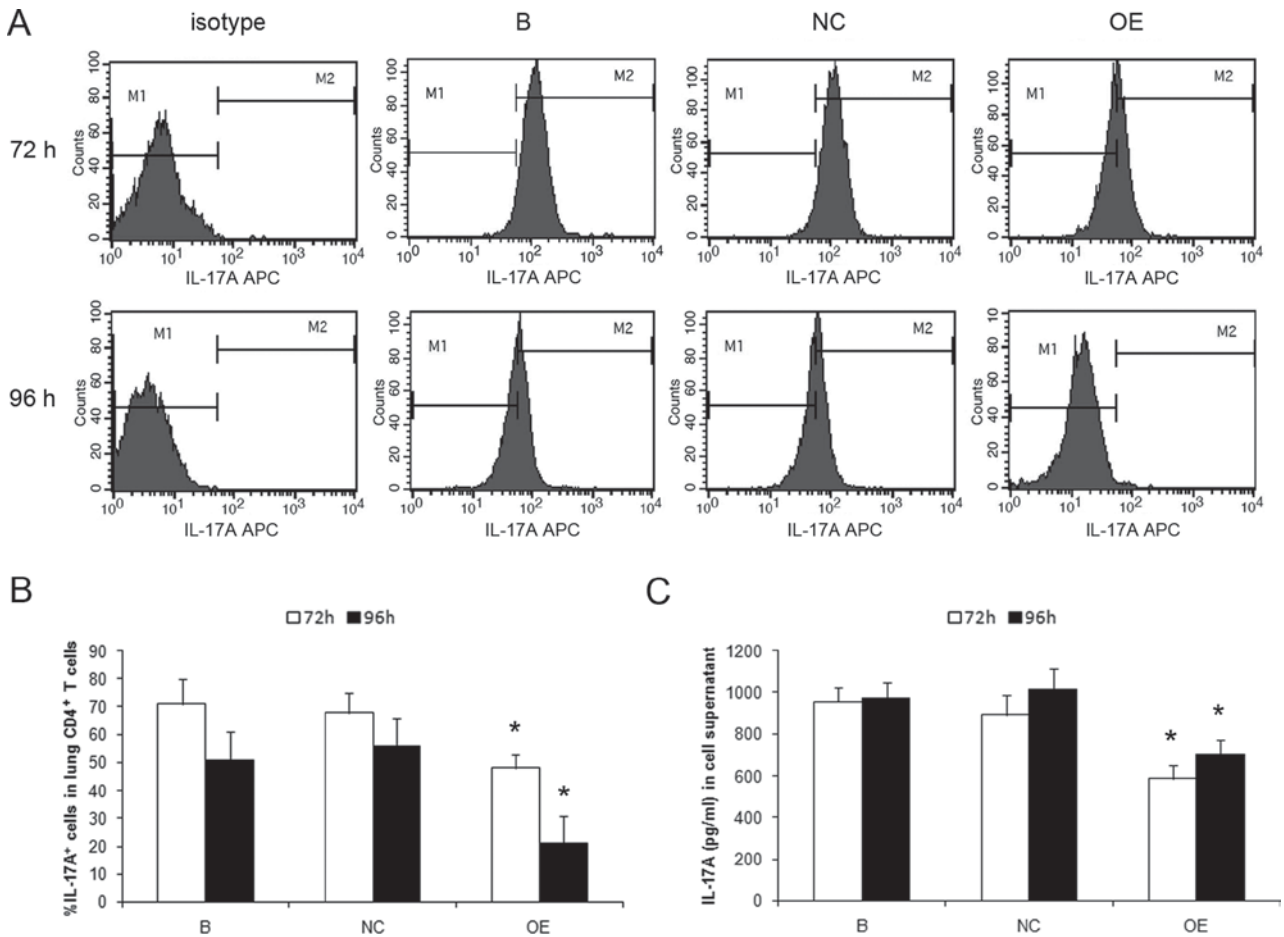

Figure 4. Suppressed Th17 responses in *Pseudomonas aeruginosa*-infected lung CD4+ T cells when stimulated by IL-23 following 72 and 96 h of lentivirus-mediated SOCS3 gene transfection. (A) Representative histograms of IL-17A+ cells by flow cytometry analysis. (B) The percentage of IL-17A+ cells in lung CD4+ T cells by flow cytometry analysis. (C) IL-17A level in the cell supernatant by ELISA. Data are presented as mean ± standard deviation of three separate experiments. *P<0.01 vs. control. B, blank control group; NC, negative control group; OE, SOCS3 overexpression group. Th17, T helper 17; IL, interleukin; SOCS3, suppressor of cytokine signaling 3.
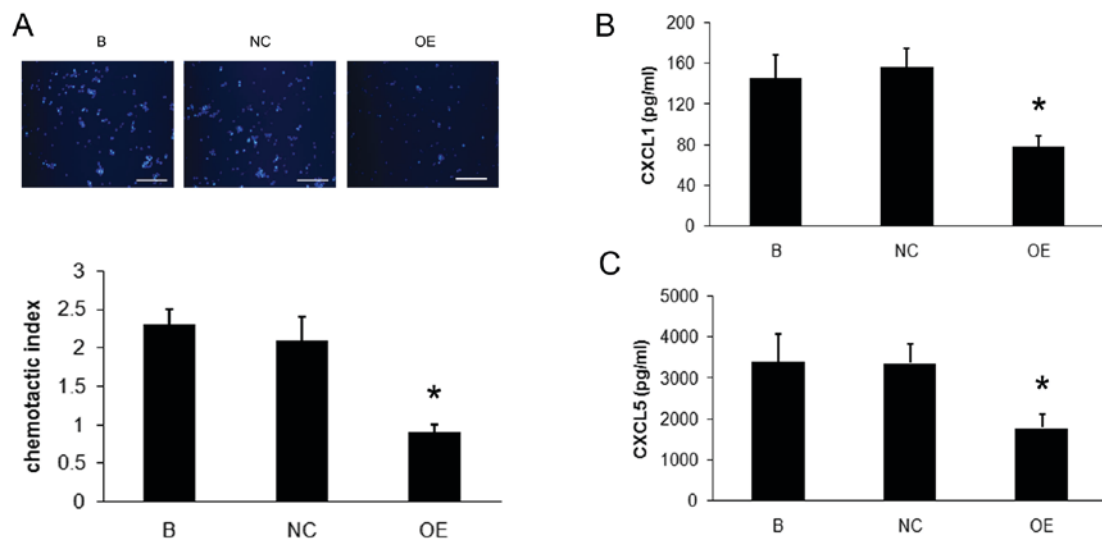
Figure 5. SOCS3 gene overexpressing lung CD4+ T cells suppresses neutrophil migration. (A) Mouse lung epithelial cell line, MLE-12, was cocultured with lung CD4+ T cells and the culture supernatant was harvested and used for a chemotaxis assay. Neutrophils that migrated in response to the culture supernatant were stained with DAPI. Significantly less neutrophil migration was observed by supernatant from SOCS3-overexpressing lung CD4+ T cells. The results are expressed as the chemotactic index, being the number of cells that migrated towards the sample divided by the number of cells that migrated towards the control medium. (B and C) CXCL1 and CXCL5 levels were analyzed in the culture supernatant by ELISA. Data shown are mean ± standard deviation of three separate experiments. *P<0.01 vs. control. B, blank control group; NC, negative control group; OE, SOCS3 overexpression group; SOCS3, suppressor of cytokine signaling 3; CXCL, chemokine (C-X-C motif) ligand.

neutrophil chemoattractants CXCL1 and CXCL5 secreted by MLE-12 cells was examined. The expressions of CXCL1 and CXCL5 were significantly lower in the supernatant of MLE-12 cells incubated with SOCS3-overexpressing cells (P<0.01; Fig. 5B and C).

Discussion

The immunological injury induced by chronic PA lung infection remains a challenge for both clinicians and scientists. So far, therapeutic interventions to control the sustained neutrophilic airway inflammation are not ideal. The aim of the present study was to investigate the role served by a cytokine-signaling negative regulator, SOCS3, in the neutrophilic airway inflammation of chronic PA lung infection. The results indicated that SOCS3 was upregulated following the STAT3/Th17 activation in a mouse model of chronic PA lung infection. *In vitro* exogenous SOCS3 gene transfer in lung CD4+ T cells decreased p-STAT3 expression and Th17 response, and suppressed the neutrophil recruitment induced by lung epithelial cells. These results suggested that SOCS3 gene therapy maybe a potential way for immunotherapy to treat neutrophillic airway inflammation in chronic PA lung infection.

It was reported previously that the integration of IL-17A into the IL-6/STAT3 signaling axis mediates lung inflammation, and that SOCS3, the feedback inhibitor of the JAK/STAT3 pathway, was increased in lungs during chronic inflammation (13). In the field of chronic PA lung infection, however, the role of SOCS3 in the regulation of STAT3/IL-17A pathway has been scarcely reported. Here, it was reported that the levels of p-STAT3 expression and Th17 response were higher in the mouse model of chronic PA lung infection than those in control mice, and SOCS3 protein and mRNA levels increased following the protein levels of p-STAT3 and RORγt became significantly higher at d5. These results suggested that

STAT3 activation and enhanced Th17 responses were related to the sustained neutrophillic airway inflammation in chronic PA lung infection, and SOCS3 may function as a negative feedback regulator of p-STAT3 to control the Th17-mediated inflammation.

Although SOCS3 expression was demonstrated to be upregulated following STAT3 activation in the mouse model of chronic PA lung infection, a strong activation of STAT3 and Th17 responses was still observed, even in the presence of high SOCS3 mRNA expression. This may be explained by the possibility that the SOCS3 expression in chronic airway inflammation may not reach a high enough level to shut off STAT3 activation completely. As reported previously, SOCS3 can be rapidly degraded by the ubiquitin-proteasome system (14), and has indicated to be phosphorylated in response to cytokine stimulation (15). Thus, to guarantee a high level of SOCS3 expression, additional expression of exogenous SOCS3 by gene transfer could be an effective way to overcome the rapid degradation and related modifications.

The inhibition of STAT3 activation by SOCS3 was presented in other human and animal studies in different inflammatory settings. In transgenic mice that overcame the inhibitory effect of SOCS3, dextran sulfate sodium induced stronger STAT3 activation and more severe colitis than in their wild-type mice, suggesting that SOCS3 serves a negative regulatory role in intestinal inflammation by downregulating STAT3 activity (16). In another study, periarticular injection of the SOCS3 adenovirus into the ankle joints of mice with arthritis drastically suppressed STAT3 activation and reduced the severity of arthritis and joint swelling compared with control groups (17). In addition, the current study observed a suppressive effect of SOCS3 on the STAT3 activation when lentivirally transferring SOCS3 genes into lung CD4+ T cells isolated from mice with chronic PA infection. The effect of SOCS3 was observed at 72 h following transinfection, and

became more obvious at 96 h. These results supported the idea that SOCS3 functioned as a negative regulator of STAT3 activation in the chronic PA lung infection, and maybe beneficial to control the airway inflammation.

Th17 cell is a proinflammatory subset of effector T cells that have been implicated in the pathogenesis of many autoimmune diseases. Activated STAT3 regulates Th17 cell differentiation by participating in the transcriptional activation of several Th17-regulatory genes, including those encoding IL-23R and RORγt (18,19). Their production of the cytokine IL-17A is known to induce epithelial cells to express a wide range of inflammatory mediators such as CXCL1 and CXCL5, which results in the local recruitment of neutrophils. In the present study, SOCS3-overexpressing lung CD4+ T cells were observed to have lower mRNA and protein levels of RORγt than control cells, and produced significantly decreased IL-17A *in vitro*. The incubation of epithelial cells with these cells induced significantly lower level of CXCL1 and CXCL5 secretion compared with controls, and recruited significantly decreased number of migrating neutrophils as indicated by chemotaxis analysis. These results indicated that SOCS3 gene transfer maybe a potential therapy for suppressing Th17-mediated neutrophilic airway inflammation.

Lung CD4+ T cells were stimulated with IL-23 *in vitro* to investigate the effect of SOCS3 overexpression on Th17 responses. IL-23 is a heterodimeric cytokine recognized as an inducer of IL-17-producing cells in the pool of activated memory T cells (20,21) and is important to maintain the Th17 phenotype (22). Activation of STAT3 appears to be the major signaling pathway of IL-23, and STAT3 binding sites were identified in the IL-23R (23). Deficiency of IL-23 was presented to be associated with lower induction of IL-17A at sites of airway inflammation observed in mucoid PA infection (24). The current results reported that the overexpression of SOCS3 in lung CD4+ T cells suppressed the IL-23-induced Th17 response, suggesting that SOCS3 served a suppressive role in regulating the IL-23/STAT3 signaling pathway involved in the Th17-related airway inflammation.

Although IL-17A is believed to be primarily produced by activated CD4+ T cells, other cells, such as activated γδ T cells, neutrophils and mast cells are also able to produce IL-17A (25). In the current study, the authors focused on the IL-17A expression produced by lung CD4+ T cells because CD4+ T cells have been suggested to be a key feature in lungs with PA infection. Both healthy individuals and patients with cystic fibrosis had robust antigen-specific memory CD4+ T cell responses to PA that not only contained a Th17 component, but also Th1 and Th22 cells (26). Inducible proliferation of Th17 with memory cell characteristics is seen in the lung draining lymph nodes of patients with cystic fibrosis (27). However, as the immune responses involved in chronic PA lung infection are complicated, the roles of IL-17A produced by other cells also need to be investigated in future studies.

Limitations of this investigation included that, in the part of *in vitro* study, lung CD4+ T cells, lung epithelial cells and neutrophils were treated outside their normal environment, and the *in vivo* exposures cannot easily be mimicked. Although these *in vitro* studies provided us with valuable insight into the influence of SOCS3 on the neutrophil migration, subsequent *in vivo* studies are required to further

testify the conclusion. Since any intervention that decreases inflammation may potentially have a detrimental effect by promoting airway infection, in future *in vivo* studies, special attention will be focused on whether the decreased amount of pulmonary neutrophils would be adequate to cope with the PA in the lungs. In addition, as many other immune cells, such as CD8+ T cells, macrophages and dendritic cells, also take part in the neutrophilic airway inflammation during chronic PA infection, investigations regarding the interaction between SOCS3-overexpressing CD4+ T cells and these immune cells are needed.

In conclusion, the present results indicated that SOCS3 was upregulated in lung CD4+ T cells in a mouse model of chronic PA lung infection and exogenous SOCS3 suppressed Th17-mediated neutrophil recruitment *in vitro*. These results suggested that SOCS3 gene therapy could be a potential way for immunotherapy to treat airway inflammation during chronic PA lung infection.

Acknowledgements

References

1. Rada B: Interactions between neutrophils and pseudomonas aeruginosa in cystic fibrosis. Pathogens 6: pii:E10, 2017.
2. McAllister F, Henry A, Kreindler JL, Dubin PJ, Ulrich L, Steele C, Finder JD, Pilewski JM, Carreno BM, Goldman SJ, *et al*: Role of IL-17A, IL-17F, and the IL-17 receptor in regulating growth-related oncogene-alpha and granulocyte colony-stimulating factor in bronchial epithelium: Implications for airway inflammation in cystic fibrosis. J Immunol 175: 404-412, 2005.
3. Tiringer K, Treis A, Fucik P, Gona M, Gruber S, Renner S, Dehlink E, Nachbaur E, Horak F, Jaksch P, *et al*: A Th17- and Th2-skewed cytokine profile in cystic fibrosis lungs represents a potential risk factor for Pseudomonas aeruginosa infection. Am J Respir Crit Care Med 187: 621-629, 2013.
4. Dubin PJ and Kolls JK: IL-23 mediates inflammatory responses to mucoid Pseudomonas aeruginosa lung infection in mice. Am J Physiol Lung Cell Mol Physiol 292: L519-L528, 2007.
5. Egwuagu CE: STAT3 in CD4+ T helper cell differentiation and inflammatory diseases. Cytokine 47: 149-156, 2009.
6. Ivanov II, Zhou L and Littman DR: Transcriptional regulation of Th17 cell differentiation. Semin Immunol 19: 409-417, 2007.
7. Harris TJ, Grosso JF, Yen HR, Xin H, Kortylewski M, Albesiano E, Hipkiss EL, Getnet D, Goldberg MV, Maris CH, *et al*: Cutting edge: An in vivo requirement for STAT3 signaling in TH17 development and TH17-dependent autoimmunity. J Immunol 179: 4313-4317, 2007.
8. Yuan K, Huang C, Fox J, Gaid M, Weaver A, Li G, Singh BB, Gao H and Wu M: Elevated inflammatory response in caveolin-1-deficient mice with Pseudomonas aeruginosa infection is mediated by STAT3 protein and nuclear factor kappaB (NF-kappaB). J Biol Chem 286: 21814-21825, 2011.
9. Yoshimura A, Naka T and Kubo M: SOCS proteins, cytokine signalling and immune regulation. Nat Rev Immunol 7: 454-465, 2007.
10. Chen Z, Laurence A, Kanno Y, Pacher-Zavisin M, Zhu BM, Tato C, Yoshimura A, Hennighausen L and O'Shea JJ: Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. Proc Natl Acad Sci USA 103: 8137-8142, 2006.
11. Baker BJ, Akhtar LN and Benveniste EN: SOCS1 and SOCS3 in the control of CNS immunity. Trends Immunol 30: 392-400, 2009.
12. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.

13. Ruwanpura SM, McLeod L, Brooks GD, Bozinovski S, Vlahos R, Longano A, Bardin PG, Anderson GP and Jenkins BJ: IL-6/Stat3-driven pulmonary inflammation, but not emphysema, is dependent on interleukin-17A in mice. Respirology 19: 419-427, 2014.
14. Cacalano NA, Sanden D and Johnston JA: Tyrosine-phosphorylated SOCS-3 inhibits STAT activation but binds to p120 RasGAP and activates Ras. Nat Cell Biol 3: 460-465, 2001.
15. Sasaki A, Yasukawa H, Suzuki A, Kamizono S, Syoda T, Kinjyo I, Sasaki M, Johnston JA and Yoshimura A: Cytokine-inducible SH2 protein-3 (CIS3/SOCS3) inhibits Janus tyrosine kinase by binding through the N-terminal kinase inhibitory region as well as SH2 domain. Genes Cells 4: 339-351, 1999.
16. Suzuki A, Hanada T, Mitsuyama K, Yoshida T, Kamizono S, Hoshino T, Kubo M, Yamashita A, Okabe M, Takeda K, et al: CIS3/SOCS3/SSI3 plays a negative regulatory role in STAT3 activation and intestinal inflammation. J Exp Med 193: 471-481, 2001.
17. Shouda T, Yoshida T, Hanada T, Wakioka T, Oishi M, Miyoshi K, Komiya S, Kosai K, Hanakawa Y, Hashimoto K, et al: Induction of the cytokine signal regulator SOCS3/CIS3 as a therapeutic strategy for treating inflammatory arthritis. J Clin Invest 108: 1781-1788, 2001.
18. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS and Dong C: STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. J Biol Chem 282: 9358-9363, 2007.
19. Zhou L, Ivanov I, Spolski R, Min R, Shenderov K, Egawa T, Levy DE, Leonard WJ and Littman DR: IL-6 programs T(H)-17 cell differentiation by promoting sequential engagement of the IL-21 and IL-23 pathways. Nat Immunol 8: 967-974, 2007.
20. Aggarwal S, Ghilardi N, Xie MH, de Sauvage FJ and Gurney AL: Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. J Biol Chem 278: 1910-1914, 2003.
21. Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA and Cua DJ: IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 201: 233-240, 2005.
22. Veldhoen M, Hocking RJ, Atkins CJ, Locksley RM and Stockinger B: TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. Immunity 24: 179-189, 2006.
23. Floss DM, Mrotzek S, Klöcker T, Schröder J, Grötzinger J, Rose-John S and Scheller J: Identification of canonical tyrosine-dependent and non-canonical tyrosine-independent STAT3 activation sites in the intracellular domain of the interleukin 23 receptor. J Biol Chem 288: 19386-19400, 2013.
24. Dubin PJ and Kolls JK: IL-23 mediates inflammatory responses to mucoid Pseudomonas aeruginosa lung infection in mice. Am J Physiol Lung Cell Mol Physiol 292: L519-L528, 2007.
25. Keijsers RR, Joosten I, van Erp PE, Koenen HJ and van de Kerkhof PC: Cellular sources of IL-17 in psoriasis: A paradigm shift? Exp Dermatol 23: 799-803, 2014.
26. Bayes HK, Bicknell S, MacGregor G and Evans TJ: T helper cell subsets specific for Pseudomonas aeruginosa in healthy individuals and patients with cystic fibrosis. PLoS One 9: e90263, 2014.
27. Chan YR, Chen K, Duncan SR, Lathrop KL, Latoche JD, Logar AJ, Pociask DA, Wahlberg BJ, Ray P, Ray A, et al: Patients with cystic fibrosis have inducible IL-17+IL-22+ memory cells in lung draining lymph nodes. J Allergy Clin Immunol 131: 1117-1129.e1-e5, 2013.