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Research Article

Suppressors of cytokine signaling 3 is essential for FcγR-mediated inflammatory response *via* enhancing CCAAT/enhancer-binding protein δ transcriptional activity in macrophages

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ABSTRACT

Compelling evidence indicates that suppressor of cytokine signaling 3 (SOCS3) plays a pivotal regulatory role in inflammation. However, the function of SOCS3 in inflammatory responses mediated by Fcγ receptor (FcγR) remains largely unknown. In the current study, we found that SOCS3 expression was greatly enhanced in peritoneal macrophages treated with IgG immune complex (IgG IC). By over-expressing SOCS3 in macrophages, we observed that SOCS3 promoted IgG immune complex-induced production of inflammatory mediators, including IL-6, TNF-α, MIP-2, and MIP-1α. In contrast, SOCS3-defective peritoneal macrophages generated less inflammatory cytokines and chemokines when compared with their wild type counterparts during IgG IC-induced inflammatory responses. We further demonstrated that CCAAT/enhancer-binding protein (C/EBP) δ transcription factor was the major downstream target of SOCS3 in macrophages. These data suggested that SOCS3 was an inflammatory enhancer in IgG IC-treated macrophages by increasing C/EBPδ activity. To elucidate the role for myeloid-derived SOCS3 in IgG IC-induced inflammation *in vivo*, *LysM-cre* $SOCS3^{fl/fl}$ mice lacking SOCS3 in macrophages and neutrophils were generated. We found that SOCS3 deficiency greatly alleviated IgG IC-induced generation of pro-inflammatory mediators in lungs, consistent with the *in vitro* data. Our current findings may provide a new theoretical basis for designing drugs for treatment of IgG IC-associated diseases.

1. Introduction

Suppressor of Cytokine Signaling 3 (SOCS3) belongs to SOCS family of proteins, and its expression can be induced by various inflammatory stimuli, such as bacterial elements, IgG immune complex, and cytokines [1–6]. SOCS3 was first found as a repressor of signal transducer and activator of transcription (STAT)/Janus kinase (JAK) signaling pathway by inhibiting JAK catalytic activity or inducing degradation of cytokine receptor/JAK [7]. Now, roles of SOCS3 in both innate and adaptive immune responses have been investigated. Depending on the distinct immune responses, SOCS3 may exert different effects on inflammation, referred to as pro- and anti-inflammatory functions. For instance, SOCS3 alleviates intestinal inflammation and rheumatoid arthritis by preventing STAT3 from hyperactivation [8,9]. Besides STAT3, SOCS3 could also repress inflammatory responses through other mechanisms. Our recent studies have proved that SOCS3 decreases IL-6 expression in osteoblasts incubated with LPS by inhibiting C/EBPβ DNA binding activity [4]. In contrast to the above reports, SOCS3 could also act as an inflammatory promoter. SOCS3 exerts its pro-inflammatory function by the interaction of its Src Homology 2 (SH2) domain with gp130, which ensures the specific interference with gp130 signaling pathways that are efficient inhibitors of LPS signaling. Thus, IL-6 challenged peritoneal macrophages lacking SOCS3 produce less TNF-α in response to LPS, and mice are resistant to LPS-induced shock in the absence of SOCS3 in macrophages and neutrophils [10]. Taken together, these studies suggest that SOCS3 may be an attractive target for therapies of inflammatory diseases.

Macrophages play a central role in innate immune responses. In both IgG IC- and LPS-induced acute pulmonary inflammation, macrophage depletion significantly attenuates generation of pro-

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inflammatory mediators [11–15]. During inflammatory responses, high level of SOCS3 is expressed by myeloid-derived cells, including macrophages [16–18], and its influence on production of pro-inflammatory mediators has been elucidated in Toll-like receptor 4 (TLR4)-activated macrophages [19,20]. However, its effect on inflammatory reactivities in macrophages upon Fcγ receptor (FcγR) cross-linking remains largely unknown. Here, by using over-expression and gene knockout technologies, we demonstrated that SOCS3 positively regulated IgG IC-induced inflammatory responses in peritoneal macrophages. In addition, we observed that myeloid-specific disruption of SOCS3 resulted in reduction of acute lung inflammation stimulated by IgG IC. Moreover, we found elevated transcriptional activity of the transcription factor-CCAAT/enhancer-binding protein (C/EBP) δ as an underlying mechanism by which SOCS3 enhanced IgG IC-induced expressions of pro-inflammatory mediators in macrophages.

2. Materials and methods

2.1. Animals and cell culture

The animal studies were conducted in accordance with guidelines approved by Southeast University and Harvard Medical School. *LysM-cre* mice were obtained from The Jackson Laboratory (Bar Harbor, ME, USA), and their Cre recombinase expressions are under the control of endogenous *Lyz2* promoter/enhancer elements. When crossed with $SOCS3^{fl/fl}$ mice (The Jackson Laboratory, Bar Harbor, ME, USA), Cre-mediated recombination resulted in deletion of *SOCS3* in myeloid cell lineage. All mice were used at the age of 8–12 weeks old. RAW264.7 cells were obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2 mM L-glutamine, and 100 units/ml penicillin–streptomycin.

2.2. RNA extraction and real time PCR

Total RNAs were isolated from cells by using Trizol obtained from Invitrogen. Then RNAs were subjected to reverse transcription, and cDNAs were synthesized. PCR were conducted with primers for SOCS3: 5′ primer, 5′-CGG GCA GGG GAA GAG ACT GT-3′ and 3′ primer, 5′-GGA GCC AGC GTG GAT CTG C-3′. The PCR universal protocol was described as follows: activation at 95 °C for 5 s and, annealing/extension at 60 °C for 30 s. Accumulation of fluorescent products was measured in real time. The relative mRNA levels were normalized to levels of GAPDH mRNA in the same sample.

2.3. Stable cell construction

Control plasmids, or human SOCS3 over-expression plasmids were transduced into RAW264.7 cells by using Fugene®6 Transfection Reagent (Roche, Indianapolis, IN). 12 h after transfection, the cells were treated with 200 μg/ml G418 (MP Biomedicals, LLC, OH), and G418-resistant colonies were selected. SOCS3 expression was detected by Western blot.

2.4. Expression vectors and promoter reporters

The mouse C/EBPδ promoter-reporter, C/EBPβ and C/EBPδ expression plasmids, were kindly provided by Richard C. Schwartz (Michigan State University). The reporter plasmid (2 × C/EBP-Luc) containing two copies of a C/EBP binding site was a kind gift from Peter F. Johnson (NCI-Frederick). NF-κB promoter-reporter was purchased from Promega. Human SOCS3 over-expression vector was kindly provided by Dr. Akihiko Yoshimura (Keio University).

2.5. Luciferase assay

Plasmids were transduced into RAW264.7 cells by by using Fugene®6 Transfection Reagent as suggested by the supplier. 48 h after transfection, the cells were treated with or without 100 μg/ml IgG immune complex. 4 h later, luciferase activity was detected by using Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) as recommended by the manufacturer.

2.6. Preparation of immune complexes

Immune complexes could be formed by incubation of 100 μg BSA (Sigma-Aldrich) in 100 μl PBS with 25 μl 2.5 mg/ml anti-BSA IgG (MP Biomedicals, LLC, OH) at 37 °C for 30 min.

2.7. Peritoneal macrophage isolation and culture

Mouse peritoneal macrophages were isolated as described previously [21]. Briefly, mice received peritoneal administration of 1 ml of 3% sterile thioglycollate. Five days later, peritoneal macrophages were isolated by instillation and aspiration of 10 ml ice-cold PBS. Cell suspension was centrifuged at 1000 rpm at 4 °C for 10 min. Then the macrophages were cultured in DMEM supplemented with 5% FBS.

2.8. IgG IC-induced acute lung inflammation

Mice were firstly anesthetized with intraperitoneal injection of 1.5% sodium pentobarbital. Then mice were treated intra-tracheally with rabbit anti-bovine serum albumin (BSA) IgG. Immediately after intra-tracheal injection of anti-BSA, 1 mg BSA was administrated intravenously. Negative control mice received intratracheal instillation of anti-BSA. 4 h later, bronchoalveolar lavage (BAL) fluids were harvested, and cell-free supernatants were subjected to ELISA.

2.9. Western blot analysis

RAW264.7 cells were lysed in cold RIPA buffer supplemented with protease inhibitors and PMSF following the manufacturer's guidelines. 30 μg proteins were subjected to electrophoresis in a 12% polyacrylamide gel and then transblotted onto a PVDF membrane followed by blocking with 5% nonfat milk solution. Rabbit anti-Myc antibody, rabbit anti-C/EBPδ antibody, rabbit anti-GAPDH antibody (Cell Signaling, Danvers, MA, USA), and rabbit anti-SOCS3 antibody (Proteintech, Wuhan, China) were applied, followed by a HRP-conjugated donkey anti-rabbit secondary IgG (GE Healthcare, Piscataway, NJ, USA). Immunoreactivity was visualized using enhanced chemiluminescence technique according to the manufacturer's direction (Thermo Fisher Scientific, Rockford, IL, USA).

2.10. ELISA

ELISA kits for mouse TNF-α, MIP-2, IL-6, MIP-1α, MCP-1 and MIP-1β were purchased from R&D Systems, and ELISA were performed following manufacture's protocols.

2.11. Gel shift assay

Gel shift assay were conducted as described previously [22,23]. Cell nuclear proteins were prepared as follows. Cells were lysed in 2 mM $MgCl_2$, 15 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 10 mM HEPES (pH 7.6), 0.5 mM PMSF, 0.1% (v/v) Nonidet P-40, and