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Adenoviral-Mediated Overexpression of SOCS3 Enhances IgG Immune Complex-Induced Acute Lung Injury

Hongwei Gao, L. Marco Hoesel, Ren-Feng Guo, Nicholas J. Rancilio, J. Vidya Sarma, and Peter A. Ward

The lung inflammatory response caused by intratracheal deposition of IgG immune complexes (IC) includes the production of IL-6, which signals through activation of STAT transcription factors. Recently, suppressor of cytokine signaling 3 (SOCS3) has been shown to be a key negative regulator of IL-6/gp130/Jak/STAT3 signal transduction. Although SOCS3 has been implicated in several inflammatory diseases, very little is known regarding its activation and its function in the lung during acute inflammation. Our previous study showed that IL-6/STAT3 activation was triggered in lungs after intrapulmonary deposition of IgG IC in rats. In the current study, we sought to determine whether SOCS3 is playing a regulatory role in the lung inflammatory response. SOCS3 induction occurred during development of inflammation in the IgG IC model of lung injury. Overexpression of SOCS3 in lung using a recombinant adenosine encoding murine SOCS3 resulted in substantial increases in lung vascular permeability and lung myeloperoxidase, together with enhanced levels of TNF-α, MIP-2, and keratinocyte-activated cytokine in bronchoalveolar lavage fluids. SOCS3 overexpression in lungs led to overproduction of bronchoalveolar lavage IL-6, but not IL-10, in this inflammatory model. We further show that activation of STAT3 was inhibited by SOCS3 overexpression as well as by anti-IL-6 treatment during IgG IC-induced lung injury, as determined by EMSA. In vitro, SOCS3 overexpression abrogated IL-6-induced activation of STAT3 in lung epithelial cells. These findings suggest SOCS3 is an important regulator of lung inflammatory injury after deposition of IgG IC. The Journal of Immunology, 2006, 177: 612–620.

The rodent lung injury model triggered by the intrapulmonary deposition of IgG immune complexes (IC) is known to induce extensive gene activation and has been used to study the roles of cytokines, chemokines, and complement in the process of acute inflammation (1). In this model, IgG IC deposition triggers complement activation and activation of residential lung macrophages via engagement of FcyRs. Lung macrophages generate and secrete a number of proinflammatory mediators, resulting in recruitment of neutrophils to the inflammatory site. Recruitment of neutrophils into inflamed lung is initiated by TNF-α, and IL-1-induced expression of adhesion molecules on the lung vascular endothelium, involving P-selectin, E-selectin, and ICAM-1 (2, 3). Moreover, TNF-α and IL-1 directly or indirectly mediate neutrophil migration into the lung interstitium and alveoli by inducing robust production of chemokines (CXC and CC) and cytokines in lung via an NF-κB-dependent mechanism (4–6). The inflammatory response induced by intrapulmonary deposition of IgG IC is also regulated by a number of endogenous anti-inflammatory cytokines. These include IL-4, IL-6, IL-10, IL-11, IL-12, and IL-13 (7). Although the molecular mechanisms by which these regulatory cytokines operate are incompletely defined, each is able to limit the extent of lung inflammation by dampening the production of TNF-α and IL-1β (8–13). Thus, a complex network of mediators plays a major role in initiating, amplifying, and regulating IgG IC-induced lung injury.

Both IL-6 and IL-10 engage receptors that recruit Jak. Activated Jaks phosphorylate the cytoplasmic domain of the receptor, creating a docking site for Src homology 2-containing proteins such as STAT transcription factors (14). Indeed, our previous work showed that STAT3 is activated in lung during IgG IC-induced acute lung injury, with both Tyr705 and Ser727 undergoing phosphorylation (15). Activation of STAT3 in lungs is macrophage and neutrophil dependent. Furthermore, we showed that activation of STAT3 requires both IL-6 and IL-10, suggesting that its activation brings into play a mechanism whereby mediator production and the inflammatory response are contained in this lung injury model. Therefore, elucidation of the regulatory mechanisms of the Jak-STAT3 pathway is important in understanding the inflammation process associated with IgG IC-induced acute lung injury. To date, at least three different classes of negative regulators exist to modulate the cytokine-Jak-STAT pathway. These include protein tyrosine phosphatases, protein inhibitors of activated STATs, and the suppressors of cytokine signaling (SOCS) (16, 17). SOCS molecules control cytokine receptor signaling by several mechanisms, including blocking of STAT3 binding to receptor chains, direct and indirect inhibition of Jak kinases, and inhibition of downstream signaling pathways (18). Moreover, SOCS molecules may have certain regulatory roles in addition to inhibition of cytokine receptor signaling. For example, very recently SOCS3 has been indicated as an important modulator of cell activation during renal inflammation by interacting with Tec tyrosine kinase and inhibiting FcγR-mediated STAT activation (19). Our previous results have shown that expression of SOCS3 in the rat lung was dramatically induced during IgG IC-induced lung injury (15). The biological relevance for this induction is not known. Although SOCS3...
has been demonstrated to play a negative regulatory role in both intestinal inflammation and inflammatory arthritis (20, 21), the function of SOCS3 during acute lung inflammation is not known. In the current study, we demonstrate that adenosivirus-mediated overexpression of SOCS3 dramatically enhances IgG IC-induced lung injury and causes reduction of STAT3 activation in the lung. The data suggest that SOCS3 by interfering with STAT3 activation may have an important regulatory role during lung inflammatory injury after deposition of IgG IC.

Materials and Methods

Reagents

Rabbit anti-TB A IgG was purchased from ICN Biomedicals. ELISA kits for mouse IL-6 and IL-10 were purchased from BioSource International. Recombinant mouse IL-6, goat anti-mouse MIP-2, anti-mouse keratinocyte-activated cytokine (KC), anti-mouse TNF-α, and anti-mouse MCP-1 IgGs were purchased from R&D Systems. Anti-mouse IL-6 and control IgG were purchased from BD Pharmingen. Rabbit anti-mouse SOCS3 and anti-mouse actin were purchased from Santa Cruz Biotechnology. Mouse monoclonal anti-phospho-STAT3 (Tyr105) and rabbit anti-mouse STAT3 were purchased from Upstate Biotechnology.

IgG IC-induced lung injury

Eight- to 10-wk-old specific pathogen-free male C57BL/6 mice were used. Mice were anesthetized i.p. with ketamine, the trachea was surgically exposed by a midline incision, and 100 μg of rabbit anti-TB A IgG in 40 μl of PBS was administered intratracheally at tracheal puncture with a 30-gauge needle. The incision was closed by two surgical clips, and 1 mg of PBS was administered intratracheally by tracheal puncture with a 30-gauge needle. The animals were housed in a humid chamber and were preceded and followed by three washes with PBS. Briefly, after a 20-min blocking step with peroxidase-blocking reagent (0.3% H2O2 in water), the tissue sections were incubated overnight at 4°C with anti-SOCS3 IgG in a dilution of 1/500 of the stock (4 μg/ml) in PBS. Thereafter, the tissue sections were incubated with a 1/500 diluted peroxidase-conjugated goat anti-rabbit IgG for 45 min. After 30-min staining in VECTASTAIN AEC Reagent, the sections were incubated with diamobenzidine peroxide substrate for 2 min (Vector Laboratories). Counterstaining was achieved with hematoxylin for 30 s. Tissue sections were fixed and cover slides were mounted with Permount medium (Fisher Scientific). Staining was documented using light microscopy and digital imaging.

Permeability index

For permeability index measurements, BSA was labeled with 125I by the chloramine T method. A trace amount of 125I-labeled BSA (sp. act., 5 μCi/μg) was added to unlabeled BSA (5 mg/ml in PBS), and 200 μl of this solution was injected i.v. to induce the IgG IC lung injury, as described above. Four hours later, mice were euthanized with ketamine (given i.p.) and blood was collected from the inferior vena cava. The thorax was opened, left atrium was incised, and the lung was perfused in situ with 3 ml of PBS via the pulmonary artery. The flushed lungs were removed, and permeability index (indicating the extent of pulmonary leakage) was determined by using a gamma counter and expressed as the ratio of cpm in the whole lung vs radioactivity in 100 μl of blood.

Myeloperoxidase (MPO) assay

Lungs were perfused via the right ventricle with 3 ml of sterile PBS, snap frozen in liquid nitrogen, and stored at −70°C. To measure MPO activity, whole lungs were homogenized and sonicated in 50 mMol/L potassium phosphate buffer containing 0.5% hexadecyltrimethylammonium bromide and 5 mMol/L EDTA. After centrifugation at 12,000 × g for 10 min at 4°C, the supernatant fluids containing MPO were incubated in a 50 mMol/L potassium phosphate buffer containing the substrate, H2O2 (1.5 mol/l). In the presence of o-dianisidine dihydrochloride (167 μg/ml; Sigma-Aldrich), the enzymatic activity was determined spectrophotometrically by measuring the change in absorbance at 460 nm over 3 min using a 96-well plate reader (Molecular Devices).

Bromochodimal lavage (BAL) fluid collection and differential white blood cell counts, and quantification of chemokine/cytokine production by ELISAs

Four hours after initiation of acute lung injury, the thorax was opened and 0.8 ml of ice-cold, sterile PBS was instilled into the lung via a tracheal incision. The recovered lavage fluid (BAL) was centrifuged at 450 × g for 6 min and the cell-free supernatants were used for chemokine and cytokine measurements by sandwich ELISA. ELISA plates were coated overnight at 4°C with 5 μg/ml capture Ab per well. After blocking with 3% BSA in PBS, the samples were added to the 96-well plates and incubated for 2 h, followed by incubation with the biotinylated secondary Ab (2 μg/ml) for 1 h. After washing, peroxidase-conjugated streptavidin was added for 30 min, followed by incubation with o-phenylenediamine dihydrochloride (peroxidase substrate) for 10 min, and the reaction was stopped with 0.5 mol/L sulfuric acid. OD was measured at 490 nm. For differential white blood cell counts, cell pellets were resuspended in 0.5 ml of PBS buffer (pH 7.4) containing 0.5% BSA. Differential cell analyses were performed by Diff-Quik-stained cytosin preparations (Dade Diagnostics) counting a total of 300 cells per slide in randomly selected high-powered fields (×400).

Proteins were preclreated with protein A/G beads overnight at 4°C with gentle rotation. Samples containing 50 μg of protein were electrophoresed in a denaturing 12% polyacrylamide gel and then transferred to a polyvinylidene difluoride membrane. Membranes were incubated with anti-SOCS3 and anti-actin Abs at a 1/500 dilution. After three washes in TBST, membranes were incubated with a 1/5000 dilution of HRP-conjugated donkey anti-rabbit IgG (Amersham Biosciences). The membrane was developed by ECL technique, according to the manufacturer’s protocol (Amersham Biosciences).

Immunohistochemical staining for SOCS3

Lungs were inflated with 0.8 ml of tissue-embedding solution (Tissue-Tek OCT compound; Fisher Scientific) before freezing to prevent alveolar collapse. Glass slides with tissue sections of 4- to 5-μm thickness were fixed in methanol for 5 min and stained using a VECTASTAIN ABC Kit (Vector Laboratories), according to the manufacturer’s instructions. All incubations took place in a humid chamber and were preceded and followed by three washes with PBS. Briefly, after a 20-min blocking step with peroxidase-blocking reagent (0.3% H2O2 in water), the tissue sections were incubated overnight at 4°C with anti-SOCS3 IgG in a dilution of 1/50 of the stock (4 μg/ml) in PBS. Thereafter, the tissue sections were incubated with a 1/500 diluted peroxidase-conjugated goat anti-rabbit IgG for 45 min. After 30-min staining in VECTASTAIN AEC Reagent, the sections were incubated with diamobenzidine peroxide substrate for 2 min (Vector Laboratories). Counterstaining was achieved with hematoxylin for 30 s. Tissue sections were fixed and cover slides were mounted with Permount medium (Fisher Scientific). Staining was documented using light microscopy and digital imaging.
Morphological assessment of lung injury

To morphologically assess lung injury 4 h after IgG IC deposition, lungs were fixed by intratracheal instillation of 1 ml of buffered (pH 7.2) formalin (10%). The lungs were further fixed in a 10% buffered Formalin solution for histological examination by tissue sectioning and staining with H&E.

Assessment of STAT3 and NF-κB activation by EMSA

Nuclear extracts of whole lung tissues were prepared, as described previously (15). Briefly, frozen lungs were homogenized in 0.6% (v/v) Nonidet P-40, 150 mM NaCl, 10 mM HEPES (pH 7.9), 1 mM EDTA, 0.5 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin. The homogenate was incubated on ice for 5 min and then centrifuged for 5 min at 3000 × g at 4°C. Proteins were extracted from the pelleted nuclei by incubation at 4°C with 420 mM NaCl, 20 mM HEPES (pH 7.9), 1.2 mM MgCl2, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM DTT, 0.5 mM PMSF, 2.5 μg/ml leupeptin, 5 μg/ml antipain, and 5 μg/ml aprotinin. Nuclear debris was pelleted by centrifugation at 13,000 × g for 30 min at 4°C, and the supernatant extract was collected and stored at −80°C. Protein concentrations were determined by Bio-Rad protein assay using BSA as a reference standard (Pierce). The EMSA probes were double-stranded oligonucleotides containing a STAT3 consensus oligonucleotide (GATCC-TCTTGGGAAAATCTCTGATC-3′; Santa Cruz Biotechnology) and an NF-κB consensus oligonucleotide (AGTTGAGGAGACTTTCCAGGC; Promega). These probes were end labeled with [γ-32P]ATP (3000 Ci/mmol at 10 Ci/μl; Amersham Biosciences). DNA-binding reactions were performed at room temperature in a 25-μl reaction mixture containing 6 μl of nuclear extract (1 μg/ml in buffer C) and 5 μl of 5× binding buffer (20% [v/v] Ficoll, 50 mM HEPES [pH 7.9], 5 mM EDTA, and 5 mM DTT). The remainder of the reaction mixture contained KCl at a final concentration of 50 mM, Nonidet P-40 at a final concentration of 0.1%, 1 μg of poly(dI-dC), 200 pg of probe (unless otherwise noted), bromophenol blue at a final concentration of 0.06% (w/v), and water to volume. Samples were electrophoresed through 5.5% polyacrylamide gels in 1× TBE (90 mM Tris base, 90 mM boric acid, and 0.5 mM EDTA) at 160 V for 2 h, dried under vacuum, and exposed to x-ray film.

Statistical analysis

All values were expressed as the mean ± SEM. Significance was assigned in which p < 0.05. Data sets were analyzed using Student’s t test or one-way ANOVA, with individual group means being compared with the Student-Newman-Keuls multiple comparison test.

Results

SOCS3 expression in IgG IC-inflamed mouse lungs

To assess changes in endogenous SOCS3 protein during the pulmonary inflammatory response, whole protein was extracted from IgG IC-inflamed lungs over a range of time (from 0 to 6 h). SOCS3 expression was analyzed by Western blot analysis; the results are shown in Fig. 1. Little, if any, expression of SOCS3 protein was detected in normal lung. SOCS3 protein expression started to increase at 2 h and increased maximally at 4 h. Sustained up-regulation was still found at 6 h. Equal loading of protein was confirmed by probing the same blot to determine β-actin levels (Fig. 1). We also harvested lungs from control mice, which received same amount of rabbit anti-BSA IgG intratracheally in the absence of an i.v. infusion of BSA. No induction of SOCS3 expression was found in lung homogenates from these mice (data not shown). We have shown previously that lung STAT3 activation (phosphorylation) during IgG IC-induced alveolitis exhibited almost the same pattern as that of SOCS3 (15). Thus, it appears that a negative feedback mechanism via SOCS3 expression may regulate signaling related to STAT3 activation during IgG IC-induced lung injury.

Expression of SOCS3 after pLP-Ad-SOCS3 administration

To elucidate the significance of SOCS3 activation in IgG IC-induced lung injury, we first examined whether exogenous administration of SOCS3 by transgene delivery could induce SOCS3 expression in the mouse lung. First, we performed initial experiments with pLP-Ad-SOCS3 to determine the timing of SOCS3 expression after intratracheal injection. SOCS3 protein expression levels were assessed by Western blot analysis of lung homogenates 24, 36, 48, 72, 96, and 120 h after in vivo pLP-Ad-SOCS3 administration (2.5–5 × 108 PFU). We observed increased SOCS3 expression levels at as early as 24 h, with peak level at 72 h of pLP-Ad-SOCS3 administration (data not shown). Because high doses of adenoviral vectors are known to cause lung inflammation, we performed dose-range experiments using 1 × 107–1 × 108 PFU of pLP-Ad-SOCS3 delivered by intratracheal injection. SOCS3 protein expression levels were assessed 72 h later after adenoviral administration. Although SOCS3 expression was observed at dose of 1 × 108 PFU (Fig. 2A, lane 3), stronger SOCS3 expression was detected at doses of 2.5 × 108 PFU or higher (Fig. 2A, lanes 4–6) when compared with control mice receiving vehicle (Fig. 2A, lane 2) or adeno-X-DsRed2 alone (Fig. 2A, lane 1). At the dose of 1 × 109 PFU, there was evidence of vascular congestion and hyperpermeability of mucus in bronchioles as shown by lung H&E staining (data not shown). At doses of 5 × 108 PFU or lower, no evidence of inflammation or increased mucus production was observed. All subsequent studies were done with 5 × 108 PFU, the dose that is able to induce strong SOCS3 expression without triggering an inflammatory response.

To localize the expression of SOCS3 protein, we performed immunohistochemical staining in lung sections after pLP-Ad-SOCS3 administration (Fig. 2, B–E). As shown in Fig. 2, B and D, mice receiving control adeno-X-DsRed2 virus exhibited low levels of SOCS3 protein expression. However, strong staining for SOCS3 protein was detected in mouse lung, especially in bronchole (Fig. 2C) and alveolar epithelium (Fig. 2E), after pLP-Ad-SOCS3 administration. Fig. 2E represents a higher magnification illustrating SOCS3 protein expression in bronchole and alveolar epithelium after administration of pLP-Ad-SOCS3 (arrowheads). To determine whether transgene expression was limited to the lung epithelium, we performed immunocytochemistry experiments using cells in BAL fluids harvested from pLP-Ad-SOCS3-infected lungs 0 and 1 h after IC deposition. SOCS3 expression was not detectable in BAL cells, including alveolar macrophages, which were the predominant lavageable cell type (data not shown). Accordingly, it appears that predominant expression of SOCS3 was limited to epithelial cells of lung.
the permeability index when compared with mice receiving only Adeno-X-DsRed2. However, administration of pLP-Ad-SOCS3 together with IgG IC deposition resulted in a significant increase (89%; \( p < 0.01 \)) in the permeability index when compared with controls receiving Adeno-X-DsRed2 together with IgG IC deposition (Fig. 3A). Increases in lung permeability correlated with increases in neutrophil accumulation in lungs as determined by MPO content (Fig. 3B). As with albumin leakage, MPO content in mice receiving pLP-Ad-SOCS3 was significantly higher (55%; \( p < 0.05 \)) when compared with values in mice receiving Adeno-X-DsRed2 during lung injury.

We further examined whether mice receiving pLP-Ad-SOCS3 exhibited enhanced lung injury by histological analyses. Fig. 4A demonstrates that mice receiving pLP-Ad-SOCS3 alone (72 h earlier) exhibited normal lung architecture with no evidence of inflammation. Lung hemorrhage, edema, fibrin deposition, and accumulation of neutrophils were observed in mice receiving control virus after IgG IC deposition (Fig. 4B); however, we observed significant increase in all of these parameters 4 h after IgG IC deposition in mice receiving pLP-Ad-SOCS3 (Fig. 4, C and D). Taken together, these data suggest the inflammatory response and evidence of injury after IgG IC-induced lung injury are intensified in the presence of virally induced SOCS3.

Effects of SOCS3 overexpression on leukocyte content in BAL fluids from IgG IC-injured lung

Cell content of BAL fluids from IgG IC-injured lungs receiving Adeno-X-DsRed2 or pLP-Ad-SOCS3 mice 72 h earlier was evaluated 4 h after IgG IC deposition. As would be expected, the cells in BAL fluids from noninjured lungs were uniformly macrophages and some lymphocytes (data not shown). In the IgG IC-injured

FIGURE 2. SOCS3 protein levels in whole lungs of adenovirus-injected mice. A, Expression of SOCS3 protein in mouse lungs 72 h after administration of different doses of pLP-Ad-SOCS3 (1 \( \times 10^8 \)–1 \( \times 10^9 \) PFU) or Adeno-X-DsRed2 (5 \( \times 10^9 \) PFU) was evaluated by Western blot using rabbit anti-SOCS3 and goat anti-actin. B–E, Representative SOCS3-immunostained lung sections obtained following administration of pLP-Ad-SOCS3 (B and D) or Adeno-X-DsRed2 (C and E). Arrowheads in E indicate SOCS3 staining in type II alveolar epithelial cells. Magnifications: B and C, \( \times 20 \); D and E, \( \times 200 \).

FIGURE 3. Effects of SOCS3 overexpression on IgG IC-induced lung injury. Lung vascular permeability and neutrophil accumulation in mice receiving pLP-Ad-SOCS3 with IgG IC deposition were compared with mice receiving Adeno-X-DsRed2 with IgG IC deposition. Controls (Ctrl) received \( \alpha \)-BSA intratracheally together with either pLP-Ad-SOCS3 or Adeno-X-DsRed2, but in the absence of i.v. administered BSA. Mouse \( ^{125}I \)-labeled albumin levels in whole lungs (A) were measured as an index for vascular leakage. MPO activity in whole lungs (B) was used as an index of neutrophil accumulation in the lungs. Results are means ± SEM for \( \geq 5 \) mice for each group.
lung, there was a 38% increase in the number of neutrophils present in the BAL fluids from pLP-Ad-SOCS3 mice (15.520 ± 1.881 \times 10^4 \text{ cells/ml}) when compared with the Adeno-X-DsRed2 control mice (11.26 ± 1.763 \times 10^4 \text{ cells/ml}) (Fig. 5). But this difference did not achieve statistical significance, probably due to the inability to efficiently lavage neutrophils enmeshed in a fibrin network (Fig. 4, C and D). As shown in Fig. 5, there was also a smaller (nonsignificant) drop of 20% (p, NS) in the numbers of macrophages in BAL fluids from the IgG IC-injured lungs receiving pLP-Ad-SOCS3 as compared with those receiving Adeno-X-DsRed2 (4.680 ± 0.4042 \times 10^4 \text{cells/ml} vs 3.762 ± 0.6639 \times 10^4 \text{cells/ml}, respectively).

Effects of SOCS3 overexpression on chemokine/cytokine levels in BAL fluids from lungs

We investigated the effects of overexpression of SOCS3 in lung on levels of proinflammatory mediators in BAL fluids after IgG IC deposition. As shown in Fig. 6, BAL fluids from lungs of mice receiving pLP-Ad-SOCS3 or Adeno-X-DsRed2 4 h after deposition of IgG IC showed significantly increased content of TNF-α as well as the CXC chemokines, MIP-2 and KC (Fig. 6, A–C) when compared with corresponding control BAL fluids from lungs treated with pLP-Ad-SOCS3 or Adeno-X-DsRed2 alone. The levels of the proinflammatory mediators in BAL fluids were significantly increased by 78, 45, and 60%, for TNF-α, MIP-2, and KC, respectively (Fig. 6) in IgG IC-injured lungs infected with pLP-Ad-SOCS3 when compared with injured mice infected with Adeno-X-DsRed2. The CC chemokine MCP-1 level was not significantly elevated in mice receiving pLP-Ad-SOCS3 or Adeno-X-DsRed2 4 h after deposition of IgG IC (Fig. 6D), suggesting that expression of CXC and CC chemokines may be differently regulated by SOCS3 during IgG IC-induced lung injury.

Overexpression of SOCS3 reduces STAT3, but not NF-κB activation

Recent studies from our laboratory have shown that both STAT3 and NF-κB are activated in the whole lung during IgG IC-induced lung injury (5, 15). To investigate the effects of SOCS3 overexpression in lung on activation of STAT3 and NF-κB after IC deposition, pLP-Ad-SOCS3 or Adeno-X-DsRed2 was administered into lungs 72 h before deposition of IgG IC. Nuclear extracts were harvested 4 h after onset of injury and were analyzed by EMSA. As expected, IgG IC deposition caused a dramatic increase in activation of both STAT3 (Fig. 7A) and NF-κB (Fig. 7B) in lungs (lanes 3 and 4) when compared with the corresponding control animals (receiving αBSA together with pLP-Ad-SOCS3 or Adeno-X-DsRed2 in the absence of systemically administered BSA) (Fig. 7, A and B, lanes 1 and 2). Administration of pLP-Ad-SOCS3 resulted in significant attenuation of STAT3 activation in the IgG IC-injured lung (Fig. 7A, lanes 5 and 6). In contrast, overexpression of SOCS3 in the lungs failed to show inhibitory effects on NF-κB activation (Fig. 7B, lanes 5 and 6), suggesting that activation of STAT3 and NF-κB mediated by IgG IC deposition is separately regulated by SOCS3 during IgG IC-induced lung injury.

SOCS3 overexpression enhances IL-6, but not IL-10, levels in BAL fluids

Our previous studies suggested that both IL-6 and IL-10 are important regulators of lung inflammatory injury after deposition of
IgG IC and appear to contain the extent of injury (9, 22). Blockade of either IL-6 or IL-10 caused dramatic reductions in STAT3 activation in IC-injured rat lungs (15). Accordingly, we sought to evaluate effects of SOCS3 overexpression on levels of both IL-6 and IL-10 in the lung injury model. As shown in Fig. 8A, IgG IC-induced IL-6 production in BAL was significantly increased by infection with pLP-Ad-SOCS3 (170% increase) \((p < 0.01)\). In contrast, there was no significant difference in BAL IL-10 levels in mice receiving either pLP-Ad-SOCS3 or Adeno-X-DsRed2 after deposition of IgG IC (Fig. 8B).

To investigate the mechanism by which overexpression of SOCS3 caused enhanced lung injury, we assessed whether anti-IL-6 Ab administered intratracheally with IgG anti-BSA would attenuate STAT3 activation induced during lung inflammatory injury. As shown in Fig. 9, the presence of anti-IL-6 Ab greatly reduced activation of STAT3 in the lung during IgG IC-induced injury.
injury. To investigate the effect of SOCS3 overexpression on IL-6 signaling, we transiently transfected mouse lung epithelial cells, C10, with pLP-Ad-SOCS3. As shown in Fig. 10, IL-6 dramatically stimulated phosphorylation (Tyr705) of STAT3 in C10 cells (lanes 3 and 4) in the presence of Adeno-X-DsRed2 control virus. When SOCS3 expression was induced by different doses of pLP-Ad-SOCS3, IL-6-induced STAT3 activation was abrogated (lanes 5–7). Taken together, these data suggest that, although there was a SOCS3-related enhancement in the IL-6 production in the IgG IC-injured lung, IL-6 signaling was perturbed by overexpression of SOCS3. Furthermore, SOCS3 enhances the intensity of lung inflammatory injury in the IgG IC model by suppressing IL-6-induced activation of STAT3.

Discussion

In the IgG IC model of acute lung injury, there is a robust upregulation of early response cytokines such as TNF-α and IL-1, which activate NF-κB and induce expression of adhesion molecules on leukocytes and on endothelial cells, all of which fits into a strong proinflammatory cascade. Simultaneously, there is an anti-inflammatory cascade in this model, featuring production of IL-6, IL-10, and IL-13, although the signal pathways mediating their effects are largely unknown. SOCS3 belongs to the family of SOCS proteins, which have been shown to be induced by a number of mediators, including LPS, TNF-α, as well as both IL-6 and IL-10 (23–25). We now provide evidence that SOCS3 is activated in lung during IgG IC-induced acute lung injury. In our model, expression of SOCS3 was induced 2 h after onset of the IgG IC-induced lung injury and became more evident at 4 and 6 h (Fig. 1). The details of signal pathways mediating IgG IC-induced SOCS3 expression are unclear. It was shown recently that both IC-induced SOCS3 expression and STAT3 activation were impaired in mesangial cells from FcεRI and/or FcεRIII-deficient mice, suggesting that FcεRI and/or FcεRIII are involved in IC-mediated responses in renal cells (19). In the same study, both tyrosine kinases and MAPKs were shown to be involved in IC-induced SOCS3 expression (19). Whether these kinases mediate SOCS3 expression and activation during IgG IC-induced lung injury is not currently known.

SOCS3 has been shown to function as a proinflammatory mediator by suppressing IL-6-gp130 signaling, interfering with its ability to inhibit LPS signaling (26, 27). For example, mice deficient in macrophages and neutrophils are resistant to LPS-induced shock, although the precise role of SOCS3 in LPS responses remains enigmatic (26). In contrast, accumulating data suggest that SOCS3 may suppress inflammatory responses in both Crohn’s disease and rheumatoid arthritis (20, 21, 26, 27). Thus, the function of SOCS3 during inflammation is dependent on the particular disease model used. In the current study, we used recombinant adenovirus to overexpress SOCS3 in lung in a mouse model of IgG IC-induced injury to examine the functional role of SOCS3 during acute lung inflammation. Our data show that pretreatment with SOCS3 transgene significantly enhances IgG IC-induced lung injury as defined by increased albumin leakage into lung and enhanced MPO content, suggesting a role for SOCS3 in neutrophil accumulation in lung (Fig. 3). Neutrophil transmigration into the alveolar compartment and lung interstitium plays a key role in the development of acute lung injury. It is assumed that the production of...
chemoattractant chemokines precedes neutrophil influx. The CXC chemokines, MIP-2 and KC, are potent chemoattractants for rodent neutrophils (28). Blocking studies with MIP-2 and KC Abs in lungs significantly decreased MPO buildup, reflective of reduced neutrophil accumulation and less lung injury in the IgG IC model (8). In the current study, we demonstrated that overexpression of SOCS3 increased CXC chemokine levels (MIP-2 and KC), but not CC chemokine level (MCP-1) in BAL fluids from IgG IC-injured lungs (Fig. 6). In addition, our preliminary data in vitro show that in mouse lung epithelial cells, overexpression of SOCS3 enhances IgG IC-induced KC production, while the opposite effect occurred with respect to KC production induced by LPS (H. Gao and P. Ward, unpublished data). In the current study, we also found that in the IgG IC-injured lung, there was no significant increase in the number of neutrophils present in the BAL fluids when SOCS3 overexpression occurred as compared with control mice (Fig. 5). This contrasts with higher levels of MPO in the same lungs (Fig. 3B), suggesting that overexpression of SOCS3 resulted in more neutrophils being recruited into lung, but the increment cannot be detected by the BAL approach either because many of the neutrophils are still in the interstitial compartment or because they are aggregated or enmeshed in fibrin deposits in the alveolar compartment. Taken together, these experiments establish a linkage between SOCS3-enhanced CXC chemokine production, neutrophil recruitment, and lung injury following IgG-IC deposition.

One of the major cellular responses in lung initiated by FcγRs cross-linking is the activation of genes encoding chemokines and cytokines important in inflammation. As indicated above, FcγRI and III are engaged by ICs, triggering proinflammatory outcomes. Whether FcγRII, which has been cloned recently, but as yet has no assigned function, is also involved is not known. Following IC deposition in lung, the resulting levels of proinflammatory mediators, anti-inflammatory mediators, and their balance dictate the magnitude of lung injury. Although the signaling pathways from FcγRs to the nucleus are not clearly defined, growing evidence indicates that the regulation of gene expression in lung is mediated by a highly intricate network of transcription factors (28, 29). Recent studies from our lab in the IgG IC-induced lung injury model have shown that several transcription factors (NF-κB, AP-1, and STAT3) are activated in both alveolar macrophages and whole lung extracts following IgG IC deposition, although peak activation in macrophages occurs in 30 min, while in whole lung extracts the peak is at 4 h (5, 15, 30). Furthermore, both IL-6 and IL-10 contribute to STAT3 activation in inflamed lung, while IL-10 and IL-13 inhibit NF-κB activation (15, 31). Moreover, both IL-6 and IL-10 have been shown to act as intrinsic regulators of lung inflammatory injury after deposition of IgG IC. The protective effects of exogenously administered IL-6 and IL-10 may be in part linked to suppressed TNF-α production (22). Thus, it is possible that IL-6 and IL-10 may exert their regulatory functions in vivo during acute lung injury by activating STAT3, which then serves as a negative regulator of the acute inflammatory response. Our current data support this possibility. When SOCS3 is overexpressed, levels of TNF-α in the lung are increased (Fig. 6A). In addition, overexpression of SOCS3 in lung reduced STAT3 activation (Fig. 7). The presence of anti-IL-6 Ab in lung reduced activation of STAT3 in mice lungs during IgG IC-induced injury (Fig. 9). These data suggest that SOCS3 functions as a negative regulator for IL-6 signaling in this lung injury model. Supporting this, we show that SOCS3 overexpression abrogated IL-6-induced activation of STAT3 in lung epithelial cells (Fig. 10). However, we cannot exclude the possibility that SOCS3 may regulate other molecules of FcγR signaling pathways in the lung to enhance injury. Indeed, a recent study shows that, although SOCS3 is not able to interact with Jak1 and Jak2 kinases in the presence of IC stimulation in human mesangial cells, there may be an intracellular association between SOCS3 and Tec tyrosine kinase (19). Interestingly, overexpression of SOCS3 does not inhibit activation of NF-κB in the IgG IC-injured lung, suggesting that IL-10-mediated inhibition of NF-κB activation observed in our previous study (31) is not linked to the STAT3/SOCS3 pathway (Fig. 7B). In support of this, a region at the COOH terminus of IL-10R intracellular domain (containing a functionally important serine residue) that is not involved in STAT3 recruitment is known to be required for anti-inflammatory activity of IL-10 (32). Deletion of this sequence does not influence STAT3 phosphorylation, but eliminates observed anti-inflammatory activities. Thus, the role of SOCS3 in acute lung injury is likely to be complicated. More details of the STAT3/SOCS3 pathway are needed for a better understanding of the regulatory mechanisms of acute lung injury.

In summary, our data demonstrate that SOCS3 is induced in the IgG IC-injured lung. Overexpression of SOCS3 in lung through adenovirus SOCS3 gene transfer enhances IgG IC-induced lung injury. This effect is most likely due to reduction of IgG IC-mediated STAT3 activation by SOCS3, representing a negative feedback inhibitor of IL-6 signaling. Further work will be needed to understand the underlying roles of SOCS3 in regulating the network of inflammatory systems in FcγR pathways in the lung.

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References


