

Status of Stat3 in an Ovalbumin-Induced Mouse Model of Asthma: Analysis of the Role of Socs3 and IL-6

Bholanath Paul^a Vani Mishra^a Bhushan Chaudhury^b Anjali Awasthi^a
Asim Bikas Das^a Urmila Saxena^a Ashok Saxena^a Lalit K. Chauhan^c
Pradeep Kumar^d Sheikh Raisuddin^e

^aImmunobiology Division, ^bCentre for Histopathology, ^cCell Biology Division and ^dCentre for Animal Facilities, Indian Institute of Toxicology Research, Mahatma Gandhi Marg, Lucknow; ^eElemental Toxicology, Jamia Hamdard University, New Delhi, India

Key Words

Stat3 · Socs3 · IL-6 · Airway inflammation · Asthma · Mice

Abstract

Background: Stat3, Socs3 and cytokines play an integral role in the coordination and persistence of inflammation. However, a clear understanding of the role played by the Stat3/IL-6 and Socs3 pathway in airway inflammation is lacking. We report the alteration in the status of expression and activation of Stat3 by ovalbumin (OVA), and establish its relationship with Socs3 and IL-6 in the lungs of mice with eosinophilic pulmonary inflammation and airway hyperresponsiveness. **Methods:** Alterations in the expression of Stat3, Socs3 and IL-6 were determined in a murine model of asthma, where Balb/c mice were sensitized and challenged with OVA (OVA/OVA) and compared with control mice sensitized and challenged with saline (SAL) (SAL/SAL) mice. The OVA/OVA mice were characterized by a moderate increase in methacholine-induced specific airway resistance, the presence of 150 µg/ml of OVA-specific IgG and 8.93 µg/ml OVA-specific IgE antibody and elevated levels of eosinophils and Th2 cytokines (IL-4 and IL-5) in the bronchoalveolar la-

vage fluid. In contrast SAL/SAL mice had low eosinophils, IL-4 and IL-5 and no OVA-specific IgG and IgE antibodies in the BALF. Stat3 and Socs3 expression profiles were monitored in OVA/OVA and Stat3- and Socs3-silenced OVA/OVA mice. Furthermore, expression of IL-6 in Stat3- and Socs3-silenced mice and the exogenous effect of IL-6 on Stat3 were studied. **Results:** The results show that expression and activation of Stat3 mRNA and proteins are significantly low in lung of OVA/OVA mice in comparison to SAL/SAL mice following OVA challenge. An increased pool of Socs3 mRNA is observed in OVA/OVA mice with or without OVA challenge and in SAL/SAL mice 24 h after OVA challenge. Transient in vivo blocking of Socs3 gene by Socs3 siRNA restores the expression of IL-6 mRNA and protein in OVA/OVA mice, and nasal administration of recombinant IL-6 to OVA/OVA mice enhanced Stat3 mRNA expression. **Conclusions:** Our data suggest that airway inflammation is associated with low expression of Stat3 and IL-6 and overexpression of Socs3 genes in a mouse model of asthma. Furthermore, IL-6 is under the influence of the Socs3 gene and may contribute to the negative regulation of Stat3 via IL-6 following a challenge with an allergen during the development of asthma.

Copyright © 2008 S. Karger AG, Basel

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2008 S. Karger AG, Basel
1018–2438/09/1482–0099\$26.00/0

Accessible online at:
www.karger.com/iaa

Correspondence to: Dr. Bholanath Paul
Immunobiology Division, Indian Institute of Toxicology Research
M.G. Marg, Lucknow 226001 (India)
Tel. +91 522 243 6144, Fax +91 522 262 8227
E-Mail bnpaul@iitr.res.in or bholanathpaul@yahoo.com

Introduction

Cytokines play a pivotal role in the development of allergic diseases by regulating the activation and proliferation of Th2 cells. Cytokine-mediated signals on effector cells are primarily transduced by the Jak-Stat signaling cascade [1]. The two major subsets of CD4⁺ T cells, Th1 and Th2 cells secrete distinct profiles of cytokines. Th1 cells secrete IL-2, interferon- γ and tumor necrosis factor- β whereas Th2 cells secrete IL-4, IL-5, IL-9, IL-10 and IL-13 [2]. The Th2 cytokines (IL-4, IL-5 and IL-13) control the majority of the contributors to the airway inflammation, including IgE class switching, recruitment and activation of eosinophils and mucous hyperproduction [2, 3]. IL-4 stimulates Jak1 and Jak3 to activate Stat6 [4], which plays an important role in regulating airway inflammation such as Th2 differentiation and IgE production [5–9]. Its role is confirmed in experiments with Stat6-deficient mice, which failed to develop allergen-induced airway inflammation and airway hyperresponsiveness (AHR) [5]. Sensitized and challenged Stat6-deficient mice failed to develop a pulmonary Th2 response, AHR, goblet cell hyperplasia, eosinophilia or IgE [9]. Presently, several groups have investigated the expression and activation of Stat6 in asthmatics [10–12] and have either found a high density of Stat6-expressing cells in the airways [11] or an elevated level of Stat6 secreted by bronchial epithelial cells [12]. Stat3, with diverse roles in biological processes including cell proliferation, survival, apoptosis and inflammation, is another cytoplasmic peptide belonging to the Stat family. It is activated by a large number of extracellular stimuli including IL-6, various other cytokines, granulocyte CSF, epidermal growth factor and IL-10 [13–16]. However, not much is known about its activation and function in the lung during airway inflammation.

The function of Stat3 is extensively studied in cell culture systems, immunological disorders and several inflammatory diseases. Its activation is implicated in the regulation of cell proliferation, differentiation, transformation and apoptosis [17]. In vivo aberrant expression of Stat3 is associated with immune tolerance [18] acute-phase response [19] septic shock [20] and constitutive activation in chronic inflammation [21]. These observations suggest that Stat3 may play an important role during airway inflammation, especially asthma. Stat3 in macrophages may play a negative role in inflammation [22] and tissue-specific disruption of Stat3 during hematopoiesis is associated with a lethal inflammatory bowel syndrome [23], suggesting that it regulates the induction of a distinct

set of target genes in different cell types. Although these studies have indicated the importance of Stat3 in inducing inflammatory response, very little is known regarding its activation and function in the lung during acute inflammation. Gao et al. [24] reported that activation of Stat3 in the lungs is macrophage dependent, and IL-6, IL-10 and C5a contribute to Stat3 activation in inflamed rat lung. Stat3 has recently been implicated in the pathogenesis of asthma in a study showing that Stat3-dependent pathways induced by IL-13 in lung myofibroblast are inhibited by the administration of an inhaled corticosteroid, fluticasone [25]. The pharmacogenetics of asthma treatment reveals Stat3 as one of the candidate genes showing association in response to corticosteroid treatment [26]. Stat3 polymorphism is significantly associated with baseline FEV₁ both in adults and children, suggesting that it may participate in inflammatory pathways and have an impact on the level of lung function [27]. Thus, there is growing evidence that Stat3 may also be associated with airway inflammation, and it would be interesting to know the status of this molecule in conjunction with Socs3 and IL-6 in airway inflammation as Socs3 is a negative regulator of Stat3 and gp130, the latter being the common signal-transducing receptor chain for IL-6-type cytokines and ubiquitously expressed in most tissues. We reveal that Stat3 mRNA expression and protein activation are significantly low in inflammatory murine lung compared with noninflamed lung; blocking Socs3 gene expression resulted in heightened expression of IL-6 and Stat3 genes. The implications of these observations are discussed in the light of the pathogenesis of asthma.

Materials and Methods

Animals and OVA Sensitization

Eight- to ten-week-old female Balb/c mice procured from Institutional Animal Facilities were used for the experiments with consent from the Committee for the Purpose of Control and Supervision on Experiments on Animals (CPCSEA), India. The mice were sensitized to OVA by intraperitoneal (i.p.) injection of 100 μ g ovalbumin (OVA; grade V; Aldrich-Sigma Chemical) emulsified in 2% alum solution on days 0 and 14 or sham-sensitized with saline (SAL). On day 14, 30 min after OVA sensitization, the animals were challenged with OVA through the nasal route. A total of 50 μ g OVA in sterile SAL was applied on the snout or the nostril in 5 cycles, 2 μ l containing 10 μ g OVA in SAL at each cycle at an interval of 2 min. On days 21, 22, 23 and 26, the mice were similarly challenged with 50 μ g OVA through the nasal route. Methacholine chloride (Mch) (Sigma) was administered intravenously on day 27 and airway hyperresponsiveness (AHR) was measured by plethysmography. Some of the animals were used for collection of blood and some for bronchoalveolar lavage

fluids (BALF). In another set of experiments, the animals were sacrificed by cervical dislocation at different time points after the final OVA exposure on day 26 and the lungs and livers dissected out. Use of sodium thiopentone (Thiosol; Neon Lab, Mumbai, India) was avoided in this group as it involves significant variations in anesthetizing time. Tissues (25–30 mg) were either stored in RNA later for future RNA isolation and one-step RT-PCR for the detection of Stat3, Socs3, IL-6 and β -actin mRNA or immediately processed for the preparation of nuclear extracts and estimation of active Stat3 protein by ELISA. Recombinant IL-6 (rIL-6) dissolved in sterile SAL, in different doses, was administered through the nasal route in the same way as OVA.

Measurement of AHR

AHR induced by different concentrations of Mch (0–40 mg/kg) was measured using a double-chambered whole-body plethysmograph (Buxco; model No. PLY 3351) [28]. The raw flow curves were acquired by sampling the signals at 2 kHz. The regularity of the breathing patterns was first assessed by qualitatively monitoring the constancy of peak flows. Irregularities in the breathing pattern were often encountered due to movement of the neck in most cases and to a lesser extent, sigh-breath and cough. Data arising from these irregularities in breathing pattern were not considered for quantitative analysis. Before the start of the experiments, the animals were conditioned to stay in the plethysmograph chamber for at least 3 or 4 sessions of 10 min duration each. It was necessary to precondition the animals to minimize irregularities in the breathing pattern during data acquisition and achieve constancy in peak thoracic and nasal flows in the shortest possible time. Both OVA/OVA and SAL/SAL mice were conditioned in a similar way to nullify the effect of preconditioning of animals on the measurement of sRaw. For each mouse, the protocol consisted of breathing in the plethysmograph for 10 min and the mean value was considered for analysis. sRaw is monitored with the *Acknowledge* software program version 3.1 (Biopac, USA). sRaw was calculated with the formula:

$$\text{sRaw (cm H}_2\text{O} \cdot \text{s)} = [(T_I + T_E)/(2 \cdot \pi) \cdot (P_{\text{atm}} - 47) \cdot 136 \cdot 2 \cdot \pi \cdot dT/(T_I + T_E)]$$

developed by Pennock et al. [29].

Collection of BALF and Sera

Mice were anesthetized with sodium thiopentone, i.p. 5 mg/0.1 ml/mouse at 2 h after the last OVA challenge for collection of BALF and blood. For collection of BALF, the trachea was exposed by midline incision in the neck region and 1 ml of PBS, pH 7.4 was injected into the lungs through the trachea and withdrawn after 10 s. The fluid recovered from each mouse was centrifuged at 2,000 rpm, for 5 min at 4°C and the supernatants were subjected to antibody and cytokine analysis [30]. The pellets were resuspended, cytocentrifuged and stained with hematoxylin and eosin (HE) and viewed under the microscope for eosinophil counts. Blood was collected from the retro-orbital sinus using nonheparinized glass capillaries, allowed to clot for 1 h and centrifuged to separate the serum.

Evaluation of Total and Antigen-Specific IgG and IgE Antibodies and Cytokines

Total and antigen-specific IgG and IgE antibodies in the BALF and sera were evaluated by quantitative ELISA following the man-

ufacturer's protocol (Mouse IgG ELISA Quantitation kit; Bethyl Laboratories, Inc., Montgomery, Tex., USA). In brief, the plates were coated with goat antimouse IgG antibody at a concentration of 10 $\mu\text{g/ml}$ to 100 μl of coating buffer (0.05 M carbonate bicarbonate, pH 9.6) and incubated for 60 min. Free sites were blocked with 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8 for 30 min, washed, analyte added and incubated for 60 min. Goat antimouse IgG conjugated with HRP was used for the detection of mouse IgG at a dilution of 1:10,000. Color was developed by adding tetramethyl benzidine substrate for 10 min. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 . The intensity of the color developed was measured on an ELISA plate reader at 450 nm. Antigen-specific IgG was determined by coating the plate with OVA in 0.05 M carbonate-bicarbonate buffer, pH 9.5, at a concentration of 10 $\mu\text{g/ml}$ and incubated at 4°C overnight. After incubation, the wells were washed with PBST and blocked with 200 μl blocking reagent (50 mM TRIS, 0.14 M NaCl, 1% BSA, pH 8) for 30 min. After 3 washes with wash buffer, 100 μl analyte was added and incubated for 2 h at room temperature. IgG antibody in the analyte was detected with the help of goat antimouse IgG antibody conjugated with HRP. Development of color and stopping of enzyme substrate reaction were performed in the same way as total IgG estimation. IL-4, IL-5 and IL-6 proteins in BALF were evaluated by solid-phase sandwich ELISA using an ELISA kit according to the manufacturer's instructions (R&D System, Minneapolis, Minn., USA).

RNA Isolation and One-Step RT-PCR

Total RNA was isolated from lung and liver tissues using RNeasy mini kit as per the manufacturer's instructions (RNeasy mini kit; Qiagen, Hilden, Germany). RT-PCR was carried out using a one-step RT-PCR kit (Qiagen, Germany). The RT-PCR conditions were 50°C for 30 min for reverse transcription; initial PCR activation at 95°C for 15 min followed by 30 cycles of denaturation at 94°C for 45 s, annealing at a 5°C below the T_m of primers for 45 s and extension of 1 min at 72°C. The reaction was stopped after a final 10-min extension at 72°C. 1 μl of template RNA containing 50 ng of total RNA was used. RT-PCR products were run on 1.2% agarose gel containing ethidium bromide and viewed and analyzed on Gel Documentation system using Gene Snap and Gene tool software for image acquisition and gel analysis, respectively (Syngene, Cambridge, UK). Molecular weight marker (0.5 μg) containing a defined quantity of each band was loaded and electrophoresed. The Syngene analysis tool was used to extrapolate the cDNA quantity from the input quantity of standard molecular weight markers and quantitated the RT-PCR products.

Primers (Operon Biotechnologies GmbH, Germany) used for RT-PCR were: Stat3: 5'-GAAGACCAAGTTCATCTGTGTG-3' (forward), 5'-GTAGCACACTCCGAGGTCAGAT-3' (reverse); Socs3: 5'-GAAGACCAAGTTCATCTGTGTG-3' (forward), 5'-GTAGCACACTCCGAGGTCAGAT-3' (reverse); IL-6: 5'-ATGA-ACTCCTTCTCCACAAGCGC-3' (forward), 5'-GAAGAGTTTCCAGGTGGACTG-3' (reverse); β -actin: 5'-GACATGGAGAA-GATCTGGCAC-3' (forward), 5'-TCCGACGCAGGATGGCG-TGA-3' (reverse).

Preparation of Nuclear Extracts and Estimation of Active Stat3 Protein

Preparation of nuclear extracts and evaluation of active Stat3 peptide was performed according to the instructions given in the technical bulletin of the mouse Stat3 activity assay kit (R&D Sys-

tem). In brief, the nuclear extracts from lung and liver tissues were prepared by solubilizing tissues in freshly prepared lysis buffer A (10 mM HEPES, pH 7.9; 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.1% NP-40, 2 mM Na₃VO₄, 5 mM NAF, 3 µg/ml aprotinin, 25 µg/ml leupeptin, 25 µg/ml pepstatin, 25 µg/ml chymostatin and 0.2 mM PMSF). Twenty-percent tissue homogenates were prepared by chopping the tissue into fine pieces, pipetting up and down and subsequently centrifuging at 16,000 g for 5 min at 2–8°C in a microcentrifuge (Sigma 2K). The nuclear pellet was solubilized in Lysis buffer B (20 mM HEPES, pH7.9; 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 25% glycerol, 2 mM Na₃VO₄, 5 mM NAF, 3 µg/ml aprotinin, 25 µg/ml leupeptin, 25 µg/ml pepstatin, 25 µg/ml chymostatin and 0.2 mM PMSF). After vortexing for 20 s, the lysate was incubated on ice for 20 min and finally centrifuged at 16,000 g for 5 min at 2–8°C. The supernatant was used for active Stat3 assay.

For estimating active Stat3, 3 µl of biotin-labeled double-stranded (ds) oligonucleotide was added to 50 µg of nuclear extract and the volume adjusted to 30 µl with Lysis buffer B and incubated at room temperature for 30 min. Biotin-labeled ds oligonucleotide without nuclear extract served as blank. Unlabeled ds oligonucleotide was used for the determination of specificity by competition. In short, 3 µl of unlabeled ds oligonucleotide was added to 3 µl of biotin-labeled ds oligonucleotide and 50 µg of nuclear extract sample and the volume adjusted to 30 µl with Lysis buffer B and incubated at room temperature for 30 min. After incubation, 200 µl of reagent diluents (5% protease free BSA in PBS containing 0.05% Tween 20, pH 7.2, 0.2 µm membrane filtered) was added to each tube and mixed gently. From each reaction, 100 µl of sample was aspirated and dispensed to wells in an ELISA plate coated with capture antibody (mouse antihuman Stat3 antibody) and incubated at room temperature for 2 h. After washing the plate three times with wash buffer (PBS containing 0.05% Tween 20, pH 7.2; 0.2 µm membrane filtered) 100 µl streptavidin conjugated to HRP diluted with reagent diluents was added and the plate incubated for 20 min at room temperature. The plate was washed again five times and 100 µl of substrate solution was added to each well and incubated for 20 min. The reaction was stopped by 2 N H₂SO₄ and the color developed read at 450 nm with a correction wavelength at 540 nm in a Power Wave XS microplate spectrophotometer (BioTek).

In vivo Inhibition of Stat3 and Socs3 Gene Expression by siRNAs

A pool of 3 strands of Stat3 or Socs3 siRNA duplexes (Santa Cruz Biotech, Calif., USA) complexed with 5% glucose solution was diluted in ExGen500 in vivo transfection reagent containing a cationic polymer, polyethylenimine (Fermentas Life Sciences, Harrington, Ont., Canada) and administered according to the manufacturer's instructions in varying concentrations. Two days later, the mice were killed by cervical dislocation and the lungs were dissected out. 25–30 mg of each tissue was stored in RNAlater (Sigma) for RNA extraction and one-step RT-PCR analysis. The sense strand sequences of the siRNA (Santa Cruz Biotech) were as follows: Stat3: 5'-GAGUGCAGGAUCUAGAACAtt-3'; 5'-CAGGACGACUUUGAUUUCAtt-3'; 5'-GAAGACACUGACUGAUGAAAtt-3'; Socs3siRNA: 5'-CAGCAUCUUUGUCGGAAAtt-3'; 5'-GUAUGAUGCUCACUUUAAAtt-3'; 5'-CCAAGUGUUGAACUUAGAA tt-3'; and the scrambled siRNA 5'-UUCUCCGAACGUGUCACGUGtdt-3' (Qiagen). The scrambled siRNA

duplex was used as the negative control. The sham control group received Exgen 500 diluted in 5% glucose solution.

Statistical Analysis

Data shown represent mean values (± SD). Student's t test was used for comparing activities of different treatments.

Results

Induction of Inflammatory and Allergic Airways in Mice

We observed low to moderate increases in Mch-induced sRaw in OVA/OVA mice in comparison to SAL/SAL mice over a dose range of 2.5–40 mg/kg but not in comparison to Mch administered to normal Balb/c mice (fig. 1A). Increased sRaw is considered to be a pathogenic state of asthma [31]. The moderate increase in Mch-induced sRaw in OVA/OVA mice suggests that the protocol adopted by us induced asthma symptoms in mice. It is not clear at the moment why SAL administration in SAL/SAL mice resulted in lower sRaw in comparison to normal mice. Possibly, saline helps in diluting and dispersing the mucous in the airways and calls for future study in this direction.

The HE-stained lung and tracheal sections revealed histologic changes in OVA/OVA mice that are not seen in SAL/SAL mice. Migration of inflammatory cells into the alveolar septum leading to its thickening (indicated by arrows) was observed in OVA/OVA mice but not in SAL/SAL mice (fig. 1B). The epithelial cells bordering the tracheal lumen were partially damaged in OVA/OVA mice but not in SAL/SAL mice. In another group of OVA/OVA mice (n = 5), 2 h after the last OVA exposure, the presence of OVA-specific IgE antibodies in BALF and sera and increased levels of IL-4 and IL-5 in BALF were observed, while SAL/SAL mice were negative for OVA-specific IgE and IgG antibodies and IL-4 and IL-5 levels were close to the basal level (table 1). A moderate elevation in the eosinophil counts in BALF was also observed in OVA/OVA mice. Thus the sensitizing and challenge protocol adopted in our experiments revealed elevation of Th2 cytokines, eosinophil counts, the presence of OVA-specific IgE, alteration in the airway architecture and AHR in OVA/OVA mice and suggests the pathological and physiological states of allergic asthma.

Stat3 Is Low and Socs3 Is High in OVA/OVA Mice

We observed high expression of Stat3 mRNA in lung of SAL/SAL mice on day 26 at 0.5, 1 and 2 h after OVA exposure in comparison to 0 h (fig. 2A). One hour after

Table 1. Comparative data on IL-4, IL-5, eosinophil and total- and OVA-specific IgG and IgE in OVA/OVA and SAL/SAL mice ~2 h after OVA exposure on day 26

	SAL/SAL	OVA/OVA
Total serum IgG, mg/ml	2.31 ± 1.89	8.83 ± 1.22*
OVA-specific serum IgG, mg/ml	2.86 ± 1.03	6.86 ± 2.57*
OVA-specific BALF IgG, µg/ml	ND	150.56 ± 12.43
Total serum IgE, µg/ml	6.87 ± 3.41	25.58 ± 5.73*
OVA-specific serum IgE, µg/ml	1.07 ± 0.50	29.35 ± 3.18*
OVA-specific BALF IgE, µg/ml	ND	8.93 ± 2.86
Eosinophils, per ml	88.6 ± 27	176.5 ± 48.4
IL-4, pg/ml	28.45 ± 16.7	105.34 ± 30.3*
IL-5, pg/ml	23.86 ± 13.9	70.63 ± 20.4

Data represent mean ± σ of values from 6 mice in each group.

* p < 0.05, statistically significant compared to SAL control.

OVA exposure appeared to be the optimum time for Stat3 expression in SAL/SAL mice; at this time a ~3 fold increase in mRNA level was observed. We then monitored the Stat3 mRNA levels in OVA/OVA mice on day 26 at 0, 1, 2, 4 and 24 h after OVA exposure and compared with the Stat3 mRNA level in SAL/SAL mice 1 h after OVA challenge. Notably, OVA/OVA mice failed to induce Stat3 to the extent seen in SAL/SAL mice at the same time points, indicating downregulation of Stat3 mRNA expression in mice sensitized and challenged with an allergen. However, β-actin mRNA expression in the lung and liver tissues was unaffected after OVA exposure at all the time points in both OVA/OVA and SAL/SAL mice, indicating that the house-keeping gene in the lung tissue is not involved in inflammatory and allergic airways. Interestingly, Stat3 expression in liver tissues of both OVA/OVA and SAL/SAL mice were also unaffected, indicating that the low expression of Stat3 is confined to the lungs of OVA/OVA mice and does not affect extra-pulmonary organs (fig. 2B). The β-actin gene in liver tissues of OVA/OVA mice was not affected as seen in lung tissues.

Tyrosine phosphorylated Stat3 is known to dimerize and subsequently translocate to the nucleus where it activates various transcription factors [32]. Therefore, the presence of active Stat3 protein in the lung nuclear extracts of OVA/OVA mice was studied and compared with SAL/SAL mice. We observed a ~5-fold increase in active Stat3 protein in lung tissues of SAL/SAL mice 1 h after OVA exposure that returned to basal level at 24 h after OVA exposure (fig. 2C). In contrast, the Stat3 peptide in

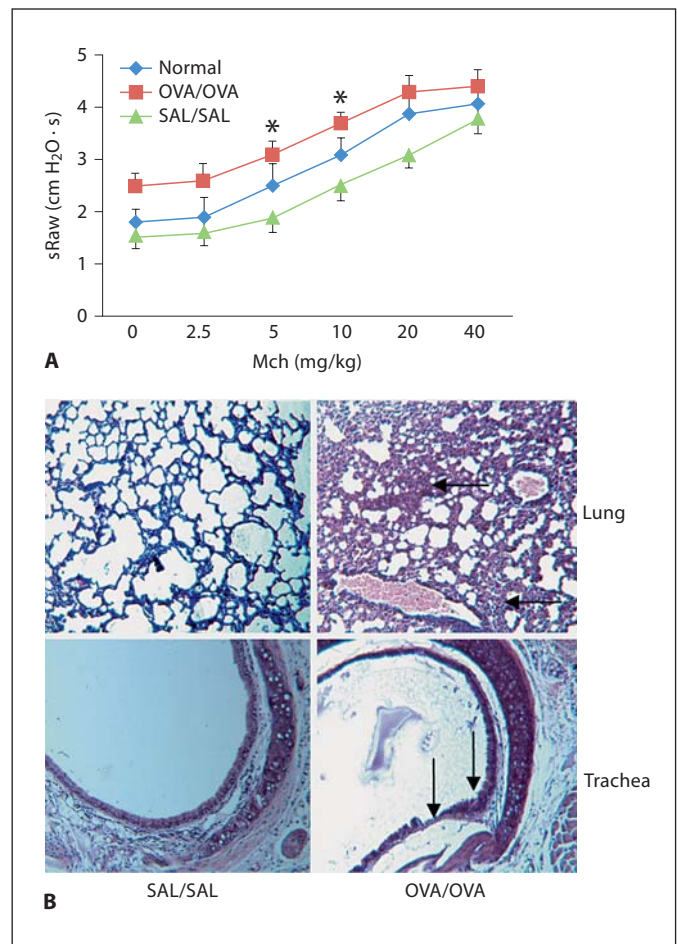


Fig. 1. sRaw is moderately elevated in OVA/OVA mice. **A** Varying concentration of Mch-induced AHR was measured in conscious OVA/OVA, SAL/SAL and normal mice by injecting Mch through the caudal vein. Means ± SEM; n = 6 mice for each time point. * p < 0.05 compared to SAL/SAL control. **B** Histopathology of lung (top) and trachea (below) of OVA/OVA and SAL/SAL mice. Arrows indicate thickening of alveolar septa in the lung and damage to the ciliated cells in the trachea.

lung nuclear extracts of OVA-exposed OVA/OVA mice was found to be close to the basal level at all the time points (fig. 2D) and is in line with the mRNA expression profile in OVA/OVA mice. In liver tissues, the induction of active Stat3 peptide in OVA/OVA mice is similar to SAL/SAL mice (fig. 2E), indicating that the extrapulmonary organs, especially the liver, are not affected in mice with inflammatory and allergic airways.

Both Stat3 and Socs3 genes are located on chromosome 11 of the Balb/c mouse and downstream of the Stat-responsive elements, the 5' upstream region of the Socs3 gene contains a GC-rich element involved in basal and

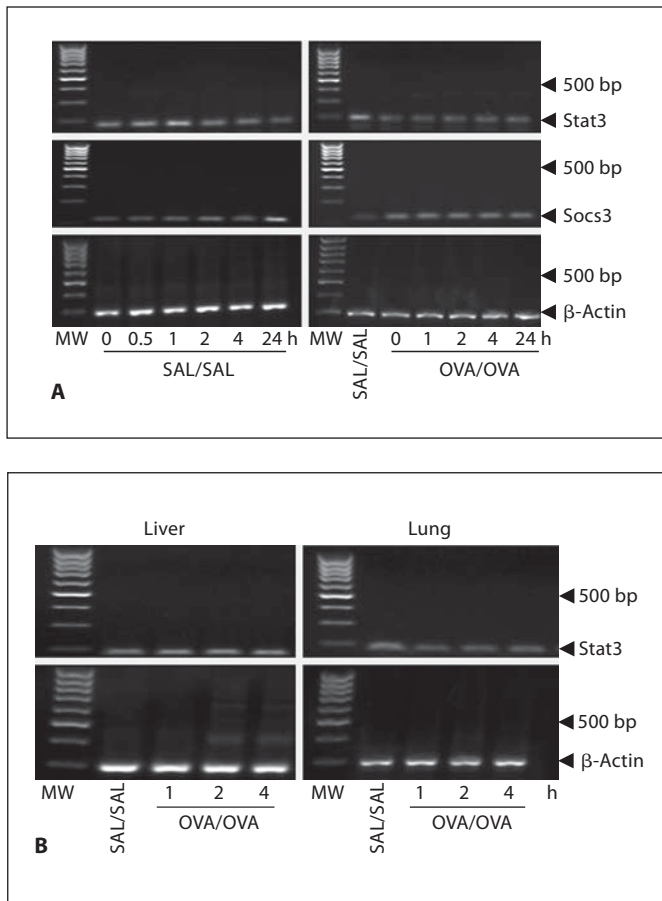
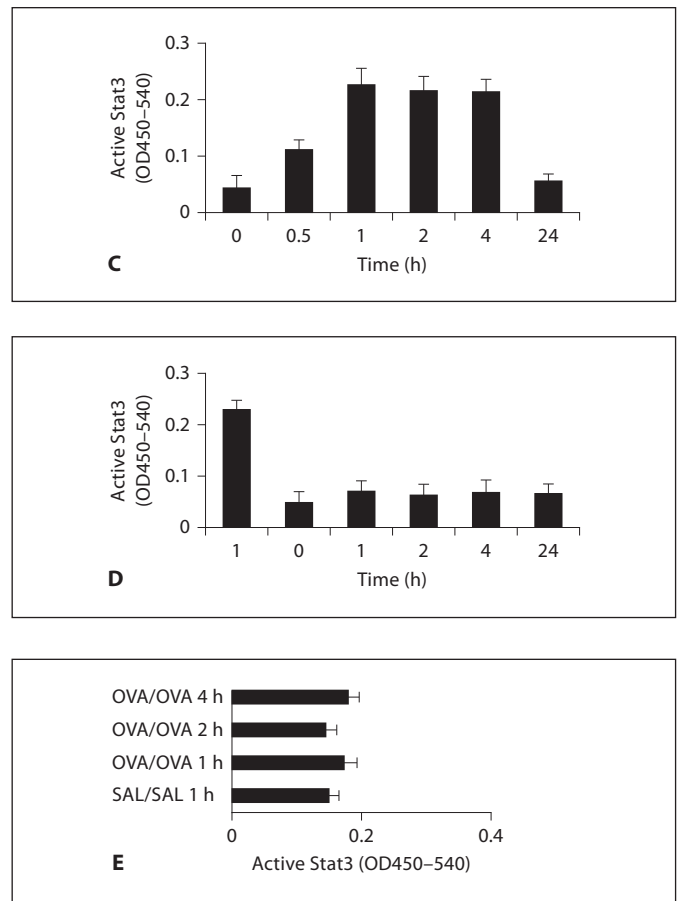


Fig. 2. Stat3 and Socs3 expression in OVA/OVA and SAL/SAL mice on day 26 after OVA challenge. **A** Stat3, Socs3 and β -actin mRNA expression profile on day 26 at different time points after OVA exposure (0, 0.5, 1, 2, 4 and 24 h) in SAL/SAL mice and (0, 1, 2, 4 and 24 h) in OVA/OVA mice. RT-PCR performed with Stat3, Socs3 and β -actin primers yield cDNA products of ~185, 112 and 300 bp, respectively. MW represents 100-bp DNA ladder. Stat3 and Socs3 mRNA levels in OVA/OVA mice are compared with the respective mRNA level in SAL/SAL mice 1 h after OVA challenge.

induced transcriptional activation of the Socs3 promoter [33]. Furthermore, Stat3 is reported to activate Socs3 mRNA transcription by associating with the 5' promoter of Socs3 [34]. In SAL/SAL mice, the basal pool of Socs3 mRNA was very low but increased dramatically 24 h after OVA exposure (fig. 2A). In contrast, Socs3 mRNA pool in OVA/OVA mice was higher than the basal pool found in the normal lung. In SAL/SAL mice, Socs3 expression was delayed compared to the Stat3 gene (fig. 2A). The basal Socs3 mRNA pool was found to be low in lung tissues of SAL/SAL mice compared to OVA/OVA mice (fig. 2A). Thus overexpression of Socs3 at all the time



B Stat3 and β -actin mRNA expression in liver and lung of OVA/OVA mice at different time points (1, 2 and 4 h) after OVA exposure on day 26 and compared with Stat3 and β -actin levels in liver and lung tissue of SAL/SAL mice 1 h after OVA challenge. Bar diagram presentation (mean $\pm$ SD; n = 5) of active Stat3 peptide in lung tissue of SAL/SAL (**C**), OVA/OVA (**D**) mice and liver tissue of OVA/OVA and SAL/SAL mice at different time points after OVA challenge (**E**).

points, including 0 h, is an interesting feature observed in OVA-sensitized and challenged mice in addition to the low expression of Stat3. Several studies have indicated that members of the Stat family of transcription factors are involved in the regulation of Socs3 expression by factors such as LIF or growth hormone [34–36] and the respective Stat-binding elements of the Socs3 promoter [34]. On the other hand, the Socs proteins, in addition to cytokine-induced phosphorylation, tightly regulate activation of Stat proteins; Socs proteins are direct target genes of Stats, which inhibit Jak/Stat signaling via a classic negative feedback loop [37]. To further delineate the

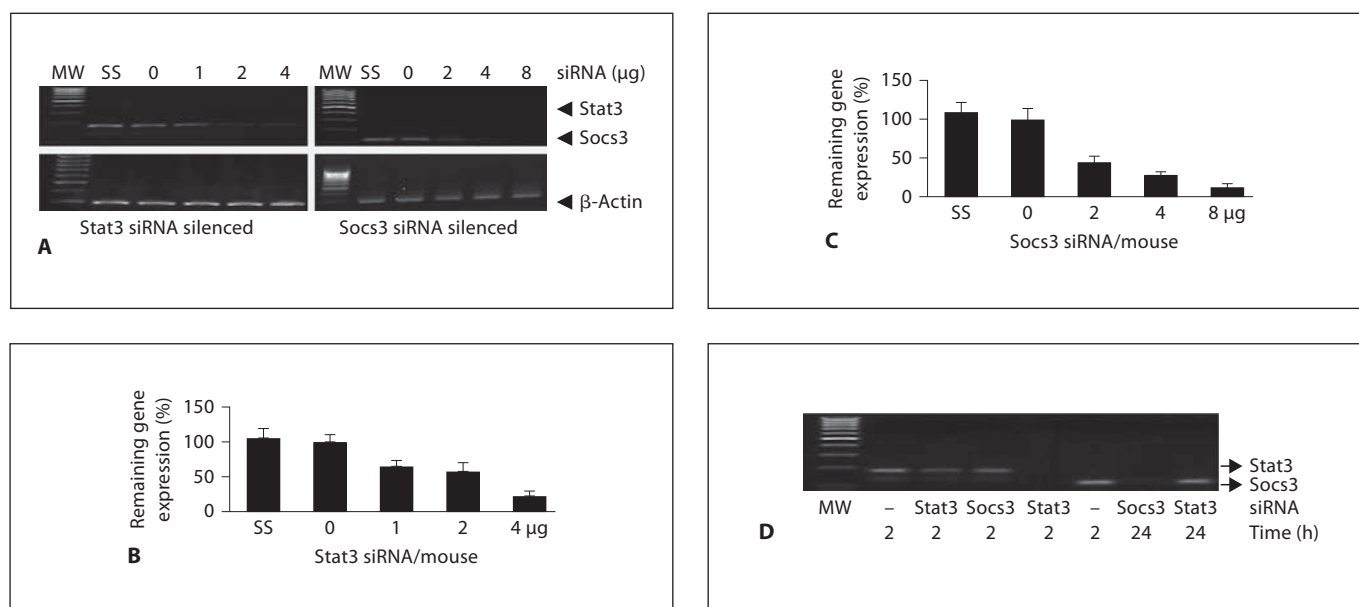


Fig. 3. siRNA-mediated silencing of Stat3 and Socs3 genes. **A** In vivo silencing of the Stat3 gene in SAL/SAL mice and the Socs3 gene in OVA/OVA mice by siRNA. SAL/SAL mice were transfected with varying doses: 0 (transfection reagent control), 1, 2 and 4 μ g Stat3 siRNA/mouse or 2 μ g/mouse scrambled siRNA (SS). Similarly, OVA/OVA mice were transfected with varying doses (0, 2, 4 and 8 μ g) Socs3 siRNA/mouse or 2 μ g/mouse scram-

bled siRNA (SS). Bar diagram presentation of percent remaining Stat3 (**B**) and Socs3 (**C**) gene expression following varying doses of siRNA transfection in SAL/SAL mice and OVA/OVA mice, respectively. **D** RT-PCR product of Stat3 and Socs3 mRNA expression profile in OVA/OVA mice transfected with Stat3 and Socs3 siRNA.

latter pathway in mice with symptoms of asthma, we blocked Socs3 gene expression by transfecting a pool of 3 synthetic siRNA duplexes and studied the status of Stat3 expression in OVA/OVA mice.

Stat3 and Socs3 Gene Silencing in OVA/OVA Mice and Modulation of Stat3 Expression

Increasing concentrations of Stat3 siRNA were administered through the nasal route in SAL/SAL mice to derive the in vivo effective dose of siRNA duplexes in silencing Stat3 gene in lung tissue. Initial studies were conducted in SAL/SAL instead of OVA/OVA mice because the level of the Stat3 mRNA pool is very low in the latter. Two days after in vivo siRNA transfection, OVA-induced Stat3 mRNA was monitored and a dose-dependent depletion of Stat3 mRNA expression was observed. Interestingly, 2 and 4 μ g of Stat3 siRNA blocked more than 75% Stat3 mRNA expression in the lungs of transfected SAL/SAL mice (fig. 3A, B). No mortality was observed in mice treated with Stat3 siRNA during the entire period of experimentation although ablation of Stat3 has been reported to lead to embryonic lethality [38] cell mortality

and apoptosis [39]. Although we did not investigate the degree of cell mortality and apoptosis in lung tissues, the possibility of these phenomena occurring in mice treated with siRNA through the nasal route remains, leading to airway remodeling without culminating into animal mortality. Transfection of scrambled sequences (SS) or transfection reagent control in SAL/SAL mice did not affect Stat3 mRNA expression. The β -actin mRNA level was unaffected following Stat3 gene silencing (fig. 3A), indicating that the siRNA used by us is gene specific. Similarly, OVA-induced Socs3 expression in Socs3 siRNA-transfected mice resulted in more than 80% inhibition of Socs3 expression at doses 4 and 8 μ g (fig. 3A, C). SS transfection and transfection reagent control in OVA/OVA mice did not affect Socs3 mRNA expression. No mortality was observed among Socs3-silenced mice. Based on these studies, the groups of mice silenced with 2 μ g Stat3 siRNA and 4 μ g Socs3 siRNA duplex pools were used in subsequent studies to check the influence of these genes on Stat3 expression in OVA/OVA mice. Interestingly, Stat3 mRNA expression was heightened in Socs3-silenced OVA/OVA mice compared to unsilenced

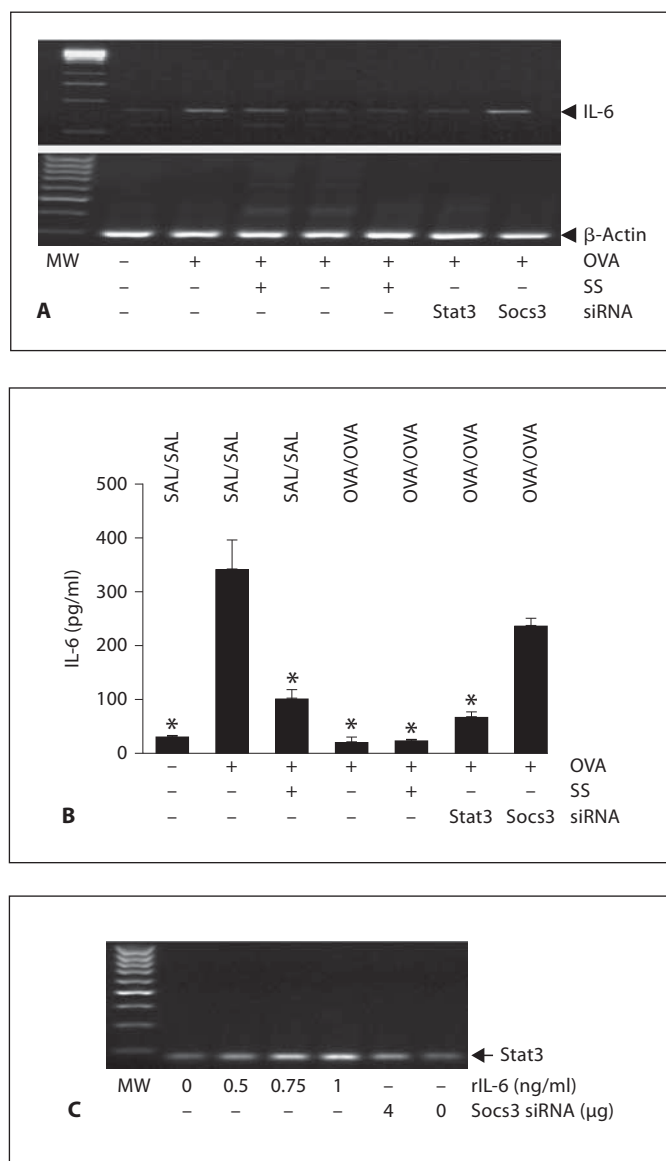


Fig. 4. Normal IL-6 mRNA expression in Socs3-silenced OVA/OVA mice. **A** RT-PCR product of IL-6 mRNA expression in lung tissues of SAL/SAL, OVA/OVA, Stat-silenced OVA/OVA and Socs3-silenced OVA/OVA mice following OVA exposure on day 26. **B** OVA-induced IL-6 protein (pg/ml) in BALF of Stat3- and Socs3-silenced OVA/OVA mice ($n = 4$) in each group. * $p < 0.05$, statistically significant compared to Stat3 mRNA expression profile in OVA/OVA mice treated with exogenous rIL-6 through the nasal route and compared with Socs3 blocked OVA/OVA mice (**C**).

OVA/OVA mice. In Stat3-silenced OVA/OVA mice, no effect was seen in the level of Socs3 mRNA (fig. 3D). It thus appears that the Stat3 gene is under the influence of Socs3 gene in mice with inflammatory and allergic airways.

Status of IL-6 in OVA/OVA Mice and Induction of IL-6 in Socs3-Silenced OVA/OVA Mice

The mechanism by which Socs3 controls Stat3 is not clear in OVA/OVA mice. As binding of a cytokine to its receptor leads to activation of Jak family members (receptor-associated kinases), which then activate Stat3 via tyrosine phosphorylation, we investigated the status of IL-6 and its role in OVA/OVA mice. On day 26, 2 h after OVA challenge, heightened expression of IL-6 mRNA was observed in SAL/SAL mice compared to unexposed SAL/SAL mice. In contrast, low expression of IL-6 mRNA was observed in lung tissues of OVA/OVA mice (fig. 4A). Similarly, nasal OVA exposure in SAL/SAL mice induced significant IL-6 protein in BALF, while OVA/OVA mice failed to secrete IL-6 to the extent seen in SAL/SAL mice (fig. 4B). Stat3 siRNA (2 μ g) transfected in OVA/OVA mice did not modulate IL-6 mRNA expression, while 4 μ g Socs3 siRNA upregulated IL-6 expression (fig. 4). Furthermore, transfection of SS or administration of transfection reagent in OVA/OVA mice failed to modulate IL-6 expression. This suggests that the induction of IL-6 is under the influence of the Socs3 gene in OVA/OVA mice and that a low level of Stat3 is associated with low expression of IL-6. Therefore, we questioned whether exogenous IL-6 can induce Stat3 in the presence of high expression of Socs3. Administration of rIL-6 through the nasal route resulted in a dose-dependent increase in Stat3 expression in OVA/OVA mice (fig. 4C). Administration of 0.5 ng of rIL-6 in OVA/OVA mice induced the same increase in Stat3 mRNA as in Socs3-silenced OVA/OVA mice. At higher doses of rIL-6 (0.75 and 1 ng/mouse) Stat3 mRNA expression increased over 1.8-fold in OVA/OVA mice. Our data suggest that IL-6 plays an important role in modulating Stat3 expression in the presence of high Socs3 expression as in OVA/OVA mice.

Discussion

In this study we investigated the role of Stat3 in pathogenesis of asthma and demonstrate low Stat3 mRNA and protein in the lungs of mice with inflamed and allergic airways and symptoms of bronchoconstriction. This is in contrast to the data obtained in the lungs without inflammation and allergy, indicating that repeated sensitization and challenge with an allergen reduces the elicitation of Stat3 molecules in the host and reflects the augmentation of a desensitizing mechanism in airways during the development of asthma. Presently, three families of proteins, the SH2-containing phosphatases (SHP), the pro-

tein inhibitors of activated Stats (PIAS), and the suppressors of cytokine signaling (Socs), inhibit specific and distinct aspects of cytokine signal transduction [40]. Both Socs1 and Socs3 are able to desensitize cells to signaling by cytokines [41]. Heightened expression of Socs3 in OVA/OVA mice may explain the underexpression of Stat3 mRNA and the low presence of active Stat3 protein in the nucleoplasm.

Socs3 has been reported to play a regulatory role in the lung inflammatory response and its induction occurs during the development of inflammation in the IgG-IC model of lung injury [42]. Overexpression of Socs3 in the lung using a recombinant adenovirus encoding murine Socs3 results in a substantial increase in lung vascular permeability and lung myeloperoxidase, together with enhanced levels of TNF- α , MIP-2, and keratinocyte-activated cytokine in BALF [42]. In this investigation, we did not investigate the levels of these molecules, but observed that overexpression of Socs3 is associated with underexpression of IL-6 in the lungs of OVA/OVA mice. Therefore, we ask whether Socs3 directly downregulates Stat3 via the classical feed-back loop or whether IL-6 is implicated in allergic and airway inflammation. In this context, low expression of IL-6 in OVA/OVA mice and high expression of IL-6 in Socs3-silenced OVA/OVA mice is very interesting and relevant as binding between IL-6 and the signal-transducing receptor chain, gp130, cannot take place and hence the Jak-Stat pathway that is necessary for Stat3 activation cannot be activated. Thus OVA/

OVA mice lack gp-130-mediated signal transduction, leading to an asthmatic state. Furthermore, normal expression of Stat3 following exogenous administration of rIL-6 in OVA/OVA mice validates our hypothesis that Socs3 specifically prevents activation of Stat3 by IL-6 and OVA or allergen-induced IL-6 expression is also under the influence of the Socs3 gene. Thus recovery of Stat3 expression following exogenous rIL-6 administration or silencing Socs3 gene documents that the IL-6/Stat3 pathway is defective and regulated by the Socs3 gene at least in the OVA-induced mouse model of asthma.

This study suggests that the varying expression levels of Stat3, IL-6 and Socs3 can serve as molecular markers for diseases such as asthma and provide new avenues for therapeutic interventions at the molecular level.

Acknowledgements

We thank Dr. C.M. Gupta, former Director and Dr. Aswini Kumar, present Director of our institute for their constant encouragement and support throughout the work. We also thank Dr. Piali Dhar Chaudhury, NYU School of Medicine, N.Y. for her critical review of the manuscript. This work is supported by the Council for Scientific and Industrial Research, New Delhi (Network Project No. SMM 0006, Allergy and Asthma Disorder Mitigation Mission Program and NWP 33; Development of Diagnostics and Molecular Target Based drug for allergy, bronchial asthma and COPD). This is IITC communication No. 2605.

References

- 1 Darnell JE: STATs and gene regulation. *Science* 1997;277:1630–1635.
- 2 Seder R, Paul W: Acquisition of lymphokine-producing phenotype by CD4+ T cells. *Annu Rev Immunol* 1994;12:635–673.
- 3 Ennis DP, Cassidy JP, Mahon BP: Prior Bordetella pertussis infection modulates allergen priming and severity of airway pathology in a murine model of allergic asthma. *Clin Exp Allergy* 2004;34:1488–1497.
- 4 Pernis AB, Rothman PB: JAK-STAT signaling in asthma. *J Clin Invest* 2002;109:1279–1283.
- 5 Kuperman D, Schofield B, Wills-Karp M, Grusby MJ: Signal transducer and activator of transcription factor 6 (Stat6)-deficient mice are protected from antigen-induced airway hyperresponsiveness and mucus production. *J Exp Med* 1998;187:939–948.
- 6 Akimoto T, Numata F, Tamura M, et al: Abrogation of bronchial eosinophilic inflammation and airway hyperreactivity in signal transducers and activators of transcription (STAT)6-deficient mice. *J Exp Med* 1998;187:1537–1542.
- 7 Miyata S, Matsuyama T, Kodama T, et al: STAT6 deficiency in a mouse model of allergen-induced abolishes eosinophilia but induces infiltration of CD8+ T cells. *Clin Exp Allergy* 1999;29:114–123.
- 8 Herrick CA, MacLeod H, Glusac E, Tigelaar RE, Bottomly K: Th2 responses induced by epicutaneous or inhalational protein exposure are differentially dependent on IL-4. *J Clin Invest* 2000;105:765–775.
- 9 Tomkinson A, Kanehiro A, Rabinovitch N, Joetham A, Cieslewicz G, Gelfand EW: The failure of STAT6-deficient mice to develop eosinophilia and airway hyperresponsiveness is overcome by interleukin-5. *Am J Respir Crit Care Med* 1999;160:1283–1291.
- 10 Miller RL, Eppinger TM, McConnell D, Cunningham-Rundles C, Rothman P: Analysis of cytokine signaling in patients with extrinsic asthma and hyperimmunoglobulin E. *J Allergy Clin Immunol* 1998;102:503–511.
- 11 Christodoulopoulos P, Cameron L, Nakamura Y, et al: Th2 cytokine-associated transcription factors in atopic and nonatopic asthma: evidence for differential signal transducer and activator of transcription 6 expression. *J Allergy Clin Immunol* 2001;107:586–591.
- 12 Mullings RE, Wilson SJ, Puddicombe SM, et al: Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium. *J Allergy Clin Immunol* 2001;108:832–838.
- 13 Zhong Z, Wen Z, Darnell JE Jr: Stat3: A Stat family member activated by tyrosine phosphorylation in response to epidermal growth factor and IL-6. *Science* 1994;264:95–98.

- 14 Darnell JE Jr: Stat and gene regulation. *Science* 1997;277:1630–1635.
- 15 Leonard WJ, O'Shea JJ: Jaks and Stats: biological implications. *Annu Rev Immunol* 1998; 16:293–322.
- 16 Akira S: IL-6 regulated transcription factors. *Int J Biochem Cell Biol* 1997;29:1401–1418.
- 17 Bromberg J, Darnell JE Jr: The role of STATs in transcriptional control and their impact on cellular function. *Oncogene* 2000;19: 2468–2473.
- 18 Cheng F, Wang H, Cuenca A, Huang M, Ghansah T, Brayer J, Kerr W, Takeda K, Akira S, Schoenberger S, Yu H, Jove R, Sotomayor E: A critical role for Stat3 signaling in immune tolerance. *Immunity* 2003;19:425–436.
- 19 Alonzi T, Maritano D, Gorgoni B, Rizzuto G, Libert C, Poli V: Essential role of Stat 3 in the control of the acute phase response as revealed by inducible gene inactivation in the liver. *Mol Cell Biol* 2001;21:1621–1632.
- 20 Andrejko KM, Chen J, Deutschman CS: Intrahepatic Stat3 activation and acute phase gene expression predict outcome after CLP sepsis in the rat. *Am J Physiol* 1998;275: 1423–1429.
- 21 Hanada T, Yoshida T, Kinjo I, Minoguchi S, Yasukawa H, Kato S, Mimata H, Nomura Y, Seki Y, Kubo M, Yoshimura AA: Mutant form of JAB/SOCS1 augments the cytokine induced JAK/STAT pathway by accelerating degradation of wild type JAB/CIS family proteins through the SOCS-box. *J Biol Chem* 2001;276:40746–40754.
- 22 Takeda K, Clausen BE, Kaisho T, Tsujimura T, Terada N, Forster I, Akira S: Enhanced Th1 activity and development of chronic enterocolitis in mice devoid of Stat3 in macrophages and neutrophils. *Immunity* 1999;10: 39–49.
- 23 Welte T, Zhang SS, Wang T, Zhang Z, Hesselein DG, Yin Z, Kano A, Iwamoto Y, Li E, Craft JE, Bothwell AL, Fikriq E, Koni PA, Flavell RA, Fu XY: Stat3 deletion during hematopoiesis causes Crohn's disease like pathogenesis and lethality: A critical role of Stat3 in innate immunity. *Proc Natl Acad Sci USA* 2003;100:1879–1884.
- 24 Gao H, Guo RF, Speyer CL, Reuben J, Neff TA, Hoesel LM, Riedemann NC, McClintock SD, Sarma JV, Rooijen NV, Zetoune FS, Ward PA: Stat3 activation in acute lung injury. *J Immunol* 2004;172:7703–7712.
- 25 Cazes E, Giron-Michel J, Baouz S, Doucet C, Cagnoni F, Oddera S, Korner M, Dasic G, Testi R, Azzarone B, Canonica GW: Novel anti-inflammatory effects of the inhaled corticosteroid fluticasone propionate during lung myofibroblastic differentiation. *J Immunol* 2001;167:5329–5337.
- 26 Tantisisira KG, Lake S, Silverman ES, Palmer IJ, Lazarus R, Silverman EK, Liggett SB, Gelfand EW, Richter B, Israel E, Gabriel S, Altshuler D, Lander E, Drazen J, Weiss ST: Corticosteroid pharmacogenetics: Association of sequence variants in CRHRI with improved lung function in asthmatics treated with inhaled corticosteroids. *Hum Mol Genet* 2004;13:1353–1359.
- 27 Litonjua AA, Tantisisira KG, Lake S, Lazarus R, Richter BG, Silverman ES, Weiss TT: Polymorphisms in signal transducer and activator of transcription 3 and lung function in asthma. *Respir Res* 2005;6:52–61.
- 28 Flandre TD, Pascal LL, Daniel J, Desmecht M: Effect of somatic growth, strain, and sex on double chamber plethysmographic respiratory function values in healthy mice. *J Appl Physiol* 2003;94:1129–1136.
- 29 Pennock BE, Cox CP, Rogers RM, Cain A, Wells J: Non-invasive technique for measurement of changes in specific airway resistance. *J Appl Physiol* 1979;46:399–406.
- 30 Sureshkumar V, Paul BN, Uthirappan M, Pandey R, Sahu AP, Lal K, Prasad AP, Srivastava S, Saxena A, Mathur N, Gupta YK: Proinflammatory and anti-inflammatory cytokine balance in gasoline exhaust induced pulmonary injury in mice. *Inhal Toxicol* 2005;17:161–168.
- 31 Geba GP, Wegner CD, Wolyniec WW, Li Y, Askenase PN: Non-atopic asthma: in vitro airway hyperreactivity adoptively transferred to naïve mice by THY-1+ and B220+ antigen- specific cells that lack surface expression of CD3. *J Clin Invest* 1997;100:629–638.
- 32 Vinkemeier U: Getting the message across Stat! Design principles of molecular signaling circuit. *J Cell Biol* 2004;167:197–201.
- 33 Ehlting C, Haussinger D, Bode JG: Sp3 is involved in the regulation of Socs 3 gene expression. *Biochem J* 2005;387:737–745.
- 34 Auernhammer C, Bousquet J, Melmed S: Autoregulation of pituitary corticotroph Socs3 expression: characterization of the murine Socs3 promoter. *Proc Natl Acad Sci USA* 1999;96:6964–6969.
- 35 Naka T, Narazaki M, Hirata M, Matsumoto T, Minamoto S, Aono A, Nishimoto N, Kajita T, Taga T, Yoshizaki K, Akira S, Kishimoto T: Structure and function of a new Stat induced Stat inhibitor. *Nature* 1997;387: 924–929.
- 36 Davey HW, McLachlan MJ, Wilkins RJ, Hilton DJ, Adams TE: Stat 5b mediates the GH-induced expression of Socs2 and Socs3 mRNA in the liver. *Mol Cell Endocrinol* 1999;158:111–116.
- 37 Alexander WS, Hilton DJ: Role of Socs proteins in regulation of the immune response. *Annu Rev Immunol* 2004;22:503–529.
- 38 Takeda K, Noguchi K, Shi W, Tanaka T, Matsumoto M, Yoshida N, Kishimoto T, Akira S: Targeted disruption of mouse Stat3 gene leads to early embryonic lethality. *Proc Natl Acad Sci USA* 1997;94:3801–3804.
- 39 Levy DE, Lee CK: What does Stat3 do? *J Clin Invest* 2002;109:1143–1148.
- 40 Wormald S, Hilton DJ: Inhibitors of cytokine signal transduction. *J Biol Chem* 2004; 279:821–824.
- 41 Wormald S, Zhang JG, Krebs DL, Mielke LA, Silver J, Alexander WS, Speed TP, Nicola NA, Hilton DJ: The comparative roles of Socs-1 and -3 in the inhibition and desensitization of cytokine signaling. *J Biol Chem* 2006;281: 11135–11143.
- 42 Gao H, Marco-Hoesel LR, Guo RF, Rancilio NJ, VidyaSarm J, Ward PA: Adenoviral-mediated overexpression of Socs3 enhances IgG immune complex-induced acute lung injury. *J Immunol* 2006;177:612–620.