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Epigenetic modification of suppressor of cytokine signaling-3 in asthmatic mouse lung: role of interleukin-6

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ABSTRACT

Interleukin-6 (IL-6) and Suppressor of cytokine signaling-3 (Socs3) play important roles in T helper cell type 2-mediated allergic responses, and are the integral components of asthma. As IL-6 regulates the activity of DNA methyl transferases, we hypothesize that asthma is fallout of epigenetic alteration in Socs3. Here, the status of CpG methylation and histone acetylation in the Socs3 promoter of asthmatic lung was studied, as was the role of IL-6 in altering these parameters. Lung tissues from asthmatic mice were cultured in presence or absence of IL-6 and CpG methylation and histone acetylation in the functional Socs3 promoter was assessed. Functional Socs3 promoter was less methylated in asthmatic lung relative to normal lung and in vitro IL-6 treatment aberrantly altered CpG demethylation sites in asthmatic lung although the net demethylation density did not change significantly (p>0.05). 5-aza-2'-dioxycytidine induced significant CpG demethylation in normal lung but did not upregulate Ovalbumin induced-Socs3 expression. Chromatin immunoprecipitation analysis with acetylated H4 antibodies revealed a significantly (p<0.05) high enrichment of Socs3 promoter in asthmatic lung compared to normal lung. In contrast, insignificant (p>0.05) enrichment was observed in IL-6 treated asthmatic lung when compared to normal lung. Our findings implicate that asthma is associated with H4 acetylation and IL-6 causes loss of acetylated histone (H4) protein. This study provides an insight into asthma pathogenesis and delineates the role of IL-6 in epigenetic regulation of the Socs3 gene in pulmonary tissue.

Key words: Asthma, Epigenesis, Socs3, Histone, acetylation, IL-6.

INTRODUCTION

Asthma is a global health problem, the prevalence of which is increasing in most countries, especially among children (Bateman et al., 2008). The disease is associated with a predominance of T helper 2 (Th2) cells that results in the elevation of allergen-specific immunoglobulin E, as well as mast cell- and eosinophil-associated inflammation (Kubo and Inoue, 2006). Suppressor of cytokine signaling 3 (Socs3) plays an important role in Th2-mediated allergic reactions by altering the balance between Th1 and Th2 cells, and its overexpression in asthma is well documented(Kubo and Inoue, 2006; Alt, 2009; Paul et al.,2009; Hartgers et al., 2008; Ozaki et al., 2005; Seki et al., 2003). IL-6, unlike Socs3 but associated with asthma, can augment (Anguita et al., 1998; Rincon et al., 1997) or inhibit (Romani et al., 1996; Ohshima et al., 1998; Ladel et al., 1997) Th2 tissue inflammation. Despite the uncertainty over whether IL-6 regulates Th2 inflammation, the pathological role of IL-6 in allergic bronchial asthma has been widely discussed (Anderson , 2006; Doganchi et al., 2005) , and its abnormally high production is reported (Konno et al., 1996; Virchow et al., 1996; Bradding et al., 1994). By contrast, several studies have demonstrated either a low expression of IL-6(Paul et al., 2009; Prieto et al., 2000) in

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asthma, or observed no change in the serum level of IL-6 in asthma patients when compared to normal controls (Wong et al., 2001). These inconsistencies suggest that IL-6 may not be directly linked to Th2 tissue inflammation, but intimately involved in other reactions associated with asthma. Regulation of Suppressor of cytokine signaling-3 (Socs3) by IL-6 in asthmatic lung has not been adequately investigated.

As IL-6 is known to regulate the activity of DNA methyl transferase (Hodge et al., 2001), enhance nuclear translocation of DNA cytosine-5-methyltransferase-1(Hodge et al., 2007), and upregulate DNA methyltransferase-mediated gene silencing in colon cancer(Foran et al., 2010), it follows that exogenous IL-6 may induce epigenetic alterations in Socs3 promoter and associate with the (re)remodeling of lung tissues in asthma pathology.

Socs3 may be silenced by an epigenetic phenomenon, namely methylation of the CpG islands within the Socs3 promoter (Isomota et al., 2005). Methylation of the cytosine residues within the promoter CpG island causes gene silencing in several cancerous diseases (Herman and Baylin, 2003; Rashid and Issa, 2004). Socs family members have distinct mechanisms of inhibition of Jak/Stat signaling (O'Shea et al., 2002). Altered Socs3 expression can also arise from chromatin protein modifications. Histone acetylation generally accounts for the increase and decrease of gene expression that occurs during normal cell differentiation or oncogenesis (Jones and Laird 1999; Nan et al., 1998; Ng and Bird, 1999;Rountree et al., 2000; Weidle and Grossman, 2000). The epigenetic changes associated with the development of asthma are not clear and therefore deciphering these in the Socs3 promoter has been undertaken to shed light on fundamental processes in asthma pathophysiology. In this study, we propose that demethylation of CpG dinucleotides in the Socs3 promoter of asthmatic mouse lungs may not be associated with Socs3 expression. Instead, we suggest that consistent hyperacetylation of H4 proteins in chromosomes may be responsible for Socs3 overexpression and that IL-6 leads to a loss of acetylated histone H4 protein in asthmatic lung tissues. This study will further the understanding of the epigenetics of asthma pathophysiology and help in designing therapeutic strategies.

METHODS

Development of mice with asthma phenotype

Asthma was induced in mice as previously described (Paul et al., 2009). The experiments were conducted with consent from the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), India. Ovalbumin (Ova) sensitized and challenged mice (Ova/Ova), while saline sensitized and challenged mice (Sal/Sal) were used in the study. Prior to lung function assessment, both Ova/Ova and Sal/Sal mice were exposed to nebulized Ova at a flow rate of 0.5 liters per minute (LPM) and a bios flow rate of 0.5 LPM for a total of 9 min over three cycles (each cycle lasted 3 min with a 3-min interval between each). The nebulizer head was loaded with 1mL saline containing 1mg Ova.

Airway hyper-reactivity induced by different concentrations of methacholine (Mch) was measured by double-chambered plethysmograph (Buxco, Wilmington, USA). Nebulized Mch was administered via inhalation route by dispensing various concentrations onto the nebulizer head and onto the nasal chamber at a flow rate 0.5 LPM for 5 min per concentration. Specific resistance of airways (sRaw) was monitored with the Biosystem XA SFT 3410 version 2.11(Buxco, Wilmington, USA). Ova-specific IgG and IgE antibodies in broncho-alveolar lavage fluid (BALF) were evaluated by ELISA. IL-4 and IL-5 proteins in BALF were evaluated by solid phase sandwich ELISA using ELISA kits according to the manufacturer's instruction (R&D System, Minneapolis, USA). BALF eosinophil and neutrophil percentages were determined by performing leukocyte differential counts on Giemsa stained slides.

Experimental design

Following evaluation of sRaw, lungs from Ova/Ova mice were cut into pieces (~1 mm × 1 mm) and cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12(Ham) (1:1) (Gibco, Auckland, NZ) containing 15 mM HEPES buffer, L-glutamine (2 mM), pyridoxine hydrochloride, and supplemented with 10% fetal bovine serum, penicillin (100 units/mL), and streptomycin (100 µg/mL). Sodium bicarbonate was used to adjust the pH between 7.2–7.4. Lung pieces were cultured in 12-well plates which were kept on a Gyrotwister (Labnet, Woodbridge, NJ) at 10 rpm inside a CO_2 incubator at 37˚C. In the case of Socs3 promoter methylation studies, cultivation was performed either in the absence or presence of 250 pg/mL rIL-6 (Sigma, St. Louis, USA) for 24 h. Sal/Sal mouse lung was cultured for a similar time period in the absence of IL-6 represent reference group. The dose of IL-6 adopted in the study is approximately five times the upper basal level found in mice. The basal level of IL-6 in Balb/c sera ranges from 15.5 to 48.5 pg/mL. IL-6 mediated histone acetylation was studied at 0, 2 and 24 h post co-culture of lung pieces with 250 pg/mL rIL-6. Lungs of Sal/Sal mice treated with 10µM of 5-aza-2'-dioxycytidine (DAC) were also studied for Socs3 promoter methylation by methylation specific PCR (MSP) and bisulphite sequencing. The in vitro effect of DAC on Ova induced Socs3 mRNA expression was studied by culturing Sal/Sal mouse lung in presence of varying concentration of DAC ranging from 0 -10µM for 24h, DAC removed by washing 3 times with PBS and thereafter cultured in presence of OVA (20µg/mL) for 2h and Socs3 mRNA quantified by RT-PCR. Ova/Ova lungs cultured in presence of OVA (20µg/mL) for 2h represent positive control.

Determination of Ova-specific antibodies by ELISA

Ova-specific IgG and IgE antibodies in BALF were evaluated by coating the plate with Ova in 0.05M carbonate-bicarbonate buffer, pH 9.5, at a concentration of 10µg/mL before incubating the plate at 4°C overnight. After incubation, wells were washed with PBST and blocked with 200 µL blocking reagent (50 mM TRIS, 0.14M NaCl, 1% BSA, pH 8.0) for 30 min.

After washing three times, 100 µL analyte was added and incubated for 2 h at room temperature. IgG and IgE antibodies in the analyte were detected with the help of HRP conjugated goat anti-mouse IgG- and IgE- antibodies, respectively (dilution of 1:10,000). Color was developed by adding tetramethyl benzidine substrate for 10 min. The reaction was stopped by adding 100 µL 2 M H_2SO_4 and the intensity of the color developed was measured on an ELISA plate reader at 450 nm.

Polymerase Chain Reaction (PCR)

RNeasy Mini kit (Qiagen, GmbH, Hilden) was used to isolate RNA and RT-PCR performed to measure Socs3 mRNA transcript levels in Sal/Sal and Ova/Ova mice according to a previously published protocol (Paul et al., 2009) using One Step RT-PCR kit (Qiagen). For MSP and ChIP assay standard PCR reactions were performed with the Hot Star Taq Master mix following instructions given in the technical bulletin (Qiagen) for the thermal cycler (ESCO, Japan).

Immunoblot analysis of Socs3 proteins

The cytosolic fraction of lung tissues was prepared using Qproteome Cell Compartment kit (Qiagen). In brief, the lung tissue pieces (~20 mg) were washed with ice cold Phosphate buffered saline (PBS), pH 7.2 and placed in a microcentrifuge tube containing 500 µL extraction buffer supplemented with protease inhibitors. After disrupting the tissues, clumps of tissues were removed by centrifuging the tissue suspension in Qiashredder at 500x g for 2 min at 4˚C. The pellets were resuspended and 500 µL extraction buffer added before incubation at 4˚C for 10 min in a shaker. After incubation, lysates were centrifuged at 4000 x g for 10 min at 4˚C and the supernatant was collected. Protein concentrations in the supernatants were measured using Biuret reagent (Spinreact, Spain). Proteins were denatured by boiling for 5 min and 50 µg was loaded onto an SDS-PAGE gel (12.5%) for electrophoresis and then transferred to nitrocellulose membranes. After blocking with 2% bovine serum albumin in TBST (137 mmole/L NaCl, 25 mmole/L tris, 1 mmole/L Na_2EDTA and 0.1% Tween 20), the membranes were incubated with rabbit anti-mouse Socs3 antibodies (Santa Cruz Biotech, California, USA) at 4°C overnight at a dilution of 1:200. After washing three times with TBST, the membranes were incubated with HRP conjugated goat anti-rabbit IgG antibody (1:2000 dilution) at room temperature for 3 h, and then washed three times with TBST before incubation with a chemiluminescent peroxidise substrate (Sigma, St. Louis, USA). Thereafter, blots were stripped and reprobed with β-Actin (C4) antibody conjugate at 1:1000 dilutions.

Bisulphite modification and MSP

DNA from lung pieces was isolated using DNeasy blood and tissue Kit (Qiagen) following instructions given in the technical bulletins. Genomic DNA was subjected to bisulphite modification using EpiTect Bisulphite Kit (Qiagen) according to the manufacturer's protocol. Bisulphite conversion efficiency of the region of our interest was measured by real time PCR from ΔCT ($C_{T\ converted}$- $C_{T\ unconverted}$) adopting the formula:

$$\text{Conversion efficiency} = [\ 2^{(-\Delta CT+1)}/2^{(-\Delta CT+1)}+1\ ] \times 100.$$

The integrity of the bisulphite converted DNA was performed by reviewing the size of both converted and unconverted PCR products after electrophoresis. Bisulphite modified DNA (50 ng) was used as templates for MSP using HotStar Taq Master Mix (Qiagen). MSP was performed using both methylation- and unmethylation-specific primers under the following conditions: initial activation for 15 min at 95°C followed by three-step cycling (denaturation at 94°C for 45 sec; annealing at 58°C for 45 sec; extension at 72°C for 1 min) for 30 cycles and a final extension at 72°C for 10 min. The methylation- and unmethylation-specific Socs3 primer pairs (Table I) were designed to amplify nucleotides -1131 to -975 and nucleotides -1128 to -973 of the Socs3 promoter region, respectively, by using MethPrimer v1.1beta software(Li and Dahiya, 2003).

Bisulphite Sequencing

Bisulphite-treated genomic DNA was amplified using HotStar Taq master mix (Qiagen) with sense and antisense primers (Table I) to amplify the region between -1050 to -747 of the Socs3 promoter, following protocols described by the manufacturer (Qiagen). The reaction conditions were the same as those employed for MSP. The PCR products were purified using a MinEluate PCR Purification kit (Qiagen, GmbH) and sent for automated DNA sequencing analysis (Sigma DNA Sequencing Service, India). The methylation status of CpG dinucleotides was analyzed and documented in a square grid using the algorithm designed by Carr et al., 2007.

X-ChIP assay

The protocol of Sakai et al., 2004 was adopted. Histone isotype specific antibodies, Ac-Histone H3 (Lys 23)-R and Ac-Histone H4 (Lys 8)-R antibody (Santa Cruz Biotech, California, USA) were used. DNA in immunoprecipitates was analyzed by standard (ESCO, Japan) or real-time quantitative PCR (Light Cycler 480, Roche). Real-time data are presented as fold enrichment of the ChIP antibody signal versus mock IgG (rabbit non-immune sera). Fold enrichment was calculated as $2^{(-\Delta\Delta Ct[Chip/mock])}$. To recover the DNA from protein A agarose/antibody/histone complexes, the histone-DNA complex was first eluted by incubating 30 µL protein A agarose/antibody/histone complex with 200 µL elution buffer (0.1 M $NaHCO_3$, 1% SDS) at room temperature for 15 min with rotation. After centrifugation at 1000 rpm for 2-3 minutes, the supernatant (150 µL) was collected and mixed with 20 µL of 5 M NaCL and heated at 65˚C for 4 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation.

Statistical analysis

Differences were evaluated for significance by Student's t-test for independent events (Microsoft Office Excel 2007)

Table 1 Sequences of primers used in different experiments

Socs3	(F) 5'TGAGCGTCAAGACCCAGTCG3'
	(R) 5' CACAGTCGAAGGCGGGGAACT3'
β-actin	(F) 5'-GACATGGAGAAGATCTGGCAC-3'
	(R) 5'-TCCAGACGCAGG ATGGCGTGA-3'
Methylation specific for MSP	(F) 5'GTTTTGGAGGTTTTAGTTTTAAGTTC3'
	(R) 5'GAATATTTACCCGAC CAATACG3'
Unmethylation specific for MSP	(F)5'TTGGAGGTTTTAG TTTTAAGTTTGT3'
	(R) 5'CAAATATTTACCCAACCAATACACC3'
Socs3 promoter (-1050 to -747)	(F) 5'GTGACTAAACATTACAAGAAG3'
for bisulphate sequencing	(R) 5' CCGTGAAGTCTACAAAGG3'

RESULTS

Relationship between Socs3 and specific resistance of the airway in asthmatic mice

Ova/Ova mice have OVA-specific IgG and IgE antibodies in BALF, which are absent in Sal/Sal mice (Table II). BALF IL-4 and IL-5 protein levels were significantly ($p<0.05$) elevated in Ova/Ova mice compared to Sal/Sal mice. Significant ($p<0.05$) increase in eosinophil percentage in the BALF cells of Ova/Ova mice was also observed compared to Sal/Sal (Table II). Changes in BALF neutrophil percentage, however, was insignificant ($p>0.05$) in Ova/Ova compared to Sal/Sal mice.

Table 2 Different parameters (IgG, IgE, IL-4, IL-5, eosinophil and neutrophil percentage) in BALF of OVA sensitized and challenged mice to reflect the asthmatic symptoms in mice.

	Sal/Sal	Ova/Ova
OVA specific IgG (µg/mL)	ND	110.6 ± 10.2
OVA specific IgE (µg/mL)	ND	4.2 ± 2.1
IL-4 (pg/mL)	35.5 ± 14.5	174.3 ± 38.2*
IL-5(pg/mL)	40.8 ± 15.5	92.5 ± 19.3*
Eosinophil (%)	1.6 ± 0.63	12.7 ± 5.39*
Neutrophil(%)	1.8 ± 0.81	4.5 ± 2.57

Data represent mean ± σ of values from 6 mice in each group; ND, not detected; Neutrophil and eosinophil (%) counts are based on Giemsa-staining. *p < 0.05, significant compared to Sal/Sal.

sRaw is a sensitive and reproducible measure of lung function. An inverse correlation exists between sRaw and lung function, whereby the higher the sRaw level, the poorer the lung function (Paul et al., 2009). Elevated levels of a varying concentration of Mch-induced sRaw were observed in Ova/Ova mice compared to Sal/Sal mice (Fig 1a). Thus, the asthma phenotype of Ova/Ova mice is established on the basis of lung physiology, BALF cytokine levels, as well as on the basis of antibody and cellular profiles. In parallel, the expression of lung Socs3 mRNA of randomly selected 5 mice from each group (Fig 1b & c) and cytosolic protein of 2 mice from each group (Fig 1d & e) was studied and observed higher expression in Ova/Ova mice

than those in Sal/Sal mice. Thus, our results reflect a good relationship between sRaw and Socs3 expression, in which heightened expression of Socs3 is associated with poorer lung function (or high sRaw). Lung tissues from Ova/Ova and Sal/Sal mice were used in subsequent studies.
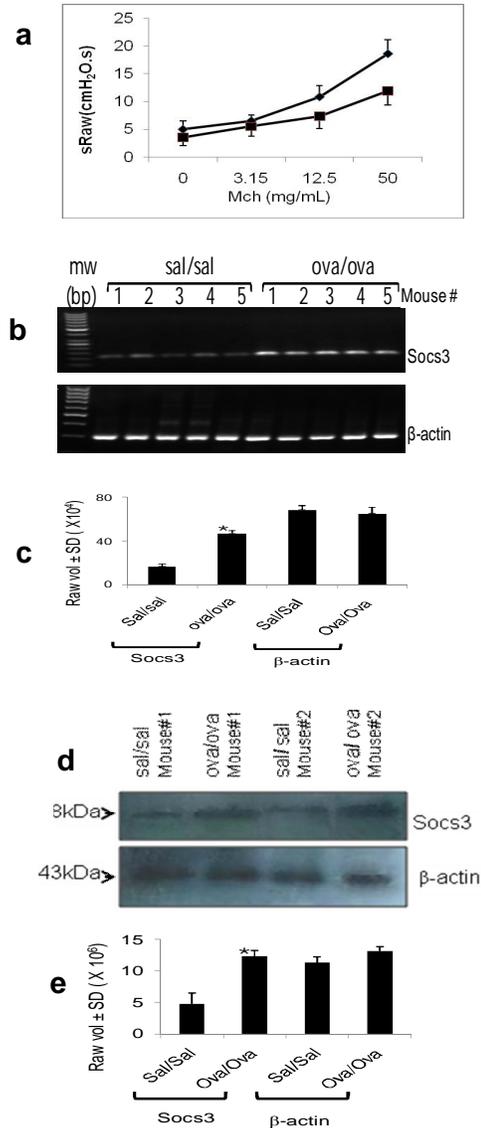
Fig 1 Relationship between lung function and Socs3 in asthmatic mice. (a) Graphical representation of Mch induced sRaw in Ova/Ova (♦) and Sal/Sal mice (■) (b) mRNA expression profile of Socs3 in Ova/Ova and Sal/Sal (n=5; in each group) mice after 30 cycles of RT-PCR. Housekeeping gene β-Actin served as internal control. 1-5 indicate the number of mice used in each group. (c) Representative western blot analysis of Socs3 and β-actin in randomly selected two Sal/Sal and two Ova/Ova mice. The 28 kDa and 43kDa bands represent Socs3 protein and β-actin respectively. The experiment shown here is representative of three experiments. (d) Bar diagram showing quantitative representation of Socs3 protein in Sal/Sal and Ova/Ova mice.

Methylation status of Socs3 promoter in asthmatic lung tissue

The epigenetic role of IL-6 was studied by assessing CpG methylation of Socs3 promoter regions in Sal/Sal, Ova/Ova and IL-6-treated Ova/Ova mouse lungs by MSP and bisulphite

sequencing. We analyzed details of the methylation status of 30 CpG motifs between -1050 to -747 of the Socs3 promoter region in normal and asthmatic mice (n=4; in each group). This region contains both functional Stat-binding sites (SBS) and a TATA box that are highly conserved in the Socs3 promoter of both mouse and rat (shown in the schema, fig 2a), and the region can function as a minimal promoter (He et al., 2003). The unmethylated and methylated primers used in MSP analysis yielded amplicons of sizes 155 and 157 bp, respectively. Sal/Sal mouse lung (n=4) lacking Socs3 expression was found to have both methylated and unmethylated bands of approximately equal intensities by MSP (Fig.2b) and hence the methylation status of normal lung cannot be drawn. In contrast, Sal/Sal lung treated with demethylating agent, DAC, revealed high intensity unmethylated amplicons relative to methylated amplicons, apparently suggesting that Socs3 gene in normal lung is under the influence of CpG methylation. However, lung tissues from Ova/Ova mice (n=4) with elevated Socs3 expression also displayed methylated and unmethylated amplicons of variable, and in some cases equal, intensities. Ova/Ova lung (n=4) cultured for 24 h in the presence of rIL-6 also showed methylated and unmethylated amplicons (Fig 2b). This implies that lungs of normal, asthmatic and IL-6 treated asthmatic mice are composed of heterogeneous cells, some of which possess the methylated Socs3 promoter and others the unmethylated promoter; thus making it difficult to come to a conclusion. We therefore performed bisulphite sequencing to delineate the status of CpG methylation in functional region of Socs3 promoter. Bisulphite conversion efficiency of the region of interest in all the groups was found between 84 to 89%. The integrity of the converted DNA was also checked by reviewing the size of the PCR products. Both the converted and unconverted PCR products have ~ similar sizes (450 bp; figure not shown) indicating that the DNAs were not degraded following bisulphite treatment. In Sal/Sal mouse lungs, the CpG motifs are mostly methylated (Fig 2c). Few cytosines (C) at positions -925, -863 and -796 were found demethylated (density $\pm$SD = 5.83$\pm$3.1 %) in the four mice studied in this group. Demethylation density is defined as the unmethylated CpGs divided by total CpGs examined in a gene promoter. In Ova/Ova mouse, however, lung Socs3 gene promoter was found to be significantly (p=0.025) demethylated (mean density of 29.9 $\pm$ 9.81%) relative to Sal/Sal lung. Interestingly, Cs at positions – 987, −975, −961, −896, −892, −833 and −808 are reproducibly unmethylated in asthmatic lung but it is not clear whether these changes are functionally active.

Both aberrant and specific alterations in the methylation of CpGs were observed in Ova/Ova lung treated with IL-6. Aberrant CpG methylation in Socs3 promoter of IL-6 treated Ova/Ova mouse lung suggest that IL-6-mediated Socs3 promoter methylation in asthmatic lung may not be playing a crucial role in transcriptional inactivation. Furthermore, the mean CpG demethylation percentages in this group is insignificant (p=0.62) in comparison to IL-6 untreated Ova/Ova lung (Fig 2d). Treatment of Sal/Sal lung with DAC (10µM) in vitro significantly (p=0.01)

increased CpG demethylation relative to Sal/Sal (Fig 2d). In spite of altered CpG demethylation, Ova-induced Socs3 mRNA expression in varying concentration of DAC pre-treated Sal/Sal lung was similar to basal mRNA level found in untreated Sal/Sal lung, and ~1/3 of the Ova induced Ova/Ova lung (Fig 2e & f). The observed inactivation of Socs3 in DAC pretreated Sal/Sal mice negates the role of CpG methylation in the regulation of lung Socs3 expression.
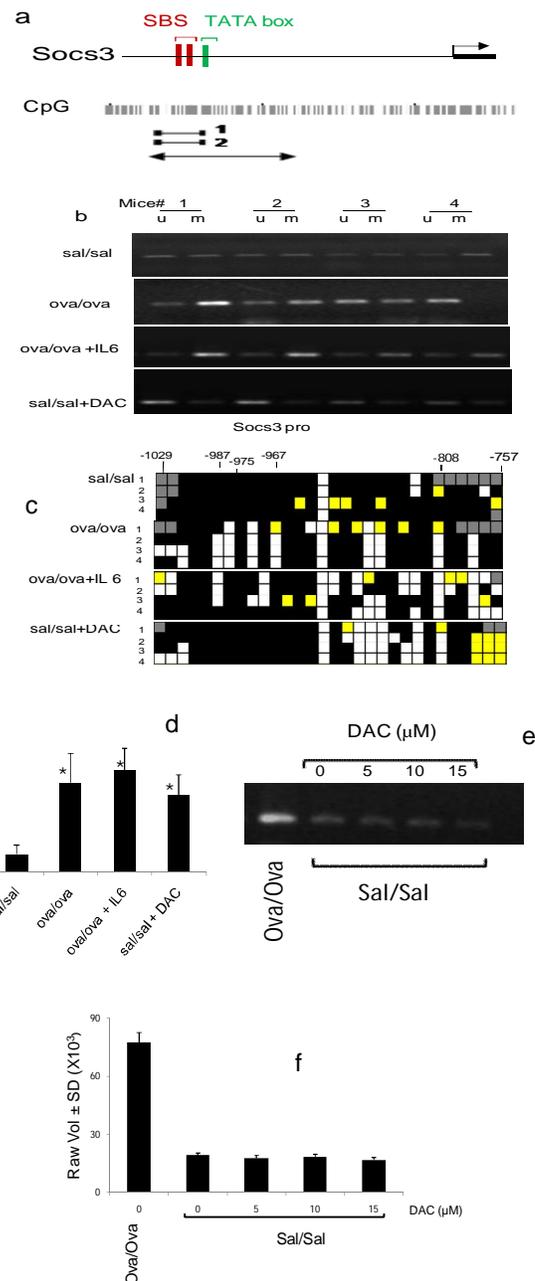
Fig 2 Correlation of methylation in the promoter region of Socs3 gene in asthmatic and normal lung tissue. (a) A schema of the 5' Socs3 promoter region and Socs3 gene. The large black bar represents the ORF of Socs3 gene with an arrow on top indicating the translation start site. The location of the two Stat binding sites (SBS) marked by red vertical bars and one TATA box (green vertical bar) in the Socs3 promoter region is indicated. Grey vertical bars represent CpG islands. The lines (1 & 2) and a double arrow below the CpG islands represent regions analyzed by MSP

(1 for methylation specific- and 2 for unmethylation specific primer region) and bisulphite sequencing, respectively. (b) MSP analysis of Sal/Sal, Ova/Ova, Ova/Ova +IL-6 and Sal/Sal + DAC mouse lung tissues. Bands (155 and 157 bp) in lanes labeled "U" and "M" represent DNA products amplified with unmethylation- and methylation-specific primers respectively. (c) Bisulphite sequencing to analyze methylation status of CpG sites of Socs3 promoter in Sal/Sal, Ova/Ova, IL-6 treated Ova/Ova, and DAC treated Sal/Sal mouse lung. PCR products amplified from bisulphite-treated genomic DNA from four different lung tissues were sequenced. The status of methylation is shown in the CpG grid. Black and white squares represent methylated (CpG), and unmethylated C (TpG or CpA), respectively; yellow represents an unidentified base (not CpG, TpG or CpA), and grey is non-aligned sequence trace. (d) Quantitative figure showing percentage demethylation in the functional region of Socs3 promoter of Sal/Sal, Ova/Ova, IL-6 treated Ova/Ova, and DAC treated Sal/Sal mouse lung. (e) Representative gel showing the effect of demethylating agent (DAC) on OVA induced Socs3 mRNA expression in Sal/Sal lung; and (f) bar diagram showing quantitative analysis of OVA-induced Socs3 expression in Sal/Sal lung pretreated with varying concentration of DAC.

Hyperacetylation of histone proteins in asthmatic lung

Transcriptionally active genes residing in chromosomal domains are characterized by an increased acetylation of the histone tail; therefore, we focused our studies on the status of acetylation of histone proteins in asthmatic and IL-6-treated asthmatic lung. Acetylated states of histone proteins (H3 and H4) were analyzed by the X-ChIP assay to determine whether gene expression changes in asthmatic lungs correlate with expected changes in histone acetylation (Fig. 3). Persistently elevated Socs3 expression in asthmatic lung tissue suggested that histones at this locus would be acetylated as compared to non-asthmatic lung. The DNA in the immunoprecipitates formed with anti-ac-H3 or anti-ac-H4 antibodies were analyzed by real-time qPCR using the Socs3 promoter-specific primers indicated in table 1.

DNA immunoprecipitated with acetylated H3 antibody showed statistically insignificant (p>0.05) signal over the background signal in all the groups (Fig 3a). By contrast, the enrichment ratio observed in Ova/Ova lung DNA immune-precipitated with acetylated H4 antibodies was significantly higher (p<0.05) than in Sal/Sal lung (Fig 3b).

However, Ova/Ova lung treated with IL-6 in vitro for 2 and 24 h showed insignificant (p>0.05) enrichment in comparison to Sal/Sal lung, indicating that histone H4 proteins are significantly acetylated in asthmatic lung, and that IL-6 treatment reduces the acetylated H4 level. Standard PCR was also performed with DNA precipitated with acetylated H4 antibodies and non immune rabbit serum (negative control) and analyzed by agarose gel electrophoresis (Fig3c). Bright bands in Ova/Ova (0 h)-group and faint bands in IL-6-treated Ova/Ova lung tissues for different time periods (2 and 24 h) were seen. Quantitative analysis from 3 independent experiments for each group (Fig 3d) further revealed significantly high intensity Socs3 promoter amplicons in Ova/Ova group relative to Sal/Sal group and negative control, and IL-6 treatment reduces Socs3promoter level, and confirmed the quantitative real-time PCR data. Thus, IL-6 treatment leads to a loss of acetylated H4 and probably results in a non-permissive environment for Socs3 gene expression.

Furthermore, in vitro IL-6 treatment of Ova/Ova lung 2 and 24h reduces the cellular Socs3 protein content significantly (Fig 3e & f).
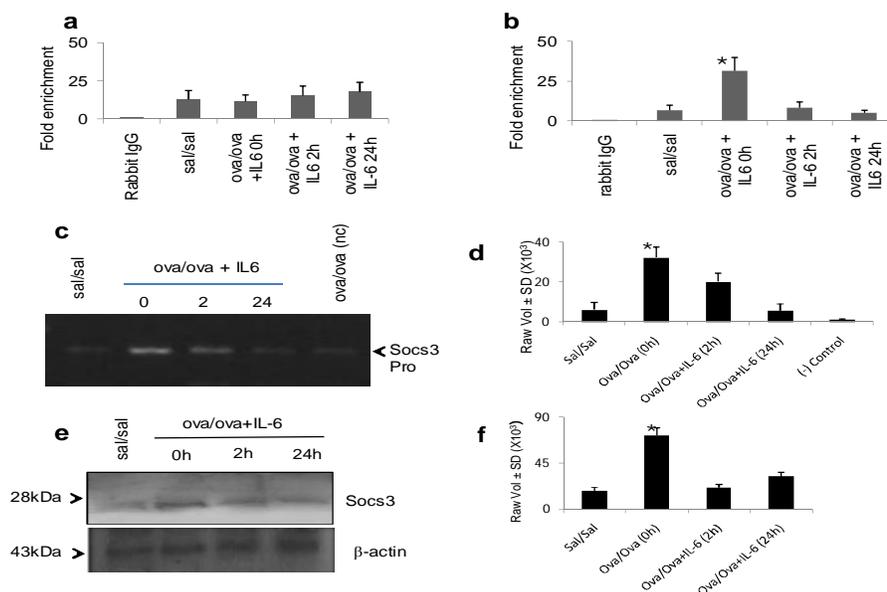
Fig 3 X-ChIP assay to analyze acetylated states of histone proteins in mouse Socs3 promoter and the effect of IL-6 on Socs3 protein expression. Anti-acetyl Histone H3 and H4 ChIP grade antibody were used to immunoprecipitate chromatin from lysates of lung tissues of Sal/Sal and Ova/Ova lung treated with IL-6 for 0, 2 and 24 h. Bar diagrams (a and b) represent real-time qPCR data showing status of acetylation of H3 and H4, respectively, in the functional Socs3 promoter. In all cases, data represent the mean of duplicate determinations of each of three independent immunoprecipitations (a total of six determinations). An asterisk indicates that the value is significantly different (p<0.05) from the Sal/Sal value. Gel picture (c) represents standard PCR performed on DNA purified from acetylated H4 antibody-mediated immunoprecipitates; Ova/Ova (nc) represents negative control i.e., PCR product with DNA from immunoprecipitate with rabbit non-immune sera. (d) Quantitative analysis of 'c' from 3 independent chromatin immunoprecipitates. Band intensities of Socs3 promoter amplicons are presented as raw volume obtained from gene tool software version3.06 (Syngene). Representative western blot analysis of Socs3 and β-actin in Ova/Ova mouse lung treated with rIL-6 for 0, 2 and 24 h is shown in (e) and quantitative representation of Socs3 protein band intensity in terms of raw volume from 3 different experiments is shown in (f).

The IL-6 dependent reduction of Socs3 expression in asthmatic lung suggests that histones at these loci would be less acetylated in comparison to asthmatic lung. Interestingly, histone H4 is less acetylated at all the time points (2 and 24h) in IL-6 treated Ova/Ova mouse lung (Fig 3b). Taken together, these data suggest that IL-6 is involved in regulating the acetylation state of chromatin in Socs3 promoter region of asthmatic lung. This is likely due to the induction of methylases and acetylases including histone deacetylases.

DISCUSSION

The present investigation attempts to delineate the association between asthma and the epigenetic alteration of functional Socs3 promoter in pulmonary tissues. Lung tissues from asthmatic mice were cultured in the presence or absence of IL-6 and the status of CpG methylation or histone acetylation in the functional Socs3 promoter was assessed. To examine whether there is any association between histone acetylation and Socs3 expression, we first determined the differences between histone acetylation status in asthmatic and non-asthmatic lung using the Chip assay. In asthmatic lungs, higher levels of acetylated histone H4 were found associated with the Socs3 promoter when compared with normal lung which supports the concept that Socs3 gene expression is associated with histone acetylation in asthma. Several lines of evidence suggest that histone acetylation plays a role in transcriptional regulation, probably by altering the chromatin structure. HDACs and histone acetyltransferases act together to modulate chromatin structure and transcriptional activity by changing the pattern of histone acetylation (Grunstein, 1997; Marks et al., 2001; Roth et al., 2001). Because of the known dynamic linkage between DNA methylation and histone deacetylation, we examined whether modulating methylation and histone acetylation could affect Socs3 expression. Exposure of normal lung tissue to the DNA methyltransferase inhibitor, DAC reduced DNA methylation but did not up regulate the Socs3 mRNA expression indicating that CpG methylation and histone decetylation are independent processes, dynamic linkage between the two may not exist.

The experimental design of our study is relevant and very close to organ-specific mechanisms, as we attempted to maintain the in vivo architecture and intercellular communication by culturing lung tissue pieces. The present study elaborates on the role of the Socs3 promoter region in maintaining heightened expression of Socs3 mRNA in asthma. The overexpression of Socs3 gene in asthma may not be associated with CpG demethylation. Instead, histone H4 acetylation seems to play a significant role, revealing an important epigenetic event during the development of asthma. Socs3 is normally activated by Janus kinase-Stat signaling in normal cells, which, in turn, inhibits Janus kinase activity and subsequent Stat3 activation. As Socs3 is persistently overexpressed in asthma (Paul et al., 2009), it may result in cell resistance to aberrant growth stimulating signals that function through the Janus kinase-Stat pathway, leading to cell death and airway tissue remodelling. In addition, our experiments

showed that IL-6-mediated loss of acetylated Histone H4 proteins in the Socs3 promoter region correlates with the underexpression of Socs3 in the lung of asthmatic mice. Therefore, overexpression of Socs3 as a result of promoter H4 protein acetylation may be associated in asthma pathogenesis. Interestingly, IL-6 can mediate epigenetic alterations of the chromatin structure in lung. In this study we explain the significance of low or unchanged levels of IL-6 in asthma pathology, as reported earlier (Paul et al., 2009; Prieto et al., 2000; Wong et al., 2001); and further provide a possible mechanism for IL-6-induced epigenetic regulation of Socs3 promoter. IL-6 is most often classified as a pro-inflammatory cytokine, although consistent data also demonstrate that it has an anti-inflammatory effect and may negatively regulate the inflammation of acute phase response by controlling the level of pro-inflammatory cytokine or increasing IL-10, IL-1 receptor antagonist and insoluble TNF-receptors [Xing et al., 1998; Petersen and Pedersen , 2005; Ropelle et al., 2010). This study demonstrates the anti-inflammatory role of IL-6 in asthma pathogenesis by epigenetic regulation of Socs3 expression and there is reason to believe that IL-6 may modify lung inflammation. In the lungs, transgenic overexpression of IL-6 results in a decrease in airway responsiveness (DiCosmo et al., 1994; Kuhn et al., 2000). Overexpression of IL-6 also protects against the acute lung injury induced by chronic hyperoxia(Ward et al., 2000) and highlights the potential use of IL-6 in therapies for the treatment of asthma and related diseases, such as chronic obstructive pulmonary disease.

CONCLUSIONS

Asthma pathophysiology is associated with epigenetic modification of Socs3 gene. Acetylation of histone protein (H4) appears to be responsible for overexpression of Socs3 and exogenous IL-6 reduces acetylated H4 proteins in asthmatic lung.

LIST OF ABBREVIATIONS

DAC, 5-aza-2'-dioxycytidine; H4, Histone 4; IL-6, Interleukin-6; SBS, Stat3 binding sites; Socs3, Suppressor of cytokine signaling3; Th2 cells, T helper cell type II; X-ChIP, crosslinked-chromatin immunoprecipitation assay

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DECLARATIONS OF INTEREST

None of the authors have a conflict of interest to declare in relation to this work.

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