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Histological Studies in Murine Model of Asthma: Effect of Stat3 siRNA

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ABSTRACT

Airway remodeling in asthma is recognized as irreversible structural change. However, several recent reports revealed that remodeling might be the process of repair from injury. Airway remodeling is increasingly recognised to be a serious consequence of chronic asthma. Stat3 and Cytokines play an integral role in the coordination and persistence of inflammation. However, exact role of Stat3 in airway inflammation is lacking. In the present study BALB/c mice were sensitized and challenged with OVA (*OVA/OVA*) after validation these mice models were further studied to check silencing effect of Stat3 .mRNA and ELISA studies revealed alteration in IL-4, IL-5, IL-13 and TGF-β in BALF and lung with blood eosinophilia also airway hyperresponsiveness in *OVA/OVA* mice. Airway hyperresponsiveness was studied by methacholine-induced specific airway resistance in a plethysmogrpah while eosinophils study was done using automatic blood analyser. Total and OVA-specific IgG and IgE antibodies depicted significant rise among mice sensitized and challenged with OVA. Studies pertaining to histology revealed fibrous tissue proliferation along with other inflammatory changes in airway structure among *OVA/OVA* mice and it are the characteristic of human model of asthma. Heightened expression of TGF-β and proliferation of fibrous tissue in lungs are directly related. On the contrary *SAL/SAL* mice revealed normal blood eosinophils. There was no change in IL-4, IL-5, IL-13 and TGF-β also OVA-specific IgG and IgE antibodies in *SAL/SAL* mice presented normal range. Our earlier studies showed down-regulation of Stat3 gene in airway tissues is related with airway inflammation in a mouse model of asthma using this background we tried to study airway histology after silencing Stat3 gene in *OVA/OVA* mice interestingly our results showed that silencing Stat3 did not help in restoring airway histology in *OVA/OVA* mice in addition to this investigations pertaining to cytokines, immunoglobulins, blood eosinophils, sRAW and mRNA studies did not depicted any sign of restoration.

Keywords: Asthma, Airway remodelling, Cytokines, Stat3, Mice.

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INTRODUCTION

Allergic asthma is a complex chronic inflammatory disease of the airways and its etiology is multifactorial. It involves the recruitment and activation of many inflammatory and structural cells, all of which release inflammatory mediators that result in typical pathological changes of asthma (Barnes *et al.*, 1998, O'Byrne and Postma, 1999). The infiltration of leukocytes, particularly eosinophils, into the lungs and release of vasoactive mediators from mast cells set the stage for asthmatic inflammation. Many cells and cellular elements, including mast cells, eosinophils, T-lymphocytes, macrophages, neutrophils and epithelial cells are involved in the process. The cells that line the airway produce excess mucus, which builds up inside the airway passage. Increased mucus production in asthma is an important cause of airflow obstruction

during severe exacerbations. Excessive mucus secretion from hyperplastic goblet cells is a feature of the asthmatic airway and might lead to the occlusion of the airways. Airway remodeling thus results in thickened airway walls in asthma, and is thought to be a determinant of airway hyper-responsiveness (AHR), as well as the accelerated loss of lung function over time described in asthma (Busse *et al.*, 1999, Elias *et al.*, 1999). 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 (Darnell, 1997). 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 hyper- production (Sender and Paul; 1994, Ennis *et al.*, 2004). Stat3, with diverse roles in biological processes including cell proliferation, survival, apoptosis and inflammation, is another cytoplasmic peptide belonging to the Stat family.   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 (Bromberg and Darnell, 2000). In vivo aberrant expression of Stat3 is associated with immune tolerance (Cheng *et al.*, 2003) acute phase response (Alonzi *et al.*, 2001) septic shock (Andrejko *et al.*, 1998) and constitutive activation in chronic inflammation (Hanada *et al.*, 2001). These observations suggest that Stat3 may play an important role during airway inflammation, especially asthma. 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.*, 2004 reported that activation of Stat3 in the lungs is macrophage dependent. 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 (Cazes *et al.*, 2001). The pharmacogenetics of asthma treatment reveals Stat3 as one of the candidate gene showing association in response to corticosteroid treatment (Tantisira *et al.*, 2004). 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. We reveal blocking Stat3 gene expression resulted elevation in blood eosinophil, rise in sRaw values, total and allergen specific IgE and IgG in serum and BALF. In addition to that many fold increase in of IL-4, IL-5.IL-13 and TGF-β. However there was no alteration in airway histology. The implications of these observations are discussed in the light of the pathogenesis of asthma.

MATERIALS AND METHOD

Animals & Mouse Model of Asthma

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 μg 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, animals were challenged with OVA through the nasal route. A total of 50 μg OVA in sterile SAL was applied on the snout or the nostril in 5 cycles, 2 μl containing 10 μg 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 μg OVA through the nasal route.

Measurement of Lung Function

Lung function was measured in terms of methacholine (Mch) induced airway hyper-reactivity (AHR) using a double-chambered whole-body plethysmograph (Buxco, USA). After a brief acclimatization period, the mice received an initial baseline challenge of saline, followed by increasing doses of Mch (3.12, 12.5 and 50 mg/ml). The specific resistance of the airways (sRaw) was recorded using BioSystem XA, version 3.1 (Buxco, USA).

Eosinophil Counting in Blood

The important clinical haematological variable in asthma episode i.e. eosinophils were measured using Abacus Haematology Analyser (model: Abacus junior 5). Blood was collected in anticoagulant coated vials and subjected to eosinophil analysis.

Collection of BALF and Sera

Blood was collected from the retro-orbital sinus of anaesthetized mice with ketamin hydrochloride (0.05ml/mice) post last OVA challenge (on 28th day) and kept at room temperature for 3h. Sera obtained after centrifugation at 3000 rpm for 5 min were stored at -20°C for ELISA. After collection of blood, the mice were sacrificed by an overdose of anaesthesia, and dissected to expose the trachea and bronchi. The left bronchi and the anterior end of the trachea were tied with surgical threads. Trachea was cannulated with 20 gauge catheter and 1 ml of PBS was injected into the left lung through trachea. The injected PBS was withdrawn after 10 sec. The collected BALF was centrifuged at 2000 rpm for 5 min at 4°C to separate the leucocytes. The supernatant was subjected to antibody and cytokine analysis. (Sureshkumar *et al.*, 2005). IL-4, IL-5, IL-13, TGF-β and IFN-γ proteins in BALF were quantified with commercial mouse ELISA kits (R&D system, Minneapolis, USA) according to manufacturer's instructions.

Evaluation of Total and Antigen specific IgG and IgE

Total and antigen specific IgG and IgE antibodies in the BALF and sera were evaluated by quantitative ELISA following the manufacturer's protocol (Mouse IgG ELISA Quantitation kit; Bethyl Laboratories, Inc; Montegomery, Tex, USA). The plates were coated with goat antimouse IgG antibody at a concentration of 10 μg/ml in a coating buffer (0.05 M carbonate bicarbonate, pH 9.6) and incubated for 60 min. Free sites were blocked with 50mM Tris, 0.14 M NaCl, 1% BSA, pH 8 for 30 min. Goat antimouse IgG conjugated with HRP was used for the detection of mouse IgG at a

dilution of 1:10,000. Colour was developed by adding tetramethyl benzidine substrate for 10 min. The reaction was stopped by adding 100µl of 2 M H_2SO_4. The intensity of the colour developed was measured by an ELISA plate reader at 450 nm. Antigen specific IgE was determined by coating the plate with OVA (10 µg/ml) in 0.05 M carbonate bicarbonate buffer, pH 9.5, and incubated at 4°C overnight. After incubation, the wells were washed with PBST and blocked with 200µl blocking reagent (50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8) for 30 min. After three washes with wash buffer, 100 µl analyte was added and incubated for 2 hrs at room temperature. IgE antibody in the analyte was detected with the help of goat antimouse IgE antibody conjugated with HRP. Development of colour and stopping of enzyme substrate reaction were performed in the same way as total IgG estimation. IL-4,IL-5,IL-13, TGF-β and IFN-γ proteins in BALF were evaluated by solid phase sandwich ELISA using ELISA kits according to the manufacturer's instructions (R& D System, Minneapolis, USA).

RNA Isolation and quantification

Total RNA was isolated from liver, lung, tracheal tissues and BALF cells using RNeasy mini kit as per the manufacturer's instructions (RNeasy mini kit; Qiagen, Hilden, Germany). The isolated RNA was quanitfied with the help of a UV spectrophotometer at 260 nm (A_{260}). Deionized water served as a blank. The difference of the absorbance of the sample and the blank was calculated and the concentration of total RNA was determined using the relationship between absorbance and concentration (1OD at A_{260} = 40 µg/ml RNA).

One Step RT-PCR

The RT-PCR was carried out using 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 45s, annealing at a 5°C below the Tm of primers for 45s and extension of 1 min at 72°C. The reaction was stopped after a final 10 min extension at 72°C. 1µl of template RNA containing 50 ng of total RNA was used. RT-PCR products were run on 1.2% agarose gel containing ethidium bromide, viewed and analysed on Gel documentation system using Gene snap and gene tool software for image acquisition and gel analysis, respectively (SynGene, Cambridge, UK). Molecular weight markers (0.5 µg) containing a defined quantity of each band was loaded and electrophoresed simultaneously. The SynGene analysis tool was used to extrapolate the cDNA quantity from the input quantity of standard molecular weight markers. Primers (Operon Biotechnologies Gmbh, Germany) used for RT-PCR were:

Stat3
5'GAAGACCAAGTTCATCTGTGTG-3' (forward)
5'GTAGCACACTCCGAGGTCAGAT-3' (reverse)
β- actin
5'GACATGGAGAAGATCTGGCAC3' (forward)
5'TCCAGACGCAGGATGGCGTGA3' (reverse)
IL-5
5'-AAGGATGCTTCTGCACTTGA-3' (forward)

5'-TATC TCTCTGAAGGACTCTGG-3' (reverse)
IL-4
5'-GACAAAAATCACTTGAGAGAGA-3' (forward)
5'-ACGAGTAATCCATTTGCATGAT-3' (reverse)
β-actin
5'-GACATGGAGAAGATCTGGC AC-3' (forward)
5'-TCCAGACGCAGGATGGCGTGA-3' (reverse)
IL-13
5'- TGGCTCTTGCTTGCCTTGG -3' (forward)
5'- GTGATGTTGCTCAGCTCCTCAAT-3' (reverse)
TGF-β
5' CCCCACTGATACGCCTGAGT 3' (forward)
5' AGCAGTGAGCGCTGAATCG 3' (reverse)
IFN-γ
5' TCTGGATCCATGAACGCTACACACTG 3' (forward)
5' CACCTCGAGTCAGCAGCGAC 3' (reverse)

Cytokines expression in Asthmatic mice

Total RNA was isolated from lung, trachea and BALF cells using RNeasy mini kit as per the manufacturer's instruction (Qiagen, Hilden, Germany). RT PCR was carried out using a QuantiTect SYBR green 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 40 cycles of denaturation at 94°C for 15 s, annealing at a 5°C below the Tm of primers for 30 s and extension of 30 s at 72°C. Melting curve analysis (not shown) was performed to verify the specificity and identity of the RT-PCR products. One micro litre of template RNA containing 50 ng of total RNA was used in a 50µL Real Time PCR cocktail. All reactions were performed in triplicates. Relative quantifications were performed in Light Cycler 480 calibrator normalised relative quantification assay in which the target concentration is calculated relative to a non-regulated reference. The results are expressed as the target/reference ratio of each sample normalised by the target/reference ratio of the calibrator using Light Cycler 480 relative quantification software release 1.2.0625.

Histopathology

The tissues from mice after sacrifice were fixed immediately in 10% neutral buffered formal saline for 72 h. Fixation was followed by washing in running water for 6-8 hrs .Fixed tissues were dehydrated by graded series of alcohol, cleared in xylene and embedded in paraffin wax. Paraffin blocks were sectioned in microtome to generate 4-5µm thick sections and collected on egg albumin coated slides. After deparaffinization sections were stained by haematoxylene and eosin (Qualigens). Histopathological studies of the sections were carried out under light microscope (Nikon, Japan).

Silencing of Stat3 in mice

The mice were *in vivo* transfected with a set of three different Stat3 duplexes (Santa Cruz Biotech, California). These Stat3 siRNA duplexes were given to mice by complexing them with 5% glucose solution in ExGen 500 (*in vivo* transfection reagent Fermentas Life Sciences, Harrington, Ont., Canada) as per the methodology of the manufacturer. ExGen 500, polyethlenimine belongs to a new category of non viral non liposal gene delivery reagents. It has better *in vivo* transfection efficiency as compared to other cationic lipids and polymers. Stat 3 siRNA of mouse is a pool

of three different strands of siRNA. The sense strand sequences of the Stat3 siRNA (Santa Cruz Biotech, USA) were:

5'GAGUGCAGGAUCUAGAACAtt3',
5'CAGGACGACUUUGAUUUCAtt3',
5'GAAGACACUGACUGAUGAAtt3'

and the scrambled siRNA sequence was 5' UUCUCCGAACGUGUCACGUdtdt 3' (Qiagen, Germany). The scrambled siRNA duplex was used as the negative control.

In vivo transfection was achieved by diluting 10 µg of siRNA in 50 µl of sterile 5% glucose solution and then mixed thoroughly by vortexing. About 1.8 µl of ExGen solution was diluted in 50 µl of sterile 5% glucose solution and vortexed. After that, 50 µl of diluted ExGen solution was added to 50 µl of diluted siRNA solution. After appropriate mixing the mixture was incubated at room temperature for 10 min. This siRNA mixture was administered in mice through intranasal route. Different concentrations of siRNA was administered to each mouse on day 26. Measurement of AHR, histopathology studies, RNA quantification and assessment of IgG & IgE levels were carried out on 28th day.

Statistical anlysis

Data shown represents mean values (± SD). Student's *t*-test with p- value <0.05 taken as indicator of statistical significance was used for comparing activities of different treatments.

RESULTS

Mouse model of asthma

Dose dependent increase in Mch-induced sRaw in *OVA/OVA* mice in comparison to *SAL/SAL* mice over a dose range of 2.5–40 mg/kg (Fig.1a). The moderate increase in Mch-induced sRaw in *OVA/OVA* mice suggests that the protocol adopted by us induced asthma symptoms in mice. Gross pathology of lungs from *OVA/OVA* mice revealed mild to mild patchy congestion. HE-stained lungs form *SAL/SAL* mice revealed normal histology (Fig.1b). Lungs from *OVA/OVA* mice depicted heavy infiltration of inflammatory cells into the alveoli, congested blood capillaries. Alveolar septa were thickened. Peribronchiolar lymphoid proliferation was present. Few alveoli depicted emphysema and majority of the alveoli were collapsed along with fibrous tissue was evident (Fig.1c). Major conducting airway i.e. trachea from *SAL/SA*L mice (Fig.1d) showed normal histology. Trachea from *OVA/OVA* mice revealed submucosal infiltration of inflammatory cells (Fig.1e). These changes were not seen in *SAL/SAL* mice. In another group of *OVA/OVA* mice (n = 6), 2 h after the last OVA exposure, significant increase in total as well as OVA-specific IgE antibodies in BALF and sera (Table1), increased levels of in BALF were observed, while *SAL/SAL* mice were negative for OVA-specific IgE and IgG antibodies. A significant elevation in the eosinophil counts in blood was also observed in *OVA/OVA* mice. (Fig.1f) 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.

Table 1: Total and OVA-specific IgG and IgE in normal and asthmatic mice.

Immunoglobulins	SAL/SAL	OVA/OVA
Total serum IgG, mg/ml	3.31 ±1.89	12.83 ± 1.22*
OVA-specific serum IgG, mg/ml	2.86 ±1.03	8.86 ± 2.57*
OVA-specific BALF IgG, µg/ml	ND	170.56 ± 12.43
Total serum IgE, µg/ml	5.87 ± 3.41	35.58 ± 5.73*
OVA-specific serum IgE, µg/ml	2.07 ± 0.50	27.35 ± 3.18*
OVA-specific BALF IgE, µg/ml	ND	11.93 ± 2.86
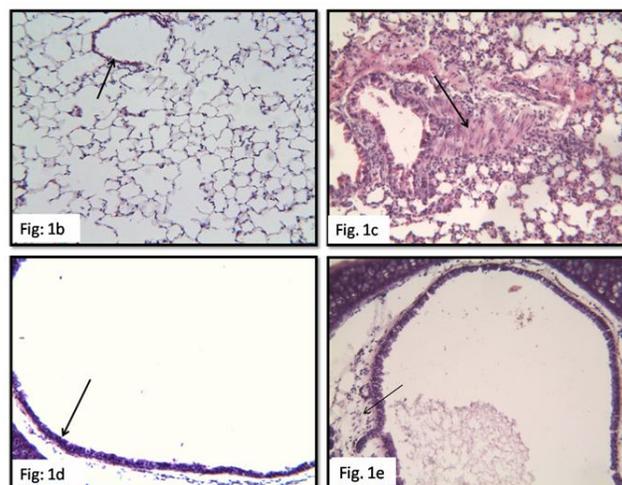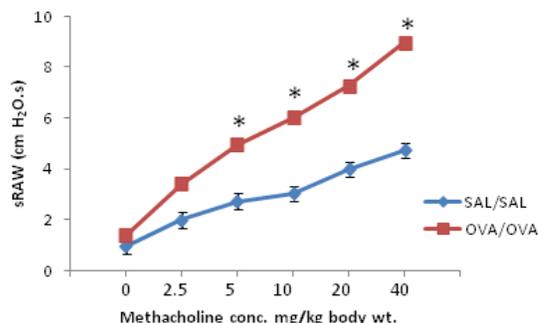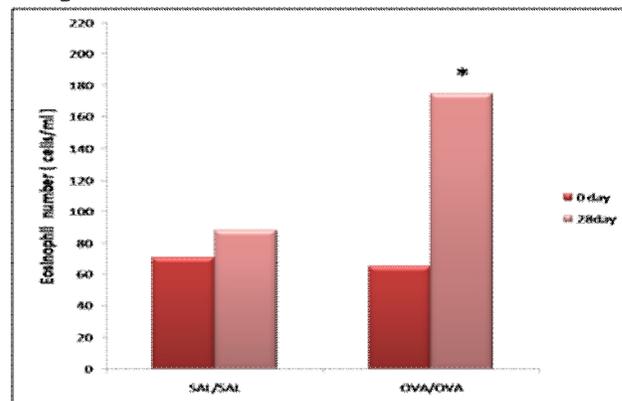
Fig. 1 Validation of asthma model **1a** Comparison of sRaw between *SAL/SAL* mice and *OVA/OVA* mice (mean ± SD,*P<0.05), **1b** Normal histology of lung, **1c** Asthmatic lung showing fibrous tissue(arrow), thickened alveolar septa, **1d** Trachea depicting normal histology, **1e** Trachea from asthmatic mice showing submucosal inflammatory cells, H & E,125 X and **1f** *OVA/OVA* mice showed significant (*P<0.05, N=6) increase in eosinophil counts in comparison to *SAL/SAL* group.

Increased expression of different cytokines in Asthmatic mice

Increased mRNA expression of IL-4, IL-5, IL-13 and TGF-β were observed in BALF, trachea and lung of asthmatic airways while IFN- γ mRNA expression was lowered in all these tissues of asthmatic airways (Fig. 2). Protein levels of IL-4, IL-5, IL-13 and TGF-β were found elevated while IFN- γ was found downregulated in BALF, trachea and lung of *OVA/OVA* mice as compared to *SAL/SAL* mice (Table 2). Thus the inflammatory cytokine was found downregulated in asthmatic mice.

Table 2: Cytokine levels (pg/ml) in BALF of *SAL/SAL* and *OVA/OVA* mice. Significant increase in IL-4,IL-5,IL-13,TGF- β among *OVA/OVA* mice on 28th day.

Cytokine	*SAL/SAL*	*OVA/OVA*
IL-4	28.45 ±16.7	105.34 ± 30.8*
IL-5	23.86 ± 13.9	70.63 ± 20.4*
IL-13	15.53 ± 7.3	83.63 ± 10.21*
TGF-β	06.31 ± 2.7	90.67 ± 8.34*
IFN- γ	10.43 ± 4.1	8.23 ± 3.31

Fig:2
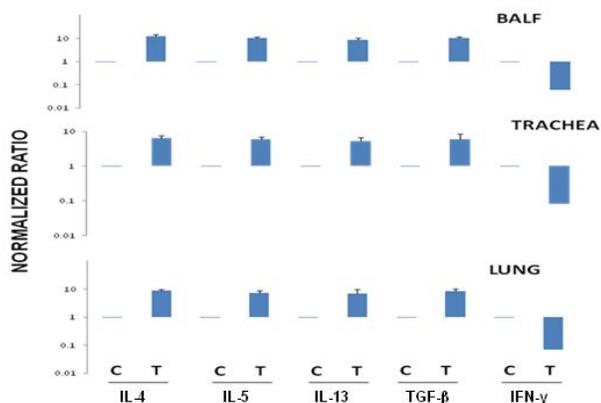
Fig. 2 Graphical representation of quantitative mRNA expression profile of various cytokines in *OVA/OVA* mice. Expression profiles (mean ± SE; n = 6) were graphically presented in terms of normalized ratio of qRT^2-PCR data. Expression data from *OVA/OVA* mice were normalized against corresponding *SAL/SAL* mice.

Low stat3 level in *OVA/OVA* mice

In our previous study we observed the low expression of Stat3 mRNA in lung, trachea and BALF of *OVA/OVA* mice on day 26 after OVA exposure in comparison to *SAL/SAL* mice indicating downregulation of Stat3 mRNA expression in mice sensitized and challenged with OVA. 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. 3a) (Paul *et al.*, 2009). Tyrosine phosphorylated Stat3 is known to dimerize and subsequently translocate to the nucleus where it activates various transcription factors (Vinkemeier., 2004). 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 about 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. In contrast, the Stat3 peptide in lung nuclear extracts of OVA-exposed *OVA/OVA* mice was found to be close to the basal level at all the time points 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. 3b), indicating that the extra-pulmonary organs, especially the liver, are not affected in mice with inflammatory and allergic airways (Paul *et al.*, 2009).

Fig:3a
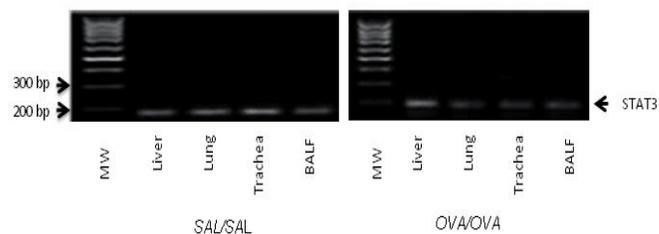
Fig:3b
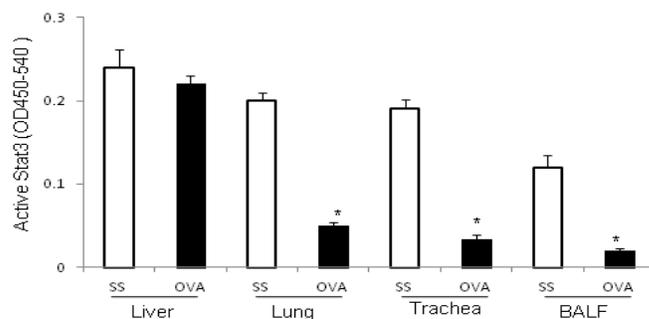
Fig. 3 Stat3 mRNA expression profile 3a in different tissues of *SAL/SAL* and *OVA/OVA* mice and 3b active Stat3 peptide (mean ± SD; n = 6) in liver, lung, trachea and BALF cell of *SAL/SAL* (SS) and *OVA/OVA* (OVA) mice. *p<0.05, statistically significant compared to *SAL/SAL* control.

Effect of Stat3 silencing on airway structure of *OVA/OVA* Mice

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 μg of Stat3 siRNA blocked more than 75% Stat3 mRNA expression in the lungs of transfected *SAL/SAL* mice (Paul *et al.*, 2009). 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 (Takeda., 1997) cell mortality. Necropsy examination of all the animals from *OVA/OVA* +SS and *OVA/OVA* + Stat3 silenced mice showed emphysematous lungs and patchy consolidation. Similar presentation of lungs was evident from Stat3 silenced normal mice which are subsequently exposed to OVA and Scramble sequence transfected normal mice which are subsequently exposed to OVA. Histopathological examination of lungs from *OVA/OVA* +scramble

sequence transfected mice depicted emphysematous alveoli, thickened alveoli and fibrous tissue proliferation (Fig.4a). Interestingly similar histopathological observations were noticed in *OVA/OVA* + stat3 siRNA transfected mice (Fig.4b). Trachea from *OVA/OVA* +Scramble sequence transfected mice (Fig.4c) and *OVA/OVA* + Stat3 siRNA transfected mice depicted submucosal infiltrations of inflammatory cells (Fig.4d). Interestingly Silencing Stat3 in *OVA/OVA* mice did not alter lung function in terms of sRaw in comparison to scramble sequence transfected *OVA/OVA* mice (Fig.4e).

Fig:4a Fig:4b Fig:4c Fig:4d

Fig:4e

Fig:4f
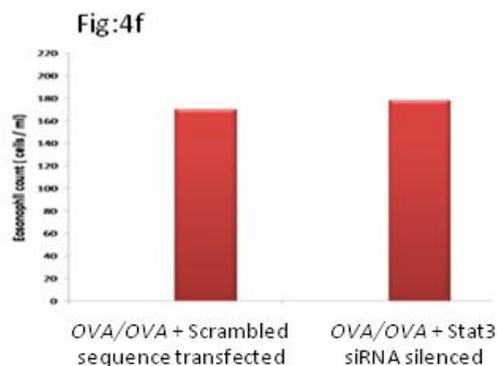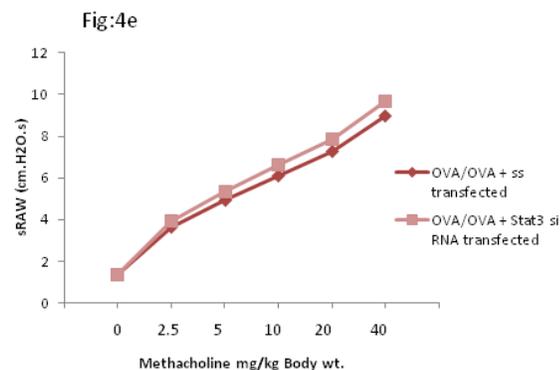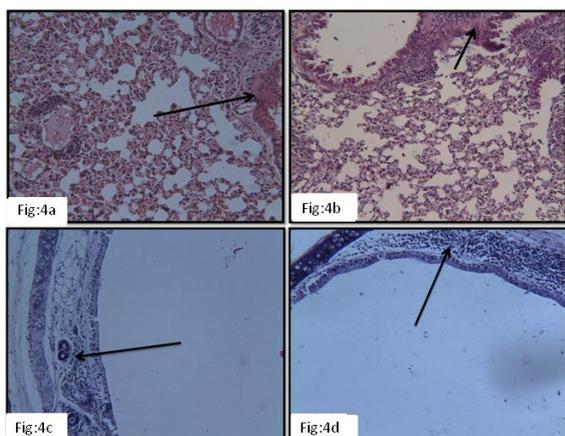
Fig. 4 Airway structure after Stat3 silencing, **4a** Lungs from *OVA/OVA* +scramble sequence transfected mice depicting emphysematous alveoli, thickened alveoli and fibrous tissue proliferation (arrow), **4b** Lungs from *OVA/OVA* +Sta3 siRNA transfected mice depicting emphysematous alveoli, thickened alveoli and fibrous tissue proliferation (arrow), **4c** Trachea from *OVA/OVA* +scramble sequence depicting submucosal infiltrations of inflammatory cells (arrow), **4d** Trachea from *OVA/OVA* +Stat3 transfected depicting submucosal infiltrations of inflammatory cells (arrow) and **4e** Effect of Stat3 siRNA on lung function in *OVA/OVA* +Stat3 siRNA transfected mice. Lung function was measured in terms of methacholine dependent sRaw **4f** Effect of Stat3 siRNA on eosinophil count in *OVA/OVA* mice (mean ± SE; n = 3).

Effect of silencing Stat3 on cytokine profile

Transfecting Stat3 siRNA on day 26 in *OVA/OVA* mice and investigation on 28th day showed more increase in the level of IL-4, IL-5, IL-13 and TGF-β.Among them TGF- β levels were more higher.Intrestingly TFN-γ revealed down-regulation as compared to other cytokines the airways and BALF of asthmatic mice (Fig.5 and Table 3).
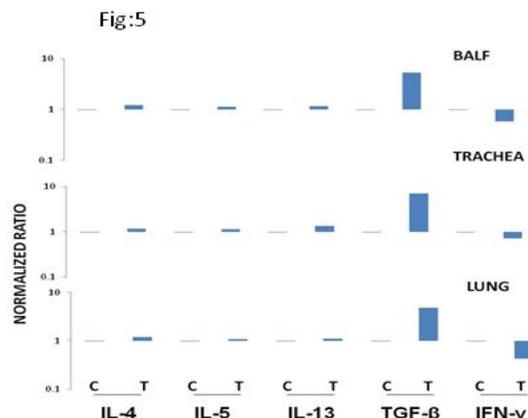
Fig:5

Fig. 5 mRNA expression profile of various cytokine in *OVA/OVA* + Stat3 siRNA transfected mice (mean ± SE; n = 3) normalized against corresponding *OVA/OVA* + scramble sequence transfected mice.

Table 3: Cytokines values represent mean of three mice per group. Mild increase in *OVA/OVA* +Stat3 siRNA transfected group in comparison with *OVA/OVA* + scramble sequence transfected mice except TGF- β (which showed exaggerated increase).

Cytokine	*OVA/OVA* +SS transfected	*OVA/OVA* + Stat3 siRNA transfected
IL-4	110.54	125.53
IL-5	68.62	74.62
IL-13	84.36	92.37
TGF-β	83.28	288.67
IFN- γ	0.39	0.33

Table 4: Total and OVA-specific IgG and IgE values in Stat3 silenced mice.

Immunoglobulins	*SAL/SAL+SS transfected*	*OVA/OVA+ Stat3 transfected*
Total serum IgG, mg/ml	14.83	16.31
OVA-specific serum IgG, mg/ml	10.86	12.21
OVA-specific BALF IgG, μg/ml	175.56	187.32
Total serum IgE, μg/ml	40.58	48.28
OVA-specific serum IgE, μg/ml	52.35	57.12
OVA-specific BALF IgE, μg/ml	14.93	18.13

Effect of Stat3 silencing on lung function, immunoglobulins and blood eosinophils

Transfecting Stat3 among *OVA/OVA* mice showed slight increase in the values of sRAW (fig.4e), imunoglobulins (Table 4) and blood eosinophils (Fig.4f) as compared to scramble sequence transfected *OVA/OVA* mice. It depicts that silencing Sat3 does not

alter clinical parameters of asthma rather it starts further elevation of inflammatory parameters.

DISCUSSION

In our experiment heightened expression of IL-4, IL-5, IL-13 and TGF-β and basal expression in case of IFN-γ among asthmatic mice was observed. Transforming growth factor-β (TGF-β) is an important fibrogenic and immunomodulatory factor that may also play a role in the structural changes observed in the asthmatic airways (Duvernelle *et al.*,2003).We suppose that probable tissue damage in the background of asthma is important factor in the entire remodeling process. IL-4, IL-5, and IL-13, are derived from T helper type 2 (Th2) cells, although they may also be derived from other cell types (Proudfoot *et al.,* 1993, Minty *et al.,* 1993). Th2 cells are recognised by their secretion of IL-4, IL-5, and IL-13, as opposed to Th1 cells, which secrete IL-2 and interferon-γ (Barnes, 2001), although a clear distinction between Th1 and Th2 cells is not as distinct in humans as in mice. Our study pertaining to IgG and IgE levels in *OVA/OVA* mice hints about the correlation of these markers in the asthma. We have earlier reported low expression of Stat3 gene in *OVA/OVA* (asthmatic) mice (Paul *et al.*, 2009). Tyrosine phosphorylated Stat3 is known to dimerize and subsequently translocate to the nucleus where it activates various transcription factors (Bromberg *et al.*, 1999). Thus down regulation of Stat3 among asthmatic mice might be the reason for other pro-inflammatory changes in airway histology. In this study we investigated the role of Stat3 siRNA in restoring airway histology in asthma. In the present study we tried to knockdown of Stat3 by antisense technology to check whether further knockdown of Stat3 shows any beneficial change in restoring the airway structure. Our results revealed that further down regulation of Stat3 in asthmatic mice aggravated AHR, immunoglobulins and blood eosinophils and cytokine profiles along with no restoration in airway histology. Stat3 in macrophages may play a negative role in inflammation (Takeda *et al.*,1999) and tissue-specific disruption of Stat3 during hematopoiesis is associated with a lethal inflammatory bowel syndrome (Welte *et al.*,2003), 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. Thus it appears that functionally Stat3 is an anti-inflammatory molecule and its depletion did not restore normal lung physiology and structure. It appears from our investigation that more studies need to be directed at the cellular, molecular and genetic levels to know and understand the negative association of Stat3 in asthma pathogenesis. Our study indicates that the varying expression levels of Stat3, cytokines can serve as molecular markers for devising new therapeutics for asthma.

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