

Titanium dioxide nanoparticles augment allergic airway inflammation and Socs3 expression via NF- κ B pathway in murine model of asthma



Vani Mishra ^{a,*}, Vikas Baranwal ^a, Rohit K. Mishra ^{b,**}, Shivesh Sharma ^b, Bholanath Paul ^c, Avinash C. Pandey ^a

^a Nanotechnology Application Centre, University of Allahabad, Allahabad 211002, India

^b Centre for Medical Diagnostic and Research, Motilal Nehru National Institute of Technology, Allahabad 211004, India

^c Immunobiology Division, Indian Institute of Toxicology Research, Lucknow 226001, India

ARTICLE INFO

Article history:

Received 19 August 2015

Received in revised form

5 March 2016

Accepted 10 March 2016

Available online 19 March 2016

Keywords:

nTiO₂

Asthma

NF- κ B

Socs3

Th2-type immune response

ABSTRACT

Titanium dioxide nanoparticles (nTiO₂) previously considered to possess relatively low toxicity both *in vitro* and *in vivo*, although classified as possibly carcinogenic to humans. Also, their adjuvant potential has been reported to promote allergic sensitization and modulate immune responses. Previously, in OVA induced mouse model of asthma we found high expression of Socs3 and low expression of Stat3 and IL-6. However, a clear understanding regarding the signaling pathways associated with nTiO₂ adjuvant effect in mouse model of asthma is lacking. In the present study we investigated the status of Stat3/IL-6 and Socs3 and their relationship with NF- κ B, with nTiO₂ as an adjuvant in mouse model of asthma. nTiO₂ when administered with ovalbumin (OVA) during sensitization phase augmented airway hyper-responsiveness (AHR), biochemical markers of lung damage and a mixed Th2/Th1 dependent immune response. At the same time, we observed significant elevation in the levels of Stat3, Socs3, NF- κ B, IL-6 and TNF- α . Furthermore, transient *in vivo* blocking of NF- κ B by NF- κ B p65 siRNA, downregulated the expression of Socs3, IL-6 and TNF- α . Our study, thus, shows that nTiO₂ exacerbate the inflammatory responses in lungs of pre-sensitized allergic individuals and that these changes are regulated via NF- κ B pathway.

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1. Introduction

Titanium dioxide (TiO₂) and its derivatives in nanoscale forms are abundantly used in number of applications including semiconductors, solar cells, photocatalyst belts, medicine as well as consumer products like sunscreen, paints and food supplements [1–3]. For long, nTiO₂ have been shown to possess relatively low toxicity as compared to other biopersistent inorganic materials and is therefore used as reference or negative control [4–7]. However, a two year inhalation study in rats resulted in lung tumors [8] and an epidemiological study in a 53 year old male, engaged in packing TiO₂ has been reported to suffer from papillary adenocarcinoma [9]. Thus based upon the recent assessment, IARC has classified TiO₂ as a human carcinogen group 2B [10] where the mode of action stands

to be lung overloading [11].

Growing evidences further indicate that ultrafine particles (<30 nm) when administered as an adjuvant in combination with Ovalbumin (OVA) as well as toluene-2,4-diisocyanate (TDI), can significantly induce pulmonary sensitization and local inflammation and modify immune responses in allergic airways [12–15]. On the contrary, Rossi et al. [16] reported recruitment of neutrophils accompanied with significant overexpression of chemokines in nTiO₂ exposed mice with inhibition in allergic pulmonary response in asthmatic mice.

Asthma is an allergic inflammatory disease of airways that is characterized by AHR, airway remodeling and eosinophil infiltration [17]. The inflammatory responses are then transmitted through a complex interplay between Th2 type cytokines, which further stimulate B cells to synthesize IgE and eosinophilic inflammation, while suppressing Th1 type immune response [18–22]. nTiO₂ have been shown to possess adjuvant effect that can prime Th2-dominant immune response with significant production of IgE, IL-4 and IL-5 in OVA-induced asthma model [20]. Taken

* Corresponding author.

** Corresponding author.

E-mail addresses: vanimish@gmail.com (V. Mishra), rohit_ernet@yahoo.co.in (R.K. Mishra).

together, the significance of Th2-driven allergic asthma and their cross talk with engineered nTiO₂ indicate an underlying cellular machinery affecting immune responses in allergic airways.

This study investigates the adjuvant effect of i.p. nTiO₂ in OVA induced mouse model of asthma. We also explored how nTiO₂ exposure to lungs modulated immune response, allergic pulmonary inflammation and AHR in OVA-induced asthma model. The expressions of key genes involved in airway inflammation were also assessed. We previously reported that airway inflammation is associated with low expression of Stat3 and IL-6 and over-expression of Socs3 [23]. nTiO₂ significantly increased NF- κ B expression in the nucleus indicating the role of NF- κ B signaling in dendritic cell maturation [24]. Several lines of evidence also indicate towards the crucial role of NF- κ B in OVA induced sensitization and challenge models in mice. These models have shown vigorous expression of NF- κ B and its rapid nuclear translocation as compared to control group [25,26]. Considering the involvement of different signaling cascades in allergic asthma and nanoparticle induced airway inflammation, it is relevant to examine the status of NF- κ B following nTiO₂ sensitization in asthmatic lungs and explore whether or not the elicitation of pro-inflammatory molecules is routed through the transcriptional activation of NF- κ B.

NF- κ B is a potent nuclear transcription factor that plays crucial role in immunity and inflammation [27–31] by regulating over 100 genes that are mainly pro-inflammatory [32]. The NF- κ B family consists of 5 members: p65/RelA, RelB, c-Rel, NF- κ B 1/p50, and NF- κ B/p52 [33]. In the resting conditions the NF- κ B dimers (predominantly p65 and p50 proteins) bind to the NF- κ B inhibitor (I κ B) proteins, specifically I κ B α and are retained in the cytosol in an inactive form. In order to achieve an active state, I κ B proteins must be phosphorylated by I κ B kinase (IKKs), which leads to ubiquitination and degradation of I κ B. Degradation of I κ B exposes the nuclear localization signal and facilitates the dimer to translocate to nucleus [33,34]. Once in the nucleus, the phosphorylated NF- κ B dimers bind to DNA with a consensus sequence to transcriptionally induce genes involved in inflammation such as IL-8, TNF- α [35,36], IL-6 [37] and Socs3 [38]. Numerous studies individually revealed the role of TLRs in nTiO₂ uptake resulting in elicitation of inflammatory responses by NF- κ B [39,40], however, the status of Stat3, Socs3 and IL-6 has not been studied in response to nTiO₂ sensitization in allergic airways. Therefore, it would be interesting to evaluate their expression profiles individually as well as in conjugation with NF- κ B in nTiO₂ sensitized lungs during development of asthma.

Our studies show that pulmonary nTiO₂ exposure results in a mixed Th2/Th1 inflammatory response and significant increase in AHR. With an increase in Th2 type cytokines, Stat3, Socs3 and NF- κ B were also elevated when mouse airways were sensitized with nTiO₂ along with OVA. Blocking NF- κ B gene expression resulted in downregulation of Socs3, IL-6 and TNF- α , indicating that inflammatory responses elicited upon nTiO₂ exposure are routed through NF- κ B pathway rather than Jak/Stat pathway, a classical route regulating asthma pathophysiology.

2. Materials and methods

2.1. Characterization of selected nanoparticles

nTiO₂ of anatase phase were purchased from PlasmaChem GmbH, Berlin. The size and shape was analyzed by transmission electron microscopy (JEM-2100HR-TEM, JEOL, Japan). The structural properties of the nTiO₂ were analyzed with XPERT-Pro X-ray diffractometer system, using CuK α radiation (λ = 0.15406 nm) in the wide angle region from 20° to 70 °C. The crystallite size was calculated using the Debye-Scherrer's equation [41]. The

hydrodynamic size and the polydispersity index (PI) [42] of the particles were measured by Differential Light Scattering also referred to as photon correlation spectroscopy (Nano ZS, Malvern Instruments Ltd., UK). The mean hydrodynamic diameter (Z-average mean) was calculated from the autocorrelation function of the intensity of light scattered from the particles (DTS Nano version 5.10; Malvern Instruments Ltd.). Compositions of the sample were determined by Energy Dispersive X-Ray Spectroscopy (EDS) (Oxford Instrument, USA) equipped with a Field emission scanning electron microscopy (FE-SEM) instrument (Zeiss ultra FE-SEM). The surface area, average pores diameter and pores volume of nTiO₂ were calculated using BET method (Quantachrome Instrument, USA) according to nitrogen adsorption after degassing at 200 °C for 1.5 h. The thermogravimetric analysis (TGA) of the nTiO₂ has been performed on TGA/DSC Analyzer (Perkin Elmer, USA). The measurement was performed in the range of room temperature to 800 °C in a step of 10 °C per minute. Three main regions of TGA curve of nTiO₂ sample according to heat profile were observed.

2.2. Animal procurement and experimental design

Eight to ten week-old Balb/c mice were procured and kept under standard animal husbandry conditions approved by institutional committee for animal care; temperature 24 $\pm$ 2 °C, relative humidity 50–55%, 12 h light and dark cycle. Following 1 week of acclimatization, mice were randomized into 4 groups (5 mice/group) to proceed with the dose administration. For preparing murine model of asthma (OVA/OVA), the protocol previously established was used [23]. Briefly, mice were sensitized to 100 μ g Ovalbumin (OVA; grade V; Aldrich-Sigma Chemical) emulsified in 2% alum solution by intraperitoneal (i.p.) injection on days 0 and 14. The OVA challenges performed on days 21, 22, 23 and 26 through nasal route. A total of 50 μ g OVA in sterile saline was applied on the snout or the nostril in 5 cycles. Fig. 1 is the detailed schematic representation of the experimental groups. Additionally, another group (OVA/TI) was sensitized with OVA in combination with 200 μ g (8 mg kg^{−1} body weight) nTiO₂ through i.p. injection on day 0. The mice were subsequently challenged by applying OVA only through nasal route. The third experimental group (OVA/TI/c) was sensitized through OVA emulsified in 2% alum solution and received nTiO₂ (100 μ g/mouse) only during the challenge phase. Control groups included animals treated with Saline only (SAL/SAL). The animals were sacrificed on day 28, after last cycle of challenge by cervical dislocation. Before sacrificing the animals, lung function was monitored in terms of Methacholine-induced sRAW by plethysmograph followed by collection of bronchoalveolar lavage fluid (BALF) from airways. Post-killing lungs and trachea were dissected out for RNA and protein isolation for the estimation of cytokines and signal molecules. A set of isolated lungs from each group were fixed in formalin for 7 days. The tissue processing involved thin sectioning of paraffin embedded lungs which were further mounted on microscopic slides and stained through hematoxylin-eosin and visualized under light microscope.

2.3. AHR evaluation

Changes in lung function were measured in terms of methacholine (Mch)-induced airway hyper-reactivity using a double-chambered whole-body plethysmograph (Buxco, USA). After a brief period of acclimatization, initial baseline measurement was performed in response to saline. Different doses of Mch (2.5, 5, 10, 20, 40 mg/ml) were nebulized for 2 min and pressure changes in the chamber were monitored. Specific resistance of airways (sRAW) was recorded using BioSystem XA, version 3.1 (Buxco, USA).

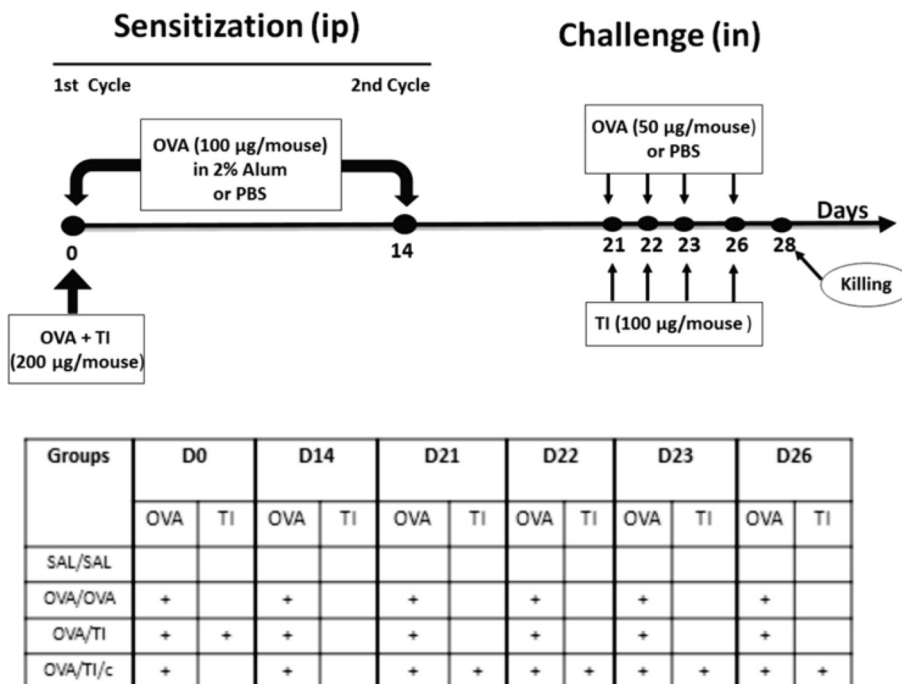


Fig. 1. Schematic representation of experimental design. The experimental setup has been divided in sensitization phase (2 cycles) followed by challenge phase (4 cycles). On day 0, Balb/c mice were sensitized with OVA alone or in conjugation with nTiO₂ by intra-peritoneal injection (i.p.) as 1st cycle. The 2nd cycle of sensitization on day 14 comprised only of OVA administration by i.p. PBS was administered similarly for control group. Further the challenge phase on day 21, 22, 23 and 26 comprised of intra-nasal (i.n.) treatment of either PBS or OVA or nTiO₂. Mice were sacrificed on day 28, i.e., after 48 h of last challenge. The table briefly summarizes the details of the experimental groups. The treatment groups include: OVA/OVA (Two cycles of OVA sensitization; OVA challenge); OVA/TI (1st cycle of OVA + nTiO₂ sensitization; 2nd cycle of OVA sensitization; OVA challenge); OVA/TI/c (Two cycles of OVA sensitization; OVA + nTiO₂ challenge). Except for the SAL/SAL group, the mice were sensitized with OVA emulsified in 2% alum solution by i.p. injection. The control group SAL/SAL included only PBS treatment.

2.4. Collection of BALF and evaluation of biochemical markers, immunoglobulins and cytokines

For collection of BALF, mice were anesthetized with sodium thiopentone, i.p. 5 mg/0.1 ml/mouse at 2 h after the last OVA challenge. 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 was centrifuged at 2000 rpm, for 5 min at 4 °C. The supernatants were subjected to biochemical assays, immunoglobulin and cytokine analysis and pellet for RNA isolation. The biochemical parameters estimated in BALF supernatant included Lactate Dehydrogenase (LDH), Alkaline Phosphatase (ALP) and Total protein as per the manufacturer's instructions. Levels of Total and OVA-specific IgE, IgG1 and IgG2a were also determined in BALF supernatant using commercially available kits according to the manufacturer's instructions (Bethyl Laboratories, Inc., Montgomery, Tex., USA). Levels of IL-4, IL-5, IL-6, IL-13 and TNF- α were assayed using ELISA kits (R&D System, Minneapolis, Minn., USA).

2.5. Isolation of RNA and mRNA quantification

Total cellular RNA was isolated using the Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The mRNA expression profile was evaluated by quantitative reverse transcription polymerase chain by using the QuantiTect SYBR green Real Time-PCR kit (Qiagen). 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 T_m 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.

Relative quantifications were performed in a Light Cycler Real-Time PCR Machine (Roche Diagnostics GmbH, Mannheim, Germany) using the Light Cycler 480 relative quantification software (version 1.2.0625). The relative gene expression was calculated as follows: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{Actin}}$ [43]. Primers (Imperial Life Science, Bangalore, India) used were: Stat3 forward 5'-GAAGACCAAGTTTCATCATCTGTGTG-3' reverse 5'-GTAGCACTCCGAG GTCAGAT-3'; Socs3 forward 5'-TGAGCGTCAA-GACCCAGTCG-3' reverse 5'-CACAGTCGAAGGCGGGAAGT-3'; NF- κ B forward 5'-TTCACCTTTCCAGACGCCCT-3' reverse 5'-ATTGTCTCTTACCGTCCCG-3'; IL-6 forward 5'-TTGCCTTCTTGGGACTGATGCT-3' reverse 5'-GTATCTCTCTGAAG-GACTCTGG-3'; IL-5 forward 5'-AAGGATGCT TCTGCATTGA-3' reverse 5'-TATCTCTCTGAAGGACTCTGG-3'; IL-4 forward 5'-GACAAAAATCACTTGAGAGAGA-3' reverse 5'-ACGAGTAATC-CATTTGCATGAT-3'; TNF- α forward 5'-CCAGACCCTCACACTCCAGAT-3' reverse 5'-AACACCCATTGCTTCACAG-3'; IL-1 β forward 5'-TGACGGACCCAAAAGATGAAG-3' reverse 5'-CTGCTTGTGAGGTGCTGATGTA-3'; β -actin forward 5'-GACATGGAGAAGATCTGGAC-3' reverse 5'-TCCAGACGCAGG ATGGCGTGA-3'.

2.6. Immunoblot analysis of protein

The nuclear and cytosolic fraction of lung tissues was prepared using Qproteome Cell Compartment kit (Qiagen). Briefly, 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 μ L extraction buffer supplemented with protease inhibitors. After disruption, clumps of tissues were removed by centrifuging the tissue suspension in Qiasredder at 500 $\times$ g for 2 min at 4 °C. The pellets were resuspended and 500 μ L extraction

buffer added before incubation at 4 °C for 10 min in a shaker. After incubation, lysates were centrifuged at 4000× 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. Blocking was performed with 2% bovine serum albumin in TBST (137 mmol/L NaCl, 25 mmol/L tris, 1 mmol/L Na₂ EDTA and 0.1% Tween 20) and the membranes were further incubated with rabbit polyclonal antibodies Stat3 (sc-7179), Socs3 (sc-9023), p65 subunit of NF-κB (sc-109), IκBα (sc-203) and β-actin (sc-81178) (Santa Cruz Biotech, California, USA) against different proteins at 4 °C overnight at a dilution of 1:200. After washing 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 peroxidase substrate (Sigma, St. Louis, USA). Blots were visualized using Gel Documentation System (Syngene, UK).

2.7. *In vivo* inhibition of NF-κB gene expression by siRNA transfection

A pool of 4 NF-κB p65 siRNA duplexes of 19–25 nts (sc-29411; SantaCruz Biotech, Calif., USA) mixed 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, mice were killed by cervical dislocation and lungs were dissected out for RNA and protein isolation and subsequent analysis by qRT-PCR and Western blot.

2.8. Statistical analysis

The results are presented as the standard error of mean (SEM). A two-way analysis of variance (ANOVA) was used to determine significant ($p < 0.05$) differences between groups.

3. Results

3.1. Characterization of selected nanoparticles

TEM analysis revealed that the particle sizes of nTiO₂ are ranging from ~4 to 8 nm range (Fig. 2A). The DLS analysis of the particles suspended in water showed two peaks. nTiO₂ particles with a hydrodynamic diameter of ~9.7 nm were detected next to the peak showing particle agglomerates with a mean hydrodynamic diameter of 244 nm (Fig. 2B) with an average size of 171.4 nm with a polydispersity index of 0.463. On a number basis, <0.01% of the particles were agglomerates or aggregates and rest of the particles had a mean hydrodynamic diameter in a range of ~3.47–7.27 nm (Fig. S1). The EDS spectrum (full scan mode) of nTiO₂ revealed in Fig. 2C,D confirms the purity of titanium metal. The spectrum shows strong peak intensity of titanium metal at 4.6 keV (94 at.%) corresponds to purity of nTiO₂. The measured N₂-BET values obtained for specific area, size and volume, respectively were 130 m²/g, 2.23 nm, 0.072 cc/g which was comparable to the values obtained from the manufacturer. We can conclude that the majority of nTiO₂ particles exist as isolated primary particles in the suspension. The XRD pattern of nTiO₂ is represented in Fig. 2E. The peaks at 2θ value of 25.53, 37.87, 48.26, 54.68, 63.44° corresponds to anatase phase of TiO₂ (JCPDS file number 89-4921) and are in good agreement and can be indexed as anatase phase of nTiO₂. The crystallinity of the particles in colloidal form calculated as per the formula was found

to be ~10 nm that remains consistent with the manufacturer's data. TGA was performed to delineate the thermal stability of nTiO₂. The first stage from 30 to 130 °C is assigned to removal of water and about 19% weight loss is observed. The second region 130–365 °C is attributed to decomposition of the organic compounds completely with around 12% weight loss. The region 365 °C onwards is almost stable showing a stable anatase phase of the nTiO₂. There is almost no weight loss on TGA curve after 365 °C which indicates that decomposition of nTiO₂ has completed (Fig. 2F).

3.2. nTiO₂ administered during sensitization, but not during challenge phase induced AHR

AHR in OVA and nTiO₂ stimulated mouse models was evaluated by Mch-induced sRAW through double-chambered whole body plethysmography. Dose dependent increase in sRAW was observed in OVA sensitized and challenged (OVA/OVA) mice indicating moderate AHR in asthmatic mice. nTiO₂ administered during first cycle of OVA sensitization (OVA/TI) significantly increased sRAW (~2.5 folds) over Mch administration as compared to control (SAL/SAL) and OVA/OVA mice ($p < 0.05$) (Fig. 3A). Interestingly, nTiO₂ exposure during OVA challenge (OVA/TI/c) did not significantly elevate AHR as compared to OVA/OVA mice, suggesting that nTiO₂ when administered as an adjuvant during OVA sensitization rather than during challenge phase could modulate AHR in asthma. Microscopic details of OVA/OVA lungs stained with Hematoxylin and Eosin (Fig. 3C) revealed infiltration of inflammatory cells causing distention of alveolar spaces and disruption of bronchial epithelial cell bordering. Such changes were not observed in sham treated mice (Fig. 3B). The concurrent histological changes and Mch-induced increase in sRAW indicated pathophysiological state of asthma in OVA/OVA mice. Lungs of OVA/TI mice revealed severe vascular congestion and inflammation with alveoli filled with eosinophilic infiltrates; such major inflammatory responses, however were absent in OVA/TI/c mice (Fig. 3D,E). The prominent differences in the data obtained from OVA/TI and OVA/TI/c mice could be accredited to prior allergen sensitization before allergen challenge.

The biochemical parameters evaluated in terms of Total Protein, ALP and LDH, further indicated major lung injuries both in OVA/OVA and OVA/TI mice. Significant changes in all three parameters were observed in both OVA/OVA as well as in OVA/TI ($p < 0.05$). OVA/TI resulted in ~4 fold and ~1.6 fold increase in LDH activity as compared to SAL/SAL mice and OVA/OVA mice respectively (Fig. 4B). The other two parameters (ALP and Total Protein) also exhibited an increase in the range of ~2 fold as compared to SAL/SAL mice (Fig. 4A,C).

3.3. nTiO₂ sensitization augmented mixed Th-2/Th-1 dominant immune response in asthma pathophysiology

The levels of total and OVA-specific IgE, OVA specific IgG1 and IgG2a were assessed to study the effect of pulmonary nTiO₂ exposure on adaptive immunity to OVA sensitization. The levels of IgE were significantly elevated in sera of both OVA/OVA and OVA/TI groups (Fig. 5A,B). Significant elevation was also observed in the level of OVA-specific IgG1, a Th2-associated immunoglobulin in OVA/TI group as compared to OVA/OVA group (Fig. 5C); Similarly OVA-specific IgG2a, a Th1-associated immunoglobulin expression showed significant elevation in OVA/OVA and OVA/TI groups as compared to SAL/SAL group. These results, thus represent a mixed Th2/Th1 immune response in asthmatic airways over nTiO₂ exposure (Fig. 5D).

To further assess the role of Th2-dependent immune response in nTiO₂ sensitized mouse, we evaluated levels of IL-4, IL-5 and IL-13,

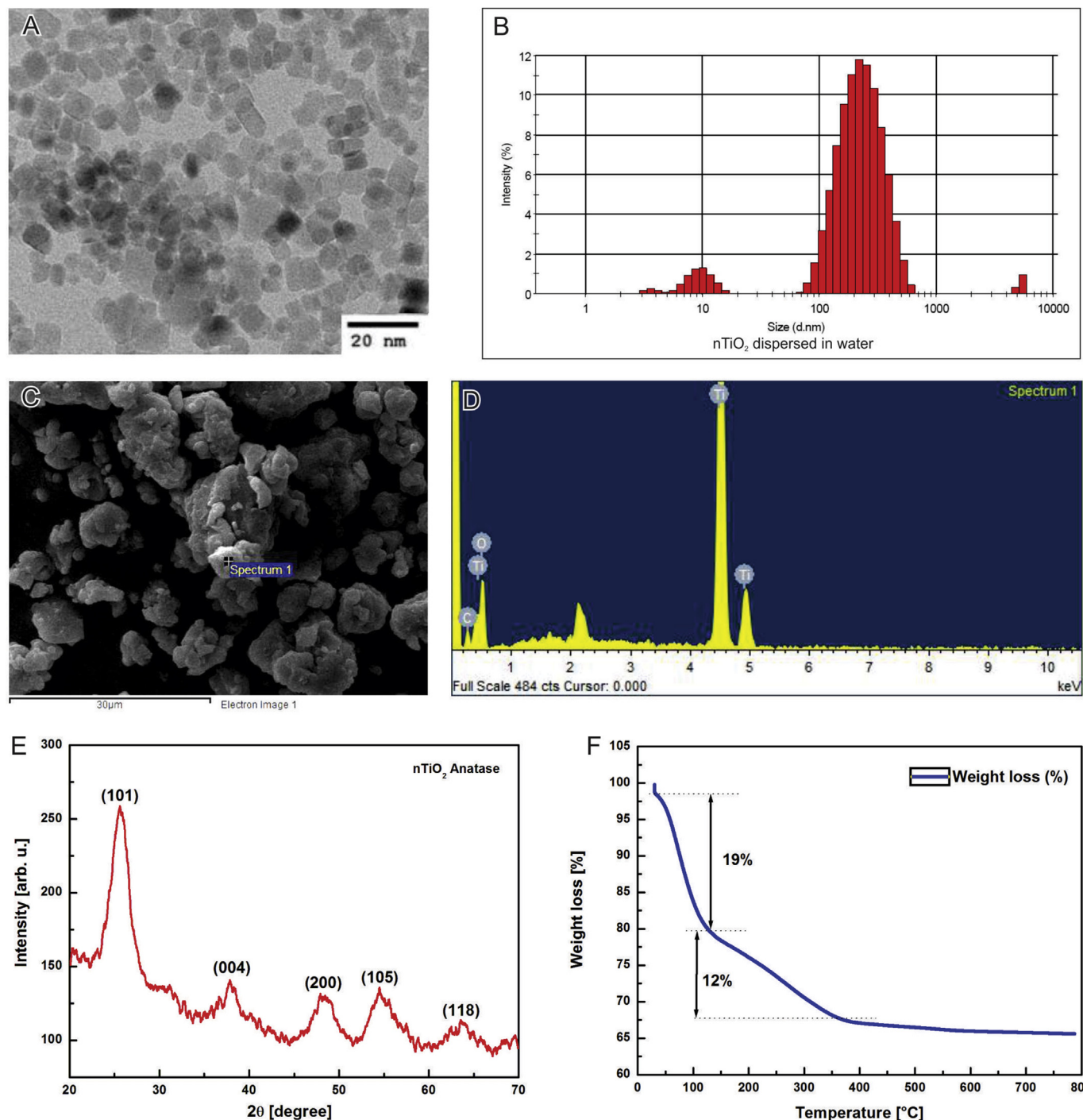


Fig. 2. Particle characteristics. (A) TEM image of nTiO₂; (B) Particle size distribution determined by DLS; (D) X-ray spectrum from the area marked in (C); (E) XRD pattern of nTiO₂ of anatase phase and (F) Thermal stability was determined by thermogravimetric analysis (TGA).

the signature cytokines of Th2 type cells through qRT²-PCR and ELISA. The relative mRNA expression of cytokine in lungs and BALF supernatant of OVA/OVA mice revealed significant increase in the levels of IL-4 and IL-13 (Fig. 6A). nTiO₂ sensitization in OVA/OVA mice further elevated the cytokine levels in both lungs and BALF. Protein expression profile was also in line with the gene expression. In OVA/OVA group IL-4 level peaked up to 126.65 ± 15.63 pg/ml, which further rose to 158.06 ± 26.14 pg/ml ($p < 0.05$) in OVA/TI group (Fig. 6B). IL-13 reached to 129.26 ± 26.51 pg/ml in OVA/TI

mice as compared to 81.26 ± 18.62 pg/ml in OVA/OVA mice ($p < 0.05$) (Fig. 6D). In the case of IL-5 similar mRNA and protein level (~ 105 pg/ml) was observed in both OVA/OVA and OVA/TI groups (Fig. 6A,C). IL-12, IL-1 α and IL-1 β , the Th-1 type cytokines were below the detection limit.

The pro-inflammatory cytokines IL-6 and TNF- α were also studied at mRNA and protein levels. TNF- α was persistently over-expressed in both OVA/OVA (34.83 ± 5.07 pg/ml) and OVA/TI groups (162.07 ± 10.12 pg/ml) as compared to the SAL/SAL group

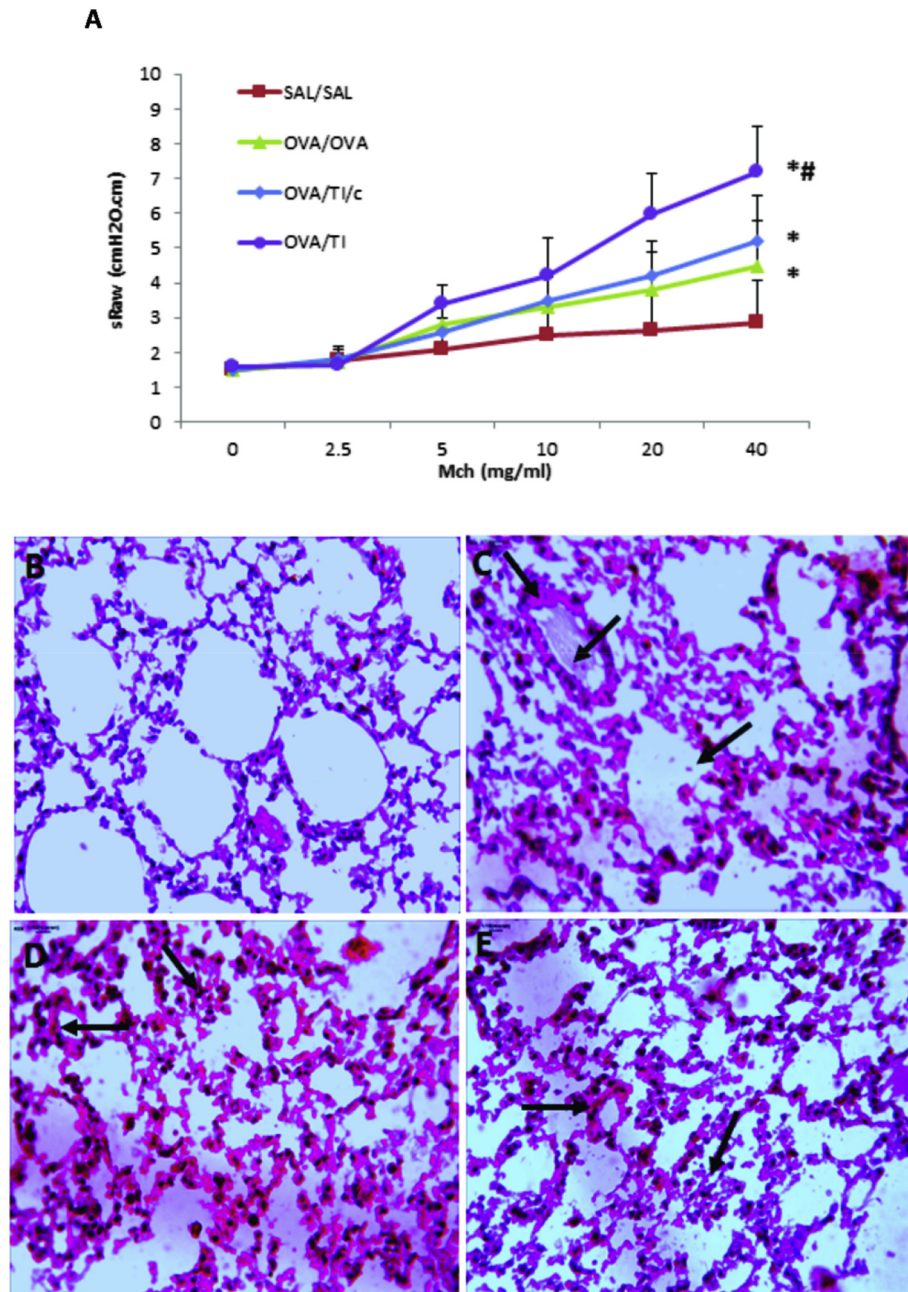


Fig. 3. Alterations in lung architecture are promoted in allergen-sensitized mice over nTiO₂ exposure. (A) The airway hyper-responsiveness was measured on day 28 (48 h after last OVA challenge) by double chambered whole-body plethysmograph in response to varying doses (2.5–40 mg/ml) of aerosolized methacholine (Mch). Data are represented as mean $\pm$ SEM for 5 mice/group, * p < 0.05 compared to SAL/SAL; # p < 0.05 compared to OVA/OVA. Photomicrograph of representative section of lungs from (B) SAL/SAL; (C) OVA/OVA; (D) OVA/TI; (E) OVA/TI/c mouse. Thickening of alveolar septa, infiltration of inflammatory cells, disrupted bronchial lining epithelium and oedema were observed over H&E staining. The identified histopathological alterations are highlighted with arrows.

(20.39 ± 3.82 pg/ml), suggestive of pulmonary inflammation (Fig. 6F). IL-6 mRNA expression in OVA/OVA mice, remained unchanged from SAL/SAL mice whereas substantial elevation in IL-6 mRNA was observed in OVA/TI mice (Fig. 6A). Indeed, IL-6 expression significantly changed in OVA/TI group at 239.65 ± 16.9 pg/ml as compared to OVA/OVA group at 49.73 ± 9.38 pg/ml (p < 0.05) (Fig. 6G). mRNA as well as protein expression did not reveal any significant change in nTiO₂ challenged (OVA/TI/c) group as compared to OVA/OVA group (data not shown).

3.4. Stat3, Socs3 and NF- κ B are persistently expressed in OVA/TI mice

Several studies have reported the influence of Stat3, Socs3 and NF- κ B in inflammatory and allergic airways; we therefore, attempted to evaluate the expression of signal molecules in nTiO₂ induced allergic lungs by qRT²-PCR and Western Blot. Relative estimation of mRNA exhibited low Stat3 (~0.557 fold) and high Socs3 (~9.89 fold) expression in OVA/OVA mice (Fig. 7A). In addition to mRNA expression, protein profiles also revealed similar expression patterns (Fig. 7B–D). We have already reported that Stat3,

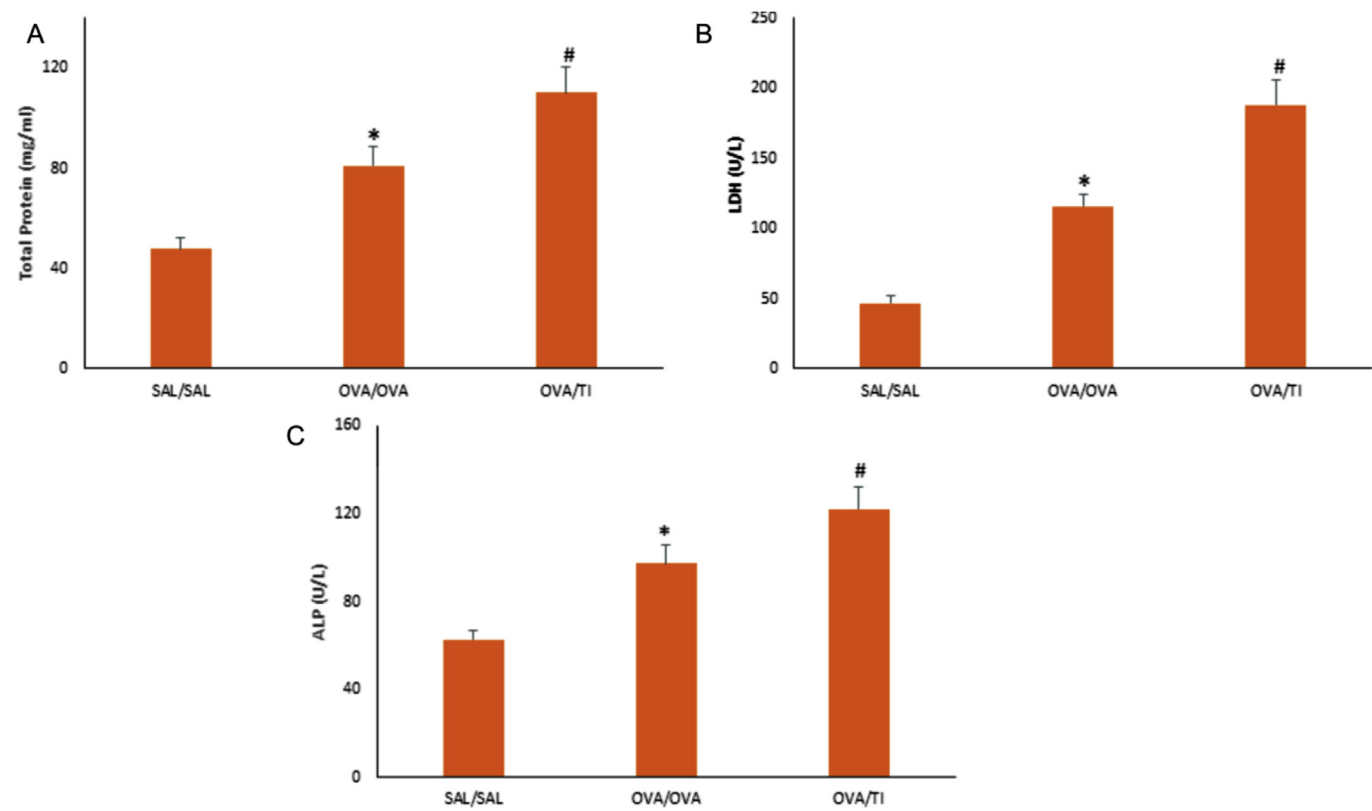


Fig. 4. Evaluation of biochemical markers of lung damage BALF supernatant from SAL/SAL, OVA/OVA and OVA/TI group was analyzed for (A) Total Protein, (B) LDH and (C) ALP content. Data are represented as mean $\pm$ SEM (N = 5 mice/group) *p < 0.05 compared to SAL/SAL; #p < 0.05 compared to OVA/OVA.

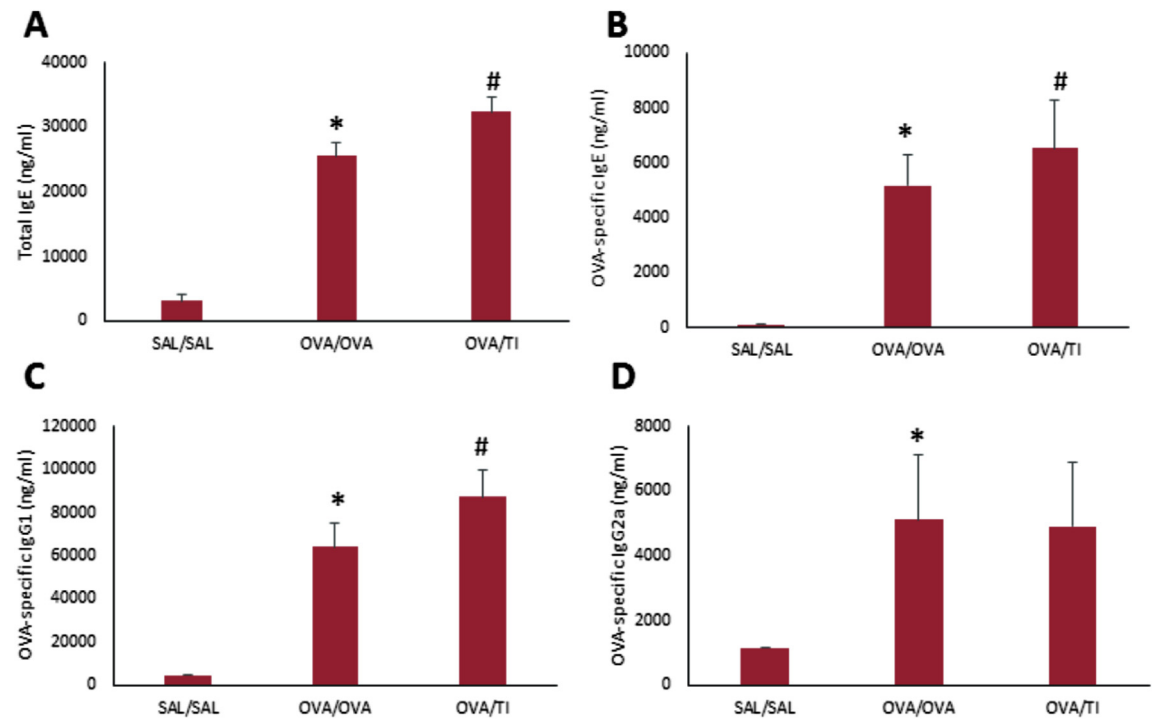


Fig. 5. nTiO₂ sensitization promotes a mixed Th1/Th2 immune response in murine model of asthma. Broncho-alveolar lavage fluid supernatant collected from mice 2 h after last challenge were analyzed for (A) Total IgE; (B) OVA-specific IgE; (C) Ova-specific IgG1; and (D) OVA-specific IgG2a. Data are represented as mean $\pm$ SEM (N = 5 mice/group) *p < 0.05 compared to SAL/SAL; #p < 0.05 compared to OVA/OVA.

Socs3 and IL-6 act as molecular markers for asthma and that Socs3 down regulates Stat3 expression via classical feedback loop

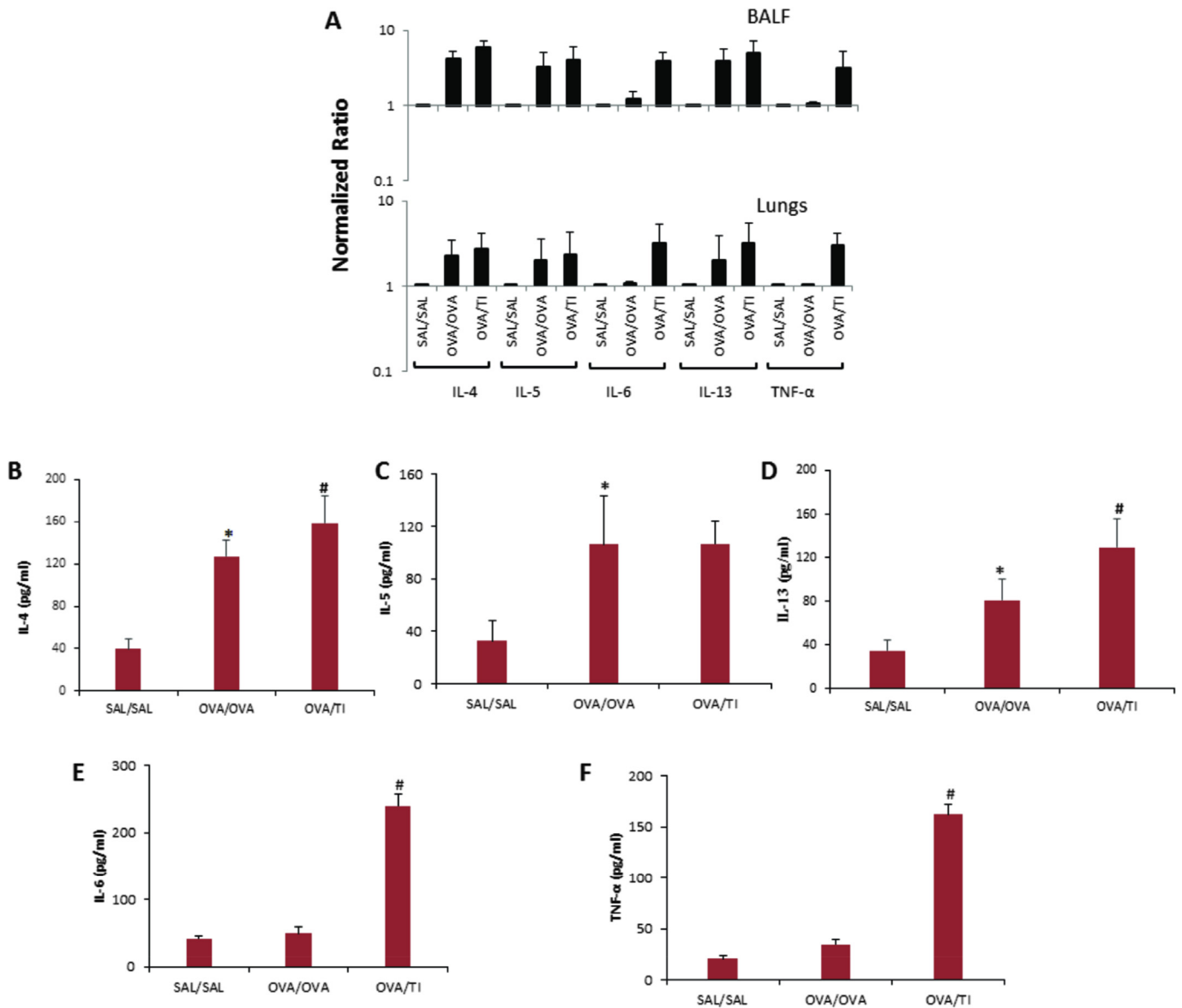


Fig. 6. Elevation of Th2 cytokines and pro-inflammatory cytokines in BALF and lungs of nTiO₂ exposed allergic mice. (A) Graphical representation of qRT²-PCR data (mean ± SE; N = 5) mRNA expression profiles of IL-4, IL-5, IL-6, IL-13 and TNF-α in BALF and lungs of SAL/SAL, OVA/OVA and OVA/TI mouse. Total RNA was processed from lungs and pellet of centrifuged broncho-alveolar lavage fluid of mice 2 h after last OVA challenge. Relative quantitation was performed against SAL/SAL as calibrator and data has been normalized to endogenous β-Actin gene expression as per ΔΔCt method. BAL fluid supernatant was assessed for (B) IL-4, (C) IL-5, (D) IL-13, (E) IL-6 and (F) TNF-α by ELISA Data are represented as mean ± SEM (N = 5 mice/group) *p < 0.05 compared to SAL/SAL; #p < 0.05 compared to OVA/OVA.

directed through Jak/Stat/IL-6. This has been validated by low IL-6 expression in OVA/OVA mice and high IL-6 expression in Socs3-silenced OVA/OVA mice (Fig. 8A).

Interestingly, persistent over expression of Stat3 (~8.19 fold), Socs3 (~7.09 fold) (Fig. 7A) and IL-6 (239.65 pg/ml) (Fig. 6E) occurred in OVA/TI group. The consistent heightened expression of Stat3 with Socs3 as well as Socs3 with Th2-type cytokine suggested that pathway regulation similar to OVA/OVA mice is absent in OVA/TI mice. Therefore, to further determine pulmonary pathophysiology associated with nTiO₂ sensitization, we evaluated the expression of transcription factor NF-κB. Relative mRNA expression revealed significant increase in the expression of NF-κB in OVA/TI mice (~8.09 fold, p < 0.05, Fig. 7A) as compared to OVA/OVA mice with relative expression of ~4.2 folds. Also, NF-κB p65 subunit and IκBα, markers of NF-κB pathway were measured in nuclear and cytoplasmic extracts of lungs of OVA/TI groups. NF-κB p65 and IκBα

was found sequestered in cytoplasm in SAL/SAL mice, with minimum expression in nuclear extract (Fig. 7B,E–G). Translocation of NF-κB p65 to nucleus leads to NF-κB activation, which further upregulates target genes by binding to their promoter regions. The level of nuclear NF-κB p65 was elevated in lungs sensitized with nTiO₂, while a downregulation of the same was observed in cytosolic content. Overall, these results indicate co-expression of Stat3, Socs3 and NF-κB activation in OVA/TI groups.

3.5. NF-κB knockdown inhibited Socs3 expression in OVA/TI mice

Stat3 is known to activate Socs3 which in turn negatively regulates Stat3 activation in asthma pathophysiology. Socs3 siRNA administered in OVA/OVA mice downregulates Stat3 expression, implying negative regulation of Socs3 over Stat3 in inflammatory and allergic airways [23]. However, Socs3 silencing did not

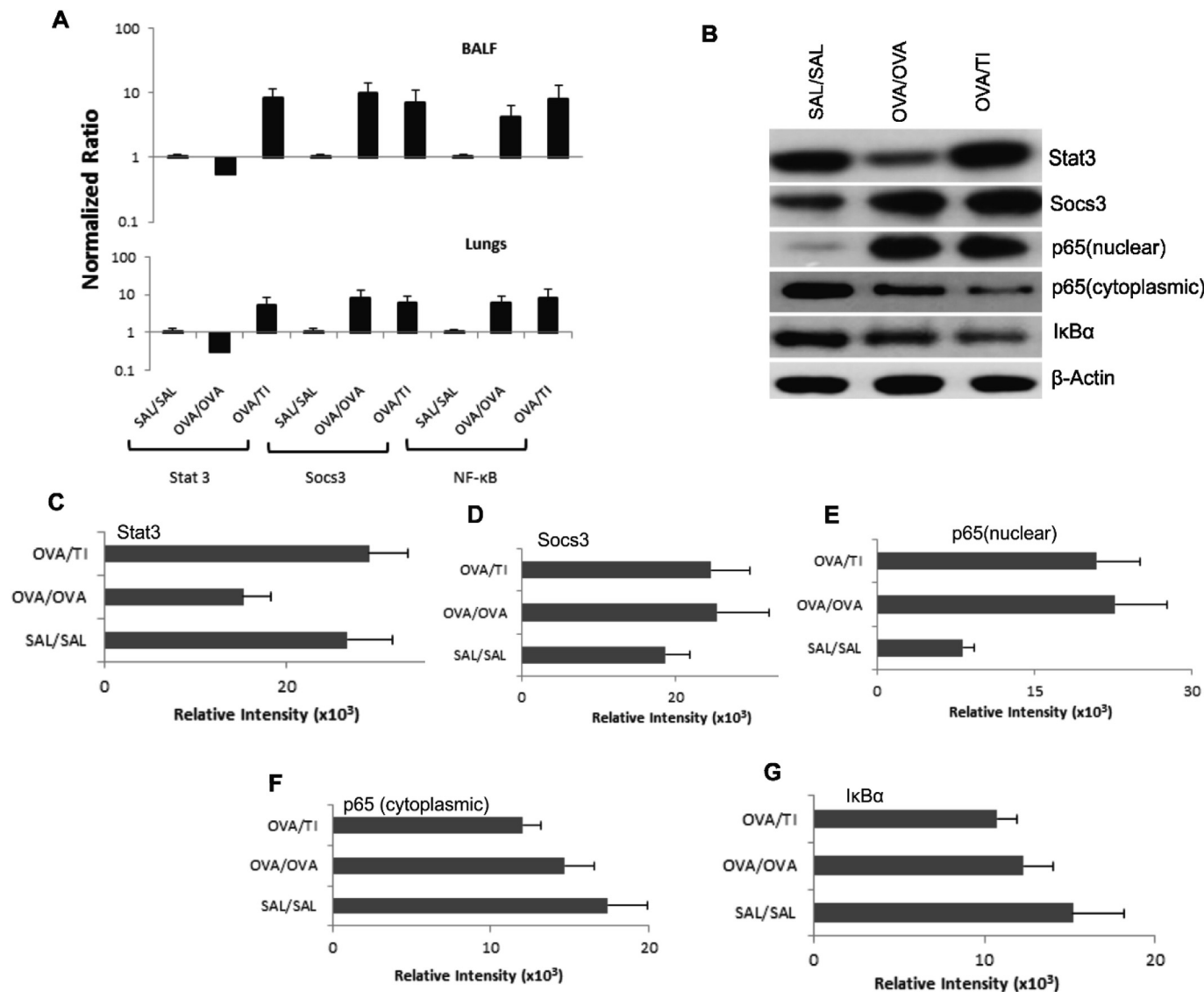


Fig. 7. nTiO₂ exposure in OVA sensitized mice upregulated Stat3, Socs3 and NF-κB expression. (A) Graphical representation of qRT²-PCR data (mean ± SEM; N = 5) mRNA expression profiles of Stat3, Socs3 and NF-κB in BALF and lungs of SAL/SAL, OVA/OVA and OVA/TI mouse. Total RNA was processed from lungs and pellet of centrifuged bronchoalveolar lavage fluid of mice 48 h after last OVA challenge. Relative quantitation was performed against SAL/SAL as calibrator and data has been normalized to endogenous β-Actin gene expression as per $\Delta\Delta C_t$ method. (B) Western Blot representing protein expression profiles of Stat3, Socs3, p65 (nuclear), p65 (cytoplasmic), IκBα (cytoplasmic) and β-Actin. (C–G) Quantitative data showing the protein expression profiles of Stat3, Socs3, p65 (nuclear), p65 (cytoplasmic) and IκBα, respectively.

influence Stat3 expression in OVA/TI mice suggesting that Socs3-mediated negative feedback loop was non operable following nTiO₂ sensitization (data not shown). Since recruitment of NF-κB p65 subunit to the promoter region of target genes activates their expression, we investigated whether loss of NF-κB affects Socs3 expression in nTiO₂ sensitized mice. In order to derive effective *in vivo* dose of siRNA duplex, NF-κB siRNA was administered in OVA/TI group through nasal route in increasing concentrations (20–100 pmol). Two days after transfection knockdown of NF-κB was measured in terms of loss of expression of nuclear NF-κB p65 subunit. Dose dependent depletion of NF-κB p65 subunit was observed with ~60% loss of expression in OVA/TI mice transfected with 50 pmol siRNA. Transfection of scrambled sequence (ss) did not affect the protein expression (Fig. 8A,B). The β-Actin expression profile also remained unaffected following siRNA transfection indicating that siRNA duplex employed is gene specific. We adopted 50 pmol/mouse dose to study the role of NF-κB in modulating

expression of Socs3. NF-κB p65 knockdown resulted in down-regulation of Socs3 expression (Fig. 8C). IL-6 and TNF-α, major downstream target genes of NF-κB pathway were also measured followed by siRNA transfection. We found that NF-κB p65 siRNA significantly suppressed IL-6 and TNF-α at protein level. Over siRNA transfection IL-6 expression in OVA/TI groups dropped ~3.1 fold, while TNF-α degraded ~1.9 fold.

4. Discussion

In the current study we have demonstrated that pulmonary exposure to nTiO₂ over OVA sensitization augments AHR as measured by whole body plethysmograph over methacholine administration and histopathology. AHR is a characteristic hall mark of asthma and its severity relates with the severity of asthma [22,44]. This feature has been substantiated in several animal models with asthma phenotype. Our results reveal similar trend

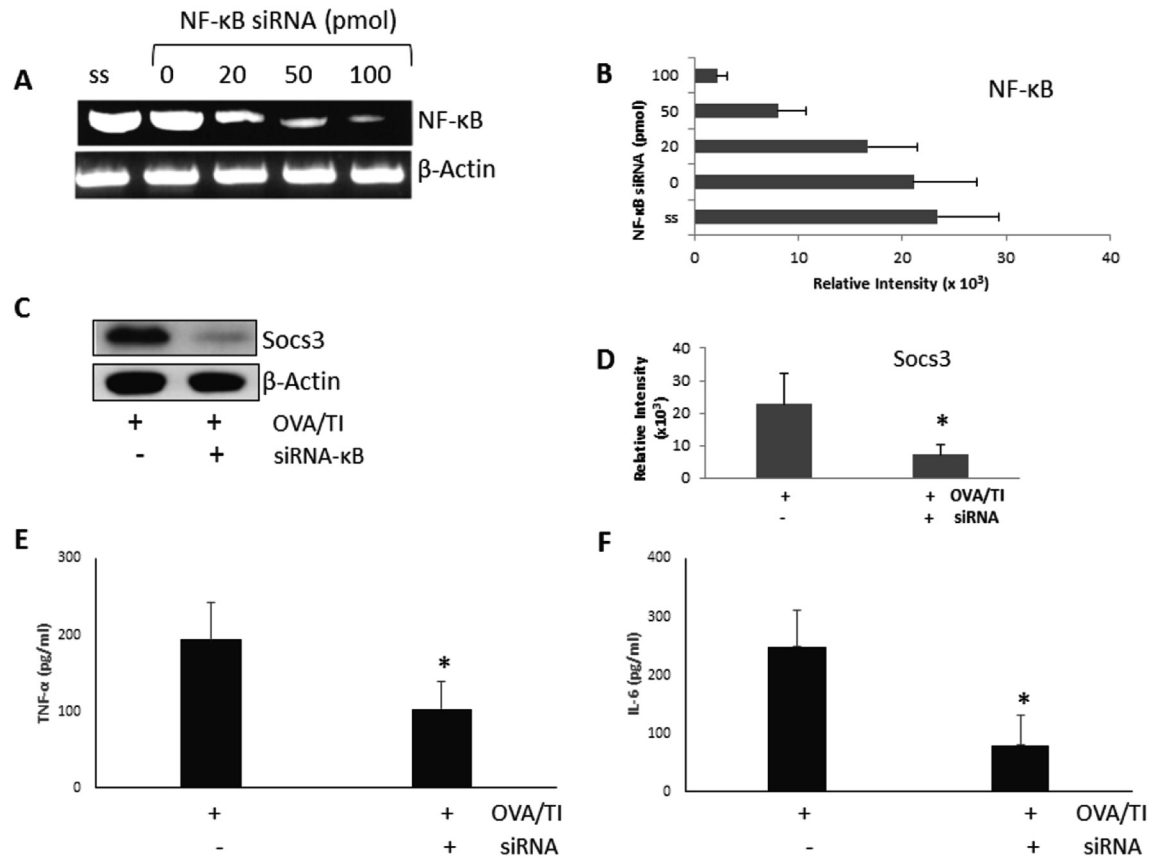


Fig. 8. NF-κB regulates the expression of target genes. (A) Gel image representation of mRNA expression profile of p65 subunit in OVA/TI group 2 days after NF-κB siRNA transfection treated with varying doses. (B) Bar diagram representing % remaining gene expression of p65 subunit over dose range of 0–100 pmol. (C) Western blot and (D) graphical representation of protein expression profiles of Socs3 and β-Actin in OVA/TI group after NF-κB siRNA transfection. (E) and (F) showing levels of TNF-α and IL-6 proteins in siRNA transfected OVA/TI group. Data are represented as mean ± SEM (N = 5 mice/group) *p < 0.05 compared to non-transfected OVA/TI mice.

with heightened Mch-induced sRAW in OVA/OVA mice. Similar features were also observed in OVA/OVA mice that induced sub-epithelial fibrosis, epithelial wall disruption and changes in extracellular matrix in form of oedema due to influx of inflammatory cells and elicitation of secondary airway inflammation. There was a prominent elevation in AHR in OVA/TI group as compared to OVA/OVA group. Interestingly, nTiO₂ exposure during OVA challenge (OVA/TI/c) did not significantly increase AHR as compared to OVA/OVA mice. These observations support the adjuvant effect of nTiO₂ when administered in combination with OVA. However, NP administration during OVA challenge did not take up or extend the inflammation in mice that subsequently results in low AHR [45]. These facts suggest that nTiO₂ exposure during sensitization phase [46] contributes towards extended AHR. Our results also indicated biochemical dysfunction in nTiO₂ exposed asthma mice marked by Total protein, LDH and ALP in BALF. LDH being a cytoplasmic enzyme released by dead cells indicates cell cytotoxicity [47] was significantly elevated in OVA/TI group as compared to both SAL/SAL and OVA/OVA groups. Similarly, Total Protein and ALP, markers for vascular permeability and injury of alveolar type II cells respectively [48] also showed significant elevation. The overall biochemical profile of BALF reflects substantial lung damage in asthmatic mice with nTiO₂ exposure. Consistent with previous reports [14], we observed significant increase in the levels of OVA-specific IgE and IgG1 in OVA/TI group as compared to OVA/OVA. Similarly, mixed Th2/Th1-driven immune response was observed with elevated expressions of IL-4, IL-5 and IL-13 in OVA/TI group. The hypermorphic expression of IL-4 and IL-5 has been associated

with Jak/Stat pathway in allergic airways [23]. Also, Stat3 has been implicated in Th2 type signaling in animal model of asthma [49] which in turn is negatively regulated by Socs3 [23,50]. In this study we observed significant over-expression of Stat3 and Socs3 in OVA/TI groups. Contradictory to the asthma pathogenesis, the negative regulation of Stat3 via Socs3 feedback loop was not observed in nTiO₂ treated asthma model. Here we hypothesize that dynamics of nTiO₂-induced pulmonary damage could be the result of independent association between Th2 type cytokine, and signal cascades without the involvement of Socs3-mediated negative regulation. This is in line with the findings that Socs3 transgenic mice increased Th2 type immune response and other hallmark pathological features of asthma. Also, dominant-negative mutant Socs3 transgenic mice as well as heterozygous deletion of Socs3 downregulated Th2 progression [51]. There is also the possibility that Socs3 has been rendered functionally defective at the molecular level and thus could not downregulate Stat3 expression [52,53]. This further supports the absence of Socs3-mediated negative feedback loop in nTiO₂ induced asthma pathophysiology. On the other hand, the persistent expression of Stat3 upon nTiO₂ exposure could be through IL-6 that is induced as a target gene of NF-κB pathway or as an auto regulatory loop operated by Stat3 itself in conjugation with CRE-binding protein [54].

In addition, our study showed elevated mRNA and protein expression of IL-6 and TNF-α upon nTiO₂ exposure with OVA. The fine and ultrafine particles have been found to induce pro-inflammatory cytokines, particularly, IL-6, TGF-β, GM-CSF, TNF-α and IL-8 by stimulating macrophages and epithelial cells

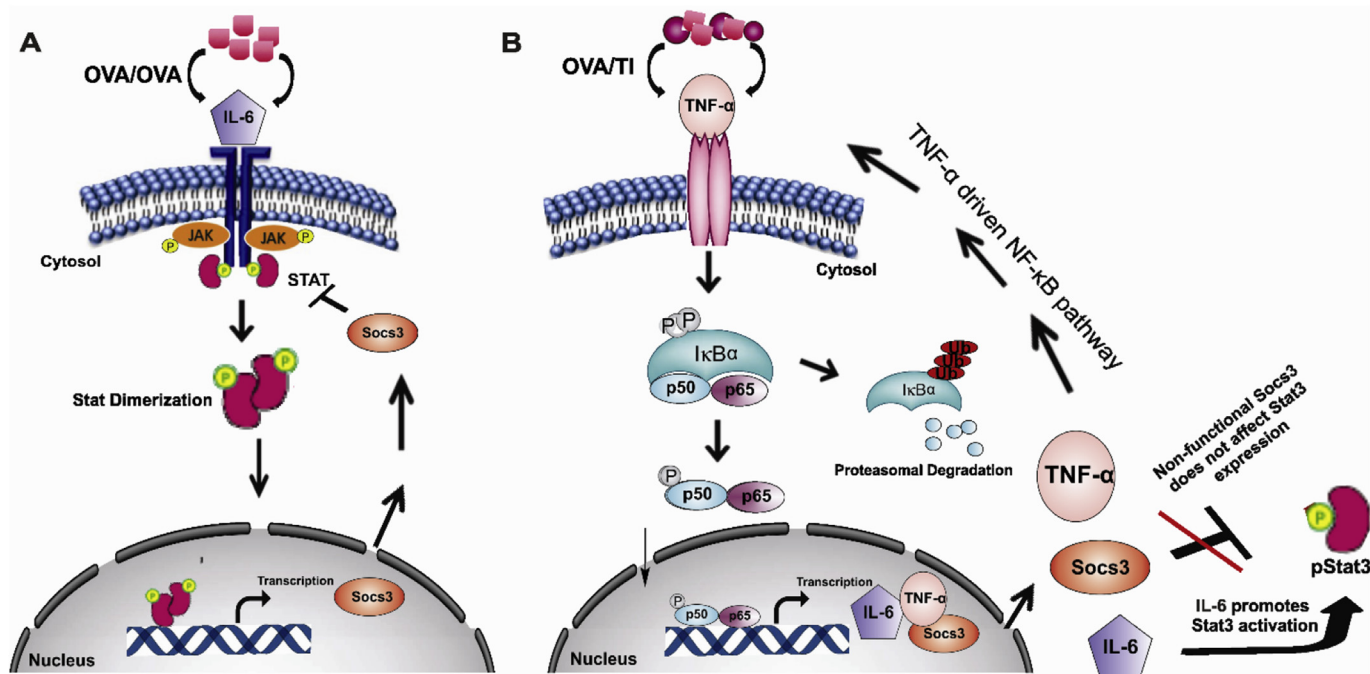


Fig. 9. Schematic representation of the pathways. (A) Conventional OVA induced Jak/Stat pathway in asthma model. (B) Modified pathway induced through nTiO₂ sensitization in asthmatic airway that is routed through NF-κB signaling cascade. The translocation of NF-κB subunits into the nucleus transcriptionally upregulates various pro-inflammatory target proteins like IL-6, TNF-α and Socs3.

[24,55–59]. Specifically, nTiO₂ has been reported to enhance the expression of pro-inflammatory cytokines in dendritic cell population and favors the activation and proliferation of CD4⁺ T cells [24,60]. TNF-α is a pleiotropic cytokine that acts as chemoattractant for eosinophils, induces oxidant stress and activates transcription factor NF-κB [61]. NF-κB activation via TNF-α occurs through the canonical pathway that allows nuclear translocation of NF-κB p65, where binding of DNA and transcriptional regulation occurs. Our study revealed significant activation of NF-κB in both OVA/OVA and OVA/Ti groups as compared to SAL/SAL group which is in conformity with the previous reports [62]. However, TNF-α expression has been found to be significantly upregulated in OVA/Ti group as compared to OVA/OVA group. This indicates that expression of NF-κB is differentially regulated in the two groups which could be attributed to nTiO₂ exposure. Socs3 expression is controlled by different signaling pathways specific of cell type and stimuli [38,63–66]. We observed that the nTiO₂-induced Socs3 expression in asthma pathophysiology is driven by NF-κB. Downregulation of Socs3 was observed over NF-κB silencing indicating that Socs3 is NF-κB induced gene. Accumulated evidences suggest that under certain conditions Socs3 expression can be regulated through NF-κB pathway. Previous reports indicate that IKK mediated NF-κB activation leads to the expression of various target genes including Socs3 [67,68]. Similarly, during viral infections, Socs3 expression in humans and murine macrophages is also regulated via NF-κB/IKK [38,69]. In addition, NF-κB knockdown was also found to be associated with decrease in IL-6 [39] and TNF-α expression, the potential downstream target genes of NF-κB pathway conferring non-recruitment of the activated NF-κB p65 subunit to the target genes.

The pathway depicted (Fig. 9A) represents a conventional JAK/Stat pathway stimulated over OVA sensitization [23]. However, Fig. 9B represents the modified signaling cascade over nTiO₂ sensitization (OVA/Ti). The modified route indicates NF-κB mediated pulmonary inflammation. Upon binding to the promoter region of the respective target molecules, NF-κB notably induces the

expression of IL-6, TNF-α and Socs3, which in turn aggravates immune response in nTiO₂ exposed allergic individuals.

5. Conclusion

To summarize, we have shown that nTiO₂ have an adjuvant effect in mice which modulates the microenvironment of the airways of experimental animals towards Th2 mediated immune response with increased IgE production and airway remodeling. We also observed constituent expression of Stat3, Socs3, NF-κB, different Th2 type cytokines (IL-4, IL-5 and IL-13) and pro-inflammatory cytokines (IL-6 and TNF-α) in OVA/Ti mice. nTiO₂ probably obliterated Socs3 mediated feedback loop (operational in asthma pathogenesis) allowing persistent expression of Stat3 in airway tissues. In addition, we report for the first time that persistent activation of Socs3 in nTiO₂-induced pulmonary inflammation during asthma pathophysiology is via NF-κB dependent mechanism which in turn upregulates the potential target genes attributed to inflammation. Further investigations are needed to establish a better understanding of the relationship between exacerbated airway inflammation and signaling pathways in nTiO₂-induced immune response in asthma pathophysiology. In light of our findings; we could conclude that nTiO₂ exposure as an adjuvant may aggravate allergic airway diseases and that the adverse health effects of NPs are highly dependent upon timing of exposure. Also NF-κB could be employed as a more efficacious target for pharmaceutical intervention for blocking/avoiding deleterious side effects of nTiO₂-induced acute as well as chronic inflammatory reactions.

Acknowledgments

The work was supported by University Grants Commission, New Delhi under the scheme DSKPDF (F.4-2/2006 (BSR)/13-813/2013), Science and Engineering Research Board (SERB), Department of

Science and Technology, New Delhi under the Fast Track Young Scientist Scheme (No. SB/YS/LS-44/2014) and Design Innovation Centre (DIC), Ministry of Human Resource Development (MHRD), India. The authors thank Prof. J.S. Singh, FNA, Emeritus Professor, Banaras Hindu University (BHU), India for critically reviewing the manuscript.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.biomaterials.2016.03.016>.

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Titanium dioxide nanoparticles augment allergic airway inflammation and Socs3 expression via NF- κ B pathway in murine model of asthma

Vani Mishra^{a*}, Vikas Baranwal^a, Rohit K. Mishra^{b*}, Shivesh Sharma^b, Bholanath Paul^c, Avinash C. Pandey^a

^aNanotechnology Application Centre, University of Allahabad, Allahabad- 211002, India.

^bCentre for Medical Diagnostic and Research, Motilal Nehru National Institute of Technology, Allahabad-211004, India.

^cImmunobiology Division, Indian Institute of Toxicology Research, Lucknow- 226001, India.

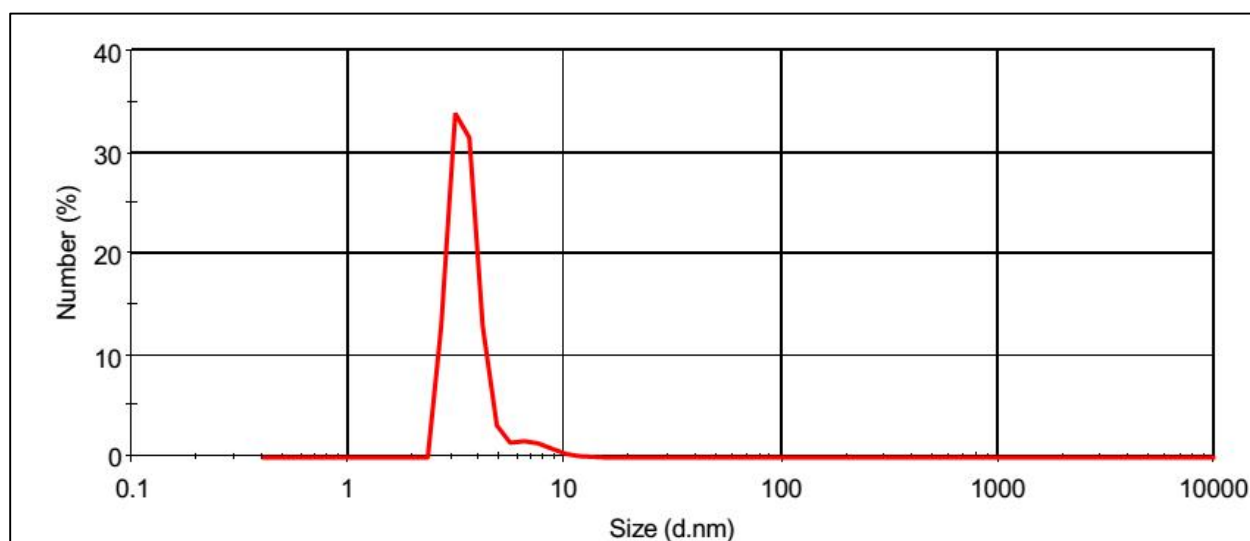


Fig S1. Size distribution analysis based upon number (%) by DLS.