

Ku70 modulation alleviates murine allergic asthma features and restores mitochondrial function in lungs

Rakhshinda Rehman^{a,b,c}, Ashish Jaiswal^{a,b,d}, Anurag Agrawal^{a,b},
Ulaganathan Mabalirajan^{a,b,d,1,*}

^a Molecular Pathobiology of Respiratory Diseases, Council of Scientific and Industrial Research (CSIR)-Institute of Genomics and Integrative Biology (IGIB), Delhi, India

^b Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India

^c Division of Pulmonary and Critical Care Medicine, Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

^d Molecular Pathobiology of Respiratory Diseases, Cell Biology and Physiology Division, CSIR-Indian Institute of Chemical Biology, Kolkata, India

ARTICLE INFO

Keywords:

Ku70

Asthma

Airway epithelium

ABSTRACT

The airway epithelium is continuously exposed to a variety of pollutants and allergens, thanks to both natural and manmade environmental pollution. With numerous protective mechanisms, the airway epithelium protects the lungs. DNA repair mechanism is one such protective response and its failure could lead to the accumulation of DNA mutations. Our lab had earlier demonstrated the dysfunctional mitochondria in airway epithelium of the asthmatic mice lungs. Here, we show that Ku70 modulation by the administration of Ku70 plasmid attenuates asthma features and reduces mitochondrial dysfunction in the lungs of allergen exposed mice. Ku70 is a key DNA repair protein with diverse roles including VDJ recombination, telomere maintenance, and maintenance of cell homeostasis. Recently, we found a reduction in Ku70 expression in asthmatic airway epithelium, and this was associated with mitochondrial dysfunction in asthmatic condition. In this study, we have shown that Ku70 overexpression in asthmatic mice attenuated airway hyperresponsiveness, airway inflammation, sub-epithelial fibrosis along with reduction in TGF- β with no effect in IL-13 levels and goblet cell metaplasia. Ku70 overexpression in asthmatic mice reduced 8-isoprostane, a marker of oxidative stress, and restored the mitochondrial function in asthmatic mice. We further found these roles of Ku70 to be independent of DNA damage as Ku70 overexpressed mice did not show any reduction in DNA tail, an index of DNA damage. Thus, our findings indicate that Ku70 can attenuate crucial features of asthma along with the restoration of mitochondrial function. This implies that Ku70 could be a therapeutic target for asthma without affecting DNA repair function.

1. Introduction

The incidence of asthma, a chronic airway inflammatory disease, is steeply increasing worldwide though we have devised numerous therapeutic strategies. This indicates the need to study novel mechanisms of its pathogenesis. Traditionally, it is believed that most of the characteristic features of asthma such as airway obstruction, airway remodeling and eosinophilia are mediated by Th1/Th2 imbalance and the resultant pro-inflammatory mediators could increase the oxidative stress and henceforth damage the structural cells (Elias et al., 2003). However, emerging evidence indicate a critical role of airway epithelium in asthma pathogenesis. These studies indicate that airway epithelium can

determine the type of airway inflammation depending on types and severity of stress they are exposed to (Swamy et al., 2010). For example, exposure to milder grade stress leads to Th2 inflammation whereas severe grade stress like smoke exposure could lead to a neutrophilic response. So airway epithelium should have sufficient mechanisms to protect the lung from these endogenous and exogenous irritants. It is well known that the airway epithelium acts as a primary barrier against these irritants (Swamy et al., 2010). In addition to this, airway epithelium secretes a specific set of cytokines such as IL-33, TSLP, and IL-25 and these mediators can initiate pro-inflammatory Th2 response by converting naïve T cells into Th2 cells (Byers, 2014; He and Geha, 2010; Swamy et al., 2010).

* Corresponding author at: Molecular pathobiology of respiratory diseases, Cell Biology and Physiology Division, CSIR-Indian Institute of Chemical Biology, Kolkata, India.

E-mail address: mabsome@yahoo.co.in (U. Mabalirajan).

¹ The authors declare no competing financial interests.

<https://doi.org/10.1016/j.mito.2020.12.005>

Received 27 October 2020; Received in revised form 1 December 2020; Accepted 14 December 2020

Available online 5 January 2021

1567-7249/© 2020 Elsevier B.V. and Mitochondria Research Society. All rights reserved.

In addition to these biochemical modes of protective mechanisms, the airway epithelium should also have an organelle mediated protection and DNA repair mechanisms. These mechanisms are not well studied in asthma pathogenesis in the context of Th2 immune response dominant airway inflammation concept. In this context, our lab and others had also shown mitochondrial dysfunction and ER stress in the airway epithelium of asthmatic lungs (Ahmad et al., 2014; Mabalirajan et al., 2008; Makhija et al., 2014). One study had also demonstrated the role of various DNA repair proteins in COPD patients (Caramori et al., 2011). Very interestingly, a number of DNA repair proteins such as Ku were found to be predominantly expressed in the airway epithelium. These indicate that these DNA repair proteins could have a role in airway epithelial homeostasis. The presence of these proteins even in normal epithelium indicates that they may be required to correct baseline DNA damage that could have occurred during normal cell division.

Ku70, a part of Ku heterodimer protein, along with Ku80, plays a critical role in non homologous end-joining DNA repair pathway, VDJ-recombination, and telomere maintenance (Downs and Jackson, 2004). The Ku heterodimer is also a part of the major component in double strand DNA repair complex called DNA protein kinase along with the catalytic subunit of DNA-PK. Recent studies including our study have shown that Ku70 is expressed even in healthy individuals and is downregulated during oxidative stress or caspase 3 activation (Rehman et al., 2020; Song et al., 2003). In addition to this, our lab had found the downregulation of Ku70 in bronchial epithelium of asthmatic mice and human samples (Rehman et al., 2020; Rehman and Mabalirajan, 2017). Ku70 is also present in mitochondria and is known to inhibit apoptosis (Song et al., 2003). Earlier, we had shown mitochondrial dysfunction along with increased airway epithelial apoptosis in asthmatic condition. Based on these, we hypothesized that Ku70 could have a beneficial role in asthma.

In the present study, we overexpressed Ku70 in asthmatic mice. We found that Ku70 over-expression reduced certain features of asthma including airway hyperresponsiveness, airway inflammation, sub-epithelial fibrosis and most importantly it restored the mitochondrial function. Therefore, Ku70 can be a good therapeutic target in asthma.

2. Materials and Methods

2.1. Mice grouping

All the mentioned methods in mice were carried out in accordance with relevant guidelines and regulations of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

All experimental protocols used for mice study were approved by the Institutional Animal Ethics Committee in IGIB, Delhi, India. The male mice (6–8 weeks, BALB/c strain 18–25 g) were procured from Central Drug Research Institute, Lucknow, India or the In-House breeding center in Institute of Genomics and Integrative biology, New Delhi (IGIB). The obtained mice were acclimatized for seven days before experiments. All protocols used for mice study were approved by the Institutional Animal Ethics Committee in IGIB, Delhi, India. Ovalbumin induced mice model of asthma was used in the study (Fig. 1). Mice were divided randomly into three groups: a) SHAM/PBS/VEH (saline sensitized, saline challenged and vehicle treated), b) OVA/OVA/Control plasmid (ovalbumin sensitized, ovalbumin challenged and control plasmid treated) c) OVA/OVA/Ku70 plasmid (ovalbumin sensitized, ovalbumin challenged and ku70 plasmid treated).

2.2. Induction of allergic airway inflammation

Mice were immunized by four intraperitoneal injections of 50 µg OVA that was adsorbed in 4 mg aluminum hydroxide (OVA/OVA/Control plasmid and OVA/OVA/Ku70 plasmid groups) or 4 mg aluminum hydroxide alone (SHAM/PBS/VEH group) on days 0, 7, 14 and 21 and after that challenged for 7 days from Day 28 to Day 34 (30 min per day) with 3% OVA in PBS or PBS alone (Mabalirajan et al., 2009b, 2010). The challenge was performed in a plexiglass chamber which had two holes and one was used to insert a nebulization tube and another was used for fresh air to enter. On days 29, 31 and 33, these mice were administered PBS or 75ug control/Ku70/plasmid intravenously and euthanized on day 35 after AHR measurement (Fig. 1). The plasmids (control or Ku70, Origene) were mixed with *in vivo*-jetPEI transfection reagent (polyethylenimine based transfection from Polyplus transfection). The vehicle given in SHAM/PBS/VEH was 10% glucose.

2.3. Plasmid

The pCMV6-entry mammalian expression vector was used as plasmid for the purpose of Ku70 over-expression system in mice. The pCMV6-Ku70 was prepared with the insertion of mouse Ku70 cDNA (insert size was 1827 bp) between SgfI-MluI restriction sites of the plasmid. The Refseq accession number of Ku70 is NM_010247. The insert sequence can be seen in catalog no MC219608 of Origene. Both pCMV6-ku70 and pCMV6 vectors were having CMV promoter to drive the gene expression and Myc-DDK tag. The details of pCMV6 can be seen in catalog no PS100001 of Origene. For more clarification, the construction design of pCMV6 has been shown in Fig. 2A. The functional elements of pCMV6

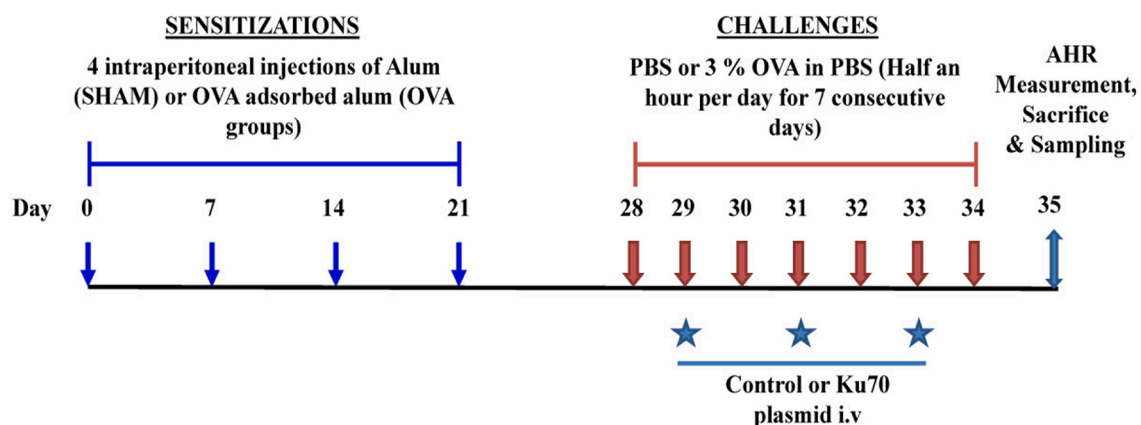


Fig. 1. Mice model of ovalbumin induced asthma and Ku70 over-expression validation: A) After acclimatization of mice to animal house environment, mice were randomly divided into three groups and sensitized with either alum (SHAM/PBS/VEH) or with Ovalbumin adsorbed in alum (OVA groups) on day 0, 7, 14 and 21 and challenged with 3% PBS or Ovalbumin in PBS from day 28 to day 35. Control plasmid or Ku70 plasmid was injected (i.v.) on day 29, 31 and 33 and on day 35 AHR measured, BAL fluid collected and mice were sacrificed and blood and lungs were collected. There were three independent set of experiments and there were 5–6 mice per group in each set of experiments.

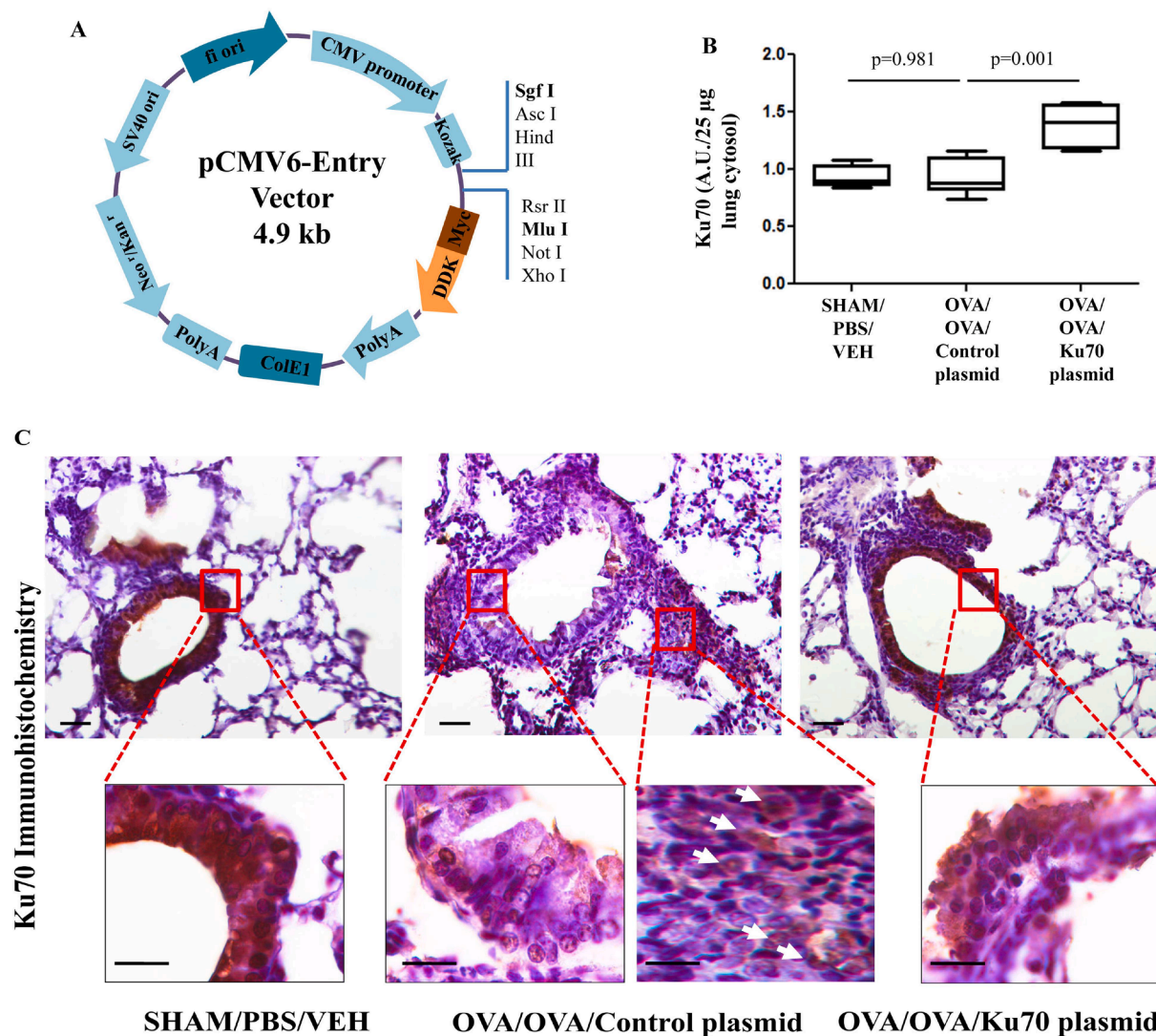


Fig. 2. Construction design of Ku70 plasmid and validation of Ku70 over-expression in mice lungs. A) pCMV6-Entry mammalian expression vector, B) After sacrifice, lungs were collected and total lung lysate was prepared and to validate the model, Ku70 was measured by ELISA. C) Immunohistochemistry was performed to determine the expression of Ku70 in lung sections. Data represents mean $\pm$ SE; $n = 5-6$ in each group for mice experiments and shown data is the representative of three independent experiments. Bars are 100 μ m (upper panel) and 50 μ m (lower panel).

were the *fi* origin of replication (*fi ori*) for E-Coli propagation, CMV promoter, the Ku70 gene, Myc-DDK tag, SV40 origin of replication, the neomycin/kanamycin resistance gene and HEK 293 T cells (Kan/Neo). The initial set of experiments was performed with GFP tagged vector to confirm the uptake of vector by lung cells. We found a significant quantity of GFP to be present 24–28 hrs post intravenous injection. The remaining sets of experiments were performed with Myc-DDK tagged vector. The pCMV6-ku70/pCMV6 vectors were administered using *in vivo*-jetPEI (commercially available *in vivo* transfection reagent that contains polymer complex of polyethylenimine which is cationic in nature and due to its cationic nature, it is able to make stable DNA complexes for successful gene delivery into the cells. Earlier experiments using *in vivo*-jetPEI had demonstrated that the lung is the primary target organ upon systemic injection (Bragonzi et al., 2000; Goula et al., 1998; Gossart et al., 2013; Song et al., 1998). In addition, maximum expression was found in the lung 12–24 hrs after systemic injection (Gossart et al., 2013). However, other organs like heart, spleen are also targeted by *in vivo*-jetPEI (Schlosser et al., 2018).

The presence of plasmid was confirmed by imaging and luciferin assay of the lung tissue extract. Indeed, intravenous injection leads to higher deposition in the lungs compared to intraperitoneal injection.

However, other organs like the heart, spleen, kidney etc also had shown the deposition of plasmid. Further, the peak of deposition in lung was found till 24 hrs after systemic injection. After 24 hrs, the quantity is gradually reduced significantly till 72 hrs after systemic injection. However, there was a significant deposition 48 hrs after injection. This is the reason why we have selected to repeat the injection every 48 h. Both ELISA and immunohistochemistry (IHC) experiments revealed that pCMV6-Ku70 plasmid administration leads to expression of Ku70 significantly in lungs (Fig. 2B & C). Though ELISA and IHC experiments revealed the presence of Ku70 protein in the lungs of Ku70 plasmid administered mice, whether this exogenous protein leads to immune response or not is really interesting. But this immune response against an exogenous protein like Ku in our model may not be possible as the clonal expansion of memory B and T cells against Ku70 would take at least two weeks whereas we administered first dose of Ku70 plasmid on day 29 and euthanized on day 35. So 6 days may not be sufficient to cause an adequate immune response.

3. Airway hyperresponsiveness (AHR) and bronchoalveolar lavage (BAL)

AHR was measured in the form of airway resistance in anesthetized mice using an airway mechanics instrument that has an inbuilt mechanical ventilator (FlexiVent, SciReq, Canada) that uses computer-controlled mouse ventilator to measure respiratory mechanics (Mabali-[lirajan et al., 2010](#)). Briefly, individual mouse was anaesthetized using xylazine and thiopentone (intraperitoneal injections) and anaesthesia was confirmed by pinching toes and once there was no response to pinching toes, tracheotomy was performed by briefly cutting the two adjacent cartilage rings. After confirmation of anaesthesia, mice were tracheotomised, cannulated and the cannula was connected to FlexiVent.

The blunt calibrated 18 G cannula was inserted into the open trachea and trachea with the cannula was tied to avoid any leakage of ventilated air. Then the cannula was connected to the ventilator with the help of Y-tubing. These mice were mechanically ventilated with 150 breaths/minute. Two maneuvers called Snapshot perturbation and Quick prime perturbation were performed to collect forced oscillation data. Single forced oscillation technique was performed to get respiratory system resistance and respiratory system compliance. And multiple frequency forced oscillation technique was introduced in constant phase model to get other respiratory parameters like Newtonian resistance, tissue damping, tissue elastance etc. As mice that have the features of allergic airway inflammation show change in respiratory system resistance compared to other parameters like elastance and compliance, we have shown the resistance to different concentrations of methacholine (Mch) and PBS. We have shown the average of resistance values obtained for 3 min for every methacholine concentration. The results were expressed assuming PBS aerosol values as 100% with an increasing dose of methacholine. After AHR measurement, BAL (bronchoalveolar lavage) fluid was collected by instilling 2 ml of PBS into the trachea and the fluid was recovered. BAL fluids were centrifuged to get BAL cells that were used for differential leukocyte count using Leishman's stain (Mabali-[lirajan et al., 2009b, 2013b](#)). Briefly, BAL fluid cells were smeared on glass slide by cytospin and fixed with methanol. Fixed cells on glass slides were stained by Leishman's stain. Cells were recognized based on morphology at 100X magnification and classified into neutrophil, eosinophil, mononuclear cells (monocytes, lymphocytes) and macrophages and the percentage of each cell type was shown. As it is difficult to distinguish monocytes and lymphocytes based on light microscopy, we calculated both monocytes and lymphocytes as mononuclear cells. However, it is relatively easy to distinguish other cell types like neutrophils, eosinophils, and macrophages based on the color of cytoplasmic granules, and shape of the cells and nuclei.

3.1. Lung histopathology

Mice were sacrificed and lungs were collected, fixed with formalin, embedded in paraffin and cut into 5 μ m sections, various staining: Haematoxylin and eosin (H & E), periodic acid-Schiff and Masson's trichrome staining were performed to assess airway inflammation, goblet cell metaplasia and sub-epithelial fibrosis, respectively (Ram [et al., 2015](#)). The Inflammation score was calculated as described earlier (Mabali-[lirajan et al., 2009b, 2013b](#)). In brief, two investigators who did not know the status of the mice groups were given randomly numbered Haematoxylin and Eosin stained lung sections for the assessment of inflammation score. Both perivascular and peribronchial inflammation regions of lung tissue sections were given zero to four scores depending on the density of the inflammatory cells. For example, score one was given if the bronchus or vessel was cuffed with single layer of recruited inflammatory cells in some places and similarly score two (one to three layers of recruited inflammatory cells), three (three to five layers of recruited inflammatory cells), four (more than five layers of recruited inflammatory cells). Score zero was given when there was no

inflammatory cell around the bronchus or vessel. Inter-observer variations were negligible. Individual peribronchial and perivascular inflammation scores were summed to get the total inflammation score.

The ImageJ software version 1.8 (<https://imagej.nih.gov/ij/download.html>) was used to perform the quantitative morphometry in MT- and PAS-stained lung sections to measure collagen deposition and airway mucin content, respectively (Mabali-[lirajan et al., 2009a](#)). Briefly, stained sections were opened through ImageJ software; images were converted into three different colors: Red, Blue and Green. Red and Green color images were selected for MT and PAS stained sections, respectively. The threshold was adjusted in each colour image in such a way that only areas representing a positive stain was visible. The positive stained area was calculated using automated particle analysis. The area was normalized by perimeter/circumference and the positive stain area was expressed as sub-epithelial collagen or airway mucin content for MT and PAS stained sections respectively.

To study apoptosis, TUNEL (terminal deoxynucleotidyl transferase dUTP nick-end labelling) assay was performed in the lung sections as described earlier (Mabali-[lirajan et al., 2013b](#)). Briefly, 5 μ m paraffin embedded lung sections were incubated with biotinylated nucleotides which can be end labeled at fragmented DNA in presence of terminal deoxynucleotidyl transferase and horseradish peroxidase-labeled streptavidin and detected with DAB/urea-H₂O₂ system (Promega, USA) as brown color. The nuclei of apoptotic cells were stained with brown color. The percentage of positive apoptotic bronchial epithelium was calculated by apoptotic index as described earlier (Mabali-[lirajan et al., 2013a](#)). Briefly, TUNEL stained lung sections were examined by experimentally blind investigators who counted the number of TUNEL positive bronchial epithelium from all possible bronchial regions in the lung sections and it was expressed as % TUNEL positive bronchial epithelia.

3.2. Enzyme linked immune sorbent assay (ELISA)

The levels of 8-isoprostane (mouse total lung proteins) were measured as per the manufacturer's instructions (Cayman Chemicals). The OVA-specific IgE and IgG2a levels were estimated in mouse sera samples as per the manufacturer's instructions (MY BIOSOURCE). The IL-13 and TGF- β 1 (BD Biosciences), Ku70 and (USCN Life Science Inc) and cytochrome C (R & D systems) were estimated in mouse total lung protein/cytosols.

3.3. Mitochondrial enzyme activities and Caspases-3 activity

Mitochondrial fractions were isolated from mice lungs as described earlier (MITOISO 1, Sigma) (Mabali-[lirajan et al., 2008](#)). Briefly, freshly dissected lung tissue was washed with HEPES isotonic buffer, homogenized in same HEPES buffer containing fat free albumin and the whole homogenate was centrifuged at 600 g for 6 min to discard the nuclei and debris and the resultant supernatants were centrifuged at 11,000 g for 12 min at 4 °C. The similar low speed and high speed centrifugations were performed again to get final mitochondrial pellets. Mitochondrial storage buffer was used to reconstitute the mitochondrial pellets that were used for various enzyme activities after protein estimation. As the type of tissue and mode of preparation are the determining factors for integrity of isolated mitochondria, it would be crucial to have similar preparation to avoid methodological errors in measuring the mitochondrial enzyme activities. So we have determined the integrity of mitochondria in a few lung tissues (CYTOCOX 1, Sigma Aldrich) in every batch by measuring the cytochrome c oxidase activity in isolated mitochondrial pellets with or without using beta dodecyl maltoside that has the capability to maintain the cytochrome c oxidase as dimer form. If there is a reduction in the outer mitochondrial membrane (OMM) integrity, exogenous cytochrome c can cross OMM to reach IMM to react with cytochrome c oxidase. In our experiments with isolated mitochondria from lung, we observed approximately 10% damage to OMM consistently (data not shown). This is expected from lung tissues as other

organs which are harder in nature would have more than 15% damage to OMM.

Complex IV (Sigma-Aldrich) and Complex I (MitoSciences) activity were measured and calculated as per the manufacturer's instructions. Briefly, mitochondria were first treated with *n*-dodecyl-D-maltoside. Then complex IV present in mitochondria oxidizes ferrocytochrome c to ferricytochrome c and this was quantified by the decrease in absorbance at 550 nm. Total Complex I activity was measured by the hydrolysis of acetyl CoA to thiols, which further reacts 5,5-dithio-bis(2-nitrobenzoic acid) to form 5-thio-2-nitrobenzoic acid, which can be quantified colorimetrically at 405 nm. Positive control experiments were performed using pure cytochrome c oxidase or citrate synthase (Sigma-Aldrich). Caspase-3 activity was assessed using lung cytosol as per the manufacturer's instruction (Biovision).

3.4. COMET assay

Comet assay was performed to study DNA damage (Trevigen). Briefly, a single cell suspension was prepared from 30 mg of lung tissue in 20 mM EDTA buffer by gently crushing the tissue on a 70 μ m cell strainer placed in 50 ml falcon. Lung cell suspension containing 50,000 cells in 0.75% low melting agarose (LMA) were placed on 0.1% LMA pre-coated slides which were solidified and lysed. The neutral/alkaline comet assay was performed as per manufacturers' protocol, propidium iodide stained slides were observed (Lieca DMI6000 B) and images were captured and analyzed by using Opencomet software (Gyori et al., 2014) which is a freely available software for automatic analysis of propidium iodide stained COMET images. For every group, there were at least 400–500 comets cumulative from the individual samples for analysis.

3.5. Statistical analysis

Unpaired Student *t*-tests were performed for comparing two groups by using Prism and JMP software packages. $p \leq 0.05$ was considered as statistically significant. The number of mice per group and other details are mentioned in the figure legends.

4. Results

4.1. Ku70 over-expression attenuated AHR

Our lab had found a reduced expression of Ku70 in the bronchial epithelium of asthmatics (Rehman et al., 2020; Rehman and Mabalirajan, 2017). To further explore the possibility of using Ku70 as a therapeutic target, we wanted to investigate whether Ku70 over-expression can modulate asthmatic features. To determine the same, we have developed a mice model of ovalbumin-induced allergic asthma as shown in Fig. 1. There were three groups as detailed in the Methods section: a) SHAM/PBS/VEH; b) OVA/OVA/Control plasmid and c) OVA/OVA/Ku70 plasmid. The design of plasmid is shown in Fig. 2A. First, we checked the levels of Ku70 by ELISA and we found increased ku70 levels in OVA/OVA/Ku70 plasmid mice as compared to OVA/OVA/Control plasmid mice (Fig. 2B). It is to be noted that we did not find any difference in the levels of Ku70 between SHAM/PBS/VEH and OVA/OVA/Control plasmid. This could be attributed to the differential expression of Ku70 in different cell types like epithelial cells and B cells. In our recent study (Rehman et al., 2020) using dual staining using B cell marker and epithelial cell marker, we found that Ku70 is also expressed in B cells where antibody production needs the help of Ku70 and these B cells had shown up-regulation in the expression of ku70 in asthmatic mice. To further confirm the effect of Ku70 over-expression plasmid on the expression of ku70 in mice lungs, immunohistochemistry (IHC) was performed in mice lung sections. IHC revealed that Sham mice had shown the strong expression of Ku70 in bronchial epithelium (Fig. 2C). Though this expression in Sham mice is surprising, earlier literature indicated such constitutive presence of various DNA repair proteins in

lungs (Rehman et al., 2020). On the other hand, bronchial epithelium of asthmatic mice had shown a reduction in the expression of ku70 (Fig. 2C) compared to Sham mice. Though asthmatic mice had shown a reduction in the expression of Ku70 in bronchial epithelia, the increased expression was found in immune cells in the regions of inflammatory foci (Lower panels in Fig. 2C). Based on our earlier reports, these inflammatory cells might be B cells. In any event, its expression in other immune cells needs more investigation. Thus, the discrepancy between ELISA (Fig. 2B that showed no change in Ku70 levels between sham and asthmatic mice) and IHC (Fig. 2C that showed a reduction in Ku70 in bronchial epithelium of asthmatic mice) seemed to be attributable to differential expression of Ku70 in epithelial and B cells. Then we have measured airway hyperresponsiveness (AHR) to observe the effect of the Ku70 plasmid in lung function using increasing concentrations of Methacholine, a nonspecific stimulus. We found that OVA/OVA/Control plasmid mice had shown a dose-dependent increase in AHR with methacholine compared to SHAM/PBS/VEH mice, which indicated that ovalbumin had caused airway obstruction. However, treatment with Ku70 plasmid significantly reduced the AHR with increasing concentrations of methacholine (Fig. 3).

4.2. Ku70 over-expression reduced airway inflammation and airway eosinophilia

As there is a tight correlation between airway inflammation and airway hyperresponsiveness and there was a reduction in AHR with Ku70 over-expression in mice model of asthma, we wanted to determine its effect on airway inflammation. We performed H&E staining in formalin-fixed lung sections of mice. While SHAM mice has shown normal lung structure without any sign of inflammation, OVA mice has shown an increased inflammation with infiltration of immune cells around the bronchial and vascular regions as compared to SHAM mice. However, OVA induced Ku70 plasmid treated mice has shown a reduction in airway inflammation (Fig. 4A). The inflammation scoring analysis, that was performed as mentioned in the Methods section, further confirms the experimental finding (Fig. 4B). Next, we wanted to determine the nature of inflammation in the airways by performing Leishman staining of the cells recovered from BAL fluid. Expectedly, there was a significant increase in the eosinophil percentage in BAL fluids of OVA-induced mice whereas there was a significant reduction in the percentage of eosinophil count in OVA/OVA/Ku70 plasmid mice as compared to OVA/OVA/Control plasmid mice (Fig. 4C). However, there was no significant increase of neutrophil percentage in OVA-induced mice and also OVA/OVA/Ku70 plasmid could not reduce the

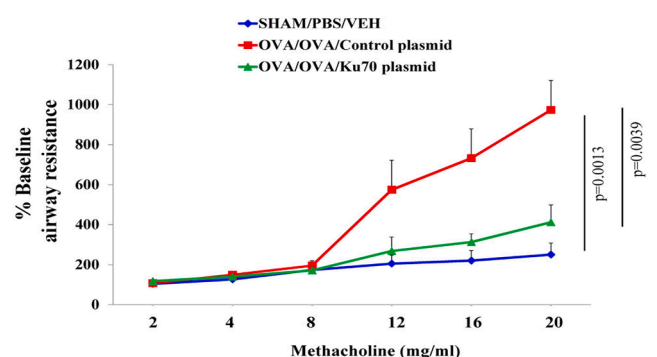


Fig. 3. Ku70 over-expression attenuated AHR. AHR was measured in mice with allergic airway inflammation that were treated with either Ku70 plasmid or control plasmid as described in Materials and Methods using invasive airway mechanic in anesthetized mice with increasing doses of methacholine. The results were expressed in percentage baseline resistance assuming PBS aerosol values as 100%. Data represents mean $\pm$ SE and the shown data is the representative of three independent experiments and there were 5–6 mice per group in each independent experiment.

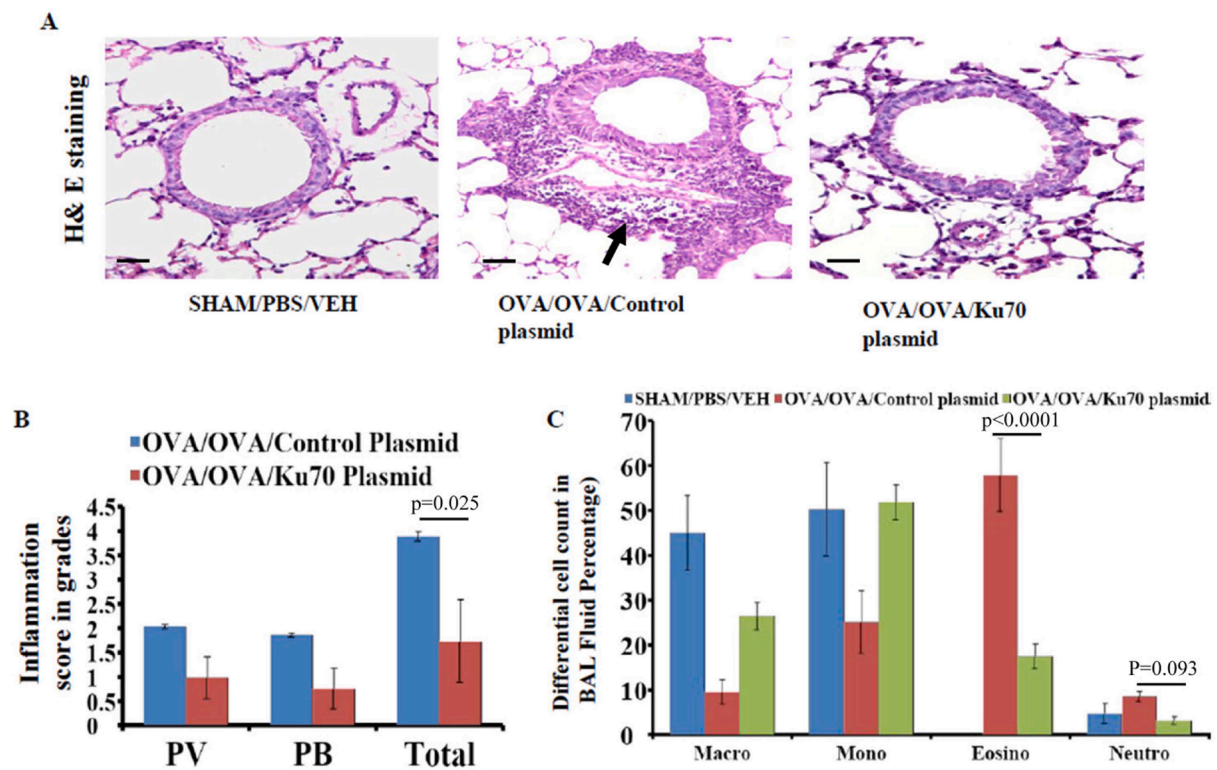


Fig. 4. Ku70 over-expression reduced airway inflammation and airway eosinophilia: A) Representative photomicrographs of lung sections of mice of different groups. Arrow indicates the infiltration of various inflammatory cells around the bronchovascular regions. Bars are 100 μ M. B) The inflammation score was determined as described in materials and methods and shown as perivascular (PV) and peribronchial (PB) and total (PV + PB). C) The differential cell count was calculated after counting the different types of cells in BAL fluid that were stained with Leishman's stain. Data represents mean $\pm$ SE and the shown data is the representative of three independent experiments and there were 5–6 mice per group.

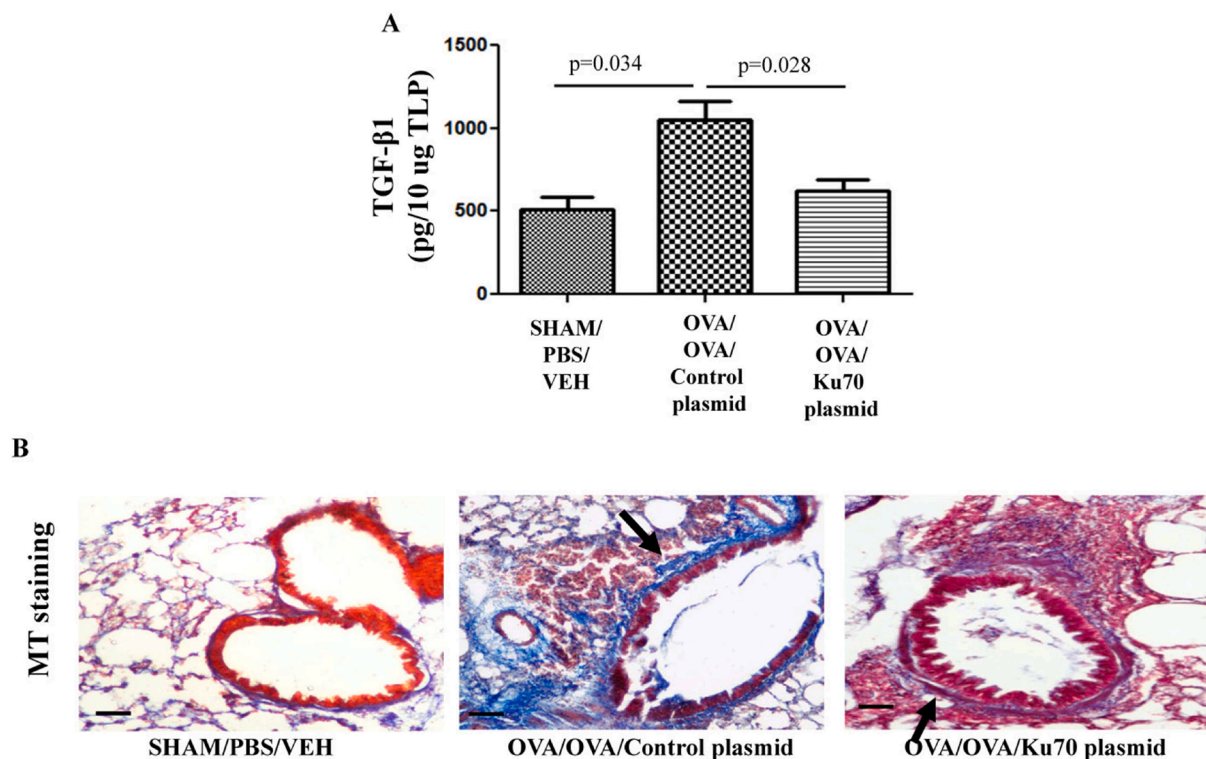


Fig. 5. Ku70 over-expression reduced sub-epithelial fibrosis: A) The levels of TGF- β 1 in total lung lysate in different mice groups. B) Lung sections were stained with MT stain to determine the sub-epithelial fibrosis. Arrows show the deposition of collagen fibers in peribronchial regions beneath the airway epithelium. Bars are 100 μ M. Data represents mean $\pm$ SE and the shown data is the representative of three independent experiments and there were 5–6 mice per group.

neutrophil percentage compared to OVA/OVA/control plasmid mice (Fig. 4C).

4.3. Ku70 over-expression reduced sub-epithelial fibrosis

As we found reduced AHR and inflammation upon Ku70 over-expression in Ova induced asthmatic mice, we wanted to determine the effect of Ku70 over-expression on sub-epithelial fibrosis. As shown in the Fig. 5A, TGF- β 1 (a cytokine that is involved in fibrosis) was significantly increased in the lungs of OVA/OVA/Control plasmid mice. Ku70 over-expression significantly reduced the levels of TGF- β as compared to OVA/OVA/Control plasmid mice. Similarly, MT staining in OVA/OVA/Control plasmid mice has shown a dense collagen deposition in the bronchovascular region; however, Ku70 over-expression in OVA mice reduced the collagen deposition as compared to OVA/OVA/Control plasmid mice (Fig. 5B).

4.4. Ku70 over-expression did not affect IL-13 levels and goblet cell metaplasia

The sub-epithelial fibrosis and goblet cell metaplasia are the two major features of asthmatic airway remodeling. So we wanted to determine the effects of Ku70 over-expression on goblet cell metaplasia. IL-13 is the key Th-2 cytokine that is involved in the pathogenesis of asthma and it is the major cytokine responsible for goblet cell metaplasia. In the lungs of OVA/OVA/Control mice IL-13 levels were found to be significantly increased as compared to SHAM/PBS/VEH mice, however it was not affected by Ku70 over-expression (Fig. 6A). To study goblet cell metaplasia, we performed PAS staining in the lung sections of mice and we also calculated the airway mucin content as described in Methods. OVA/OVA/Control plasmid mice showed increased goblet cell metaplasia but goblet cell metaplasia was not reduced in Ku70 plasmid treated OVA mice and quantitative morphometry also confirmed these findings (Fig. 6B& 6C). It is to be noted that though there was a drastic

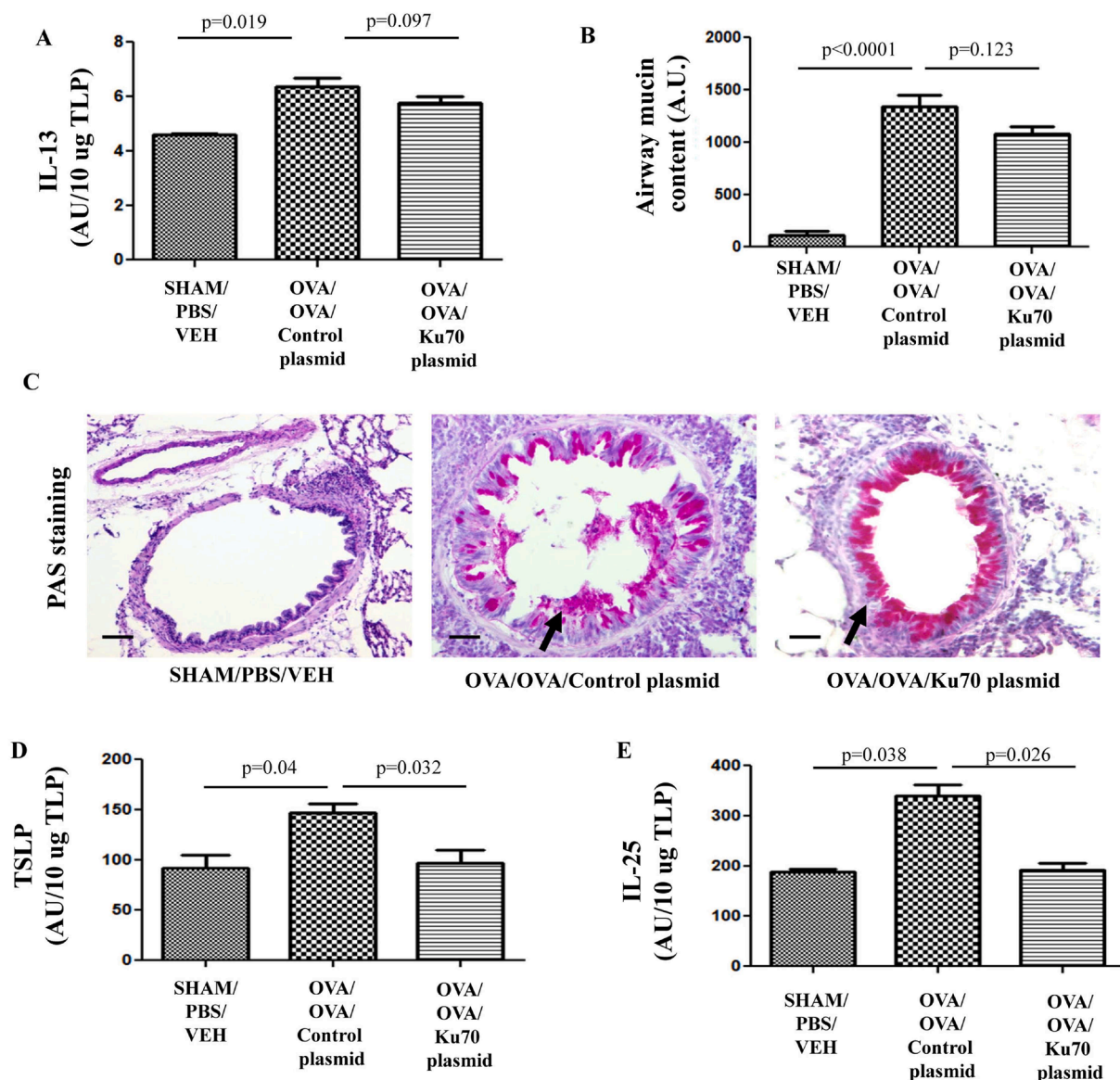


Fig. 6. Ku70 over-expression did not affect IL-13 levels and goblet cell metaplasia: A) IL-13 levels in total lung lysates of different mice groups. B& C) Lung sections were stained with PAS staining to determine the goblet cell metaplasia and PAS stained slides were assessed by morphometry to show the airway mucin content in arbitrary units (A.U.). Magenta colour indicates presence of mucin. Bars are 100 μ m. The levels of TSLP (D) and IL-25(E) in total lung lysates. Data represents mean $\pm$ SE; and the shown data is the representative of three independent experiments and there were 5–6 mice per group. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

increase of goblet cells in OVA mice lung sections, IL-13 seems to be modestly elevated in these lungs. This could be due to the involvement of other contributing factors like IL-4 and other cytokines.

Though there is a reduction of airway inflammation that was found with the Ku70 over-expression, there was no reduction in goblet cell metaplasia. This indicates that Ku70 over-expression might have improved cell survival as surviving epithelium is required for the generation of goblet cell metaplasia. So, it seems that Ku70 over-expression might have reduced the airway epithelial stress. Therefore we have measured the levels of lung epithelial secretory cytokines like IL-25 and TSLP (Byers, 2014; He and Geha, 2010). Expectedly, OVA/OVA/Control plasmid mice has shown upregulation in the levels of these two cytokines; indicating that the airway epithelium in this model might be under stress. However, Ku70 over-expression in Ova-induced asthmatic mice reduced the levels of both TSLP and IL-25 (Fig. 6D and 6E). Though both IL-25 and TSLP are strong indicators in the secreted form, tissue levels of these cytokines could also be good indicators of lung tissue function as demonstrated by the earlier studies wherein a good positive correlation exists between tissue levels and BAL fluid levels of both IL-25 and TSLP (Hams et al., 2014; Lee et al., 2017).

4.5. Effects of Ku70 over-expression on OVA specific immunoglobulins and 8-isoprostane

IL-13 is the key cytokine in asthma pathogenesis as it can induce number of asthma features and we found no reduction of IL-13 in Ku70 plasmid mice. On one hand IL-13 is responsible for the IgE class switching and on the other hand, Ku70 is required for VDJ recombination. So, it would be interesting to see the effects of Ku70 over-expression on the levels of OVA specific IgE. As shown in the Fig. 7A, OVA specific IgE was found to be increased in OVA induced asthmatic mice whereas it was found to be significantly reduced in the sera samples of OVA/OVA/Ku70 plasmid mice as compared to the control plasmid treated mice (Fig. 7A). But OVA-specific IgG2a was unaffected with Ku70 over-expression (Fig. 7B).

As Ku70 plasmid reduced the features of allergic airway inflammation, we wanted to see the effects on 8-isoprostane, a marker for oxidative stress. 8-isoprostane was found to be significantly increased in OVA/OVA/Control plasmid treated mice as compared to SHAM/PBS/VEH mice, however, it was reduced drastically in OVA/OVA/Ku70 plasmid mice (Fig. 7C).

4.6. Ku70 over-expression restores mitochondrial function and reduces bronchial epithelial apoptosis

While Ku70 is known to inhibit apoptosis via sequestration of Bax from the mitochondria, mitochondrial dysfunction is well known in asthma pathogenesis (Mabalirajan et al., 2008; Reddy, 2011). So, we wanted to determine the effects of Ku70 over-expression on key features of mitochondrial dysfunction like activities of complex IV & complex I in lung mitochondria and caspase-3 activity and cytochrome C in the lung cytosol. As shown in Fig. 8A, complex I activity in OVA/OVA/control plasmid mice was found to be reduced significantly as compared to SHAM/PBS/VEH mice; however, Ku70 over-expression restored it. Similarly, complex IV activity was reduced in OVA/OVA/Control plasmid mice as compared to SHAM/PBS/VEH and restored by Ku70 over-expression (Fig. 8B). Though we have not measured activities of other complexes (like II and III), future investigations are required to measure these. We have measured complexes I and IV as our lab had demonstrated the reduction in the activities of complex I and IV (Mabalirajan et al., J. Immunol. 2008) along with mitochondrial ultra-structural changes in bronchial epithelia of asthmatic mice. However, other labs had shown the alteration in other activities like complex III (Aguilera-Aguirre et al., 2009) in asthmatic mice.

As shown in Fig. 8C, caspase-3 activity in lung cytosol was increased in OVA/OVA/Control plasmid as compared to SHAM/PBS/VEH mice and restored by Ku70 over-expression in asthmatic mice. Cytochrome C levels in lung cytosol were found to be increased in OVA/OVA/Control plasmid mice (Fig. 8D), however Ku70 over-expression in OVA mice reduced it significantly as compared to OVA/OVA/Control plasmid mice

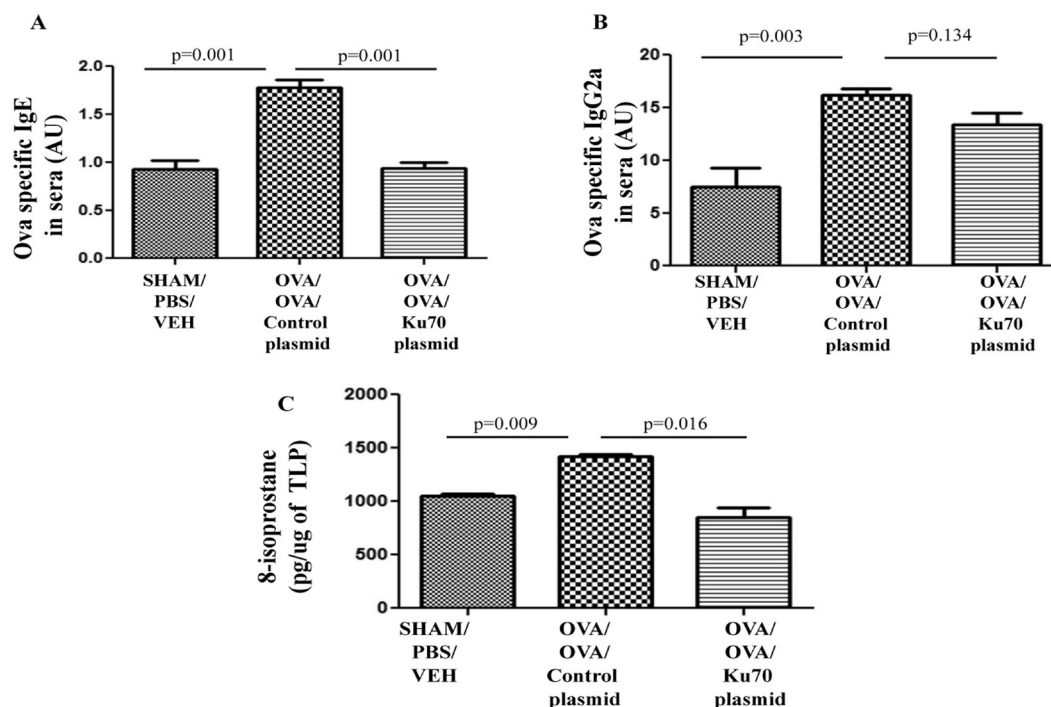


Fig. 7. Ku70 over-expression reduced ova specific IgE and 8-isoprostane but did not affect ova specific IgG2a: The levels of OVA specific IgE (A) and IgG2a (B) in sera of different mice groups. C) The 8-isoprostane was measured in total lung protein. Data represents mean $\pm$ SE; and the shown data is the representative of three independent experiments and there were 5–6 mice per group.

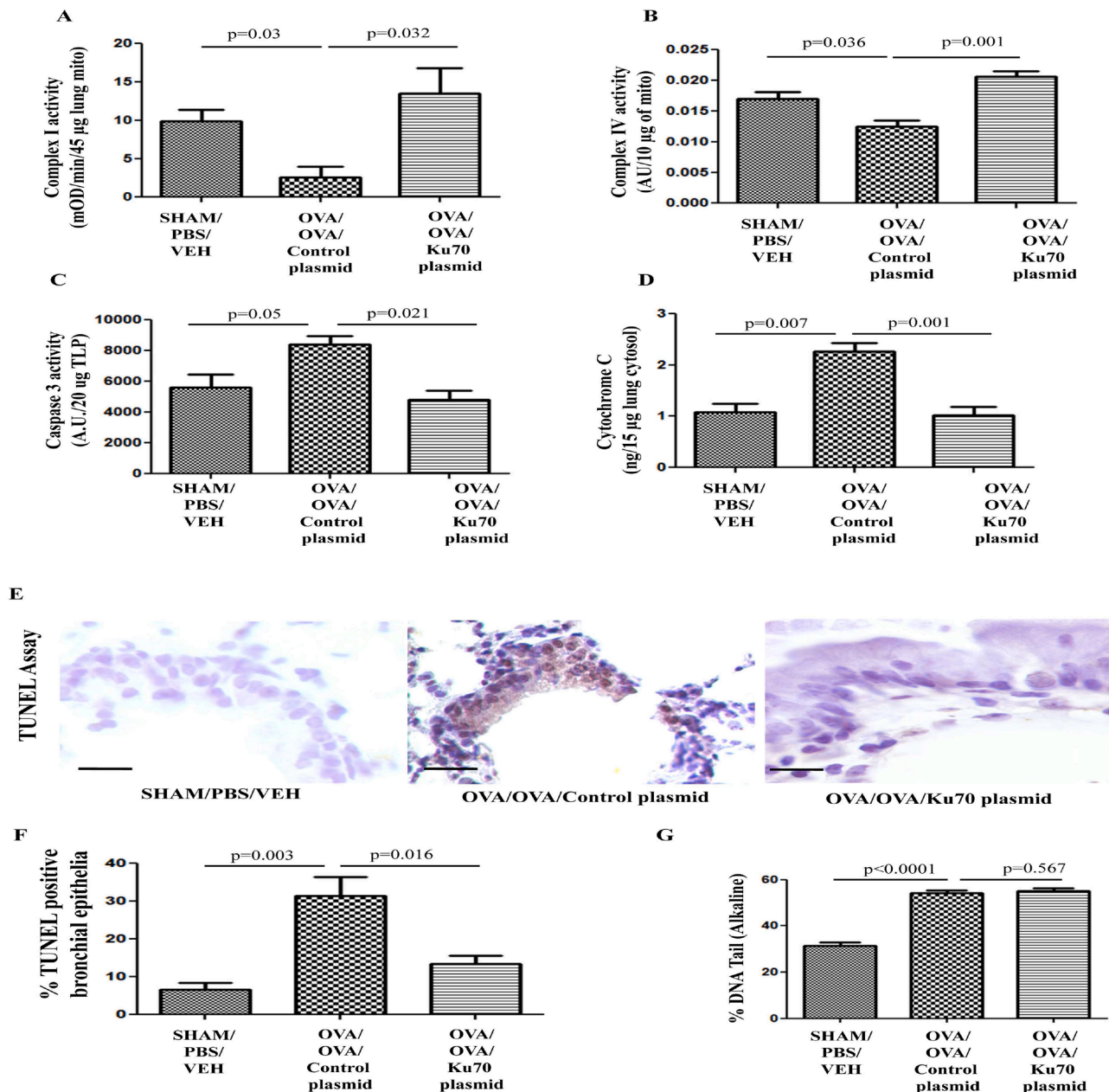


Fig. 8. Ku70 over-expression restores mitochondrial function and reduced the apoptosis of bronchial epithelium. The activities of Complex I (A) and complex IV (B) in lung mitochondria and caspase-3 (C) in lung cytosol were measured in different mice groups. D) The cytochrome C was measured using lung cytosol by ELISA. E) TUNEL staining was performed in formalin fixed lung tissue sections to determine the apoptosis. Brown color indicates the presence of damaged DNA. Bars are 50 µm. F) Apoptotic index was calculated based on the brown color present in airway epithelia. G) The DNA damage was assessed in single cell suspensions derived from the lungs of different mice groups and shown as percentage DNA tail. Data represents mean $\pm$ SE; and the shown data is the representative of three independent experiments and there were 5–6 mice per group.

(Fig. 8D). To examine whether Ku70 over-expression affects apoptosis of bronchial epithelium, TUNEL assay was performed. As shown in Fig. 8E, OVA/OVA/Control plasmid mice had shown increased bronchial epithelial apoptosis (Fig. 8E as indicated by brown color), however apoptosis was found to be reduced in OVA/OVA/Ku70 overexpressed mice (Fig. 8E). The apoptotic index confirmed these findings (Fig. 8F).

4.7. Ku70 over-expression did not affect DNA damage in asthmatic mice:

To know whether restoration of certain features of asthma by Ku70 over-expression is dependent or independent of DNA damage, we performed comet assay to assess the status of DNA damage. As shown in the Fig. 8G, OVA/OVA/Control plasmid mice have shown increased DNA

damage compared to SHAM/PBS/VEH control mice. In any event, Ku70 over-expression in OVA mice did not reduce the DNA damage.

5. Discussion

The homeostatic maintenance of organs that are directly exposed to the environment is very crucial for overall body health. The lung is one such organ that is continuously exposed to the environment and airway epithelium is the major cell type to respond to any exogenous irritants and allergens. So airway epithelium should have potent safeguard molecular mechanisms to maintain lung health. Its determining role in asthma pathogenesis is described in many studies (Byers, 2014; He and Geha, 2010; Mabalirajan et al., 2008; Swamy et al., 2010). One such

mechanism is the DNA repair mechanism. Report on the constitutive expression of various DNA repair proteins in bronchial epithelium (Caramori et al., 2011) indicates two things: a) the expression of these DNA repair proteins could be essential to repair DNA damage that could happen at baseline levels and b) there could be other moonlighting functions of these DNA repair proteins. As the end result of DNA damage/repair process is cell survival/death, various DNA repair proteins cross-talk with cell death related pathways. DNA damage is shown to be increased in asthma. This raises another interesting question whether the accumulated mutations due to DNA damage in asthmatic lungs could lead to lung cancer. Though this needs large scale epidemiologic studies, it would also open the avenue for using these DNA repair proteins as therapeutic target. But its role in DNA repair may preclude one to select Ku70 as a therapeutic target. Emerging literature indicates such moonlighting roles of DNA repair proteins in various diseases. Thus, it seems that the role of DNA repair proteins in asthma is not straightforward. For example, recently it has been reported that pro-inflammatory role of DNA-PK in asthma seems to be independent of either DNA repair role or Ku70 (Ghonim et al., 2018). These indicate that DNA repair proteins could have a moonlighting role in contrast to earlier belief. This kind of DNA repair independent role of DNA repair proteins also open a therapeutic avenue to target these proteins as it may not interfere with DNA repair functions. This also adds more complexity in devising therapeutic strategies against DNA repair proteins. So it seems that benefits of targeting DNA repair protein could be dependent on nature of the individual DNA repair protein.

Ku70 is one such DNA repair protein that also prevents the translocation of Bax from mitochondrial membrane and therefore acts as an anti-apoptotic protein (Amsel et al., 2008). Ku70 also has been shown to act as a target of sirtuins like SIRT3 and SIRT1 in such a way that these sirtuins can deacetylate Ku70 to promote the interaction of Ku70 with Bax and as a result apoptosis can be prevented (Amsel

et al., 2008). This indicates that Ku70 can also be targeted indirectly via sirtuins. Similarly, HDAC inhibitors, that are used to induce apoptosis in various cancer cells, also lead to Ku70 acetylation to cause apoptosis by the release of Bax (Jeong et al., 2007). Thus, being targets of various crucial mediators like HDAC inhibitors, sirtuins, Ku70 seems to be a potential target in various diseases where cell fate is the deciding factor in disease pathophysiology.

As our lab had demonstrated mitochondrial dysfunction in asthmatic condition (Mabalirajan et al., 2008), it would be interesting to see the role of Ku70 in asthma features. Recently, we have found that Ku70 is reduced in asthmatic airway epithelium. In this study, we have observed that Ku70 over-expression in asthmatic mice attenuated many features of asthma. As our lab and others have found the constitutive expression of Ku70 protein (Amsel et al., 2008; Rehman et al., 2020; Rehman and Mabalirajan, 2017), this indicates its possible role in epithelial homeostasis. We verified this by knocking down Ku70 using specific siRNA as Ku70 siRNA administered mice had shown spontaneous asthma like features (Rehman et al., 2020). However, in this study, we have demonstrated the beneficial role of Ku70 over-expression on asthma features. First, we checked the effects of Ku70 over-expression plasmid on AHR. Ku70 over-expression in OVA mice decreased AHR. Though the exact mechanism for reduction in AHR and asthma features in Ku70 plasmid treated mice is unclear now, we believe this could be attributable to this reason: Ku70 over-expression might have compensated the reduction in Ku70 in bronchial epithelium of asthmatic mice and thus improved its cell survival and this survived epithelium might have led to a reduction in AHR due to various known mechanisms (Fig. 9). Because injured airway epithelium leads to activation of fibroblast, this initiates sub-epithelial fibrosis that plays a role in AHR. In this regard, we found that Ku70 over-expression reduces sub-epithelial fibrosis possibly via reducing TGF-beta. In addition, we also found that Ku70 over-expression restores mitochondrial function and reduces the bronchial epithelial

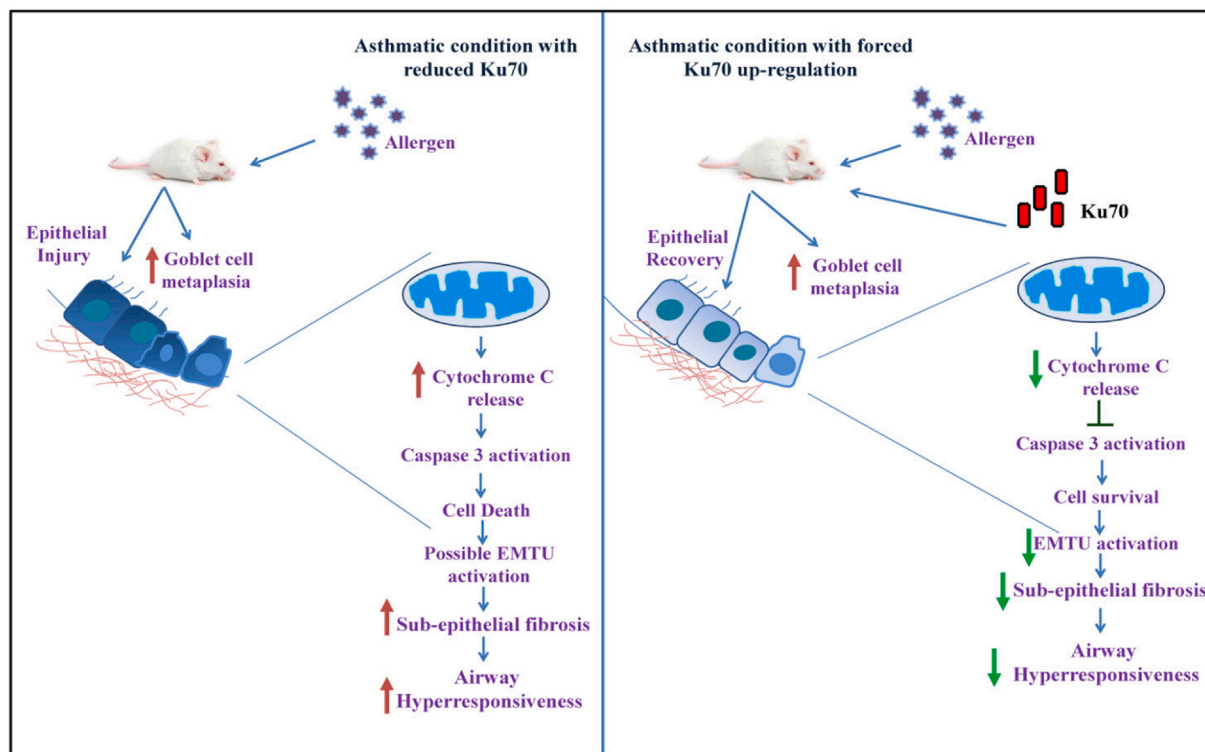


Fig. 9. Scheme diagram to illustrate the possible role of Ku70 in asthma pathogenesis and how Ku70 over-expression may reduce asthma features. EMTU, epithelial mesenchymal trophic unit. In asthmatic condition, reduced expression of Ku70 in bronchial epithelium may lead to the epithelial cell injury through sequestering Bax from the mitochondria and the resultant epithelial injury could be attributable to increased airway hyperresponsiveness (AHR) by causing sub-epithelial fibrosis. However, epithelial injury and its subsequent events including AHR may have reduced with Ku70 over-expression in asthmatic mice.

apoptosis in asthmatic mice. Various reports had demonstrated the presence of dysfunctional mitochondria in asthmatic airway epithelia (Mabalirajan et al., 2008; Reddy, 2011). Indeed, severe asthma has been shown to be associated with the mitochondrial haplogroup. In addition, a number of molecular mechanisms like increased expression of 15-lipoxygenase in inflammatory cells like macrophages that release metabolites called 13-S-HODE could cause increased mitochondrial calcium overload in bronchial epithelium to cause cell death (Mabalirajan et al., 2013a, 2013b). On the other hand, the replacement of dysfunctional mitochondria present in injured bronchial epithelial cells in asthmatic mice with healthy mitochondria by mesenchymal cells is sufficient to restore many features of asthma (Ahmad et al., 2014). This indicates that restoration of bioenergetics of airway epithelia could act as an anti-inflammatory strategy. In addition, both mitochondrial dysfunction and ER stress can lead to severe asthma by inducing inflammasome pathway. This also indicates the presence of a crosstalk between sub-cellular organelles and immune response. Restoration of mitochondria could be the possible mechanism for attenuation of certain features of asthma by Ku70 over-expression. However, DNA repair role of Ku70 for the reduction in asthma features is yet to be investigated. Further, one can also expect increased IgE with Ku70 overexpression as B cells need Ku70 for IgE class switching (Downs and Jackson, 2004). In contrast, we found the downregulation in OVA specific IgE in OVA/OVA/Ku70 plasmid mice. We believe that DNA repair role of ku70 might have not participated in these events as heterodimerization of Ku70 and Ku80 is required for DNA repair role (Downs and Jackson, 2004) whereas anti-apoptotic role of Ku70 does not need Ku80 (Downs and Jackson, 2004). While DNA repair role of Ku70 happens in the nucleus as a heterodimer, anti-apoptotic role happens in cytoplasm. So the attenuation of asthma features in Ku70 overexpressed mice might be independent of DNA repair role of Ku70.

Very interestingly, Ku70 over-expression did not affect goblet cell metaplasia. There may be two reasons behind this, since Ku70 is found to increase cell survival and reduce bronchial epithelial apoptosis, thus it could increase goblet cell metaplasia, and, we also found Ku70 over-expression did not affect IL-13, which is a key regulator for goblet cell metaplasia (Kondo et al., 2006). Because the goblet cell metaplasia needs two steps: a) epithelial cell survival and b) conversion of survived epithelial cells into goblet cell. Thus, we can conclude from our findings that Ku70 over-expression attenuates certain features of asthma in mice by restoring mitochondrial dysfunction. This shows therapeutic implication for Ku70 in asthma and requires detailed study. In contrast to mitochondrial dysfunction in asthmatic airway epithelia, hyperactive mitochondria have been reported in asthmatic airway smooth muscle and there is a good correlation between this mitochondrial hyperactivity and airway smooth muscle hypertrophy. Thus, mere general improvement of mitochondrial function may not be sufficient to improve the lung function. However, Ku70 over-expression mediated reduction in asthma features could be attributable to its predominant expression in airway epithelia compared to airway smooth muscle. In any event, it needs more investigations. In addition, we found DNA damage by performing COMET assay in total lung cells. While it would be ideal to perform COMET in individual cells like airway epithelium, certain immune cells, we have performed it in total lung cells. As we found a higher expression of 8-OHdG in asthmatic airway epithelium compared to other cell types in asthmatic lung (data not shown), the heightened DNA damage observed in asthmatic lung could be attributable to the heightened DNA damage in the airway epithelium. This also needs more investigation in order to understand the entire phenomena in detail.

We also found that Ku70 over-expression alone is sufficient to attenuate certain asthmatic features independent of DNA damage, which indicates the role of Ku70 other than DNA repair. Ku70 over-expression in asthmatic mice reduced the levels of 8-isoprostane indirectly, indicating that Ku70 over-expression reduces oxidative stress in asthmatic mice.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This study was supported by the projects MLP137 and MLP 126 at CSIR-Indian Institute Chemical Biology, Council of Scientific and Industrial Research, Govt. of India. RR, and AJ acknowledge Academy of Scientific and Innovative Research (AcSIR), Ghaziabad, India, for their Ph.D registrations.

References

- Aguilera-Aguirre, L., Bacs, A., Saavedra-Molina, A., Kurosky, A., Sur, S., Boldogh, I., 2009. Mitochondrial dysfunction increases allergic airway inflammation. *J. Immunol.* 183, 5379–5387.
- Ahmad, T., Mukherjee, S., Pattnaik, B., Kumar, M., Singh, S., Rehman, R., Tiwari, B.K., Jha, K.A., Barhanpurkar, A.P., Wani, M.R., Roy, S.S., Mabalirajan, U., Ghosh, B., Agrawal, A., 2014. Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. *EMBO J.* 33, 994–1010.
- Amsel, A.D., Rathaus, M., Kronman, N., Cohen, H.Y., 2008. Regulation of the proapoptotic factor Bax by Ku70-dependent deubiquitination. *PNAS* 105 (13), 5117–5122. <https://doi.org/10.1073/pnas.0706700105>.
- Bragonzi, A., Dina, G., Villa, A., Calori, G., Biffi, A., Bordignon, C., Assael, B.M., Conese, M., 2000. Biodistribution and transgene expression with nonviral cationic vector/DNA complexes in the lungs. *Gene Ther.* 7 (20), 1753–1760. <https://doi.org/10.1038/sj.gt.3301282>.
- Byers, D.E., 2014. Defining the Roles of IL-33, Thymic Stromal Lymphopoietin, and IL-25 in Human Asthma. *Am. J. Respir. Crit. Care Med.* 190 (7), 715–716. <https://doi.org/10.1164/rccm.201408-1539ED>.
- Caramori, G., Adcock, I.M., Casolari, P., Ito, K., Jazrawi, E., Tsaprouni, L., Villetti, G., Civelli, M., Carnini, C., Chung, K.F., Barnes, P.J., Papi, A., 2011. Unbalanced oxidant-induced DNA damage and repair in COPD: a link towards lung cancer. *Thorax* 66 (6), 521–527. <https://doi.org/10.1136/thx.2010.156448>.
- Downs, J.A., Jackson, S.P., 2004. A means to a DNA end: the many roles of Ku. *Nat. Rev. Mol. Cell Biol.* 5 (5), 367–378. <https://doi.org/10.1038/nrm1367>.
- Elias, J.A., Lee, C.G., Zheng, T., Ma, B., Homer, R.J., Zhu, Z., 2003. New insights into the pathogenesis of asthma. *J. Clin. Invest.* 111 (3), 291–297. <https://doi.org/10.1172/JCI17748>.
- Ghonim, M., Pyakurel, K., Luu, H., Okpechi, S., Ju, J., 2018. The catalytic subunit of DNA-PK has a unique function in inflammation independently of Ku70 and DNA repair: a new opportunity to target the enzyme without interfering with DNA repair. *J. Immunol.* 200 (1 Supplement), 173.18.
- Goula, D., Benoist, C., Mantero, S., Merlo, G., Levi, G., Demeneix, B.A., 1998. Polyethylenimine-based intravenous delivery of transgenes to mouse lung. *Gene Ther.* 5 (9), 1291–1295. <https://doi.org/10.1038/sj.gt.3300717>.
- Gyori, B.M., Venkatachalam, G., Thiagarajan, P.S., Hsu, D., Clement, M.-V., 2014. OpenComet: An automated tool for comet assay image analysis. *Redox Biol.* 2, 457–465. <https://doi.org/10.1016/j.redox.2013.12.020>.
- Hams, E., Armstrong, M.E., Barlow, J.L., Saunders, S.P., Schwartz, C., Cooke, G., Fahy, R. J., Crotty, T.B., Hirani, N., Flynn, R.J., Voehringer, D., McKenzie, A.N.J., Donnelly, S. C., Fallon, P.G., 2014. IL-25 and type 2 innate lymphoid cells induce pulmonary fibrosis. *Proc. Natl. Acad. Sci.* 111 (1), 367–372. <https://doi.org/10.1073/pnas.1315854111>.
- He, R., Geha, R.S., 2010. Thymic stromal lymphopoietin. *Ann. N. Y. Acad. Sci.* 1183, 13–24.
- Gossart J.-B., Kédinger V., Guérin-Peyrou G., Erbacher P., A.-L.B.-B., 2013. Application Note: Bioimaging of Gene Delivery with In Vivo-jetPEI, PerkinElmer, Inc.
- Jeong, Jaemin, Juhn, Kyungmi, Lee, Hansoo, Kim, Sang-Hoon, Min, Bon-Hong, Lee, Kyung-Mi, Cho, Myung-Haeng, Park, Gil-Hong, Lee, Kee-Ho, 2007. SIRT1 promotes DNA repair activity and deacetylation of Ku70. *Exp. Mol. Med.* 39 (1), 8–13. <https://doi.org/10.1038/emmm.2007.2>.
- Kondo, Mitsuko, Tamaoki, Jun, Takeyama, Kiyoshi, Isono, Kazuo, Kawatani, Kiyomi, Izumo, Takehiro, Nagai, Atsushi, 2006. Elimination of IL-13 Reverses Established Goblet Cell Metaplasia into Ciliated Epithelia in Airway Epithelial Cell Culture. *Allergol. Int.* 55 (3), 329–336. <https://doi.org/10.2332/allergolint.55.329>.
- Lee, Jong-Uk, Chang, Hun Soo, Lee, Hyeon Ju, Jung, Chang An, Bae, Da Jeong, Song, Hyun Ji, Park, Jong Sook, Uh, Soo-Taek, Kim, Young Hoon, Seo, Ki-Hyun, Park, Choon-Sik, 2017. Upregulation of interleukin-33 and thymic stromal lymphopoietin levels in the lungs of idiopathic pulmonary fibrosis. *BMC Pulm. Med.* 17 (1) <https://doi.org/10.1186/s12890-017-0380-z>.
- Mabalirajan, Ulaganathan, Ahmad, Tanveer, Leishangthem, Geeta Devi, Joseph, Duraisamy Arul, Dinda, Amit Kumar, Agrawal, Anurag, Ghosh, Balam, 2010. Beneficial effects of high dose of L-arginine on airway hyperresponsiveness and airway inflammation in a murine model of asthma. *J. Allergy Clin. Immunol.* 125 (3), 626–635. <https://doi.org/10.1016/j.jaci.2009.10.065>.

- Mabaliarajan, U., Ahmad, T., Rehman, R., Leishangthem, G.D., Dinda, A.K., Agrawal, A., Ghosh, B., Sharma, S.K., 2013a. Baicalein Reduces Airway Injury in Allergen and IL-13 Induced Airway Inflammation. *PLoS One* 8, e62916.
- Mabaliarajan, Ulaganathan, Aich, Jyotirmoi, Agrawal, Anurag, Ghosh, Balam, 2009a. Mepacrine inhibits subepithelial fibrosis by reducing the expression of arginase and TGF- β 1 in an extended subacute mouse model of allergic asthma. *Am. J. Physiol.-Lung Cell. Mol. Physiol.* 297 (3), L411–L419. <https://doi.org/10.1152/ajplung.00138.2009>.
- Mabaliarajan, Ulaganathan, Dinda, Amit Kumar, Kumar, Sarvesh, Roshan, Reema, Gupta, Pooja, Sharma, Surendra Kumar, Ghosh, Balam, 2008. Mitochondrial Structural Changes and Dysfunction Are Associated with Experimental Allergic Asthma. *J. Immunol.* 181 (5), 3540–3548. <https://doi.org/10.4049/jimmunol.181.5.3540>.
- Mabaliarajan, Ulaganathan, Dinda, Amit Kumar, Sharma, Surendra Kumar, Ghosh, Balam, 2009b. Esculetin Restores Mitochondrial Dysfunction and Reduces Allergic Asthma Features in Experimental Murine Model. *J. Immunol.* 183 (3), 2059–2067. <https://doi.org/10.4049/jimmunol.0900342>.
- Mabaliarajan, Ulaganathan, Rehman, Rakhshinda, Ahmad, Tanveer, Kumar, Sarvesh, Singh, Suchita, Leishangthem, Geeta D., Aich, Jyotirmoi, Kumar, Manish, Khanna, Kritika, Singh, Vijay P., Dinda, Amit K., Biswal, Shyam, Agrawal, Anurag, Ghosh, Balam, 2013b. Linoleic acid metabolite drives severe asthma by causing airway epithelial injury. *Sci. Rep.* 3 (1) <https://doi.org/10.1038/srep01349>.
- Makhija, Lokesh, Krishnan, Veda, Rehman, Rakhshinda, Chakraborty, Samarpana, Maity, Shuvadeep, Mabaliarajan, Ulaganathan, Chakraborty, Kausik, Ghosh, Balam, Agrawal, Anurag, 2014. Chemical Chaperones Mitigate Experimental Asthma by Attenuating Endoplasmic Reticulum Stress. *Am. J. Respir. Cell Mol. Biol.* 50 (5), 923–931. <https://doi.org/10.1165/rcmb.2013-0320OC>.
- Ram, A., Mabaliarajan, U., Jaiswal, A., Rehman, R., Singh, V.P., Ghosh, B., 2015. Parabromophenacyl bromide inhibits subepithelial fibrosis by reducing TGF- β 1 in a chronic mouse model of allergic asthma. *Int. Arch. Allergy Immunol.* 167, 110–118.
- Reddy, P.H., 2011. Mitochondrial Dysfunction and Oxidative Stress in Asthma: Implications for Mitochondria-Targeted Antioxidant Therapeutics. *Pharmaceuticals* 4, 429–456.
- Rehman, Rakhshinda, Mabaliarajan, Ulaganathan, 2017. Proficient DNA repair in house dust mite-induced asthma? *J. Allergy Clin. Immunol.* 139 (2), 713–714. <https://doi.org/10.1016/j.jaci.2016.07.045>.
- Rehman, R., Vijayakumar, V.E., Jaiswal, A., Jain, V., Mukherjee, S., Karuthedath Vellarikkal, S., Dieffenbach, P.B., Fredenburgh, L.E., Prakash, Y.S., Ghosh, B., Agrawal, A., Mabaliarajan, U., 2020. Non-canonical role for Ku70/80 in the prevention of allergic airway inflammation via maintenance of airway epithelial cell organelle homeostasis. *Am. J. Physiol. Cell. Mol. Physiol.* 319, L728–L741.
- Schlosser, K., Taha, M., Deng, Y., Stewart, D.J., 2018. Systemic delivery of microRNA mimics with polyethylenimine elevates pulmonary microRNA levels, but lacks pulmonary selectivity. *Pulm. Circ.* 8, 2045893217750613.
- Song, Ji Yeon, Lim, Joo Weon, Kim, Hyeyoung, Morio, Tomohiro, Kim, Kyung Hwan, 2003. Oxidative Stress Induces Nuclear Loss of DNA Repair Proteins Ku70 and Ku80 and Apoptosis in Pancreatic Acinar AR42J Cells. *J. Biol. Chem.* 278 (38), 36676–36687. <https://doi.org/10.1074/jbc.M303692200>.
- Song, YK, Liu, F, Liu, D, 1998. Enhanced gene expression in mouse lung by prolonging the retention time of intravenously injected plasmid DNA. *Gene Ther.* 5 (11), 1531–1537. <https://doi.org/10.1038/sj.gt.3300770>.
- Swamy, Mahima, Jamora, Colin, Havran, Wendy, Hayday, Adrian, 2010. Epithelial decision makers: in search of the 'epimmunome'. *Nat. Immunol.* 11 (8), 656–665. <https://doi.org/10.1038/ni.1905>.