House dust mite-induced asthma causes oxidative damage and DNA double-strand breaks in the lungs
House Dust Mite-induced Asthma Causes Oxidative Damage and DNA Double Strand Breaks in the Lungs

Tze Khee Chan, BSc, Xin Yi Loh, BSc, Hong Yong Peh, BSc, W. N. Felicia Tan, BSc, W. S. Daniel Tan, BSc, Na Li, PhD, Ian J. J. Tay, BEng, W. S. Fred Wong, PhD, Bevin P. Engelward, Sc.D.

1 Department of Pharmacology, Yong Loo Lin School of Medicine, National University Health System, Singapore
2 Immunology Program, Life Science Institute, National University of Singapore
3 Singapore-MIT Alliance for Research and Technology (SMART), Infectious Diseases Interdisciplinary Research Group, Singapore
4 Agency for Science, Technology and Research Graduate Academy, Singapore
5 Department of Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

*Corresponding author:
W.S. Fred Wong
Department of Pharmacology, Yong Loo Lin School of Medicine
Block MD3, 16 Medical Drive, Level 4, #04-01,
National University of Singapore, Singapore 117600
Tel: 65-6516-3266
Fax: 65-6873-7690
E-mail: phcwongf@nus.edu.sg
Acknowledgement of fundings:

This work was partly supported by a NMRC grant NMRC/CBRG/0027/2012 from the National Medical Research Council of Singapore, by Singapore-MIT Alliance for Research and Technology (SMART), and by CA26731 and the MIT NIEHS Center for Environmental Health Sciences (P30-ES002109).
Abstract

BACKGROUND: Asthma is related to airway inflammation and oxidative stress. High levels of reactive oxygen and nitrogen species (RONS) can induce cytotoxic DNA damage. Nevertheless, little is known about the possible role of allergen-induced DNA damage and DNA repair as modulators of asthma-associated pathology.

OBJECTIVE: To study DNA damage and DNA damage responses induced by house dust mite (HDM) \textit{in vivo} and \textit{in vitro}.

METHODS: We measured DNA double strand breaks (DSBs), DNA repair proteins and apoptosis in HDM-induced allergic asthma model and in lung samples from asthma patients. To study DNA repair, we treated mice with DSB repair inhibitor NU7441. To study the direct DNA damaging effect of HDM on human bronchial epithelial cells, we exposed BEAS-2B cells to HDM and measured DNA damage and ROS.

RESULTS: HDM challenge increased lung levels of oxidative damage to proteins (3-nitrotyrosine), lipids (8-isoprostane) and nucleic acid (8-oxoguanine). Immunohistochemical evidence for HDM-induced DNA DSBs was revealed by increased DSB marker, $\gamma$H2AX foci in bronchial epithelium. BEAS-2B exposed to HDM showed enhanced DNA damage as measured by comet assay and $\gamma$H2AX staining. In human asthma lung tissue, we observed increased levels of DNA repair proteins and apoptosis as shown by caspase-3 cleavage, caspase-activated DNase (CAD) level and TUNEL staining. Notably, NU7441 augmented DNA damage and cytokine production in bronchial epithelium and apoptosis in the allergic airway, implicating DSB as an underlying driver of asthma pathophysiology.
CONCLUSION: This work calls attention to RONS and HDM-induced cytotoxicity, and to a potential role for DNA repair as a modulator of asthma-associated pathophysiology.

Key messages
- HDM-induced allergic asthma causes significant increase in DNA double strand break in lung tissue, and specifically in bronchial epithelium.
- Direct exposure of human bronchial epithelial cells to HDM leads to the production of ROS and increased DNA damage in cells.
- Defect in DNA double strand break repair augments DNA damage, proinflammatory cytokine production and apoptosis in bronchial epithelium.

Capsule summary
HDM induces DNA DSBs in mouse and human lung epithelial cells in vitro and in vivo, and inefficient DNA repair enhances cell death and inflammation, pointing to DNA damage as a susceptibility factor for asthma.

Key words
HDM, Asthma, Oxidative stress, DNA Double strand breaks, BEAS-2B, Cell death, DNA repair, NU7441
Abbreviations used

3-NT: 3-nitrotyrosine
8-OHdG: 8-hydroxy-2-deoxyguanosine
8-oxoG: 8-oxoguanine
ATM: Ataxia-telangiectasia mutated
BAL: Bronchoalveolar lavage
CC10: Club cell 10 kD protein
CAD: Caspase-activated DNase
DCFH-DA: 2,7-Dichlorodihydrofluorescein diacetate
DDR: DNA damage response
DNAPKcs: DNA-dependent protein kinase catalytic subunit
DSB: Double strand break
EPO: Eosinophil peroxidase
H2AX: Histone 2AX
HDM: House dust mite
HR: Homologous recombination
MPO: Myeloperoxidase
NHEJ: Non-homologous end joining
PARP-1: Poly ADP-ribose polymerase 1
PAR: Poly ADP-ribose
PIKKs: PI3 kinase-related protein kinases
RONS: Reactive oxygen and nitrogen species
ROS: Reactive oxygen species
SSBs: Single-strand breaks
**Introduction**

Asthma is characterized by chronic inflammation of conducting airways resulting in bronchial obstruction and airway hyper-responsiveness \(^1\). Allergic asthma, the most common form of asthma, can be triggered by allergens such as house dust mite (HDM) \(^2\). In asthma, immune cells generate genotoxic reactive oxygen and nitrogen species (RONS), such as hydroxyl radicals (HO\(^-\)), superoxide (O\(_2^--\)), peroxides, peroxynitrite (ONOO\(^-\)) and nitric oxide (NO). RONS can be measured in peripheral blood, induced sputum and bronchoalveolar lavage (BAL) fluid of asthmatic lung \(^3,4\). RONS can damage lipids, proteins and nucleic acids \(^5\) and contribute to increased asthmatic severity \(^3\). Here, we reveal the genotoxic effects of aeroallergen-induced airway inflammation and oxidative stress on lung cells, and a direct DNA damaging effect of aeroallergen on bronchial epithelium.

We have previously shown that NO-induced base lesions and ONOO\(^-\)-induced single-strand breaks (SSBs) are central to large scale sequence rearrangements \(^6,7\). Among these DNA lesions, DNA double strand break (DSB) is one of the most cytotoxic forms and, if they are not properly repaired, they can lead to genomic rearrangements and cell death \(^8\). DNA DSBs are predominantly repaired by two major DNA repair pathways, homologous recombination (HR) and non-homologous end joining (NHEJ). HR ensures accurate repair of broken DNA by using a homologous strand as a template to restore lost genetic information \(^9\). This involves resection of the DNA end and formation of Rad51 nucleoprotein filament that is capable of homology searching. In contrast, NHEJ involves detection and direct joining of DNA ends, for which the DNA-dependent protein kinase catalytic subunit (DNAPKcs) is essential \(^8\). Defective DNA DSB repair plays a causative role in several
human diseases, including infectious disease, cancer risk, immune dysfunction and neurodegeneration. Here, we investigated the role DNA repair in asthma pathogenesis.

The DNA damage response (DDR) involves the actions of sensors, transducers and effectors, that together modulate DNA repair, cell cycle arrest and cell death. A key early step in response to DNA DSBs is the modification of chromatin. Specifically, the histone variant H2AX is phosphorylated to become γH2AX in the vicinity of the breakpoint, which is then able to recruit downstream damage response and DNA repair factors. DNA damage also causes induction of DNA repair proteins such as Rad51 and Ku70. Rad51 and Ku70 are key components in HR and NHEJ repair pathways, respectively. Indeed, cells that are deficient in either Rad51 or Ku70 have an increased sensitivity to DNA damage-induced cytotoxicity.

Here, we show that HDM-induced asthma leads to a significant increase in DNA damage in lung tissues, especially in the bronchial epithelium. Furthermore, direct exposure of human bronchial epithelial cells to HDM allergen in vitro induced ROS production and DNA damage. Inhibition of DNA repair by NU7441 worsened DNA damage in airway epithelial cells and augmented apoptosis in lung cells. Taken together, our findings suggest that DNA damage and repair impact asthma pathogenesis.
Methods

Animals. Female BALB/c mice 6-8 weeks old (Animal Resources Centre, Canning Vale, WA, Australia) were sensitized on days 0 and 7 and challenged on day 14 with 40 μl of 25 μg purified house dust mite (HDM) protein extract (*Dermatophagoides pteronyssinus*; Greer Laboratories, Lenoir, NC) via intratracheal administration. NU7441 (Axon Medchem, The Netherlands) was dissolved in 40% PEG400 and given at 10 mg/kg intraperitoneally twice daily on days 13, 14, and on days 1 and 2 post-HDM challenge. 40% PEG400 in saline was administered as vehicle control. Animal procedures were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

BAL fluid cells count. Mouse bronchoalveolar lavage (BAL) fluid was collected as previously described. Differential immune cell count was determined using BD Fortessa FlowCytometer and analyzed with Flowjo software (Treestar Inc., San Carlos, CA). Immune cells were identified as CD45+, macrophage as CD11c+/Siglec F+, eosinophils as CD11c-/Siglec F+, neutrophils as GR-1+/CD11b+ and T cells as CD3+/CD19-. The number per treatment group are n = 9-13 for saline control (S), 1, 3, and 5 days post-challenge (d.p.c) and n = 5 for 10 d.p.c.

2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay and detection of 8-OHdG/8-oxoG, 8-isoprostane and 3-nitrotyrosine (3-NT). For DCFH-DA assay, BAL fluid cells were incubated with 10 μM DCFH-DA (Invitrogen, Grand Island, NY) for 20 min at 37°C. BAL fluid was then spun down, re-suspended in RPMI medium (Invitrogen) and measured by a spectrophotometer with excitation at 492 nm and emission at 525 nm. To measure oxidative damage in lung, BAL fluid levels of 8-
isoprostane, 8-OHdG/8-oxoG (Cayman Chemical, Ann Arbor, MI) and lung level of 3-
NT (Cell Biolabs, San Diego, CA) were measured using enzyme immunoassays.
There are 13-15 mice for each treatment group.

**Human sample.** Paraffin-embeded human lung sections were purchased from
United State Biological (Salem, MA). Asthma lung sections (T5595-5112M) was
obtained from a 63-year-old female with a clinical diagnosis of asthma with unknown
cause of death. Normal lung section (T5595-4977) was obtained from a 83-year-old
female who passed away due to disease not related to lung. Human lung lysate were
purchased from Genetex (San Antonio, TX). Asthmatic lung lysate (GTX25167) was
obtained from a 85-year-old female patient that had been diagnosed with asthma for
25 years, and died of asthma. Donor for normal lung lysate is a 38-year-old female
whose lung has been found negative for HbsAg, HIV 1/1 and HCV. All other
information are inaccessible.

**Immunofluorescence staining.** Mouse lung tissues were processed as described
by Cheng *et al.* and immunofluorescence staining was performed as described by
Yamada *et al.* For immunocytochemical staining, human bronchial epithelial cells
BEAS-2B were cultured in RPMI supplemented with 10% fetal bovine serum (Atlanta
Biologicals) and 100 units/ml penicillin-streptomycin (Invitrogen). Cells were seeded
onto chamber slides and exposed to HDM (2, 20 or 200 μg/ml) for 6 hr at 37°C.
Slides were probed with antibodies targeted at γH2AX (Cell Signaling Technologies,
Danvers, MA), 8-OHdG (Abcam, Cambridge, MA), or Club Cell 10 kDa protein
(CC10, Santa Cruz Biotechnology, Dallas, TX), followed by DAPI staining. To
measure ROS production in cells, cells were stained with CellROX Green (Invitrogen).
Stained slides were mounted in SlowFade reagents (Invitrogen). Images were captured using the Zeiss fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY, USA) and were quantified using the Bitplane Imaris 5.7 software (Bitplane AG, Zurich, Switzerland). Quantification of images was performed on n = 3 mice per group for immunofluorescence staining where 10 images per lung section were quantified.

**Immunoblotting.** Mouse lungs were isolated and cleaned of connective tissues and bronchial lymph nodes before being snapped frozen in liquid nitrogen. Immunoblotting was performed as described previously. Antibodies used were targeted at Ku70, γH2AX, cleaved caspase-3 (Cell Signaling Technologies), Rad51 (Santa Cruz), PARP (Abcam), PAR (Calbiochem, San Diego, CA), or β-actin (Abcam).

**Comet assay.** BEAS-2B and human alveolar epithelial cell A549 were obtained from American Type Culture Collection (ATCC, Rockville, MD), and cultured in RPMI. BEAS-2B cells were exposed to HDM (2, 20, 200 μg/ml) for 6 hr at 37°C and alkaline CometChip assay was performed as described by Wood et al. As positive control for NU7441 activity, A549 were exposed to 50 μM NU7441 for 2 hr at 37°C and then irradiated with 50 Gy ionizing radiation using a Cobalt-60 irradiator (Gammacell 220 Excel, MDS Nordion, Ottowa, Canada). Neutral CometChip assay was performed as described by Weingeist et al.
Cytokine profiling with ELISA

BEAS-2B cells were seeded on 96-well plate and left to adhere overnight before being treated with HDM (2, 20 or 200 μg/ml) for 20 hr at 37°C. For NU7441 treatment, BEAS-2B cells were pretreated with NU7441 (2.5 μM) for 2 hr, washed off before exposing to HDM. As vehicle control, same concentration of DMSO was used to replace NU7441 treatment. Cell culture supernatant was collected and the levels of human cytokine were determined using sandwich ELISA kits for IL-4, IL-5, IFNγ (OptEIA, BD Biosciences), IL-13, IL-33 and TSLP (Duoset, R&D system, Minneapolis, MN, USA), according to manufacturer’s instruction. All samples were assayed in duplicate.

TUNEL and cell death staining

Apoptotic cells were detected in situ on lung sections using Apotag Peroxidase TUNEL assay (Merck Millipore, Billerica, MA). In vitro, cell death was studied by staining cells with Alexa Fluor® 488 annexin V/ Dead cell apoptosis kit (Thermo Scientific). BEAS-2B cells were exposed to HDM with the presence or absence of NU7441. Same concentration of DMSO was added in HDM control. BEAS-2B was harvested together with supernatant and washed once with PBS followed by staining with Annexin V and PI. Cell death was quantified using BD Fortessa FlowCytometer and analyzed with Flowjo software (Treestar Inc.). Dead cells were identified as Annexin V +/ PI – (early apoptosis) and Annexin V +/- PI + (late apoptosis) cells.

Measurement of airway hyperresponsiveness (AHR)

Development of AHR was measured by increased airway resistance (RI) and decreased dynamic lung compliance (Cdyn) in response to methacholine (1 - 32
mg/ml; Sigma-Aldrich) recorded using a wholebody plethysmograph chamber (Buxco, Sharon, CT). AHR was measured on Day 3 post-HDM challenge, as described in Cheng et al. 25.

Statistical analysis. All data are expressed as mean ± SEM. Statistical comparison of multi-group data was analyzed by one-way ANOVA followed by the Tukey’s for post-hoc analysis using GraphPad PRISM.
**Results**

**HDM induces airway eosinophilic inflammation.** Mice were sensitized to HDM on days 0 and 7, and challenged with HDM on day 14 to induce inflammatory responses to HDM (Fig 1, A) 20. We examined the inflamed airways up to 10 days post-challenge. HDM increased total infiltrated immune cell counts in BAL fluid, with rapid neutrophil influx followed by moderate increase in alveolar macrophages (Fig 1, B). The observed influx of neutrophils and their gradual replacement by eosinophils, which persisted up to 5 days post-challenge (Fig 1, B), resembles human allergic responses 26. Histological analysis revealed classical features of bronchial inflammation with a high cell infiltration score surrounding the bronchioles on 1, 3, and 5 days post-challenge (Fig 1, C).

**HDM induces airway oxidative damage.** HDM exposure caused a significant increase oxidative stress in BAL fluid (Fig 2), as measured by oxidation-sensitive fluorescent probe, 2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) 27. When the levels of RONS overwhelm antioxidant capacity, damage to cellular macromolecules may ensue 3. 8-isoprostone, an indicator of oxidative damage to lipids 28 was significantly increased on 3 and 5 days post-challenge. 3-nitrotyrosine (3-NT) is formed by the reaction of proteins with ONOO⁻, nitrogen dioxide (NO₂), or oxidation of nitrite (NO₂⁻) by myeloperoxidase (MPO) or eosinophil peroxidase (EPO), both of which are found in abundance in asthma 29. Results showed that the levels of tissue 3-NT were high on 1 and 3 days post-challenge (Fig 2), indicative of significant nitrosative protein damage in lung cells. Finally, to quantify oxidative damage to nucleic acids, we measured 8-OHdG/8-oxoG levels in BAL fluid and in lung tissue.
Levels of 8-OHdG were markedly increased on 1, 3 and 5 days post-challenge using both approaches.

**HDM induces DNA DSBs in bronchial epithelium.** To learn about DNA damage in allergic airways, we measured the frequency of cells that are positive for DNA DSBs marker γH2AX. Extensive phosphorylation flanking the DSB enables visualization of DSBs using immunofluorescence \(^{30}\). Bleomycin, known to induce DSBs, caused a significant increase in γH2AX (green) (Fig 3, A). Club cells, also known as Clara cells, are nonciliated secretory epithelial cells lining the pulmonary airway \(^{31}\). Co-staining with Club Cell 10 kD protein (CC10) showed that most of the DNA damage was in the bronchial epithelium. CC10 was used here as a marker to identify the airway epithelium layer.

HDM exposure significantly increased the frequency of γH2AX-positive cells, especially in airway epithelium. Higher magnification revealed that most γH2AX positive cells had a punctate pattern, indicative of repair foci (Fig 3, A, inset). The percentage of γH2AX-positive cells in asthmatic lung increased from day 1 to day 5 post-challenge (Fig 3, B). Immunoblotting of whole lung lysates showed a similar increase in the levels of γH2AX (Fig 3, C). Thus, both qualitative and quantitative analysis show a clear increase in DNA damage, as indicated by γH2AX, in HDM-induced asthma.

**DNA DSBs are evident in lung tissues from human asthma.** Immunostaining of paraffin-embedded lung sections obtained from patient who suffered from chronic asthma revealed higher levels of DNA DSBs shown by γH2AX staining (Fig 3, C).
Quantitative results showed a significant increase in the frequency of γH2AX-positive cells in human asthmatic lung (Fig 3, E). As these lung sections do not cut across bronchial airways, we could only confirm DNA DSBs in human peripheral lung. Nevertheless, immunoblotting of normal and asthmatic human lung lysates showed a marked increase in the levels of γH2AX (Fig 3, F).

**HDM directly induces DNA DSBs in human bronchial epithelial cells.** To elucidate the direct DNA damaging effect of HDM on bronchial epithelial cells, BEAS-2B cells were exposed to HDM (2, 20 and 200 μg/ml). DNA damage was analyzed by alkaline comet assay and staining of γH2AX. Alkaline comet assay is a single-cell gel electrophoresis assay for evaluation of a broad spectrum of DNA lesions in cells including SSBs and abasic site. HDM dose-dependently induced DNA damage in BEAS-2B cells, as revealed by the percentage of DNA in the comet tail (the amount of DNA in the tail is a reflection of DNA damage; Fig 4, A). Furthermore, HDM directly induced DNA DSBs in cells, as shown by γH2AX foci (green) staining (Fig 4, B). Quantification of γH2AX-positive cells (≥10 foci per nucleus) revealed more than 80% of the cells was severely damaged by HDM (200 μg/ml). These results clearly demonstrate the DNA damaging potential of HDM allergen on bronchial epithelial cells.

To investigate the ability of HDM to induce the production of ROS in BEAS-2B cells, we measure ROS through fluorescence emission of CellROX dye. Strong green signal was detected within the cells after cells were exposed to H₂O₂, which serves as a positive control. Bright green signal was also observed in cells after HDM exposure, suggesting that HDM stimulated cellular production of ROS (Fig 4, C).
These results suggest that, even without the presence of immune cells, HDM on its own can trigger the ROS in bronchial epithelial cells. Importantly, HDM-induced ROS have the potential to induce DNA damage.

**Asthma increases lung levels of DNA repair proteins.** To learn about DNA repair responses to asthma-induced DNA damage, we assessed the levels of two key DNA repair proteins, Rad51 and Ku70 (involved in HR and NHEJ, respectively). HDM challenge resulted in a robust increase in both Rad51 and Ku70 in mouse lung tissues (Fig 5, A). In response to SSBs, poly ADP-ribose polymerase 1 (PARP1) catalyzes the synthesis of poly ADP-ribose (PAR) chain. Notably, the levels of both PARP-1 and PAR were strongly elevated in HDM-challenged lungs (Fig 5, A). In parallel, we analyzed protein lysates from normal and asthmatic human lungs. There was a significant increase in the levels of Rad51, Ku70 and PAR (for technical reasons, PARP1 was not analyzed in the human samples) (Fig 5, B). Taken together, asthma significantly augmented the levels of key DNA repair proteins.

**NU7441 inhibition of NHEJ augments DNA DSBs in allergic airways.** DSBs repair NHEJ pathway is initiated by the binding of Ku70/80 heterodimer to the broken DNA ends, and forming complex with DNAPKcs. The observation that Ku70 protein levels were elevated in asthmatic lung (Fig 5, A and B) raises the possibility that NHEJ may be important in repairing DSBs during asthma. We used NU7441, a well-characterized inhibitor of DNAPKcs to study this possibility. Neutral comet assay was used to confirm the potential of NU7441 in inhibiting DNA DSB repair. Ionizing radiation is a potent inducer of DNA DSBs (see tail length at time zero in Fig 6, A). Irradiated cells repaired damaged DNA rapidly as indicated by the reduction in
tail length within 1 hr. Cells treated with NU7441 were unable to repair DNA DSBs (Fig 6, A).

In HDM mouse asthma, NU7441 was given twice daily for 2 days before and after the final HDM challenge (Fig 6, B). NU7441 markedly increased γH2AX level in allergic airway epithelium. Co-staining with CC10 indicated that DSBs were present in epithelial cells, and quantification revealed an increase in the frequency of γH2AX-positive cells, rising to significance on day 5 post-challenge (Fig 6, C and D).

We next studied the impact of NU7441 on the levels of DNA repair proteins. Immunoblotting of lung lysates revealed lower γH2AX levels in naive mice exposed to NU7441 alone, which is consistent with previous studies showing that inhibition of DNAPKcs can prevent phosphorylation of H2AX (Fig 6, E) \(^ {11}\). In contrast, HDM asthma mice treated with NU7441 showed an increase in the levels of γH2AX. While one might expect these DSBs are hard to be detected, given the fact that NU7441 inhibits DNAPKcs’s ability to phosphorylate H2AX, results shown in Fig 6, E are consistent with redundant PI3 kinase-related protein kinases (PIKKs) (e.g., Ataxia-telangiectasia mutated (ATM)) that also phosphorylate H2AX, leading to a strong γH2AX signal under conditions where DSB levels are high. In addition, the levels of Rad51 were further elevated in NU7441-treated HDM asthma mice (Fig 6, E), raising the possibility Rad51 facilitates HR repair of DSBs when NHEJ pathway is inhibited\(^ {35}\). Additionally, NU7441 reduced HDM-induced Ku70 levels (Fig 6, E), which is consistent with the possibility that NU7441 blocks a stabilizing interaction between DNAPKcs and Ku70, potentially leading to Ku70 degradation \(^ {36}\). Finally, NU7441 had
no effect on PARP-1 levels, which primarily impacts SSB repair, rather than DSB repair.

**NU7441 augments HDM-induced cytokine production in bronchial epithelial cells.** NU7441 significantly increased IL-4, IL-5, IL-13 and IL-33 production in BEAS-2B exposed to HDM, as compared to cells exposed to HDM alone (same concentration of DMSO was added as vehicle control) (Fig 7, A). These cytokines are epithelial cell-derived cytokines that promote Th2 responses in asthma. IL-4 is crucial for allergic sensitization and IgE production, IL-5 promotes eosinophil survival, IL-13 has a central role in airway remodeling, while, IL-33 activates lung DC. Notably, HDM was unable to trigger the production of TSLP and IFN-γ in BEAS-2B cells, and NU7441 did not alter the level of these two cytokines. Nevertheless, this result suggests that inhibition of NHEJ repair pathway by NU7441 in BEAS-2B potentiated inflammatory responses, highlighting the importance of DNA repair in suppressing airway inflammation.

**NU7441 enhances HDM-induced cell death in bronchial epithelial cells.** Exposure to HDM dose-dependently and time-dependently induced cell death in bronchial epithelial cells (Fig 7, B). Importantly, HDM-induced cell death was further enhanced by NU7441 (HDM + NU). NU7441 treatment alone (NU alone) did not induce cell death, suggesting that DNA repair inhibition was fatal only when the cells were exposed to HDM. One possible explanation is, HDM induced DNA damage in bronchial epithelial cells (Fig 3, A and Fig 4, B) and DNA damage activates DNA repair. When DNA repair was inhibited by NU7441, DNA damage induced by HDM...
was not efficiently repaired and hence, activated cell death pathway. We have shown that efficient DNA repair protected bronchial epithelial from HDM-induced cell death.

NU7441 enhances allergic airway inflammation-induced caspase 3 activation and cell death. To investigate the effects of NU7441 in asthma-induced lung cell death via apoptosis, we measured the levels of cleaved caspase-3 and caspase-activated DNase (CAD). HDM increased the lung levels of cleaved caspase-3 and CAD on 1 and 3 days post-challenge (Fig 8, A). NU7441 treatment resulted in a small but consistent increase in the levels of cleaved caspase-3 in HDM mouse asthma (Fig 8, B). Further, analysis using TUNEL staining showed much higher levels of apoptosis in HDM-challenged mice than control. Consistent with in vitro data, NU7441 led to an even greater increase in HDM-induced lung apoptosis, especially evident on 5 days post-challenge (Fig 8, C and D).
Discussion

It is estimated that 300 million people suffer from asthma worldwide leading to enormous socioeconomic costs. Despite its prevalence, little is known about the cytotoxic effects of aeroallergen-induced asthma on the airways. HDM is one of the most common aeroallergens, affecting approximately 50%~85% of asthmatic patients. Being the most problematic allergen, our studies focus on HDM-induced allergic asthma. Here, we showed that HDM exposure causes airway inflammation, oxidative damage, DNA damage and cell death in the airway. In addition, direct HDM exposure to bronchial epithelial cells in vitro induces DNA damage accompanied by increased ROS production. In human and mouse asthma lung tissue, we observed increased levels of DNA repair proteins and apoptosis. When HDM asthma mice were treated with a chemical inhibitor of DSB repair, DNA damage levels and apoptosis are further enhanced. In addition, when DSB NHEJ repair protein, DNAPK was inhibited in bronchial epithelial cells in vitro, we observed significant increase in HDM-induced production of proinflammatory cytokines and cell apoptosis. These studies point to a potential role of DNA DSBs and DNA repair in regulating asthma pathophysiology.

It is well established that asthma causes infiltration of immune cells, including eosinophil and neutrophils that are capable of producing high levels of RONS. Lysosomal enzyme MPO, the most abundant protein stored in neutrophil granules, together with EPO from eosinophils, catalyse the oxidation of halides by $\text{H}_2\text{O}_2$ to form hypochlorous acid (HOCl) and hypobromous acid (HOBr) respectively. Hypohalous acids react with $\text{O}_2^{-}$ to form $\text{OH}^-$, an extremely reactive radical that is capable of damaging cellular molecules. The influx of neutrophils on day 1 post-challenge, followed by eosinophil influx 2 days later, filled the HDM allergic airway with
significant amount of MPO and EPO. We observed a sharp rise in ROS levels in BAL fluid and increased levels of RONS-induced damage to macromolecules in HDM allergic airway. These data suggest that during allergic airway inflammation, ROS produced by inflammatory cells could lead to oxidative damage in lungs.

Although most RONS-induced DNA lesions are base damages (such as 8-oxoG, 8-nitroG and deamination products), RONS can directly induce SSBs. Importantly, SSBs formed either chemically or enzymatically can be converted to DSBs if in close proximity to opposing strands, or if encountered by a replication fork. The observation that asthma is associated with γH2AX foci is consistent with the induction of DSBs and possibly stalled replication forks. Our results have extended those reported by Chapman et al. which showed that asthma could induce systemic genotoxicity, as measured by micronucleus formation and protein nitration in peripheral blood. Results described here indicate that HDM-challenged bronchial epithelial cells show induction of DNA damage and repair, in mouse cells, mouse asthmatic lung and in human asthmatic lung.

To learn the potential biological relevance of asthma-induced DSBs, we explored the impact of DSB repair on asthma pathology by using the DNA DSB repair inhibitor NU7441. The catalytic activity of DNAPKcs is essential for NHEJ pathway which is inhibited by NU7441. Indeed, DSB repair was greatly impaired in alveolar epithelial cells pre-treated with NU7441. In HDM asthma mouse treated with NU7441, we observed a significant increase in γH2AX, which is consistent with defective repair leading to increased levels of DSBs. In addition, NU7441 further increased apoptosis, in both mice asthmatic lungs and *in vitro* bronchial epithelial cells exposed to HDM.
These studies point to a possible role for DNA repair in protecting against asthma-induced DNA damage and apoptosis.

While the major focus of this work has been on DNA DSBs, SSBs also can play an important role in tissue pathology. SSBs are recognized by PARP, which can poly-ADP-ribosylate downstream substrates, depleting the NAD+ pool. Here, we observed that PARP activity was induced by asthma, as shown by the high levels of PAR. Excessive DNA damage leads to high levels of PAR, and the resultant energy depletion can lead to necroptosis. Interestingly, necroptosis during airway inflammation has recently been shown to contribute to severe asthma development. Our results are consistent with the possibility that RONS-induced SSBs trigger energy depletion and resultant pathologies.

DSBs are formed not only by exposure to genotoxic agents, but they are also formed endogenously during V(D)J recombination by immature B- and T-lymphocytes, enabling hypermutation that is necessary for the adaptive immune response. However, V(D)J recombination only takes place in lymphoid tissues, such as the bone marrow, thymus and lymph nodes, and only after B and T lymphocytes have completed V(D)J recombination, mature lymphocytes will migrate into the lungs. In our experiments, we carefully trimmed lymph node and excess tissue and fat from lungs before freezing them, hence, it is unlikely that the DSBs observed in asthmatic lung are from V(D)J recombination.

DNAPK, not only plays a role in NHEJ repair, it is also crucial in maintaining lymphocytes V(D)J recombination and immunoglobulin class switching. Therefore,
using animal model to study the effects of NU7441 on airway inflammation will have
unavoidable confounding factor, as NU7441 affects T cell development. Hence, to
directly elucidate the biological consequences of DNA repair inhibition, specifically, in
bronchial epithelial cell, we performed in vitro experiment and measured
proinflammatory cytokine production by BEAS-2B cells. We revealed that NU7441
significantly augmented the levels of IL-4, IL-5, IL-13 and IL-33 in BEAS-2B cells
exposed to HDM. These cytokines play key roles in driving the important features of
allergic airway inflammation and airway remodeling in asthma. These data
demonstrate that a defect in DNA repair exacerbates the inflammatory response
during allergen exposure. As the inflammatory response is a well-established cause
of asthma-induced pathology, these data provide direct evidence for the importance
of DNA repair in suppressing asthma-induced pathology.

After demonstrating the potential of DNA repair in suppressing proinflammatory
responses in bronchial epithelial cells, we wish to learn about the potential
importance of DNA repair during an acute asthmatic attack. We exposed animals to
NU7441 and subsequently measured airway hyperresponsiveness (AHR) in
response to HDM (Fig E1). Interestingly, we did not detect worsening of lung function
in HDM-induced asthma. AHR is caused by multiple factors and DNA damage may
just be one of the many factors that regulate AHR. Another possible explanation for
not observing an impact of NU7441 on AHR may be the short duration (only 4 days)
of DNA repair inhibition. Moreover, this is an acute asthma model. Our data point to
relevance in a chronic asthma condition where persistent inflammation contributes to
pathology, hence, further studies are warranted.
Interestingly, two other studies by Ghonim et al. and Misha et al. have demonstrated that long term inhibition of DNAPK has beneficial effects on asthma\(^{52,53}\). Ghonim et al. showed that DNAPK inhibition reduced airway inflammation and AHR\(^{52}\). On the other hand, Mishra et al. showed that DNAPK inhibition in dendritic cells reduced airway inflammation\(^{53}\). While both of these studies focused on the anti-inflammatory aspect of DNAPK inhibition, here, we focused on the acute effect of DNAPK inhibition by NU7441. Specifically, we studied DNA damage in bronchial epithelium and revealed that efficient DNA repair is important in preventing DNA damage induced by asthma. We have shown that, in bronchial epithelial cells, defect in DNA repair exacerbates inflammatory responses. We also examined lung cell death when DNAPK is inhibited, both of which were not studied in these two papers. We revealed that DNAPK inhibition increased lung cell death in both HDM asthma mouse lung and isolated human bronchial epithelial cells exposed to HDM. This could be an important but unrecognized side effects of DNAPK inhibitor. Indeed, long-term inhibition of DNA DSB repair in humans is known to pose risk to disease development\(^{54}\). For example, 70% of Ataxia Telangiectasia Mutated (ATM) patients, who suffer from chronic depletion of DNA repair, developed lung diseases, which are the main causes of death in ATM patients\(^{54}\). Reversible airway obstruction, one of the characteristics of asthma, has also been reported in ATM patients\(^{55}\). This suggests that long-term inhibition of DNA DSB repair may have an adverse impact on airway function.

DNA repair may also play a role in preventing asthma comorbidities. The observation that HDM induces DNA damage in epithelial cells raises the possibility that DNA damage contributes to defective epithelial tight junctions, which have been observed in asthmatic biopsies\(^{56}\). Being the first line of defense against pathogens, impairment
of barrier function is thought to impact the susceptibility of asthmatic airways to respiratory viral or microbial infections. Further studies are warranted to learn more about the possible roles of DNA repair in asthma pathogenesis. In addition to HDM allergen, other aeroallergens such as cockroach allergen extract, ragweed pollen extract and *Aspergillus fumigatus* fungal, which have demonstrated different degree of genotoxicity on bronchial epithelial cells (data not shown), will be studied in the future as well.

Taken together, these studies show that HDM-induced airway inflammation is associated with increased RONS, increased DNA DSBs, changes in DNA repair protein expression, and increased apoptosis. Further, direct exposure to HDM leads to potentially cytotoxic levels of DNA damage in bronchial epithelial cell in both *in vivo* and *in vitro*. While further studies are needed to ascertain the specific impact of unrepaired DSBs, these studies point to the possibility that DNA repair plays a role in modulating susceptibility to asthma-associated pathologies. In particular, damage to DNA in epithelial cells raises the possibility that barrier function may be compromised. Finally, we have shown that the impact of asthma on DNA damage and apoptosis is conserved from mice to humans. Taken together, these studies suggest a possible role for DNA repair in maintaining and restoring airway function during asthma.

**Acknowledgements**

We would like to thank Dr Orsolya Kiraly for the helpful discussion and comments. We would also like to thank Dr Yin Lu for his help in quantifying our lung sections.
Reference:


40. Collins JA, Schandi CA, Young KK, Vesely J, Willingham MC. Major DNA fragmentation is a late event in apoptosis. J Histochem Cytochem 1997; 45:923-34.


57. Busse WW, Lemanske RF, Jr., Gern JE. Role of viral respiratory infections in asthma and asthma exacerbations. Lancet 2010; 376:826-34.

**Figure legend**

**FIG 1.** HDM induces inflammatory cell recruitment to the airway. **A,** HDM-induced airway inflammation model. **B,** Quantification of immune cells in BAL fluid on 1, 3, 5 and 10 day post-challenge (d.p.c). Total immune cells (Total), alveolar macrophage (Φ), eosinophil (Eos), neutrophil (Neu), and T lymphocyte (T cells), were quantified. Values are the mean ± SEM. **C,** Representative images of H&E-stained lungs. Quantification of inflammatory cell infiltration in lungs according to Bao Z, *et al.* Micrographs were taken at 200x magnification. *P < 0.01, #P < 0.05, significant difference from saline control (S).*

**FIG 2.** Increased levels of oxidative damage in BAL fluid and lung tissues. Estimated level of ROS in the BAL fluid obtained from mice on 3 day post-challenge (d.p.c), measured using DCFH-DA. Levels of 8-isoprostane, 3-nitrotyrosine and 8-OHdG/8-oxoG were measured using ELISA and immunofluorescence staining of lung sections (bottom right). Negative control (-ve) indicates secondary antibody alone. Values are the mean ± SEM. *P < 0.01, #P < 0.05, significant difference from saline control (S).*

**FIG 3.** Asthma induces DNA DSBs in asthmatic mouse and human lungs. **A,** Analysis of γH2AX (green), CC10 (red) and DAPI (blue) in lung sections. Negative control (-ve) indicates secondary antibody alone and positive control is lung treated with bleomycin (Bleo). **B,** Quantification of γH2AX-positive cells (≥ 5 foci). **C,** γH2AX protein levels measured by immunoblotting. **D,** Analysis of γH2AX (green) in human normal and asthmatic lung tissue. **E,** Quantification of γH2AX-positive cells (≥ 5 foci). **F,** γH2AX protein levels quantified using immunoblotting. Values are the mean of n=3 ± SEM. *P < 0.01, significant difference from healthy control.*
FIG 4. HDM induces direct DNA DSBs in BEAS-2B cells. A, Representative images of alkaline comets. Percentage of DNA intensity in the comet tail, which reflects the level of DNA damage, was quantified. B, Cells were stained for γH2AX (green) and nuclei are stained with DAPI (blue). Cells with ≥10 foci were counted as positive. C, Cells were stained with CellROX to measure oxidative stress level. All experiments were repeated at least 3 times and data presented was mean ± SEM. * P < 0.01, # P < 0.05 significant difference from untreated.

FIG 5. Asthma induces DDRs as indicated by increased levels of Rad51, Ku70, PARP-1, and PAR. A, Lung protein extract analyzed by western for Rad51, Ku70, PARP-1 and PAR. B, Human lung lysate from normal or asthmatic lung were immunoblotted with Rad51, Ku70 and PAR antibodies. β-actin serves as an internal control. Images show representative results for one of three or more experimental replicates.

FIG 6. DNA repair inhibitor increases DNA DSBs and DDRs. A, Analysis of DNA DSBs using neutral comet analysis after exposure to γIR. Tail length reflects the frequency of DNA DSBs 32. B, NU7441 treatment timeline. C, Lung sections were stained for γH2AX (green), CC10 (red) and DAPI (blue). D, Quantification of cells positive for γH2AX (≥ 5 foci), excluding cells with pan-nuclear γH2AX staining. E, Lung protein extracts were analyzed by immunoblotting for γH2AX, Ku70, Rad51, and PARP-1. Experiments were repeated at least three times. * P < 0.01 significant difference from HDM-only control.

FIG 7. NU7441 increases proinflammatory cytokine production and induces cell death in BEAS-2B exposed to HDM. A, Levels of cytokine IL-4, IL-5, IL-13, IL-33, TSLP, IFNγ in culture supernatant were quantified using ELISA. B, Cell death assay was performed using
Annexin V/PI staining. Cells with Annexin V +/ PI – and Annexin V +/ PI + were dead cells. All experiments were repeated at least 3 times and values shown are mean ± SEM. * $P < 0.01$, # $P < 0.05$, significant difference from HDM-alone control.

**FIG 8.** Asthma-induced cell death is enhanced by NU7441. A, Immunoblotting of cleaved caspase-3 and CAD. B, Immunoblotting of cleaved caspase-3 in HDM asthma lungs with and without NU7441 treatment. C, Analysis of apoptosis by TUNEL staining, where apoptotic cells are stained brown. Negative control (-ve) indicates staining without TdT enzyme. Positive control (+ve) indicates lungs treated with DNase I. D, Quantification of TUNEL-positive cells. Values shown are mean ± SEM. * $P < 0.01$, significant difference from HDM-only control.
Figure 1

A

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Day | Day post-challenge (d.p.c)

B

- Saline (S)
- 1 d.p.c
- 3 d.p.c
- 5 d.p.c
- 10 d.p.c

Cell count ($10^5$)

- Total
- Alveo $\Phi$
- Eos
- Neu
- T cells

C

- Saline (S)
- 1 d.p.c
- 3 d.p.c
- 5 d.p.c

Inflammation score

- S
- 1
- 3
- 5

d.p.c
Figure 2

DCFH-DA Fold Change (%)

Saline (S) 3 d.p.c

8-isoprostane

pg/ml BAL fluid

S 1 3 5
d.p.c

3-nitrotyrosine

pM/mg protein

S 1 3 5
d.p.c

8-OHdG/8-oxoG

pg/ml BAL fluid

S 1 3 5
d.p.c

8-OHdG IF

+ve count/image

-ve S 1 3 5
d.p.c
Figure 3

**Mouse Lung**

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**Human Lung**

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Mouse Lung

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Mouse lung lysate

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Human Lung

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Human lung lysate

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Human lung lysate

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Figure 5

A  Mouse Lung Lysate  B  Human Lung Lysate

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Healthy  Asthma

Rad51  Ku70  PAR  β-actin
Figure 6

A. DNA Double Strand Breaks

![Graph showing DNA Double Strand Breaks over time with treatments DMSO, NU7441, and HDM.]

B. Sample collection timeline

![Timeline illustrating sample collection days with RNA extraction and HDM exposure.]

C. Immunofluorescence images of γH2AX, CC10, and DAPI

5 d.p.c and 5 d.p.c + NU

D. Bar graphs showing % γH2AX positive

![Bar graphs with % γH2AX positive for different treatment conditions and days post challenge (d.p.c).]

E. Western blot analyses

![Western blot images with bands for γH2AX, β-actin, Ku70, Rad51, PARP-1, and β-actin from different treatment conditions and days post challenge.]
Figure 7 A  Cytokine Expression

IL-4 (pg/ml)  IL-5 (pg/ml)  IFN-γ (pg/ml)
Un-treated NU alone  HDM (μg/ml)  #
2  20  200  #

IL-13 (pg/ml)  IL-33 (pg/ml)  TSLP (pg/ml)
Un-treated NU alone  HDM (μg/ml)  NU
2  20  200  2  20  200

B  Cell Death

6 hours

Untreated  NU alone  Control
HDM alone  HDM + NU
2 μg/ml  20 μg/ml  200 μg/ml

20 hours

Untreated  NU alone  Late apoptotic  Early apoptotic
HDM alone  HDM + NU
2 μg/ml  20 μg/ml  200 μg/ml

Annexin V

% cell death
Un-treated NU alone  HDM (μg/ml)
2  20  200  2  20  200

% cell death
Un-treated NU alone  HDM (μg/ml)
2  20  200  2  20  200

* Indicates significant difference.
Supplementary Figure E1

Cdyn (Fold change over baseline)

Methacholine (mg/ml)

PBS 1 2 4 8 16 32

Saline HDM HDM + NU

RI (Fold change over baseline)

Methacholine (mg/ml)

PBS 1 2 4 8 16 32

Saline HDM HDM + NU