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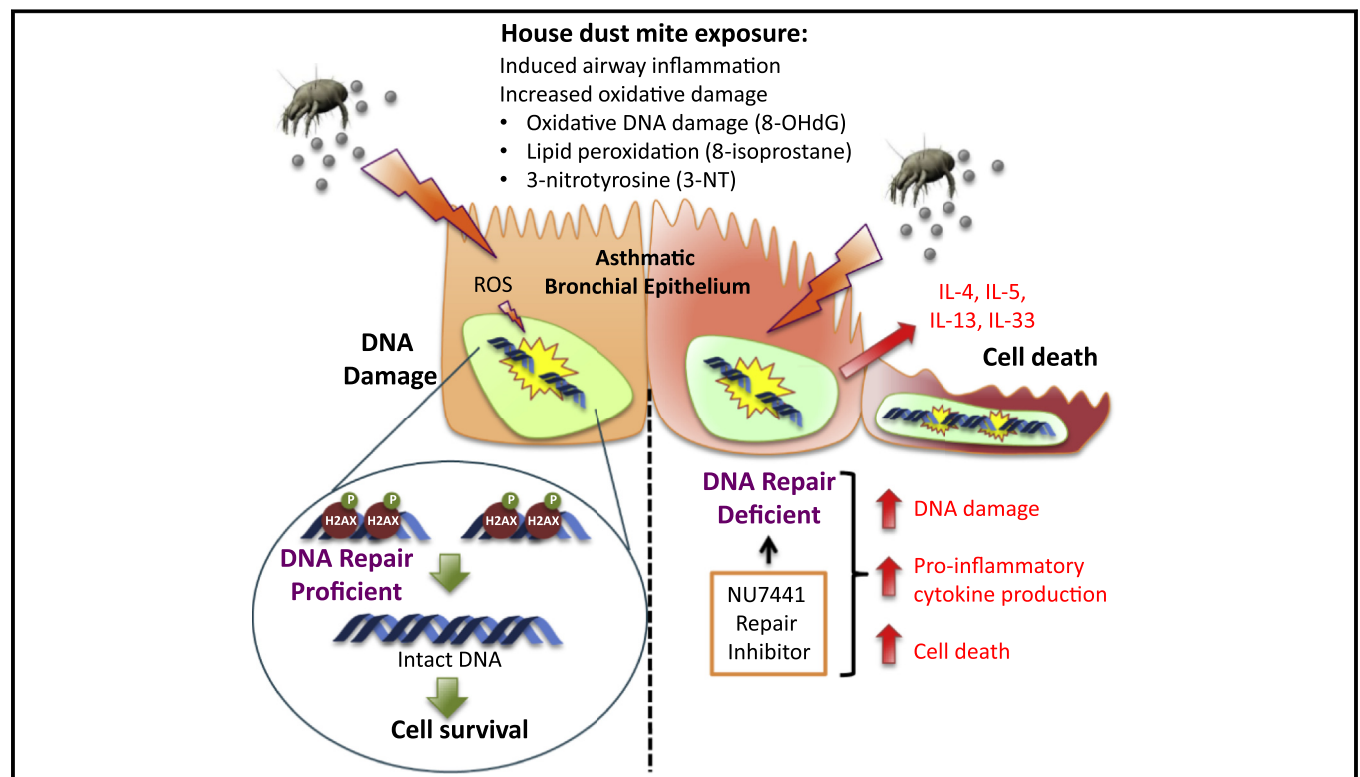
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House dust mite-induced asthma causes oxidative damage and DNA double-strand breaks in the lungs

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GRAPHICAL ABSTRACT



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Background: Asthma is related to airway inflammation and oxidative stress. High levels of reactive oxygen and nitrogen species 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:** We sought to study DNA damage and DNA damage responses induced by house dust mite (HDM) *in vivo* and *in vitro*. **Methods:** We measured DNA double-strand breaks (DSBs), DNA repair proteins, and apoptosis in an HDM-induced allergic asthma model and in lung samples from asthmatic patients. To study DNA repair, we treated mice with the 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 reactive oxygen species levels. **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 levels of the

DSB marker γ Histone 2AX (H2AX) foci in bronchial epithelium. BEAS-2B cells exposed to HDM showed enhanced DNA damage, as measured by using the comet assay and γ H2AX staining. In lung tissue from human patients with asthma, we observed increased levels of DNA repair proteins and apoptosis, as shown by caspase-3 cleavage, caspase-activated DNase levels, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining. Notably, NU7441 augmented DNA damage and cytokine production in the bronchial epithelium and apoptosis in the allergic airway, implicating DSBs as an underlying driver of asthma pathophysiology.

Conclusion: This work calls attention to reactive oxygen and nitrogen species and HDM-induced cytotoxicity and to a potential role for DNA repair as a modulator of asthma-associated pathophysiology. (J Allergy Clin Immunol 2016;■■■:■■■-■■■.)

Key words: House dust mite, asthma, oxidative stress, DNA double-strand breaks, BEAS-2B, cell death, DNA repair, NU7441

Asthma is characterized by chronic inflammation of the conducting airways resulting in bronchial obstruction and airway hyperresponsiveness (AHR).¹ Allergic asthma, the most common form of asthma, can be triggered by allergens, such as house dust mite (HDM).² In asthmatic patients, immune cells generate genotoxic reactive oxygen and nitrogen species (RONS), such as hydroxyl radicals, superoxide, peroxides, peroxynitrite, and nitric oxide. RONS can be measured in peripheral blood, induced sputum, and bronchoalveolar lavage (BAL) fluid from the lungs of asthmatic patients.^{3,4} RONS can damage lipids, proteins, and nucleic acids⁵ and contribute to increased asthma 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 aeroallergens on bronchial epithelium.

We have shown previously that nitric oxide-induced base lesions and peroxynitrite-induced single-strand breaks (SSBs) are central to large-scale sequence rearrangements.^{6,7} Among these DNA lesions, DNA double-strand breaks (DSBs) are one of the most cytotoxic forms of damage, and if they are not properly repaired, they can lead to genomic rearrangements and cell death.⁸ DNA DSBs are predominantly repaired by 2 major DNA repair pathways: homologous recombination (HR) and nonhomologous 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 the Rad51 nucleoprotein filament, which 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, immune dysfunction, and neurodegeneration.¹⁰ Here we investigated the role of DNA repair in asthma pathogenesis.

The DNA damage response involves the actions of sensors, transducers, and effectors, which together modulate DNA repair, cell-cycle arrest, and cell death.¹¹ A key early step in response to DNA DSBs is the modification of chromatin. Specifically, the histone variant H2AX is phosphorylated to become γ H2AX in the vicinity of the breakpoint, which is then able to recruit downstream damage response and DNA repair factors.¹² DNA

Abbreviations used

AHR:	Airway hyperresponsiveness
ATM:	Ataxia-telangiectasia mutated
BAL:	Bronchoalveolar lavage
CC10:	Club cell 10 kDa protein
DCFH-DA:	2,7-Dichlorodihydrofluorescein diacetate
DMSO:	Dimethyl sulfoxide
DNAPK:	DNA-dependent protein kinase
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:	Nonhomologous end-joining
3-NT:	3-Nitrotyrosine
8-OHdG:	8-Hydroxy-2-deoxyguanosine
8-oxoG:	8-Oxoguanine
PAR:	Poly ADP-ribose
PI:	Propidium iodide
RONS:	Reactive oxygen and nitrogen species
ROS:	Reactive oxygen species
SSB:	Single-strand break

damage also causes induction of DNA repair proteins, such as Rad51 and Ku70.¹³⁻¹⁵ Rad51 and Ku70 are key components in the HR and NHEJ repair pathways, respectively.^{16,17} Indeed, cells deficient in either Rad51 or Ku70 have an increased sensitivity to DNA damage-induced cytotoxicity.^{18,19}

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 reactive oxygen species (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 affect asthma pathogenesis.

METHODS

Animals

Female 6- to 8-week-old BALB/c mice (Animal Resources Centre, Canning Vale, Australia) were sensitized on days 0 and 7 and challenged on day 14 with 40 μ L of 25 μ g of purified HDM protein extract (*Dermatophagoides pteronyssinus*; Greer Laboratories, Lenoir, NC) by means of intratracheal administration.²⁰ NU7441 (Axon Medchem, Groningen, The Netherlands) was dissolved in 40% PEG400 and administered at 10 mg/kg intraperitoneally twice daily on days 13 and 14 and on days 1 and 2 after HDM challenge. PEG400 (40%) in saline was administered as a vehicle control. Animal procedures were approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

BAL fluid cell count

Mouse BAL fluid was collected, as previously described.⁵ The differential immune cell count was determined by using the BD Fortessa flow cytometer and analyzed with FlowJo software (TreeStar, San Carlos, Calif). Immune cells were identified as CD45⁺, macrophages as CD11c⁺Siglec-F⁺, eosinophils as CD11c⁺Siglec-F⁺, neutrophils as GR-1⁺CD11b⁺, and T cells as CD3⁺/CD19⁻ cells. The number per treatment group was 9 to 13 mice for the saline control group and the groups 1, 3, and 5 days after challenge and 5 mice for the group 10 days after challenge.

2,7-Dichlorodihydrofluorescein diacetate assay and detection of 8-hydroxy-2-deoxyguanosine/8-oxoguanine, 8-isoprostane, and 3-nitrotyrosine

For the 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) assay, BAL fluid cells were incubated with 10 $\mu\text{mol/L}$ DCFH-DA (Invitrogen, Grand Island, NY) for 20 minutes at 37°C. BAL fluid was then spun down, resuspended in RPMI medium (Invitrogen), and measured by using a spectrophotometer with excitation at 492 nm and emission at 525 nm. BAL fluid levels of 8-isoprostane and 8-hydroxy-2-deoxyguanosine (8-OHdG)/8-oxoguanine (8-oxoG; Cayman Chemical, Ann Arbor, Mich) and lung levels of 3-nitrotyrosine (3-NT; Cell Biolabs, San Diego, Calif) were measured with enzyme immunoassays to measure oxidative damage in the lung. There were 13 to 15 mice for each treatment group.

Human samples

Paraffin-embedded human lung sections were purchased from United State Biological (Salem, Mass). Lung sections from an asthmatic patient (T5595-5112M) were obtained from a 63-year-old woman with a clinical diagnosis of asthma and unknown cause of death. A normal lung section (T5595-4977) was obtained from an 83-year-old woman who died of disease not related to the lung. Human lung lysates were purchased from GeneTex (San Antonio, Tex). A lung lysate from an asthmatic patient (GTx25167) was obtained from an 85-year-old woman with a diagnosis of asthma for 25 years who died of asthma. The donor for normal lung lysates was a 38-year-old woman whose lung tissue had been found to be negative for HbsAg, HIV-1/1, and HCV. All other information was inaccessible.

Immunofluorescence staining

Mouse lung tissues were processed, as described by Cheng et al.,²¹ and immunofluorescence staining was performed, as described by Yamada et al.²² For immunocytochemical staining, human bronchial epithelial BEAS-2B cells were cultured in RPMI supplemented with 10% FBS (Atlanta Biologicals, Lawrenceville, Ga) and 100 U/mL penicillin-streptomycin (Invitrogen). Cells were seeded onto chamber slides and exposed to HDM (2, 20, or 200 $\mu\text{g/mL}$) for 6 hours at 37°C. Slides were probed with antibodies targeted at γH2AX (Cell Signaling Technologies, Danvers, Mass), 8-OHdG (Abcam, Cambridge, Mass), or Club cell 10 kDa protein (CC10; Santa Cruz Biotechnology, Dallas, Tex), followed by 4'-6-diamidino-2-phenylindole dihydrochloride staining. Cells were stained with CellROX Green (Invitrogen) to measure ROS production in cells. Stained slides were mounted in SlowFade reagents (Invitrogen). Images were captured with a Zeiss fluorescence microscope (Carl Zeiss, Thornwood, NY) and quantified with Bitplane Imapris 5.7 software (Bitplane AG, Zurich, Switzerland). Quantification of images was performed on 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 snap-frozen in liquid nitrogen. Immunoblotting was performed, as described previously.⁵ Antibodies used were targeted at Ku70, γH2AX , cleaved caspase-3 (Cell Signaling, Danvers, Mass), Rad51 (Santa Cruz Biotechnology, Dallas, Tex), PARP (Abcam, Cambridge, United Kingdom), poly ADP-ribose (PAR; Calbiochem, San Diego, Calif), or β -actin (Abcam).

Comet assay

BEAS-2B cells and the human alveolar epithelial cell line A549 were obtained from American Type Culture Collection (Rockville, Md) and cultured in RPMI. BEAS-2B cells were exposed to HDM (2, 20, and 200 $\mu\text{g/mL}$) for 6 hours at 37°C, and the alkaline CometChip assay (Trevigen, Inc, Gaithersburg, Md) was performed, as described by Wood et al.²³ As a positive control for NU7441 activity, A549 cells were exposed to 50 $\mu\text{mol/L}$ NU7441 for 2 hours at 37°C and then irradiated with 50 Gy of ionizing radiation with a Cobalt-60 irradiator (Gammacell 220 Excel; MDS Nordion, Ottawa, Canada). The neutral CometChip assay was performed, as described by Weingeist et al.²⁴

Cytokine profiling with ELISA

BEAS-2B cells were seeded on a 96-well plate and left to adhere overnight before being treated with HDM (2, 20, or 200 $\mu\text{g/mL}$) for 20 hours at 37°C. For NU7441 treatment, BEAS-2B cells were pretreated with NU7441 (2.5 $\mu\text{mol/L}$) for 2 hours and washed before exposure to HDM. As a vehicle control, the same concentration of dimethyl sulfoxide (DMSO) was used to replace NU7441 treatment. The cell-culture supernatant was collected, and levels of human cytokine were determined by using sandwich ELISA kits for IL-4, IL-5, IFN- γ (OptEIA, BD Biosciences), IL-13, IL-33, and thymic stromal lymphopoietin (DuoSet; R&D Systems, Minneapolis, Minn), according to the manufacturer's instructions. All samples were assayed in duplicate.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling and cell death staining

Apoptotic cells were detected *in situ* on lung sections by using the ApoTag Peroxidase TUNEL assay (Merck Millipore, Billerica, Mass). *In vitro* cell death was studied by staining cells with the Alexa Fluor 488 Annexin V/Dead cell apoptosis kit (Thermo Scientific, Waltham, Mass). BEAS-2B cells were exposed to HDM with the presence or absence of NU7441. The same concentration of DMSO was added in HDM control. BEAS-2B cells were harvested together with supernatants and washed once with PBS, followed by staining with Annexin V and propidium iodide (PI). Cell death was quantified with the BD Fortessa flow cytometer and analyzed with FlowJo software (TreeStar). Dead cells were identified as Annexin V⁺PI⁺ (early apoptosis) and Annexin V⁺PI⁺ (late apoptosis) cells.

Measurement of AHR

Development of AHR was measured by increased airway resistance and decreased dynamic lung compliance in response to methacholine (1-32 mg/mL; Sigma-Aldrich, St Louis, Mo) recorded by using a whole-body plethysmograph chamber (Buxco, Sharon, Conn). AHR was measured on day 3 after HDM challenge, as described by Cheng et al.²⁵

Statistical analysis

All data are expressed as means \pm SEMs. Statistical comparison of multigroup data was analyzed by using 1-way ANOVA, followed by Tukey *post hoc* analysis with 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 after challenge. HDM increased total infiltrated immune cell counts in BAL fluid, with rapid neutrophil influx followed by a moderate increase in alveolar macrophage counts (Fig 1, B). The observed influx of neutrophils and their gradual replacement by eosinophils, which persisted up to 5 days after challenge (Fig 1, B), resembles the human allergic response.²⁷ Histologic analysis revealed classical features of bronchial inflammation with a high cell infiltration score surrounding the bronchioles at 1, 3, and 5 days after challenge (Fig 1, C).²⁶

HDM induces airway oxidative damage

HDM exposure caused a significant increase in oxidative stress in BAL fluid (Fig 2), as measured by using the oxidation-sensitive fluorescent probe DCFH-DA.²⁸ When the levels of RONS overwhelm antioxidant capacity, damage to cellular macromolecules can ensue.³ We found that levels of 8-isoprostane, an indicator of oxidative damage to lipids,²⁹ were significantly increased on 3 and 5 days after challenge. Furthermore, 3-NT is formed by the reaction of proteins with peroxynitrite, nitrogen dioxide, or

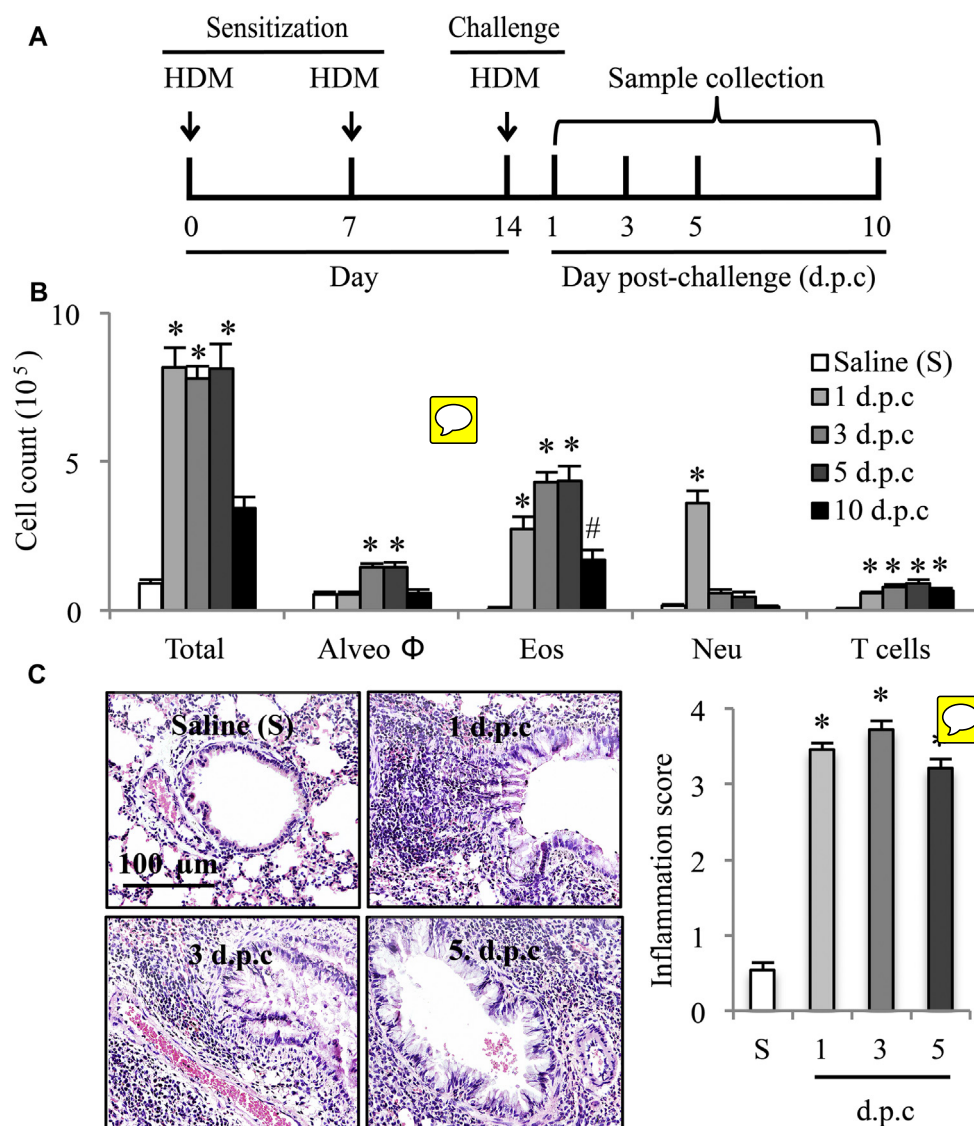


FIG 1. HDM induces inflammatory cell recruitment to the airway. **A**, HDM-induced airway inflammation model. **B**, Quantification of immune cells in BAL fluid 1, 3, 5, and 10 days after challenge (d.p.c). Total immune cells (Total), alveolar macrophages (Alveo Φ), eosinophils (Eos), neutrophils (Neu), and T lymphocytes (T cells) were quantified. Values are means \pm SEMs. **C**, Representative images of hematoxylin and eosin-stained lungs. Quantification of inflammatory cell infiltration in lungs was done according to the method of Bao Z et al.²⁶ Micrographs were taken at $\times 200$ magnification. * $P < .01$ and # $P < .05$, significant difference from saline control (S).

oxidation of nitrite by myeloperoxidase (MPO) or eosinophil peroxidase (EPO), both of which are found in abundance in asthmatic patients.³⁰ Results showed that tissue 3-NT levels were high 1 and 3 days after challenge (Fig 2), which is 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 lung tissue. Levels of 8-OHdG were markedly increased 1, 3, and 5 days after challenge, as determined by 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 the DNA DSB marker

γ H2AX. Extensive phosphorylation flanking the DSB enables visualization of DSBs by using immunofluorescence.³¹ Bleomycin, which is known to induce DSBs, caused a significant increase in γ H2AX levels (green; Fig 3, A). Club cells, also known as Clara cells, are nonciliated secretory epithelial cells lining the pulmonary airway.³² Costaining with CC10 showed that most of the DNA damage was in the bronchial epithelium. CC10 was used here as a marker to identify the airway epithelial layer.

HDM exposure significantly increased the frequency of γ H2AX-positive cells, especially in airway epithelium. Higher magnification revealed that most γ H2AX-positive cells had a punctate pattern, which is indicative of repair foci (Fig 3, A, inset). The percentage of γ H2AX-positive cells in lungs of asthmatic patients increased from days 1 to 5 after challenge (Fig 3, B).

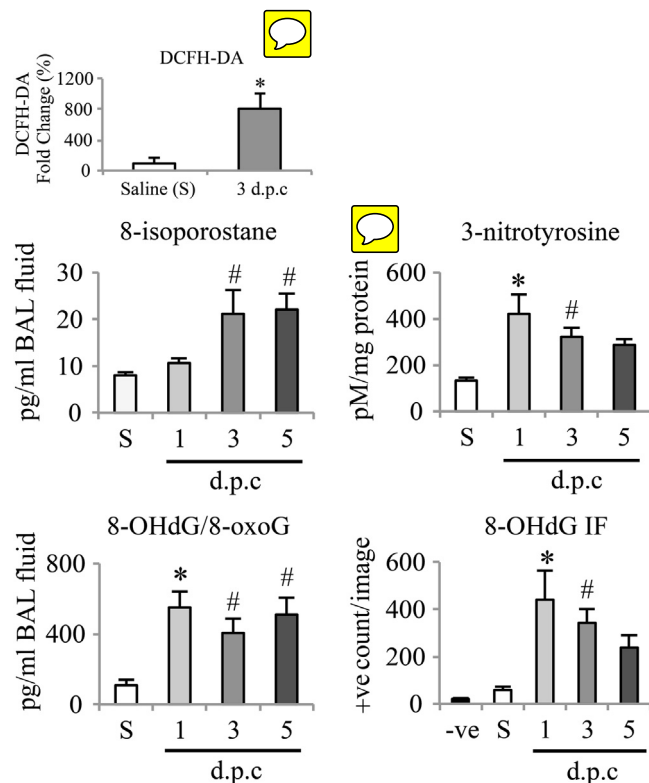


FIG 2. Increased levels of oxidative damage in BAL fluid and lung tissues. Estimated level of ROS in BAL fluid obtained from mice 3 days after challenge (*d.p.c.*), as measured by using the DCFH-DA assay. Levels of 8-isoprostane, 3-NT, and 8-OHdG/8-oxoG were measured by using ELISA and immunofluorescence staining of lung sections (*bottom right*). The negative control (*-ve*) indicates secondary antibody alone. Values are means \pm SEMs. * $P < .01$ and # $P < .05$, significant difference from saline control (*S*).

Immunoblotting of whole lung lysates showed a similar increase in γ H2AX levels (Fig 3, C). Thus both qualitative and quantitative analysis showed a clear increase in DNA damage, as indicated by γ H2AX, in HDM-induced asthma.

DNA DSBs are evident in lung tissues from human asthmatic patients

Immunostaining of paraffin-embedded lung sections obtained from a patient with chronic asthma revealed higher levels of DNA DSBs, as shown by γ H2AX staining (Fig 3, C). Quantitative results showed a significant increase in the frequency of γ H2AX-positive cells in the lungs of asthmatic patients (Fig 3, E). Because 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 γ H2AX levels (Fig 3, F).

HDM directly induces DNA DSBs in human bronchial epithelial cells

BEAS-2B cells were exposed to HDM (2, 20, and 200 μ g/mL) to elucidate the direct DNA-damaging effect of HDM on bronchial epithelial cells. DNA damage was analyzed by using the alkaline comet assay and γ H2AX staining. The 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 sites.³³ 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).³³ Furthermore, HDM directly induced DNA DSBs in cells, as shown by γ H2AX foci (green) staining (Fig 4, B). Quantification of γ H2AX-positive cells (≥ 10 foci per nucleus) revealed that more than 80% of the cells were severely damaged by HDM (200 μ g/mL). These results clearly demonstrate the DNA-damaging potential of HDM allergen on bronchial epithelial cells.

To investigate the ability of HDM to induce ROS production in BEAS-2B cells, we measured ROS through fluorescence emission of CellROX dye. Strong green signal was detected within the cells after cells were exposed to H_2O_2 , 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 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 2 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 levels in mouse lung tissues (Fig 5, A). In response to SSBs, poly ADP-ribose polymerase 1 (PARP-1) catalyzes the synthesis of the PAR chain.³⁴ Notably, both PARP-1 and PAR levels were strongly increased in HDM-challenged lungs (Fig 5, A). In parallel, we analyzed protein lysates from normal and asthmatic human lung samples. There was a significant increase in the levels of Rad51, Ku70, and PAR (for technical reasons, PARP-1 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

The NHEJ pathway is initiated by the binding of Ku70/80 heterodimer to the broken DNA ends, forming a complex with DNAPKcs.¹⁷ The observation that Ku70 protein levels were increased in the lungs of asthmatic patients (Fig 5, A and B) raises the possibility that NHEJ might be important in repairing DSBs during asthma. We used NU7441, a well-characterized inhibitor of DNAPKcs, to study this possibility.³⁵ The neutral comet assay was used to confirm the potential of NU7441 in inhibiting DNA DSB repair.³³ Ionizing radiation is a potent inducer of DNA DSBs (see tail length at time zero in Fig 6, A).³³ Irradiated cells repaired damaged DNA rapidly, as indicated by the reduction in tail length within 1 hour. Cells treated with NU7441 had severe reduction in the repair DNA DSBs (Fig 6, A).

For the HDM mouse asthma model, NU7441 was administered twice daily for 2 days before and after the final HDM challenge (Fig 6, B). NU7441 markedly increased γ H2AX levels in the epithelium of mice with allergic airways. Costaining with CC10 indicated that DSBs were present in epithelial cells, and quantification revealed an increase in the frequency of γ H2AX-positive cells, increasing to significance on day 5 after challenge (Fig 6, C and D).

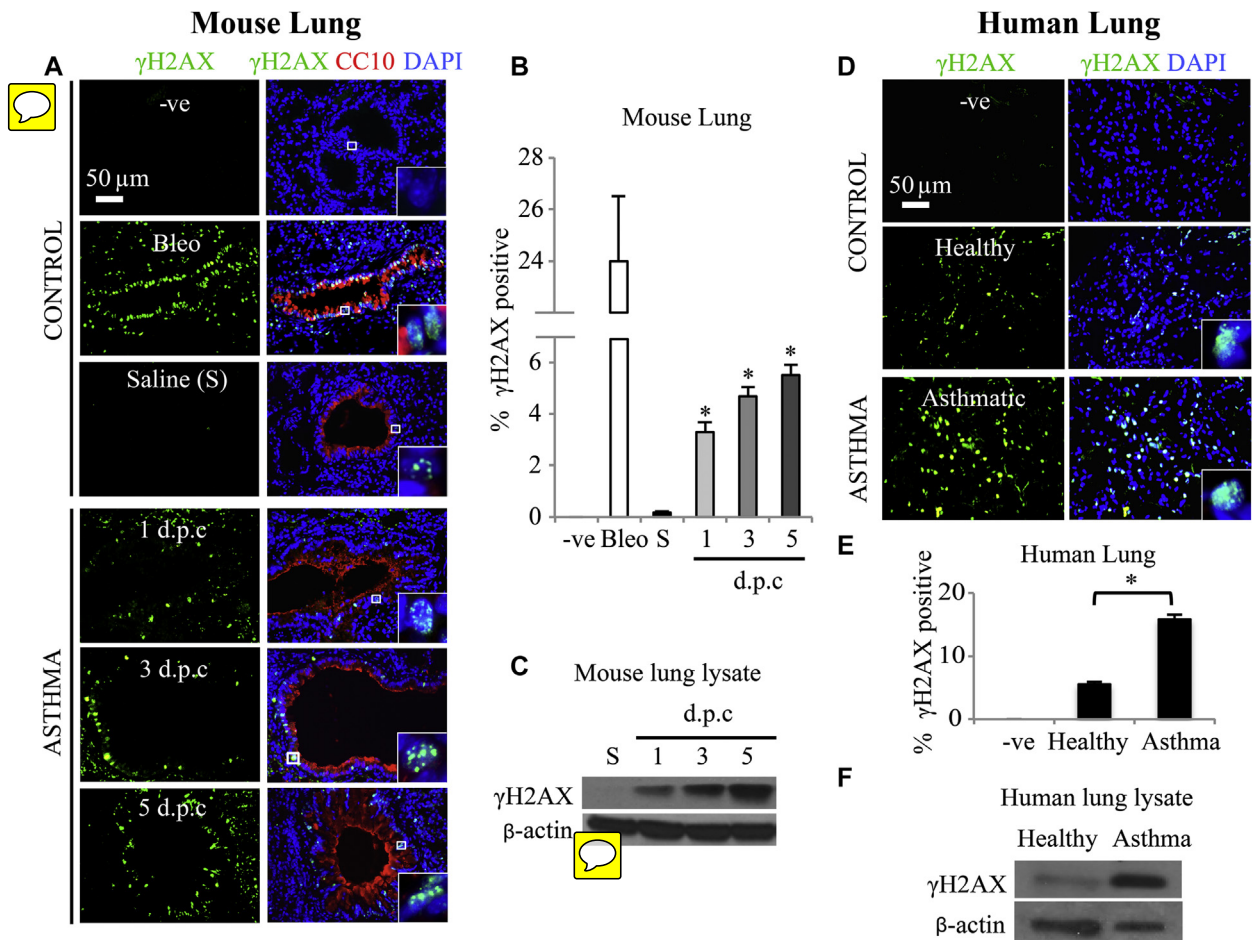


FIG 3. Asthma induces DNA DSBs in lungs of asthmatic mice and human subjects. **A**, Analysis of γ H2AX (green), CC10 (red), and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue) in lung sections. The negative control (-ve) indicates secondary antibody alone, and the positive control indicates lung tissue treated with bleomycin (Bleo). **B**, Quantification of γ H2AX-positive cells (≥ 5 foci). **C**, γ H2AX protein levels measured by means of immunoblotting. **D**, Analysis of γ H2AX (green) in human normal and asthmatic lung tissue. **E**, Quantification of γ H2AX-positive cells (≥ 5 foci). **F**, γ H2AX protein levels quantified by using immunoblotting. Values are means of $3 \pm$ SEMs. * $P < .01$, significant difference from healthy control tissue. d.p.c, Days after challenge.

We next studied the effect of NU7441 on levels of DNA repair proteins. Immunoblotting of lung lysates revealed lower γ H2AX levels in naive mice exposed to NU7441 alone, which is consistent with previous studies showing that inhibition of DNAPKcs can suppress phosphorylation of H2AX (Fig 6, E).¹¹ In contrast, mice with HDM-induced asthma treated with NU7441 showed an increase in γ H2AX levels. Although one might expect DSBs to be difficult to detect, 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 (eg, ataxia-telangiectasia mutated [ATM]), which also phosphorylate H2AX, leading to a strong γ H2AX signal under conditions in which DSBs are high. In addition, Rad51 levels were further increased in NU7441-treated mice with HDM-induced asthma (Fig 6, E), raising the possibility that Rad51-associated HR repair of DSBs is augmented when the NHEJ pathway is inhibited.³⁶ 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.³⁷ Finally, NU7441 had no effect on PARP-1 levels, which primarily affects 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 cells exposed to HDM compared with cells exposed to HDM alone (the same concentration of DMSO was added as a vehicle control; Fig 7, A). These cytokines are epithelial cell-derived cytokines that promote T_H2 responses in asthmatic patients.^{38,39} IL-4 is crucial for allergic sensitization and IgE production, IL-5 promotes eosinophil survival, IL-13 has a central role in airway remodeling, and IL-33 activates lung dendritic cells.^{39,40} Notably, HDM was unable to trigger the production of thymic stromal lymphopoietin and IFN- γ in BEAS-2B cells, and NU7441 did not alter the levels of these 2 cytokines. Nevertheless, this result suggests that inhibition of the NHEJ repair pathway by NU7441

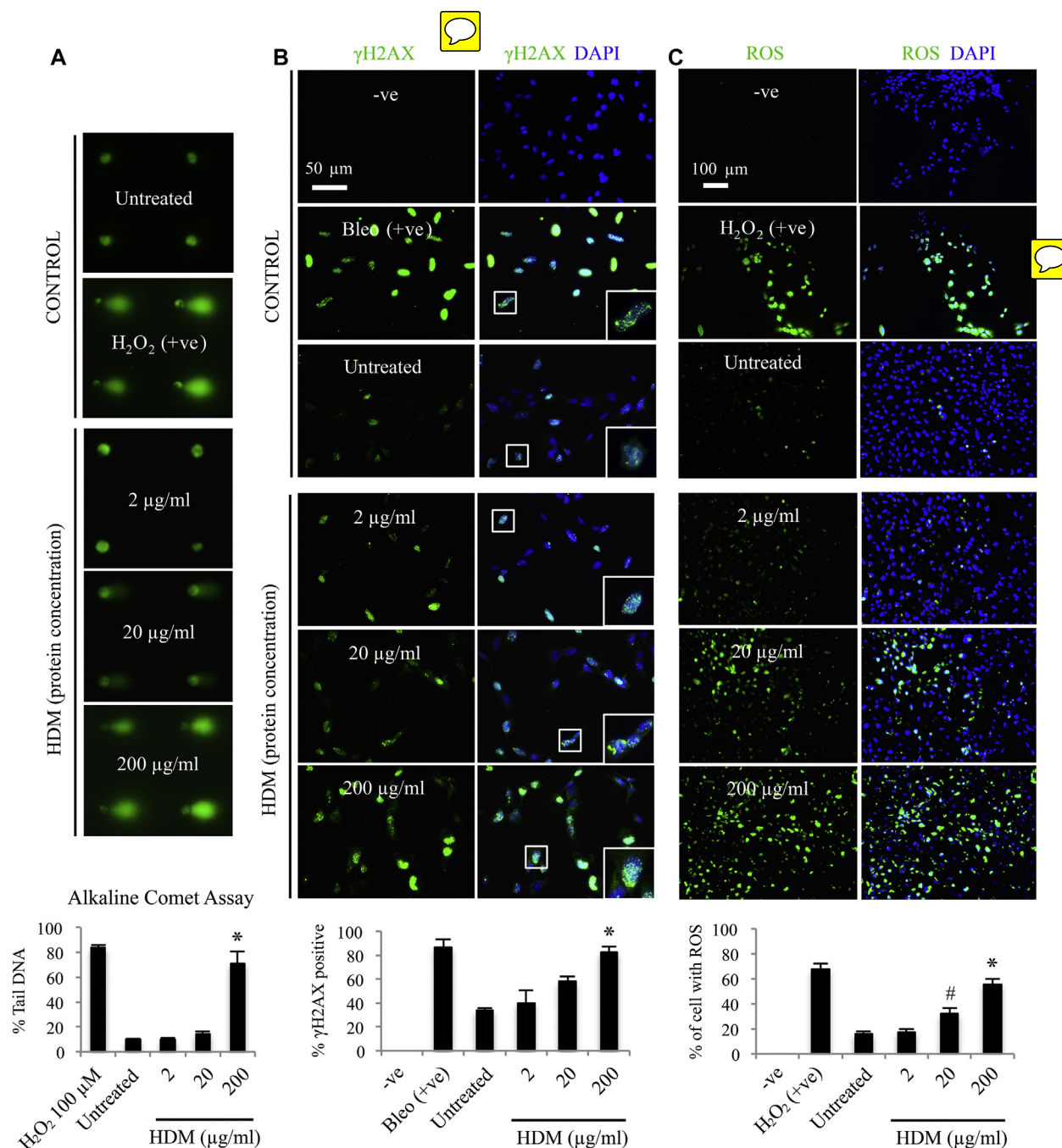


FIG 4. HDM induces direct DNA DSBs in BEAS-2B cells. **A**, Representative images of alkaline comets. Percentage of DNA intensity in the comet tail, which reflects the level of DNA damage, was quantified. **B**, Cells were stained for γ H2AX (green), and nuclei were stained with 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). Cells with 10 or more foci were counted as positive. **C**, Cells were stained with CellROX to measure oxidative stress levels. All experiments were repeated at least 3 times, and data are presented as means \pm SEMs. * $P < .01$ and # $P < .05$, significant difference from untreated.

in BEAS-2B cells potentiates 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- and time-dependently induced cell death in bronchial epithelial cells (Fig 7, B). Importantly,

HDM-induced cell death was further enhanced by NU7441 (HDM plus 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 that HDM induces DNA damage in bronchial epithelial cells (Figs 3, A, and 4, B) and that DNA damage activates DNA repair. When DNA repair was inhibited by NU7441, DNA damage induced by HDM was not efficiently repaired and hence can potentiate cell death. Thus we have shown

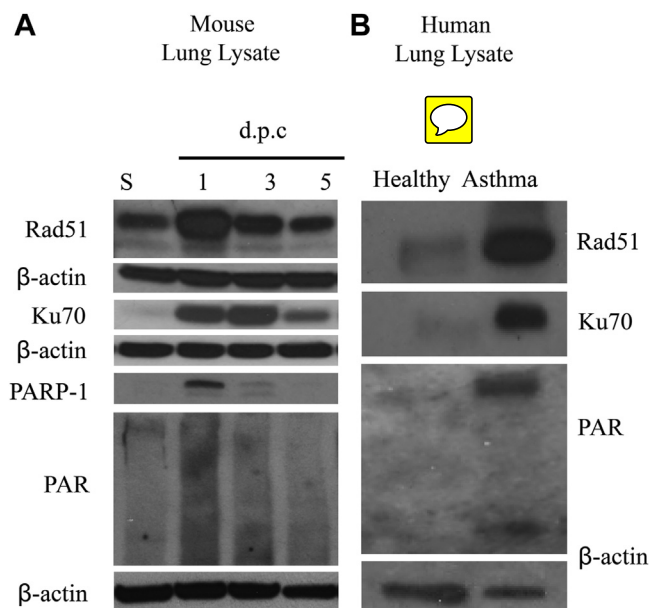


FIG 5. Asthma induces DNA damage responses, as indicated by increased Rad51, Ku70, PARP-1, and PAR levels. **A**, Lung protein extract analyzed by means of Western blotting for Rad51, Ku70, PARP-1, and PAR. **B**, Human lung lysates from normal or asthmatic lung tissue were immunoblotted with Rad51, Ku70, and PAR antibodies. β -Actin serves as an internal control. Images show representative results for one of 3 or more experimental replicates. d.p.c., Days after challenge.

that efficient DNA repair protects 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 through apoptosis, we measured levels of cleaved caspase-3 and caspase-activated DNase. HDM increased lung levels of cleaved caspase-3 and caspase-activated DNase 1 and 3 days after challenge (Fig 8, A). NU7441 treatment resulted in a small but consistent increase in the levels of cleaved caspase-3 in mice with HDM-induced asthma (Fig 8, B). Furthermore, analysis with terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling⁴¹ showed much higher levels of apoptosis in HDM-challenged mice than control mice. Consistent with the *in vitro* data, NU7441 led to a higher level of HDM-induced lung apoptosis, which is especially evident 5 days after challenge (Fig 8, C and D).

DISCUSSION

It is estimated that 300 million persons suffer from asthma worldwide, leading to enormous socioeconomic costs.⁴² 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% to 85% of asthmatic patients.⁴³ 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 lung tissue from human subjects and mice with

asthma, we observed increased levels of DNA repair proteins and apoptosis. When mice with HDM-induced asthma were treated with a chemical inhibitor of DSB repair, DNA damage levels and apoptosis were enhanced further. In addition, when the NHEJ DSB repair protein DNA-dependent protein kinase (DNAPK) was inhibited in bronchial epithelial cells *in vitro*, we observed a significant increase in HDM-induced production of proinflammatory cytokines and cell apoptosis. These studies point to a potential role for DNA DSBs and DNA repair in regulating asthma pathophysiology.

It is well established that asthma causes infiltration of immune cells, including eosinophils and neutrophils, that are capable of producing high levels of RONS.³ The lysosomal enzyme MPO, the most abundant protein stored in neutrophil granules, together with EPO from eosinophils, catalyzes the oxidation of halides by H_2O_2 to form hypochlorous acid and hypobromous acid, respectively.³ Hypohalous acids react with superoxide to form OH^\cdot , an extremely reactive radical that is capable of damaging cellular molecules.³ The influx of neutrophils on day 1 after challenge, followed by eosinophil influx 2 days later, filled the airways of subjects with HDM allergy with a significant amount of MPO and EPO. We observed a sharp increase in ROS levels in BAL fluid and increased levels of RONS-induced damage to macromolecules in the airways of subjects with HDM allergy. These data suggest that during allergic airway inflammation, ROS produced by inflammatory cells could lead to oxidative damage in the lungs.

Although most RONS-induced DNA lesions are base damages (eg, 8-oxoG, 8-nitroG, and deamination products), RONS can directly induce SSBs. Importantly, SSBs formed either chemically or enzymatically can be converted into DSBs if 2 SSBs are in close proximity or if SSBs encountered and collapsed a DNA replication fork.⁴⁴ The observation that asthma is associated with γ H2AX foci is consistent with the induction of DSBs and possibly stalled replication forks.⁴⁵ Our results have extended those reported by Chapman et al,⁴⁶ which showed that asthma could induce systemic genotoxicity, as measured based on micronucleus formation and protein nitration in peripheral blood. Results described here indicate that HDM induces DNA damage and repair in human bronchial epithelial cells and in lungs of mice and human subjects with asthma.

To learn the potential biological relevance of asthma-induced DSBs, we explored the effect of DSB repair on asthma pathology by using the DNA DSB repair inhibitor NU7441. The catalytic activity of DNAPKs, which is essential for the NHEJ pathway, is inhibited by NU7441.³⁵ Indeed, DSB repair was greatly impaired in alveolar epithelial cells pretreated with NU7441. In mice with HDM-induced asthma treated with NU7441, we observed a significant increase in γ H2AX levels, which is consistent with defective repair, leading to increased DSBs. In addition, NU7441 further increased apoptosis, both in lungs of asthmatic mice and *in vitro* in 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.

Although the major focus of this work has been on DNA DSBs, SSBs can play an important role in tissue pathology. SSBs are recognized by PARP, which can poly-ADP ribosylate downstream substrates, depleting the NAD^+ pool.⁴⁷ Here we observed that PARP activity was induced by asthma, as shown by the high levels of PAR. Excessive DNA damage can lead to high PAR levels, and the resultant energy depletion can lead to

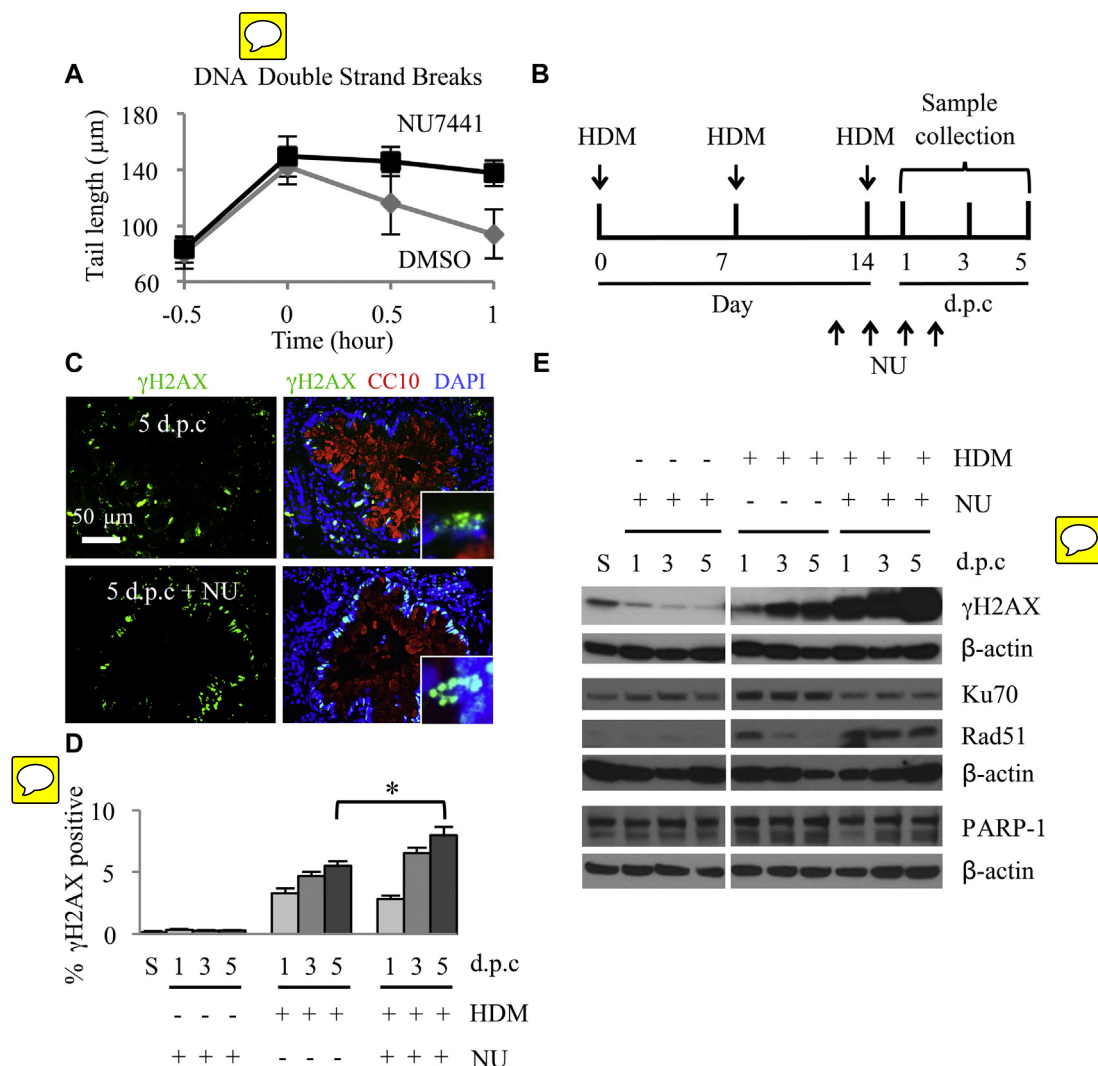


FIG 6. DNA repair inhibitor increases DNA DSBs and DNA damage responses. **A**, Analysis of DNA DSBs by using neutral comet analysis after exposure to γIR . Tail length reflects the frequency of DNA DSBs.³³ **B**, NU7441 treatment timeline. **C**, Lung sections were stained for γH2AX (green), CC10 (red), and 4'-6-diamidino-2-phenylindole dihydrochloride (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 3 times. * $P < .01$, significant difference from HDM-only control. d.p.c, Days after challenge.

necroptosis.⁴⁸ Interestingly, necroptosis during airway inflammation has recently been shown to contribute to severe asthma development.^{49,50} 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 also endogenously during V(D)J recombination by immature B and T lymphocytes, enabling hypermutation, which is necessary for the adaptive immune response.⁵¹ However, V(D)J recombination only takes place in lymphoid tissues, such as the bone marrow, thymus, and lymph nodes, and it is only after B and T lymphocytes have completed V(D)J recombination that mature lymphocytes will migrate into the lungs.⁵² In our experiments we carefully trimmed lymph nodes and excess tissue and fat from lungs before freezing them, and hence it is unlikely that the DSBs observed in the lungs of asthmatic mice are from V(D)J recombination.

DNAPK not only plays a role in NHEJ repair but is also crucial in maintaining lymphocyte V(D)J recombination and

immunoglobulin class-switching.¹¹ Therefore using animal models to study the effects of NU7441 on airway inflammation will be an unavoidable confounding factor because NU7441 affects T-cell development.⁵³ Hence to directly elucidate the biological consequences of DNA repair inhibition specifically in bronchial epithelial cells, we performed *in vitro* experiments and measured proinflammatory cytokine production by BEAS-2B cells. We revealed that NU7441 significantly augmented IL-4, IL-5, IL-13, and IL-33 levels 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 the setting of asthma.^{38,40} These data demonstrate that a defect in DNA repair exacerbates the inflammatory response during allergen exposure. Because 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 for suppressing proinflammatory responses in bronchial epithelial

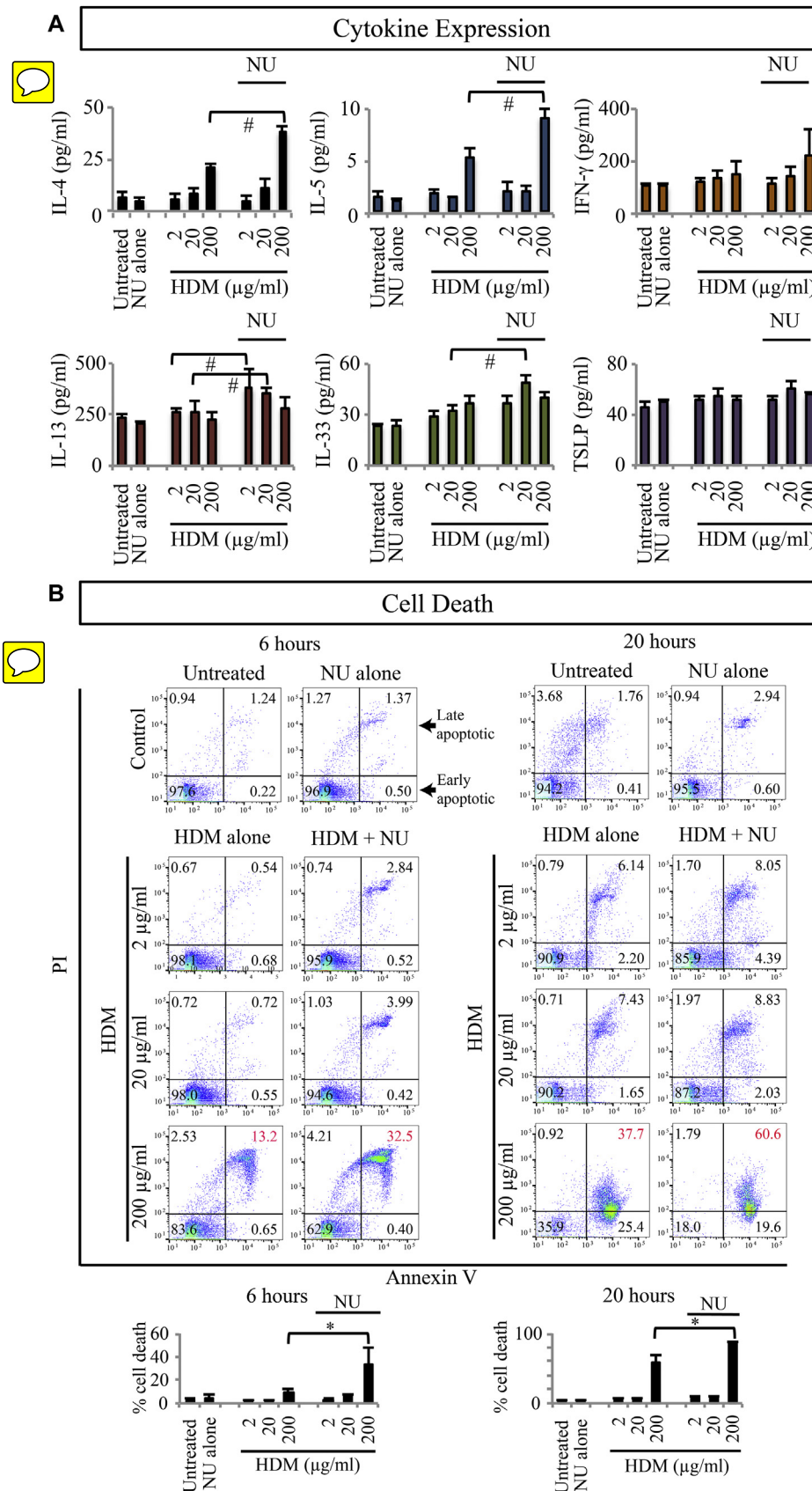


FIG 7. NU7441 increases proinflammatory cytokine production and induces cell death in BEAS-2B cells exposed to HDM. **A**, IL-4, IL-5, IL-13, IL-33, thymic stromal lymphopoietin (*TSLP*), and IFN- γ levels in culture supernatants were quantified by using ELISA. **B**, Cell death assay was performed with Annexin V/PI staining. Annexin V⁺PI⁺ cells were considered dead cells. All experiments were repeated at least 3 times, and values are shown as means \pm SEMs. * $P < .01$ and # $P < .05$, significant difference from HDM-alone control.

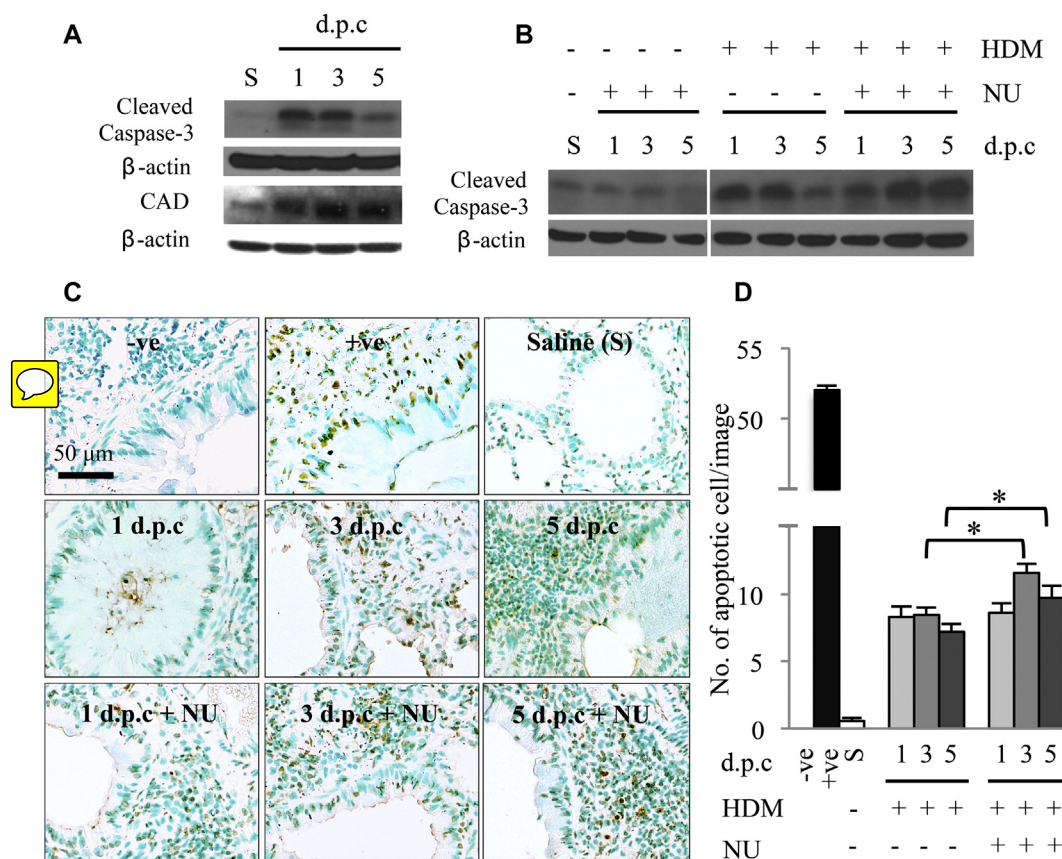


FIG 8. Asthma-induced cell death is enhanced by NU7441. **A**, Immunoblotting of cleaved caspase-3 and caspase-activated DNase (CAD). **B**, Immunoblotting of cleaved caspase-3 in lungs of subjects with HDM-induced asthma with and without NU7441 treatment. **C**, Analysis of apoptosis by using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining, where apoptotic cells are stained brown. The negative control (-ve) indicates staining without terminal deoxynucleotidyl transferase enzyme. The positive control (+ve) indicates lungs treated with DNase I. **D**, Quantification of TUNEL-positive cells. Values are shown as means \pm SEMs. * $P < .01$, significant difference from HDM-only control. d.p.c, Days after challenge.

cells, we sought to learn about the potential importance of DNA repair during an acute asthma attack. We exposed animals to NU7441 and subsequently measured AHR in response to HDM (see Fig E1 in this article's Online Repository at www.jacionline.org). Interestingly, we did not detect worsening of lung function in the setting of HDM-induced asthma. AHR is caused by multiple factors, and DNA damage might be one of the many factors that regulate AHR. Another possible explanation for not observing an effect of NU7441 on AHR could 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 in which persistent inflammation contributes to pathology, and hence further studies are warranted.

Interestingly, 2 other studies by Ghonim et al⁵³ and Mishra et al⁵⁴ have demonstrated that long-term inhibition of DNAPK has beneficial effects on asthma. Ghonim et al⁵³ showed that DNAPK inhibition reduced airway inflammation and AHR. On the other hand, Mishra et al⁵⁴ showed that DNAPK inhibition in dendritic cells reduced airway inflammation. Although 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 the DNA damage induced by asthma. We have shown that in bronchial epithelial cells a defect in DNA repair exacerbates inflammatory responses. We also examined lung cell death when DNAPK is inhibited, neither of which was studied in these 2 articles. We revealed that DNAPK inhibition increased lung cell death in both lung tissue of mice with HDM-induced asthma and isolated human bronchial epithelial cells exposed to HDM. This could be an important but unrecognized side effect of DNAPK inhibitor. Indeed, long-term inhibition of DNA DSB repair in human subjects is known to pose a risk to disease development.⁵⁵ For example, 70% of patients with ATM who have chronic depletion of DNA repair had lung diseases, which are the main causes of death in patients with ATM.⁵⁵ Reversible airway obstruction, one of the characteristics of asthma, has also been reported in patients with ATM.⁵⁶ This suggests that long-term inhibition of DNA DSB repair might have an adverse effect on airway function.

DNA repair might 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 biopsy specimen from asthmatic patients.⁵⁷ Being the first line of defense against pathogens, impairment of

barrier function is thought to affect the susceptibility of asthmatic airways to respiratory tract viral or microbial infections.⁵⁸ 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*, which have demonstrated different degrees 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 levels, increased DNA DSBs, changes in DNA repair protein expression, and increased apoptosis. Furthermore, direct exposure to HDM leads to potentially cytotoxic levels of DNA damage in bronchial epithelial cells in both *in vivo* and *in vitro*. Although further studies are needed to ascertain the specific effect 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 might be compromised. Finally, we have shown that the effect of asthma on DNA damage and apoptosis is conserved from mice to human subjects. Taken together, these studies suggest a possible role for DNA repair in maintaining and restoring airway function during asthma.

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Key messages

- HDM-induced allergic asthma causes a significant increase in DNA DSBs in lung tissue and specifically in the bronchial epithelium.
- Direct exposure of human bronchial epithelial cells to HDM leads to ROS production and increased DNA damage in cells.
- A defect in DNA DSB repair augments DNA damage, proinflammatory cytokine production, and apoptosis in the bronchial epithelium.

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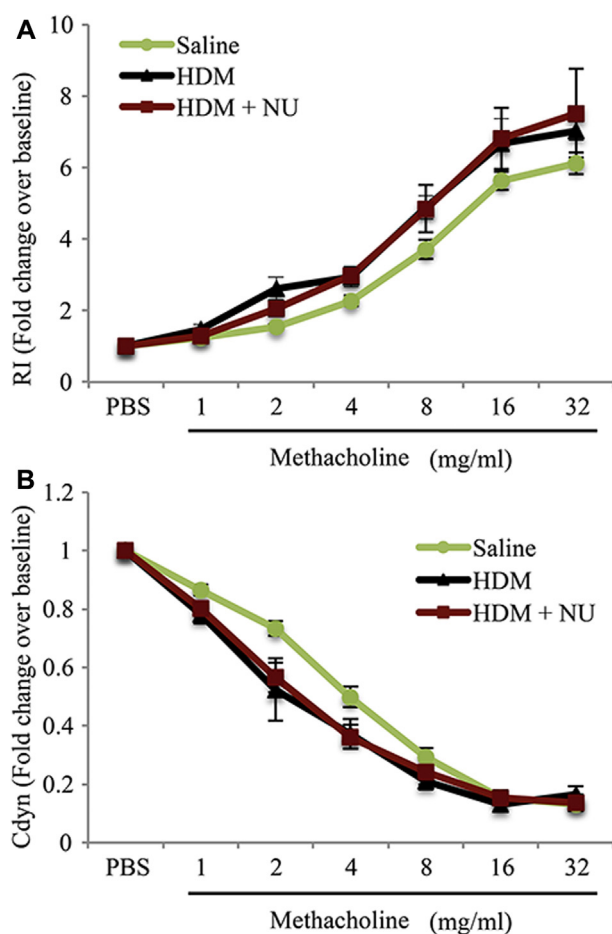


FIG E1. Effects of NU7441 on HDM-induced AHR. AHR of mechanically ventilated mice in response to aerosolized methacholine was measured 3 days after the last saline or HDM challenge with or without NU7441 treatment. AHR is expressed as fold change over baseline level. **A**, Lung resistance (*RL*, *n* = 6 mice) defined as the pressure driving respiration divided by flow. **B**, Dynamic compliance (*Cdyn*, *n* = 6 mice), referring to the dispensability of the lung and defined as the change in lung volume produced by a change in pressure across the lung. Values are shown as means \pm SEMs.