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Excision release of 5-hydroxycytosine oxidatively induced DNA base lesions from the lung genome by cat dander extract challenge stimulates allergic airway inflammation

Koa Hosoki¹, Pawel Jaruga², Toshiko Itazawa¹, Leopoldo Aguilera-Aguirre³, Erdem Coskun², Tapas K. Hazra^{4,5}, Istvan Boldogh^{3,5}, Miral Dizdaroglu², and Sanjiv Sur^{1,5}

¹Department of Internal Medicine, Division of Allergy and Immunology, University of Texas Medical Branch, Galveston, Texas ²Biomolecular Measurement Division National Institute of Standards and Technology, Gaithersburg, Maryland ³Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas ⁴Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine, University of Texas Medical Branch, Galveston, Texas ⁵Sealy Center for Molecular Medicine, University of Texas Medical Branch, Galveston, Texas

Abstract

Background: Ragweed pollen extract (RWPE) induces TLR4-NFκB-CXCL-dependent recruitment of ROS-generating neutrophils to the airway and OGG1 DNA glycosylase-dependent excision of oxidatively induced 8-OH-Gua DNA base lesions from the airway epithelial cell genome. Administration of free 8-OH-Gua base stimulates RWPE-induced allergic lung inflammation. These studies suggest that stimulation of innate receptors and their adaptor by allergenic extracts initiates excision of a set of DNA base lesions that facilitate innate/allergic lung inflammation.

Objective: To test the hypothesis that stimulation of a conserved innate receptor/ adaptor pathway by allergenic extracts induces excision of a set of pro-inflammatory oxidatively induced DNA base lesions from the lung genome that stimulate allergic airway inflammation.

Methods: Wild-type (WT), *Tlr4KO*, *Tlr2KO*, *Myd88KO*, and *TrifKO* mice were intra-nasally challenged once or repeatedly with cat dander extract (CDE), and innate or allergic inflammation and gene expression were quantified. We utilized GC-MS/MS to quantify a set of oxidatively induced DNA base lesions after challenge of naïve mice with CDE.

Results: A single CDE challenge stimulated innate neutrophil recruitment that was partially dependent on TLR4 and TLR2, and completely on Myd88, but not TRIF. A single CDE challenge stimulated MyD88-dependent excision of DNA base lesions 5-OH-Cyt, FapyAde, and FapyGua

Correspondence Sanjiv Sur, The University of Texas Medical Branch, Galveston, TX. sasur@UTMB.EDU; ssur@bcm.edu.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

from the lung genome. A single challenge of naïve WT mice with 5-OH-Cyt stimulated neutrophilic lung inflammation. Multiple CDE instillations stimulated MyD88-dependent allergic airway inflammation. Multiple administrations of 5-OH-Cyt with CDE stimulated allergic sensitization and allergic airway inflammation.

Conclusions and Clinical Relevance: We show for the first time that CDE challenge stimulates MyD88-dependent excision of DNA base lesions. Our data suggest that the resultant-free base(s) contribute to CDE-induced innate/allergic lung inflammation. We suggest that blocking the MyD88 pathway in the airways with specific inhibitors may be a novel targeted strategy of inhibiting amplification of innate and adaptive immune inflammation in allergic diseases by oxidatively induced DNA base lesions.

1 | INTRODUCTION

Exposure of the airway epithelial cells to ragweed pollen extract (RWPE) induces intracellular reactive oxygen species (ROS).¹ ROS generated in cells induce a plethora of DNA base lesions.² Maintaining the genomic integrity in the face of assault by oxidative stress is a constant challenge for living organisms.³ The DNA base excision repair (BER) pathways are the primary pathways that excise these oxidatively induced DNA base lesions.² BER is generally thought to play an important role in preventing mutations associated with 7,8-dihydro-8-oxo-guanine (8-OH-Gua).³ However, we discovered a new role of BER in allergen-induced innate and allergic lung inflammation. RWPE challenge in sensitized mice activates DNA repair enzyme 8-oxoguanine DNA glycosylase-1 (OGG1)-mediated excision of 8-OH-Gua from the genome of airway epithelial cells.⁴ The free base recombines with OGG1, initiating OGG1-Ras-GTPase-mediated activation of NF- κ B, which drives mRNA transcriptome responses and allergic airway inflammation.⁴⁻⁶ This earlier work⁴⁻⁶ suggests that allergenic extracts likely excise a set of DNA base lesions that facilitate innate/allergic lung inflammation. A major objective of this manuscript was to utilize a unique GC-MS/MS technology to identify this set of DNA base lesions that are modulated in the lung genome after exposure to an allergenic extract.

Innate neutrophil recruitment is a hallmark of an innate immune response.⁷ Neutrophil recruitment to the skin facilitates the induction of OVA-induced allergic skin inflammation.⁸ We reported that RWPE stimulates Toll-like receptor 4 (TLR4) and myeloid differentiation protein-2 (MD2)-dependent NF- κ B activation mediated recruitment of ROS-generating neutrophils to the lungs.^{9,10} Repeated challenge with RWPE shifts the immune response to a TLR4-dependent allergic inflammation.⁹ Adoptive transfer of purified neutrophils together with RWPE reconstitutes allergic inflammation in *Tlr4KO* mice.⁹ These studies highlight the contribution of innately recruited neutrophils to allergic inflammation. Recently, we reported that TLR4 as an innate receptor involved in pollen and cat dander-induced oxidative stress and DNA damage in airways.¹¹ Based on these studies, here we hypothesized that stimulation of a conserved innate receptor/adaptor pathway by allergenic extracts induces innate neutrophilic inflammation that excises a set of oxidatively induced DNA base lesions from the genome, and these free bases in turn further stimulate innate/allergic lung inflammation.

To test this hypothesis while ascertaining that the base excision properties are not limited to unique properties of RWPE,¹ it was important to perform the studies with an allergenic extract that is linked to human disease and yet is quite distinct from RWPE.¹ Large-scale epidemiological studies have identified cat dander as one of the most common indoor allergens that induce allergic sensitization and disease,^{12–22} and as a risk factor for prevalence, severity, and hospitalization from asthma.^{23–26} Being an animal extract, cat dander extract (CDE) is very different from RWPE and yet shares the ability of RWPE to induce oxidative stress and DNA damage.¹¹ However, very little is known about the innate receptors/adaptors involved in CDE-induced innate and allergic airway inflammation. For these reasons, we selected CDE as a model allergenic extract in the present study. To test our hypothesis, we performed CDE challenges in wild-type (WT) mice and mice lacking various innate receptors/adaptors, quantified innate and allergic inflammation to identify a conserved innate adaptor and utilized a sensitive GC-MS/MS technology to accurately quantify a repertoire of oxidatively induced DNA base lesions that are excised from the lung genome after CDE challenge in WT mice and mice lacking the identified adaptor.

2 | MATERIALS AND METHODS

2.1 | Mice

Eight- to 12-wk-old male WT mice (C57BL/10SNJ or C57BL/6J), *Tlr4KO* mice (C57BL/10ScNJ), T7r2KO mice (B6.129-Tlr2^{tm1Kir/J}), *Myd88KO* mice (B6.129P2(SJL)-*Myd88*^{tm1.1Defi/J}), and *TrifKO* mice (C57BL/6J-*Ticam1*^{Lps2/J}) from Jackson Laboratory (Bar Harbor, ME) were used for our studies. All mice were maintained in a pathogenfree environment at the University of Texas Medical Branch (UTMB Galveston, TX). Animal experiments were approved by UTMB IACUC (Institutional Animal Care and Use Committee) (approval no. 9708038B) and were performed according to the NIH Guide for Care.

2.2 | Allergenic extracts

Lyophilized CDE (lot# 253320) was purchased from Greer Labs (Lenoir, NC). All allergenic extracts had less than 0.1 pg endotoxin/ 1 µg protein using LAL chromogenic endotoxin quantitation kit (Thermo Scientific, Hudson, NH).

2.3 | Animal study protocols

Mice were anesthetized with low dose intraperitoneal xylazine and ketamine for intranasal challenge or sensitization and sacrificed by lethal overdose of intraperitoneal xylazine and ketamine.¹

2.3.1 | Single-challenge model—WT, *Tlr4KO*, *Tlr2KO*, *Myd88KO*, and *TrifKO* mice were challenged intranasally with a single dose of CDE (100 µg/60 µL or 1 µg/60 µL), uracil (Ura), cytosine (Cyt), 5-hydroxyuracil (5-OH-Ura), or 5-hydroxy-cytosine (5-OH-Cyt) (60 µL of 1 µmol/L solution)⁵ and sacrificed 2 or 16 hours post challenge (Figure S1A). In some experiments, 2 hours before CDE challenge, WT mice were treated with intraperitoneal 1 mg of the MyD88 inhibitor T6167923 (Aobious, Gloucester, MA),²⁷ challenged with CDE (100 µg/60 µL), and sacrificed (Figure S1B).

2.3.2 | Multiple-challenge model—WT or *Myd88KO* mice were sensitized by five intranasal doses of CDE (100 µg/60 µL) on days 0, 1, 2, 3, and 4, then challenged intranasally with PBS or CDE on day 11 and sacrificed after 2, 4, 16, or 72 hours post-challenge.^{9,10} In some experiments, WT mice were challenged with five intranasal low doses of CDE (1 µg/60 µL) with or without 5-OH-Cyt on days 0, 1, 2, 3, 4, and, 11 and sacrificed after 72 hours post-challenge (Figure S1C).

2.4 | Processing of BALF and lung samples

Bronchoalveolar lavage fluid (BALF) and lung samples for biochemical and histological examinations were taken as previously described.^{9,10} Briefly, to collect the BALF, trachea of mice was cannulated, and lungs were lavaged with ice-cold PBS (Corning, Manassas, VA). Mice were thoracotomized and the blood in pulmonary circulation flushed with PBS, and the lungs were excised. A portion of the lungs was frozen for RNA and DNA extraction. The remaining lungs were perfused and fixed with Zn fixative (BD Biosciences, San Jose, CA) for at least 24 hours and then embedded in paraffin, sectioned at a thickness of 4 µm, and stained with periodic acid-Schiff (PAS) for mucus staining. Total and differential cell counts were performed in BALF as previously described.^{9,10} In the single-challenge model, CXCL1/2, and in the multiple-challenge model, IL5, IL13, TSLP, and IL33 (Figure S1A and S1C) were assayed in BALF with ELISA development kits (R&D Systems, Minneapolis, MN). In some experiments, butylated hydroxytoluene (0.5 mmol/L; Sigma-Aldrich, St. Louis, MO) was added to BALF to prevent further lipid oxidation in some aliquots. The levels of GSSG in the BALF were measured with infinite 200 (Tecan, Durham, NC) using a glutathione/GSSG-412 kit (Bioxytech; OXIS, Portland, OR).¹

2.5 | Epithelial Mucin Score

Two investigators who were blinded to the treatment groups assessed mucin production using a minor modification of a method reported^{9,10} on a subjective scale of 0, 1, 2, 3, and 4 corresponding to none, mild, moderate, marked, or severe mucin deposition, respectively. The data were expressed as mean of score recorded by two blinded investigators.

2.6 | Measurement of CDE-specific IgE in serum

Ninety-six-well plates were coated with 100 µg/mL of cat dander protein overnight at room temperature and blocked for 2 hours with sea block buffer. Plate was applied with serum from mice overnight at room temperature. After washing, biotin-conjugated rat anti-mouse IgE (BD Biosciences, San Jose, CA) followed by avidin-alkaline phosphatase (Sigma-Aldrich, St. Louis, MO) was added. Fluorescence intensity was analyzed using AttoPhos substrate solution (Promega, Madison, WI).

2.7 | Measurement of DNA base lesions in lung genomic DNA

Genomic DNA from mouse lungs was extracted using Qia-gen Genomic-tip 500/G and Genomic DNA buffer set (Qiagen, Valencia, CA) according to manufacturer's protocol. We utilized gas chromatography-tandem mass spectrometry (GC-MS/MS) with isotope dilution using stable isotope-labelled analogues of analytes as internal standards to positively identify and accurately quantify the following DNA base lesions in lung genomic DNA:5-

OH-Cyt, 5-OH-Ura, thymine glycol (ThyGly), 5-hydroxy-5-methylhydantoin (5-OH-5-MeHyd), 4,6-diamino-5-formamidopyrimidine (FapyAde), 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), and 8-OH-Gua. Aliquots of 5-OH-Cyt- ^{13}C , $^{15}\text{N}_2$, 5-OH-Ura- $^{13}\text{C}_4$, $^{15}\text{N}_2$, ThyGly- $^2\text{H}_4$, 5-OH-5-MeHyd- ^{13}C , $^{15}\text{N}_2$, FapyAde- ^{13}C , $^{15}\text{N}_2$, FapyGua- ^{13}C , $^{15}\text{N}_2$, and 8-oxo-Gua- $^{15}\text{N}_5$ were added as internal standards to 50 μg of DNA samples. After drying in a SpeedVac, DNA samples were dissolved in 50 μL of an incubation buffer consisting of 50 mmol/L phosphate buffer (pH 7.4), 100 mmol/L KCl, 1 mmol/L EDTA, and 0.1 mmol/L dithio-threitol, and then incubated with 2 μg of *Escherichia coli* Nth and 2 μg of *E. coli* Fpg for 1 hour at 37°C in a water bath to release DNA base lesions from DNA. Subsequently, DNA was precipitated with ethanol, and supernatant fractions were separated, lyophilized, and trimethylsilylated as described.²⁸ Derivatized samples were analyzed by GC-MS/MS with multiple reaction monitoring (MRM). The following mass transitions were used: m/z 343 $\rightarrow$ m/z 342 and m/z 346 $\rightarrow$ m/z 345 for 5-OH-Cyt and 5-OH-Cyt- ^{13}C , $^{15}\text{N}_2$, respectively; m/z 344 $\rightarrow$ m/z 343 and m/z 350 $\rightarrow$ m/z 349 for 5-OH-Ura and 5-OH-Ura- $^{13}\text{C}_4$, $^{15}\text{N}_2$, respectively; m/z 448 $\rightarrow$ m/z 259 and m/z 452 $\rightarrow$ m/z 262 for ThyGly and ThyGly- $^2\text{H}_4$, respectively; m/z 346 $\rightarrow$ m/z 331 and m/z 349 $\rightarrow$ m/z 334 for 5-OH-5-MeHyd and 5-OH-5-MeHyd- ^{13}C , $^{15}\text{N}_2$, respectively; m/z 369 $\rightarrow$ m/z 354 and m/z 372 $\rightarrow$ m/z 357 for FapyAde and FapyAde- ^{13}C , $^{15}\text{N}_2$, respectively; m/z 457 $\rightarrow$ m/z 368 and m/z 460 $\rightarrow$ m/z 371 for FapyGua and FapyGua- ^{13}C , $^{15}\text{N}_2$, respectively; m/z 455 $\rightarrow$ m/z 440 and m/z 460 $\rightarrow$ m/z 445 for 8-oxoGua and 8-oxoGua- $^{15}\text{N}_5$, respectively. GC-MS/MS conditions were as described previously.²⁹

2.8 | qRT-PCR analysis

Total RNA was extracted from mouse lungs and cDNA synthesized using an RNeasy mini kit and cDNA Synthesis kit (Qiagen, Valencia, CA), and subjected real-time PCR on an ABI 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) using RT² Profiler PCR Arrays for Mouse Allergy & Asthma PCR Array or Mouse Innate & Adaptive Immune Responses PCR Array (catalogue number PAMM-067Z and PAMM-052Z, respectively; SABiosciences, Valencia, CA) using SYBR Green Kit (Qiagen, Valencia, CA). Primer sequences for *Hprt*, *Cxcl1*, and *Cxcl2* were as described.⁹ Primer sequences for *Il1b* and *Icam1* were as follows: mouse *Il1b*, forward: 5'-CTCTTGTTGATGTGCTGCTG-3', reverse, 5'-GACCTGTTCTTTG AAGTTGACG-3'; mouse *Icam1*, forward, 5'-GGTCCTTGCTACTT GCTG-3', reverse, 5'-CTGTGCTTTGAGAACTGTGG-3'.

These primers were purchased from Integrated DNA Technologies (Coralville, IA).

2.9 | Hierarchical cluster and PANTHER analyses

Hierarchical clusters were constructed with Morpheus (<https://software.broadinstitute.org/morpheus/>). In the hierarchical clustering analysis (Figures 2A and 4A; Figure S2B), red represents increased expression, green represents decreased expression, and black depicts no change in expression. The PCR array data were analyzed by PANTHER (version 9.0, Protein ANalysis THrough Evolutionary Relationships, (<http://www.pantherdb.org/>)).

2.10 | Statistical analysis

The results of this study are presented as mean of measurements of independent samples in a treatment group, and the uncertainties are expressed as standard deviations, Std.

Comparisons of two groups were done by unpaired *t* test, and multiple comparisons by ANOVA. We utilized the software package GraphPad Prism 6 (GraphPad Software, San Diego, CA) to perform analysis of variance followed by the statistically stringent rigid Bonferroni-Dunn posthoc analysis. All statistical analyses considered data significant at $P < 0.05$. The degrees of significant *P* values are designated with the following asterisk: $*=P < 0.05$, $**=P < 0.01$, $***=P < 0.001$, and $****=P < 0.0001$.

3 | RESULTS

3.1 | A single challenge with cat dander extract induces Myd88-dependent innate inflammation and oxidative stress

Because RWPE induces the TLR4/MD2-dependent CXCL chemo-kine pathway that almost entirely mediates innate neutrophil recruitment and allergic inflammation,^{9,10} we initially examined the contribution of TLR4 in CDE-induced innate neutrophilic inflammation. Compared to WT mice, a single CDE challenge (CDE single-challenge model, Figure S1A) in *Tlr4KO* elicited 50% less innate neutrophil recruitment (Figure 1A). These data implied that a second TLR also participates in CDE-induced innate neutrophil recruitment; we hypothesized that TLR2 may be involved in this CDE-induced innate response. We tested this hypothesis; compared to WT mice, a CDE single-challenge model in *Tlr2KO* mice elicited 50% less innate neutrophil recruitment (Figure 1B). Together these data indicate that CDE-mediated innate neutrophil recruitment is dependent on both TLR2 and TLR4. Since the common adaptor shared by TLR2 and TLR4 is MyD88, we hypothesized that MyD88 might comprehensively regulate CDE-induced innate neutrophil recruitment. Consistent with our hypothesis, a CDE single-challenge model in *Myd88KO* mice failed to induce any innate neutrophil recruitment (Figure 1C). By contrast, a CDE single challenge in *TrifKO* mice stimulated CDE-induced innate neutrophil recruitment (Figure 1C). The small molecule MyD88 inhibitor T6167923 disrupts MyD88 signalling by inhibiting homodimer formation²⁷ and protects mice against lethal staphylococcal enterotoxin B-induced toxic shock.²⁷ Administration of this MyD88 inhibitor to WT mice before CDE single-challenge model (Figure S1B) attenuated CDE-induced innate neutrophil recruitment (Figure 1D). We have previously reported that a single RWPE challenge in mice increases TLR4-dependent lung *Cxcl1/2* mRNA expression and secretion of CXCL1 and CXCL2 in BALF, which stimulates CXCR2-dependent innate neutrophilic inflammation.⁹ Similar to this effect of RWPE,⁹ challenging naïve mice with CDE increased Myd88-dependent lung *Cxcl1* and *Cxcl2* mRNA expression (Figure 1E), and secretion of CXCL1 and CXCL2 in BALF (Figure 1F). We recently reported that a single CDE challenge in mice increases TLR4-dependent cell-free BALF concentration of oxidized glutathione (GSSG).¹¹ Likewise, a CDE single-challenge challenge in WT mice increased cell-free BALF concentration of GSSG 16 hours after challenge (Figure 1G). This increase in BALF levels of GSSG was not observed in *Myd88KO* mice. Taken together with our earlier reports,^{9–11} these results indicate that MyD88 is the critical TLR adaptor that mediates CDE-induced innate neutrophilic inflammation and oxidative stress.

Building on the observation that CDE utilizes a shared MyD88 pathway for inducing innate inflammation and oxidative stress, we focused our efforts on CDE as a model allergen for additional mechanistic studies on the role of Myd88-dependent gene expression and DNA base lesion studies. We performed qPCR arrays for lung mRNAs of 84 innate immune response genes (Table S1) 2 hours after a CDE single-challenge model in WT and *Myd88KO* mice (Figure S1A) and subjected the data to hierarchical clustering and volcano plot analysis. Hierarchical clustering analysis rendered a dendrogram with four clusters of transcripts (Figure 2A); cluster 2 showed upregulation in WT, but not *Myd88KO* mice (Figure 2A). Compared to PBS challenge, a single CDE challenge in WT mice increased lung mRNA expression of 22 genes: *C5ar1*, *Ccl12*, *Ccr8*, *Cd4*, *Cd80*, *Csf2*, *Cxcl10*, *Icam1*, *Ifnar1*, *Ifnb1*, *Il10*, *Il17a*, *Il1a*, *Il1b*, *Il23a*, *Il6*, *Nfkb1*, *Nfkb1a*, *Nlrp3*, *Nod2*, *Tlr2*, and *Tnf*, largely in cluster 2 (Figure 2B). The expression levels of the remaining 62 genes were not significantly changed. Subjecting the gene expression data to PANTHER analysis predicted that CDE challenge activates eight signalling pathways in WT mice (Figure 2C). Compared to WT mice, CDE challenge in *Myd88KO* mice elicited significantly decreased lung mRNA expression of 12 of these 22 genes: *Ccl12*, *Ccr8*, *CD4*, *Cd80*, *Cxcl10*, *Icam1*, *Il23a*, *Il1b*, *Nfkb1*, *Nfkb1a*, *Nod2*, and *Tnf* (Figure 2D; Figure S2A), but not *Ifnb1* (Figure 2D). These data suggest one or more of these 12 genes may contribute to CDE-induced innate inflammation.

3.2 | A single challenge with cat dander extract induces excision of 5-OH-Cyt, FapyAde, and FapyGua from lung genome

Building on these observations that MyD88 regulates CDE-induced oxidative stress and innate inflammation, we hypothesized that allergenic extracts may stimulate MyD88-dependent excision of oxidatively induced DNA base lesions. Consistent with our hypothesis, a single CDE challenge (Figure S1A) in WT mice innately decreased the levels of 5-OH-Cyt, FapyAde, and FapyGua, but not 8-OH-Gua, in the lung genome 16 hours after challenge (Figure 3A). By contrast, a CDE challenge in *Myd88KO* mice failed to decrease the levels of these base lesions (Figure 3A).

3.3 | 5-OH-Cyt challenge induces innate lung inflammation

Because exposure of the lungs of WT mice to 8-oxoGua stimulates innate neutrophilic inflammation,⁵ we hypothesized that excised DNA base lesions observed in the present study may also stimulate innate neutrophilic inflammation. To test our hypothesis, we selected 5-OH-Cyt as a model oxidized DNA base. A single i.n. 5-OH-Cyt challenge, but not other control bases such as Cyt, Ura, or 5-OH-Ura, stimulated neutrophil recruitment to the airways of naive WT mice (Figure 3B), validating our hypothesis, and demonstrating the specificity of 5-OH-Cyt. Building on our observation that CDE challenge stimulates MyD88-dependent increase in expression of *Il-1b* and ICAM 1 in the lungs (Figure 2D) and these genes play an important role in the pathogenesis of asthma,^{30,31} we next examined and elucidated that a single i.n. 5-OH-Cyt challenge in the naive mice increases the expression of *Icam1* and *Il1b* mRNA expression in the lungs (Figure 3C).

3.4 | CDE requires MyD88 to induce allergic airway inflammation

Building on our observations that MyD88 regulates CDE-induced innate mRNA expression and neutrophil recruitment, taken together with earlier reports that innate neutrophil recruitment facilitates allergic inflammation,^{8,9} we hypothesized that MyD88 should modulate CDE-induced allergic inflammation. To test this hypothesis, we performed a CDE multiple-challenge model (Figure S1C).^{9,10} As expected, these challenges induced allergic inflammation in WT mice (Figure 4A-G). This was characterized by an increase in total cells (Figure 4A), eosinophils (Figure 4B), and neutrophils (Figure 4C) in BALF, stimulation of mucin in airway epithelial cells (Figure 4D,E), increase in serum levels of cat dander-specific IgE (Figure 4F), and increase in BALF levels of IL5, IL13, IL33, and TSLP at 72 hours post-challenge (Figure 4G). All these increases were either abolished or attenuated in *Myd88KO* mice (Figure 4A-G). These observations indicate that CDE requires MyD88 for inducing allergic inflammation.

We next utilized CDE as a model allergen to identify allergy genes modulated by CDE multiple-challenge model (Figure S1C). After the sixth challenge (Figure S1C), lung mRNA expression of 84 allergy and asthma-associated genes (Table S1) was quantified at different time-points and subjected to hierarchical clustering analysis. A dendrogram for CDE challenges to WT mice 2, 4, and 16 hours post-challenge showed six major clusters of transcripts (Figure S2B). Cluster 4 (Figure 5A) contained 35 transcripts with mostly upregulated (red colour) genes at 4 hours post-challenge. Compared to PBS challenge, volcano plot analyses identified that the sixth CDE challenge in WT mice modified 42 of these 84 genes, increasing the expression of 39 genes: *Adrb2*, *Areg*, *Ccl11*, *Ccl12*, *Ccl17*, *Ccl22*, *Ccl26*, *Ccl4*, *Cd40lg*, *Chill*, *Clca3*, *Csf2*, *Csf3r*, *Ear11*, *Ear5*, *Ets1*, *Icos*, *Ifng*, *Ifngr2*, *Il10*, *Il13*, *Il13ra1*, *Il13ra2*, *Il17a*, *Il21*, *Il25*, *Il2ra*, *Il3*, *Il4*, *Il4ra*, *Il9*, *Mmp9*, *Pdcd1*, *Retnlg*, *Stat5a*, *Tgfb1*, *Tnfrsf4*, *Tnfsf4*, and *Tslp* (Figure 5B). Panther analysis revealed activation of 16 signalling pathways (Figure 5C). Compared to WT mice, CDE challenge in *Myd88KO* mice failed to increase in the mRNA expression of 20 of these 39 upregulated genes: *Areg*, *Ccl17*, *Ccl22*, *Ccl4*, *Chill*, *Clca3*, *Csf2*, *Csf3r*, *Icos*, *Ifngr2*, *Il10*, *Il13ra1*, *Il17a*, *Il21*, *Il2ra*, *Il4ra*, *Mmp9*, *Retnlg*, *Stat5a*, and *Tgfb1* (Figure 5D, Figure S2C). These data suggest that one or more these 20 genes contribute to MyD88-dependent allergic sensitization/inflammation.

3.5 | 5-OH-Cyt facilitates CDE-induced allergic airway inflammation

Building on the observation that a single challenge with 5-OH-Cyt stimulates neutrophil recruitment (Figure 3B) and the expression of lung *Icam1* and *Il1b* (Figure 3C), we hypothesized that repeated administration of 5-OH-Cyt with CDE may facilitate allergic sensitization and inflammation. To minimize the effect of neutrophil recruitment induced by CDE challenge, we first identified a low dose (1 µg) of CDE that does not induce neutrophil recruitment in WT mice (Figure 6A). Next, we examined the effects of repeated intranasal administrations of this low dose of CDE together with 5-OH-Cyt or PBS (Figure S1C). Compared to repeated administration of this low dose of CDE, repeated administration of CDE+5-OH-Cyt stimulated allergic sensitization, as indicated by an increase in the serum levels of cat dander-specific IgE (Figure 6B), and augmented allergic inflammation, characterized by an increase in total cells (Figure 6C) and eosinophils (Figure 6D) in BALF.

4 | DISCUSSION

We recently reported that stimulation of TLR4 innate receptor RWPE and CDE induces rapid oxidative stress and DNA damage in airways.¹¹ Here, we extend these observations by demonstrating for the first time that a single challenge with an allergenic extract induces MyD88-dependent excision of oxidatively induced DNA base lesions such as 5-OH-Cyt, FapyAde, and FapyGua from the lung genome. MyD88 is an essential adaptor for TLRs signalling and mediates innate chemokine secretion and neutrophil recruitment.^{32–34} To our knowledge, this is the first report of using GC-MS/MS to accurately quantify oxidatively induced DNA base lesions on lung genome after allergen exposure. Because CDE challenge in WT but not *Myd88KO* mice recruits ROS-generating neutrophils to the airways, it is likely that oxidative stress generated by these recruited neutrophils contributes to excision of these DNA base lesions.

We previously reported that RWPE challenge activates OGG1-mediated excision of 8-OH-Gua from the genome, and the free base recombines with OGG1, initiating OGG1-Ras-GTPase-mediated activation of NF- κ B, which drives mRNA transcriptome responses and allergic airway inflammation.^{4–6} Thus, the ability of 5-OH-Cyt to stimulate innate neutrophil recruitment and facilitate allergic airway inflammation in the present study suggests that MyD88-dependent excision release of specific DNA base lesions by CDE challenge contributes to innate and allergic airway inflammation. In the present study, we show that CDE challenge stimulates MyD88-dependent increase in expression of *Il-1b* and *Icam 1* in the lungs. Prior studies have shown that inhibiting IL-1 β signalling or ICAM-1 attenuates airway hyperresponsiveness and eosinophilic inflammation in animal models of asthma.^{30,31} Taken together, these results suggest that CDE challenge stimulates excision release of 5-OH-Cyt from the lung genome, and the free 5-OH-Cyt base stimulates IL-1 β and ICAM-1-mediated allergic lung inflammation.

A previous study reported that recombinant *Felis domesticus* 1 (Fel d 1), the major allergen in cat dander, binds lipopolysaccharide (LPS) and stimulates TLR4 in cultured HEK293 cells,³⁵ leading to the question whether endotoxin contamination of the CDE used in the present study could explain the oxidative stress and DNA damage repair induced by exposure to CDE. In the present study, CDE had <0.1 pg endotoxin/1 μ g allergenic extract protein. In several peer-reviewed publications, we have documented that such low levels of endotoxin in allergenic extracts cannot stimulate the innate and allergic immune responses.^{9–11} First, adoptive transfer of neutrophils from WT donor mice together with multiple intranasal RWPE challenges reconstitutes RWPE-induced allergic inflammation in *Tlr4 KO* mice that lack the receptor to respond to LPS.⁹ Second, forcing recruitment of neutrophils in *Tlr4KO* mice by administration of a superoxide generator also reconstitutes RWPE-induced allergic inflammation in *Tlr4KO* mice.⁹ Third, unlike the innate effects of LPS that are dependent on CD14, RWPE, and CDE utilize a CD14-independent pathway to induce an innate neutrophilic airway inflammation.¹⁰ Fourth, in TLR4^{Hi}-MD2 cells that overexpress TLR4 and MD2 but lack CD14 and cannot bind or respond to very high concentrations of LPS, RWPE and CDE can bind to the receptor and induce CXCL chemokine secretion.¹⁰ Finally, allergenic extracts stimulate intracellular ROS generation in TLR4^{Hi} cells, whereas LPS even at very high (1 μ g/mL) concentration fails to induce ROS generation.¹¹ For all

these reasons, the results of the present study cannot be attributed to endotoxin contamination of allergenic extracts.

A single CDE challenge induced MyD88-dependent innate immune response that increased expression of several genes in the present study, including *Il1b*, *Il23a*, *Cxcl10*, and *Tnf*. Because neutrophil recruitment is crucial for the induction of allergic airway inflammation and sensitization,⁹ it is possible that MyD88-mediated IL1 β and TNF secretion recruits neutrophils and stimulates neutrophil-mediated allergic sensitization.^{36,37} Likewise, since TNF secretion can be IL23-dependent,³⁸ CDE-induced IL23 secretion could contribute to CXCL10 secretion and induce neutrophil recruitment³⁹ and allergic sensitization.⁹

Multiple-challenge with CDE induced MyD88-dependent increases in the expression of *Ccl17*, *Il17a*, and *Mmp9* in the present study. Several studies indicate a role of CCL17, IL17a, and MMP9 in allergic inflammation. Inhibition of CCL17 by anti-CCL17 antibody decreases airway hyperresponsiveness, allergic airway inflammation, and secretion of Th2 cytokines into the BALF in an OVA-induced allergic asthma model.⁴⁰ Mice lacking *Mmp9* demonstrate less secretion of CCL17 and decreased allergic airway inflammation and sensitization.⁴¹ In human subjects, IL17 levels correlate with the severity of asthma,^{42,43} IL17a enhances neutrophil recruitment through CXCR2 signalling and exacerbates airway hyperresponsiveness in mouse airway inflammation.⁴⁴ Taken together with our results, these reports suggest that MyD88-dependent upregulation in *Ccl17*, *Mmp9*, and *Il17a* may contribute to CDE-induced allergic airway sensitization/inflammation.

In summary, CDE challenge stimulates MyD88-dependent innate and allergic lung inflammation, and excision of 5-OH-Cyt, FapyAde, and FapyGua from the lung genome. 5-OH-Cyt stimulates innate neutrophil inflammation. Administration of 5-OH-Cyt with CDE stimulates allergic airway inflammation. These observations indicate that excision of oxidatively induced bases such as 5-OH-Cyt by CDE contributes to CDE-induced innate and allergic airway inflammation. We suggest that blocking the MyD88 pathway in the airway with specific inhibitors or inhibiting oxidative stress-induced excision of 5-OH-Cyt from the lung genome may be a novel targeted strategies of inhibiting allergic inflammation in allergic diseases such as allergic rhinitis and asthma.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

MyD88 mediates CDE-induced innate lung inflammation. A-G, BALF innate immune response and lung mRNA expression in CDE-induced single-challenge model (Figure S1A). A-D, BALF neutrophil counts in (A) WT and *Tlr4*KO mice (n = 5 per group); (B) WT and *Tlr2*KO mice (n = 3-4 per group); (C) WT, *Trif*KO, and *Myd88*KO mice (n = 3-5 per group); (D) WT mice treated with a MyD88 inhibitor (Figure S1B) prior to CDE challenge (n = 4 per group) (E) CDE-induced *Cxcl1* and *Cxcl2* lung mRNA expression in WT and *Myd88*KO mice (n = 3-4 per group). (F) CDE-induced CXCL1 and CXCL2 secretion into the BALF in WT and *Myd88* KO mice (n = 4-6 per group). (G) BALF level of oxidized glutathione (GSSG) in WT and *Myd88* KO mice in single-challenge model (Figure S1A) (n = 4-5 per group). Data are expressed as means ± standard deviations, Std. *=*P* < 0.05, **=*P* < 0.01, ***=*P* < 0.001, and ****=*P* < 0.0001, analyzed by *t* test or ANOVA and posthoc test Bonferroni

FIGURE 2.

CDE challenge induces MyD88-dependent innate gene expression. A-D, Innate gene expression in the lung after a PBS or CDE single-challenge model (Figure S1A) ($n = 3-4$ per group). A, Hierarchical clustering analysis showing single CDE challenge-induced fold change of mRNA over PBS control in WT mice (mouse #1-8) and *Myd88*KO mice (mouse #9-14) after 2 h post challenge. B,D, Volcano plot showing fold difference of mRNA in the lung and p value between 2 h post CDE challenge vs PBS control in WT mice (B), and between WT mice and *Myd88*KO mice (D) 2 h post CDE challenge. C, Pie chart showing predicted signalling pathways activated in WT mice after a single CDE challenge

FIGURE 3.

CDE challenge induces MyD88-dependent excision of DNA base lesions. A, The content of 5-OH-Cyt, FapyAde, FapyGua, and 8-OH-Gua in the lung genomic DNA of WT or *Myd88KO* mice 16 h after a single CDE challenge (Figure S1A) (n = 3 per group). B, BALF neutrophil counts in WT mice 16 h after a single challenge (Figure S1A) with PBS, 60 μ L of 1 μ mol/L solution cytosine (Cyt), 5-OH-Cyt, uracil (Ura), or 5-OH-Ura (n = 3-5 per group). C, 5-OH-Cyt-induced *Il1b* and *Icam1* lung mRNA expression in WT mice (n = 4 per group). Data are expressed as means $\pm$ standard deviations, Std. * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$, and **** = $p < 0.0001$, analyzed by *t* test or ANOVA and post-hoc test Bonferroni

FIGURE 4.

MyD88 mediates CDE-induced allergic sensitization and inflammation. A-G, CDE multiple-challenge model in WT and *Myd88* KO mice (Figure S1C) (n = 5-7 per group). Number of total inflammatory cells (A), eosinophils (B), and neutrophils (C), in BALF, and mucin secreting airway epithelial cells (D, E). Original magnifications, $\times 400$. F, Serum CDE-specific IgE. G, BALF level of IL5, IL13, IL33, and TSLP. Data are expressed as means $\pm$ standard deviations, Std. $*=P < 0.05$, $**=P < 0.01$, $***=P < 0.001$, and $****=P < 0.0001$, analyzed by ANOVA and posthoc test Bonferroni

FIGURE 5.

Lung mRNA expression in CDE multiple-challenge model. A-D, Gene expression and signalling pathways in CDE multiple-challenge model (Figure S1C) (n = 3 per group). Cluster 4 (A) of the hierarchical clustering analysis (Figure S2B) showed the greatest change of lung mRNA expression 2, 4, and 16 h after CDE challenge in WT mice. (B, D) Volcano plot showing fold difference of lung mRNA expression and p value 4 h post CDE challenge. B, Comparison between CDE vs. PBS in WT mice. D, Comparison between WT mice and *Myd88KO* mice. C, Pie chart showing predicted signalling pathways activated in CDE multiple-challenge model

FIGURE 6.

5-OH-Cyt facilitates CDE-induced allergic airway inflammation. A, BALF neutrophil counts in WT mice 16 h after a single challenge (Figure S1A) with PBS or 1 µg CDE or 100 µg CDE (n = 4-5 per group). B-D, Allergic sensitization or allergic inflammation induced by multiple administration 1 µg CDE with or without 60 µL of 1 µmol/L solution 5-OH-Cyt in WT mice (Figure S1C) (n = 6-7 per group). B, Serum CDE-specific IgE. Number of total inflammatory cells (C) and eosinophils (D) in BALF. Data are expressed as means ±

standard deviations, Std. $*=P<.05$, $**=P<0.01$, $***=P<0.001$, and $****=P<0.0001$, analyzed by t test or ANOVA and post-hoc test Bonferroni