

Pollen-induced oxidative DNA damage response regulates miRNAs controlling allergic inflammation

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Abstract

A mucosal oxidative burst is a hallmark response to pollen exposure that promotes allergic inflammatory responses. Reactive species constituents of oxidative stress signal via the modification of cellular molecules including nucleic acids. One of the most abundant oxidative genomic base damage is 8-oxo-7,8-dihydroguanine (8-oxoG), which is removed from DNA by 8-oxoguanine DNA glycosylase1 (OGG1). OGG1 in complex with 8-oxoG acts as a GDP-GTP exchange factor and induces acute inflammation; however, the mechanism(s) by which OGG1 signaling regulates allergic airway inflammation is not known. Here, we postulate that the OGG1 signaling pathway differentially altered the levels of small regulatory RNAs and increased the expression of T helper 2 (Th2) cytokines in ragweed pollen extract (RWPE)-challenged lungs. To determine this, the lungs of sensitized mice expressing or lacking OGG1 were challenged with RWPE and/or with OGG1's excision product 8-oxoG. The responses in lungs were assessed by next-generation sequencing, as well as various molecular and histological approaches. The results showed that RWPE challenge induced oxidative burst, damage to DNA and activated OGG1 signaling, resulting in the differential expression of 84 microRNAs, which then exacerbated antigen-driven allergic inflammation and histological changes in the lungs. The exogenous administration of the down-regulated let-7b-p3 mimetic or inhibitors of up-regulated miR23a or miR27a decreased eosinophil recruitment, mucus and collagen production via controlling the expression of IL4, IL5, and IL13. Together, these data demonstrate the roles of OGG1 signaling in the regulation of antigen-driven allergic immune responses via differential expressions of microRNAs upstream of Th2 cytokines and eosinophils.

Introduction

In organs and tissues affected by environmental allergens one of the most referenced forms of oxidatively damaged DNA bases is 8-oxo-7,8-dihydroguanine (8-OH-Gua = 8-oxoG) and its open-ringed form 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua = FapyG) from both human patients and experimental animal models (19, 29, 33, 38, 54, 58). Comprehensive studies have also shown that the length of exposure and disease intensity correlates well with increased levels of the genomic 8-oxoG and repair by product of OGG1 (the free base 8-oxoG) in sputum, serum, urine and bronchoalveolar lavage fluid (BALF) (38, 58). Despite these close correlations genomic or the free 8-oxoG base is traditionally considered as a marker of oxidative stress, and whether these lesion(s) contribute to the development, maintenance and/or exacerbation of allergic disease processes remains unknown (8, 13, 26).

8-OxoG is potentially mutagenic and is preferentially repaired by the DNA base excision repair (BER) pathway (28, 49), which primarily occurs via 8-oxoguanine DNA glycosylase1 (OGG1). OGG1 excises the lesion by cleaving its N-glycosidic bond to release the 8-oxoG base, followed by endonucleolytic cleavage and filling of nucleoside gaps by DNA polymerases the DNA strand is then sealed by ligases (28, 49). Deficiency in OGG1-BER leads to the accumulation of 8-oxoG, which is conventionally considered mutagenic and associated with various pathological changes that accelerate aging and age-associated diseases (25). Unexpectedly, the lack of OGG1 activity in *Ogg1* knockout mice (*Ogg1*^{-/-}) and the consequent increases in the level of genomic 8-oxoG did not alter embryogenesis or life span, and mice showed no increased tumor frequency or marked pathological changes (41, 48). Among the phenotypic changes in *Ogg1*^{-/-} mice best defined thus far are aberrant immune responses to bacterial infections, as well as lipopolysaccharide (LPS) exposures as shown by lower levels of Th1 chemokines, cytokines and interleukin expression (45, 62). In line with these observations, decreased ovalbumine-induced allergic immune responses were documented in *Ogg1*^{-/-} mice partially due to low levels of phosphorylation of nuclear factor-κB (NF-κB) and STAT6, which

decreased the expression of Th1 and Th2 cytokines (44). Silencing *Ogg1* expression in the lungs of ragweed pollen extract (RWPE)-sensitized mice resulted in the accumulation of genomic 8-oxoG, but lower allergic inflammatory responses, as documented by the decreased expression of Th2 cytokines (IL-4, IL-5 and IL-13), eosinophilia and airway hyper-responsiveness (10). Decreased immune responses in OGG1 deficient mice could be explained by both a lack of OGG1 and free 8-oxoG base. Follow up work has documented that the free 8-oxoG base is bound by cytosolic OGG1, and that the formed complex functions as a guanine nucleotide exchange factor to activate small GTPases, including Kirstein (K)-RAS (1, 15, 32). Down-stream from KRAS, the *v-raf* murine leukemia viral oncogene homolog 1 (RAF1), mitogen activated kinases (MEK1/2, ERK1/2), mitogen-stress related kinase-1 (MSK-1), and phosphatidylinositol-4,5-bisphosphate 3-kinases (PI3K) pathways are activated, resulting in the phosphorylation and nuclear translocation of transcription factors such as NF- κ B (1), which as a key regulator of inflammation in asthma and other chronic pulmonary diseases (16, 60).

Signaling pathways down-stream from small GTPases are known to regulate the expression of both coding and non-coding RNAs including miRNAs (3, 18). This study, used next generation sequencing, and system level analysis supported by molecular approaches, to demonstrate that OGG1 signaling pathways regulate the expression of miRNAs that contribute to RWPE-induced allergic responses in an experimental mouse model. The modulation of miRNA levels in the lungs by the administration of mimics (or inhibitors) decreased RWPE-induced allergic immune and histological changes in the lungs. These data are the first to show that OGG1-signaling differentially modulates the expression of microRNAs in airways, which leads to the overexpression of Th2 cytokines and the exacerbation of antigen-driven allergic immune and tissue responses. The pharmacological inhibition DNA damage-repair signaling or the topical administration of specific miRNAs could have clinical utility.

Materials and Methods

Animals and treatment

Animal experiments were performed according to the NIH Guide for the Care and Use of Experimental Animals and approved by the University of Texas Medical Branch (UTMB) Animal Care and Use Committee (approval no. 0807044A). Eight-week-old BALB/c mice (Jackson Laboratory, Bar Harbor, ME, USA) were used for these studies. Parallel groups of mice were sensitized (on day 0 and 4 via the intraperitoneal route) using LPS-free ragweed (*Ambrosia artemisiifolia*) pollen extract (RWPE; 100 µg mixed with alum) (14). Allergic immune and tissue responses in OGG1-proficient and -deficient airways with or without miRNA mimics (or their inhibitors) were induced by LPS-free RWPE (50 µg in 60 µl saline; n = 5 per group) (2, 14). Randomly selected groups of mice were challenged i.n. on day 0 or days 2 and 4 with 60 µL of pH-balanced 8-oxoG (Cayman Chemicals, Ann Arbor, MI) solution (pH: 7.4; 0.0005 mg per kg) or saline (3). MiRNA mimics or inhibitors were introduced into airways via i.n. using Lipofectamine[®] RNAiMAX (Invitrogen). Depletion of target gene 8-oxoguanine DNA glycosylase1 (*Ogg1*) was performed using stealth RNAi[™] (Cat # MSS237431; Invitrogen Life Technologies) in RNAi transfection reagent (Cat# 201–10G New York, NY) at -72 h and -24 h (1).

Assessment of oxidative stress

MLE-12 (immortalized mouse lung epithelial cells) were cultured in RPMI 1640 medium per the instructions of the American Type Culture Collection (ATCC). Cells were loaded with 2'-7'-dihydro-dichlorofluorescein diacetate (H₂DCF-DA; Molecular Probes) and were mock or challenged with nicotinamide adenine dinucleotide phosphate oxidase (NOX) active RWPE (RWPE^{NOX}) or heat-inactivated (56°C for 30 min) RWPE (RWPE^{HI}). Changes in DCF fluorescence were determined at 480 nm and 526 nm (excitation and emission, respectively)

using a BioTek FLx800 fluorometer (11, 14). Oxidized (carbonylated) protein levels were assessed prior to and after RWPE challenge. Lung lysates were derivatized with 4-dinitrophenylhydrazine (DNPH; Sigma/Millipore), and carbonylated proteins were detected by anti-DNP antibody (MAB2223, clone 9H8.1) according to manufacturer recommendations and as we published (2).

Levels of genomic 8-oxoG in the airways of mock and RWPE^{NOX} challenge OGG1^P and OGG1^D mice were determined by quantitative (q) PCR. DNAs were isolated at 1 h post-challenge (mock or RWPE^{NOX}) using the Qiagen Genomic-tip 20/G kit per the manufacturer's recommendations. To prevent aerial oxidation during genomic DNA isolation, 100 μ M TEMPO (2,2,6,6-tetramethylpiperidine-*N*-oxyl) was added to the buffers (21). The DNA was quantitated by Pico Green (Molecular Probes), and gene-specific qPCR of DNA was performed after mock or recombinant (r)OGG1 (Novus Biologicals; 5 ng per sample) digestion in a 100- μ l reaction buffer (50 mmol/l Tris-HCl [pH 7.4], 50 mM NaCl, 1 mmol/l EDTA, 1 mM dithiothreitol, 1 mg/ml bovine serum albumin) at 37°C for 15 min. After digestion, DNA was extracted and 20 ng was subjected to q-PCR. A 3.2-kb strand of the DNA polymerase beta (*Pol b*) gene was amplified using gene-specific primers. The primers were designed using <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>. The primers were synthesized by Integrated DNA Technologies (IDT, Coralville, Iowa USA), and the amplified product was validated by sequencing. The base excision activity of rOGG1 was determined using a 40-mer oligonucleotide (100 fmol per reaction) containing an 8-oxoG labeled at the 3' end with Cy5 (5'-AGAGAAGAAGAAGAAGAA /8oxodG/AGATGGGTTATTCGAAGCTAGC/3Cy5Sp/-3'), as we previously described (32).

Western blot analysis

Lung were homogenized by using a TissueMiser (Fisher Scientific) homogenizer (25,000 rpm, on ice) in lyses buffer containing 25 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1

mM EDTA, 1% NP-40, 1 mM DTT, 5% glycerol, protein inhibitor cocktail (Cat # P0044; Sigma-Aldrich, Inc). Tissue homogenates were clarified by centrifugation at 12,000 x g and the supernatants collected and 20 µg proteins per lane were separated by 4-20% SDS-polyacrylamide gel electrophoresis (Mini-Protean Gel, Cat # 546-1094, Bio-Rad Laboratories), and proteins were subsequently transferred onto nitrocellulose membranes (Amersham Biosciences). The membranes were then blocked with 3% BSA in Tris-buffered saline (TBS) containing 0.1% Tween (TBS-T) for 3h at room temperature. Primary antibody (rabbit anti-OGG1; Cat # ab124741; AB Chem Inc) diluted in 3% BSA in TBS-T (1:3000) and incubated with the membrane overnight at 4°C as we described previously (9, 52). The membranes were washed then blocked with 3% BSA as above for 3h and incubated with horseradish peroxidase-conjugated secondary (goat anti-rabbit Ab (Cat # 4050-05; Southern BioTech). The chemiluminescent signals were detected by using the Western Blotting Detection Reagents (GE Life Sciences) and autoradiography. Membranes were stripped (Restore PLUS Western Blot Stripping buffer (Cat # 46430; Thermo Scientific) and re-probed using anti GAPDH antibody (Cat # 2118S; Cell Signaling Technology).

RNA isolation, sequencing and analysis

Excised lungs (100 mg wet weight) were homogenized and RNAs were extracted using an RNeasy kit per the manufacturer's instructions (Qiagen, Valencia, CA, USA). Ten ug RNA from each sample were provided to UTMB's Next-Generation Sequencing Core Facility (Director: Dr. Thomas G. Wood), and sequencing analysis was performed using an Illumina HiSeq 1000 sequencing system (Illumina Inc., San Diego, CA, USA). Base call conversion to sequence reads was performed using CASAVA-1.8.2. Sequence data were analyzed with the Bowtie2, TopHat, and Cufflinks programs using NCBI's mouse (*Mus musculus*) genome build reference mm10. RNA-Seq data have been deposited in the NCBI's Gene Expression Omnibus (GEO), accession numbers GSE61095 and GSE65031 (3, 4).

Visual exploration of RNA-sequencing data was undertaken using the GENE-E matrix visualization and analysis platform (<https://software.broadinstitute.org/GENE-E/>). For comparison and visualization of biological lists to create Venn diagrams, the Venny online software v2.1 was utilized (<http://bioinfogp.cnb.csic.es/tools/venny/>). To predict target(s) and functional annotations of microRNAs, the online miRNA database (<http://mirdb.org/miRDB/>) was utilized. GeneCards (www.genecards.org), an integrated database (an authoritative compendium of annotative information about human and mouse genes), was utilized to identify potential allergy-associated genes.

Quantitative reverse-transcription PCR

Excised lungs were homogenized and RNAs were extracted using an RNeasy kit per the manufacturer's instructions (Qiagen, Valencia, CA, USA). Total RNA (1 µg) was reverse-transcribed using a SuperScript® III First-Strand Synthesis System (Invitrogen Life Technologies). mRNA levels were determined by quantitative RT-PCR (qRT-PCR). The commercially (Integrate DNA Technology, Inc.) validated primers (for the mRNA) were *Il4*, Cat # Mm.PT.58.7882098; *Il5*, Cat # Mm.PT.58.41498972; *Il13*, Cat # Mm.PT.58.31366752; *Ogg1*, Cat #: MP231384; *Hprt*, Cat # Mm.PT.58.32092191; *Actb*, Mm.PT.58.33540333 and *Gapdh*, Mm.PT.39a.1. *Collagen I*: F: 5'-TGG CCA AGA AGA CAT CCC TGA AGT-3', R, 5'-ACA TCA GGT TTC CAC GTC TCA CCA-3'; qRT-PCR was performed on an ABI7000 thermal cycler, and transcript levels were evaluated using the $\Delta\Delta C_t$ method (2).

Evaluation of airway inflammation

Broncho-alveolar lavage fluids (BALF) were collected at 72 h post-challenge, total cell numbers were determined using a hemocytometer, and cytospin preparations were stained with Modified Wright–Giemsa for HEMA-TEK 2000 Slide Stainer (Protocol™) for differential cell counts as previously described (2, 14). For histology, lungs were fixed in 10% neutral buffered

formalin overnight and embedded in OCT for frozen sectioning. Four micrometer lung sections were stained with hematoxylin and eosin (for general morphology and to visualize inflammatory cell infiltrates), periodic acid–Schiff stain (to visualize epithelial cell differentiation to the mucus: neutral glycoproteins producing cells), and Masson's trichrome stain (to observe collagen depositions) at the Research Histopathology Core at UTMB (Director; G. Valbuena, MD).

Collagen levels were determined by assessing the hydroxyproline content of the mouse lungs using the colorimetric detection assay (Cat #; MAK008-1KT) as recommended by the manufacturer (Sigma Aldrich). In brief, lung parenchyma (free extrapulmonary airways and major blood vessels) was homogenized in 1.0 ml of PBS and acid-hydrolyzed at 110°C for 24 hours (34). Hydroxyproline concentrations were determined by the reaction of hydroxyproline with 4-(Dimethylamino) benzaldehyde at 560 nm using the BioTek FLx800 fluorometer.

Standard curves were generated for each experiment using hydroxyproline reagent as a standard. The results were expressed as micrograms of hydroxyproline contained in total lung tissue.

Mucin-5 subtype AC (MUC5AC) levels were determined by enzyme-linked immunosorbent assay (ELISA) as we previously described (2). In brief, BALF were serially diluted in coating buffer (50 mM Na₂CO₃, 50 mM NaHCO₃), and 100-μl aliquots were added to 96-well plates for “coating”. Primary mouse MUC5AC antibody was used (1:16000; Cat #: MA5-12178; Invitrogen, Thermo Scientific). Bound primary antibody was assessed by peroxidase substrate (tetramethylbenzidine) and peroxidase-conjugated goat anti-mouse secondary antibody (1:5000) (Amersham). Incubations were performed at 37°C. Changes in absorbance were determined at 450 nm using a SpectraMax 190 ELISA plate reader (Molecular Devices, Sunnyvale, CA).

Statistical analysis

256 Statistical analysis was performed using Student's *t*-test or ANOVA, followed by post hoc
257 tests: Bonferroni's and Dunnett's T3 with SPSS 14.0 software. The data are presented as the
258 means $\pm$ the standard error of the mean. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.
259

Results

Pollen antigens-induced allergic responses are exacerbated by OGG1 signaling

Ragweed pollen extract containing intrinsic NADPH oxidases (NOX, RWPE^{NOX}) that were documented to generate reactive oxygen species (ROS) (11, 14), induced robust recruitment of eosinophils into the airways after challenge of sensitized OGG1-expressing (OGG1-proficient; OGG1^P) mice (Fig. 1A). In controls, OGG1^P mice were challenged with heat-inactivated (HI) RWPE (RWPE^{HI}; 56°C for 30 min, a treatment that decreases NOX activity (14). RWPE^{HI} caused a significantly lower eosinophil recruitment into lungs, as assed by numerating cells in BALF (Fig. 1A). RWPE^{NOX} challenge of lungs oxidatively modifies macromolecules including DNA (10). To determine if repair of oxidatively damaged DNA and consequent activation of the OGG1 signaling pathway plays a role in eosinophilic inflammation, OGG1 expression was silenced in the lungs (OGG1^D; Fig. 1B,C; Materials and Methods). OGG1 silencing resulted in a ~90% (Fig. 1B, lanes #1,2,3) decrease in protein levels when compared to OGG1^P ones (Fig. 1B, #4,5,6). Parallel groups of OGG1^P and OGG1^D mice were then challenged with RWPE^{NOX} or RWPE^{HI} and the inflammatory response was determined. Compared to OGG1^P mice, those lacking OGG1 showed significantly decreased numbers of eosinophils (Fig. 1A) after challenge with RWPE^{NOX}. Interestingly, numbers of eosinophils in RWPE^{HI}-challenged groups of OGG1^P and OGG1^D mice were similar (Fig. 1A) implying a profound role of RWPE^{NOX}-ROS in allergen-induced inflammatory response. To document differences in the capacities of RWPE^{NOX} and RWPE^{HI} to alter levels of intra-cellular ROS, we showed that it was increased by RWPE^{NOX} but not by RWPE^{HI} in cultured airway epithelial cells (Fig. 1D). To exclude the possibility that decreased inflammatory responses in lungs of OGG1^D mice were due to an increased antioxidant capacity, we show that changes in the levels of oxidative stress markers (carbonylated protein, Fig. 1E) and fold changes in genomic 8-oxoG (Fig. 1F) are similar after RWPE^{NOX}-challenge. We note that in non-sensitized OGG1^P mice,

RWPE^{NOX} challenge induced a robust transient innate immune response (IIR), while challenge of OGG1^D mice, a poor IIR was observed, which is consistent with previous findings (11, 14).

To test further the potential role(s) of the OGG1-pathway in exacerbation of allergic inflammatory responses, we administered 8-oxoG base (0.00005 mg/kg, a dose that may be generated by OGG1-BER upon oxidative stress (1)) along with either RWPE^{NOX} or RWPE^{HI} to OGG1^P mice. The co-administration of the 8-oxoG base with RWPE^{NOX} was insignificant but consistently enhanced eosinophil recruitment into the lungs. In contrast, co-challenge of OGG1^P mice with RWPE^{HI} and 8-oxoG significantly increased the allergic immune responses (Fig. 1A). In controls, co-challenge of OGG1^D mice with 8-oxoG and RWPE^{NOX} or RWPE^{HI} did not significantly alter eosinophil counts in BALF (Fig. 1A). These results suggest that both OGG1 and free base 8-oxoG (the product of OGG1-BER) are required for robust antigen-driven, allergic immune responses.

OGG1 signaling induces differential expression of miRNAs

To obtain an overall insight into the mechanism by which the activation of the OGG1-pathway could be implicated in allergic inflammatory responses, we mimicked OGG1-BER (byproduct of which is free 8-oxoG base) by directly instilling 8-oxoG into the lungs of OGG1^P mice. We administered 0.00005 mg/kg 8-oxoG once or repeatedly (3 times; 48-h intervals (3)). Single 8-oxoG challenge (sCh serving as a control) was used to mimic the impact of the OGG1-pathway immediately after RWPE^{NOX} challenge (10, 11). Repeated challenges were intended to mimic prolonged OGG1 signaling, which may occur under chronic oxidative stress induced by patho-physiological immune and tissue responses upon allergen exposures (10, 37). The 8-oxoG base is rapidly taken up by cells and bound by cytosolic OGG1. The formed complex then activates small GTPases, and downstream signaling (1, 15, 32). To assess cellular responses to 8-oxoG challenge, lungs were harvested and total RNAs were isolated at 0, 30, 60 and 120 min sCh and after the last challenge in an rCh model. Changes at RNA levels were determined

by unbiased RNA sequencing (Materials and Methods). Analysis of RNA sequencing data showed differential expression of 1,592 and 2,080 coding mRNAs after sCh and rChs, respectively (1, 3, 8). Among non-coding transcripts, 118 differentially expressed miRNAs were identified. Fold changes in the levels of each miRNA as a function time and their database ID number are summarized in *Supplementary Table 1*. Levels/expressions of 84 of 118 miRNAs were significantly (>3-fold) changed (Fig. 2A). rCh challenges modulated the levels of 73 miRNAs, of which 51 were upregulated (Fig. 2B) and 22 were down-regulated (Fig. 2C). After sCh, the levels of most miRNAs were increased (22 miRNAs of which 10 overlapped with those enhanced by rCh), while 3 were decreased (Fig. 2A, B, C). These data suggest that activation OGG1-signaling in lungs modulates the levels of miRNAs and robust allergic inflammation in OGG1^P mice may be due to the changes in miRNA expressions/levels.

Replenishment of miRNAs down-regulated by OGG1-signaling decreases allergic inflammation

Taking into account the roles of miRNAs in gene expression both at post-transcriptional and translational levels (7), we used the miRNA database and GeneCards (Materials and Methods) to select potential allergic inflammation-relevant miRNAs among those most significantly altered by OGG1 signaling. These included *let-7b*, which was up-regulated (6.4-fold) by sCh, but down-regulated (8.5-fold, nearly 90%) compared to unchallenged controls) after rCh. *In silico* analysis showed that *let-7b* has 56 allergy-relevant targets including mRNAs of interleukin (Il) *Il4*, *Il5*, *Il13*, and *KRas*. *miR-23a* and *miR-27a* were up-regulated in lungs by both sCh (21.7-fold) and rCh (9.9-fold) after challenge and had 29 and 34 targets, respectively (*Supplementary Table 2*). *miR-23a* and *miR-27a* are potentially implicated in the modulation of allergic immune responses via the regulation of T cell functions (22). To provide evidence that the OGG1 signaling-induced miRNAs (Fig. 2) play a role, we determined whether their

replenishment by synthetic mimics (and inhibitors) modulated RWPE^{NOX}-induced recruitments of eosinophils into the lungs of OGG1^P mice.

MiRNA mimics and inhibitors of *let7b-3p*, *let7b-5p*, *miR-23a* or *miR-27a* were individually introduced into the lungs of parallel groups of sensitized OGG1^P mice at 1 h before RWPE^{NOX} challenge. An additional dose was applied at 35 h post-challenge (Materials and Methods). These time points were selected based on our previous studies that examined kinetics changes in the levels of IL-4, IL-5 and IL-13 in BALF after RWPE^{NOX} exposure (10). Control groups of mice received RWPE^{NOX} or solvent (saline). At 72 h post-exposure, the lungs were lavaged and the numbers of eosinophils were determined. The *let-7b-3p* mimic significantly ($p<0.001$) decreased the number of eosinophils in BALF (Fig. 3A), while its inhibitor increased ($p<0.01$) their counts. When the *let-7b-3p* mimic and its inhibitor were administered together, there was no significant effect. Interestingly, the *let-7b-5p* mimic or its inhibitor did not significantly change the number of eosinophils in BALF induced by RWPE^{NOX} (Fig. 3A). Mimics of *miR-23a* and *miR-27a* had no effect; however, their inhibitors decreased ($p<0.01$) the RWPE^{NOX}-induced accumulation of eosinophils (Fig. 3B). The addition of miRNA mimics or inhibitors alone to sensitized mice induced insignificant levels of neutrophilia (determined at 16 h post-challenge; data not shown). In support of the impact of *let-7b-3p* on RWPE^{NOX}-induced BALF eosinophil counts, we show that its administration of the *let-7b-3p* inhibitor increased, while its mimetic decreased RWPE^{NOX}-induced tissue infiltration of eosinophils compared to RWPE^{NOX} challenge alone (Fig. 3C). Although miR-23a and miR-27a inhibitors decreased the RWPE^{NOX}-induced accumulation of eosinophils in BALF, there were no significant observable differences at the levels of tissue infiltration (data not shown). These data together suggest that the recruitment of eosinophils into BALF and lung tissues of OGG1^P mice was significantly due to the down-regulation of *let-7b* by OGG1 signaling.

OGG1-signaling increases Th2 cytokines levels via the down-regulation of *let-7b-3p*

Integrated signals generated by interleukin (IL) -13, -4, and -5 are primarily responsible for immune and histo-pathological changes documented in airways exposed to allergens (14, 64). The *let-7b-3p* mimic significantly decreased the number of eosinophils in the lungs (Fig. 3), which may be due to decreased levels of its target mRNAs including interleukin (IL) *IL4*, *IL5* and *IL13*. Notably, *let-7b-3p* also targeted mRNA of *KRas*, which encodes for a small GTPase and is directly activated by OGG1-8-oxoG complex (1). To determine if the *let7b-3p* mimic alters the mRNA levels of *IL4*, *IL5*, and *IL13*, mice were RWPE^{NOX}-challenged with and without installation of the *let-7b-3p* mimic and total RNAs were isolated. The transcript levels of *IL4*, *IL5*, and *IL13* were determined by qRT-PCR. RWPE^{NOX} challenge alone induced a rapid (1 h) increase in the transcript levels of *IL4* and *IL13*. At 36 h post-challenge with RWPE^{NOX}, the expression levels of *IL4* showed only a modest increase (>3-fold, Fig. 4A), while *IL5* (9.8 ± 2.5 -fold) and *IL13* (>22 ± 9.5 -fold) were significantly elevated (Fig. 4B,C). The addition of *let-7b-3p* mimic significantly ($p < 0.001$) decreased the RWPE^{NOX}-induced transcript levels of *IL4*, *IL5*, and *IL13*, in line with its predicted targets (*Supplementary Table 2*) (Fig. 3, Fig. 4). These results suggest that OGG1 signaling increased the inflammatory response via the down-regulation of the miRNA *let-7b* expression in the lungs of RWPE^{NOX}-challenged sensitized mice.

IL-4 and IL-13 are documented to be pleiotropic cytokines playing a central role in the pathogenesis of asthma including increases in goblet cell differentiation (23). IL-4 and IL-13 also induce the expression of transforming growth factor alpha (TGF- α) by mucosal epithelial cells and via autocrine signaling, resulting in mucous metaplasia (36); the latter is a characteristic of RWPE-induced allergic tissue responses (14). To investigate the effects of the *let-7b-3p* mimic on RWPE^{NOX}-induced mucus production, lung sections were examined for neutral glycoproteins (mucus) -producing cells using periodic acid-Schiff stain (PAS), and MUC 5A was also determined in BALF. The results summarized in Fig. 4D show excessive PAS staining in RWPE^{NOX} and *let-7b-3p* inhibitor+RWPE^{NOX}-challenged lungs (Fig. 4D, left panel), while *let-7b-3p* mimic plus RWPE^{NOX} challenge significantly decreased PAS staining (Fig. 4D, far right

panel) and the levels of MUC5A in BALF (Fig. 4E). These results together imply that the activation of OGG1 signaling via the down-regulation of *let-7b-3p* exacerbates mucous cell metaplasia and the consequent over-secretion of mucus, which are key features of both acute and chronic allergic lung inflammation in asthma (35).

Collagen deposition is a hallmark of asthma in animal models and in asthmatic patients (30, 36, 39, 50). Our *in silico* analysis identified that *let-7b-3p* targets both transforming growth factor beta receptor 1 (*Tgfb1*) and transforming growth factor beta (*Tgfb*), which are documented to be involved in collagen expression (61). To determine whether OGG1 signaling upregulates TGF β -driven collagen production, lung sections were Masson's trichrome-stained. Compared to control (saline challenge), RWPE^{NOX} challenge induced extensive collagen depositions in subepithelial regions and alveolar parenchyma (Fig. 4F, left panels). The inhibitor of *let-7b-3p* further increased collagen deposition, while *let-7b-3p* mimic administration decreased the levels of collagen deposition induced by RWPE^{NOX} nearly to those observed in saline-challenged lungs (Fig. 4F, far left and far right panels). To support the histological observations, the hydroxyproline levels were determined in the total lung extracts. The lungs of the saline-treated mice had 61 ± 14.4 $\mu\text{g}/\text{gram}$ collagen content as assessed by levels of hydroxyproline. RWPE^{NOX}–challenge of lungs resulted in 177 ± 17.9 $\mu\text{g}/\text{gram}$ hydroxyproline (collagen) content. The installation of *let-7b-3p* inhibitor to RWPE^{NOX}-challenged lungs further increased (233 ± 17.9 $\mu\text{g}/\text{gram}$) hydroxyproline levels (Fig. 4G). Lung extracts from *let-7b-3p* mimic and RWPE^{NOX} challenge showed significantly decreased levels of hydroxyproline (89 ± 22 $\mu\text{g}/\text{gram}$) compared to those challenged only with RWPE^{NOX}. Changes in hydroxyproline (collagen) levels support the histological observations and suggest that OGG1 signaling increases collagen levels via *let-7b* depletion in RWPE-induced allergic airway inflammation.

Discussion

Allergic immune and tissue responses are manifested via a highly complex interplay between dysregulated airway epithelial cells, mast cells, basophils, dendritic cells, B and T cells, neutrophils, and eosinophils, involving a multitude of soluble mediators and ROS (19, 55). Upon exposure to allergen-containing entities, e.g., pollens, molds, pet dander, and others, ROS levels are increased in all cell types, where they function as signaling entities and oxidatively modify various macromolecules (24). Most ROS-modified molecules are degraded; however, oxidatively damaged DNA is repaired to maintain genomic integrity (28, 49). Here, for the first time, we document that the repair of the most abundant DNA base lesion 8-oxoG by OGG1 and down-stream signaling exacerbate RWPE^{NOX}-induced allergic inflammation via modulating levels of miRNAs. To prove this hypothesis, the administration of miRNA mimics or inhibitors modulated by OGG1-signaling significantly modulated RWPE^{NOX}-induced allergic immune and histological changes. These results suggest that the pharmacological modulation of OGG1 signaling or the topical administration of specific miRNAs could have clinical utility.

ROS generated immediately after RWPE^{NOX} challenge has been shown to be key in the exacerbation of allergic immune and tissue responses (11-14). Accordingly, RWPE^{HI} (lacking NADPH oxidase activity) induced significantly less inflammation compared to RWPE^{NOX}. Intriguingly, the addition of a physiologically relevant dose of 8-oxoG base together with RWPE^{HI} increased inflammation to the level induced after RWPE^{NOX} challenge and allowed us to propose that OGG1-BER plays a key role in allergic immune responses. Although the exact mechanism is yet to be determined, these observations may mean that down-stream signaling from OGG1-BER, including the activation of small GTPases (1, 15), exacerbates antigen-induced allergic inflammation. These results also raise the possibility that increased 8-oxoG base levels in the serum, sputum, and BALF of allergic human subjects could have pathological relevance, although it is traditionally considered a biomarker of oxidative stress and diseases processes (8, 57, 58).

To obtain insight into the etiological role of OGG1-8-oxoG signaling in allergic immune and tissue responses and to identify the potential mechanism, we utilized sensitized mice lacking OGG1 expression in lungs (1, 10). The rationale for topical OGG1 silencing is that *Ogg1*^{-/-} mice cannot be efficiently sensitized: they generate significantly less (~10-fold) allergen-specific IgE antibodies compared to those of their wild-type counterparts. These observations are in line with OGG1's role in regulating both innate and adaptive immune responses (1, 4, 8, 10). To test the potential role of OGG1 signaling, mouse lungs were challenged repeatedly with 8-oxoG to mimic reoccurring oxidative DNA damage repair under chronic inflammatory conditions such as those that occur in asthma (51). A system-level analysis of the entire transcriptome identified differentially modulated coding RNAs of which protein products are potentially involved in atopic, non-atopic asthma, mucus production, and AHR (3, 4, 8, 13). RNA sequencing analysis also identified a large number of non-coding RNAs including miRNAs. The latter observation implies that complex events induced by allergens in RWPE are exacerbated by the OGG1 pathway, which may involve gene regulation at both the transcriptional and post-transcriptional levels.

MicroRNAs, originally discovered in *C. elegans*, are highly conserved classes of small RNAs that regulate gene expression via RNA silencing and decreasing translation efficiency (7). Signaling down-stream from OGG1-BER modulated levels of over eighty differentially expressed miRNAs. Several of these target mRNAs encode for proteins implicated in allergic immune and tissue responses (27, 53). Based on their potential targets, we selected *let-7b* (up-regulated by sCh but down-regulated by rCh), *miR-23a*, and *miR-27a* (upregulated by both sCh and rCh). The administration of *let-7a-3p* significantly decreased RWPE^{NOX}-induced allergic airway inflammation and histological changes. These data are in line with those found using the OVA model of allergic airway inflammation (43, 53). *Let-7b-3p* also decreased collagen deposition in the subepithelial region and reticular basement membrane, which was documented to occur in asthma (30, 39). Decreased RWPE^{NOX}-induced immune and tissue

responses by *let-7b-3p* were due to the down-regulation of *Il4*, *Il5*, and *Il13* expression at the post-transcriptional level. As expected, an inhibitor of *let-7b-3p* exacerbated RWPE^{NOX}-induced allergic responses. We note that *let-7b* contributes to the regulation of numerous biological processes including innate and adaptive immune responses. For instance, they regulate the expression of the *IL-6* and *IL-10* genes upon bacterial and protozoan infection of macrophages or exposure to LPS (5, 27). *Let-7b-5p* had no significant effect, which is in line with previous studies showing that when *let-7b-5p* interacts with its target elements (e.g., 5'UTR mRNA), it stimulates, rather than inhibits, gene transcription and protein translation (40).

MiR-23a and miR-27a belong to the *mir23-27-24* microRNA cluster and are involved in a variety of biological processes (22, 63, 65). Although their level is increased by OGG1 signaling, miR-23a and miR-27a mimics did not alter RWPE^{NOX}-induced eosinophilia. Interestingly, these inhibitors decreased the numbers of eosinophils in BALF. Their role in RWPE^{NOX}-induced inflammatory responses warrants determination in future studies. We note that miR-27a is an important regulator of inflammatory response in sepsis (63), while miR-23a has diverse functions including the regulation of TGF- β -induced epithelial-mesenchymal transition by targeting E-cadherin in lung cells (20). Of note, our primary goal was to prove the role of OGG1-signaling in modulating miRNAs levels relevant to allergic airway inflammation; therefore, we tested few of these. We note that among the 84 miRNAs altered by OGG1-signaling, there are several that are considered to be disease markers (6), while others are etiologically involved in disease pathogenesis (27, 47, 56).

Questions remain regarding how down-stream signaling from OGG1-BER regulates the expression of various miRNA families. MiRNA promoters are under positive and negative control of transcription factors (TF), enhancers, and silencing elements, which are also affected by chromatin modifications, as well as DNA methylation (17, 46). DNA encoding for miRNA are located in intergenic regions, exons, or introns of genes and are primarily transcribed by RNA polymerase II and processed by multi-protein complexes in the nucleus (e.g., Drosha

microprocessor complex). It is then exported to the cytoplasm by the exportin-5/Ran-GTP complex, where it is further processed to generate miRNAs (rev (17)). TFs that regulate miRNA transcription largely overlap with those that control protein coding genes, e.g., NF- κ B, Tumor Protein 53 (TP53), V-Myc Avian Myelocytomatosis Viral Oncogene Homolog (MYC), Specificity Protein 1 (SP1), Early Growth Response 1 (EGR1), Kruppel family of transcription factor 4 (KLF4), and RE1 silencing TF (a KLF family protein that is associated with promoter regions) controlling miRNAs (42, 59). Intronic promoters also contain binding sites for TFs, such as Forkhead Box Protein A1-2-3, GATA Binding Protein 1, and Forkhead-Related TF2 (17, 42, 59). Although we did not perform mechanistic studies, our previous Ingenuity Pathways Knowledge Base analysis of microarray (Affymetrix GeneChip) data showed that in airway epithelial cells, (A549) OGG1-RAS signaling modulated networks regulated by TFs, including NF- κ B, TP53, KLF4, MYC, and others (31). Thus, we speculate that OGG1-BER-small GTPases [(H-, K-, N-RAS) RAF-1-MEK1/2, ERK1/2, and p-PI3K(p85)] and the consequent activation of TFs and/or OGG1-mediated facilitation of TF binding (1, 9, 15, 32, 52) could be involved in modulating miRNA expression.

In conclusion, allergic asthma is a complex disorder of the airways involving chronic inflammation, decline in airway function, and tissue remodeling. Disease severity is linked to genetic susceptibility and co-exposures to environmental agents including gases, particles, or infection. *Although many pathways are identified, the overarching* mechanism common to all asthma phenotypes is not well understood. Oxidative stress and oxidatively modified DNA bases and strands are common and represent the earliest signs of allergen exposure and immune reactions; their levels and quality are biomarkers of allergic diseases and are well documented. The most referenced biomarker of allergic immune responses is genomic 8-oxoG, which is primarily repaired by OGG1 and in complex with its repair by product activates small GTPases. Here, we are the first to demonstrate that OGG1-driven cell signaling exacerbates pollen antigens-induced allergic inflammation via the downregulation of specific miRNAs

expression/levels, which are targeted against mRNA coding for Th2 cytokines as well as signal generators, such as small GTPases KRAS. These results and those from previous studies support the notion that cell signaling down-stream from OGG1 BER leads to multilayered transcriptional and post-transcriptional control over immune and tissue responses. The results are in line with decreased allergic responses in *Ogg1* KO mice, which lack both OGG1 activity and the BER product 8-oxoG. Thus, one may propose that the topical modulation of OGG1's activity by small molecule inhibitors or the application of a miRNA mimetic could have clinical utility in the treatment of pollen allergens-driven diseases.

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Conflict of interest

The authors declare no conflicts of interest.

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Figure Legends

Figure 1

OGG1 expression exacerbates pollen-induced allergic inflammation. **A**, OGG1 silencing in lungs decreases the accumulation of eosinophils (gray columns) compared to those expressing OGG1 (empty columns). Sensitized OGG1 expressing (OGG1^P) and OGG1^D mice were challenged with RWPE^{NOX}, heat-inactivated (RWPE^{HI}), RWPE^{NOX}+8-oxoG or RWPE^{HI}+8-oxoG. Seventy two h later, lungs were lavaged and the numbers of BALF eosinophils were determined. **B,C**, Expression of OGG1 at protein (B) and RNA (C) levels in the lungs of sensitized mice prior and after OGG1 silencing. OGG1 was silencing in airways is described in Materials and Methods. Protein and RNA levels in lungs of OGG1^D and OGG1^P mice were determined 72 h after RWPE challenge. **D**, Intrinsic NADPH oxidase of RWPE (RWPE^{NOX}) increases cellular ROS levels in cultured airway epithelial (AML-12) cells (empty columns). Heat-inactivated RWPE (RWPE^{HI}) had no effect on cellular ROS levels (gray columns). Changes in ROS levels were determined by oxidation of H₂DCF (Material and Methods). **E, F**, RWPE^{NOX} challenge increased the protein carbonyl (E) and genomic 8-oxoG (F) levels in lungs of OGG1^P and OGG1^D. Data are presented as the means $\pm$ SEM of 6 individual mice. ** $p < 0.01$, *** $p < 0.001$.

Figure 2

Differential expression of miRNAs in lungs induced by 8-oxoG the product of OGG1-BER. **A**, Hierarchical clustering of miRNAs. The heat map was generated using the GENE-E matrix visualization and analysis platform. **B**, List of miRNAs upregulated after single and repeated challenges of airways. **C**, List of down-regulated (>50%) miRNAs after single and repeated challenges of lungs.

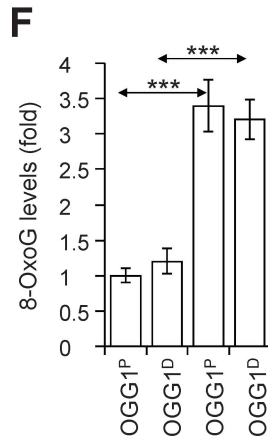
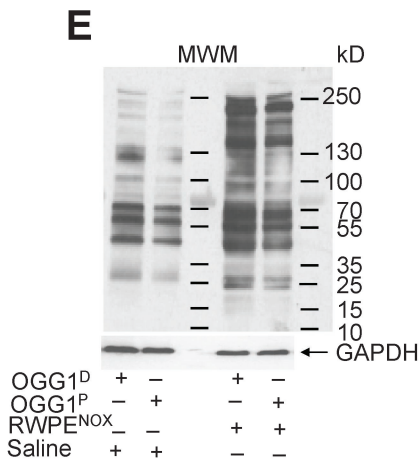
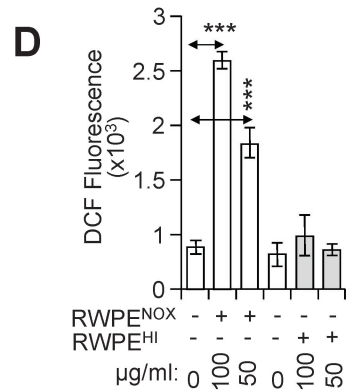
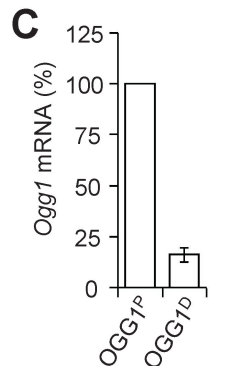
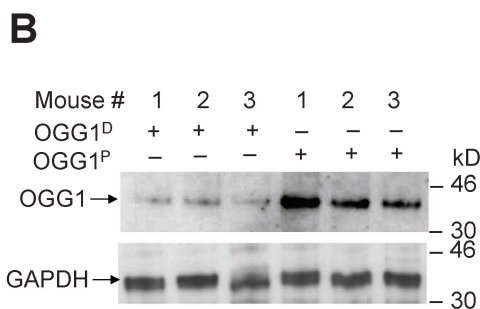
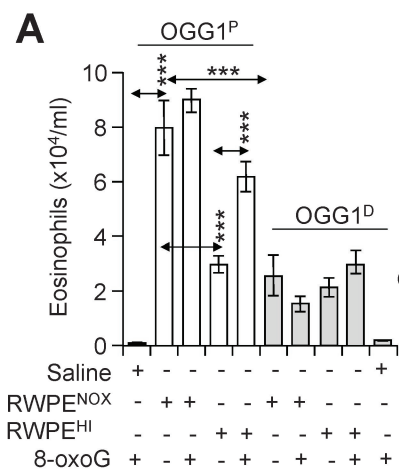
Figure 3

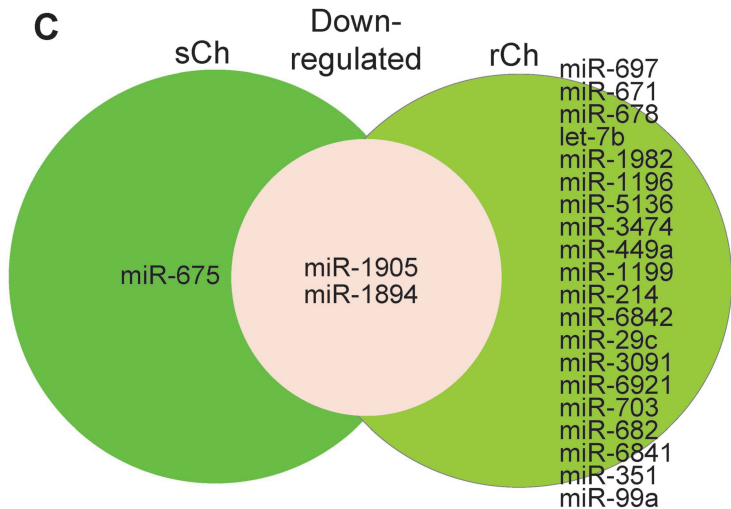
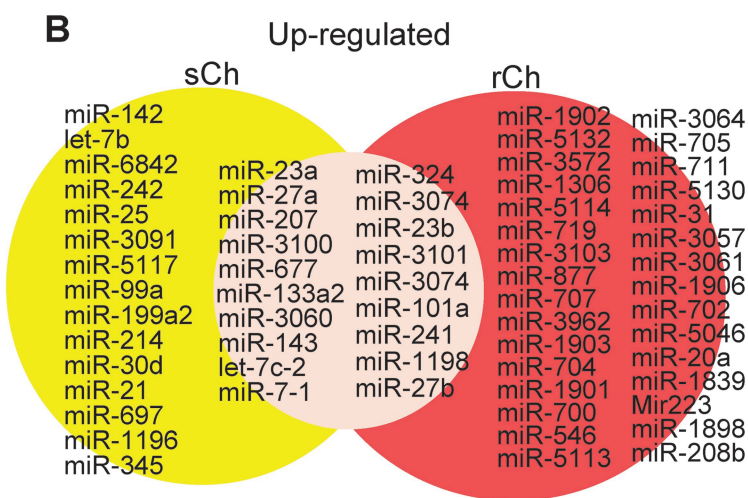
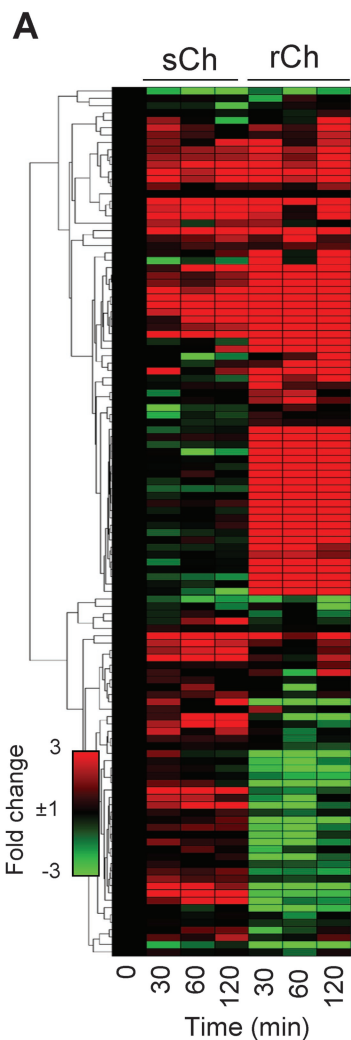
Modulation of RWPE-induced accumulation of eosinophils by miRNA mimics and inhibitors in sensitized OGG1^P mice. **A, B**, Effect of miRNA mimics or inhibitors on RWPE^{NOX}-induced eosinophilia. Individual miRNAs (*let-7b-3p*, *let-7b-5p*, *miR-23a*, or *miR-27a*) were introduced into lungs 1 h before and 35 h after RWPE^{NOX} challenge in sensitized mice. Differential cell counts in BALF were determined at 72 h post challenge with RWPE^{NOX}. **C**, *let-7b-3p* mimic decreased RWPE^{NOX}-induced eosinophil accumulation. Accumulation of inflammatory cells (eosinophils) in peribronchial regions of RWPE^{NOX}-challenged lungs (hematoxylin and eosin staining of lung

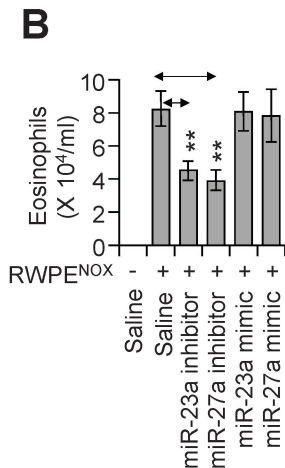
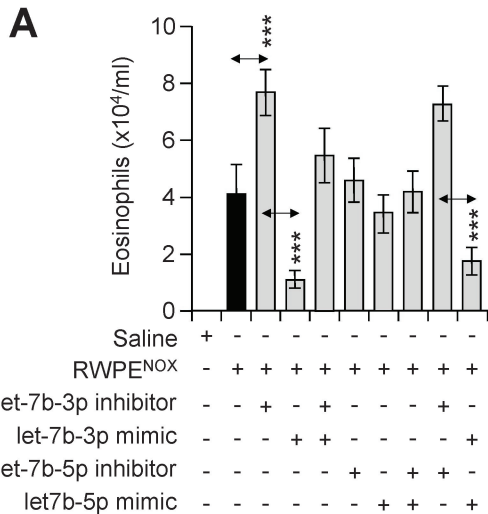
sections) is shown. *let-7b-3p* inhibitor increased while its mimic decreased the density of eosinophils peribronchial regions of lungs. Upper panels: magnification 20x; lower panels: magnification 60x. Histological images show representative staining of 6 sections per mice for each treatment. Data are presented as the means $\pm$ SEM of 6 mice, $**p < 0.01$, $***p < 0.001$.

Figure 4

Replenishment of mirlet7b-3p decreases the RWPE^{NOX}-induced expression of Th2 cytokines and histological changes. **A**, *Let-7b-3p* mimic decreases mRNA levels of *Il4*, *Il5* (and *Il13*). The *let-7b-3p* mimic or its inhibitor were installed 1 h before and 35 h after RWPE^{NOX} challenge. Mice were sacrificed one h after miRNA addition and total RNAs were isolated. One μ g RNA from individual lungs reverse-transcribed were subjected to qRT-PCR analysis (Materials and Methods) for the expression of *Il4* (**A**), *Il5* (**B**), and *Il13* (**C**). **D**, **E**, *let-7b-3p* mimic decreased the mucus levels induced by RWPE^{NOX} challenge. The *let-7b-3p* inhibitor increased while its mimic decreased the density of magenta-colored lung epithelial cells (D). Changes in Periodic acid–Schiff staining are consistent with MUC5A levels in BAL fluid (E). **F**, **G**, The *let-7b-3p* mimic decreased the density of collagen deposition induced by RWPE^{NOX} in OGG1^P mice (F). Lungs were subjected to acid hydrolysis, and the resulting hydroxyproline contents (G) were determined as in Materials and Methods. A, B, C, E, F: Data are presented as the means $\pm$ SEM of 5 mice, $***p < 0.05$, $***p < 0.001$. D, F: histological images show representative staining of 6 sections per mouse for each treatment.







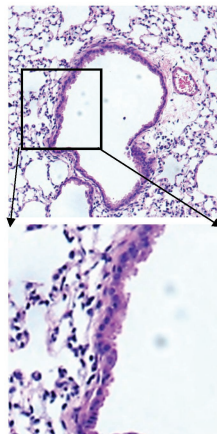
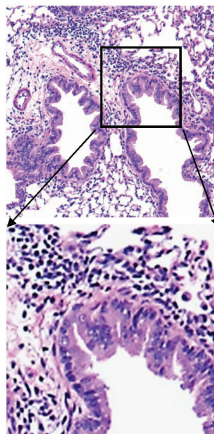
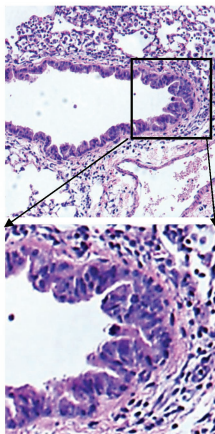
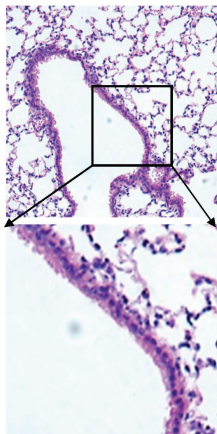
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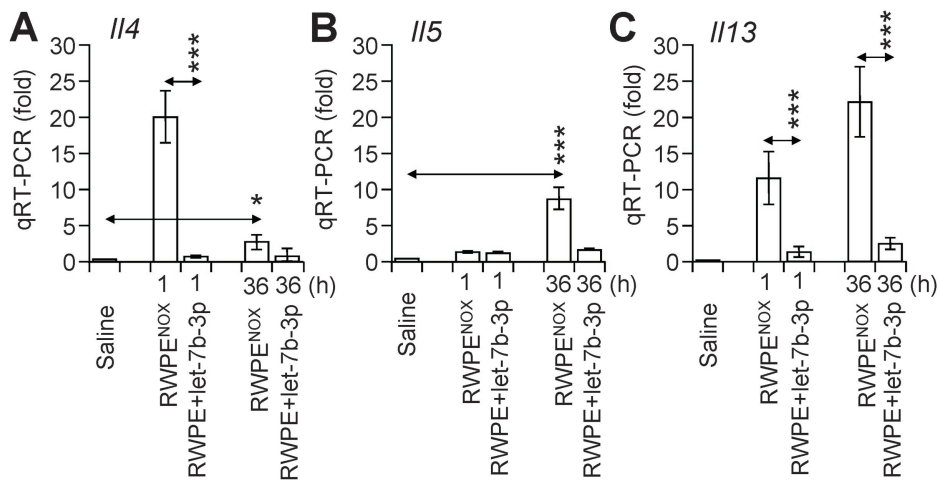
Saline

RWPE^{NOX}

let-7b-3p
inhibitor

let-7b-3p
mimic





Saline RWPE^{NOX}

