Title
Asthmatic Responses via DUOX Activation Following Exposure to Fine Particulate Matter

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Asthmatic Responses via DUOX Activation Following
Exposure to Fine Particulate Matter

By

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DAVIS

Approved

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Kent E. Pinkerton, Chair

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Committee in Charge

2021

ABSTRACT

High levels of particulate pollution are becoming commonplace worldwide. For those with respiratory symptoms, greater air pollution levels can exacerbate respiratory disease. One possible driver of the enhanced asthmatic responses upon fine particulate matter ($PM_{2.5}$) exposure is the activation of dual oxidase (*DUOX*) genes involved in neutrophil recruitment and inflammation.

To determine whether *DUOX* gene activation plays a role in the severity of PM-induced lung inflammation, *DUOX*-knockout (KO) and wildtype (WT) mice were exposed to house dust mite (HDM) allergen to produce an allergic mouse model and ambient $PM_{2.5}$ collected from Sacramento, California. The study was designed to determine if exposure to $PM_{2.5}$+HDM would cause an exacerbated asthmatic response in either KO or WT mice compared to HDM alone, PM alone or sham controls. S129 mice 12-to-14-weeks of age were administered via intranasal instillation phosphate-buffered saline (PBS), $PM_{2.5}$, HDM, or HDM+$PM_{2.5}$ on days 1, 3, and 5 (sensitization period) and PBS or HDM on days 12, 13, and 14 (challenge period). On the 15th day of the experiment, mice were then examined for the degree of allergic response using pulmonary function testing, the degree of lung inflammation by bronchoalveolar lavage, and lung histopathology. The KO mice were found to exhibit less elevated levels of asthmatic-like symptoms when compared to treatment-matched WT mice, suggesting *DUOX* gene expression plays a critical role in the induction of asthma-like symptoms.

INTRODUCTION

Asthma is a widespread respiratory condition afflicting more than 339 million people worldwide (4). It is well-known particulate matter (PM), especially fine particulate matter ($PM_{2.5}$), can exacerbate asthma symptoms and promote airway hyper-responsiveness (AHR; 5). AHR is associated with airway narrowing due to bronchoconstriction resulting in difficulty breathing, and increased risk of new onset asthma. Moreover, AHR severity is directly associated with the severity of asthmatic-like symptoms (6). One gene that has been suggested to contribute to such pathogenesis is dual oxidase (*DUOX*) (2). The *DUOX* genes are found in airway epithelial cells and are members of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase family that releases hydrogen peroxide (H_2O_2) in the respiratory tract. H_2O_2 plays a role in neutrophil influx to allergic airways (2). Specifically, *DUOX* 1 has been implicated in the augmentation of mucin expression in the airways and promotion of epithelial wound healing, both of which can lead to pathologic airway remodeling in response to chronic inflammation (2). Additionally, when exposed to $PM_{2.5}$ the expression of *DUOX* is enhanced, causing oxidative stress resulting in higher levels of inflammation (7).

Previous studies have been conducted with animals to test inflammation and gene expression linked to *DUOX* activation upon $PM_{2.5}$ exposure. In our laboratory, a study by Zhang et al. (2019) investigated *DUOX* gene expression using BALB/C mice to demonstrate that exposure to PM can enhance *DUOX* in the lung, thus activating a type 2 immune response during allergic asthma (11). A type 2 immune response is strongly connected with asthma and is mediated through the influx of eosinophils, mast cells,

basophils, T_H2 cells, and group 2 innate lymphoid cells (16). Findings from Zhang et al. (2019) demonstrated mice exposed to PM and house dust mite (HDM) which is an asthmatic allergen, exhibited increased levels of airway resistance, eosinophils in bronchoalveolar lavage fluid (BALF), and lung tissue inflammation. However, this study included only wild-type (WT) mice and did not test whether the results would be similar if the *DUOX* genes were inactivated.

In the present study, *DUOX*-knockout (KO) mice and WT mice of the s129 strain were exposed to $PM_{2.5}$ and HDM to determine whether *DUOX* gene activation affects the severity of inflammation. In the *DUOX* knockout mouse model, using conventional or constitutive knockout mice, the target gene is permanently inactivated in every cell of the organism. $PM_{2.5}$ for this study was collected in downtown Sacramento on the rooftop of a two-story building at the northeast corner of T and 13th Streets during the summer of 2011 (10). During that time, Sacramento was ranked as the 14^{th} city in the nation with the highest short-term particle air pollution (10). For this study eight different groups of mice, including four KO and four WT groups, were exposed to 1) phosphate buffered saline (PBS) as the control, 2) $PM_{2.5}$, 3) HDM, or 4) $PM_{2.5}$ + HDM. HDM was used to induce asthma-like responses, therefore, mice exposed to $PM_{2.5}$ + HDM were hypothesized to exhibit exacerbated asthmatic responses. After the mice were randomly assigned to their respective groups and received treatment, they were examined and compared to one another to evaluate the impact of each respective exposure on the respiratory system. Our overall hypothesis was exposure to $PM_{2.5}$+ HDM would result in exacerbated asthmatic symptoms in WT mice, but not in KO mice.

METHODS

Animal Care and Use

Male and female s129 mice were used in the present study and equally distributed in each group. The s129 strain was chosen because of their wide use in targeted mutation studies. The KO mice were obtained from the laboratory of Dr. Richart Harper at the University of California, Davis, and the WT mice were obtained from Jackson Laboratory (Sacramento, CA). All mice were acclimated for 1 week prior to beginning the experiment and were 12-14 weeks of age at the time of euthanasia. A total of 22 KO and 26 WT mice were randomly assigned to the following groups: 1) PBS (n=4 KO and 8 WT), 2) PM (n=6 each), 3) HDM (n=6 each), or 4) HDM+PM (n=6 each). All experiments and animal protocols were approved by the Institutional Animal Care and Use Committee at the University of California, Davis.

PM Sample Collection, Extraction and Chemical Characterization

Ambient PM was collected as previously described (10) during the summer of 2011 at an urban sampling site located on the rooftop of a two-story building at the northeast corner of T St. and 13th St. in downtown Sacramento, CA. The sampling site is surrounded by a mixture of residential, commercial and industrial sources and within a quarter mile of a major freeway interchange. In brief, summertime $PM_{2.5}$ in Sacramento was dominated by organic carbon (49% composition by mass), including PAHs and nonaromatic hydrocarbons, and water soluble inorganic ions (21% composition by mass). Elemental carbon accounted for 1.4% of PM mass, and various metals ranging from lithium to lead were detected at levels significantly above detection

limits. PM samples were collected in field studies conducted in an urban setting using a high-volume $PM_{2.5}$ sampler (Tisch Environmental Inc., TE-6070V-2.5-HVS), operating at a flow rate of 40 cfm. The fine PM fraction (PM2.5 ¼ Dp50 < 2.5 mm) was collected using Teflon coated borosilicate glass microfiber filters (Pall Corporation, TX40H120WW-8 × 10) followed by a multisolvent extraction method (20,21). Lipopolysaccharide (LPS) levels were quantified by the Lonza Kinetic Chromogenic LAL Endotoxin Assay (Basel, Switzerland). Endotoxin levels in the collected PM sample were found to be below the limit of detection (LOD) of < 0.005 endotoxin units.

Intranasal Instillation

All mice were administered their respective test materials (HDM, PM, or PBS) via intranasal instillation over a 14-day period which included sensitization (days 1, 3 and 5) and challenge (days 12, 13 and 14). To perform intranasal instillation, mice were sedated with isoflurane gas in an exposure chamber prior to instilling respective test materials. Each mouse was exposed for 3 min to ensure they would not disrupt the installation. PM was sonicated for 30 min immediately prior to administration and all test materials were vortexed prior to administration. The HDM was administered at a concentration of 1 μg/μL in a 25 μL suspension. The PM was prepared as a suspension in milliQ water to a final concentration of 1 μg/μL and was administered at a concentration of 1 μg/μL in a 33.3 μL suspension. The PBS (Sigma Aldrich, St Louis, Missouri) was administered as a 33.3 μL solution.

Mice were sensitized on days 1, 3, and 5 and challenged on days 12, 13, and 14. On the days of sensitization, the mice received PBS or HDM as one dose and a second

dose of either PBS or PM with a 30-min interval between each instillation. On the days of challenge, the mice received only a single dose of either PBS or HDM. This 14-day regimen of sensitization and challenge has been previously reported (1,3,10,17,18) with a strong allergic response. The exposure regimen used for this study has been found to be highly effective in eliciting an adaptive immune response upon allergen challenge and reproducibility (1,3,10,17,18). Figure 1 provides a schematic of the experimental design for this study.

Pulmonary Function Test

On day 15 (the day after the final challenge instillation), the mice were weighed and then deeply anesthetized with a 50-mg/kg telazol and 1-mg/kg dexdomitor cocktail via intraperitoneal (IP) injection. The mice were then intratracheally cannulated and a Flexivent system (SCIREQ, Montreal, Canada) was used to perform pulmonary function test (PFT) measurements with increasing doses of methacholine (MCh) to measure changes in pulmonary dynamics. MCh is a potent bronchoconstrictor in animals and humans and is used to measure airway sensitivity due to a variety of conditions including the relative severity of asthmatic-like conditions. To perform studies to measure physiological lung measures using the Flexivent system, the diaphragm of the mice was paralyzed with an intramuscular injection of succinylcholine (1mg) to prevent involuntary reflexes. Lung resistance (measure of airflow due to presence of airway secretion and/or hyper-reactive airway smooth muscle tone), compliance (measure of distensibility of the lung), and elasticity (measure of the recoil of the lungs due to the elasticity of the lung tissue) were measured using forced oscillation perturbation for the

complete respiratory system (17). All PFT measurements were recorded and averaged for each mouse. EC200RL (effective concentration that leads to a two-fold increase in airway resistance) was calculated from the MCh challenge using increasing doses (0.25, 0.5, 1, 2, 4, 8 mg/ml of MCh) to determine AHR. PFT measurements for each mouse were stopped once a consistent EC200RL was reached.

BALF and Cell Differentials

Following PFT measurements, each mouse was euthanized by IP injection of Beuthanasia-D (1 mg/mL). The trachea was recannulated, the thorax opened and left mainstem bronchus clamped, and two 0.6-mL aliquots of PBS instilled via the tracheal cannula to lavage the right lung lobes for collection of bronchoalveolar lavage fluid (BALF). The bronchoalveolar lavage (BAL) procedure involved instillation and withdrawal of each aliquot of PBS three times prior to final collection. The final total volume of PBS withdrawn and collected from the lungs was recorded. Each BALF sample was centrifuged at 4°C for 15 minutes at 2500 Rotations Per Minute to form a cell pellet. The BALF supernatant was decanted from the cell pellet and stored in a -80°C freezer for future analysis. The cell pellet was resuspended in 0.5 mL of PBS and analyzed for cell counts and viability using a hemocytometer filled with 100 µL in 10 µL of trypan blue stain (Sigma-Aldrich). Once cell counts were finalized, three cytospin slides were prepared using 100 µL of cell suspension/slide placed in a Shandon Cytospin 4 (ThermoScientific, Kalamazoo, MI). One slide from each animal was stained with DiffQuik (American Mastertech, Lodi, CA) for cell differential analysis. A total of 500

BAL cells were counted for each mouse to determine the relative percentages and cells/mL for macrophages, neutrophils, lymphocytes, and eosinophils.

Collection of Right and Left Lung Lobes

Immediately following the collection of BALF, the left lung was unclamped, and the right lungs were tied off with a suture. The right lung lobes were excised and placed in a -80°C freezer. The left lung was removed along with the cannulated trachea and inflation-fixed with 4% paraformaldehyde at 30 cm hydrostatic pressure for one hour and stored in 4% paraformaldehyde for 24 hours in the refrigerator. The left lung was subsequently transferred into 70% ethanol and maintained in the refrigerator until ready for tissue processing and embedment.

Preparation of Left Lung Tissue Sections for Semi-Quantitative Histopathological Assessment

The left lung from each animal was used for histological analysis. The left lung was cut into four transverse slices. These four transverse slices represented virtually the entire left lung from above the left main-stem bronchus to the diaphragmatic level, thus allowing for complete examination of the left lung. The four lung slices/animal were placed into a single cassette and processed using an Autotechnicon Tissue Processor over a period of 24 hours with final embedment in paraffin wax. The embedded blocks were cut into 5-μm sections using a Microtome and then placed onto a slide. The slides were stained with hematoxylin and eosin (H&E) for semi-quantitative histopathological analysis for the severity and extent of inflammation in the alveolar, pleural, bronchiolar,

and perivascular regions of the lung. A semi-quantitative scoring system was used. This scoring system has been routinely used in our laboratory (3). Table 1 provides a description of the scoring rubric.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism 9 software (San Diego, CA). Data is shown in the present document as arithmetic means and experimental results two standard deviations away from mean were excluded. One-way analysis of variance (ANOVAs) was performed along with Tukey's Honest Significant Difference post-hoc tests to determine all statistically significant differences between specific groups. Statistical significance was set at a p-value of ≤ 0.05.

RESULTS

Pulmonary Function Tests

Airway resistance only demonstrated statistically significant differences between the PBS and PM for the WT mice at 1.0 mg/mL ($p<0.01$) and 2.0 mg/mL ($p<0.01$) (Figure 2A). However, WT mice in the HDM+PM group didn't have any data points past 0.5 mg/mL and with the trend it was on it potentially could have demonstrated a statistically significant difference compared to PBS. At 0.5 mg/mL PBS and HDM+PM demonstrated the greatest difference at this point at a 1.3-fold difference (Figure 2A). There were no statistically significant differences in airway resistance among the various KO mouse groups at any of the tested MCh concentrations (Figure 2B). There were also no statistically significant differences in airway resistance comparing WT and KO groups. WT mice did show statistically significant differences for AHR between PBS and PM ($p<0.001$), PBS and HDM ($p<0.001$), and for PBS and HDM+PM ($p<0.001$) (Figure 3A). WT results had PBS demonstrating EC200RL at the highest MCh concentration of any group and HDM+PM requiring the lowest (Figure 3A). AHR had no statistically significant differences between any of the KO groups (Figure 3B). The KO results also didn’t follow a directional trend; PBS was expected to need the highest concentration of MCh to reach EC200RL but ended up needing the lowest. Thus, the greatest difference in AHR was observed between the WT HDM+PM and PBS groups, with the EC200RL of the latter approximately 13-fold decrease than the former. Notably, their KO mice counterparts demonstrated a 1.7-fold difference in the opposite direction

Cell Differentials and Lavage Analysis

For total cells/mL in the BALF, WT mice demonstrated statistically significant differences between PBS and HDM+PM ($p<0.01$) and for PM and HDM+PM ($p<0.01$) for total cell counts (Figure 4A). KO mice demonstrated no statistically significant differences between any of their groups for total cells/mL in the BALF (Figure 4B). Both WT and KO mice demonstrated their greatest differences between their PBS and HDM+PM group. The WT mice had a 12-fold difference whereas the KO demonstrated a 9-fold difference. There were no statistically significant differences in total cells/mL comparing WT and KO groups. Cell differential analysis demonstrated statistically significant differences for WT mice in the amount of eosinophils, neutrophils, and lymphocytes (Figure 5A). When comparing the amount of eosinophils PBS and HDM+PM ($p<0.01$) and PM and HDM+PM ($p<0.02$) demonstrated statistically significant differences (Figure 4A). Neutrophils were also statistically significant between PBS and HDM+PM ($p<0.001$) and for PM and HDM+PM ($p<0.01$) (Figure 5A). Lymphocytes were statistically significant between PBS and HDM ($p<0.02$), PBS and HDM+PM ($p<0.02$), PM and HDM ($p<0.05$), and for PM and HDM+PM ($p<0.05$) (Figure 5A). KO mice demonstrated no statistically significant differences for cell differential analysis in the BALF (Figure 5B). There were also no statistically significant differences for cell differential analysis in the BALF when comparing WT and KO groups.

Lung Histopathology

Semi-quantitative histopathological scoring demonstrated statistical significance at all regions of the lung for WT mice (Figure 6A). The alveolar demonstrated significance between PBS and HDM ($p<0.001$), PBS and HDM+PM ($p<0.001$), PM and HDM ($p<0.001$), and for PM and HDM+PM ($p<0.001$) (Figure 6A). The perivascular region demonstrated significance between PBS and HDM ($p<0.001$), PBS and HDM+PM ($p<0.001$), PM and HDM ($p<0.001$), and PM and HDM+PM ($p<0.001$) (Figure 6A). The bronchiolar had significance between PBS and HDM+PM ($p<0.001$) and for PM and HDM+PM ($p<0.001$) (Figure 6A). The pleural had significance between PBS and HDM ($p<0.05$), PBS and HDM+PM ($p<0.001$), PM and HDM ($p<0.05$), and for PM and HDM+PM ($p<0.001$) (Figure 6A). Polymorphonuclear leukocytes (PMNs) were visible in both the HDM and HDM+PM group at all regions for the WT mice (Figure 6A, 7, and 8). KO mice demonstrated statistical significance in the alveolar and perivascular regions. The alveolar demonstrated statistical significance between PBS and HDM+PM ($p<0.02$), PM and HDM ($p<0.05$), and for PM and HDM+PM ($p<0.01$) (Figure 6B). The perivascular region had statistical significance between PBS and HDM ($p<0.01$), PBS and HDM+PM ($p<0.001$), PM and HDM ($p<0.01$), and for PM and HDM+PM ($p<0.001$) (Figure 6B). PMNs were only seen in the alveolar of the HDM+PM group for the KO mice (Figure 12).

When comparing between WT and KO mice for treatment effects, statistically significant differences were noted for all lung regions for WT and KO exposed to HDM+PM. Alveolar ($p<0.01$), bronchiolar ($p<0.01$), perivascular ($p<0.001$), and plural

($p<0.01$) regions all demonstrated statistically significant differences when comparing WT mice to KO mice for the HDM+PM groups. No other treatment conditions demonstrated statistically significant differences in the histopathology of the inflammatory scores when comparing between the WT and KO groups. These findings suggest the DUOX gene plays a critical role in PM effects to exacerbate the effects of HDM+PM treatment.

DISCUSSION

In the present study, we examined the effects of PM exposure on an asthma model with and without the *DUOX* genes. The sensitization and challenge procedure we performed to introduce the asthma allergen (HDM) and PM (Figure 1) has been repeated in our laboratory many times and has been successful in producing an adaptive immune response upon allergen challenge (1,13,10,17,18). Previous studies have shown that when the *DUOX* genes are activated by an asthmatic response, there is a correlation between an increased amount of *DUOX* expression in the lungs and an induced airway immune response (11). Allergic asthma can be characterized by AHR, eosinophilic inflammation, and an influx of inflammatory cells (2,5,13). In the present study, we showed that when the *DUOX* genes can't be activated the immune response provoked by PM and HDM isn't statistically significant when compared to the control group but when the *DUOX* genes can be activated the demonstrated responses are different.

KO mouse exposure to PM, HDM, and HDM+PM demonstrated no significant change to AHR (Figure 3B). The EC200RL was not consistent for the KO mice and fluctuated up and down between the groups. PBS reached EC200RL at the lowest concentration on average which could have skewed the data due to the control being expected to need the highest concentration of MCh for EC200RL, but there was still no statistical significance between any of the other groups. These results suggest that exposure to the treatment groups had no effects on the constriction of the airways for the KO mice. Which also agrees with their airway resistance results as those

demonstrated no significance either (Figure 2B). However, the WT mice did show a significance for AHR as each treatment group demonstrated statistical significance when compared to the control group (Figure 3A). These results however didn't agree with the airway resistance results because only the PM group demonstrated statistical significance. The HDM+PM group potentially could have shown statistical significance as its trend was going the highest, but no values were recorded after EC200RL was reached (Figure 2A). The magnitude of change in airway resistance and AHR between PBS and HDM+PM demonstrated in WT mice was also much greater than that of the KO mice (Figure 2 and 3). These results suggest the WT mice will have their airways constricted when exposed to the treatment groups, but the KO mice won’t demonstrate a comparable constriction compared to their control group.

An influx of the total amount of immune cells and more specifically the amount of eosinophils and neutrophils were exhibited by the WT mice. WT mice did not demonstrate statistically significance in the total amount of cells in the BALF until after the HDM was introduced along with PM (Figure 4A), and the same results can be demonstrated by the amount of eosinophils and neutrophils found in the BALF (Figure 5A). The WT HDM+PM group had an increase of 86.8% more eosinophils found in the BALF compared to their HDM group leading to its statistical significance compared to the control. These results deemed to be statistically significant, however the same can't be said for the KO mice. The amount neutrophils in the KO mice did not increase enough to reach statistical significance in the HDM or HDM+PM groups which is expected since *DUOX* genes signal for neutrophil recruitment into allergic airways with

its activation (2), but the amount of eosinophils were not statistically significant either. While the amount of eosinophils still increased in the KO mice when comparing the HDM and HDM+PM, an increase of 32%, neither of these results were statistically significant compared to the amount found in the control. These results suggest that, given the same exposures, changes in BALF cell differentials were less severe in KO versus WT mice exposed to HDM or HDM+PM versus PBS (Figure 4,5).

Histopathological inflammatory scores in the KO mice showed some statistical significance in the alveolar and perivascular regions but were seen statistically significant in almost all regions for the HDM group and all regions in the HDM+PM for the WT mice (Figure 6). Both KO and WT mice showed statistically significant differences for alveolar inflammation compared to their controls, but the WT mice reached much higher levels of inflammation (Figure 6). WT mice demonstrated an average score of 4 for the HDM+PM group which correlates to most of the alveolar exhibiting PMNs; while the KO mice only had an average score of 2.1, which would still demonstrate PMNs but not nearly as much as the WT mice (Figure 11 and 12). *DUOX* genes are widely expressed in the alveoli (15) and the KO mice didn't have any signs of eosinophils in the alveolar in any groups besides the HDM+PM group (Figure 12), while the WT mice had a much higher influx of cells and eosinophils in both the HDM and HDM+PM group (Figure 9 and 11). KO mice also didn’t show any statistical significance at the bronchiolar region between any of their groups, but the WT mice did. KO mice did however have much higher levels of inflammation in the control groups, which could potentially be attributed to the initial innate immune response that the *DUOX* genes

would have provided (15). The severity of inflammation observed in the bronchiolar of the KO controls stayed consistent with the other test groups with little to no eosinophils observed (Figure 6B, 8,10,12). The KO HDM+PM group had a score of 2.6 which correlates to PMNs being present in about ⅓ of the bronchiolar (Figure 12). The difference in score between the KO mice PBS and the HDM+PM group was only 0.91. WT mice however, had a much higher level of inflammation in the bronchiolar, and eosinophils were seen in both the HDM and HDM+PM groups (Figure 6A,9,11). The WT HDM+PM group had a score of 5.95 which correlates to about half of the bronchiolar having thickened tissue and a severe influx of PMNs (Figure 11). The difference in score between the WT PBS and HDM+PM group was 4.8. When comparing the WT and KO mice all regions of the lung were statistically significant between the HDM+PM groups, signifying just how much more of an effect HDM+PM had on the WT compared to the KO mice.

An HDM-driven atopic response wasn't seen in all the end points for the WT or KO mice. The only endpoints statistically influenced by HDM in the KO mice were the histopathological scores for the alveolar and perivascular regions. Even with the introduction of the PM to exacerbate its effects no new statistically significant findings were observed in the KO mice. The WT mice however showed much more divergence when HDM was introduced but only AHR and some histopathological scores demonstrated statistical significance. It wasn’t until HDM was introduced with the addition of PM that the total cell count, amount of eosinophils, and bronchial inflammation would become statistically significant. These results suggest that the HDM

was able to create asthmatic-like symptoms in the WT mice, but with the introduction of HDM+PM together they increased the number of parameters that would be expected of an asthmatic-like response. This response was not demonstrated by the KO mice.

When comparing the WT and KO mice the only statistically significant data was demonstrated by the semi-quantitative histopathological scores. This could potentially suggest that the inactivity of the *DUOX* genes had no effect on suppressing the severity of an asthmatic-like response. When considering the fold-change in some of the data and the severity exhibited by histopathology images we plan to perform different statistical tests in the future to attempt to statistically signify the change exhibited when comparing the two groups.

CONCLUSION

The *DUOX* genes are found in airway epithelial cells and function by providing an innate immune response, however these genes also release a detrimental amount of oxidants during lung inflammation. PM enhances both the effects of HDM and *DUOX* which would typically increase the levels of AHR, and inflammation found in the lungs. In the present study, we demonstrated that the introduction of HDM+PM can enhance the total influx of cells, amount of eosinophils in the BALF, AHR, and inflammation found throughout the entire lung. However, using that same HDM and PM treatment regimen on KO mice, which lack *DUOX* genes, produced no statistically significant changes from the control in any of the aforementioned parameters besides the inflammation observed in the alveolar and perivascular regions. With all of this taken into account we conclude that when the *DUOX* genes cannot be activated an asthmatic response to PM can be minimized.

FIGURES

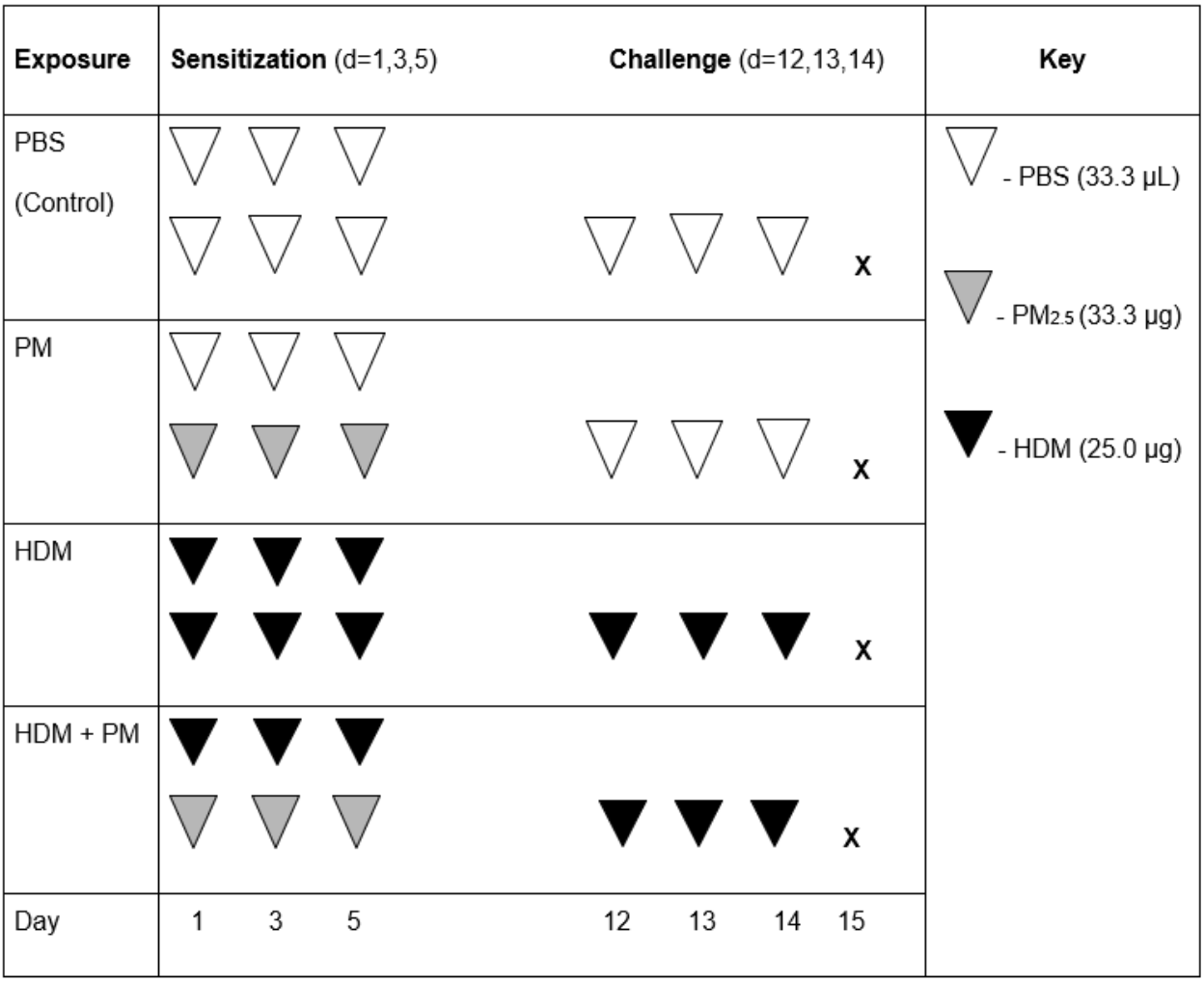
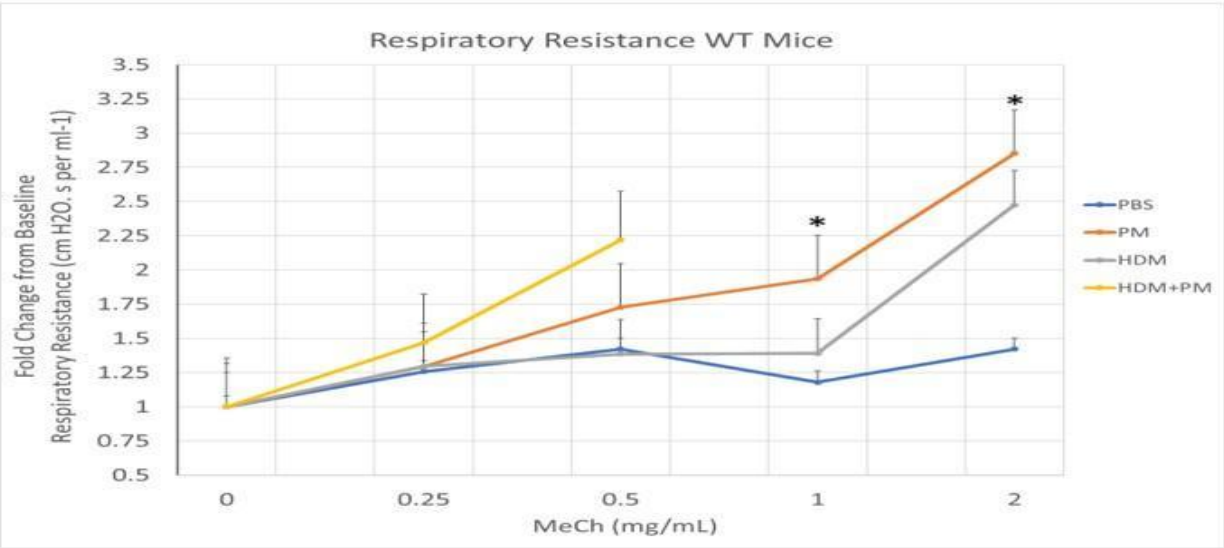


Figure 1: Diagram of the experimental timeline for allergic sensitization and challenge exposures performed with mice via intranasal instillation. During the sensitization exposures (days 1, 3, and 5), mice were instilled twice/day with PBS (33.3 µL/instillation; white triangle), PM (33.3 µg/instillation; grey triangle), and/or HDM (25.0 µg/instillation; black triangle). During the challenge exposures (days 12,13, and 14), they were instilled once per day with PBS or HDM. Necropsies occurred on day 15 (noted in the figure by an "**X**") after pulmonary function testing.

A.



B.

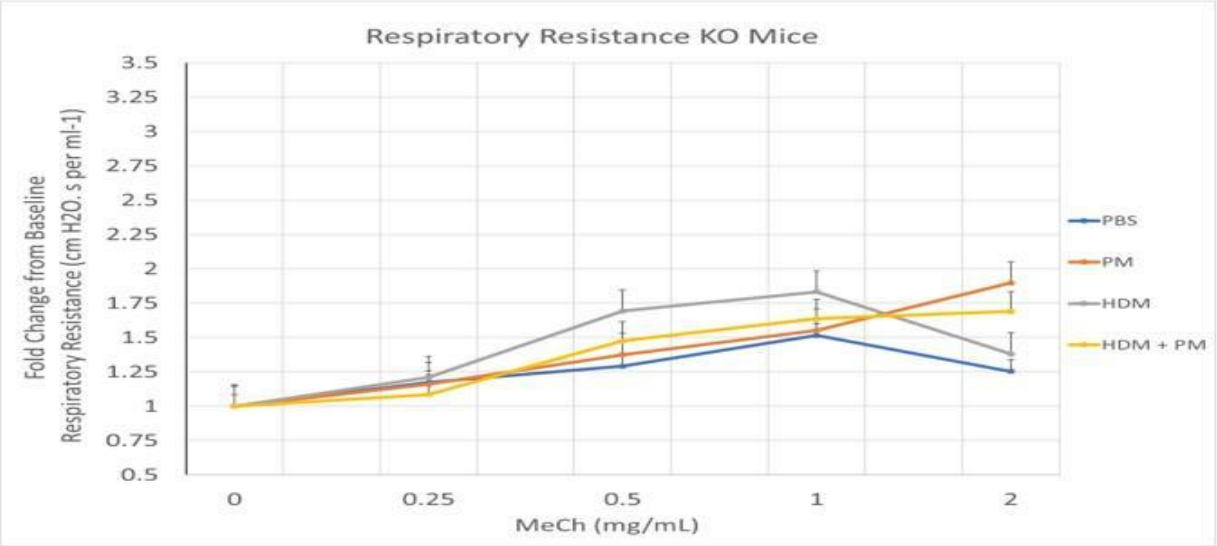


Figure 2: Airway resistance in WT (A) and *DUOX* KO mice (B). Airway resistance is represented as a fold-change from the baseline control value (0 mg/ml of MCh). Measurements for each mouse were stopped once a consistent EC200RL was reached.

One-way ANOVA followed by Tukey's Honest Significant Difference Post-Hoc Test was used to determine statistical significance within groups (WT and KO) of mice. The asterisk (*) represents significance from the respective control value ($p \leq 0.05$).

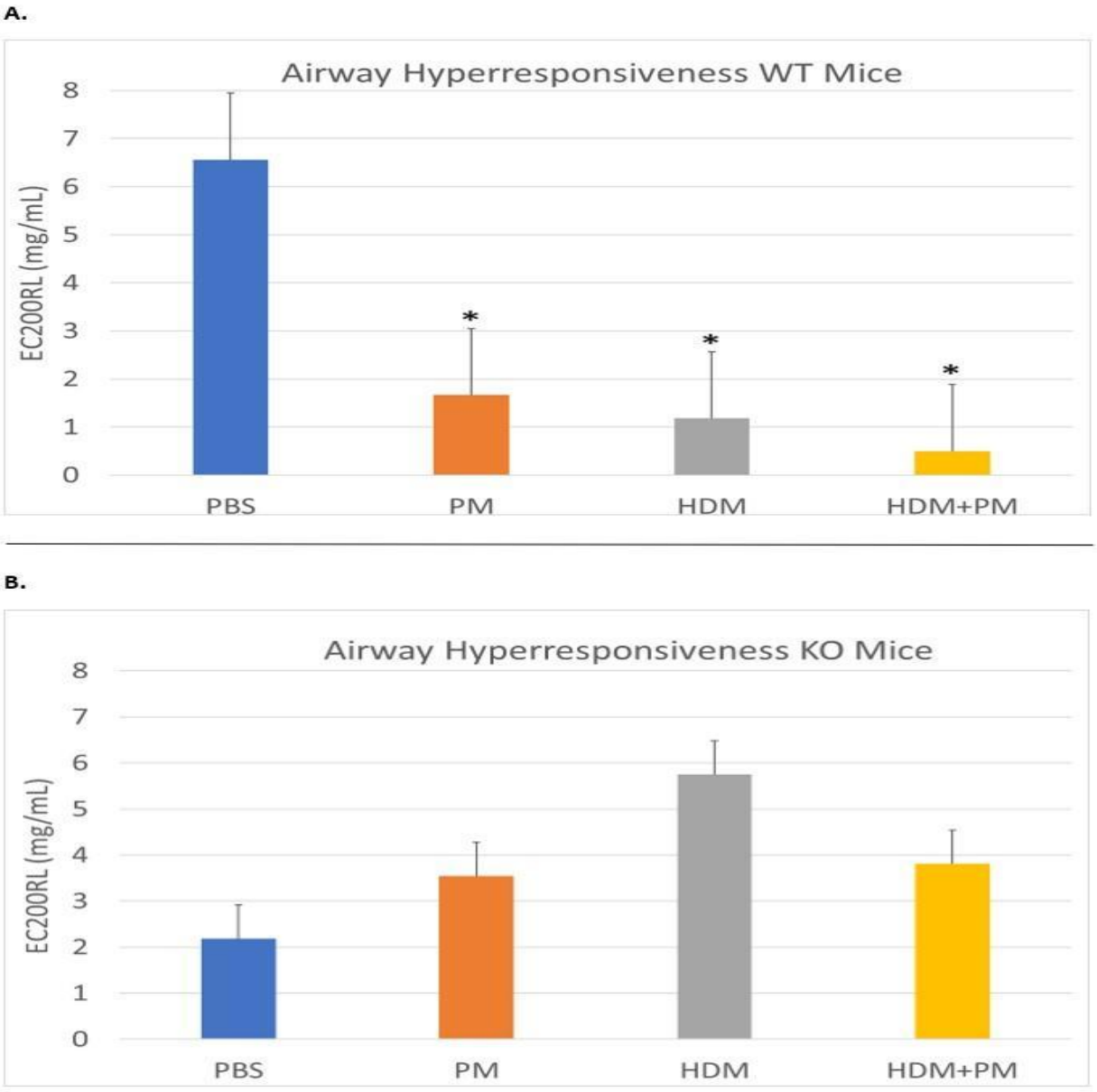


Figure 3: Airway hyperresponsiveness in WT (A) and *DUOX* KO mice (B). AHR was measured as the dose of MCh required to double airway resistance (EC200RL). One-way ANOVA followed by Tukey's Honest Significant Difference Post-Hoc Test was used to determine statistical significance between groups. The asterisk (*) represents a significant difference from the control ($p \leq 0.05$).

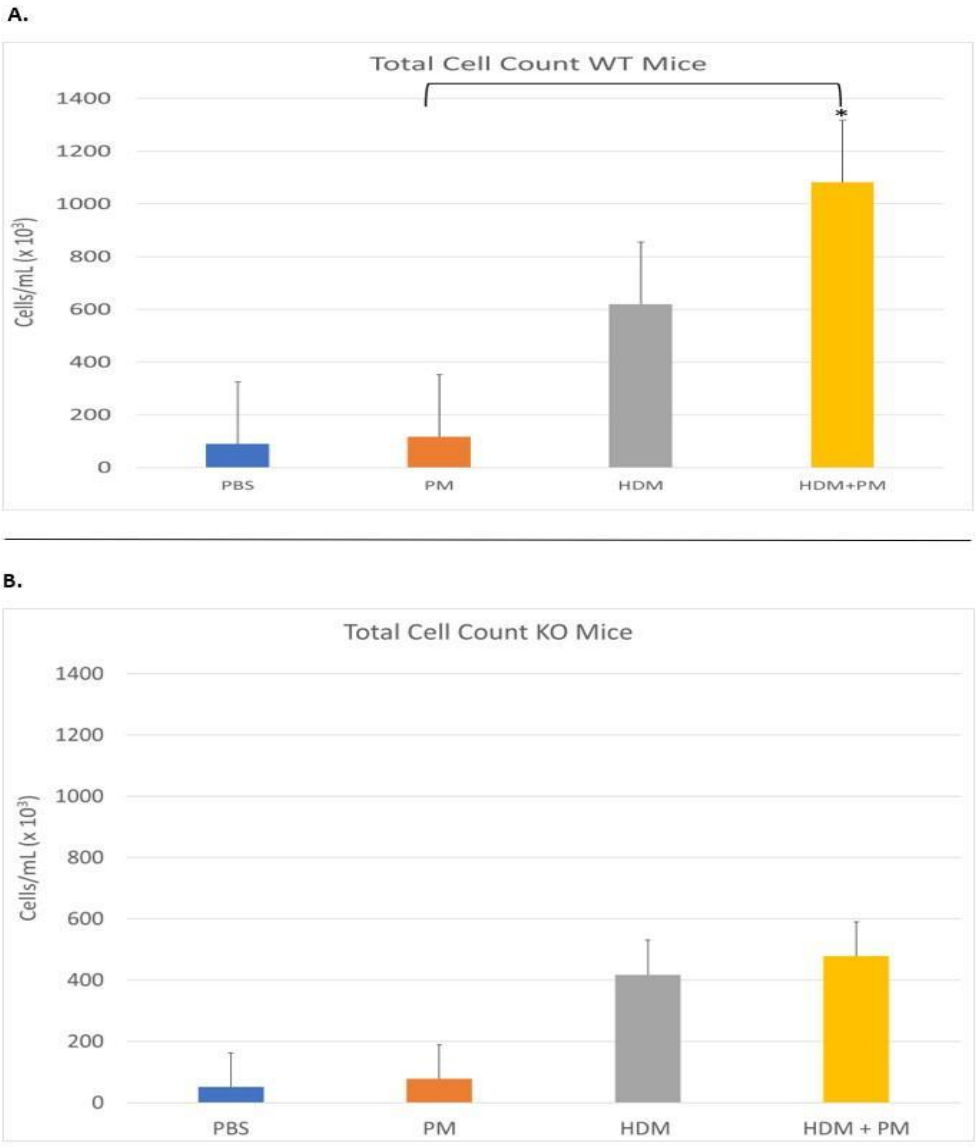
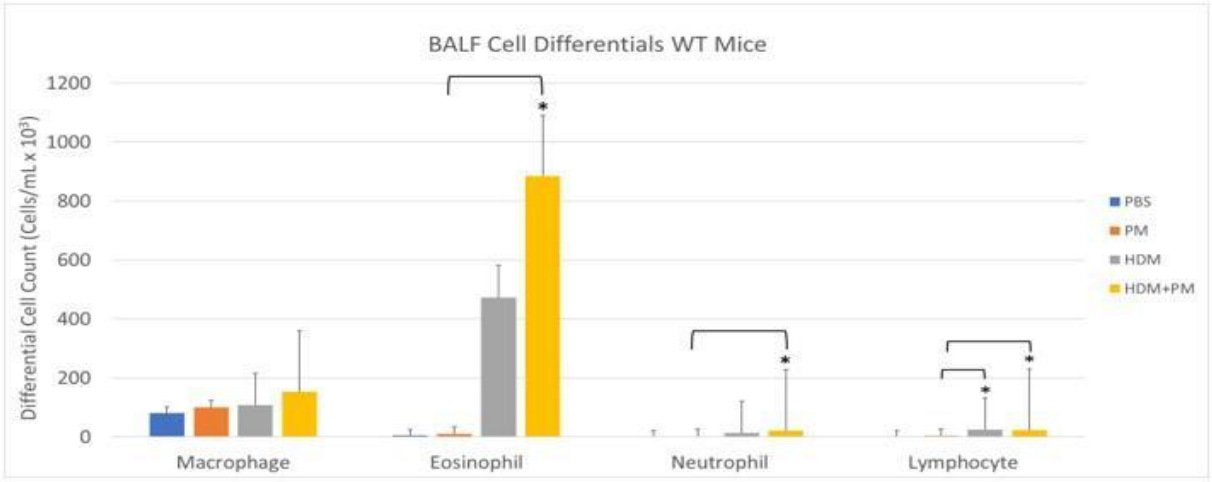


Figure 4: Total cell counts from BALF in WT (A) and *DUOX* KO mice (B). Total cells were calculated based on the amount of cells counted per mL of BALF collected. One-way ANOVA followed by Tukey's Honest Significant Difference Post-Hoc Test was used to determine statistical significance between groups. The asterisks (*) represent significance from the control and brackets represent significance between exposure groups ($p \leq 0.05$).

A.



B.

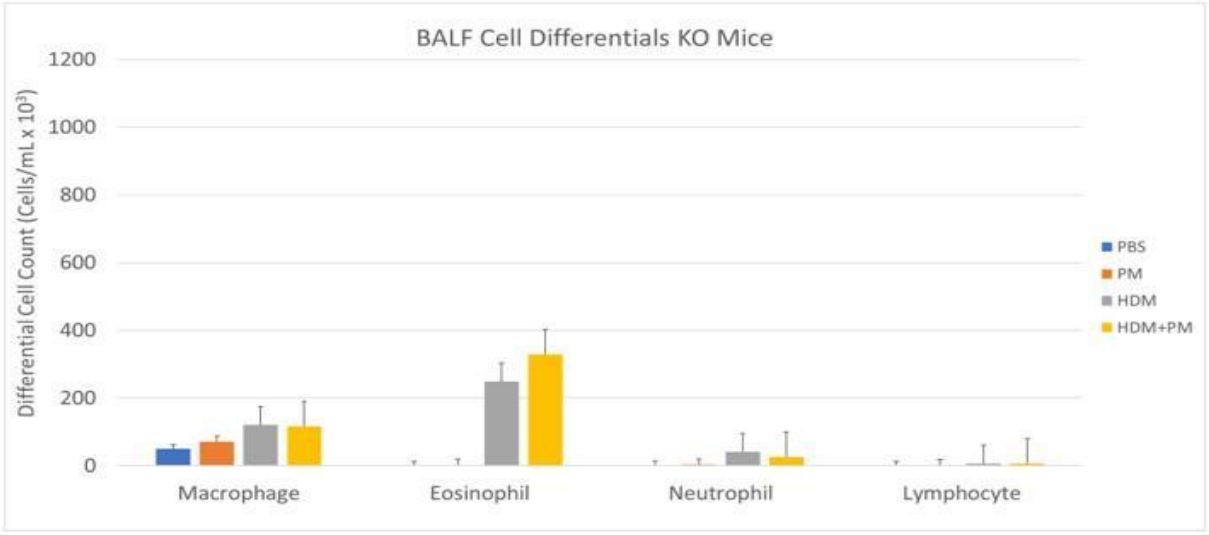


Figure 5: Cell differentials collected from BALF in WT (A) and *DUOX* KO mice (B). Cell differentials were calculated after counting 500 cells per BALF. One-way ANOVA followed by Tukey's Honest Significant Difference Post-Hoc Test was used to determine statistical significance between groups. The asterisks (*) represent significance from the control and brackets represent significance between exposure groups ($p \leq 0.05$).

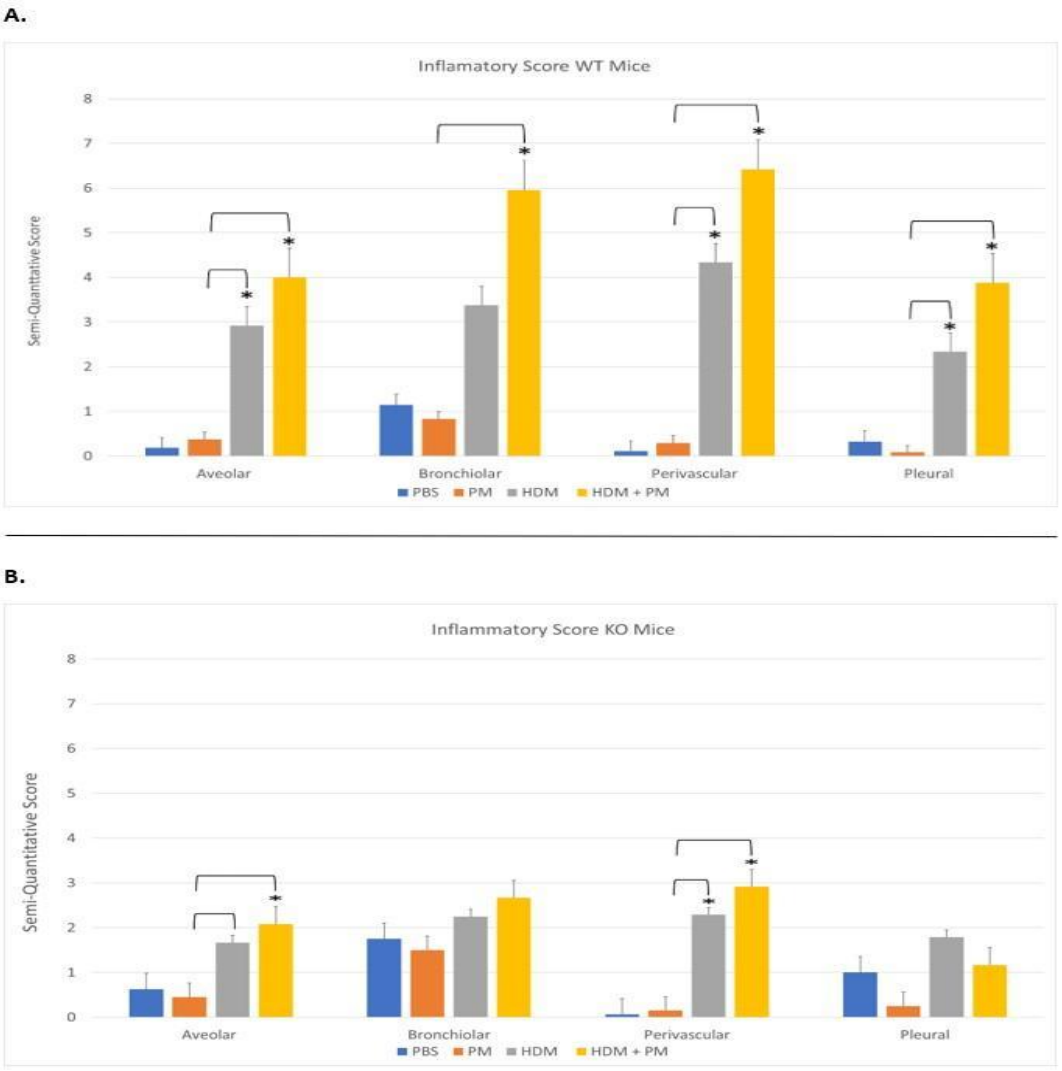


Figure 6: Semi-quantitative histopathological scores of inflammation in four regions of the lung after exposure in WT (A) and *DUOX* KO mice (B). Semi-quantitative scores are measured using a scoring rubric and the values represent the means of the severity and extent multiplied. One-way ANOVA followed by Tukey's Honest Significant Difference Post-Hoc Test was used to determine statistical significance between groups. The asterisks (*) represent significance from the control and brackets represent significance between exposure groups ($p \leq 0.05$).

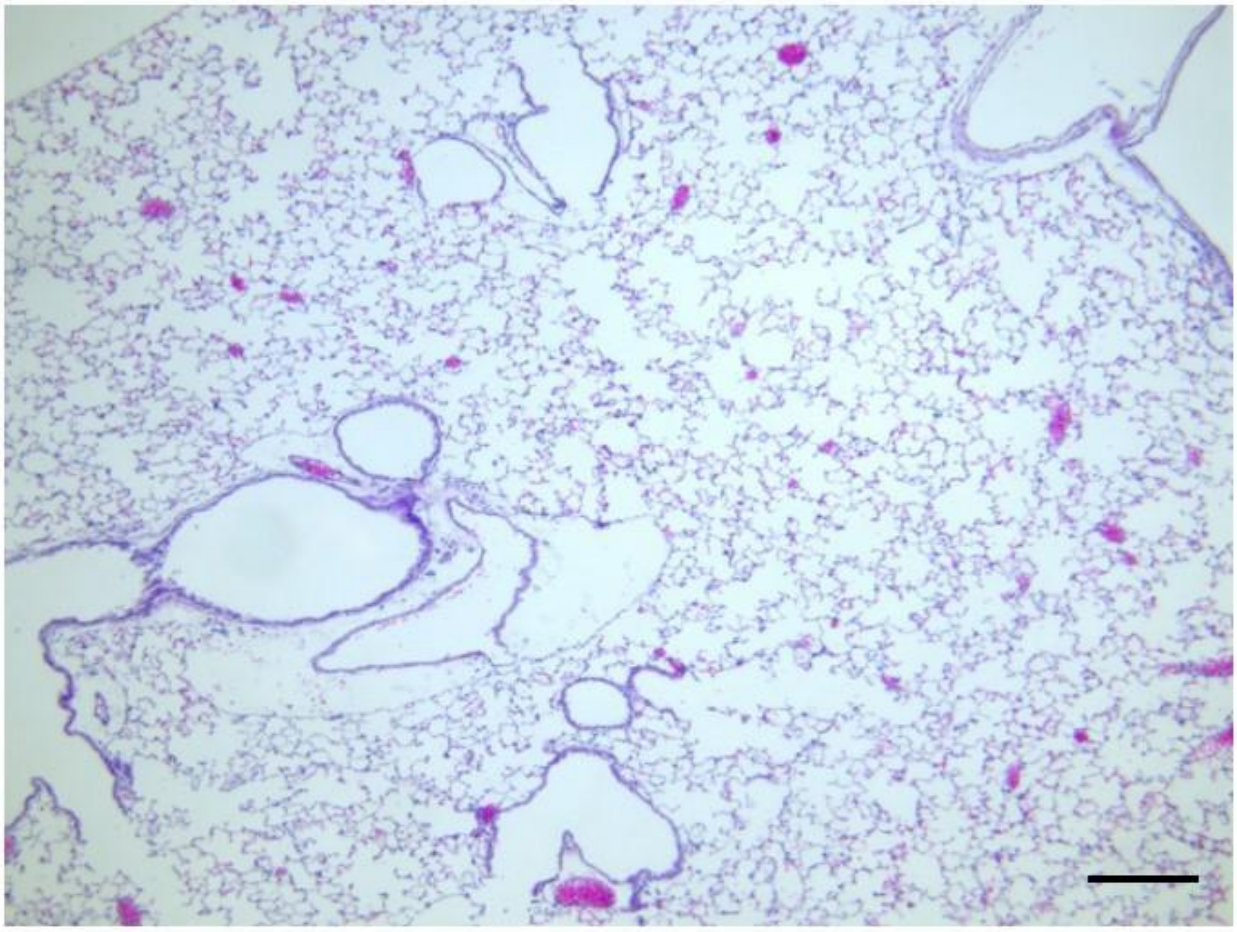


Figure 7: Hematoxylin and Eosin stained lung tissue from a WT mouse exposed to PBS. The image is by brightfield microscopy using a Zeiss Axio Lab.A1 microscope. Scale bar is 50μm.

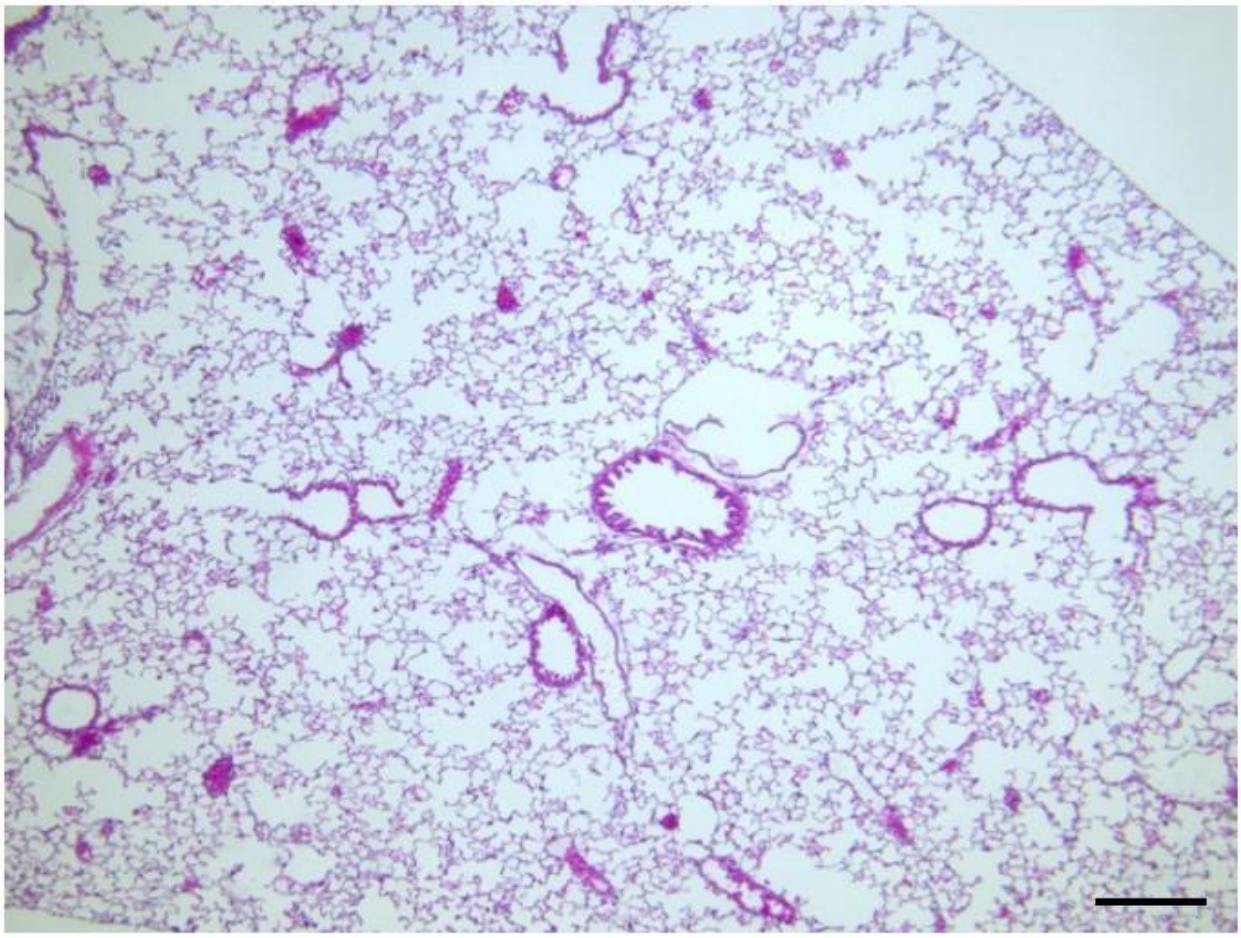


Figure 8: Hematoxylin and Eosin stained lung tissue from a KO mouse exposed to PBS. The image is taken by brightfield microscopy using a Zeiss Axio Lab.A1 microscope. Scale bar is 50µm.

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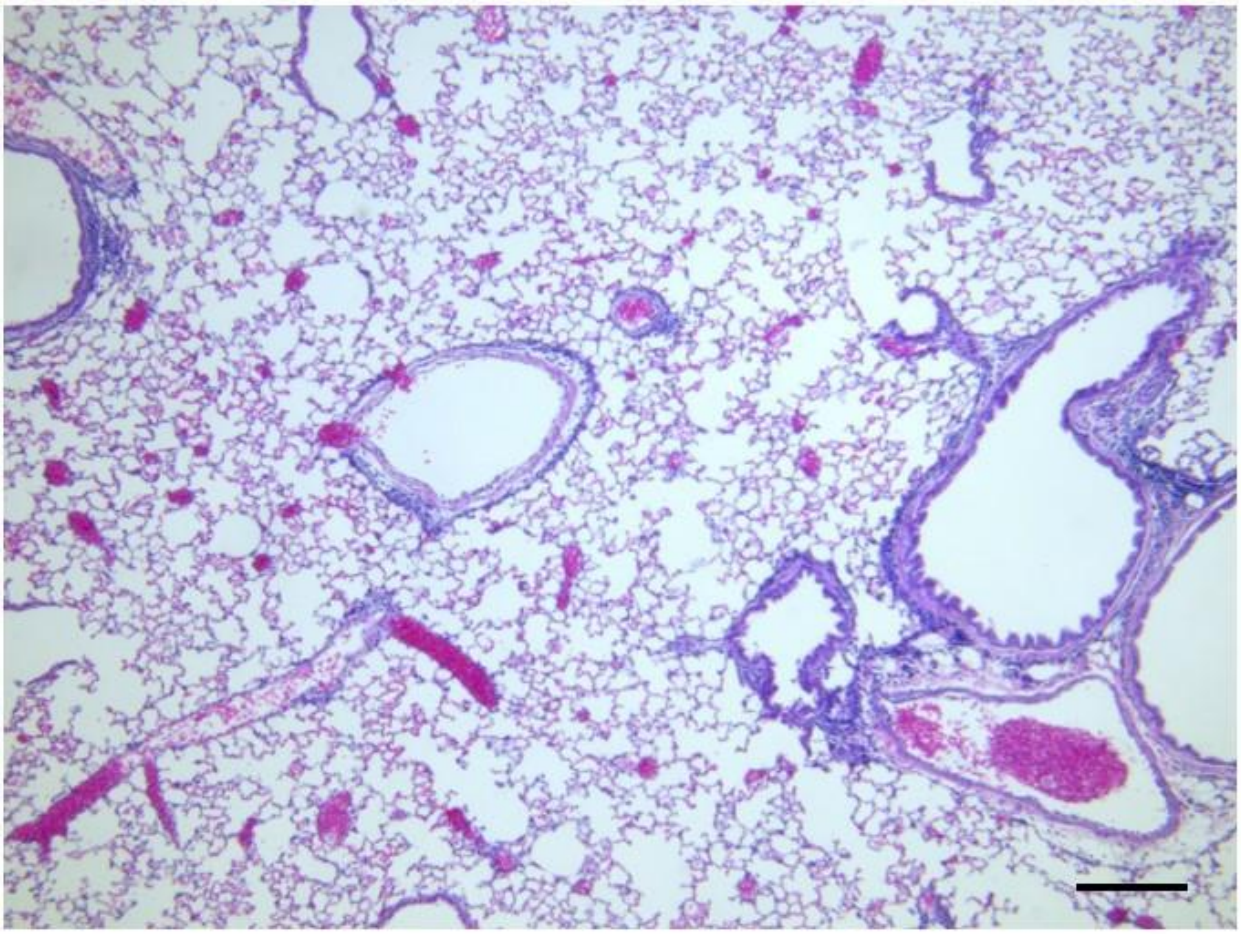

Figure 9: Hematoxylin and Eosin stained lung tissue from a WT mouse exposed to HDM. The image is taken by brightfield microscopy using a Zeiss Axio Lab.A1 microscope. Scale bar is 50µm.

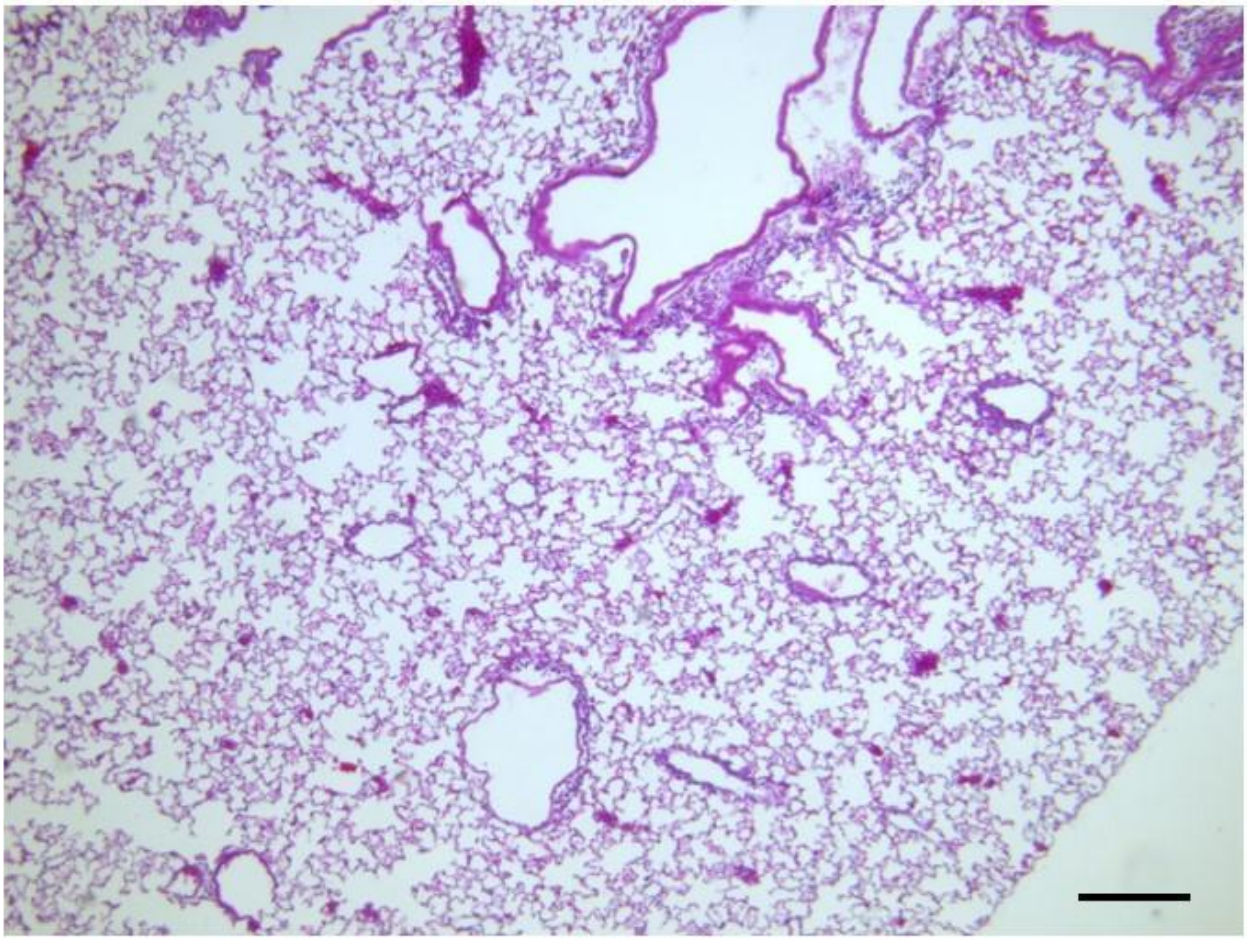

Figure 10: Hematoxylin and Eosin stain of left lung slices of KO mouse exposed to HDM. The image is taken by brightfield microscopy using a Zeiss Axio Lab.A1 microscope Scale bar is 50µm.

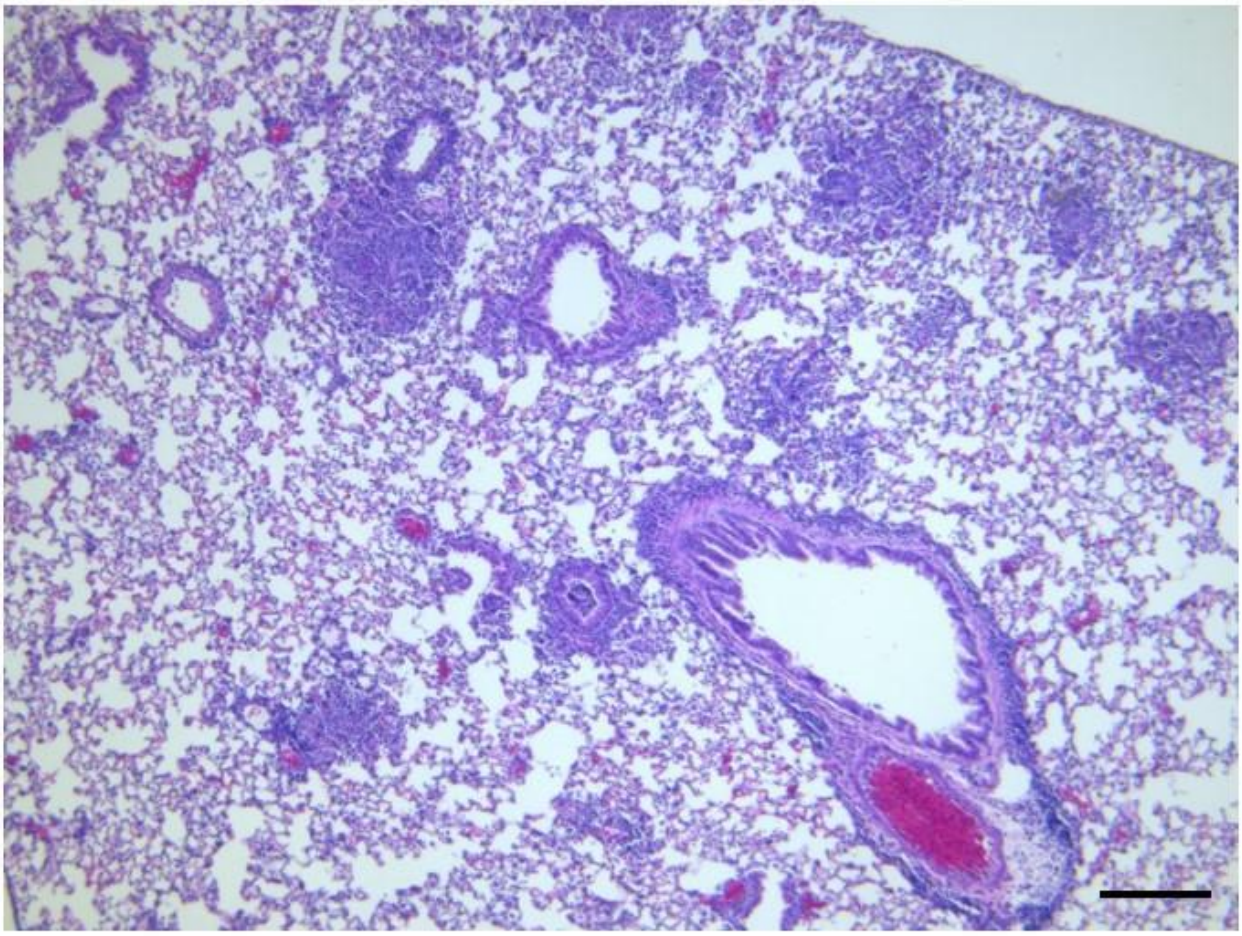

Figure 11: Hematoxylin and Eosin stained lung tissue from a WT mouse exposed to HDM+PM. The image illustrates extensive inflammation of the airways and alveoli. Focal areas of alveoli are completely filled with inflammatory cells. The image is taken by brightfield microscopy using a Zeiss Axio Lab.A1 microscope. Scale bar is 50µm

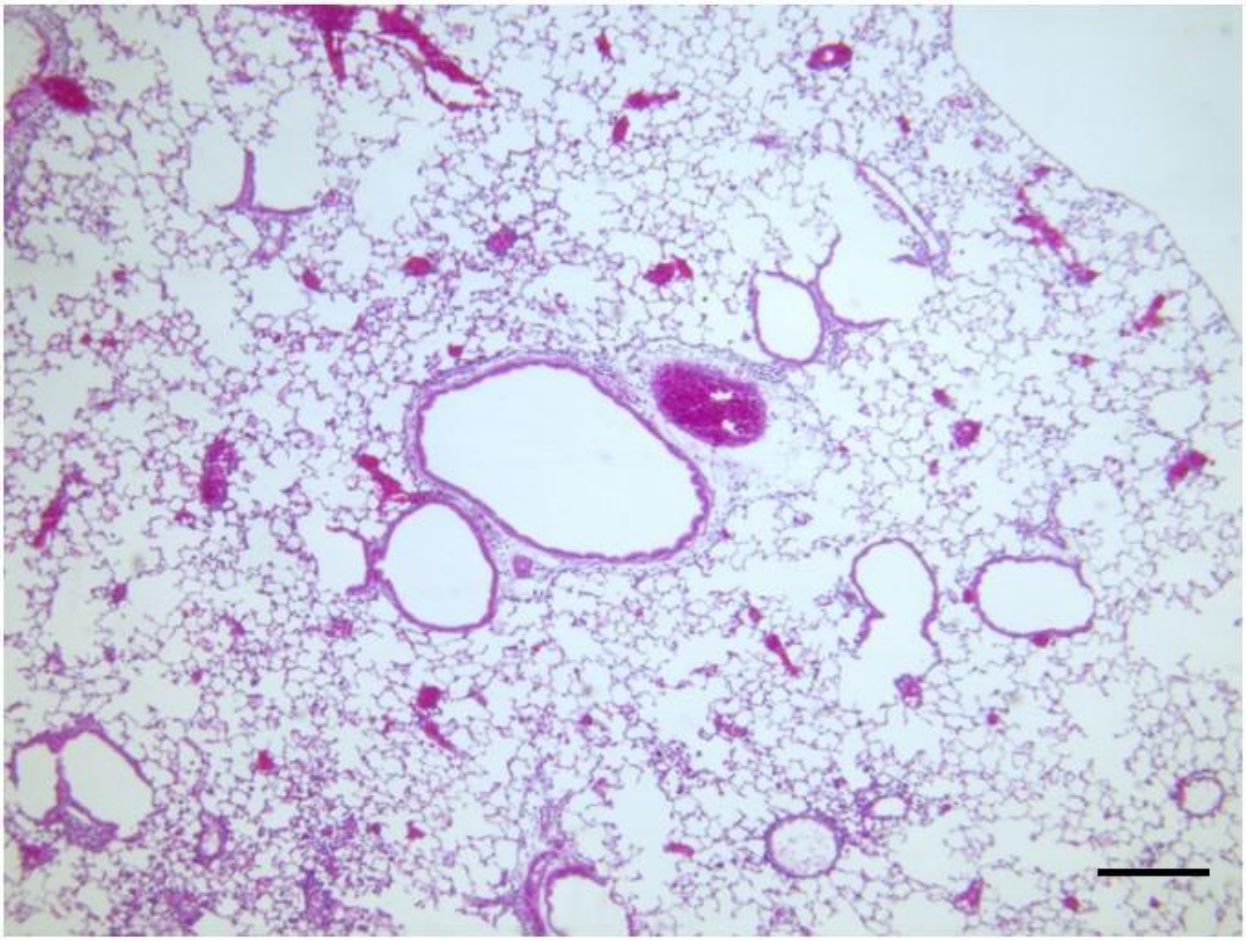

Figure 12: Hematoxylin and Eosin stain of left lung slices of KO mouse exposed to HDM+PM. In contrast to WT mice, the degree of inflammation is notably less in KO mice exposed to HDM+PM. The image is taken by brightfield microscopy using a Zeiss Axio Lab.A1 microscope Scale bar is 50µm.

TABLE

Score Type	Score Value			
	0	1	2	3
Severity	Little to no inflammatory cells visible	Slightly increased cellularity but no polymorphonuclear leukocytes (PMNs)	Moderately increased cellularity with PMNs present	Marked influx of cells accompanied by thickened tissue
Extent	Normal Tissue	About a third of the lung section is affected by inflammation	About half of lung section affected by inflammation	More than three-quarters of lung section affected by inflammation

Table 1: Semi-quantitative scoring rubric for histopathological analysis. Alveolar, bronchiolar, pleural, and perivascular regions of the lung were all scored separately for extent and severity. The extent and severity scores are then multiplied to obtain a final score.

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