



Original Contribution

Dual oxidase regulates neutrophil recruitment in allergic airways



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ABSTRACT

Enhanced reactive oxygen species production in allergic airways is well described and correlates with increased airway contractions, inflammatory cell infiltration, goblet cell metaplasia, and mucus hypersecretion. There is also an abundance of interleukin-4/interleukin-13 (IL-4/IL-13)- or interleukin-5-secreting cells that are thought to be central to the pathogenesis of allergic asthma. We postulated that the dual oxidases (DUOX1 and DUOX2), members of the nicotinamide adenine dinucleotide phosphate oxidase family that release hydrogen peroxide (H₂O₂) in the respiratory tract, are critical proteins in the pathogenesis of allergic airways. DUOX activity is regulated by cytokines, including IL-4 and IL-13, and DUOX-mediated H₂O₂ influences several important features of allergic asthma: mucin production, IL-8 secretion, and wound healing. The objective of this study was to establish the contribution of DUOXs to the development of allergic asthma in a murine model. To accomplish this goal, we utilized a DUOX-deficient mouse model (*Duoxa*^{-/-}) that lacked maturation factors for both DUOX1 and DUOX2. Our results are the first to demonstrate evidence of DUOX protein and DUOX functional activity in murine airway epithelium. We also demonstrate that DUOX maturation factors are required for airway-specific H₂O₂ production and localization of DUOX to cilia of fully differentiated airway epithelial cells. We compared wild-type and *Duoxa*^{-/-} mice in an ovalbumin exposure model to determine the role of DUOX in allergic asthma. In comparison to DUOX-intact mice, *Duoxa*^{-/-} mice had reduced mucous cell metaplasia and lower levels of T_H2 cytokine levels in bronchoalveolar fluid. In addition, increased airway resistance in response to methacholine was observed in *Duoxa*^{+/-} mice, as expected, but was absent in *Duoxa*^{-/-} mice. Surprisingly, *Duoxa*^{-/-} mice had decreased influx of neutrophils in bronchoalveolar fluid and lung tissue sections associated with a lower level of the chemotactic cytokine IL-6. These findings suggest that DUOX-derived H₂O₂ has an important role in signaling neutrophils into allergic airways.

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Introduction

Allergic asthma is characterized by airway hyperresponsiveness, eosinophilic inflammation, an increase in smooth muscle mass, abnormal deposition of extracellular matrix, and goblet cell metaplasia [1]. There is compelling evidence in animal models that a predominance of interleukin-4 (IL-4)¹/interleukin-13 (IL-13)- or interleukin-5 (IL-5)-secreting CD4⁺ T lymphocytes is characteristic of the allergic asthma phenotype [2]. Recently, the respiratory tract epithelium, in conjunction with dendritic cells, has been recognized to be primarily responsible for driving this skewed T_H2 milieu [3]. Importantly, there is direct evidence that T_H2 cytokines

can promote all the primary features of allergic asthma [2] and that the severity of asthma correlates directly with the number of T_H2 cells in the airway [4]. The mechanisms responsible for altered cytokine expression in allergic asthma have not been clearly established, but the pattern recognition receptors of the Toll-like receptor family (TLRs), in particular TLR-3, -4, and -5, have been implicated in epithelial-derived allergic sensitization [5–8].

In parallel, reactive oxygen species (ROS) in airway epithelium are thought to exacerbate the response to allergen challenge [9–11]. Enhanced ROS production has long been noted in allergic airways and correlates with increased airway contractions, inflammatory cell infiltration, goblet cell metaplasia, and mucus hypersecretion [12–14]. There are several potential sources for the ROS that affect asthma symptoms, but there is mounting evidence that two epithelial-specific enzymes, dual oxidase 1 and 2 (DUOX1 and DUOX2), are critical for promoting the allergic airway response. DUOX1 and DUOX2 are members of a seven-member NADPH oxidase family that produce hydrogen peroxide (H₂O₂) in airway

Abbreviations: IL, interleukin; TLR, Toll-like receptor; T_H, T-helper cell; ROS, reactive oxygen species; DUOX, dual oxidase; BALF, bronchoalveolar lavage fluid; L-T₄, levothyroxine; DPI, diphenyleneiodonium

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epithelial cells [15–17]. Several recent reports implicate either DUOX1- or DUOX2-mediated H_2O_2 generation as necessary for TLR signaling in response to house dust mite exposure, bacterial infection, or viral infection [18–21]. DUOX1 has also been implicated in augmenting mucin expression in the airway, a hallmark feature of allergic asthma [22,23], and promoting epithelial wound healing [24,25] that may lead to pathologic airway remodeling in response to chronic inflammation and injury. The observation that DUOX1 is regulated by T_H2 cytokines IL-4 and IL-13 [26,27] suggests that DUOX may be part of a positive feedback loop in which TLR-induced IL-4/13 production further enhances DUOX-mediated TLR activation of T_H2 cytokines.

To better understand the role of DUOX in allergic asthma, we utilized a *Duoxa*^{-/-} knockout mouse model that does not express functional DUOX1 or DUOX2 [28]. Because both DUOX1 and DUOX2 have been implicated in models of allergic asthma or inflammatory signaling pathways, it was important to interrogate a model system that lacked both DUOX isoforms. In addition, this model excluded the possibility of one isoform compensating for the loss of the other. Using an ovalbumin exposure model that recapitulates several features of allergic asthma [29], we examined airway hyperresponsiveness, eosinophilic inflammation, and goblet cell metaplasia in *Duoxa*^{-/-} versus *Duoxa*^{+/+} mice that differentially expressed functional DUOX in the airway. We hypothesized that DUOX was primarily responsible for epithelial activation of allergic responses and that lack of a functional DUOX system will lead to decreased features of allergic disease.

Materials and methods

Animals

All procedures with mice were performed in accordance with the University of California at Davis (UC Davis) Institutional Animal Care and Use Committee. *Duoxa*^{-/-} knockout mice were generated as described previously [28]. Mice utilized for our experiments were acquired through subsequent breeding of these breeding pairs at the UC Davis facility. All mice were of 129Sv6 background and maintained in HEPA-filtered laminar-flow cage racks with a 12-h light/dark cycle and allowed free access to food (Purina Rodent Chow) and water. Mice were housed and cared for by the veterinary staff of the UC Davis Animal Resource in AALAC-accredited facilities. Genotypes for all mice were verified from tail snips using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MO, USA) per the manufacturer's protocol. Because *Duoxa*^{-/-} mice are severely hypothyroid without hormone replacement [28], mice were injected with 40 ng L-T₄/g body wt from birth to weaning (L-thyroxine; Sigma). After weaning, *Duoxa*^{-/-} mice were provided with albuminized drinking water containing 0.26 ng/μl L-T₄ to maintain thyroid hormone supplementation. This hormone replacement regimen produced animals with normal growth characteristics and normal adult organ and body weight compared with wild-type animals as previously shown [28]. Mice were euthanized at the end of an experiment with an intraperitoneal overdose of pentobarbital. Anesthesia and euthanasia procedures were performed according to UC Davis IACUC-approved protocols.

H_2O_2 measurements

Duoxa^{+/+} and *Duoxa*^{-/-} mouse tracheas were isolated and perfused with 0.2% protease (Sigma; P5147-1G) overnight. Tracheas were then flushed with 0.0125% fetal bovine serum (FBS; Gemini, 100-106) in Opti-MEM (Gibco, 11058-021). Isolated epithelial cells were then centrifuged at 1200g and the cell pellet was washed three times in Opti-MEM to ensure removal of

protease and FBS. After the third wash, cell pellets were resuspended in 150 μl of Opti-MEM and H_2O_2 production was measured after 30 min using the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Molecular Probes, Eugene, OR, USA) as previously described [26]. Diphenyliodonium (DPI)—1 μM for 10 min before H_2O_2 measurement—was used to determine flavin protein-specific H_2O_2 production. Relative fluorescence units were converted to micromolar H_2O_2 using a standard curve generated in parallel. Horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine were added in excess to ensure H_2O_2 was the limiting substrate. Eight tracheas from *Duoxa*^{+/+} or *Duoxa*^{-/-} mice were isolated separately and divided into two tubes to measure H_2O_2 from DPI-treated and DPI-untreated samples. Results for each treatment condition represent the mean and SEM for three measurements from one experiment.

Western blots

A novel anti-DUOX monoclonal antibody was designed to recognize the amino acid sequence (DTDPPQEIRR) between the EF-hand and the second transmembrane domain of murine DUOX1 (Abmart, Shanghai, China). Cell pellets used for H_2O_2 measurements from the airway epithelium were lysed in 4 °C RIPA buffer (Pierce, Rockford, IL, USA) supplemented with Sigma protease inhibitor cocktail and phenylmethanesulfonyl fluoride (Sigma) on ice. The lysate was centrifuged for 10 min at 14,000 rpm at 4 °C, and the supernatant was transferred into a fresh tube and stored at -80 °C. Total protein concentration was determined using the Bio-Rad DC Protein Assay Kit. Samples were combined with Laemmli buffer (2% SDS, 100 mM dithiothreitol) and heated to 65 °C for 10 min and placed on ice. Samples—20 μg protein/lane—were resolved on a 7% NuPAGE Novex Tris-acetate gel (Invitrogen) and then transferred to a polyvinylidene difluoride membrane. Blots were blocked in 5% fat-free dry milk in Tris-buffered saline and incubated with mouse anti-DUOX primary antibody (1:1000; Abmart) at 4 °C for 24 h and with secondary goat anti-mouse horseradish peroxidase-coupled antibodies (1:8000; R&D Systems) at 25 °C for 1 h. Detection was achieved by enhanced chemiluminescence (HyGlo; Denville Scientific, South Plainfield, NJ, USA). The intensity of DUOX protein on the Western blot was normalized to β-tubulin using MultiGauge version 2.3 software (Fujifilm, Cypress, CA, USA).

Ovalbumin asthma model

We generated an ovalbumin allergic asthma model as previously described [30]. Briefly, 9- to 11-week-old male and female knockout, heterozygous, and wild-type mice were sensitized via intraperitoneal injection of 10 μg/0.1 ml ovalbumin (grade V, ≥98% pure; Sigma) with alum adjuvant at days 0 and 14. The mice were then challenged with 10 ml aerosolized 1% ovalbumin (OVA; 10 mg/ml) in phosphate buffered saline (PBS) for 2 weeks with six exposures every other day starting from day 28. Aerosolized exposures of approximately 45 min were conducted with a side-stream nebulizer (Invacare Corp., Elyria, OH, USA) and Passport compressor (Invacare). Matched controls received an intraperitoneal injection of ovalbumin and alum and were provided filtered air, whereas the experimental animals received OVA aerosolization.

Lung function evaluation

Immediately after the last aerosolized ovalbumin challenge on day 42, lung function was evaluated as previously described [30]. Using a plethysmograph for restrained mice (Buxco, Troy, NY, USA) dynamic compliance and resistance were measured while the mice were anesthetized and sedated with medetomidine, 0.75 mg/kg

(Domitor; Orion Pharma, Espoo, Finland) and tiletamine/zolpidem, 37.5 mg/kg (Telazol; Fort Dodge Laboratories, Fort Dodge, IA, USA). Mice were ventilated with a volume of 125 μ l at 150 strokes per minute (MiniVent; Harvard Apparatus, Cambridge, MA, USA). Nebulized saline and methacholine (0.1–2.0 mg/ml) were administered for 3 min with a 3-min recovery period after each dose. Dynamic compliance (ml/cm H₂O) and resistance (cm H₂O · s/ml) measurements were made at baseline and immediately after each administration of saline or methacholine.

Bronchoalveolar lavage sample collection and processing

After lung function testing, mice were euthanized via an overdose of pentobarbital and dilantin. The lung was then lavaged two times with 1 ml sterile PBS (pH 7.4) to collect bronchoalveolar lavage fluid (BALF). BALF was centrifuged at 2000 rpm for 10 min and supernatant was collected and stored at –80 °C. The resulting BALF cell pellet was resuspended in ACK/RBC lysis buffer to remove red blood cells and recentrifuged at 2000 rpm for 10 min. The resulting pellet was resuspended in PBS for white blood cell counts. Live cell concentrations were estimated by counting trypan-blue-excluding cells on a hemacytometer. To determine BALF cell differentials, cytocentrifuge preparations were stained with a Hema3 kit as described in the manufacturer's instructions (Fisher Scientific, Kalamazoo, MI, USA) and sealed using Cytooseal 60 (Richard-Allen Scientific, Kalamazoo, MI, USA). Cell percentage differentials were calculated by counting 10 fields at 400 $\times$ magnification and classifying cell types as alveolar macrophage, neutrophil, eosinophil, lymphocyte, or "other" based upon standard morphological characteristics and staining profiles. Totals of each cell type per milliliter of bronchoalveolar lavage fluid were calculated using the differential percentages.

Lung histology

After BALF collection, lungs were inflated at 30 cm H₂O with 1% paraformaldehyde (PFA) in PBS for approximately 30 min and then fixed in 1% PFA for at least 24 h. Trachea, left lung, and right lung were then separated, placed in 70% ethanol, and embedded in paraffin wax. The left lungs were cut into 5 μ m-thick sections, placed on glass microscope slides, and dried. Next, the sections were deparaffinized with xylene and rehydrated with ethanol for further processing. To visualize DUOX protein localization in lung sections, paraffin-embedded left lung sections from ovalbumin-treated animals were incubated with a mouse anti-DUOX antibody followed by addition of goat anti-mouse secondary antibody conjugated to peroxidase (Vectastain ABC Kit, Cat. No. PK-4002) utilizing 3,3'-diaminobenzidine (DAB) substrate detection.

Tissue cell differentials were determined from hematoxylin and eosin (HE)-stained tissue slices using a semiquantitative method. Five fields at 1000 $\times$ magnification were analyzed and cell types were classified as alveolar macrophage, neutrophil, eosinophil, lymphocyte, or "other" based upon standard morphological characteristics and staining profile. Peribronchiolar, vascular, perivascular, and alveolar compartments were compared between groups. To visualize the neutrophil spatial arrangement in lung sections, paraffin-embedded left lung sections were incubated with a neutrophil-specific Ly-6 G antibody 1A8 (BD Pharmingen; Cat. No. 551459) followed by a secondary antibody conjugated to peroxidase (Vectastain ABC Kit; Cat. No. PK-4004) utilizing DAB substrate detection. Tissue sections were sealed using Cytooseal 60 (Richard-Allen Scientific). To visualize mucin production, periodic acid-Schiff base (PAS) staining was utilized and semiquantitative scoring was used to compare groups. PAS-stained tissues were examined under light microscopy for PAS-positive staining in the upper airway epithelium in five fields at 400 $\times$ magnification. PAS-

positive epithelial cells of the total epithelial cells were counted to obtain a percentage and compare groups.

Enzyme-linked immunosorbent assay (ELISA)

The supernatant fraction of the BALF was thawed on ice and used in ELISA. The mouse homologs of human IL-8, keratinocyte-derived cytokine (KC), and macrophage inflammatory protein (MIP)-2 were detected using ELISA (R&D Systems; Product Nos. MKC00B and MM200, respectively). Sample levels were compared to a standard curve provided by the supplier.

Multiplex

The supernatant fraction of the BALF was used in multiplex analyses (Milliplex; Cat. No. MPXMCYTO-70K-22) per the manufacturer's instructions. Analysis was followed per the manufacturer's protocol and as previously described [31]. Briefly, 50 μ l of BALF supernatant was incubated with antibody-coupled beads for analysis. Quantitation of antigen-antibody binding was performed using a flow-based Luminex 100 suspension array system (Bio-Plex 200; Bio-Rad). Known reference cytokine concentrations included in the Milliplex kit were utilized to create a standard curve and sample cytokine concentrations were calculated using the Bio-Plex Manager software. Concentrations below the sensitivity limit of detection (LOD) of the method were calculated as LOD/2 for statistical comparisons.

Statistics

All data were processed using Prism 5 software (GraphPad Software, San Diego, CA, USA). The unpaired Student *t* test was used unless otherwise noted. Males and females were evaluated individually and were not statistically different; thus they were compiled in results unless otherwise noted. Data were deemed statistically significant at *p* ≤ 0.05.

Results

DUOX location and function in mouse airway epithelium

To confirm that DUOX was present in mouse airway epithelial cells, we isolated murine airway epithelial cells from *Duoxa*^{+/+} and *Duoxa*^{–/–} mice and identified DUOX using Western blot analysis (Fig. 1A). Densitometry analyses demonstrated that *Duoxa*^{+/+} and *Duoxa*^{–/–} mice had similar levels of DUOX protein (ratio of 0.52 versus 0.39 normalized to β -tubulin, respectively). However, functional DUOX activity was substantially impaired in *Duoxa*^{–/–} mouse airways. Using Amplex red to measure H₂O₂ production in isolated airway epithelium, *Duoxa*^{+/+} mice had a 15-fold increase in DPI-inhibitable H₂O₂ production from unstimulated cells compared to *Duoxa*^{–/–} mice (0.31 μ M H₂O₂/μg protein/30 min versus 0.02 μ M H₂O₂/μg protein/30 min, respectively; Fig. 1B). These data are consistent with previous observations that DUOX proteins require their respective maturation factors for normal functional activity.

To further evaluate the effect of *Duoxa* knockout on DUOX expression, we performed immunohistochemical analysis on lung sections obtained from *Duoxa*^{+/+} and *Duoxa*^{–/–} mice. We found that both genotypes treated with ovalbumin had predominant DUOX expression in the airway epithelium, but the cellular location of DUOX was substantially different between the wild-type and the knockout animals. In *Duoxa*^{+/+} mice, DUOX was localized to the cilia of the epithelial cell with minimal cytoplasmic staining. *Duoxa*^{–/–} mice, in contrast, had diffuse protein expression dispersed throughout the cytoplasm without observable staining in

cilia (Fig. 1C). This suggests an inability of DUOX protein to be properly escorted to the plasma membrane in mice that lack DUOXA.

Duoxa knockout has variable effects on allergic asthma

To determine the role of functional DUOX in allergic asthma, we first compared changes in airway hyperresponsiveness after ovalbumin exposure in *Duoxa*^{+/+} versus *Duoxa*^{-/-} mice. At the end of the sensitization protocol, the mice were anesthetized and tracheostomized followed by measurement of dynamic compliance and resistance on a mouse ventilator. Serial administrations of increasing doses of aerosolized methacholine, to a maximum dose of 2 mg/ml, were performed to determine changes in

baseline compliance and resistance. Compared to filtered air controls, ovalbumin-exposed *Duoxa*^{+/+} mice had a significant reduction in compliance and corresponding increase in resistance after exposure to methacholine, consistent with features of a successful allergic airway model. In contrast, the ovalbumin-exposed *Duoxa*^{-/-} mice did not demonstrate significantly increased hyperresponsiveness compared to matched air controls even at the highest dose of methacholine tested (Fig. 2).

We next analyzed lung tissues for mucin production by PAS staining and found a significant reduction in mucin-positive cells in ovalbumin-exposed *Duoxa*^{-/-} mice compared with *Duoxa*^{+/+} mice (Fig. 3A). Animals exposed to filtered air alone had negligible PAS staining. To minimize biased sampling and quantify our

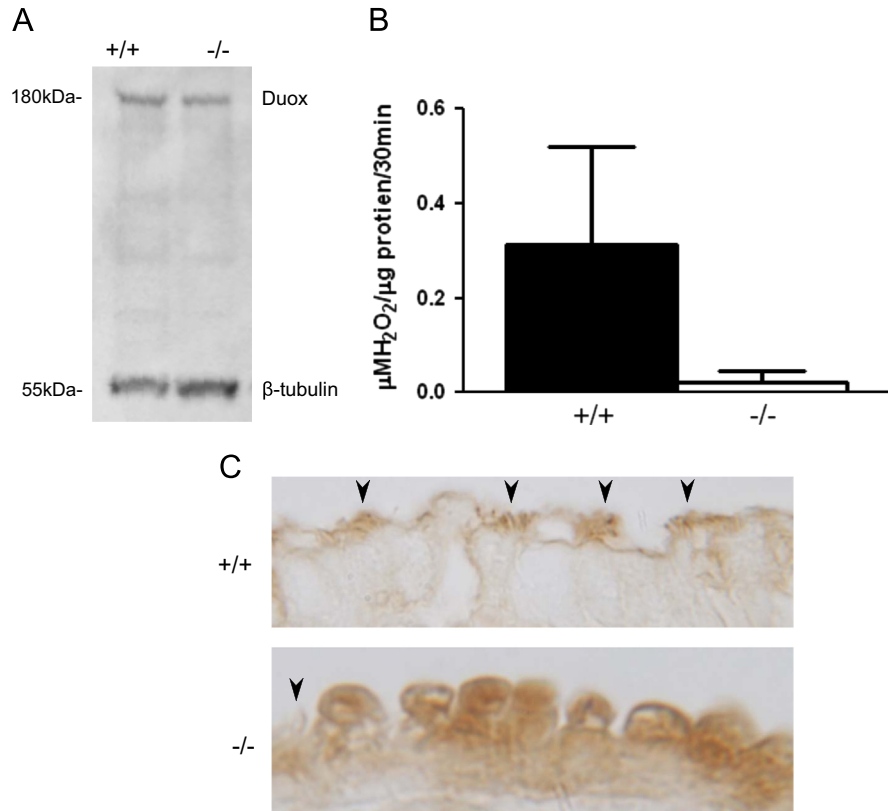


Fig. 1. DUOX expression and activity in isolated mouse airway cells. (A) Western blot analysis confirmed DUOX expression in isolated epithelial cells from *Duoxa*^{+/+} and *Duoxa*^{-/-} mice. (B) *Duoxa*^{+/+} and *Duoxa*^{-/-} mouse airway cells were isolated from tracheas and immediately analyzed for DPI-inhibitable H₂O₂ production by Amplex red analysis. (C) Immunohistochemistry on airway epithelial cells using a mouse anti-DUOX antibody in ovalbumin-treated animals (arrowheads indicate cilia).

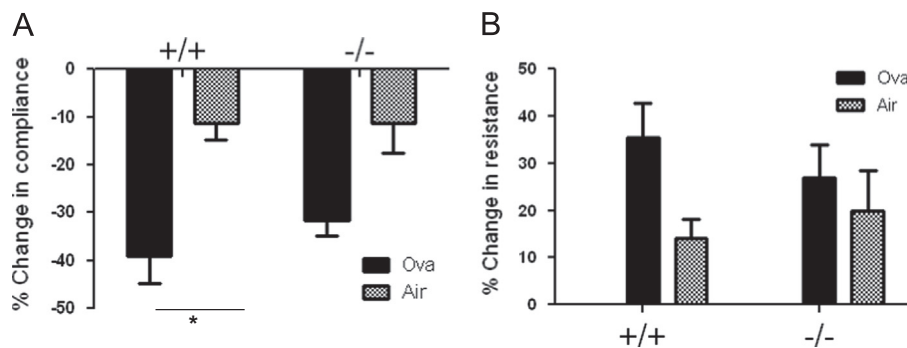


Fig. 2. DUOX is necessary for increased airway hyperresponsiveness. Percentage change in (A) compliance or (B) resistance was determined in *Duoxa*^{+/+} (+/+) and *Duoxa*^{-/-} (-/-) mice exposed to 2 weeks of either filtered air or ovalbumin. Percentage change values were calculated by dividing the remainder of compliance or resistance determined at the highest dose of methacholine (2 mg/ml) minus compliance or resistance determined after exposure to aerosolized saline (baseline value) by the baseline value times 100. Data are shown as means ± SEM from six mice in each group; **p* < 0.05.

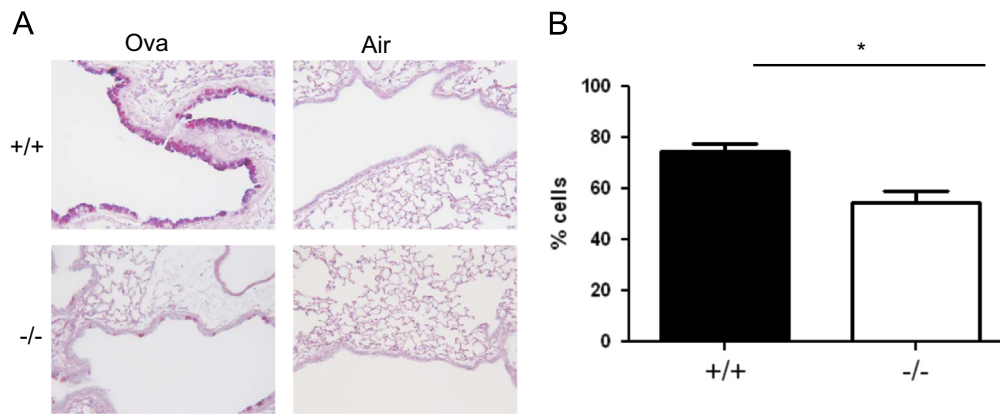


Fig. 3. DUOX induces mucin expression in mouse lung epithelium after allergen challenge. (A) PAS staining of paraffin-embedded lung sections obtained from $Duoxa^{+/+}$ ($+/+$) and $Duoxa^{-/-}$ ($-/-$) male mice after 2 weeks of aerosolized ovalbumin exposure in sensitized animals. Similar airway levels are shown to ensure equivalent representation of hyperplastic potential between groups (original magnification $200\times$). (B) Quantification of PAS-positive staining was determined by counting the number of PAS-positive epithelial cells normalized to total epithelial cells for $Duoxa^{-/-}$ ($-/-$) and $Duoxa^{+/+}$ ($+/+$) mice. Five fields incorporating the upper airway were randomly chosen for counting, $*p < 0.05$.

results, we determined the number of PAS-positive epithelial cells normalized to the total number of epithelial cells in randomly selected airway sections and found similar results (Fig. 3B).

However, the lack of functional DUOX protein had minimal effects on eosinophilic inflammation. After the last challenge with aerosolized ovalbumin or filtered air, we lavaged the lungs of $Duoxa^{+/+}$ and $Duoxa^{-/-}$ mice to determine total cell counts and cell differentials. In both $Duoxa^{+/+}$ and $Duoxa^{-/-}$ mice, animals that received nebulized ovalbumin had significant elevations in total live cell counts compared to filtered air-exposed animals (Fig. 4A). Sex or genotype had no significant impact on total cell counts or number of dead cells recovered from BALF for each treatment group (data not shown). The predominant cell type in the $Duoxa^{+/+}$ mice was eosinophils, consistent with the expected allergic asthma phenotype as shown previously (Fig. 4B) [32,33]. Unlike the effect of functional DUOX protein on airway hyperresponsiveness and mucus hyperplasia, however, both $Duoxa^{+/+}$ and $Duoxa^{-/-}$ mice demonstrated predominant eosinophil infiltration (approximately 60%) and similar numbers of eosinophils between the two groups after ovalbumin exposure (Fig. 4B). Together, these data suggest that DUOX plays a significant role in two major features of allergic asthma, airway hyperresponsiveness and mucus hyperplasia, but that eosinophilic inflammation is due to a separate, DUOX-independent mechanism.

DUOX mediates neutrophil influx during allergic inflammation

Although neutrophils are not a predominant feature of allergic inflammation, our model induced approximately 20% neutrophil influx into the airways of $Duoxa^{+/+}$ mice. $Duoxa^{-/-}$ mice, in contrast, had minimal neutrophilic inflammation in BALF after ovalbumin exposure (Fig. 4B). When we corrected for the total cell numbers between the two groups of mice, absolute neutrophil counts in the alveolar compartment between $Duoxa^{+/+}$ and $Duoxa^{-/-}$ mice remained statistically significant (Fig. 4C).

To determine if this difference in neutrophil recruitment correlated with the number of neutrophils in lung tissue, we stained paraffin-embedded lung sections with hematoxylin and eosin to localize neutrophils based on morphology. To ensure only neutrophils were counted, we performed immunohistochemical staining using the 1A8 antibody against the neutrophil-specific marker Ly-6G (Fig. 5). Consistent with the BALF data, there was a significant reduction in neutrophils seen in the alveolar compartment of $Duoxa^{-/-}$ mice compared to $Duoxa^{+/+}$ mice (Fig. 5A). Similarly, lower numbers of neutrophils were seen in the peribronchial and

perivascular compartments of $Duoxa^{-/-}$ mice (Figs. 5B and C). There was no significant difference in neutrophil counts between $Duoxa^{+/+}$ and $Duoxa^{-/-}$ mice treated with filtered air (data not shown).

To better quantify the change in neutrophil numbers throughout the lung, we counted cells in the peribronchiolar, vascular, perivascular, and parenchyma compartments (see Materials and methods). Cell types were determined based on morphologic appearance, and the number of neutrophils for each compartment was determined as a percentage of the total cells counted. As shown in Fig. 6, neutrophil numbers were reduced in $Duoxa^{-/-}$ versus $Duoxa^{+/+}$ mice for all four compartments examined in the lung despite similar levels of circulating neutrophils in both groups (Supplementary Fig. S1). These data suggested that DUOX influenced retention of neutrophils in the lung after allergen exposure.

DUOX-mediated changes in cytokine expression

To examine the events driving lower neutrophil infiltration found in $Duoxa^{-/-}$ compared to $Duoxa^{+/+}$ mice, we examined changes in 2 major chemokines involved in neutrophil chemotaxis, KC and MIP-2 [34–36]. KC and MIP-2 levels were measured in BALF from $Duoxa^{+/+}$ and $Duoxa^{-/-}$ mice by ELISA (Fig. 7). There was a nonstatistical trend toward higher KC and MIP-2 levels in $Duoxa^{-/-}$ mice, which suggested that KC- or MIP-2-mediated signaling was not the predominant mechanism for neutrophil recruitment in this model. Based on this finding, we utilized multiplex analyses to probe for other potential proinflammatory cytokines that were modulated by DUOX during allergic inflammation. As shown in Figs. 8, 5 cytokines were highly expressed in $Duoxa^{+/+}$ compared to $Duoxa^{-/-}$ mice. Differences between mice for 2 of these 5 cytokines, IL-5 and eotaxin, were statistically significant. The remaining 17 cytokines that were measured were either similar in both groups of mice after ovalbumin exposure or undetected (Supplementary Table S1).

Discussion

As previously demonstrated, our ovalbumin exposure model was able to generate several characteristic features of allergic asthma including airway hyperresponsiveness, goblet cell metaplasia, and eosinophilic inflammation [32,33]. These features correlated with a robust increase in airway expression of IL-4, IL-13, IL-5, and eotaxin, consistent with the known roles of these

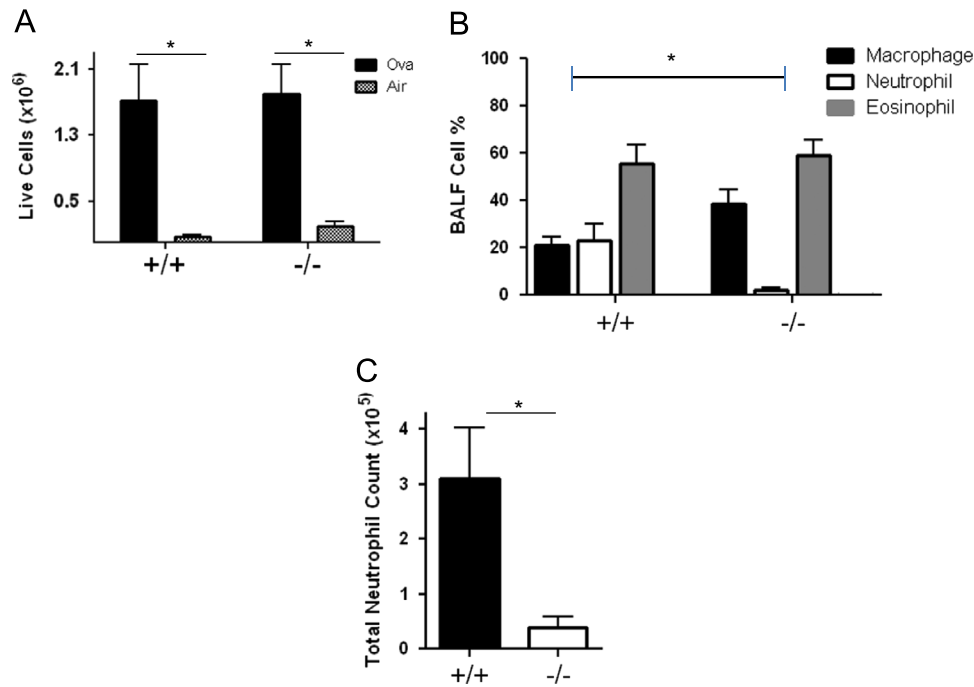


Fig. 4. DUOX affects neutrophilic, but not eosinophilic, airway inflammation after allergen challenge. (A) Leukocytes were collected from the airway compartment by bronchoalveolar lavage (BAL) and the number of live cells was determined by trypan blue exclusion. The number of live cells was compared between ovalbumin-exposed (Ova) and filtered air-exposed (Air) *Duoxa*^{-/-} (-/-) and *Duoxa*^{+/+} (+/+) mice. (B) Leukocytes were collected from the airway compartment by bronchoalveolar lavage after 2 weeks of aerosolized ovalbumin exposure and cell differentials were determined visually based on cell morphology. The percentage of each cell type was compared between ovalbumin-exposed *Duoxa*^{-/-} (-/-) and *Duoxa*^{+/+} (+/+) mice. (C) Absolute neutrophil counts in ovalbumin-exposed *Duoxa*^{-/-} (-/-) and *Duoxa*^{+/+} (+/+) mice were calculated by multiplying neutrophil percentage by total cell number from BAL. Data are shown as means $\pm$ SEM from six mice in each group; **p* < 0.05 using two-way ANOVA and Bonferroni posttest correction.

cytokines in the generation of allergic asthma [37]. Lack of functional DUOX protein attenuated airway hyperresponsiveness, goblet cell metaplasia, and T_H2 cytokine levels in response to ovalbumin exposure. Together, these data suggested DUOX is a critical enzyme that modulates the epithelial response to allergens and the development of several asthmatic features.

Although we cannot exclude contributions from other cell types with potential DUOX activity [42], our data support the notion that airway epithelium-derived DUOX is responsible for our observations. Certainly, differences in PAS staining between wild-type and *Duoxa*^{-/-} mice suggest direct local effects from lung epithelium consistent with human airway DUOX. Importantly, we provide evidence that functional DUOX exists in the mouse airway epithelium, in contrast to a previous report [29]. We postulate that the reason for this discrepancy is a difference in mouse strains or the use of a mouse-specific anti-DUOX antibody. Additional studies in various mouse backgrounds may shed light on strain differences in airway DUOX expression. Based on our findings, there seem to be several important parallels to human airway DUOX, including the requirement of the maturation factor for functional activity [38] and localization to ciliated cells [39]. Using an estimate that airway epithelial cells contain close to 150 μ g of protein/10⁶ cells [40], we observed H₂O₂ production rates of approximately 0.70 nM H₂O₂/10 min/10⁶ cells from unstimulated murine airway epithelium. This degree of H₂O₂ production is similar to that seen in unstimulated human airway cultures [41], although it is difficult to directly compare without performing parallel experiments in mouse and human airway tissues.

The lack of functional DUOX in the airway epithelium had no impact on eosinophilic inflammation. The *Duoxa*^{-/-} and the *Duoxa*^{+/+} mice had similar levels of eosinophilic inflammation in response to ovalbumin exposure. This discordant relationship between eosinophilic inflammation and other features of allergic asthma is consistent with previous findings [43] and suggests that

DUOX-mediated signaling is a key factor responsible for these differences. Unexpectedly, we observed lower levels of eotaxin in the *Duoxa*^{-/-} animals despite similar eosinophil numbers between the two groups of mice. This suggests that eotaxin is not primarily responsible for recruiting eosinophils to the lung in our mouse model [44], or that the eotaxin levels expressed in the knockout mice were above a threshold required to amply recruit eosinophils.

The difference we observed in neutrophil infiltration between the *Duoxa*^{-/-} and the *Duoxa*^{+/+} mice in response to allergen was unexpected. Although we observed predominant eosinophilic inflammation, over 20% of the cells identified in BALF of DUOX-intact animals were neutrophils. In the absence of functional DUOX, there was a greater than 80% reduction in the number of neutrophils recruited to the lung. Histologic evaluation revealed that this reduced number of neutrophils was observed in the vascular, perivascular, peribronchiolar, and alveolar compartments. These data suggest that the primary deficit is due to impaired endothelial–neutrophil interactions or reduced numbers of circulating neutrophils in the *Duoxa*^{-/-} mice. Peripheral blood counts from *Duoxa*^{-/-} and *Duoxa*^{+/+} mice demonstrated similar levels of circulating neutrophils (Supplementary Fig. S1), implying that DUOX-mediated signaling is important for activation of either circulating neutrophils or pulmonary endothelium.

Reactive oxygen species are known to regulate signaling molecules and proteins [45,46]. Therefore, it is likely that DUOX-derived hydrogen peroxide or hydrogen peroxide-derived metabolites directly recruit neutrophils to the airway [47–49] or regulate molecules involved in neutrophil chemotaxis [19,21,50]. Because DUOX is important for regulating IL-8 in airway epithelial cells [19,21,50], we examined differences in mouse homologs of IL-8, MIP-2, KC, and LIX, in the BALF of *Duoxa*^{-/-} and *Duoxa*^{+/+} mice and did not observe any significant difference between groups (see Fig. 7 and Supplementary Table S1). This is in contrast to a recent study that demonstrated that *Duox2* knockout resulted in

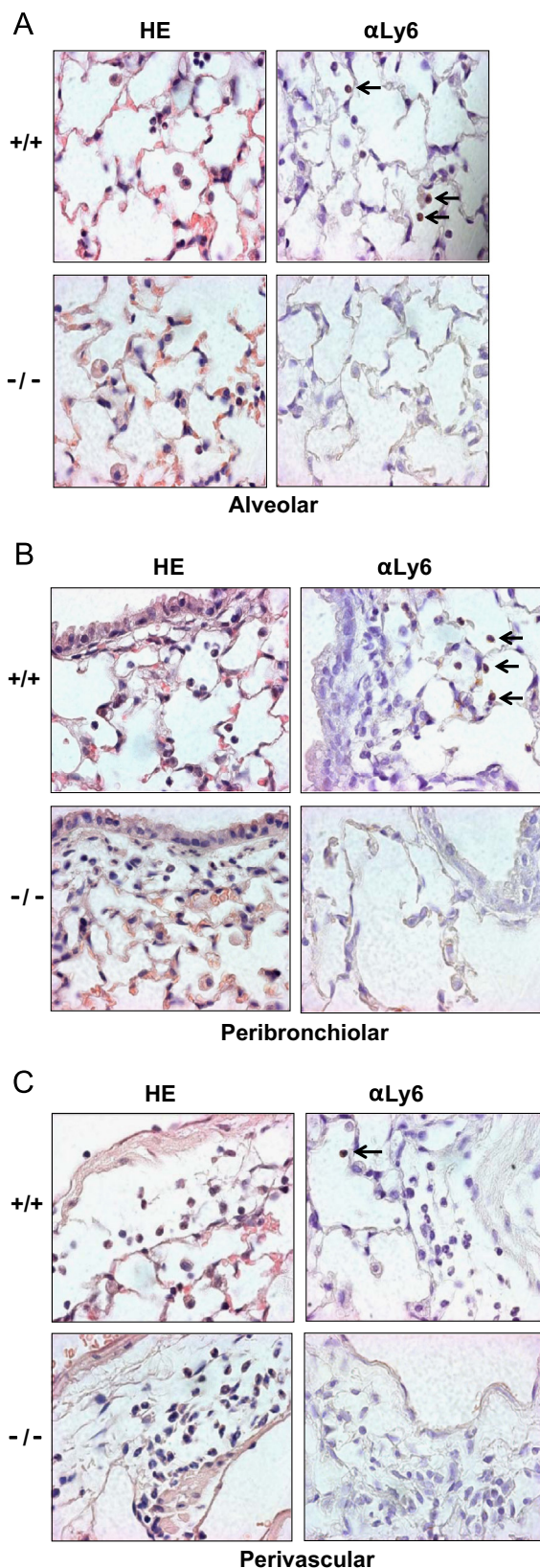


Fig. 5. *Duoxa*^{-/-} mice had lower neutrophil influx into multiple compartments of airway tissue. Paraffin-embedded lung sections were obtained from ovalbumin-sensitized *Duoxa*^{+/+} (+/+) and *Duoxa*^{-/-} (-/-) male mice after 2 weeks of aerosolized ovalbumin exposure. Lung sections were stained with hematoxylin and eosin (HE) for morphometric analysis of neutrophilic influx. The presence of neutrophils was verified by immunohistochemical staining using with rat anti-Ly-6 antibody (αLy6). Representative images (original magnification 400 ×) of the (A) alveolar, (B) peribronchiolar, and (C) perivascular compartments are shown. Black arrows highlight positive neutrophil staining.

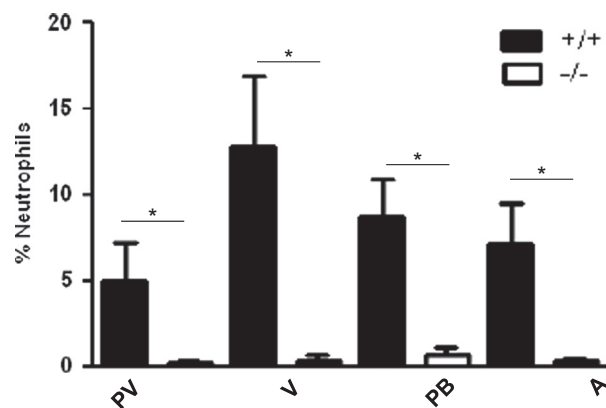


Fig. 6. Neutrophilic inflammation was similarly attenuated in all lung compartments from *Duoxa*^{-/-} mice. Paraffin-embedded lung sections were obtained from ovalbumin-sensitized *Duoxa*^{+/+} (+/+) and *Duoxa*^{-/-} (-/-) male mice after 2 weeks of aerosolized ovalbumin exposure. Lung sections were stained with HE to determine the percentage of neutrophils in each of four lung compartments (see Materials and methods). Leukocyte populations in 10 high-powered fields for each compartment were counted and neutrophil percentage was determined by dividing the total neutrophils counted by the total number of leukocytes. Data are shown as means ± SEM for the perivascular (PV), vascular (V), peribronchiolar (PB), and alveolar (A) compartments, **p* < 0.05.

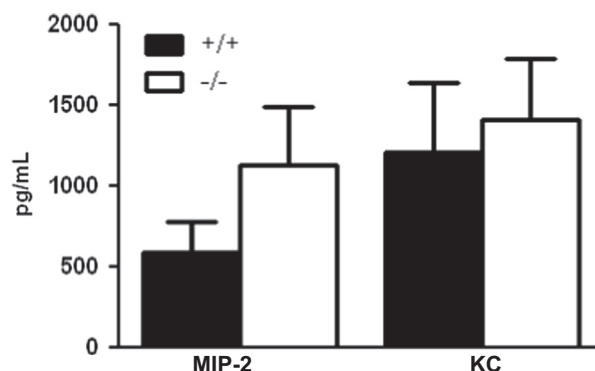


Fig. 7. DUOX does not influence expression of IL-8 homologs after ovalbumin exposure. BALF was obtained from ovalbumin-sensitized *Duoxa*^{+/+} (+/+) and *Duoxa*^{-/-} (-/-) mice immediately after 2 weeks of aerosolized ovalbumin exposure. ELISA was used to determine the levels of MIP-2 and KC in BALF supernatant. Data represent means ± SEM from six animals in each group.

lower levels of MIP-2 after nasal flagellin exposure [19]. Given the substantial differences between the two models, it is difficult to speculate on the reason for the discordance between the two studies. However, it will be important to challenge our model with flagellin or bacteria to see what differential effects our double-DUOX-knockout model may exhibit.

Given that IL-8 homologs were not required for DUOX-mediated neutrophil recruitment, we utilized multiplex analyses to analyze alternative signaling pathways that potentially were responsible for the differences we characterized. We observed decreased levels of IL-6 and G-CSF, cytokines increased by activated neutrophils, in the *Duoxa*^{-/-} mice. Because both cytokines are produced by activated neutrophils, lower levels of both cytokines in *Duoxa*^{-/-} mice may simply confirm our histologic findings. However, IL-6 is also known to recruit and activate neutrophils as well [51]. Based on the lower level of neutrophils in the vascular compartment of *Duoxa*^{-/-} mice, we speculate that DUOX-mediated hydrogen peroxide or hydrogen peroxide-derived metabolites from the epithelial compartment activate an IL-6-dependent pathway necessary for endothelial activation and retention of neutrophils. Further study with these mice will

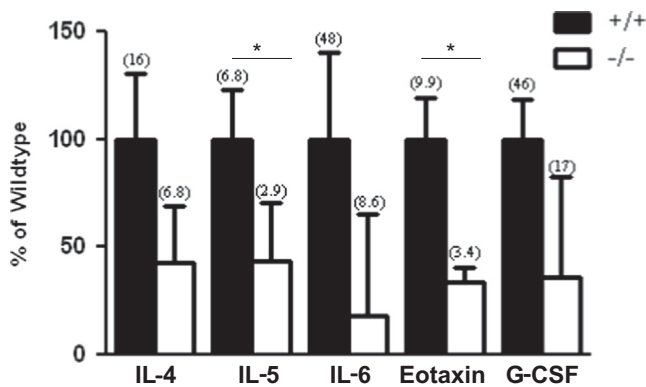


Fig. 8. T_H2 cytokine levels are reduced in *Duoxa*^{-/-} mice. Multiplex analyses were used to determine cytokine levels in BALF supernatant from ovalbumin-exposed *Duoxa*^{+/+} (+/+) and *Duoxa*^{-/-} (-/-) mice. For each cytokine, cytokine levels from *Duoxa*^{-/-} mice are shown as a percentage of wild-type levels. Data represent means ± SEM from six animals in each group. Mean cytokine levels in pg/ml from each group are shown in parentheses. **p* < 0.05.

determine if this postulate is correct and the mechanisms by which this occurs.

In addition, our results confirm mounting evidence that DUOX is essential for signaling events in response to allergen or infection. Ryu et al. [18] found that DUOX2 differentially mediated β-glucan-induced TLR2 signaling in the nose and lipopolysaccharide-induced TLR4 signaling in the lungs of knockout mice. Flagellin-mediated activation of TLR5 induced mucus hypersecretion and IL-8 production occurs through a DUOX2-mediated signaling cascade in vitro [19,20]. And, DUOX2-mediated signaling is important for TLR3-mediated soluble TNFR1 shedding in vitro [20]. Similarly, DUOX1 seems to be important for the induction of IL-8 via multiple TLR ligands [21] in vitro, and both DUOX1 and DUOX2 are upregulated in polyp tissue of patients with chronic rhinosinusitis [52]. Because we used a *Duoxa*^{-/-} knockout model, we are unable to determine which DUOX is predominantly essential for the development of asthma features versus neutrophil influx, but based on previous data both seem to be important for various components of lung immunity. Given the differential regulation of both DUOX1 and DUOX2 [26], it will be essential to determine the contribution of each isoform to lung immunity and response to allergen.

Based on our data, it is highly plausible that DUOX activity could worsen asthma symptoms or be partially responsible for severe asthma phenotypes characterized by neutrophilic inflammation. The driving forces behind neutrophil infiltration in an asthmatic lung have not yet been elucidated [53,54], and it is often associated with more severe forms of asthma, which are less responsive to standard therapeutic strategies [55,56]. We postulate that further characterization of the mechanisms behind DUOX-mediated neutrophil recruitment after allergen challenge will lead to a deeper understanding of this important clinical problem.

Conclusion

Our study suggests that DUOX has an important role in modulating several features of allergic asthma including airway hyperactivity and mucus metaplasia independent of eosinophilic inflammation. Also, DUOX is necessary to sufficiently target neutrophils into the airways in response to allergic challenge. Based on the known function of DUOX enzymes, it is likely that DUOX-derived hydrogen peroxide is a part of the important signaling events leading to allergy-initiated inflammation. Specifically, DUOX may be an important target for certain subsets of

asthma patients that mechanistically are unique from most allergic asthma patients. It is possible that DUOX activity contributes to the mechanism behind neutrophil infiltration in certain forms of asthma and future study may lead to novel therapeutic strategies for this patient population.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.freeradbiomed.2013.06.012>.

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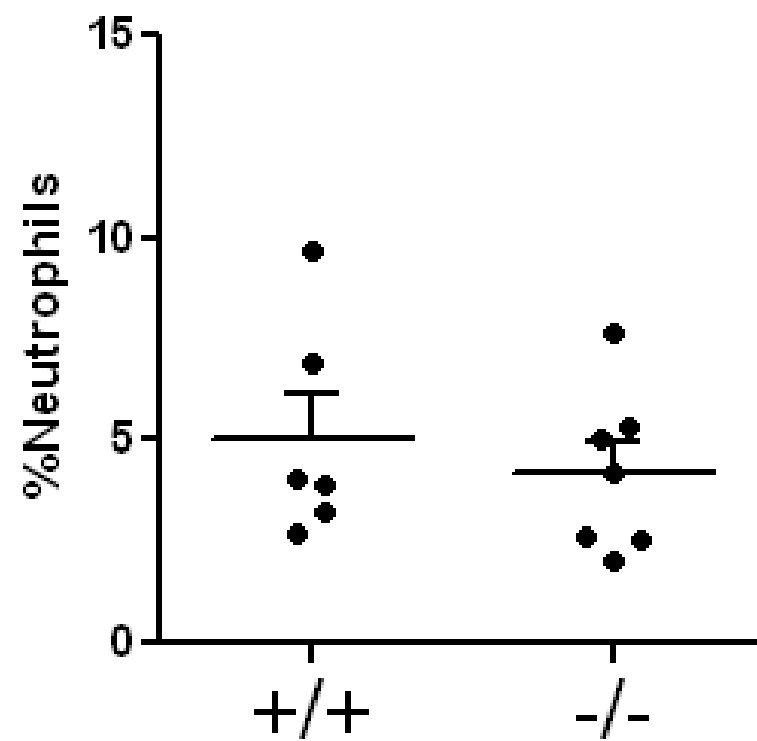


Figure S1

Cytokine	+/+ OVA	+/+ air	-/- OVA	-/- air
Eotaxin	9.91 ± 1.82	<LD	3.37 ± .67	.64 ± .39
G-CSF	46.02 ± 13.16	<LD	16.76 ± 8.06	1.38 ± 1.10
IL-13	1.92 ± 1.03	<LD	<LD	<LD
IL-10	1.00 ± .42	<LD	.46 ± .40	<LD
IL-1alpha	1.61 ± .25	1.16 ± .11	1.66 ± .30	<LD
LIX	6.19 ± 3.81	<LD	9.81 ± 6.22	<LD
IL-5	6.77 ± 1.52	<LD	2.93 ± .84	2.16 ± 2.04
IL-6	48.14 ± 18.68	<LD	8.62 ± 4.25	0.30 ± 0.10
IL-2	.027 ± .0020	<LD	.051 ± .021	<LD
MCP-1	1.93 ± 0.578	<LD	1.92 ± 0.92	0.69 ± 0.16
IL-4	15.95 ± 4.91	.0043 ± 0.0043	6.79 ± 1.78	0.050 ± 0.35
MIP-1alpha	34.66 ± 11.28	1.25 ± 0.24	29.26 ± 7.11	2.70 ± 1.19
VEGF	4.04 ± 0.53	3.75 ± 0.33	3.51 ± 0.43	3.22 ± 0.31
TNF-alpha	53.71 ± 25.37	<LD	55.04 ± 21.36	.051 ± .026
RANTES	0.18 ± .069	.0058 ± .00083	.12 ± .051	.01 ± .0041
MIP-1beta	63.02 ± 29.84	1.31 ± .19	57.45 ± 19.53	1.64 ± .52
IL-12	<LD	<LD	<LD	<LD
GM-CSF	<LD	<LD	<LD	<LD
IL-17	<LD	<LD	<LD	<LD
IFNgamma	<LD	<LD	<LD	<LD
IL-15	<LD	<LD	<LD	<LD
IL-1beta	<LD	<LD	<LD	<LD
•KC	1208.44±435.54	9.69±3.26	1412.14±381.00	-17.69±30.58
•MIP-2	594.53±191.75	-1.48±.077	1127.57 ±368.80	.90±1.62

Table S1