

# *P. aeruginosa* Lipopolysaccharide-Induced MUC5AC and CLCA3 Expression Is Partly through Duox1 *In Vitro* and *In Vivo*

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## Abstract

**Background:** We have previously found that reactive oxygen species (ROS) are involved in *Pseudomonas aeruginosa* lipopolysaccharide (*PA-LPS*) induced MUC5AC in airway epithelial cells. Dual oxidase1 (Duox1), a member of NADPH oxidase(Nox), is known to be responsible for ROS production in respiratory tract epithelial cells. Our aim was to clarify whether Duox1 was also involved in the *PA-LPS*-induced MUC5AC and calcium dependent chloride channel 3(Clca3), another recognized marker of goblet cell hyperplasia and mucus hyper-production.

**Methods:** *PA-LPS*-induced Duox1 mRNA levels were examined in A549 cells, primary mouse tracheal epithelial cells (mTECs) and lung tissues of mice. Nox inhibitors diphenyleneiodonium chloride (DPI) and Duox1 siRNA were used to investigate whether Duox1 is involved in *PA-LPS*-induced MUC5AC and Clca3 expression both in vitro and in vivo.

**Results:** Duox1 is induced by *PA-LPS* in A549 cells, primary mTECs and lung tissues of mice. DPI significantly inhibited *PA-LPS*-induced up-regulation of Duox1, Muc5ac and Clca3 in primary mouse trachea epithelial cells and lung tissues of mice. Knockdown of Duox1 markedly inhibited *PA-LPS*-induced MUC5AC expression via a ROS-TGF- $\alpha$  cascade in A549 cells. Furthermore, DPI significantly inhibited *PA-LPS*-induced increases in inflammatory cells accumulated in mouse lungs.

**Conclusions:** We demonstrate for the first time that *PA-LPS*-induced MUC5AC and Clca3 expression is partly through Duox1, and provide supportive evidence for Duox1 as a potential target in treatments of mucin over-production diseases.

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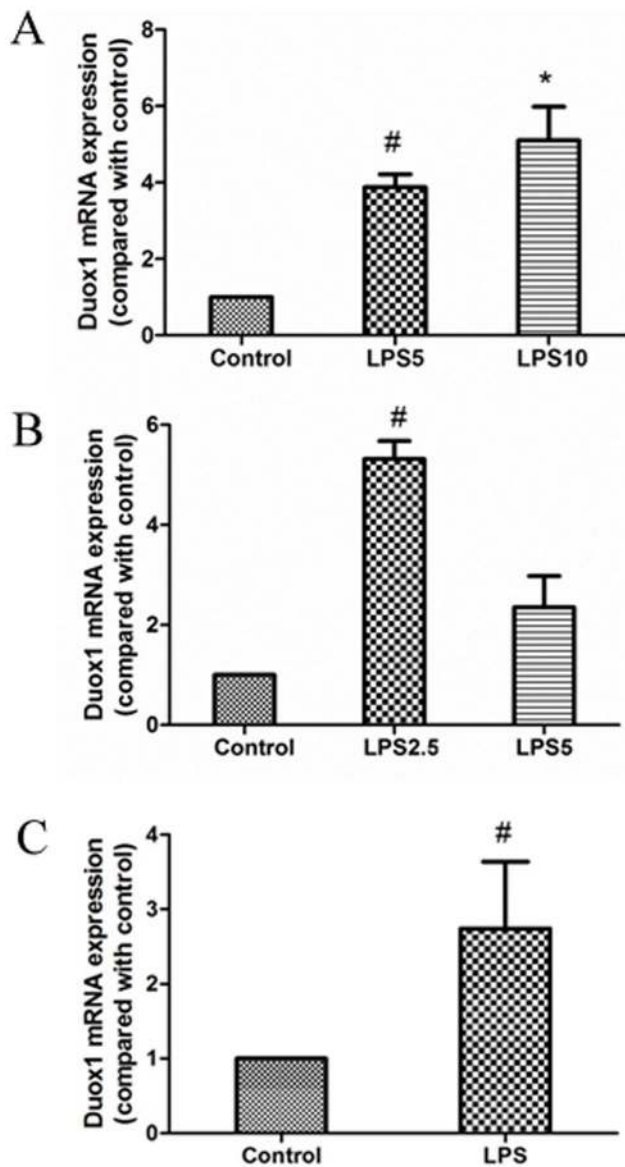
9 These authors contributed equally to this work.

## Introduction

Mucus hypersecretion is commonly observed in chronic inflammatory airway diseases such as asthma, chronic obstructive pulmonary disease, and cystic fibrosis. Excessive production of mucus contributes to morbidity and mortality in these diseases by plugging the airways and causing recurrent infections [1,2]. MUC5AC mucin is the major component of airway mucus [3,4]. A number of in vitro and in vivo studies have been carried out to explore the signaling mechanisms underlying the regulation of MUC5AC expression induced by many different stimuli [5,6,7,8,9,10]. *Pseudomonas aeruginosa* (*PA*) infection is common in chronic inflammatory airway diseases [11], especially in cystic fibrosis. It has been previously shown that *Pseudomonas aeruginosa* lipopolysaccharide (*PA-LPS*) significantly up-regulates MUC5AC

mucin expression in airway epithelial cells [12,13,14]. However, the underlying molecular mechanisms remain largely unknown.

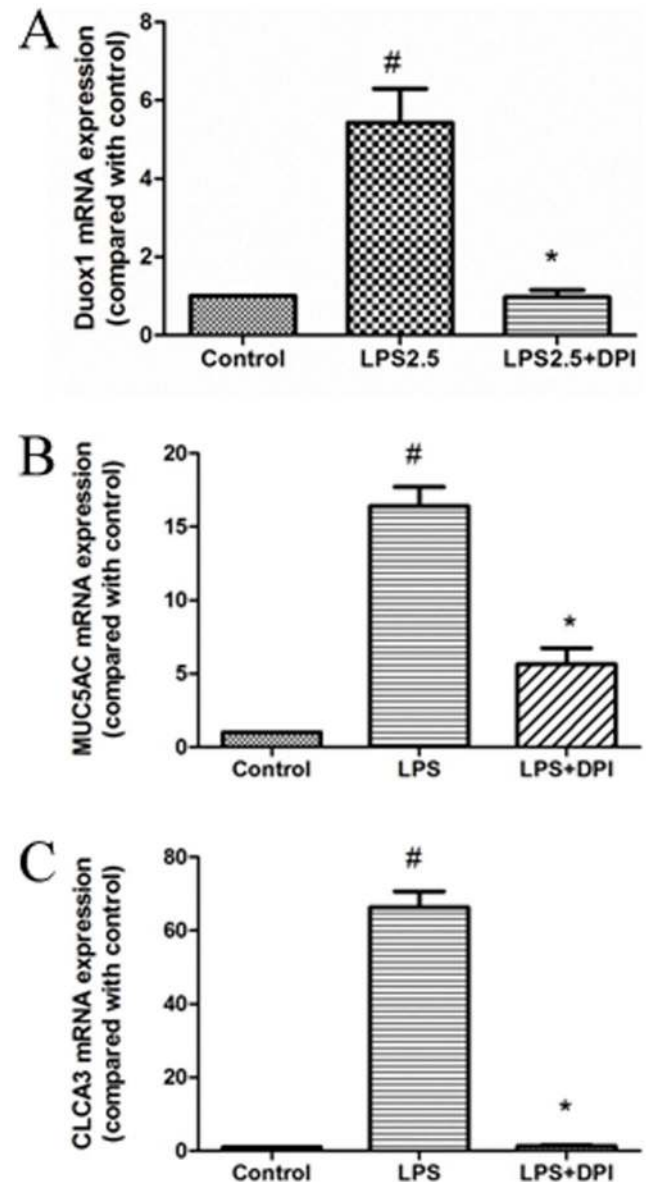
Reactive oxygen species (ROS) have been found to play important roles in cigarette smoke, neutrophil elastase and phorbol 12-myristate 13-acetate (PMA) induced MUC5AC mucin expression [15,16,17]. In addition, we have previously shown that ROS are involved in *PA-LPS* induced MUC5AC production [18]. Dual oxidases (Duox), members of the NADPH oxidase (Nox) family, originally identified and cloned from the epithelium of the thyroid gland [19,20,21], were initially found to be responsible for ROS production, which is involved in the anti-microbial activity of lactoperoxidase in respiratory tract epithelial (TBE) cells [22]. The two Duox isoforms Duox1 and Duox2 have high structural similarity. While they were differentially regulated by a variety of



**Figure 1. PA-LPS induced Duox1 expression in A549 cells, primary mouse trachea epithelial cells and in mouse lungs.** A: PA-LPS(5  $\mu$ g/mL and 10  $\mu$ g/mL) significantly up-regulated Duox1 mRNA expression in A549 cells; B: PA-LPS(2.5  $\mu$ g/mL) significantly up-regulated Duox1 mRNA expression in primary mTECs; C: PA-LPS (100  $\mu$ g) significantly up-regulated Duox1 mRNA expression in lung tissues of mice; # $P$ <0.05 compared with control, \* $P$ <0.05 compared with control. doi:10.1371/journal.pone.0063945.g001

proinflammatory factors, Duox1 is induced by Th2 cytokines IL-4 and IL-13, whereas Duox2 is induced by Th1 cytokines IFN- $\gamma$  and poly(I:C) [23]. In addition, Duox1 and Duox2 have distinct functions in airway epithelium. Duox2 is mainly involved in inflammation, whereas Duox1 is mainly responsible for mucus production [17]. Nadel et al [17] have recently reported that Duox1 mediated neutrophil elastase- and PMA-induced MUC5AC mucin expression in airway epithelial cells. However, it is still unclear if Duox1 could also be involved in LPS-induced MUC5AC mucin expression. We hypothesized that Duox1 may also mediate PA-LPS-induced MUC5AC expression via controlling ROS production in airway. Based on our previous finding that the ROS-TGF- $\alpha$  signaling

pathways mediate PA-LPS-induced MUC5AC expression in NCI-H292 cells [18], we explored whether Duox1 controls the production of ROS and TGF- $\alpha$  in airway epithelial cells. To further confirm the specific role of Duox1 in mucins regulation, we also investigated whether knockdown of Duox2 can influence MUC5AC expression in A549 cells. Furthermore, because we previously showed that ROS scavenger dimethylthiourea (DMTU) inhibited PA-LPS-induced MUC5AC expression in vitro, we sought to further investigate whether DMTU also plays a similar role in vivo.



**Figure 2. Suppression of PA-LPS-induced Duox1, Muc5ac and Clca3 expression by DPI in primary mouse trachea epithelial cells.** A: Diphenylene iodonium(2.5  $\mu$ M) significantly inhibited PA-LPS-induced Duox1 mRNA expression in primary mTECs. B: Diphenylene iodonium(2.5  $\mu$ M) significantly inhibited PA-LPS-induced Muc5ac mRNA expression in primary mTECs. C: Diphenylene iodonium(2.5  $\mu$ M) significantly inhibited PA-LPS-induced Clca3 mRNA expression in primary mTECs. # $P$ <0.05 compared with control, \* $P$ <0.05 compared with PA-LPS. doi:10.1371/journal.pone.0063945.g002

Calcium dependent chloride channels (CLCA) of airway epithelial cells play an important role in the regulation of mucus production [24,25]. The expression of human CLCA1 (hCLCA1) is increased in patients with asthma [26] and COPD [27] in which mucus was excessively produced in the airway. Similarly, the expression of calcium dependent chloride channel 3 (Clca3), the mouse homolog of hCLCA1, markedly increased and was closely correlated with up-regulation of MUC5AC during mucus overproduction in the airway of mice [28,29], and we thus investigated whether Clca3 can be also induced by *PA-LPS*, and regulated by duox1 during *PA-LPS* induced MUC5AC expression. Furthermore, because *PA-LPS*-induced MUC5AC production is often accompanied by the increased airway inflammation, we investigated whether Duox1 may also regulate the number of total inflammatory cells and neutrophils in BALF of mice treated with *PA-LPS*.

To test our hypothesis, we investigated whether Duox1 is induced by *PA-LPS* in A549 cells, primary mouse tracheal epithelial cells (mTECS) and lung tissues of mice respectively, and Nox inhibitors diphenyleiiodonium chloride (DPI) inhibits *PA-LPS*-induced Duox1, MUC5AC and Clca3 expression in vitro and in vivo. The roles of Duox1 and Duox2 in *PA-LPS*-induced MUC5AC expression were also further verified by knockdown of Duox1 and Duox2 in A549 cells using small interfering RNA (siRNA). In the present study, we demonstrated for the first time that Duox1 is partly involved in *PA-LPS*-induced MUC5AC and Clca3 expression.

## Materials and Methods

### Materials

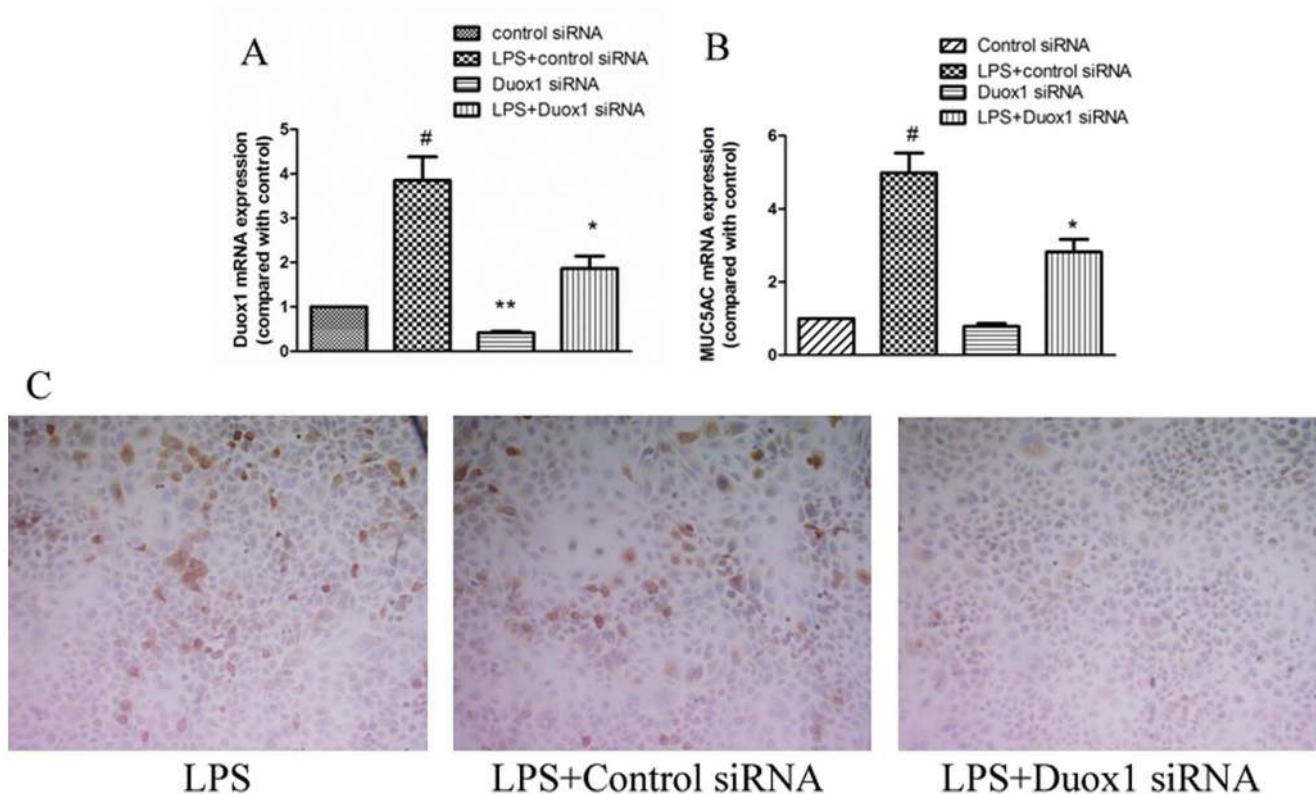
A549 cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA). Diphenyleiiodonium chloride (DPI) and dimethylthiourea (DMTU) were from Calbiochem. *PA-LPS* from serotype 10 was from Sigma.

### Animals

8–10 week-old C57BL/6 mice were purchased from the Experimental Animal Center of Zhejiang University, animal protocols and procedures were approved by the Ethical Committee for Animal Studies at Zhejiang University, China. Mice were once administered *PA-LPS* (100  $\mu\text{g}/50 \mu\text{L}$ ) by tracheal cannula. After 30 minutes of *PA-LPS* administration, mice were injected (intraperitoneal injection, i.p) with DPI (1 mg/kg) or DMTU (1 mg per mice) once a day, and sacrificed after 6 days. The BALF of mice was collected to count numbers of inflammatory cells. The lungs were fixed in 10% buffered formalin and stained with Alcian blue/periodic acid-Schiff (AB/PAS) and Hematoxylin & Eosin (H&E) staining.

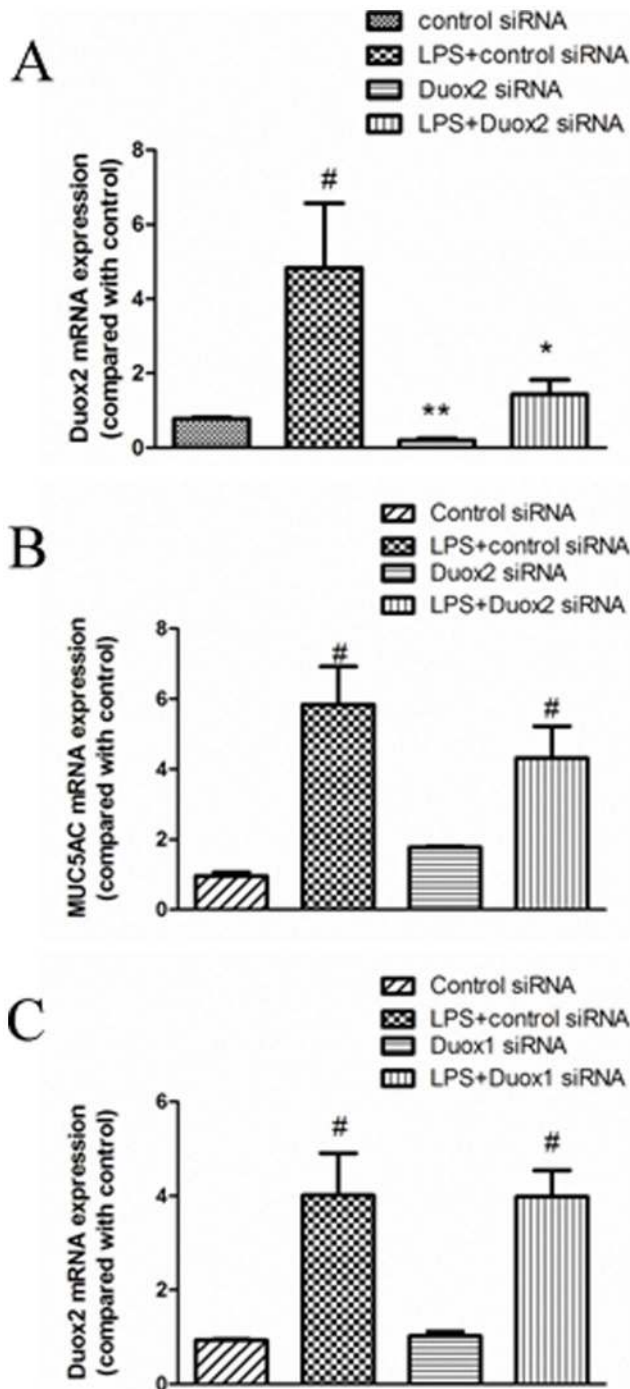
### Primary Mouse Tracheal Epithelial Cells Culture

Mouse trachea was isolated from C57BL/6 mice under sterile conditions, and digested with 10 mL 0.15% Pronase solution overnight at 4°C. Then tracheal epithelial cells were harvested and submerged-cultured with mTECS proliferation medium (DMEM/F12 basic media add HEPES, glutamine solution, NaHCO<sub>3</sub>, heat-inactivated FBS, Retinoic acid, Insulin, Epidermal growth factor



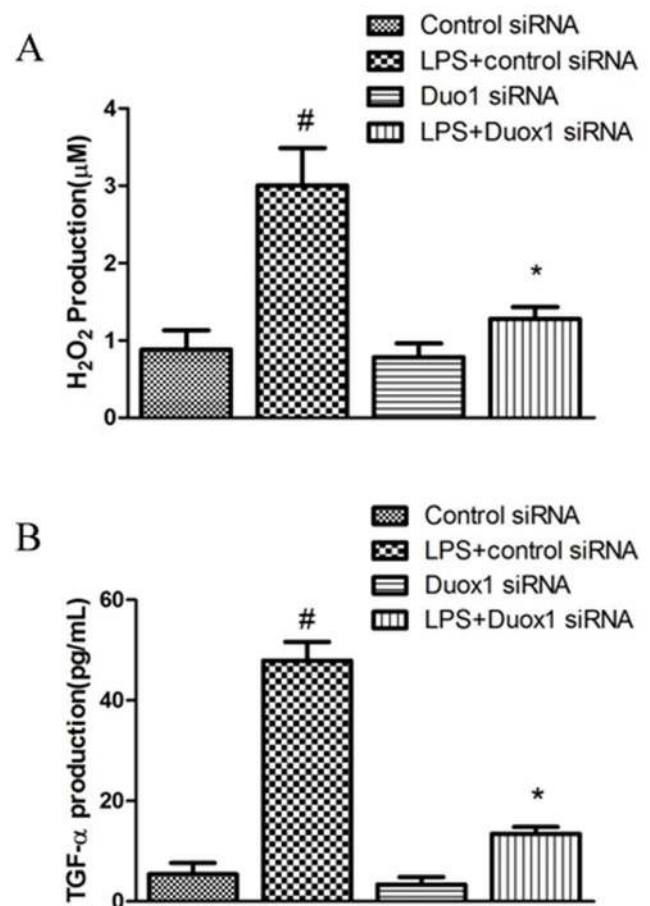
**Figure 3. Duox1 small interfering RNA inhibited *PA-LPS*-induced MUC5AC expression in A549 cells.** A: Duox1 siRNA(100 nM) significantly inhibited Duox1 mRNA expression in normal as well as *PA-LPS*-stimulated(10  $\mu\text{g}/\text{mL}$ ) A549 cells. B: Duox1 siRNA(100 nM) significantly inhibited *PA-LPS*-induced MUC5AC mRNA expression in A549 cells; C: Duox1 siRNA(100 nM) significantly inhibited *PA-LPS*-induced MUC5AC protein expression in A549 cells ( $\times 200$ ). # $P < 0.05$  compared with control siRNA. \* $P < 0.05$  compared with *PA-LPS*+control siRNA. \*\* $P < 0.05$  compared with control siRNA. doi:10.1371/journal.pone.0063945.g003





**Figure 4. Role of Duox2 small interfering RNA in PA-LPS-induced MUC5AC expression in A549 cells.** A: Duox2 siRNA(100 nM) significantly inhibited Duox2 mRNA expression in normal as well as PA-LPS-stimulated(10 µg/mL) A549 cells. B: Duox2 knockdown exerted no considerable effects on PA-LPS -incuded MUC5AC mRNA expression in A549 cells; C: Duox1 knockdown has no effects on Duox2 expression in normal as well as PA-LPS-stimulated(10 µg/mL) A549 cells. #*P*<0.05 compared with control siRNA. \**P*<0.05 compared with PA-LPS+control siRNA. \*\**P*<0.05 compared with control siRNA. doi:10.1371/journal.pone.0063945.g004

solution, bovine pituitary extract, Transferrin) in transwell plates(Corning, NY) for 10–14 days. When cells were confluent, the medium in apical side was removed and air-liquid interface



**Figure 5. Duox1 is required for PA-LPS-induced ROS and TGF-α production in A549 cells.** A: Duox1 siRNA(100 nM) significantly inhibited PA-LPS-incuded H<sub>2</sub>O<sub>2</sub> production in A549 cells. B: Duox1 siRNA(100 nM) significantly inhibited PA-LPS-incuded TGF-α production in A549 cells. #*P*<0.05 compared with control siRNA. \**P*<0.01 compared with PA-LPS+control siRNA. doi:10.1371/journal.pone.0063945.g005

(ALI) culture began. After 1-week of ALI culture, cells were stimulated by adding PA-LPS (2.5 µg/mL) and DPI (5 µM) in the basal wells for 24 hours.

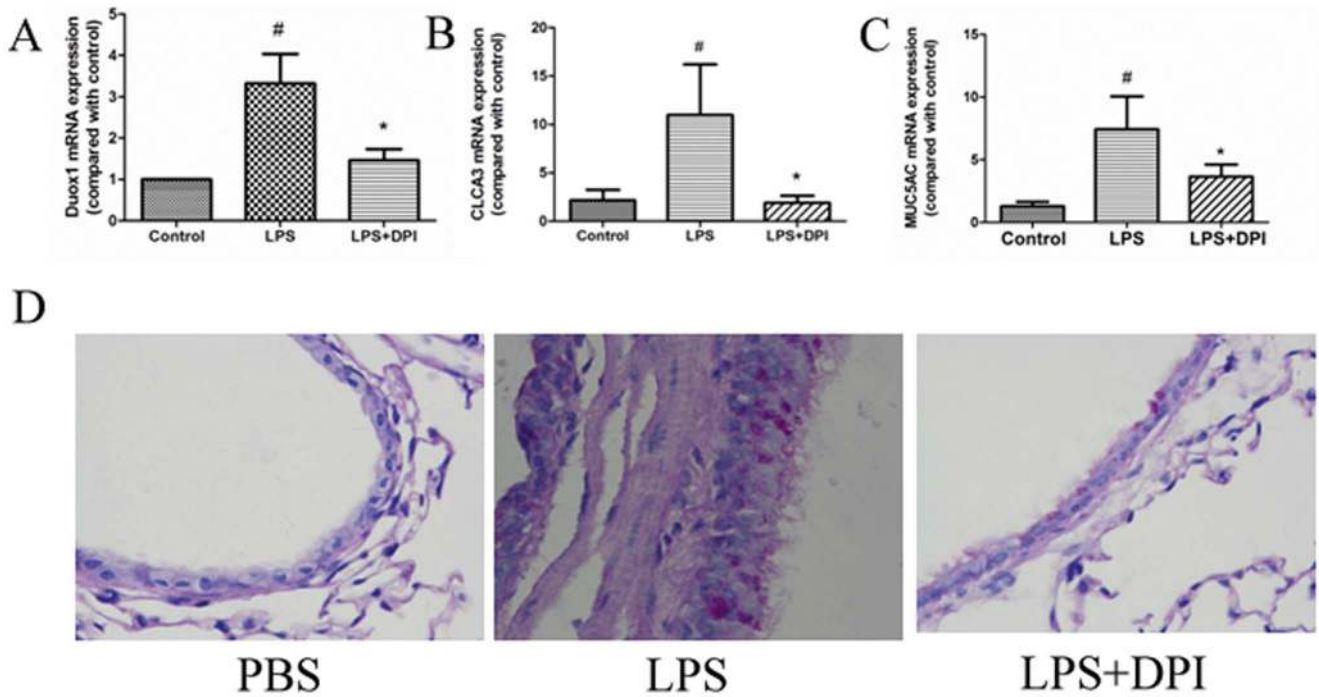
#### A549 Cells Culture

A549 cells were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) fetal bovine serum. Before experiments, confluent A549 cells were serum-starved for 24 h to maintain low basal levels of MUC5AC expression.

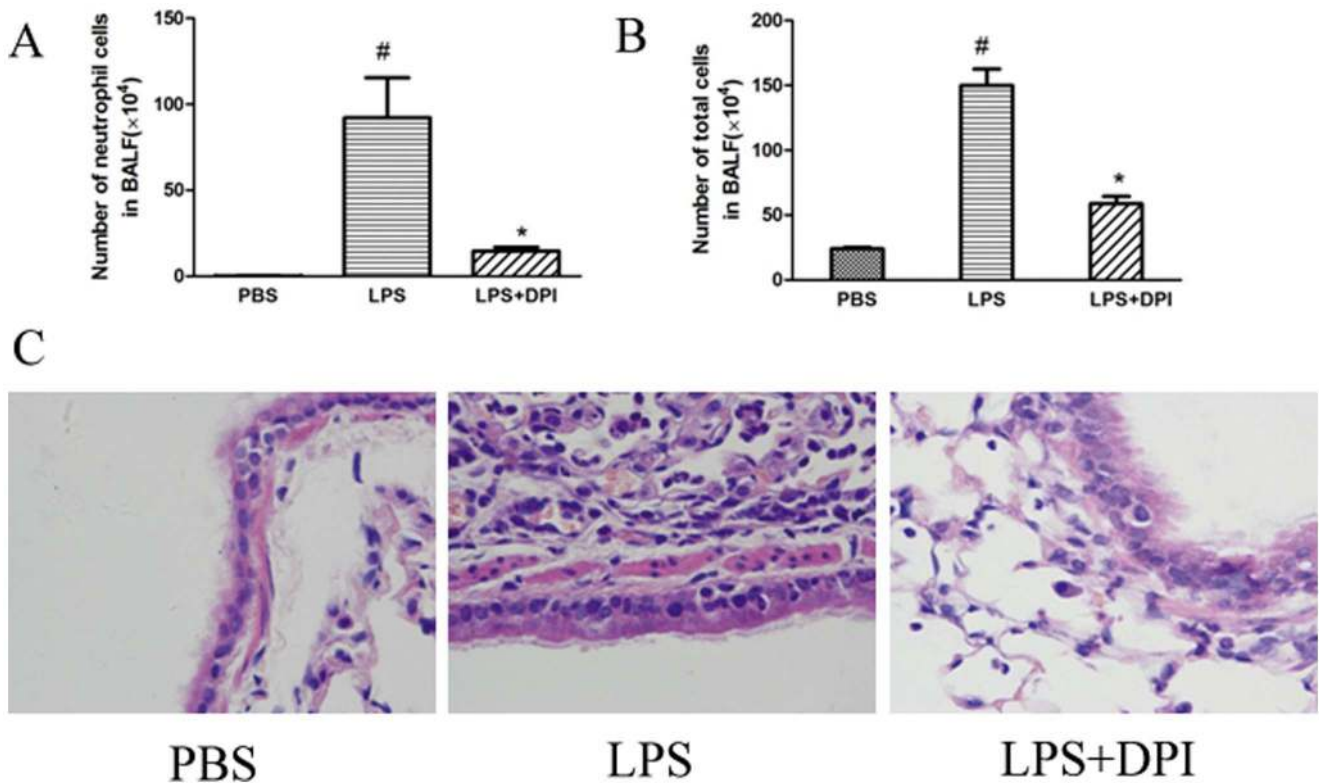
#### Real-time PCR

Total RNA was isolated from cells and mice lung tissues using TRIzol Reagent (Invitrogen) according to the manufacturer's instruction. For RT-PCR, cDNA was generated by reverse transcription using 2 µg total RNA. The expression levels of mClca3 and MUC5AC mRNA were determined by quantitative real-time PCR using the SYBR Green system (Takara) on a spectrofluorometric thermal cycler (iCycler; Bio-Rad). The PCR primers are as follows: human MUC5AC: forward: GGACTTCAATATCCAGCTACGC, reverse: CAGCTCAACAAC-TAGGCCATC; mouse Muc5ac: forward: GGACTTCAA-TATCCAGCTACGC, reverse: GGACTTCAATATCCAGCTACGC; human Duox1: forward:





**Figure 7. Effects of DPI on PA-LPS-induced Duox1, Muc5ac and Clca3 production in vivo.** A–C: DPI (1 mg/kg) blocked PA-LPS-induced Duox1, Clca3 and Muc5ac mRNA expression in the lung of mice; D: DPI (1 mg/kg) inhibited PA-LPS-induced mucin production in the lung of mice (PAS  $\times 400$ ). # $P < 0.05$  compared with control, \* $P < 0.01$  compared with PA-LPS. doi:10.1371/journal.pone.0063945.g007



**Figure 8. DPI reduced PA-LPS-induced inflammatory cells in BALF and lung tissues of mice.** A–B: DPI (1 mg/kg) significantly inhibited PA-LPS-induced neutrophils and total cells in BALF of mice. C: DPI (1 mg/kg) significantly reduced PA-LPS-induced inflammatory cells in lung tissues of mice (H&E  $\times 400$ ). # $P < 0.05$  compared with PBS, \* $P < 0.01$  compared with PA-LPS. doi:10.1371/journal.pone.0063945.g008



## Results

### *PA-LPS* Induced Duox1 Expression in Primary Mouse Trachea Epithelial Cells and in Mouse Lungs

As an initial approach to explore the possible role of Duox1 in mediating *PA-LPS* induced mucus production, the induction of Duox1 by *PA-LPS* in A549 cells, mTECs and mouse lung tissues was analyzed. As expected, *Duox1* mRNA was markedly increased in A549 cells, mTECs and mouse lungs treated with *PA-LPS* (Figures 1A–1C), though there was no dose-dependent effect for such an induction. These data suggested that Duox1 might positively regulate the mucus production in context of *PA-LPS* treatment.

### Suppression of *PA-LPS*-induced Muc5ac and mClca3 Expression by DPI through Decreasing the Expression of Duox1 in Primary Mouse Trachea Epithelial Cells

To clarify the role of Duox1 in *PA-LPS*-induced mucus production, we utilized an NADPH oxidase inhibitor DPI which is known to inhibit the function of Duox1 [17], as genetic approaches are difficult to be used in primary epithelial cultures. As shown in Figures 2A–2C, DPI (2.5  $\mu$ M) significantly inhibited the *PA-LPS* (2.5  $\mu$ g/mL) induced mRNA transcripts of Duox1, and also reduced the expression of Muc5ac and Clca3 mRNA, both of which are well recognized markers of goblet cell hyperplasia and mucus hyper-production. These results showed that DPI inhibited *PA-LPS* induced-Muc5ac and Clca3 expression partly through decreasing the expression of Duox1 in primary mTECs.

### Duox1 Small Interfering RNA Inhibited *PA-LPS*-induced MUC5AC Expression in A549 Cells

To further confirm whether Duox1 is required for *PA-LPS*-induced MUC5AC expression in airway epithelial cells, we examined the effect of Duox1 knockdown on *PA-LPS*-induced MUC5AC expression in A549 cells by real-time PCR and immunohistochemistry. As shown in Figure 3A, Duox1 siRNA (100 nM) significantly inhibited Duox1 mRNA expression in A549 cells, and knockdown of Duox1 (100 nM Duox1 siRNA) significantly reduced *PA-LPS*-induced expression of MUC5AC mRNA in A549 cells (decreased by 44% compared with control,  $P < 0.05$ ) (Figure 3B). Furthermore, using mouse monoclonal antibody to MUC5AC (clone 45 M1, 1:100), we confirmed that 100 nM Duox1 siRNA markedly inhibited MUC5AC protein expression in A549 cells (Figure 3C). These results suggested that *PA-LPS*-induced MUC5AC expression was Duox1-dependent in airway epithelial cells.

### Duox2 Small Interfering RNA Exerted no Considerable Effects on *PA-LPS*-induced MUC5AC expression in A549 Cells

To investigate whether Duox2 is also involved *PA-LPS*-induced MUC5AC expression, we transfected Duox2 siRNA into A549 cells and showed that Duox2 siRNA could not significantly inhibit *PA-LPS*-induced MUC5AC expression (Figure 4A–4B). Furthermore, we showed that Duox1 siRNA transfection had no obvious effects on Duox2 expression (Figure 4C). These data supported that Duox2 function is different from Duox1 in airway epithelial cells, especially in term of mucin regulation.

### Duox1 is Required for *PA-LPS*-induced ROS and TGF- $\alpha$ Production in A549 Cells

On the basis of our previous finding showing the involvement of the ROS-TGF- $\alpha$  cascade in *PA-LPS*-induced MUC5AC expression [18], we further investigated whether Duox1 mediates the production of ROS and TGF- $\alpha$ . As shown in Figure 5A and 5B, 100 nM Duox1 siRNA significantly reduced *PA-LPS*-induced ROS and TGF- $\alpha$  production in A549 cells. Taken together, these data demonstrated that Duox1 is involved *PA-LPS*-induced MUC5AC expression via a ROS-TGF- $\alpha$ -dependent mechanism.

### Effects of DMTU and DPI on *PA-LPS*-induced Muc5ac and mClca3 Production *in vivo*

We have demonstrated both ROS and Duox1 play important roles in *PA-LPS*-induced MUC5AC expression *in vitro*, whether same effects could exist *in vivo* have yet to be addressed. We previously showed that ROS scavenger DMTU significantly decreased *PA-LPS*-induced MUC5AC production in NCI-H292 cells [18]. Here we further showed that DMTU (1 mg per mouse) significantly blocked *PA-LPS*-induced Muc5ac mRNA expression in the lung tissues of mice (Figure 6A), and also inhibited mucin over-production as demonstrated by Alcian blue/periodic acid-Schiff (AB/PAS) staining (Figure 6B). As shown in figure 2A–2C, DPI significantly suppressed *PA-LPS*-induced Duox1, Muc5ac and mClca3 expression in mTECs. Similarly, DPI (1 mg/kg) also inhibited *PA-LPS*-induced Duox1, Clca3 and Muc5ac mRNA, and Muc5ac mucin production in mouse lung tissues (Figure 7A–7D). These data demonstrated that Duox1 is partly involved in *PA-LPS*-induced up-regulation of Muc5ac and Clca3 *in vivo*.

### DPI Reduced *PA-LPS*-induced Inflammatory Cells in BALF and Lung Tissues of Mice

*PA-LPS*-induced airway mucin over-production is usually accompanied by an increase in inflammatory cells, especially neutrophils. To determine the effects of Duox1 on *PA-LPS*-induced inflammatory cells in lung of mice, we further examined neutrophils and total inflammatory cells in BALF and lung tissues of mice treated by *PA-LPS*. As shown in figure 8A and 8B, DPI significantly reduced *PA-LPS*-induced neutrophils and total cells in BALF of mice, and also reduced inflammatory cells in lung tissues demonstrated by H&E staining (8C), thereby suggesting that Duox1 may be involved in the regulation of *PA-LPS*-induced airway inflammation in mice.

## Discussion

To the best of our knowledge, this is the first study to demonstrate that *PA-LPS*-induced up-regulation of MUC5AC and Clca3 is partly through Duox1 *in vitro* and *in vivo*. Here we showed Duox1 is induced by *PA-LPS* both *in vitro* and *in vivo*, and DPI (specific inhibitors of Nox) significantly inhibited *PA-LPS*-induced up-regulation of MUC5AC and Clca3 partly through decreasing Duox1 expression in mTECs and lung tissues of mice. In addition, we also confirmed that knockdown of Duox1 markedly inhibited *PA-LPS*-induced MUC5AC expression via a ROS-TGF- $\alpha$  cascade in A549 cells. Furthermore, we demonstrated that DPI significantly inhibited *PA-LPS*-induced increase in total inflammatory cells and neutrophils accumulated in BALF and lung tissues of mice. On the basis of our previous data that ROS scavengers DMTU reduced *PA-LPS*-induced MUC5AC production in NCI-H292 cells, we further confirmed that DMTU significantly inhibited *PA-LPS*-induced Muc5ac *in vivo*.

Initially identified and cloned from the thyroid gland, Duox are known to be expressed on the surface of ciliated airway epithelial cells [30], and Duox1 is specifically expressed in large airways [31], and its expression is 5-fold higher than that of Duox2 in normal airway epithelium [23]. Harper et al [23] found Duox1 mRNA was specially but moderately increased (by approximately four fold) by Th2 cytokines IL-4 and IL-13. Boots et al [32] showed LPS (10  $\mu\text{g}/\text{mL}$ ) significantly increased Duox1 mRNA expression in immortalized human bronchial epithelial (HBE1) cells. In this study we showed Duox1 mRNA was up-regulated by approximately 5-fold in vitro and 2.5-fold in vivo by *PA-LPS*. Interestingly Rada B et al [33] showed *pyocyanin* produced by *Pseudomonas aeruginosa* inhibited Duox1 activation induced by Th2 cytokines in primary normal human bronchial cells and NCI-H292 cells, suggesting the regulation of Duox1 is different in various microenvironments. Rigutto et al demonstrated activation of Duox1 is  $\text{Ca}^{2+}$  dependent [34], and whether *PA-LPS* also activates Duox1 via  $\text{Ca}^{2+}$  signaling needs to be further investigated.

Human Duox1 and Duox2 are highly similar trans-membrane proteins, while they have distinct function in airway epithelium. Duox2 is mainly involved in responses to infection and inflammation, whereas Duox1 plays an important role in defense and mucus production [17]. Nadel et al [17] confirmed PMA and neutrophil elastase-induced MUC5AC expression was Duox1-dependent in vitro. In this study we found for the first time that *PA-LPS*-induced MUC5AC production is partly through Duox1, not Duox2, thus providing supportive evidence for the role of Duox1 in mucus production in airway, and supporting that Duox2 function is different from Duox1 in airway epithelial cells, especially in term of mucins regulation. It should be noted that it is the first time to demonstrate that Duox1 was involved in mucus regulation in vivo. Kim H et al [35] demonstrated that Nox4 is involved in  $\text{H}_2\text{O}_2$ -induced MUC5AC over-production in normal nasal epithelial (NHNE) cells, and independent of Duox1. Thus Duox1 may be more specific for the bronchial epithelial cells in the regulation of MUC5AC expression. On the other hand, in agreement with our previous data in vitro, we showed DMTU significantly inhibit *PA-LPS*-induced MUC5AC production in vivo, further suggesting that ROS play important roles in *PA-LPS*-induced MUC5AC expression.

Clca3 is another known marker of goblet cells in mice airway, and its expression has significantly correlated with MUC5AC. Here we found Clca3 can not only be significantly induced by *PA-LPS*, but also regulated by Duox1 in *PA-LPS*-induced Muc5ac

expression in mTECs and mice. In contrast to our data that Clca3 and MUC5AC could be both induce by *PA-LPS* and both regulated by Duox1, Thai P et al [36] showed that MUC5AC and Clca3 were differentially induced and regulated by IL-13 in primary tracheobronchial epithelial (TBE) cells. Thus the regulation of MUC5AC and Clca3 was distinct in response to different stimuli. In this study we confirmed the role of Duox1 in *PA-LPS*-induced MUC5AC expression in A549 cells by RNA interference, and demonstrated the function of Duox1 in mice by DPI. Its role has to be further investigated by using Duox1-deficient mice.

Except the role of Duox1 in mucus regulation, we additionally found DPI significantly reduced neutrophils of BALF and lung tissues in mice, which suggested Duox1 may play an important role in *PA-LPS*-induced airway inflammation. Our data is in light with some previous studies. For example, Nakanaga T et al [37] showed Duox1 mediated *PA-LPS*-induced IL-8 production in airway epithelial cells, and Kong X et al [38] showed NADPH oxidase-deficiency ( $\text{Gp}^{91\text{phox-/-}}$  mice) significantly reduced LPS-induced sepsis in  $\text{Nrf2}^{-/-}$  mice. However, Zhang WJ et al [39] demonstrated genetic deficiency of NADPH oxidase does not diminish, but rather enhances LPS-induced acute inflammatory responses in mice injected ip with 50  $\mu\text{g}$  LPS, and Gao XP et al [40] showed NADPH oxidase-deficiency ( $\text{p}^{47\text{phox-/-}}$  and  $\text{Gp}^{91\text{phox-/-}}$  mice) significantly increased neutrophils infiltration in lung tissues of mice injected ip by *E. coli* ( $2 \times 10^8$  live *E. coli*/100  $\mu\text{L}$ ). One of the possible reasons for the discrepancy between these studies may be due to different animal models and protocols that were used.

## Conclusions

In summary, we firstly demonstrated that *PA-LPS*-induced MUC5AC and Clca3 expression are partly through Duox1 in vitro and in vivo, and provide supportive evidence for Duox1 as a potential target in treatments of mucin over-production diseases.

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## Author Contributions

Conceived and designed the experiments: WL FY HS. Performed the experiments: WL FY HZ XL YW CC. Analyzed the data: WL FY ZC HS. Contributed reagents/materials/analysis tools: NZ ZC JL. Wrote the paper: WL FY HS.

## References

- Lundgren JD, Shelhamer JH (1990) Pathogenesis of airway mucus hypersecretion. *J Allergy Clin Immunol* 85: 399–417.
- Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, et al. (2004) The nature of small-airway obstruction in chronic obstructive pulmonary disease. *N Engl J Med* 350: 2645–2653.
- Hovenberg HW, Davies JR, Carlstedt I (1996) Different mucins are produced by the surface epithelium and the submucosa in human trachea: identification of MUC5AC as a major mucin from the goblet cells. *Biochem J* 318: 319–324.
- Hovenberg HW, Davies JR, Herrmann A, Lindén CJ, Carlstedt I (1996) MUC5AC, but not MUC7, is a prominent mucin in respiratory secretions. *Glycoconj J* 13: 839–847.
- Belcher CE, Drenkow J, Kehoe B, Gingeras TR, McNamara N, et al. (2000) The transcriptional responses of respiratory epithelial cells to *Bordetella pertussis* reveal host defensive and pathogen counter-defensive strategies. *Proc Natl Acad Sci USA* 97: 13847–13852.
- Chokki M, Yamamura S, Eguchi H, Masegi T, Horiuchi H, et al. (2004) Human airway trypsin-like protease increases mucin gene expression in airway epithelial cells. *Am J Respir Cell Mol Biol* 30: 470–478.
- Song KS, Lee WJ, Chung KC, Koo JS, Yang EJ, et al. (2003) Interleukin-1 beta and tumor necrosis factor-alpha induce MUC5AC overexpression through a mechanism involving ERK/p38 mitogen-activated protein kinases-MSK1-CREB activation in human airway epithelial cells. *J Biol Chem* 278: 23243–23250.
- Wang B, Lim DJ, Han J, Kim YS, Basbaum CB, et al. (2002) Novel cytoplasmic proteins of nontypeable *Haemophilus influenzae* up-regulate human MUC5AC mucin transcription via a positive p38 mitogen-activated protein kinase pathway and a negative phosphoinositide 3-kinase-Akt pathway. *J Biol Chem* 277: 949–957.
- Lora JM, Zhang DM, Liao SM, Burwell T, King AM, et al. (2005) Tumor necrosis factor-alpha triggers mucus production in airway epithelium through an I $\kappa$ B kinase beta-dependent mechanism. *J Biol Chem* 280: 36510–36517.
- Shen H, Yoshida H, Yan F, Li W, Xu F, et al. (2007) Synergistic induction of MUC5AC mucin by nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae*. *Biochem Biophys Res Commun* 365: 795–800.
- Boucher RC, Knowles MR, Yankaskas JR (2000) Cystic fibrosis. In: Murray JF, Nadel JA, editors. *Textbook of Respiratory Medicine*. Philadelphia: W.B. Saunders Co. 1291–1323.
- Shao MX, Ueki IF, Nadel JA (2003) Tumor necrosis factor alpha-converting enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 100: 11618–11623.
- Kohri K, Ueki IF, Shim JJ, Burgel PR, Oh YM, et al. (2002) *Pseudomonas aeruginosa* induces MUC5AC production via epidermal growth factor receptor. *Eur Respir J* 20: 1263–1270.



14. Li JD, Dohrman AF, Gallup M, Miyata S, Gum JR, et al. (1997) Transcriptional activation of mucin by *Pseudomonas aeruginosa* lipopolysaccharide in the pathogenesis of cystic fibrosis lung disease. *Proc Natl Acad Sci USA* 94: 967–972.
15. Shao MX, Nakanaga T, Nadel JA (2004) Cigarette smoke induces MUC5AC mucin overproduction via tumor necrosis factor- $\alpha$  converting enzyme in human airway epithelial (NCI-H292) cells. *Am J Physiol Lung Cell Mol Physiol* 287: L420–L427.
16. Shao MX, Nadel JA (2005) Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF- $\alpha$ -converting enzyme. *J Immunol* 175: 4009–4016.
17. Shao MX, Nadel JA (2005) Dual oxidase 1-dependent MUC5AC mucin expression in cultured human airway epithelial cells. *Proc Natl Acad Sci USA* 102: 767–772.
18. Yan F, Li W, Jono H, Li Q, Zhang S, et al. (2008) Reactive oxygen species regulate *Pseudomonas aeruginosa* lipopolysaccharide-induced MUC5AC mucin expression via PKC-NADPH oxidase-ROS-TGF- $\alpha$  signaling pathways in human airway epithelial cells. *Biochem Biophys Res Commun* 366: 513–519.
19. Caillou B, Dupuy C, Lacroix L, Nocera M, Talbot M, et al. (2001) Expression of reduced nicotinamide adenine dinucleotide phosphate oxidase (ThoX, LNOX, Duox) genes and proteins in human thyroid tissues. *J Clin Endocrinol Metab* 86: 3351–3358.
20. De Deken X, Wang D, Many M-C, Costagliola S, Libert F, et al. (2000) Cloning of two human thyroid cDNAs encoding new members of the NADPH oxidase family. *J Biol Chem* 275: 23227–23233.
21. Dupuy C, Ohayon R, Valent A, Noel-Hudson M-S, Deme D, et al. (1999) Purification of a novel flavoprotein involved in the thyroid NADPH oxidase. Cloning of the porcine and human cDNAs. *J Biol Chem* 274: 37265–37269.
22. Geiszt M, Witta J, Baffi J, Lekstrom K, Leto TL (2003) Dual oxidases represent novel hydrogen peroxide sources supporting mucosal surface host defense. *FASEB J* 17: 1502–1504.
23. Harper RW, Xu C, Eiserich JP, Chen Y, Kao CY, et al. (2005) Differential regulation of dual NADPH oxidases/peroxidases, Duox1 and Duox2, by Th1 and Th2 cytokines in respiratory tract epithelium. *FEBS Lett* 579: 4911–4917.
24. Knowles MR, Clarke LL, Boucher RC (1991) Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis. *N Engl J Med* 325: 533–538.
25. Groneberg DA, Eynott PR, Oates T, Lim S, Wu R, et al. (1998) The relationship of chronic mucin secretion to airway disease in normal and CFTR-deficient mice. *Am J Respir Cell Mol Biol* 19: 853–866.
26. Hoshino M, Morita S, Iwashita H, Sagiya Y, Nagi T, et al. (2002) Increased expression of the human Ca<sup>2+</sup>-activated Cl<sup>-</sup> channel 1(CaCC1) gene in the asthmatic airway. *Am J Respir Crit Care Med* 165: 1132–1136.
27. Fujimoto K, Morita S, Iwashita H, Kimura H, Gono H, et al. (2002) Gene expression of calcium-dependent chloride channel (CLCA) family in the sputum cells from COPD (abstract). *Am J Respir Crit Care Med* 165: A599.
28. Kuperman DA, Huang X, Koth LL, Chang GH, Dolganov GM, et al. (2002) Direct effects of interleukin-13 on epithelial cells cause airway hyperreactivity and mucus overproduction in asthma. *Nat Med* 8: 885–889.
29. Nakanishi A, Morita S, Iwashita H, Sagiya Y, Ashida Y, et al. (2001) Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. *Proc Natl Acad Sci USA* 98: 5175–5180.
30. Schwarzer C, Machen TE, Illek B, Fischer H (2004) NADPH oxidase-dependent acid production in airway epithelial cells. *J Biol Chem* 279: 36454–36461.
31. Fischer H (2009) Mechanism and function of DUOX in epithelia of the lung. *Antioxid Redox Signal* 11: 2453–2465.
32. Boots AW, Hristova M, Kasahara DJ, Haenen GR, Bast A, et al. (2009) ATP-mediated activation of the NADPH oxidase DUOX1 mediates airway epithelial responses to bacterial stimuli. *J Biol Chem* 284: 17858–17867.
33. Rada B, Lekstrom K, Damian S, Dupuy C, Leto TL (2008): The *Pseudomonas* toxin pyocyanin inhibits the dual oxidase-based antimicrobial system as it imposes oxidative stress on airway epithelial cells. *J Immunol* 181: 4883–4893.
34. Rigutto S, Hoste C, Grasberger H, Milenkovic M, Communi D, et al. (2009) Activation of dual oxidases (Duox1 and Duox2): Differential regulation mediated by PKA and PKC-dependent phosphorylation. *J Biol Chem* 284: 6725–6734.
35. Kim HJ, Park YD, Moon UY, Kim JH, Jeon JH, et al. (2008) The role of Nox4 in oxidative stress-induced MUC5AC overexpression in human airway epithelial cells. *Am J Respir Cell Mol Biol* 39: 598–609.
36. Thai P, Chen Y, Dolganov G, Wu R (2005) Differential regulation of MUC5AC/Muc5ac and hCLCA-1/mGob-5 expression in airway epithelium. *Am J Respir Cell Mol Biol* 33: 523–530.
37. Nakanaga T, Nadel JA, Ueki IF, Koff JL, Shao MX (2007) Regulation of interleukin-8 via an airway epithelial signaling cascade. *Am J Physiol Lung Cell Mol Physiol* 292: L1289–1296.
38. Kong X, Thimmulappa R, Kombairaju P, Biswal S (2010) NADPH oxidase-dependent reactive oxygen species mediate amplified TLR4 signaling and sepsis-induced mortality in Nrf2-deficient mice. *J Immunol* 185: 569–577.
39. Zhang WJ, Wei H, Frei B (2009) Genetic deficiency of NADPH oxidase does not diminish, but rather enhances, LPS-induced acute inflammatory responses in vivo. *Free Radic Biol Med* 46: 791–798.
40. Gao XP, Standiford TJ, Rahman A, Newstead M, Holland SM, et al. (2002) Role of NADPH oxidase in the mechanism of lung neutrophil sequestration and microvessel injury induced by Gram-negative sepsis: studies in p<sup>47phox</sup>-/- and gp<sup>91phox</sup>-/- mice. *J Immunol* 168: 3974–3982.