Increased expression of miR146a dysregulates TLR2 signaling in airway epithelial cells from patients with chronic obstructive pulmonary disease

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Abstract

Toll-like receptors (TLR) play a pivotal role in stimulating innate immune responses of airway epithelial cells to bacterial and viral infections. Airway epithelial cells from patients with chronic obstructive pulmonary disease (COPD) show suboptimal responses to non-typeable *H. influenzae* (NTHi) and TLR2 ligands despite expressing TLR2 similar to normal airway epithelial cells, but the underlying mechanisms are not well-known. Here we demonstrate that compared to normal, mucociliary-differentiated COPD airway epithelial cell cultures show impaired expression of β-defensin (HBD2) when infected with NTHi or treated with TLR2 agonists. Apical secretions from TLR2 agonist-treated normal but not COPD airway epithelial cells efficiently kills NTHi. Knockdown of HBD2 significantly reduced NTHi killing by apical secretions of normal airway epithelial cells. Interestingly, compared to normal, COPD bronchial epithelial cell cultures showed a significant reduction in the expression of interleukin-1 receptor-associated kinase (IRAK)-1, a downstream component of TLR2 signaling. Attenuated IRAK-1 levels were associated with increased expression of microRNA (miR)146a. Treatment with miR146a antagonist increased the expression of IRAK-1 and improved the expression of HBD2 in response to TLR2 agonists in COPD airway epithelial cells. Further, inhibition of miR146a also enhanced the killing of bacteria by apical secretions of FSL-1-stimulated COPD airway epithelial cells. Reduced expression of IRAK-1 was also observed in the bronchial epithelium of COPD patients. These results suggest that reduced levels of IRAK-1 due to increased expression of miR146a may contribute to dysregulation of TLR2 signaling and impaired expression of HBD2 in COPD airway epithelial cells.

Introduction

Acute exacerbations are the primary cause of morbidity and mortality in chronic obstructive pulmonary disease (COPD) [1]. Respiratory infections are responsible for 80% of the acute exacerbations in COPD [2], and ½ of which are associated with bacterial infections. Non-typeable *H. influenzae* (NTHi) is one of the frequently detected bacteria during acute exacerbations in these patients [3]. The severity of the acute exacerbations depends on the interaction of host defense mechanisms and infecting bacteria. Previous literature has demonstrated impaired responses to NTHi infection in COPD airway epithelial cells [4] and lung macrophages [5–9].

Airway epithelial cells produce antimicrobial peptides in response to bacterial infection, thus contributing to innate immunity. Bronchial epithelial cells from COPD patients express reduced levels of antimicrobial peptides (AMP), including human β-defensin 2 (HBD2), in response to NTHi or *P. aeruginosa* infection [4,10]. Moreover, HBD2 expression was reduced in the bronchial airway epithelium of COPD patients [11]. In addition, HBD2 has antimicrobial activity against NTHi [12]. Activation of toll-like receptor (TLR)2 signaling pathway in response to bacterial infection contributes to AMP expression in airway epithelial cells [13]. Previously, we demonstrated that despite expressing TLR2 similar to normal airway epithelial cells, COPD cells show attenuated IL-8 response to TLR2 agonists [14]. These observations indicate dysregulated TLR2 signaling rather than expression, but the underlying mechanisms are not well known.

Upon recognizing ligand, TLR2 heterodimerizes with either TLR1 or TLR6, which initiates recruitment of myeloid differentiation primary response gene 88 (MyD88) adaptor to the TLR-IL1R interaction domain. MyD88 then complexes with interleukin-1 receptor-associated kinase (IRAK) family members, and this complex is referred to as Myddasome [15]. During Myddasome formation, IRAK4 phosphorylates IRAK-1 initiating activation, autophosphorylation and release of IRAK-1 from Myddasome [16,17]. Release of IRAK-1 is essential for ubiquitination of TNF receptor-associated factor (TRAF) leading to activation of IKK complex and translocation of NF-κB or AP-1 translocation via activation of serine threonine-specific protein kinases [18]. TLR2 signaling in airway epithelial stimulates HBD2 via activation of NF-κB [19]. There is no defect in IKK driven NF-κB activation in the bronchial epithelium of COPD patients [20]. Based on this literature, we hypothesized that dysregulated TLR2 signaling might be due to defects in the expression or activation of adaptor proteins, MyD88 or IRAKs.

MicroRNA (miR) is a class of non-coding small RNA molecules. miRs carry out their biological functions by binding to the 3' untranslated regions of their target mRNAs, thereby repressing translation, degrading the target mRNAs or inducing target mRNAs. Previous studies have demonstrated that several miRs, including miR-21, miR-146, miR-155 and Let-7 family target TLRs or adaptor proteins in TLR signaling pathways (reviewed in [21]. Profiling of miRs in the whole lungs identified several differentially expressed miRs between healthy smokers and smokers with COPD with upregulated expression of miR146a in COPD patients [22]. miR146a inhibits MyD88-dependent TLR and IL-1 receptor (IL-1R) signaling via attenuation of IRAK-1 and TNF receptor-associated factor (TRAF)-6 expression [23], and degradation of signal transducer and activator of transcription (STAT)-1 [24]. Therefore, increased expression of miR146a may contribute to dysregulated TLR2 signaling in COPD airway epithelial cells.

This study examined the mechanisms underlying the dysregulation of TLR2 signaling and HBD2 and IL-8 expression in COPD using mucociliary-differentiated bronchial epithelial cell cultures established from COPD and normal airway basal cells.

Results

COPD airway epithelial cells are attenuated in reducing bacterial load. Mucociliary-differentiated normal and COPD airway epithelial cell cultures were apically infected with NTHi at multiplicity of infection (MOI) of 3, incubated for 24 h and bacterial density was measured. Compared to normal, COPD cells showed significantly more bacteria (Fig. 1A) indicating the inability of COPD cells to control bacterial replication.

COPD cells show attenuated expression of HBD2 in response to NTHi infection. Mucociliary-differentiated normal and COPD airway epithelial cell cultures were infected as above, and mRNA expression of *DEFB4*, a gene encoding human HBD2, was determined at 3, 24 and 48 h post-infection. Both normal and COPD cultures showed variability in the expression of *DEFB4* under unstimulated conditions and after NTHi stimulation (Fig. 1B). Therefore, we performed paired analysis between PBS-treated and NTHi-infected cultures. Normal airway epithelial cell cultures showed an increasing trend in *DEFB4* expression following infection with NTHi as early as 3h (Fig. 1A), which increased significantly at 24h. In contrast, COPD

cultures did not show a significant increase in the expression of *DEFB4* either at 3 or 24 h post-NTHi. In addition, the expression of *DEFB4* was significantly lower in NTHi-infected COPD cell cultures than the similarly infected normal cultures at both time points (Supplemental Fig. 1). At 48h infection, there was a significant cell death (data not shown) particularly in COPD cell cultures; therefore, we did not determine the expression of *DEFB4*. Next, we examined the HBD2 protein levels in apical secretions and basolateral medium at 3 and 24h post-infection. A detectable level of HBD2 was observed only in the apical secretions at 24h post-infection but not in the basolateral medium. However, the HBD2 levels were significantly lower in COPD cells than in normal (Fig. 1C). In the subsequent experiments, HBD2 protein was measured only in the apical secretions.

We also determined the expression of another AMP, cathelicidin, which is expressed by airway epithelial cells. NTHi infection did not stimulate this AMP even at 24h post-infection (Supplemental Fig. 2), therefore this study was focused on HBD2.

COPD cell cultures show attenuated HBD2 in response to TLR2 agonists. Since NTHi binds to TLR2 (14), and TLR2 activation contributes to HBD2 expression in response to bacterial infection, we assessed whether treating with TLR2 ligands stimulates HBD2 in airway epithelial cells. We used the synthetic ligands Pam3CSK4 and FSL-1, which respectively stimulate TLR2/TLR1 and TLR2/TLR6 heterodimerization, the first step in TLR2 activation. Initially, we conducted time-course experiment with FSL-1 using one normal and one COPD culture to determine the optimal time for *DEFB4* expression. Maximum expression of *DEFB4* was observed at 3h in both normal and COPD cultures and HBD2 protein expression was higher at 6 h (data not shown). Therefore, we determined the expression of *DEFB4* at 3h, and HBD2 protein levels after 6h of incubation. Treating with either FSL-1 stimulated the expression of *DEFB4* in both normal and COPD epithelial cell cultures, but it was significantly lower in COPD (Fig. 2A and 2B and Supplemental Fig. 3). Treatment with Pam3CSK4-induced *DEFB4* in normal but not in COPD cells. Both COPD and normal cultures showed an increase in HBD2 protein levels in response to FSL-1 ligand, but it was significantly lower in COPD cell cultures than in normal cell cultures (Fig. 2C). Consistent with mRNA expression, Pam3CSK4- induced HBD2 protein in normal, but not in COPD cells. These results indicate that TLR2 signaling that regulates HBD2 expression is dysregulated in COPD.

Apical secretions from COPD cell cultures are defective in killing NTHi.

HBD2 has antimicrobial effects against NTHi [12]. Therefore, we examined the antibacterial effects of apical secretions from unstimulated and FSL-1-stimulated normal and COPD cell cultures against NTHi. FSL-1 was chosen for this set of experiments because it induced the highest amounts of HBD2 protein (Fig. 2C). Apical secretions from unstimulated normal and COPD cultures did not reduce the bacterial counts (Fig. 3A). On the other hand, while apical secretions from FSL-1-stimulated normal cultures reduced the viable bacterial counts by 2 to 3 logs, secretions from similarly stimulated COPD cultures reduced the viable counts by ≤ 1 log. These results indicate that antimicrobial factors stimulated by the TLR2 signaling pathway kill NTHi, and this process is defective in COPD airway epithelial cell cultures.

To confirm the contribution of antimicrobial activity of TLR2-stimulated HBD2 against NTHi, HBD2 was genetically inhibited by using DEFB4 shRNA in normal airway epithelial cells, stimulated with FSL-1, and the apical secretions were examined for its ability to kill NTHi. Compared to NT-transduced controls, the apical secretions from HBD2-inhibited cells showed a significantly reduced ability to kill bacteria (Fig. 3B). HBD2 protein levels in the apical secretion were significantly lower in HBD2 shRNA- transduced cells than in NT shRNA-transduced control cells (Fig. 3C), indicating the efficient knockdown of HBD2. These results confirm that TLR2-stimulated HBD2 in airway epithelial cells plays an essential role in killing NTHi.

TLR2 expression is similar in COPD and normal airway epithelial cells.

Next, we determined the expression of TLR2 by Western blot analysis. As observed previously [14], we did not observe a difference in the expression of TLR2 between normal and COPD bronchial epithelial cells (Fig. 4A and 4B). These results indicate that the defect in TLR2 signaling is not due to the attenuated expression of TLR2 in COPD.

Expression of IRAK-M is not altered in COPD

IRAK-M is an endogenous inhibitor and inhibits MyD88-dependent TLR signaling by binding to the IRAK-1/IRAK-4 dimer in Myddasome, thereby preventing dissociation of hyperphosphorylated IRAK-1from the Myddasome and activation of TRAF-6 [25]. By Western blot analysis, there was no difference in the expression of IRAK-M between normal and COPD cultures (Fig. 4C and 4D), indicating IRAK-M may not contribute to the observed dysregulation of TLR2 signaling in COPD bronchial epithelial cells.

IRAK-1 expression is reduced in COPD cells

Next, we examined the expression of adaptor proteins MyD88, IRAK-4 and IRAK-1 between COPD and normal by Western blot analysis. There was no difference in the expression of MyD88 or IRAK-4 between COPD and normal cells (Fig. 5A to 5C). However, COPD cells showed significantly lower expression of IRAK-1 than normal bronchial epithelial cell cultures (Fig. 5D).

Next, we examined whether the expression of IRAK-1 is also affected in the bronchial epithelium of COPD patients by immunofluorescence microscopy. Paraffin lung sections containing secondary bronchi were prepared from the lungs of COPD and normal individuals, immunostained with antibody to IRAK-1 and observed under the fluorescence microscope. Compared to normal, bronchial epithelium in the lungs of COPD patients showed a substantial reduction in the expression of IRAK-1 (Fig. 6). These results indicate that the observed defect in TLR2 signaling may be due to a reduction in the expression of IRAK-1 in COPD bronchial epithelial cells.

Knockdown of IRAK-1 reduces TLR2-mdediated HBD2 expression

To examine the contribution of IRAK-1 in the expression of HBD2, IRAK-1 was genetically inhibited in normal epithelial cell cultures by using IRAK-1 shRNA (13). IRAK-1 knockdown significantly reduced NTHi, or TLR2 ligand-induced HBD2 expression (Fig. 7A). Knockdown of IRAK-1 was confirmed by Western blot analysis (Fig. 7B). These results confirmed the contribution of IRAK-1 in HBD2 expression through activation of TLR2 signaling.

miR146a expression is increased in COPD airway epithelial cells. Given the role of miR146a in attenuating the expression of IRAK-1, we examined the expression of miR146a in normal and COPD airway epithelial cells. COPD cells showed a small but significant increase in miR146a expression (Fig. 8A). Genetic inhibition of miR146a in COPD epithelial cells increased the expression of IRAK-1 (Fig. 8B) and restored NTHi and TLR2 ligands-stimulated HBD2 expression (Fig. 8C to 8E). Apical secretions from COPD cells transduced with miR146a inhibitor and stimulated with FSL-1 killed NTHi more efficiently than the secretions from similarly-treated cells transduced with scrambled shRNA (Fig. 8F). These results indicate the contribution of miR146a in inhibiting HBD2 expression via attenuation of IARK-1 expression in COPD cells.

Effect of miR146a inhibition on NTHi or TLR2 ligands induced IL-8 production.

Previously, we demonstrated that compared to normal, COPD cell cultures show reduced IL-8 production in response to stimulation with TLR2 ligands agonists. To determine the role of miR146a in TLR2-induced IL-8, we genetically inhibited miRNA in COPD cells and examined IL-8 response to FSL-1. As observed previously, COPD cell cultures showed higher levels of IL-8 compared to normal under unstimulated conditions (PBS-treated) (Fig. 9A) [26,27]. However, normal, but not COPD cells showed increased expression of IL-8 following treatment with FSL-1 as observed previously[14]. Inhibition of miR146a increased the IL-8 response to FSL-1 in COPD cells (Fig. 9B) indicating that miR146a not only inhibits TLR2 signaling-induced HBD2, but also pro-inflammatory response.

Next, we examined whether miR146a also affects NTHi-induced IL-8 in COPD cell cultures. COPD cell cultures showed higher IL-8 levels than normal under unstimulated conditions (Fig. 9C), which further increased following NTHi infection, unlike COPD cells stimulated with FSL-1. IL-8 response to NTHi infection further increased in COPD cells transduced with miRZip miRNA146a inhibitor (Fig. 9D). These results may indicate that NTHi stimulates IL-8 by other pathways in addition to TLR2 signaling.

In summary, these results indicate that reduced IRAK-1 expression may contribute to dysregulation of TLR2 signaling in COPD bronchial epithelium leading to attenuated expression of HBD2 in response to NTHi infection.

Discussion

Epithelium lining the upper respiratory tract is the first line of defense against inhaled pathogens; therefore, it is equipped with powerful innate immune defense mechanisms. AMPs such as defensins and cathelicidins represent a pivotal part of airway epithelial innate defense mechanisms because of their

broad-spectrum activity against bacteria, fungi and enveloped viruses [28]. Inducible AMPs in airway mucosa provide protection against bacterial challenge in a mouse model of airway infection [29]. Previous studies have shown that the COPD airway epithelial cells are deficient in expressing an inducible antimicrobial peptide, HBD2, which correlated with the defective killing of NTHi by these cells [4,10]. This report highlights one of the mechanisms underlying the defective HBD2 expression in COPD airway epithelial cells.

TLRs are evolutionarily conserved pathogen recognition molecules, and airway epithelial cells express all TLRs at the protein level except for TLR8 [30]. TLR2 and TLR4 are required for optimal clearance of bacteria in a mouse model of NTHi infection, bacteria-induced early chemokine and cytokine production, and the development of adaptive immune responses [31,32]. Alveolar macrophages from COPD patients show reduced responses to TLR2/4 ligands and NTHi despite expressing TLR2/4 similar to healthy smokers [33,34]. Moreover, in human middle ear epithelial cells, activation of the MyD88-dependent signaling pathway via TLR2, but not TLR4 was demonstrated to be required for NTHi-induced expression of HBD2 [35]. Cigarette smoke causes dysregulation of TLR2 signaling by inducing TLR2 hyposensitivity [36]. Previously, we have shown that TLR2 signaling is dysregulated in COPD tracheobronchial epithelial cell cultures despite expressing TLR2, similar to normal cells [14]. For the first time, this study demonstrates that attenuated expression of IRAK-1 via upregulated expression of miR146a contributes to dysregulated TLR2 signaling in COPD airway epithelial cells.

Here, we show that NTHi-induces the expression of *DEFB4*, a gene encoding HBD2 in normal, but not in COPD mucociliary-differentiated cultures. We also observed significantly attenuated levels of secreted HBD2 in NTHi-infected COPD epithelial cells at 24 h post-infection compared to similarly infected normal cultures. This was associated with a higher bacterial load in COPD cells than normal cultures. In the previous report the difference in HBD2 protein levels between normal and COPD cells was not observed until 72 hours post-infection and this may be due to the use of UV-inactivated NTHi, which is replication deficient [4]. In the present study, we used live NTHi, which can induce HBD2 within 24h. However, prolonged incubation that is beyond 24h caused cell death due to increased bacterial load.

Interestingly, TLR2 agonists, Pam3CSK4 and FSL-1 induced HBD2 in normal, but not in COPD cells, indicating the contribution of TLR2 to HBD2 expression. Apical secretions from FSL-1 -treated normal, but not COPD cultures effectively killed NTHi. Furthermore, genetic inhibition of HBD2 in normal cells significantly attenuated the ability of apical secretions to kill NTHi, indicating the pivotal contribution of HBD2 to antimicrobial activity against NTHi in airway epithelial cells. These observations indicate dysregulation of a TLR2 signaling pathway in COPD bronchial epithelial cells. Since there was no difference in the expression of TLR2 between normal and COPD bronchial epithelial cell cultures, defect in downstream signaling may contribute to the dysregulation of TLR2 signaling in COPD.

IRAK-M is an endogenous inhibitor of MyD88-dependent TLR signaling. The mRNA expression of IRAK-M is increased in sputum cells obtained from COPD patients, particularly in patients with frequent

exacerbations [37]. However, in this study, there was no difference in the expression of IRAK-M between normal and COPD bronchial epithelial cells. The observed discrepancy may be due to differences in the cell types and the microenvironment. The sputum is enriched in innate immune cells, such as alveolar macrophages, monocytes, neutrophils, T cells and others. Therefore, increased IRAK-M expression may correspond to these cells and not epithelial cells. COPD patients, especially those with a history of exacerbations, are treated with inhaled corticosteroids [38], and this can potentially increase IRAK-M expression, particularly in macrophages and other innate immune cells [39]. The bronchial epithelial cells used in this study were obtained from patients with end-stage lung disease who may have treated with corticosteroids. The fact that there was no difference in the expression of IRAK-M between normal and COPD cells indicates that corticosteroids' effect may not persist for an extended period. These observations also suggest that the observed dysregulation of TLR2 in COPD epithelial cells is not due to IRAK-M.

Intriguingly, the assessment of Myddasome adaptor proteins indicated significant reduction in the expression of IRAK-1, but not IRAK-4 or MyD88. Moreover, knockdown of IRAK-1 abrogated TLR2 ligands or NTHi-induced HBD2, indicating the contribution of IRAK-1 in this process. Several mechanisms regulate IRAK-1 expression. For instance, after activating MyD88-dependent signaling, IRAK-1 is rapidly degraded to terminate TLR signaling and maintain homeostasis [40,41]. TLR2/TLR4 activation induces the expression of miR146a, which acts as a negative feed-back regulator of IRAK-1 to dampen the magnitude of innate immune activation [42–44]. Our previous studies have indicated that compared to normal, COPD airway epithelial cells show higher protein expression of IL-6, CXCL-1, and CXCL-8 under unstimulated conditions [14,26,27], indicating persistent activation of TLR or other innate immune signaling pathways. Such persistent activation may modulate the expression of negative regulators of the TLR pathway, such as miR146a, to dampen the excessive activation of innate immune signaling. Consistent with this notion, we observed significantly higher expression of miR146a in COPD than in normal bronchial epithelial cells. Inhibition of miR146a restored IRAK-1 expression and NTHi or TLR2 agonist-stimulated HBD2 and antimicrobial activity in COPD epithelial cells. These observations suggest the contribution of miR146a in the dysregulation of the TLR2 signaling pathway in COPD bronchial epithelial cells.

It should be noted that IRAK-1 participation in TLR and IL-1R signaling is dependent on both species and cell type. For instance, IRAK-1 plays a predominant role in driving TLR and IL-1R signaling in human but not in murine macrophages (20). Moreover, TLR signaling in fibroblasts from an infant with fatal inherited IRAK-1 deficiency showed impaired response to TLR ligands but not IL-1β (21). In contrast, IRAK-1 deficiency did not affect TLR or IL-1R signaling in peripheral blood monocytic cells. Since like fibroblasts, epithelial cells are non-hematopoietic cells, and IRAK-1 expression is reduced in cultured COPD bronchial epithelial cells as well as COPD bronchial epithelium, it is conceivable that reduced IRAK-1 may significantly contribute to impaired TLR2 signaling, but not IL-1 signaling in COPD.

In addition to stimulating HBD2, activation of TLR2 signaling also induces pro-inflammatory cytokines. We observed that unlike HBD2, NTHi-induced IL-8 was not completely attenuated in COPD cells. This is

not surprising because NTHi induces pro-inflammatory responses not only via TLR2 signaling but also by other pathways [45,46]. However, inhibition of miR146a further enhanced NTHi-induced IL-8 in COPD cells, indicating the contribution of TLR2 signaling in this process.

One of the limitations of this study is that the bronchial epithelial cells were obtained from patients with end-stage lung disease. Since this population in general are afflicted with a higher frequency and severity of clinical exacerbations, cells from these patients may show increased dysregulation of TLR2. Bronchial epithelial cells from patients with mild to moderate disease may show variable expression of IRAK-1 and TLR2 desensitization. Moreover, all the patients had stopped smoking at least 6 months prior to double lung transplantation; therefore, it is hard to predict the effect of smoking. It will be interesting to compare the expression of IRAK-1, TLR2 desensitization in patients with mild to moderate disease, current and former smokers, but such studies require prospective collection of tissues, which is beyond the scope of the present study. In summary, although impaired TLR2 signaling in COPD has been known for some time, as far as we know, this is the first report to elucidate one of the underlying mechanisms.

Methods

Bronchial epithelial cell cultures. Bronchial tissue segments from normal lung donors and explanted lungs from COPD patients were collected at the time of lung transplantation. COPD patients provided a written informed consent forms to use their explanted lungs for research. Donors of normal lungs had standing consent to use their organs for organ transplant and research after their demise. The collection and use of the tissue samples were approved by Temple University institutional review board. All methods were performed in accordance with the relevant guidelines and regulations. Patient characteristics are provided in Table 1. Airway basal cells from bronchial segments were isolated and expanded in the Bronchial Life medium (Lifeline Cell Technology, Frederick, MD). The basal cells at passage 1 were cultured in 12 mm transwells at an air/liquid interface to promote differentiation of cells into mucociliary phenotype as previously described [14,47].

Table 1
Characteristics of patients with COPD and healthy non-smokers

No	Age (Yr)	Gender	FEV1 (% predicted)	Smoking history (pk years)
COPD				
15	*45*	*Female*	*17*	*31.2*
17	*64*	*Female*	*29*	*45*
713	73	Male	19	70
1190	68	Male	42	84
1209	79	Male	34	50
1236	59	Male	18	30
Healthy non-smokers				
53	*54*	*Male*		
31	*60*	*Female*		
396	46	Male		
1201	70	Male		
1220	67	Female		
1259	77	Male		

Samples in italics were obtained from University of Michigan Hospital, and rest of them were obtained through Biobank, Department of Thoracic Medicine and Surgery, Temple University Hospital.

Inhibition of HBD2, IRAK-1 and miR146a in COPD airway epithelial cells

Normal airway basal cells were transduced with lentiviral vectors expressing DEFB4 or scrambled non-targeting (NT) shRNA (Santa Cruz Biotechnology, Inc., Dallas, TX) as previously described [47]. Normal airway basal cells were transduced with lentiviral vectors expressing IRAK-1 and scrambled shRNA (Santa Cruz Biotechnology, Inc., Dallas, TX). Transduced cells were selected on puromycin, and then grown at air/liquid interface to promote mucociliary differentiation.

COPD bronchial epithelial cell cultures were transduced basolaterally with a lentiviral vector expressing miRZip scrambled hairpin RNA control or miRZip 146a microRNA inhibitor using TransDux Max transducing reagent (System Biosciences Inc., Mountain View, CA). Briefly, basolateral medium was replaced with fresh culture medium containing TransDux and MAX enhancer and then lentiviral particle expressing miRZip scrambled hairpin RNA control or miRZip 146a microRNA inhibitor (50 ul of medium containing 5 x 10^6 pfu equivalent to 5 MOI) was added to the basolateral medium. Cell cultures were

incubated for 3 days and the expression of miR146a target IRAK-1 was determined by Western blot analysis.

Infection of cell cultures with NTHi

Clinical isolate of NTHi, 5P54HI was isolated at the time of exacerbation from COPD patients (kindly provided by Dr. Murphy, University Buffalo, Buffalo, NY). For infection, 5P54HI was subcultured on a chocolate agar plate and incubated overnight at 37°C/5% CO2. Bacterial colonies were suspended in PBS and centrifuged at 1000 x g for 5 min. The bacterial pellet was finally suspended in PBS, and OD_{600} was adjusted to 1, which corresponds to 1–3 x 10^9 CFU/ml by dilution plating. The apical surface of the cell cultures was washed once with 0.15% sodium bicarbonate and rinsed with PBS to remove the mucus and other secretions. Cell cultures were transferred to a new receiver plate containing fresh medium with no antibiotics. They were immediately infected with 50 µl of NTHi (1–3 x 10^7 CFU/ml) suspension or treated with 50 µl PBS (control) and incubated for 24h. The cells were washed and lysed in 0.1% sterile triton X-100, lysates were serially diluted and plated to determine the bacterial density.

Treatment with TLR2 ligands

TLR2 ligands Pam3CSK4 and FSL-1 were purchased from InvivoGen (San Diego, CA) and dissolved in sterile PBS. The apical surface of the cell cultures was washed and then the wells were transferred to new receiver plates as above and treated with 50 µl of 1 µg/ml Pam3CSK4 or 10 ng/ml FSL-1 as recommended by the manufacturer, or PBS (control), and incubated for 3, 6 or 24h.

Killing of bacteria by apical secretions

After appropriate treatment, 500 µl of PBS was added to the apical surface of the cultures, incubated for 15 minutes, the apical secretions were collected and homogenized by mixing with pipette. NTHi (100 µl containing 1x 10^6 CFU) was added to 100 µl apical secretions, incubated for 2h at 37°C, serially diluted, and plated on chocolate agar plates to determine the viable bacterial counts.

Hbd2 Elisa

Apical secretions and basolateral medium were used to determine the protein levels of HBD2 by ELISA following manufacturers instructions (MyBioSource.com, San Diego, CA). The sensitivity of the assay was 15.6 pg/ml. IL-8 levels were determined in the basolateral medium by ELISA (R&D systems, Minneapolis, MN).

RNA isolation and qPCR

After appropriate treatment, cell cultures were washed three times with PBS and lysed in 0.7 ml TRIZOL. Total RNA was isolated from Trizol lysates of the cell cultures (Zymo Research, Irvine, CA), cDNA was synthesized using a High-Capacity cDNA reverse transcription kit (ThermoFisher Scientific, Waltham, MA) and subjected to probe-based qPCR. Primetime probe-based assays were purchased from Integrated DNA

Technologies (Coralville, IA). Expression of genes of interest was presented as a fold change over house-keeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [48]. miR146a expression was determined by using a TaqManTM microRNA assay kit (ThermoFisher Scientific, Waltham, MA) and the results were expressed as a fold change over RNU44 (control).

Western blot analysis

Total proteins from normal and COPD cell cultures were isolated by lysing cells with RIPA buffer containing protease and phosphatase inhibitors. Equal amounts of protein were subjected to Western blot analysis with antibodies to TLR2, IRAK-1, IRAK-4, IRAK-M, MyD-88 (all from Santa Cruz Biotechnology, Santa Cruz, CA) or GAPDH (Sigma Aldrich). Specific bands were quantified by densitometry using ImageJ and expressed as fold change over GAPDH.

Immunofluorescence microscopy

Paraffin Lung sections were deparaffinized and subjected to immunofluorescence staining with IRAK-1 antibody (Santacruz Biotechnology) using tyramide signal amplification kit (Thermoscientific) as previously described [49]. Briefly, deparaffinized sections were subjected to antigen retrieval in a boiling citric acid buffer, endogenous peroxidase activity quenched with 3% hydrogen peroxide, and blocked in 5% normal horse serum. The sections were incubated with IRAK-1 antibody (1:2000 dilution). The bound antibody was detected using antirabbit polymeric IgG conjugated with HRP (VectorLabs, Burlingame, CA) and tyramide signal amplification kit, counter stained with DAPI, and visualized under the fluorescence microscope. Sections stained with non-specific IgG served as control and these sections were used to determine the exposure time to detect specific signals. The IRAK-1 antibody used in this study gives a single band by Western blot analysis of airway epithelial cell lysis (data not shown).

Statistical analysis

Statistical significance for normally distributed data was assessed by unpaired student t test (for comparisons between 2 groups) or by analysis of variance (ANOVA) with Tukey-Kramer post-hoc test (for comparisons between 3 or more groups). If the data were not normally distributed non-parametric tests: Wilcoxon Rank Sum test to compare between 2 groups, and ANOVA on ranks with Kruskal-Wallis H test for comparing 3 or more groups. For paired analysis, the statistical significance was determined by Wilcoxon signed-rank test. A p value of ≤ 0.05 was considered as statistically significant.

Declarations

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AUTHOR CONTRIBUTIONS

HRV, Designed and conducted the experiments with primary cells and analyzed the samples; YK, isolated RNA and analyzed the samples by qPCR; CR, performed immunofluorescence on the human lung tissue sections; US, conceived the project, performed final analysis of the data and prepared the manuscript.

DATA AVAILABILITY STATEMENT

All the data are available from the corresponding author on reasonable request.

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Figures

Figure 1

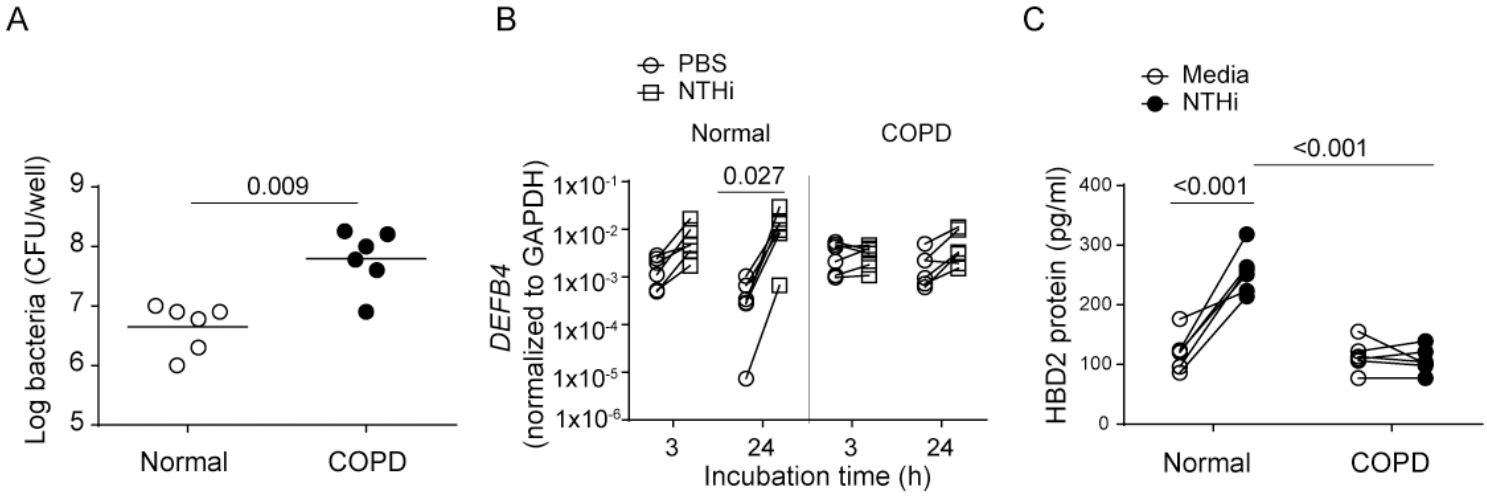

Figure 1

NTHi-infected COPD epithelial cell cultures show increased bacterial load and attenuated HBD2 expression. COPD and normal mucociliary-differentiated bronchial epithelial cell cultures (6 normal and 6 COPD) were infected with NTHi or treated with PBS (control) and incubated for 3 or 24h. (A) Cells were washed at 24 h post-infection, lysed in Triton X-100 and estimated bacterial density. (B) Total RNA was isolated and the expression of *DEFB4* and *GAPDH* was determined by RT-qPCR using gene-specific primers. *DEFB4* expression was normalized to *GAPDH*. (C) After 24 h incubation, measured HBD2 protein levels in the apical wash. (A and B) Data represent range with median and the statistical significance calculated by ANOVA on ranks with Kruskal-Wallis H. (C) Paired analysis with Wilcoxon signed-rank test was performed to determine the statistical significance.

Figure 2

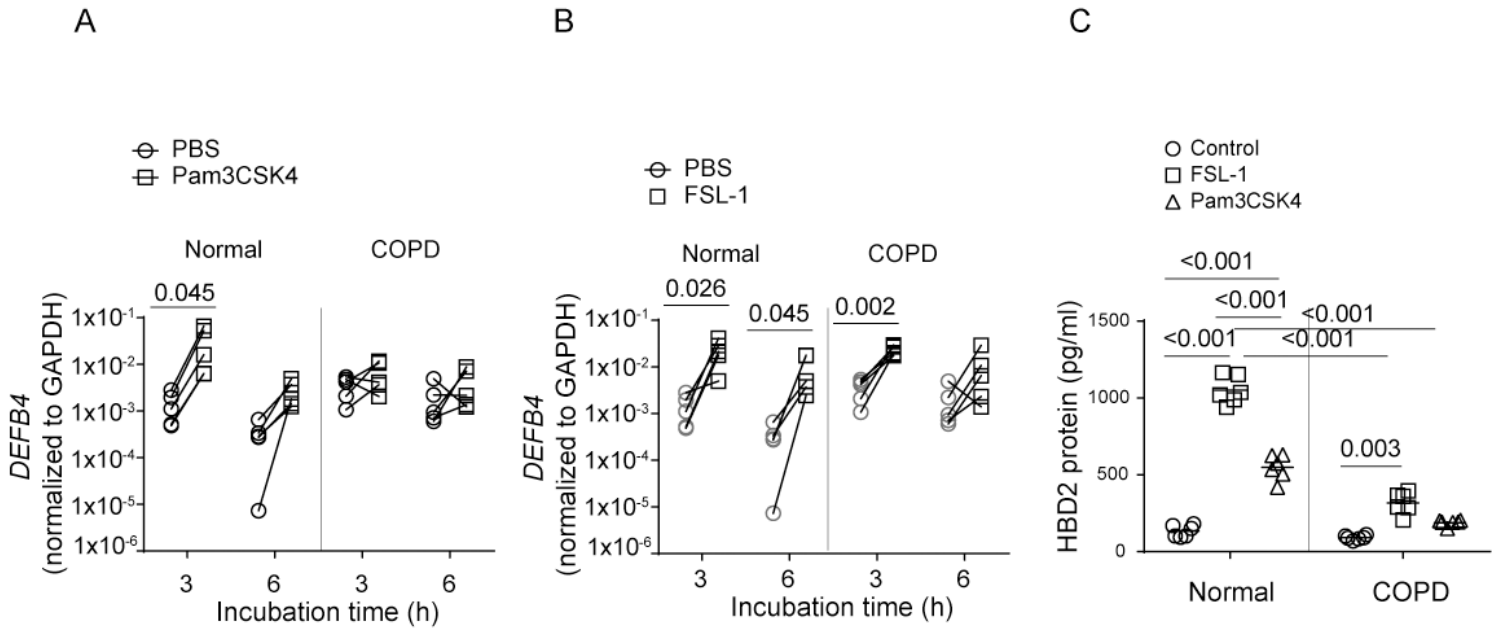

Figure 2

TLR2 agonists-induced HBD2 is lower in COPD epithelial cell cultures than normal cells. Mucociliary-differentiated COPD and normal bronchial epithelial cell cultures (6 normal and 6 COPD) were treated with Pam3CSK4, FSL-1 or PBS (control). (A and B) After 3 or 6h of incubation total RNA was isolated and determined the expression of *DEFB4* and *GAPDH* by RT-qPCR. The data represent *DEFB4* expression levels normalized to *GAPDH*. Paired analysis with Wilcoxon signed-rank test was performed to determine the statistical significance. (C) After 24 h incubation, HBD2 protein levels were measured in apical wash. Data represent range with median, and the statistical significance calculated by ANOVA on ranks with Kruskal-Wallis H post-hoc test.

Figure 3

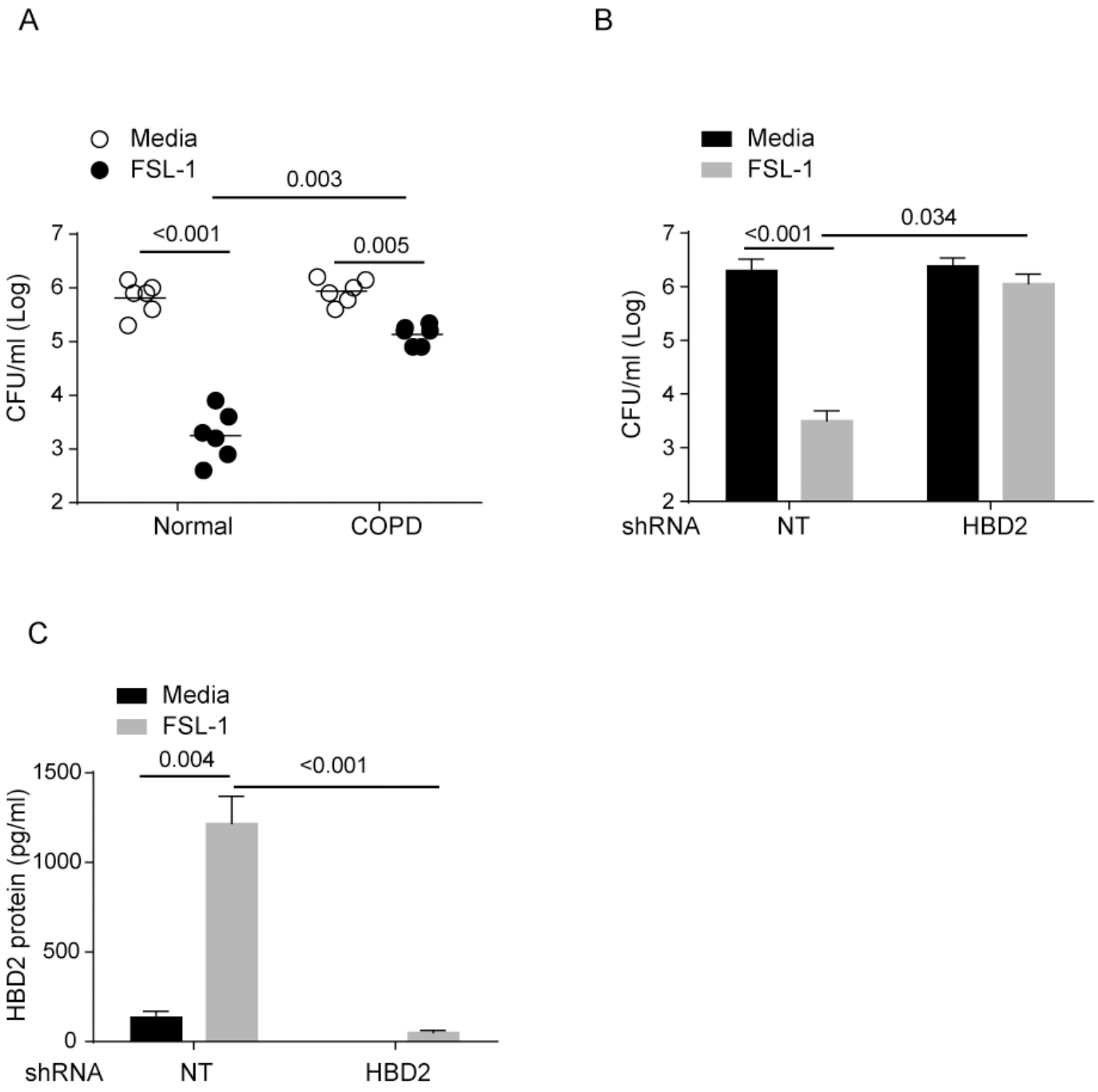

Figure 3

Secreted HBD2 shows antibacterial activity against NTHi. Normal and COPD bronchial epithelial cell cultures (established from 6 normal and 6 COPD patients) were treated with FSL-1 or PBS (control) and incubated for 24 h. (A) The apical secretions were then collected, incubated with NTHi (1 x 10^6 CFU) for 2 h, and the number of viable bacteria was determined by dilution plating. Data represent median with range and the statistical significance analyzed by ANOVA on ranks with Kruskal-Wallis H post-hoc

test. (B) Normal bronchial epithelial cell cultures transduced with non-targeting (NT) or HBD2 shRNA were treated with PBS (control) or FSL-1 and the antibacterial activity of apical secretions was measured as described above. (C) Levels of HBD2 protein in the apical secretions was measured by ELISA. (B and C) Data represent mean ± SD calculated from 2 independent experiments conducted in triplicates. Statistical significance was calculated by ANOVA with Tukey's post-hoc test.

Figure 4

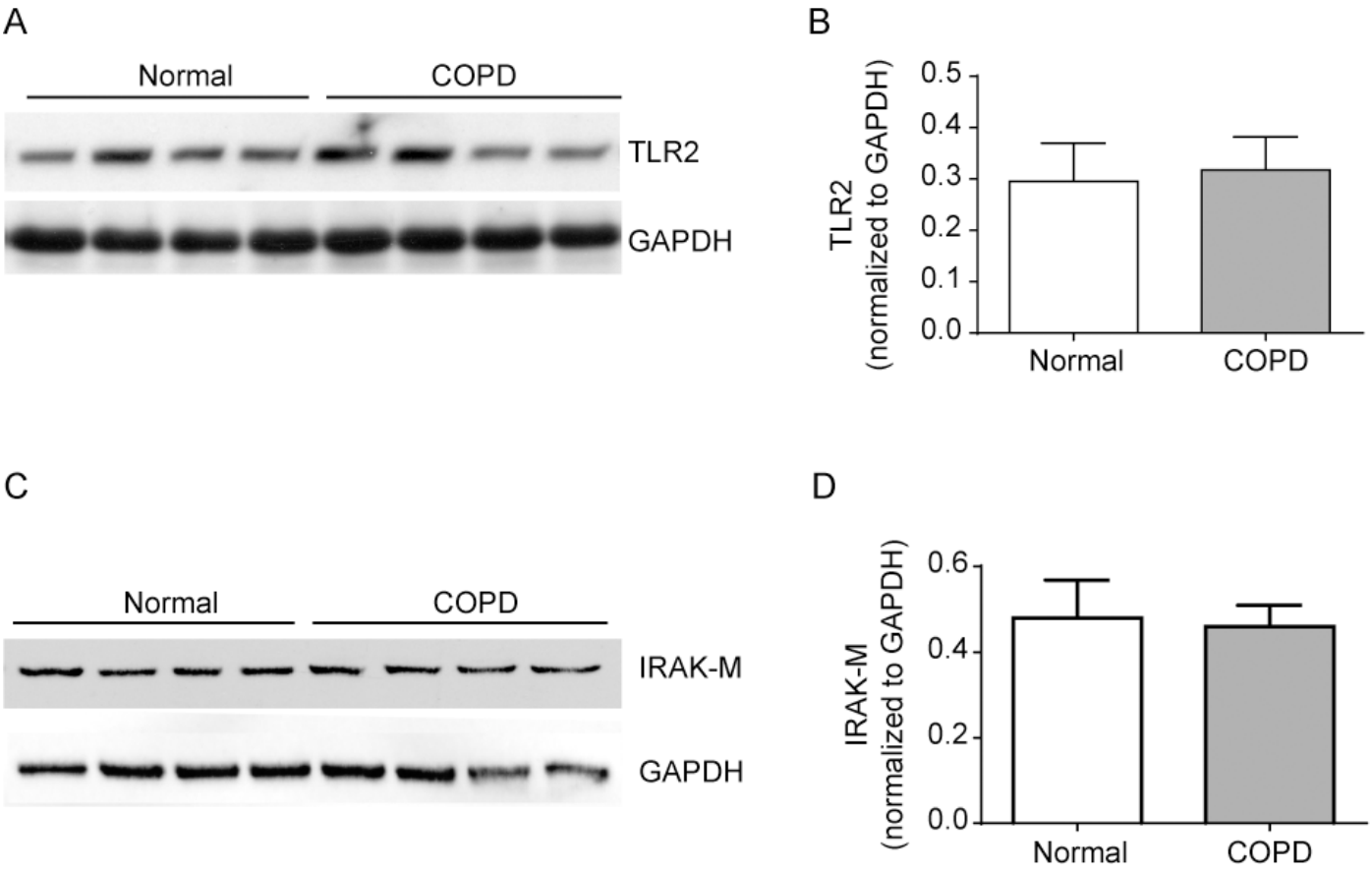

Figure 4

TLR2 and IRAK-M expression is not altered in COPD bronchial epithelial cell cultures. (A and C) Total protein isolated from COPD and normal mucociliary-differentiated cell cultures was subjected to Western blot analysis with TLR2, IRAK-M and GAPDH antibodies (n=4). (B and D) The band intensities were quantified by ImageJ software, and data were expressed as fold change over GAPDH. Data represent mean ± SD calculated from 4 normal and 4 COPD cultures.

Figure 5

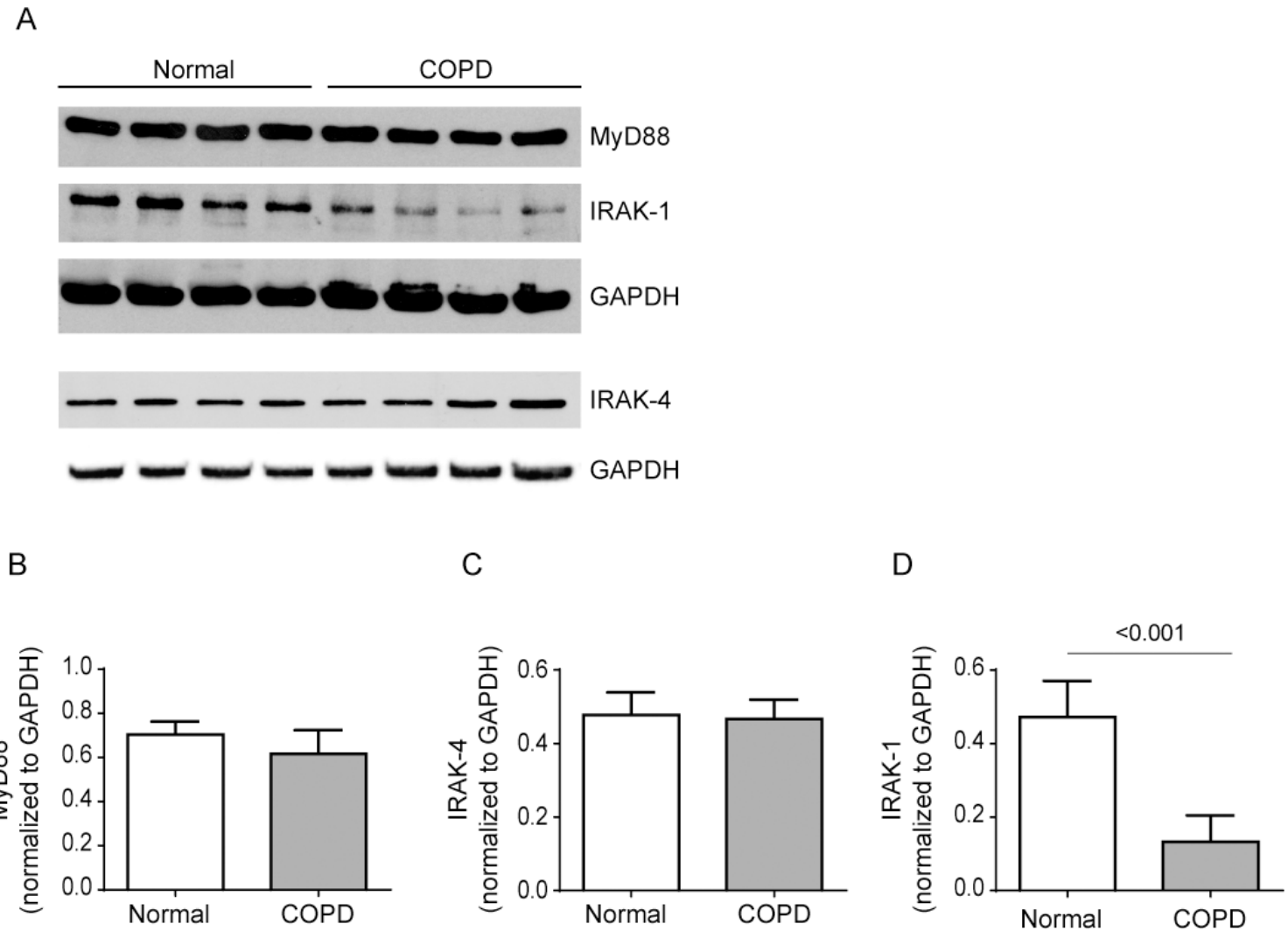

Figure 5

IRAK-1 expression is reduced in COPD bronchial epithelial cells. (A) Total protein isolated from normal and COPD mucociliary-differentiated cultures was subjected to Western blot analysis with MyD88, IRAK4, IRAK-1 and GAPDH antibodies. (B, C and D) The intensity of the bands was quantified by ImageJ software and levels of MyD88, IRAK4 and IRAK-1 were expressed as fold change over GAPDH. Data represent mean ± SD calculated from 4 normal and 4 COPD cultures, and statistical significance calculated by unpaired T test.

Figure 6

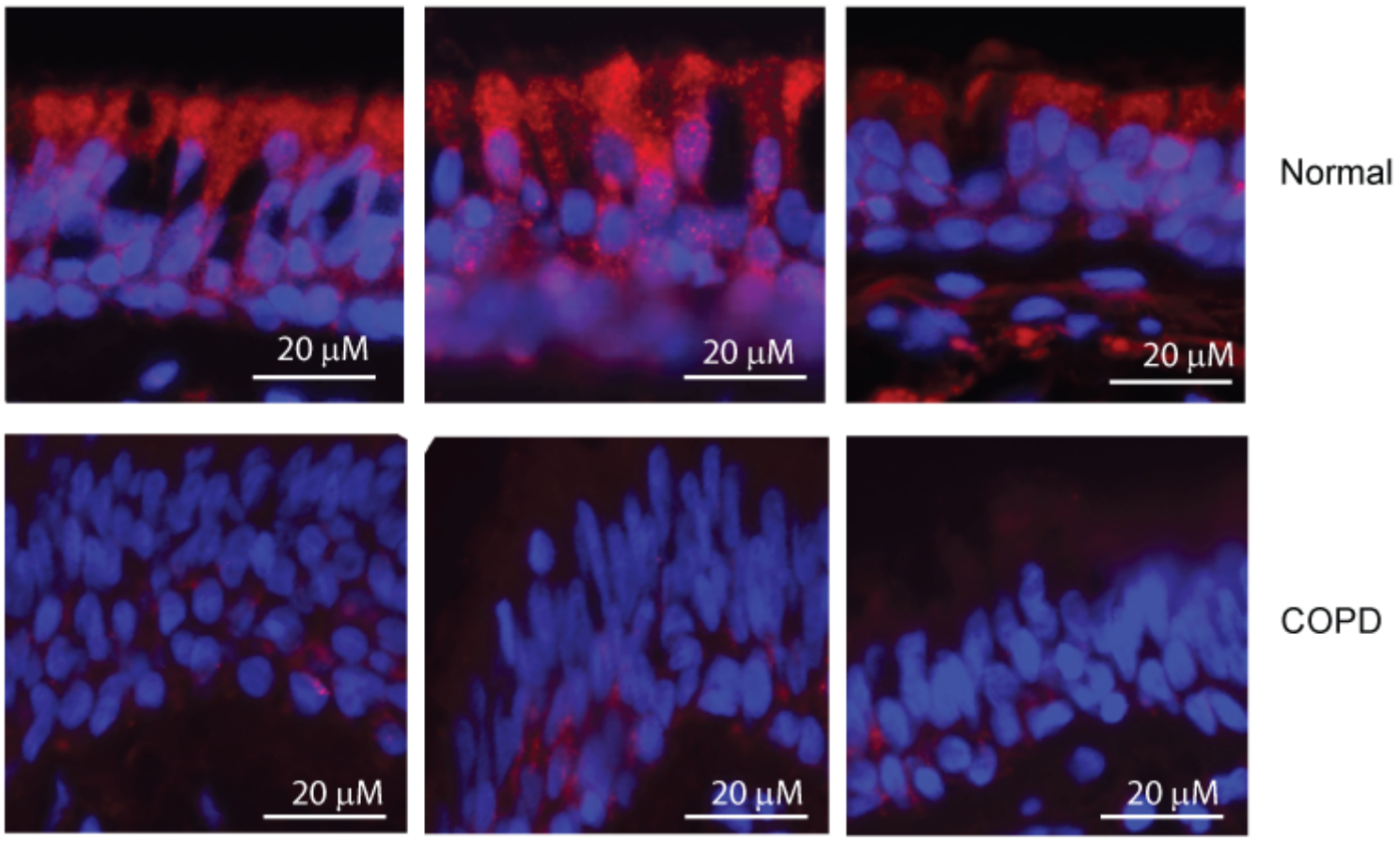

Figure 6

COPD bronchial epithelium show attenuated expression of IRAK-1. Paraffin sections of COPD and normal lung sections were deparaffinized, subjected to antigen unmasking and incubated with antibody to IRAK-1. The bound antibody was detected by a second antibody conjugated with HRP followed by signal amplification with tyramide conjugated with AlexaFlour 598 and then counterstained with DAPI (n=3; Red, IRAK-1 and blue, Nuclei).

Figure 7

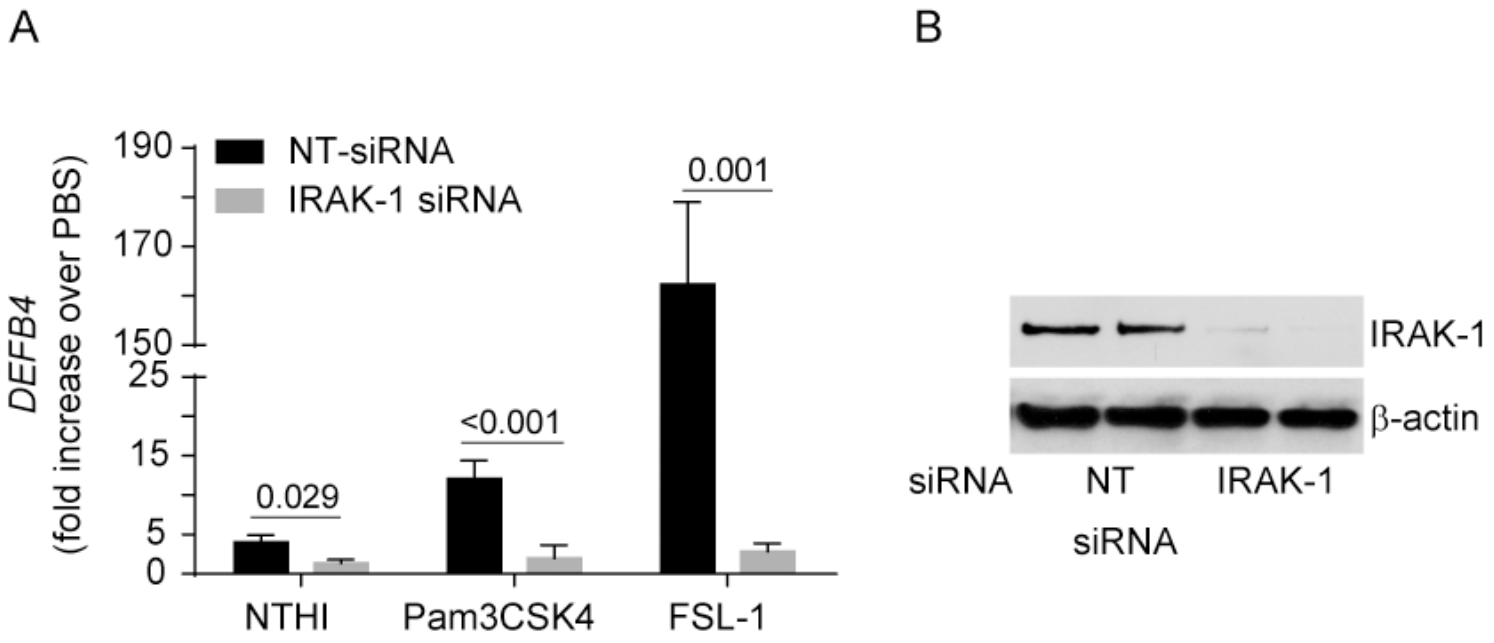

Figure 7

IRAK-1 is required for *DEFB4* response in airway epithelial cell cultures. (A) Normal basal cells transduced with non-targeting or IRAK-1 shRNA were grown as mucociliary-differentiated cultures, challenged with NTHi, or TLR2 ligands, and examined *DEFB4* expression by RT-qPCR. The expression levels of *DEFB4* mRNA were normalized to *GAPDH* and presented as fold change over PBS-treated cultures. Data represent mean ± SD calculated from 2 independent experiments conducted in triplicates. Statistical significance was calculated by ANOVA with Tukey's post-hoc test. (B) Western blot analysis confirmed successful IRAK-1 knockdown in both experiments.

Figure 8

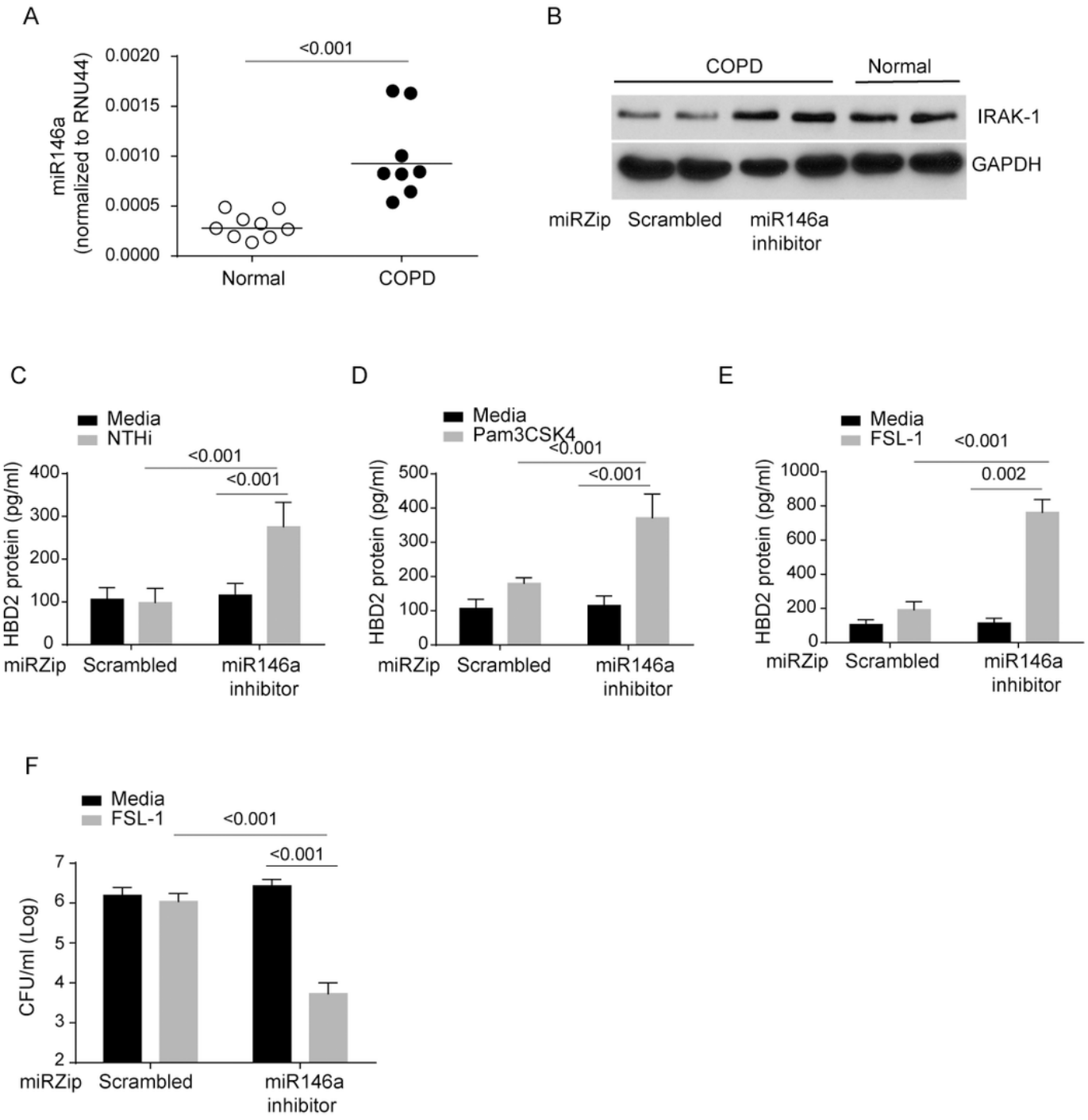

Figure 8

Enhanced expression of miR146a contributes to the attenuated expression of IRAK-1 and NTHI-stimulated HBD2 in COPD epithelial cells. (A) Total RNA isolated from normal and COPD bronchial epithelial cell cultures was subjected to RT-qPCR to determine the levels of miR146a and RNU44. Data represent median with range (n=8 to 9), and the statistical significance calculated by Mann-Whitney test. (B) Total protein isolated from normal bronchial epithelial cell cultures and COPD bronchial epithelial

cell cultures transduced with scrambled or miR146a inhibitor were subjected to Western blot analysis with antibodies to IRAK-1 and GAPDH. (C to E) COPD epithelial cells transduced with control or miR146a inhibitor were infected with NTHi or treated with PBS (control), Pam3CSK4 or FSL-1, and the HBD2 protein levels were measured in the apical secretions by ELISA. (F) Apical secretions from FSL-1 treated COPD epithelial cells transduced with control or miR146a inhibitor was incubated with NTHi for 2h, and the viability of bacteria was measured by dilution plating. Data in C to F represent mean ± SD calculated from 2 independent experiments conducted in triplicates and the statistical significance calculated by ANOVA with Tukey post-hoc test.

Figure 9

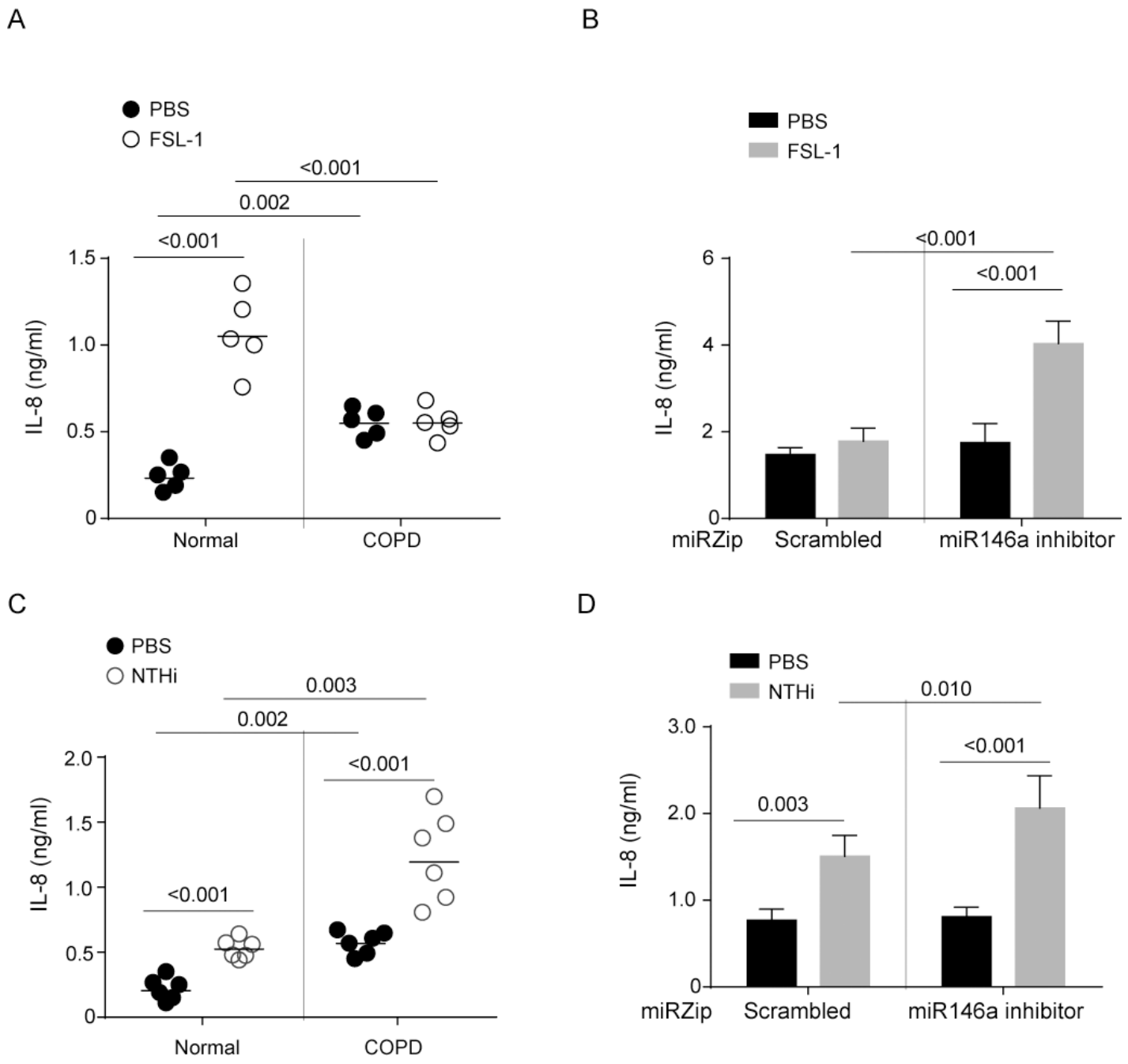

Figure 9

Inhibition of miR146a restores TLR2 agonist-induced IL-8 expression in COPD epithelial cells. (A and C) Mucociliary-differentiated normal and COPD epithelial cell cultures were treated with FSL-1 or infected with NTHi, incubated for 24 h and IL-8 levels were measured in the basolateral medium by ELISA. Data represent range with medium (n=6) and statistical significance calculated by ANOVA on ranks with Kruskal-Wallis H. (B and D) COPD epithelial cells transduced with control or miR146a inhibitor were

infected with NTHi or treated with PBS (control), or FSL-1, and the IL-8 protein levels were measured by ELISA after 24h. Data represent mean ± SD calculated from 2 independent experiments conducted in triplicates and the statistical significance was calculated by ANOVA with Tukey post-hoc test.

Supplementary Files

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