Contribution of Dipeptidyl peptidase 4 to nontypeable \textit{H}. \textit{influenzae}-induced lung inflammation in COPD

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\textbf{Clinical Perspectives}
Infection with non-typeable *H. influenzae* (NTHi) causes acute exacerbations in COPD, sometimes leading to persistent lung inflammation and progression of lung disease, but the underlying mechanisms are not well-known.

We demonstrate that in COPD, pulmonary macrophages show dipeptidyl peptidase (DPP)4-dependent exaggerated pro-inflammatory responses to NTHi. In a mouse model of COPD, NTHi infection causes DPP4-dependent sustained lung inflammation even in the absence of detectable bacteria indicating an important role of DPP4 in persistent lung inflammation.

Therefore, treatment with DPP4 inhibitors may be effective in preventing NTHi-induced sustained lung inflammation and preventing progression of lung disease in COPD.
Abstract

Dipeptidyl peptidase 4 (DPP4) expression is increased in the lungs of chronic obstructive pulmonary disease (COPD). DPP4 is known to be associated with inflammation in various organs, including LPS-induced acute lung inflammation. Since non-typeable H. influenzae (NTHi) causes acute exacerbations in COPD patients, we examined the contribution of DPP4 in NTHi-induced lung inflammation in COPD. Pulmonary macrophages isolated from COPD patients showed higher expression of DPP4 than the macrophages isolated from normal subjects. In response to NTHi infection, COPD, but not normal macrophages show a further increase in the expression DPP4. COPD macrophages also showed higher expression of IL-1β, and CCL3 responses to NTHi than normal, and treatment with DPP4 inhibitor, diprotin A attenuated this response. To examine the contribution of DPP4 in NTHi-induced lung inflammation, COPD mice were infected with NTHi, treated with diprotin A or PBS intraperitoneally, and examined for DPP4 expression, lung inflammation and cytokine expression. Mice with COPD phenotype showed increased expression of DPP4, which increased further following NTHi infection. DPP4 expression was primarily observed in the infiltrated inflammatory cells. NTHi-infected COPD mice also showed sustained neutrophilic lung inflammation and expression of CCL3, and this was inhibited by DPP4 inhibitor. These observations indicate that enhanced expression of DPP4 in pulmonary macrophages may contribute to sustained lung inflammation in COPD following NTHi infection. Therefore, inhibition of DPP4 may reduce the severity of NTHi-induced lung inflammation in COPD.
INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is highly prevalent worldwide and by 2030, it is expected to affect more than 210 million people (1). Acute exacerbations are the major cause of morbidity and mortality in these patients (2). Eighty percent of the acute exacerbations are due to respiratory infections with bacteria and viruses (3). Bacterial infections account for about ½ of all the respiratory infections-related acute exacerbations in COPD. Non-typeable *H. influenzae* (NTHi) is one of the major organisms detected during acute exacerbations (4) in these patients.

The severity of the acute exacerbations depends on the interaction of host defense mechanisms and the infecting organism (5). Accumulating evidence indicates a dysregulated host response to infection in COPD, which may influence respiratory infection's overall outcome. For example, alveolar macrophages isolated from COPD patients show defect in phagocytosis, bacterial killing, and expression of cytokines in response to bacterial infection *ex vivo* (6-11). There is also limited literature showing reduced migratory accuracy of neutrophils towards the targets (12), and this may lead to inefficient removal of bacteria by neutrophils. A more recent study shows that neutrophil clearance is also significantly reduced in COPD lungs (13), leading to neutrophil accumulation and tissue damage. Airway epithelial cells from COPD patients also show impaired antimicrobial expression in response to NTHi challenge (14). Such dysregulated host innate immune responses may promote bacterial persistence and lung inflammation.

Smoking is thought to be the primary risk factor for the development of COPD. Smoking causes long-term changes in structure and function of airway epithelium, and impairs the innate immune responses of epithelial cells and macrophages. *In vitro* exposure of epithelial cells and
macrophages to cigarette smoke attenuates antimicrobial responses and bacterial phagocytosis, respectively (14, 15). In the in vivo models, smoking was associated with increased lung inflammation following NTHi challenge despite augmented bacterial clearance (16, 17). Furthermore, one of the mechanisms of neutrophilic lung inflammation was shown to be dependent on IL-17A through enhanced IL-1 receptor activation (18).

Dipeptidyl peptidase 4 (DPP4), also known as CD26, is a serine peptidase and cleaves peptides with alanine and proline in the second position at the N-terminus (19). Two well-known endogenous physiological substrates for DPP4 are glucose-dependent insulinotropic polypeptide and glucagon-like peptide 1 (GLP-1), and thus inhibitors of DPP4 are clinically used to improve glucose tolerance in diabetic patients (20). DPP4 inhibitors also have favorable effects on cardiovascular disorders (21). In addition, DPP4 inhibitors attenuate lipopolysaccharide-induced inflammation in a mouse model of acute lung injury (22) and lung inflammation caused by P. aeruginosa (23).

DPP4 is expressed in all the organs of the body. In the lungs, DPP4 is primarily expressed in the alveolar epithelial cells and rarely in the bronchial epithelial cells (24). However, in patients with COPD, expression of DPP4 was observed not only in the alveolar epithelial cells but also in the bronchial epithelium, submucosal glands and macrophages (24, 25). Since DPP4 plays a role in LPS or P. aeruginosa-induced lung inflammation, and DPP4 is increased in COPD, we hypothesized that DPP4 contributes to NTHi-induced exaggerated inflammatory changes in COPD.

In this report, we demonstrate that the alveolar macrophages from COPD patients express higher levels of DPP4 and show exaggerated expression of CCL3 and IL-1β expression in response to
NTHi challenge. Furthermore, using a mouse model of COPD, we show that NTHi infection increases lung inflammation dependent on DPP4 expression by infiltrated inflammatory cells.

METHODS AND MATERIALS

Bacteria and infection

NTHi isolates 7P49HI, 5P54HI, and 6P5HI were isolated at the time of exacerbation from COPD patients (kindly provided Dr. Murphy, University Buffalo, Buffalo, NY). For infection, 7P49HI 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.

Human bronchoalveolar lavage cells and NTHi infection

Bronchoalveolar lavage (BAL) cells were obtained from lavaging lungs from healthy donors, which were rejected for transplantation, and explanted lungs from COPD subjects at the time of double lung transplantation. Donor's lungs were from Gift of Life program and the donors had standing consent for the use of their organs for transplantation and research after their demise. COPD subjects provided written informed consent for the use of their tissue for research purposes. Biobank maintained by Lung Center collected the donor and COPD lungs with the approval from Temple University Institutional Review Board IRB#4407 and deidentified the lung tissue before transferring to the research lab with no information to identify the donors. Therefore, research with these tissue samples was considered to be non-human subjects research.
by the Temple Institutional review board, IRB Waiver #23201. Details of the lung donors are provided in Table 1.

To isolate macrophages and other infiltrated inflammatory cells, 50 ml of PBS was instilled into the right lobe, incubated for 10 min, and then the fluid was aspirated. This process was repeated three times, and all the washes were pooled and centrifuged. Macrophages were separated from other cell types using an EasySep™ Human Monocyte enrichment kit without CD16 depletion (STEMCELL Technologies Inc., Cambridge, MA (26). The isolated macrophages were cultured in RPMI containing 10% fetal bovine serum, penicillin/streptomycin and 0.25 μg of fungizone. After 6 h, the nonadherent cells were removed along with the medium and replaced with fresh RPMI medium containing 5% serum with no antibiotics or fungizone and further incubated for 18 h. Cells from one well from each donor were harvested and counted to determine the number of cells. The adherent cells were infected with NTHi at 1 MOI suspended in serum and antibiotic-free RPMI medium, or sham-infected with medium alone, and incubated for 3 h. The infection medium was replaced with fresh medium containing penicillin/streptomycin to prevent excessive growth of bacteria and the incubation continued for another 20 h. Cells were harvested and total RNA was isolated from the cells to determine the mRNA expression of inflammatory cytokines by qPCR. In some experiments, cells were infected with NTHi and incubated in the presence of diprotin A (25 μM) (Sigma-Aldrich Inc., St. Louis, MO) or vehicle (PBS). The sample size was determined based on our preliminary experiment, in which the difference in the expression of DPP4 between normal and COPD was determined.

Animals
All the experimental procedures were approved by the Animal Care and Use Committee of Temple University, Philadelphia, and the studies were conducted in compliance with the approved protocols #4599. FVB/N mice were bred in the Temple University Laboratory for Animal Research Facility and the mice were maintained in a specific pathogen-free environment. Six to eight weeks old male mice were exposed to a combination of cigarette smoke and heat-killed NTHi (isolate 5P54HI) as described previously to induce mild COPD-like lung disease (27). Briefly, mice were subjected to whole-body exposure to cigarette smoke for 2 h a day, 5 days a week for 10 weeks. Mice were briefly anesthetized with inhaled isoflurane and treated intranasally with heat-killed NTHi (equivalent to 5 x 10^6 CFU) at the end of 1 and 3 weeks of cigarette smoke exposure. These mice are referred to as COPD mice. Mice exposed to room air and similarly treated with heat-killed NTHi were used as controls, and we designated these mice as normal mice. Treatment of mice with heat-killed NTHi did not induce mucosal or peripheral antibodies to NTHi, as determined by slot blot immunoassay using 3 different isolates of NTHi, 5P54HI, 6P5HI and 7P49HI.

Infection of mice

Normal and COPD mice were briefly anesthetized with inhaled isoflurane and infected with 50 μl of NTHi (PBS containing 5 x 10^6 CFU) by the intranasal route (28). Control mice in each group were sham-infected with PBS alone. Mice were sacrificed at 7 days post-infection by asphyxiation. In some experiments, COPD mice were injected intraperitoneally with DPP4 inhibitor, 100 μl of diprotin A (5 nmol) for 6 days starting from the day of NTHi infection. COPD mice injected with 100 μl of sterile PBS served as experimental controls. The diprotin A dose was based on the previous study, which demonstrated 5 nmol of diprotin A was sufficient to inhibit DPP4 effects in a mouse model of vascular disorder (29).
Bronchoalveolar lavage fluid and lung homogenates

Mouse lungs were lavaged with ice-cold sterile PBS as previously described without opening the thoracic cavity (27, 30). Briefly, 1 ml sterile PBS was administered into the lungs via intubated trachea and aspirated. This procedure was repeated 5 times. The first wash was collected separately, and an aliquot was plated on to chocolate agar plate to determine the bacterial load. The remaining BALF was centrifuged, the supernatant was immediately stored at -70°C. The cell pellet was mixed with remaining washes, centrifuged again, and the cell pellet was used to determine the total and differential cell counts (30) or for immunostaining. The remaining cells were lysed in TRIZOL for total RNA isolation.

Lavaged lungs were collected and homogenized in sterile PBS. Aliquots (50 μl) of lung homogenates was dilution plated on chocolate agar plates to determine the bacterial load. The remaining lung homogenates were used for total RNA isolation.

RNA isolation and qPCR

Total RNA was isolated from Trizol lysates of the lungs and BAL cells (Zymo Research, Irvine, CA), cDNA was synthesized using LunaScript® RT SuperMix Kit (New England Biolabs, Ipswich, MA) and subjected to probe-based qPCR. All the Primetime probe-based assays were purchased from Integrated DNA Technologies (Coralville, IA).

ELISA

BAL or lung homogenate supernatants were used for immunoassays. Custom-made Multiplex luminex immunoassay (R & D Systems, Minneapolis, MN) was used to determine CXCL-1, CXCL2, CCL2, CCL3, TNF-α, and IL-1β according to the manufacturer’s instructions. DPP4,
IL-17, and neutrophil elastase (all assays from R & D Systems) levels were measured by ELISA. The levels of DPP4, IL-8 in spent medium from human alveolar macrophages was measured by ELISA (R & D Systems).

**Histology and immunofluorescence staining**

Peripheral lung tissue from the left lobe of the lungs from normal and COPD patients was fixed and embedded in paraffin. The details of lung donors is provided in Table 1. Mouse lungs were inflation-fixed in 10% neutral buffered formalin and embedded in paraffin. Five micron thick lung sections were deparaffinized and stained with hematoxylin and eosin to assess morphological changes.

Five-micron thick mouse or human lung sections were deparaffinized and subjected to immunofluorescence staining with DPP4 antibody and tyramide signal amplification kit as previously described (31). Briefly, after deparaffinization, the 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 DPP4 antibody (ThermoFisher Scientific, Waltham, MA) (1:2000 dilution for mouse sections, and 1:5000 for human sections) or similarly diluted normal rabbit IgG (VectorLabs, Burlingame, CA, negative control). The bound antibody was detected using antirabbit polymeric IgG conjugated with HRP (VectorLabs) and tyramide signal amplification kit (ThermoFisher Scientific), counter stained with DAPI, and visualized under the fluorescence microscope. In some experiments, mouse lung sections immunostained with DPP4 antibody were incubated with 1:500 diluted FITC-labeled F4/80 antibody (BioLegend, San Diego, CA) or normal mouse IgG...
labeled with FITC (negative control) to detect lung macrophages. Negative controls were used to set the exposure time on the fluorescence microscope, in order to detect the specific signals.

Cytospins prepared from mouse lungs were fixed in 1% paraformaldehyde in PBS and subjected to immunofluorescence staining using DPP4 antibody (1:5000 dilution) or normal rabbit IgG (negative control), antirabbit IgG and tyramide signal amplification kit as described above.

**Statistical analysis**

Data were expressed as mean ± SD or median with range. Data were analyzed by using SigmaStat statistical software (Systat Software, San Jose, CA). One way ANOVA with Tukey post-hoc test, ANOVA on ranks with Kruskal-Wallis H test or unpaired t test were performed as appropriate to compare groups and a p value ≤ 0.05 was considered to indicate significant differences.

**Results**

**Alveolar macrophages isolated from COPD patients show increased expression of DPP4 following NTHi infection**

Initially, we determined the expression of DPP4 in the lung sections of normal and COPD subjects by immunofluorescence. Lung sections from normal subjects showed minimal expression of DPP4 (Figure 1A). In contrast, there was an abundant expression of DPP4 in COPD lung sections, particularly in the cells accumulated in the alveolar space (Figure 1B). Compared to normal, isolated BAL macrophages from COPD patients showed increased mRNA expression of DPP4 (Figure 1C).
To assess the effect of NTHi infection on DPP4 expression in BAL macrophages, normal and COPD macrophages were infected with NTHi, and the expression of DPP4 protein in the spent medium was determined by ELISA after 24 h. COPD macrophages showed higher levels of DPP4 than normal under basal conditions, which further increased following NTHi infection (Figure 1D). Both COPD and normal macrophages showed increases in IL-8, IL-1β and CCL3 mRNA expression after NTHi infection. However, only NTHi-induced IL-1β and CCL3 expression were much higher in COPD than normal (Figure 1E to 1G), indicating differential regulation of certain pro-inflammatory cytokines in COPD alveolar macrophages. Treatment of COPD macrophages with diprotin A, a DPP4 inhibitor, attenuated NTHi-induced expression of IL-1β and CCL3, but not IL-8 (Figure 2). These results indicate the contribution of DPP4 in modulating the expression of cytokines that were specifically upregulated in NTHi-infected COPD macrophages.

**NTHi-infected COPD mice show sustained lung inflammation and increased DPP4 expression**

Next, we examined whether DPP4 expression is associated with NTHi-induced lung inflammation *in vivo* by using mice displaying mild to moderate COPD-like lung disease (27, 32, 33). Our previous studies demonstrated that normal mice clear all the bacteria by 3 days post-infection and resolve bacteria-induced inflammation by day 7 (27, 30). Therefore, to differentiate the responses between normal and COPD mice, we examined bacterial load, lung inflammation and DPP4 expression at 7 days post-infection. Both normal and COPD mice cleared bacteria by 7 days post-infection. As observed previously, sham-infected COPD mice showed mild peribronchiolar and parenchymal inflammation (27) (Figure 3A and 3C). At 7 days
post-NTHi infection, normal mice showed very mild lung inflammation, whereas COPD mice showed heightened lung inflammation (Figure 3B and 3D).

Total and differential cell counts in the BAL were determined to quantify inflammation. Compared to normal mice, COPD mice showed an increase in total cell counts, the number of macrophages, neutrophils, T cells and eosinophils as observed previously (27). All the cell types except for eosinophils further increased after NTHi infection in COPD mice (Figure 4A-E).

Additionally, we observed a significant increase in neutrophil elastase in the BAL fluid of NTHi-infected COPD mice compared to all the other groups (Figure 4F). On the contrary, normal mice infected with NTHi showed a significant increase in total cell counts, but there was no difference in the number of neutrophils, macrophages, T cells or eosinophils, or the levels of neutrophil elastase between sham and NTHi-infected normal mice. These results indicate that COPD mice, despite clearing bacteria, fail to resolve neutrophilic lung inflammation.

Evaluating DPP4 expression by immunofluorescence microscopy, we found that normal mice, irrespective of infection, showed minimal expression of DPP4 (Figure 5A and 5B). In contrast, COPD mice showed substantial DPP4 expression, primarily in the cells that are present in the alveolar space (Figure 5C). NTHi infection further increased DPP4 expression in COPD mice particularly in the cells present in the inflammatory foci and surrounding inflamed parenchyma (Figure 5D). Lung sections of NTHi-infected COPD mice were co-stained with antibodies to DPP4 and F4/80 (macrophage marker) to determine whether DPP4 is expressed in macrophages. DPP4 was expressed primarily in F4/80 positive macrophages in the inflammatory foci (Figure 5E). However, not all the F4/80 positive cells expressed DPP4 indicating expression of DPP4 in some subtypes of macrophages. Since most of the DPP4 expression was found to be associated with infiltrated inflammatory cells, we quantified the DPP4 mRNA expression in BAL cells.
Compared to normal, BAL cells from COPD mice showed increased expression of DPP4 mRNA, which further increased following NTHi infection (Figure 5F). These results indicate that DPP4 expression in the alveolar macrophages may contribute to NTHi-induced sustained lung inflammation in COPD mice.

Inhibition of DPP4 reduces NTHi-induced sustained lung inflammation in COPD mice

To examine the contribution of DPP4 in NTHI-induced lung inflammation, COPD mice were infected with NTHi and treated with diprotin A or PBS for 6 days by the intraperitoneal route. Mice were sacrificed at 7 days post-infection and examined for lung inflammation. Initially, we determined the inflammation by performing total and differential cell counts in the BAL. Compared to PBS-treated mice, diprotin A-treated NTHI-infected COPD mice showed a reduced trend in total cell counts (Figure 6A). There was no difference in the number of macrophages or T cells between PBS and diprotin A-treated NTHI-infected COPD mice (Figure 6B and 6C). On the other hand, diprotin A-treated mice showed one log fewer neutrophils than the PBS-treated mice, and this was associated with the reduced neutrophil elastase content in the BAL fluid (Figure 6D and 6E).

We also assessed the lung inflammation by histology. Vehicle-treated NTHi-infected COPD mice treated with vehicle showed severe lung inflammation similar to untreated NTHi-infected COPD mice (Figure 7A). Compared to vehicle-treated mice, diprotin A-treated mice showed substantially less inflammation (Figure 7B) and this was associated with significantly decreased DPP4 protein expression (Figure 7C and 7D) and DPP4 mRNA expression in BAL cells (Figure 7E). The observed decrease in DPP4 expression may not be due to the direct effect of diprotin A, but rather a reduction in inflammatory cells in the lungs that express DPP4. These results
indicate that DPP4 expressed by infiltrated inflammatory cells, possibly macrophages, may contribute to NTHi-induced neutrophilic inflammation in COPD mice.

**Inhibition of DPP4 reduces CCL3 and CCL20 expression in NTHi-infected COPD mice**

Since DPP4 inhibitor reduced NTHi-induced neutrophil accumulation in the lungs, we examined the levels of inflammatory cytokines, which can recruit neutrophils, such as CXCL-1, CXCL2, CCL2, CCL3, TNF-α, IL-1β, and IL-17 in the BAL fluid by either multiplex or individual ELISA assays. We also determined the expression of CCL20, which attracts activated neutrophils that express CCR6 (34). At 7 days post-infection, levels of all the measured cytokines except for CCL3, CCL20 and IL-17 were below the detection limit. NTHi-infected COPD mice showed significant increases in protein levels of CCL3 and CCL20 compared to other groups (Figure 8A and 8B). In contrast, IL-17 level was not altered by NTHi infection in either COPD or normal mice (Figure 8C). Analysis of mRNA expression of DPP4 in BAL cells (infiltrated cells) and the lavaged lungs (structural cells) indicate that BAL cells are the primary source of CCL3 (Figure 8D and 8E), whereas CCL20 mRNA expression was only detected in structural cells (Figure 8F). NTHi-infected COPD mice treated with diprotin A showed a significantly attenuated CCL3 and CCL20 mRNA expression in BAL and lung structural cells, respectively, compared to vehicle-treated mice (Figure 8G and 8H).

Taken together, our findings suggest an essential role for DPP4 in NTHi-induced persistent lung inflammation in COPD.

**Discussion**
The present study highlights one of the mechanisms underlying NTHi-induced prolonged lung inflammation in COPD. We show that DPP4 expression is increased in COPD patients’ lungs, primarily in the cells accumulated in the alveolar space. Compared to normal, macrophages isolated from COPD patients show increased expression of DPP4 and DPP4-dependent exaggerated expression of IL-1β and CCL3 in response to NTHi infection. DPP4 expression was predominantly observed in the infiltrated inflammatory cells in a mouse model of COPD, which further increased following NTHi infection. This was associated with enhanced lung inflammation even in the absence of detectable bacteria. Treatment with DPP4 inhibitor reduced NTHi-induced sustained neutrophilic inflammation. These results indicate that DPP4 contributes to sustained lung inflammation in COPD following NTHi infection.

Airway neutrophilic inflammation is a prominent feature of COPD (35). Neutrophil recruitment to the airways is increased during exacerbations, particularly in bacteria-associated exacerbations and correlates with rapid decline in lung function (2, 5). Neutrophilic inflammation occurs due to increased recruitment of neutrophils to the airways and impaired clearance of neutrophils (13, 36). Previously, IL-17A was demonstrated to mediate exaggerated neutrophilic inflammation at 12 h post-NTHi infection in mice with COPD phenotype (18). In this study, IL-17 was measured 7 days post-infection and there was no difference in IL-17 levels between NTHi and sham-infected COPD mice. IL-17 is an acute response to bacterial infection and may not be present during the late phase of infection, explaining the observed discrepancy. Therefore, we speculate that neutrophilic inflammation at 7 days post-infection is driven by mechanisms other than IL-17.

In recent years, DPP4 has been demonstrated to cause inflammation in various organs, including the lungs. For example, in a mouse model of acute injury, the expression of DPP4 was increased
compared to normal mice (22, 37). Treatment with DPP4 inhibitor substantially reduced LPS-induced lung inflammation by attenuating oxidative stress via positive regulation of NRF2. DPP4-knockout rats showed reduced inflammation following *Pseudomonas aeruginosa* infection indicating the contribution of DPP4 to bacteria-induced lung inflammation (23). The current study demonstrates that NTHi infection causes sustained neutrophilic inflammation in COPD mice. DPP4 inhibition significantly reduced numbers of neutrophils, implying the contribution of DPP4 to persistent neutrophilia. Interestingly, neutrophil elastase levels were also increased indicating activation or necrosis of neutrophils, which was also considerably reduced by DPP4 inhibition.

As reported previously (24, 25), lungs from COPD patients showed increased expression of DPP4. However, we observed DPP4 expression primarily in lung macrophages. Similar to that observed in COPD patients, COPD mice also showed increased expression of DPP4 compared to normal mice. The DPP4 levels further increased at 7 days post-infection in COPD mice, but not in normal mice, and this was associated with increased numbers of macrophages, T cells and neutrophils. Interestingly, DPP4 expression was primarily observed in macrophages in these mice. Although DPP4 inhibition reduced DPP4 expression in macrophages, DPP4 does not appear to have a role in the recruitment of either macrophages or T cells. Macrophages play a significant role in clearing apoptotic cells, including apoptotic neutrophils, and macrophages from COPD patients show impairment in efferocytosis (38). Alveolar macrophages from COPD patients colonized with bacteria show a further reduction in efferocytosis (39). This was attributed to a reduction in surface receptors responsible for the recognition of apoptotic cells. We speculate that DPP4 being a peptidase may cleave the cell surface receptors, thus, contributing to impaired clearance of apoptotic neutrophils leading to persistent neutrophilia.
Apoptotic neutrophils eventually undergo necrosis and release intracellular cellular contents including elastase and this may partially contribute to increased neutrophil elastase in our study.

Sustained neutrophilia may also occur due to increased levels of chemoattractants. Consistent with this notion, NTHi-infected COPD mice showed increased levels of CCL3 and CCL20, both of which recruit neutrophils to the lungs. Since inhibition of DPP4 reduces the expression of CCL3 and CCL20, it is conceivable that DPP4 also contributes to the recruitment of neutrophils by increasing the levels of these CCL chemokines. Furthermore, CCL20 has also been shown to recruit CCR6 expressing activated neutrophils (34), and this can also contribute to the observed increased elastase levels in NTHi-infected COPD mice.

A DPP4-dependent exaggerated expression of NTHi-induced CCL3 was also observed in lung macrophages isolated from COPD patients. In these cells, CCL20 was below the detection limit, and this is not surprising because CCL20 is primarily expressed by epithelial cells. Accordingly, we found that CCL3 is expressed by both BAL cells (macrophage and other hematopoietic cells) and lavaged lungs (epithelial cells and other structural cells), whereas CCL20 was observed only in lavaged lungs. However, there was no change in the expression of DPP4 in the lavaged lungs, whereas inhibition of DPP4 reduced the CCL20 levels, which may be due to paracrine action of DPP4 released by macrophages. Since activated neutrophils also express CCL3, reduction in number of neutrophils may also contribute to reduced levels of this chemokine in DPP4 inhibitor-treated NTHi-infected COPD mice. We also observed exaggerated DPP4-dependent IL-1β responses to NTHi infection in COPD lung macrophages, and this was not observed in COPD mice at 7 days post-NTHi infection. Since IL-1β is an acute response to bacterial infection, this cytokine may not be expressed at 7 days post-infection. These observations
indicate that DPP4 inhibition may attenuate exaggerated and sustained responses to infection, thus reducing lung inflammation.

Previous studies have shown that DPP4 increases the expression of pro-inflammatory cytokines via activation of NF-κB (40). DPP4 expression may also regulate pro-inflammatory cytokine expression by suppressing the expression of glucagon peptide-1 like (GLP-1) receptor expression in macrophages (41). Interestingly, treatment with GLP-1R agonist has been shown to reduce inflammation in mouse models of allergic asthma and obstructive lung disease (42, 43). In patients with COPD, GLP-1R expression is markedly decreased in airway smooth muscle cells, and overexpression of GLP-1R in isolated airway smooth cells reduces expression of pro-inflammatory cytokines and proliferation of cells (44). Our ongoing studies indicate that GLP-1R is reduced in the lungs of COPD patients as well as in the COPD mouse model. Based on these observations, it is plausible that DPP4 reduces CCL3, CCL20 and IL-1β via suppression of GLP-1R, which will be explored in the future.

Some COPD patients who experience exacerbations related to bacterial infections show loss of lung function even after treatment with antibiotics. This may be due to altered responses of DPP4-expressing macrophages to bacterial infection. Since DPP4 inhibitor reduces exaggerated pro-inflammatory responses of COPD macrophages to NTHi, and alleviates NTHi-induced lung inflammation in the mouse model of COPD, treatment with DPP4 inhibitor may prevent accelerated lung function decline in these patients.

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Conflict of interest
None

Data Availability Statement

All the data are presented in the manuscript.
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Table 1

Characteristics of patients with COPD and healthy non-smokers

| No   | COPD Age (Yr) | Gender | Healthy non-smokers Age (Yr) | Gender |
|------|---------------|--------|------------------------------|--------|
| 713  | 73            | Male   | 312                          | 58     | Male |
| 1018 | 57            | Male   | 366                          | 46     | Male |
| 1030 | 57            | Female | 396                          | 46     | Male |
| 1054 | 75            | Female | 1201                         | 70     | Male |
| 1076 | 62            | Male   | 1220                         | 67     | Female |
| 1290 | 62            | Male   | 1259                         | 77     | Male |

All tissue samples were obtained from the Biobank, Department of Thoracic Medicine and Surgery, Temple University Hospital.
Figure Legends

Figure 1. DPP4 expression is increased in COPD, which further increases after NTHi infection. Paraffin sections of normal (A) and COPD (B) subjects were deparaffinized, subjected to antigen retrieval, endogenous peroxidase quenched, blocked, and incubated with DPP4 antibody at 4°C for 16 h. The slides were washed, incubated with second antibody conjugated with HRP followed by signal amplification with tyramide conjugated with AlexaFlour 598 and then counterstained with DAPI. Red (arrow), DPP4 and blue, nuclei. Images are representative of lung tissues obtained from 3 normal and 3 COPD subjects. (C) COPD and normal macrophages were plated in 24 well plates, incubated for 24 hours, total RNA was isolated and subjected qRT-PCR. Data was normalized to G3PDH and represent median with range (Mann-Whitney U Test). (D to G) COPD and normal macrophages were infected with NTHi or sham-infected with medium alone and medium was collected after 24 h incubation to determine DPP4 levels by ELISA (D). Total RNA was isolated from the cells and subjected qRT-PCR and data normalized to G3PDH (E to G). Within group paired comparison between sham and NTHi-infected cultures and between group comparison was conducted by the signed-rank test and Mann-Whitney U test, respectively. Data represent median with range calculated using macrophages obtained from 6 COPD and 6 normal subjects.

Figure 2. Treatment with diprotin A inhibits NTHi-stimulated IL-1β and CCL3. COPD macrophages were infected with NTHi or sham-infected with medium and incubated in the presence of diprotin A or vehicle (PBS) for 24 h. Total RNA was isolated and subjected to qRT-PCR and data was normalized to G3PDH. Within-group paired comparison between sham and NTHi infected cultures and between group comparison was conducted by the signed-rank test.
and Mann-Whitney U test, respectively. Experiments were performed in triplicates with macrophages obtained from 3 COPD subjects.

Figure 3. Mice with COPD phenotype show sustained lung inflammation after NTHi infection. Normal and COPD mice were sham-infected with PBS or infected with NTHi and sacrificed after 7 days. (A to D) Lungs were fixed, embedded in paraffin and lung sections were stained with H & E (arrows in C and D represent peribronchiolar and perivascular inflammation). Experiment was conducted 2 times with two to three mice per group (n=5 - 6 per group).

Figure 4. NTHi-infected COPD mice show persistent increase in inflammatory cells. Normal mice or mice with COPD phenotype were sham-infected with PBS or infected with NTHi and sacrificed 7 days post-infection. (A to E) BAL was performed, centrifuged, cytospins of BAL cells were stained with DiffQuick stain, and the total and different cell types were counted under microscope. (F) The BAL supernatant was used to determine the levels of neutrophil elastase by ELISA. Experiment was conducted 2 times with three to four mice per group (n=6-7 per group). Data in A to E represents median with range and the statistical difference was determined by ANOVA on Ranks with Kruskal-Wallis H test. Data in F represent mean ± SD (ANOVA with Tukey post-hoc test).

Figure 5. Inflammatory cells show increased expression of DPP4 in COPD mice following NTHi infection. Normal mice and mice with COPD phenotype were sham-infected with PBS or infected with NTHi, sacrificed 7 days post-infection, and lungs were inflation fixed or subjected to BAL. (A to D) Five-micron thick paraffin sections were deparaffinized, subjected to antigen retrieval, and immunostained with DPP4 as described under Figure 1. Green (arrow), DPP4 and blue, nuclei. Images are representative of 3 normal and 3 COPD mice. (E) Paraffin section from
NTHi-infected COPD mice was co-immunostained with antibodies to DPP4 and F4/80 to detect DPP4 and macrophage, respectively. Green, DPP4; red, macrophage; yellow co-localization of DPP4 with F4/80, and blue, nuclei. Image is representative of 3 mice. (F) From some mice BAL cells were isolated, total RNA extracted and subjected to qRT-PCR. Data was normalized to β-actin and expressed as mean ± SD from 6 to 7 mice per group (ANOVA with Tukey test). (G) Cytospins of BAL cells were fixed in 4% paraformaldehyde containing 0.05 Triton X-100 and immunostained with DPP4 antibody as described above. Images are representative of 6 to 7 animals per group.

Figure 6. Treatment with diprotin A reduces neutrophil accumulation in NTHi-infected COPD mice. Mice with COPD phenotype were infected with NTHi and treated with diprotin A or vehicle (PBS) by intraperitoneal route daily for 7 days. Sham-infected COPD mice served as experimental control. Mice were sacrificed and BAL was performed. (A to D) Cytospins of BAL cells were stained with DiffQuick to quantify total and differential cell counts. Data represent median with range and statistical significance was determined by ANOVA on Ranks with Kruskal-Wallis H test. (E) Neutrophil elastase in BAL fluid was determined by ELISA. Data represent mean ± SD calculated from 6 mice per group (ANOVA with Tukey post-hoc test).

Figure 7. Diprotin A attenuates NTHi-induced inflammation in COPD mice. Mice with COPD phenotype were infected with NTHi and treated with diprotin A or vehicle (PBS) by intraperitoneal route daily for 7 days. Mice were sacrificed and lungs were fixed and embedded in paraffin. (A and B) Paraffin sections were deparaffinized and stained with H & E. (Arrows represent peribronchiolar inflammation). (C and D) Paraffin sections were deparaffinized and subjected to immunostaining with DPP4 as described in Figure 1. Green, DDP4 and blue, nuclei.
Images are representative of 3 animals per group. (E) Total RNA isolated from BAL cells was subjected to qRT-PCR to determine DPP4 mRNA expression. Data was normalized to β-actin and expressed as mean ± SD from 6 mice per group (ANOVA with Tukey test).

Figure 8. Diprotin A treatment inhibits CCL3 and CCL20 expression in COPD mice infected with NTHi. (A to F) Normal and COPD mice were infected with NTHi or sham-infected with PBS. (A to C) Mice were sacrificed 7 days post-infection and BAL performed. Levels of CCL3, CCL20 and IL-17 protein in clarified BAL fluid was measured by ELISA. (D to F) Total RNA isolated from BAL cells was subjected to qRT-PCR and data was normalized to β-actin. (G and H) COPD mice infected with NTHi were treated with diprotin A or PBS (vehicle) for 7 days. Mice were sacrificed, BAL was performed to obtain BAL cells. Total RNA was isolated from BAL cells and subjected to qRT-PCR and data was normalized to β-actin. Sham-infected mice served as experimental control. Data in all the panels represent mean ± SD calculated from 2 experiments with three to four mice per group (n=6 -7; ANOVA with Tukey post-hoc test).
Figure 1

A

Healthy non-smoker

B

COPD Exsmoker

C

DPP4 mRNA
(normalized to GAPDH)

1x10^-0

1x10^-1

1x10^-2

p=0.002

Normal

COPD

D

DPP4 protein (ng/ml)

4

3

2

1

0

p=0.002

p=0.018

p=0.009

Normal

COPD

E

IL-8 mRNA
(normalized to GAPDH)

p=0.001

p=0.031

Medium

NTHi

F

IL-1β mRNA
(normalized to GAPDH)

p=0.002

p=0.005

Medium

NTHi

G

CCL3 mRNA
(normalized to GAPDH)

p=0.008

p=0.002

Medium

NTHi

Normal

COPD
Figure 2

- **IL-1β mRNA (normalized to GAPDH)**
  - PBS: Medium: p=0.045, NTHi: p=0.008
  - DiprotinA: Medium: p=0.513, NTHi: p=0.016

- **CCL3 mRNA (normalized to GAPDH)**
  - PBS: Medium: p=0.045
  - DiprotinA: Medium: p=0.013, NTHi: p=0.050
Figure 3

A  Normal sham

B  Normal NTHI

C  COPD sham

D  COPD NTHI

100 μM
Figure 6

A. Total cells/Lung (Log)

B. Total macrophages/Lung (Log)

C. Total T cells/Lung (Log)

D. Total neutrophils/Lung (Log)

E. Neurophil elastase (pg/ml)

- Vehicle vs. Diprotin A
- PBS vs. NTHi

Statistical significance:
- <0.001
- 0.002
- 0.005
- 0.014
- 0.220
- 0.426
- 0.767
- 0.873
Figure 7

A. PBS

B. Diprotin A

C. DPP4 mRNA in BAL cells (normalized to β-actin x 1x 10^-5)

E. Comparison of DPP4 mRNA levels between PBS and NTHi groups:

- PBS: 0.087
- NTHi: 0.001 and 0.004

D. Diprotin A

(Images show histological and fluorescent images with scales 100 μM and 50 μM respectively.)
