



Expression of Toll-like Receptor 2 and 4 in Peripheral Blood Neutrophil Cells from Patients with Chronic Obstructive Pulmonary Disease

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ABSTRACT

Objectives: Chronic obstructive pulmonary disease (COPD) is one of the leading causes of morbidity and mortality around the world. Preliminary studies have evaluated the association between innate immunity including Toll-like receptors (TLRs) and airway samples of patients with COPD. The role of TLRs in peripheral blood neutrophils is poorly understood. Hence, this study aimed to investigate the role of TLR2 and TLR4 in peripheral blood neutrophils of COPD patients. **Methods:** A total of 101 COPD cases and an equal number of healthy controls participated in this case-control study. Peripheral blood neutrophils were isolated from all participants and cultured for 24 hours through lipopolysaccharide (LPS) stimulation. The gene expressions of TLR2 and TLR4 were assessed by real-time polymerase chain reaction. The protein levels of interleukin (IL)-8 and matrix metalloproteinase (MMP)-9 were measured in neutrophils cell culture supernatants using enzyme-linked immunosorbent assay (ELISA). **Results:** The levels of IL-8 and MMP-9 were significantly higher in patients with COPD compared to healthy controls. Similarly, the gene expression of TLR2 and TLR4 were increased in LPS stimulated peripheral blood neutrophils of patients with COPD. Smoke pack years was positively correlated with IL-8 levels and negatively correlated with forced expiratory volume in the first second % ($r = -0.33$; $p = 0.023$) and FEV1/forced vital capacity (FVC) ($r = -0.27$; $p = 0.011$). **Conclusions:** The increased expression of TLR2 and TLR4 suggests its role in disease pathogenesis of COPD. Smoke pack years was negatively associated with spirometric parameters in COPD patients. This may help to predict the smokers without COPD who risk developing the condition in the future.

Chronic obstructive pulmonary disease (COPD) refers to a group of lung diseases characterized by airway inflammation and tissue destruction with non-reversible airflow limitation. Airflow limitation is generally progressive and associated with an abnormal response of the lungs to noxious particles or gases such as cigarette smoke, coal mining dust, diesel exhaust particles, and fumes from burning biomass fuels for cooking or heating that leads to chronic airway inflammation.¹ There is a broad range of inflammatory cells like macrophages, neutrophils, T and B lymphocytes, eosinophils, and epithelial cells involved in the inflammatory process

of COPD.² Today COPD is acknowledged as a systemic disease that not only affects the airways and lungs.³ Neutrophils are one of the main effector cells in COPD and during exacerbations, which are frequently caused by bacterial infections. Neutrophils are rapidly recruited to the site of action and play a significant role in tissue damage and killing bacteria.^{4,5}

Neutrophils, together with other innate immune cells, are involved in inflammation, interact with pathogens and/or damage associated molecular patterns (PAMPs and/or DAMPs). These molecular patterns are sensed through highly conserved pattern-recognition receptors (PRRs), called Toll-like receptors (TLRs). TLR2 recognizes lipoproteins

from Gram-positive bacteria with other wide range of components like glycopeptides, peptidoglycan, and lipoarabinomannan.⁶ TLR4 recognizes lipoproteins from Gram-negative bacteria and binds lipopolysaccharide (LPS) and lipooligosaccharide (LOS).^{7,8} A signaling cascade is initiated upon activation of PRRs that lead to the activation of nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- κ B), which up-regulate the production of inflammatory mediators, chemokines, and cytokines.^{9,10} Both TLR2 and TLR4 are widely studied and appear to have both endogenous and exogenous ligands. It has been shown that patients with COPD express higher TLR2 and TLR4 than healthy controls from airway samples,¹¹ but in peripheral blood neutrophils, these receptors have been lesser studied. TLR2 and TLR4 are believed to have a regulating role as a snare receptor in host innate immune response against pathogens. Therefore, it results in a negative regulation of cytokines and chemokines.

Environmental exposures are also triggering the innate immune response in COPD patients. A persistent innate immune activation is associated with COPD.¹² This persistent immune activation is concerned to the interaction of PRRs with pathogens, reactive oxygen species, and damaged cells, which lead to the development and exacerbations in COPD.^{13,14} There is an imbalance between proteases and antiproteases which leads to excessive proteinase activity that can cause host tissue damage in COPD.¹⁵ Neutrophils and macrophages are the major source of matrix metalloproteinase-9 (MMP-9) and neutrophil elastase (NE). Neutrophils get activated and release proteinase (including NE, MMP-9 and MMP-8), which have been shown to be increased in COPD.¹⁶ The neutrophil cells are also associated with interleukin-8 (IL-8) mediated neutrophilic inflammatory responses in the airways of COPD patients.¹⁷ Altered activities of neutrophilic cells are found in the peripheral blood of patients with stable COPD and upregulation of inflammatory genes¹⁸ with increased respiratory burst during exacerbations.¹⁹

Our study aimed to analyze the expression of innate immune receptors in peripheral blood neutrophils of patients with COPD compared to their healthy counterparts. However, there is no proper predictive marker for the early diagnosis of airway obstructions in patients with COPD.

Therefore, we examined the innate immune response in circulating neutrophils stimulated with TLR 2/4 agonist, LPS, and associated expression of IL-8 and MMP-9 while controlling for the effect of smoking, and the presence and severity of airway obstruction. To examine these effects, we also assessed the relationship between smoking status and airflow obstruction.

METHODS

This study was conducted at the Department of Respiratory Medicine, King George's Medical University, Lucknow, India and was approved by the ethics committee of the institution. All participants gave their written informed consent before inclusion in this study. We enrolled (n = 101) patients with COPD who were referred to our respiratory disease outpatient clinic and healthy controls (n = 101) having forced expiratory volume in the first second (FEV1) > 80% of predicted value and without any other respiratory disease in the study. All the patients were diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD).¹ Patients with COPD had to be in a stable state (no exacerbations in the last six weeks) for inclusion into the study. Patients with confirmed history of smoking and a FEV1/forced vital capacity (FVC) ratio of < 70% after salbutamol administration were enrolled in the study. Inclusion criteria for the healthy control group were the absence of COPD confirmed by history, physical evaluation, and spirometry. Patients were excluded if they had any other respiratory disease, acute infections, and other inflammatory diseases. Demographic characteristics, clinical assessment, medical history (including smoking status) of the studied participants were recorded. Peripheral blood samples were obtained from all participants.

Pulmonary function tests were performed using pulmonary function equipment (Cosmed, Italy) by a single technician. After three consecutive tests, the best test was accepted. According to American Thoracic Society guidelines, FEV1, FVC, and FEV1/FVC were measured and COPD staging was done according to GOLD 2013 criteria.¹

Peripheral blood neutrophils isolation was performed as per methodology previously used by Clark and Nauseef.²⁰ Neutrophil cells were resuspended in RPMI 1640 (Gibco Invitrogen,

Table 1: Primers used for Toll-like receptor 2 (TLR2), TLR4, and β -actin.

Gene	Forward primer	Reverse primer
TLR2	5'-TGCTTTCCTGCTGGAGATTT-3'	5'-TGTAACGCAACAGCTTCAGG-3'
TLR4	5'-TTCAAGACCAAGCCTTTCAG-3'	5'-CATAGTCCTTCCATGATAGA-3'
β -actin	5'-ATGGGTGAGAAGGACTCCTACG-3'	5'-AGTGGTACGACCAGAGGCATAC-3'

USA) supplemented with 10 mM HEPES, 1% fetal calf serum and 1% antibiotics (penicillin/streptomycin). Neutrophil cells were cultured at 1×10^6 cells/mL \pm LPS (100 ng/mL *Escherichia coli* LPS, Sigma, USA) at 37 °C (5% CO₂) for 24 hours. Cell-free supernatants were prepared for enzyme-linked immunosorbent assay (ELISA) and cell pellets stored in RLT buffer (Qiagen, Germany) at -80 °C for RNA extraction.

Total RNA was extracted from peripheral blood neutrophils stimulated by LPS using a commercially available RNA isolation kit (RNeasy Mini kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions. After purification of RNA we performed DNase I treatment to assure highly pure RNA without genomic DNA contamination. The quality and quantity of extracted RNA was determined by nanodrop. High capacity cDNA Reverse Transcription kit (Applied Biosystems Foster city CA, USA) was used reverse transcribed to cDNA from 100 ng of RNA. Real-time polymerase chain reaction (PCR) was performed by ABI 7500 real-time PCR machine (Applied Biosystem USA) using an ABI Power SYBR Green Master mix (Applied Biosystems, USA). The

primers used for TLR2, TLR4, and β -actin were shown in Table 1. The results were calculated using 2^{- Δ ct} method normalizing to the internal calibrator Beta-actin.

IL-8 and MMP-9 levels were determined in cell culture supernatant of peripheral blood neutrophils stimulated by LPS using the ELISA technique according to the manufacturer instructions (Ray Bio, Human IL-8/MMP-9 ELISA Kit, Norcross, USA).

Data were analyzed using GraphPad Prism version 5 (GraphPad software Inc.; La, Jolla, CA, USA). All data were expressed as mean \pm standard error of the mean (SEM). The chi-square test was used for categorical data and groups were compared by unpaired *t*-test or one-way analysis of variance (ANOVA). The Bonferroni test was applied for multiple comparisons. Spearman's rank correlation tests were used for determining associations between data. Data with $p < 0.050$ were considered significant.

RESULTS

Patients with moderate ($n = 15$), severe ($n = 65$) and very severe ($n = 21$) COPD were enrolled in the study according to the GOLD criteria. The

Table 2: Clinical characteristics of patients in the COPD and healthy control groups.

Subject characteristics	Control (n = 101)	COPD (n = 101)	p-value
Age, mean \pm SEM, years	52.9 \pm 1.1	55.6 \pm 0.9	0.070 ^a
Gender, M/F	69/32	85/16	0.008 ^b
Height, mean \pm SEM, cm	161.1 \pm 0.9	160.2 \pm 0.9	0.460 ^a
Weight, mean \pm SEM, kg	62.8 \pm 1.3	50.6 \pm 1.0	0.001 ^a
BMI, mean \pm SEM, kg/m ²	24.1 \pm 0.4	19.8 \pm 0.4	0.001 ^a
Smoking, never/smoker	68/33	28/73	0.001 ^b
Pack years, mean \pm SEM	18.7 \pm 1.3	32.8 \pm 1.8	0.001 ^a
Post FVC, mean \pm SEM, %	88.7 \pm 0.9	59.7 \pm 1.4	0.001 ^a
Post FEV1 predicted, mean \pm SEM, %	89.8 \pm 0.7	37.6 \pm 1.0	0.001 ^a
Post FEV1/FVC, mean \pm SEM, %	101.6 \pm 0.7	61.2 \pm 0.7	0.001 ^a

Data were expressed in mean \pm standard error of the mean (SEM). $p < 0.050$ was considered significant.

^aUnpaired *t*-test, ^bchi-square test; FVC: forced vital capacity; BMI: body mass index; FEV1: forced expiratory volume in the first second; COPD: chronic obstructive pulmonary disease.

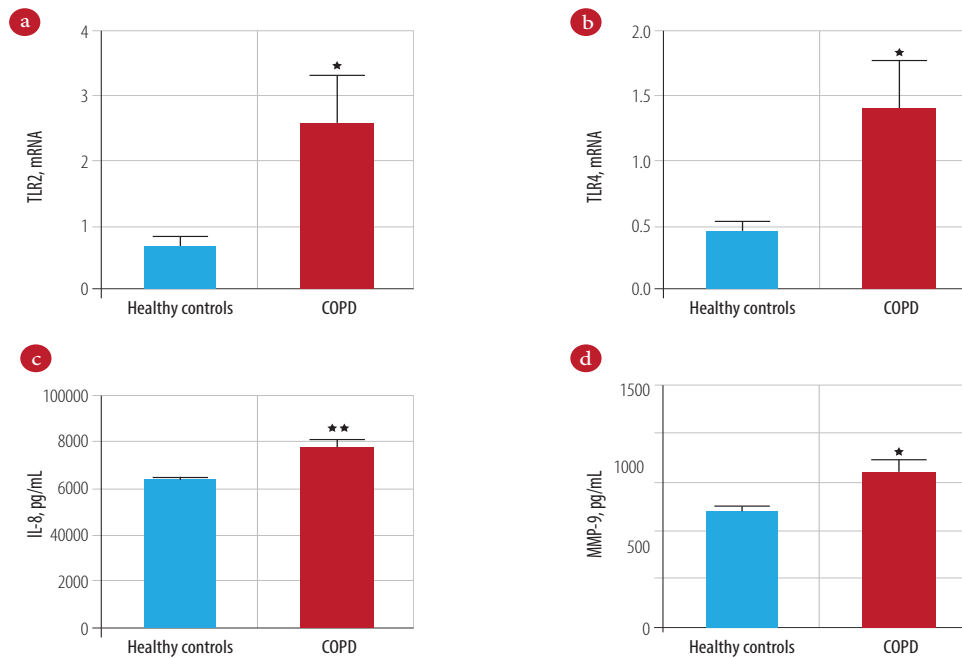


Figure 1: Comparison of innate immune mediators between the chronic obstructive pulmonary disease (COPD) and control groups. Gene expression of Toll-like receptor 2 (TLR2) (a) and Toll-like receptor 4 (TLR4) (b) measured by real-time polymerase chain reaction (PCR) in COPD (n = 101) and controls (n = 101). Protein level of interleukin-8 (IL-8) (c) and metalloproteinase-9 (MMP-9) (d) measured by enzyme-linked immunosorbent assay (ELISA) in COPD (n = 101) and controls (n = 101). Data presented as mean ± standard error of the mean (SEM) and compared by unpaired *t*-test. **p*-values < 0.050 are considered significant. ***p*-values < 0.010 vs. the healthy control group.

demographic and clinical characteristics of all the participants are shown in Table 2. The mean age of patients in the COPD and control group were not significantly different ($p = 0.070$). The male/female sex ratio of COPD patients was higher compared to the healthy control group ($p = 0.008$). A significant difference was found in smoking history and pack years smoked ($p = 0.001$). In the control group, it was 18.7 ± 1.3 pack years and 32.8 ± 1.8 pack years in the COPD group. COPD patients had significantly lower lung function compared to healthy controls. The pulmonary function tests of patients in the COPD group showed predicted mean post-bronchodilator FEV1 of $37.6 \pm 1.0\%$ and a predicted FVC of $59.7 \pm 1.4\%$ with a mean FEV1/FVC ratio of $61.2 \pm 0.7\%$. The healthy controls showed FEV1 of $89.8 \pm 0.7\%$ and FVC $88.7 \pm 0.9\%$ with a mean FEV1/FVC ratio of $101.6 \pm 0.7\%$.

The gene expression of TLR2 on peripheral blood neutrophils was significantly higher in the COPD group compared to the control group [Figure 1a; $p = 0.012$]. Similarly, TLR4 gene expression was also increased in the COPD group compared to the control group [Figure 1b; $p = 0.015$]. Patients in

the COPD group had significantly higher levels of IL-8 [Figure 1c; $p = 0.004$] and MMP-9 [Figure 1d; $p = 0.010$] protein than patients in the control group.

The effect of smoking was investigated by further analysis of innate immune mediators in smokers and nonsmokers in both the COPD and control groups. Patients with COPD that smoked had significantly higher TLR2 gene expression [Figure 2a; $p = 0.047$] and TLR4 [Figure 2b; $p = 0.012$] and significantly increased levels of IL-8 [Figure 2c; $p = 0.016$] and MMP-9 [Figure 2d; $p = 0.043$] compared to smokers in the control group and all nonsmokers. However, there was a strong correlation observed between the smoking history (pack years) with IL-8 marker and measures of airflow obstructions. Smoking pack years was significantly positively correlated with IL-8 levels ($p = 0.002$) and negatively correlated with FEV1% predicted ($p = 0.023$) and FEV1/FVC ($p = 0.011$) [Table 3].

To examine the effect of severity in COPD, we compared patients with very severe (n = 21) and severe COPD (n = 65) to patients with moderate COPD (n = 15). We found the gene expression of TLR2 was higher in very severe COPD, but

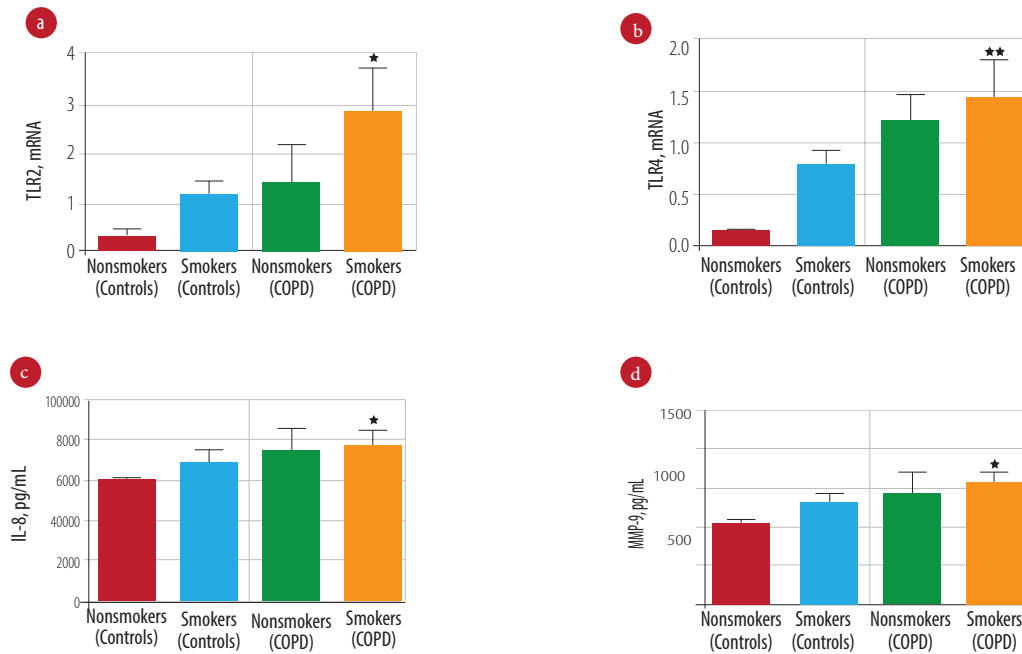


Figure 2: Comparison of innate immune mediators between patients in the chronic obstructive pulmonary disease (COPD) (smokers and nonsmokers) and control groups (smoker and nonsmokers). Gene expression of Toll-like receptor 2 (TLR2) (a) and Toll-like receptor 4 (TLR4) (b) measured by real-time polymerase chain reaction (PCR) in COPD (smokers (n = 73), nonsmokers (n = 28)) and control group (smokers (n = 33) and nonsmokers (n = 68)). Protein level of interleukin-8 (IL-8) (c) and metalloproteinase-9 (MMP-9) (d) measured by enzyme-linked immunosorbent assay (ELISA) in COPD (smokers (n = 73) and nonsmokers (n = 28)) and control group (smokers (n = 33) and nonsmokers (n = 68)). Data presented as mean± standard error of the mean (SEM) and compared by one-way analysis of variance (ANOVA) test followed by Bonferroni post-test for multiple comparisons. ***p*-values < 0.010 vs. the nonsmokers control group. **p*-values < 0.050 vs. the nonsmokers control group.

this was not statistically different from severe to moderate COPD patients [Figure 3a; *p* = 0.354]. Reduced expression of TLR4 gene was found in very severe COPD patients [Figure 3b; *p* = 0.041]. There was a significantly elevated level of IL-8 in very severe COPD patients compared to severe and moderate COPD patients [Figure 3c; *p* = 0.026]. However, increased levels of MMP-9 in very severe

COPD patients was not statistically significant [Figure 3d; *p* = 0.058].

DISCUSSION

Inflammation and inflammatory mediators are important components of occurrence and progression of COPD. Neutrophils are the primary component of the innate immune response and play a key role in the COPD-associated inflammatory process. In this study, circulating neutrophils was isolated from patients with COPD and healthy controls and the role of TLR2, TLR4, IL-8, and MMP-9, in the context of smoking and COPD severity, was evaluated. The increased expression of TLR2/4 in circulating neutrophils of COPD indicated chronic activation of innate immune responses. Similarly, the increased levels of IL-8 and MMP-9 in COPD patients may suggest that the COPD is an inflammatory condition [Figure 1]. Furthermore, we also observed a correlation of

Table 3: Correlation between pack years, IL-8 level, and spirometry parameters in peripheral blood neutrophils of patients with COPD.

Characteristics	Smoke pack years	
	r	<i>p</i> -value
IL-8 level	0.44	0.002
Post FEV1%	-0.33	0.023
Post FEV1/FVC	-0.27	0.011

IL-8: interleukin 8; COPD: chronic obstructive pulmonary disease; FEV1: forced expiratory volume in the first second; FVC: forced vital capacity. Coefficient of correlation. *p* < 0.050 was considered significant.

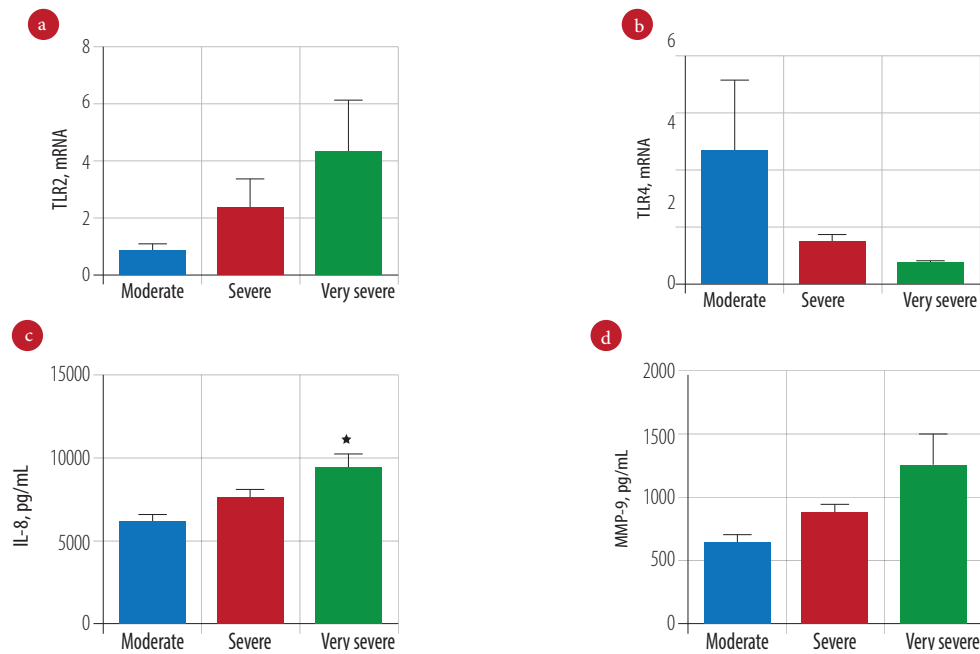


Figure 3: Comparison of innate immune mediators according to the severity of chronic obstructive pulmonary disease (COPD). Gene expression of Toll-like receptor 2 (TLR2) (a) and Toll-like receptor 4 (TLR4) (b) measured by real-time polymerase chain reaction (PCR) in moderate (n = 15), severe (n = 65), and very severe COPD patients (n = 21). Protein level of interleukin-8 (IL-8) (c) and metalloproteinase-9 (MMP-9) (d) measured by enzyme-linked immunosorbent assay (ELISA) in moderate (n = 15), severe (n = 65), and very severe COPD patients (n = 21). Data presented as mean \pm standard error of the mean (SEM) and compared by one-way analysis of variance (ANOVA) test followed by Bonferroni post-test for multiple comparisons. **p*-values < 0.050 are considered significant.

smoke pack years with inflammatory markers and pulmonary function in terms of FEV1% and FEV1/FVC. There is an association between smoking and more inflammation in COPD when these patients were compared with nonsmokers. The level of all the studied inflammatory markers is higher in smokers with COPD. Further, smoke pack years is positively correlated with IL-8 levels and negatively correlated with FEV1% and FEV1/FVC. This results support that the inflammatory neutrophil granulocyte is one cause of COPD and smoking is driving this factor [Figure 2 and Table 3]. We also found an association between the severity of COPD and inflammation. However, this was not statically significant, possibly due to the small sample size [Figure 3].

Cigarette smoking is a known risk factor for COPD and our study exhibits a greater history of smoking in patients with COPD [Table 2]. Cigarette smoke induces an inflammatory response that includes macrophage, neutrophil, monocytes, T-lymphocyte attracting factor, proinflammatory mediators, and proteolytic enzymes.^{21,22} Many previously conducted studies have suggested

the importance of upregulated cytokine and inflammatory genes; also establishing the migration and cytotoxic responses of systemic neutrophils in COPD.^{11,18,19,23–25} Neutrophils may release an extensive quantity of IL-8, which adds to the positive feedback circle in COPD. We observed that the level of IL-8 was higher in smokers with COPD compared to nonsmoking healthy controls. We also observed a significant increase in the level of IL-8 with increasing COPD severity. The results of the current study are consistent with previous studies in serum and bronchoalveolar fluids from patients with mild, moderate, and severe COPD.^{26,27} They found that IL-8 levels increased with increasing severity of COPD. An increased level of IL-8 in peripheral blood neutrophils of COPD patients suggests a systemic inflammatory effect on these patients.

MMP-9 is one of the most important MMP in its family and is responsible for tissue repair and remodeling.^{28,29} The increased level of MMP-9 may significantly increase the elastolytic load in the lungs, which accelerate the loss of lung functions. An Egyptian study demonstrated that MMP-9 level

was increased in patients with COPD and smokers in the healthy control group compared with healthy nonsmokers.³⁰ Similarly, another study showed the levels of MMP-9 were significantly higher in COPD and the levels were increasing in severe to very severe stages.³¹ Our study showed MMP-9 levels were significantly higher in smokers with COPD compared to nonsmoking healthy controls. MMP-9 levels also increase in very severe stages of COPD compared to patients with severe and moderate forms of the disease. However, the difference was not statically significant between these groups. This increased level of MMP-9 may lead to the destruction of the extracellular matrix in airways and may contribute to decline the lung function of COPD.

The role of TLRs in COPD pathogenesis is an emergent interest, along with its association between altered expression of TLRs and cigarette smoke exposure.³² TLR2 and TLR4 upon activation, activate the NF- κ B pathway that regulates neutrophil activation, migration, and survival.³³ Decreased expressions of TLR2 have been reported in macrophages from cigarette smokers and patients with COPD.³⁴ In addition, LPS stimulation on macrophages also does not increase TLR2 mRNA and protein expression. Alternatively, other studies have reported an upregulation of TLR2 in circulating blood neutrophils and peripheral blood-derived monocytes of patients with COPD.^{11,35} Similarly, Simpson et al,³⁶ also reported an increased expression of TLR2 in COPD, which increases in severe to very severe stages. In accordance with the above-mentioned studies, our results also demonstrate increased expression of TLR2 in peripheral blood neutrophils of smokers with COPD.^{11,35,36} It is also increasing parallel and proportional to the disease severity. However, the difference was statistically insignificant. This may be due to the severity induced upregulation of TLR2 receptors in a small number of subjects in this group. These findings indicate that upon activation, neutrophils generate an innate immune response in an enhanced manner that is important in COPD pathogenesis.

The role of TLR4 has been linked to COPD exacerbations in the context of bacterial infections and inflammations. Previous studies conducted on neutrophils and monocyte-derived macrophages demonstrating the involvement of TLR4 have reported the upregulated expression upon LPS

stimulation in COPD.^{11,37} Similarly, another study reported a correlation between cigarette smoke exposure and the increased gene expression of TLR4 and TLR9 in addition to excess production of cytokine.³⁸ Downregulation of TLR4 gene expressions was reported in the nasal epithelium of smokers and severe COPD patients compared to nonsmoking controls and patients with less severe COPD.³⁹ We found the gene expression of TLR4 is higher in smokers with COPD compared to nonsmoking healthy controls. Interestingly, we found that the TLR4 expression dips during very severe COPD compared to moderate COPD; though it was still higher than nonsmoker controls. Although the response mechanism of innate immunity to cigarette smoke is unclear, it has been projected that injured airways epithelium produces a danger signal or DAMPs that may act as ligands for TLRs such as TLR2 and TLR4.⁴⁰ This activates the transcription of proinflammatory cytokines such as IL-1, IL-6, and IL-8.³⁹ We also observe the number of pack years smoked is positively correlated with inflammatory mediator IL-8 and negatively correlated with the severity of airflow obstruction. These results indicate that cigarette smoking may trigger the release of chemoattractant from epithelial cells. These chemoattractant may promote the neutrophils recruitment, which is related to the production of innate immune mediators that results in the decline of pulmonary functions.

CONCLUSION

This study demonstrates that LPS stimulation in peripheral blood neutrophil increases innate immune response, showing upregulation of TLR2 and TLR4 mRNA with increased release of IL-8 and MMP-9 protein in COPD. This outcome supports the possibility of activation of innate immune response, which is an important mechanism of disease pathogenesis. Henceforth, TLR2 and TLR4 may be considered as a diagnostic marker in COPD patients. Thus, we believe that TLR2 and TLR4 may be exploited in the future for therapeutic target discovery.

Disclosure

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