Increased neutrophil expression of pattern recognition receptors during COPD exacerbations


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ABSTRACT
Previously, we observed increased serum levels of damage associated molecular patterns (DAMPs) during COPD exacerbations. Here, gene expression of DAMP receptors was measured in peripheral blood neutrophils of COPD patients during stable disease and severe acute exacerbation. The expression of TLR2, TLR4 and NLRP3 was significantly increased, while serum levels of the decoy receptor sRAGE were decreased during exacerbation. Together, these data indicate that increased DAMP signaling contributes to activation of neutrophils during COPD exacerbations.
Chronic obstructive pulmonary disease (COPD) is a progressive pulmonary disease characterized by persistent airflow limitation and an enhanced chronic airway inflammatory response, and is caused by chronic exposure to noxious gases and particles. Exacerbations, the sudden worsening of symptoms, are amongst the most important drivers of accelerated lung function decline and loss of quality of life in COPD. Acute, severe exacerbations regularly lead to hospital admissions and increased morbidity and mortality. The pathophysiology of COPD exacerbations is still largely unknown, and few effective treatment options are available. Recently, it has been proposed that the suppression of the innate immune system may be key in the treatment of COPD exacerbations. An increase in airway neutrophils is frequently observed during exacerbations, and neutrophil numbers correlate with the decrease in lung function during exacerbation.

Evidence for a role of damage associated molecular patterns (DAMPs) in the pathophysiology of COPD is emerging. DAMPs are molecules released from damaged or dead cells that activate the innate immune system by binding to pattern recognition receptors (PRRs), e.g. toll like receptor (TLR)2, TLR4, TLR9, the receptor for advanced glycation end-products (RAGE) and the NLR family, pyrin domain containing 3 (NLRP3) inflammasome. Activation of PRRs by DAMPs can induce recruitment and activation of immune cells, including neutrophils. In COPD patients, the levels of several DAMPs have been shown to be increased compared to smoking and non-smoking controls, both systemically and locally in the lungs. Of interest, the \textit{AGER} gene encoding the RAGE receptor was identified as a susceptibility gene for lung function decline and onset of COPD. RAGE is present as a transmembrane receptor, and as a soluble form (sRAGE), the latter acting as an immuno-suppressive decoy receptor for RAGE ligands. Recently, we have shown that serum levels of the RAGE-activating DAMPs HMGB1, S100A9 and LL-37 are increased in COPD patients during exacerbation compared to stable disease. These DAMPs can activate PRRs on neutrophils to induce their recruitment.

No studies have been performed investigating PRRs in severe acute exacerbations necessitating hospitalization. In the current study, we hypothesized that the expression of PRRs on neutrophils is increased in COPD patients during acute exacerbations compared to stable disease.

Therefore, we analyzed the mRNA expression of well-known PRRs, i.e. TLR2, TLR4, TLR9, NLRP3 and AGER, in neutrophils isolated from whole blood of COPD patients during exacerbation and stable disease. Furthermore, we measured the levels of sRAGE in serum of these patients. To this end, we included fourteen patients from a prospective trial on acute COPD exacerbations requiring hospital admission. An exacerbation was defined as a worsening of respiratory symptoms from the stable disease state that is beyond normal day-to-day variations and requires additional treatment. All patients were hospitalized for the exacerbation and were treated with systemic steroids and additional salbutamol/ipratropium by nebulizer. We collected serum and blood samples during hospitalization and again 42 days later during stable disease. For patient characteristics see Figure 1A. This study was approved by the medical ethics committee of the University Medical Center Groningen (Groningen, the Netherlands) and is registered in the WHO and ICMJE approved Dutch trial registry (NTR4600). All participants signed informed consent.

Peripheral blood granulocytes were isolated using Lymphoprep™ density gradient medium (Fresinius Kabi, Bad-Homburg, Germany), and consisted of ≥90% neutrophils for all samples, as assessed by the Sysmex XT-1800iV (Sysmex, Hyogo, Japan). RNA was isolated from neutrophils using TRIzol (ThermoFisher Scientific, Waltham, MA) and cDNA was synthesized using the \textit{iScript} cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantification of PRR gene expression was performed using TaqMan technology with the ABI 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) and primer/probes sets specific for the target genes (Invitrogen Life Technologies, Carlsbad CA). sRAGE was measured using the human RAGE DuoSet ELISA (R&D Sytems, Minneapolis).

The mRNA expression of TLR2, TLR4 and NLRP3 was significantly increased in neutrophils of COPD patients during acute exacerbation compared to stable disease (Figure 1B, C, E), while the mRNA expression of TLR9 and AGER did not differ significantly between stable disease and exacerbation (Figure 1D, F). In contrast, the serum levels of sRAGE were significantly decreased during COPD exacerbations compared to stable disease (Figure 1G).
The increased expression of TLR2, TLR4 and NLRP3 on neutrophils may lead to increased sensitivity of peripheral blood neutrophils to DAMPs released during COPD exacerbations. Previously, we have shown that during COPD exacerbations, levels of circulating HMGB1, S100A9 and LL-37 are increased.\(^8\) In addition to binding RAGE, both HMGB1 and S100A9 have been shown to activate TLR2 and TLR4. The activation of TLR2/4 induces activation and migration of neutrophils and may thus contribute to the increased airway inflammation during COPD exacerbations.\(^{10}\) Furthermore, a positive feedback loop has been described in which DAMPs not only activate TLR4, but also induce TLR4 upregulation indicating that the increased expression of TLRs may be a consequence of DAMP release.\(^8\) Thus, increased neutrophilic expression of TLR2 and TLR4 in combination with increased DAMP levels may contribute to DAMP-induced neutrophilic airway inflammation during COPD exacerbations. In addition to DAMPs, TLR2 and TLR4 are also receptors for pathogen-associated molecular patterns (PAMPs). Therefore, PAMPs may also contribute to the inflammatory reaction during airway infection-associated exacerbations. NLRP3 is known to be activated by ATP, a DAMP shown to be increased in BAL fluid of COPD patients.\(^5\) NLRP3 activation on neutrophils leads to release of the pro-inflammatory cytokines IL-1β and IL-18, and has been implicated in the development of COPD.\(^5,11\)

Our findings on reduced sRAGE levels during exacerbation are in line with literature, showing reduced plasma levels of sRAGE during COPD exacerbations.\(^12\) In contrast, our previous study did not show a significant difference in serum sRAGE levels between stable disease and exacerbation,\(^8\) which may be due to the lower severity of the exacerbations in that study. The decrease in circulating sRAGE along with the increase in RAGE agonists,\(^8\) but without alterations in AGER expression on neutrophils, may lead to increased RAGE signaling-mediated activation of neutrophils during COPD exacerbations.

Although our study strengthens the hypothesis that DAMPs and PRRs are involved in the pathophysiology of COPD exacerbations, there are some limitations to this study. We cannot exclude the possibility that the use of corticosteroids has affected the outcome of our studies. Multiple studies have shown that corticosteroid treatment downregulates TLR2 and TLR4 expression on airway epithelial cells, fibroblasts and monocytes, suggesting that the observed TLR upregulation was not induced by corticosteroid treatment.\(^{13-14}\) Nevertheless, another study showed that corticosteroid treatment increases TLR2 expression on airway epithelial cells.\(^15\) Furthermore, cigarette smoking may affect TLR expression. Since only four out of fourteen subjects in our study were current smokers, it was not possible to assess the effect of cigarette smoking in our study. Previously, it has been shown that nasal epithelial TLR4 expression levels are decreased in smokers compared to non-smokers, while the expression of TLR2 was unchanged.\(^16\) On the other hand, it has been shown that TLR4 expression was increased in bronchial and nasal epithelial cells by CSE exposure in vitro.\(^{17-18}\) Obviously, monocytes and macrophages may also contribute to the DAMP-induced inflammatory reaction during COPD exacerbations, inducing the attraction of neutrophils upon activation by DAMPs. Furthermore, the sample size in our study was relatively small and therefore we were unable to differentiate between various causes of COPD exacerbations, e.g. bacterial or viral airway infection or unknown. In future studies, it will be of interest to assess whether the inhibition of specific PRRs during COPD exacerbations decreases neutrophil activation and migration in in vitro and in vivo models.

In conclusion, we show for the first time that TLR2, TLR4 and NLRP3 expression in neutrophils is increased during acute exacerbations of COPD compared to stable disease. Furthermore, we show that circulating sRAGE levels are decreased during acute COPD exacerbations. These data strengthen the hypothesis that DAMP signaling plays an important role in the activation of neutrophils during COPD exacerbations, which provides novel avenues for therapeutic strategies aimed at DAMP receptors.

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**Figure 1: The mRNA expression of PRRs during COPD exacerbations and stable disease.**

A) Patient characteristics. Data shown as mean (±SD). Three out of the fourteen patients had a bacterial and a viral infection simultaneously during exacerbation. BMI: Body Mass Index; FEV₁: Forced Expiratory Volume in 1 second; FVC: Forced Vital Capacity. B-F) The mRNA expression ($2^{\Delta\Delta CT}$) of TLR2, TLR4, TLR9, NLRP3 and AGER in peripheral blood neutrophils of COPD patients during exacerbation and stable disease. G) The levels of sRAGE in serum of COPD patients during exacerbation and during stable disease. Median ± interquartile range are indicated. Fourteen patients were included, for three patients RNA samples during stable disease were unavailable. The GOLD stage of the patients is indicated using colors, black for GOLD stage IV, dark grey for GOLD stage III, light grey for GOLD stage II and white for GOLD stage I. Significance was tested using a Wilcoxon Signed Rank test, *= P<0.05, **=P<0.01, ***=P<0.001.
References


