Exacerbations of COPD correlate with down-regulation of cytokine-inducible genes in peripheral blood neutrophils

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Abstract

Chronic obstructive pulmonary disease (COPD) is associated with systemic inflammation, which is in part mediated by increased levels of inflammatory cytokines/chemokines and by (pre)activation of peripheral blood neutrophils. To investigate systemic inflammation during acute exacerbations of the disease, we analyzed the expression of cytokine-induced inflammatory genes in peripheral blood neutrophils from frequently exacerbating COPD patients during a stable phase of the disease compared to an exacerbation. Data were obtained by using real time reverse transcriptase-polymerase chain reaction.

The expression of several pro-inflammatory genes, including CD83, IL-1β, IL-1RA, IL-8 and MIP-1β was increased in patients with moderate to severe COPD (Gold class IIa and III). In contrast to our expectation the expression of these genes was actually decreased in peripheral blood neutrophils during an exacerbation of the disease to levels similar to that of neutrophils obtained from normal donors. This was most evident for CD83 and, therefore, we utilized this marker for an in vitro mechanistic study. TNFα induced CD83 mRNA in a dose and time dependent manner in neutrophils in vitro. Interestingly, CD83 was dramatically down-regulated by simultaneous incubation of neutrophils with granulocyte-macrophage colony-stimulating factor (GM-CSF) and TNFα. This effect was dose dependent and optimal at 100 pM GM-CSF. This GM-CSF-induced effect is independently of IκBα phosphorylation. Thus, acute exacerbations of COPD are associated with a clear down-regulation of inflammatory genes in circulating neutrophils, which might be caused by a change in the levels of systemic inflammatory mediators.
Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic inflammatory disease of the airways, which is characterized by the presence of chronic airflow obstruction and chronic inflammation in the lower airways. Several studies have monitored local inflammation in COPD by measuring granulocyte activation at the site of inflammation (1-3). Additional studies have measured cytokine levels in the sputum to monitor disease progress (4-6).

In contrast to this relatively well-studied local inflammation, little is known concerning the systemic inflammatory response in COPD. A number of studies provide evidence that local inflammation is reflected by cytokine release into the systemic circulation. However, several inflammatory mediators such as tumor necrosis factor (TNF)-α are difficult to detect in the peripheral blood because of their short half-life, binding to soluble receptors, and renal clearance from the peripheral blood (7-9). Recent studies showed that levels of soluble TNF-receptor (sTNF-R)55 and sTNF-R75 were increased in the peripheral blood of patients with COPD, which is thought to reflect TNFα induced inflammation (9-11).

Other studies reported increased levels of eosinophil cationic protein (ECP) and myeloperoxidase (MPO) in the serum of COPD patients (12), which are indirect measurements for pre-activation of granulocytes. Indeed, initial data have shown that inflammatory cells in the peripheral blood of patients with chronic pulmonary diseases are characterized by a primed phenotype (13-15). Despite the increasing evidence that granulocytes are activated in the peripheral blood from patients with COPD, little is known regarding the identity of the inflammatory mediators and the type and kinetics of the inflammatory response. Even less is known regarding systemic inflammation during exacerbations of COPD.

In this study the activation status of peripheral blood neutrophils was examined as read-out for the chronic inflammatory response during acute exacerbations of COPD. This was achieved by analysis of the expression of inflammatory genes in peripheral blood neutrophils in vivo and in vitro using real time RT-PCR. We compared gene profiles of neutrophils, stimulated with the pro-inflammatory cytokine TNFα in vitro with the gene profiles of neutrophils isolated from patients with COPD before and at time of exacerbation of their disease. Pro-inflammatory genes such as CD83, IL-1β and MIP-1β were upregulated in neutrophils obtained from severe COPD patients. These genes are typically induced by TNF in vitro (see Chapter 2). Surprisingly, these genes were down-regulated in peripheral blood neutrophils during acute exacerbation of the disease. These findings demonstrate a change in systemic inflammation during an acute exacerbation of COPD.
Materials and Methods

Reagents
Formyl-methionyl-leucyl-phenylalanine (fMLP) and LPS were purchased from Sigma (St. Louis, MO, USA). Recombinant human GM-CSF was from Genzyme (Boston, MA, USA). Recombinant human TNF-α was purchased from Boehringer Mannheim (Mannheim, Germany). IL-8 was purchased from Peprotech (Rocky Hill, NJ, USA). Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands). RPMI 1640 medium with Glutamax was purchased from Life Technologies (Breda, The Netherlands). Ficoll-Paque was from Pharmacia (Uppsala, Sweden). Anti-phospho-IκBα (ser32) was from New England Biolabs (Beverly, MA, USA). Actin (I-19) polyclonal antiserum was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Subjects
Six patients were enrolled into the study with unstable moderate to severe (class IIb and III) COPD according to the inclusion criteria of the GOLD guidelines (16). All patients had in the year prior to the inclusion to the study at least two exacerbations requiring hospital admission. Patients also had to meet the following additional inclusion criteria: a FEV₁ < 80% and a reversibility of the airway obstruction of less than 10% or less than 200 ml after inhalation of a β2-agonist and a FEV1/FVC of less than 70%. A history of other concomitant confounding diseases such as diabetes mellitus, lung carcinoma, cardiovascular disease and bronchiectasis were excluded from the study. Also patients with a history of asthma or atopy were excluded. Patients were on standardized therapy with a muscarinic receptor antagonist (b.i.d), long acting β2-agonist (b.i.d.), inhaled cortico-steroids if necececcary, and they were allowed to use a short acting β2-agonist for rescue medication. These COPD patients were followed for nine months and disease status was monitored every two weeks during a visit to the out patient clinic. During every visit a medical history, Borg score (17), lung function (FEV₁ and FVC), and routine laboratory tests were performed. Blood samples were taken during these visits to investigate peripheral blood neutrophil gene expression during an exacerbation(s) before treatment of the exacerbation and during stable phases of their disease. The presence of an acute disease exacerbation was diagnosed according to Anthonisen (18), being an increase of dyspnoea, cough, or sputum production resulting in hospital admission. During exacerbations patients were treated with antibiotics (levofloxacin 500 mg once a day for fourteen days) and glucocorticosteroids intravenously (Di-Adreson F-aquosum 50 mg for 7 days). A stable phase was defined as at least 4 weeks before or after an acute exacerbation and no presence of a respiratory tract infection or respiratory complaints during 4 weeks prior or after sampling. The mean age of the patient group was 63.3 ±4.9
The mean FEV1 was 29.8 ± 3.4% (range 19.1%-39.9%). Eleven healthy subjects with a normal lung function were enrolled as controls. The study was approved by the Medical Ethics Committee of the University Medical Center, Utrecht, and informed consent was obtained from all subjects.

Isolation of human neutrophils

Blood was obtained from healthy volunteers and COPD patients. Mixed granulocytes were isolated from 50-100 ml blood (normal volunteers) or 20 ml (COPD patients), which was anti-coagulated with 0.32% sodium citrate. Blood was diluted 2.5:1 with PBS containing 0.32% sodium citrate and human pasteurized plasma-protein solution (4 g/L). Mononuclear cells were removed by centrifugation over Ficoll-Paque for 20 minutes at 2000 rpm. The erythrocytes were lysed in isotonic ice-cold NH₄Cl solution followed by centrifugation at 4°C. Granulocytes were allowed to recover for 30 minutes at 37°C in Hepes buffered RPMI 1640 medium, supplemented with L-glutamine and 0.5% HSA. All preparations contained >97% neutrophils.

RNA isolation and cDNA synthesis.

RNA isolation was performed as previously described (19). Briefly, 2×10^7 cells were washed with ice-cold PBS and lysed in 0.4 mL of GIT solution (6 mol/L guanidine thiocyanate, 25 mmol/L sodium citrate, 0.5% N’-lauroyl sarcosine, 100 mmol/L β-mercaptoethanol). After storage at −20°C, phenol extraction was performed followed by ethanol precipitation. Total RNA was resuspended in a small volume of H₂O and treated with DNase for half an hour. After DNase treatment RNA was again extracted with phenol, precipitated with ethanol and RNA was then eluted in 10 µl of RNAse-free H₂O. cDNA was synthesized using MMLV reverse transcriptase and oligo(dT) primers. Samples containing 1 µg of total RNA were heated for 3 minutes at 65°C and quickly chilled on ice. A mixture of 25 µl containing 20 µg/ml oligo(dT) primers, 5 µl of 5x RT buffer, 20 mM DTT, 2 mM each deoxynucleoside triphosphate, 0.8 U/µl of RNAse inhibitor and 400 units of MMLV reverse transcriptase was added and incubated at 37 °C for 90 minutes, followed by RT inactivation for 10 min at 65°C. cDNA was stored at -20°C before further use. All reagents used for cDNA synthesis were obtained from Life Technologies (Breda, The Netherlands)

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

CD83 gene was analyzed by Taqman real time pcr as described in Chapter 2. All the other genes were analyzed by real time pcr using SYBR green I (Chapter 2). Amplification and detection of both Taqman and SYBR green I pcr were performed with an ABI Prism 7700 sequence detection system (PE Applied Biosystems, Nieuwerkerk a/d ijssel, The Netherlands) under the following conditions: 2 minutes at 50°C, 10 minutes at 95°C to
activate AmpliTaq Gold DNA polymerase, and 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. During amplification, the ABI Prism sequence detector monitored real-time PCR amplification by quantitatively analyzing fluorescence emissions. The signal of the dye was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations occurring from well to well. Results were normalized for the housekeeping gene β-actin and GAPDH. A reference sample of cDNA on every 96 wells plate allowed correction of differences between plates. Results were expressed as fold regulation.

**Western blotting**
Cytosolic extracts were prepared by the same method as for obtaining nuclear extracts in the section EMSA. Samples were normalized for protein content and boiled for 5 min at 95°C in the presence of Laemmli sample buffer. Proteins were analyzed on 10% SDS-polyacrylamide gels and transferred to Immobilon-P. The blots were blocked in hybridization buffer (10 mM Tris, 150 mM NaCl and 0.3% Tween-20) containing 5% BSA for 1 hour followed by incubation with polyclonal anti-phospho-IκBα (ser32) (1/2000) in hybridization buffer with 0.5% BSA for 1 hours at room temperature. Second antibody was incubated in hybridization buffer for 1 hour. The western blots for hybridization with anti-actin were blocked in hybridization buffer containing 5% non-fat milk for 1 hour followed by incubation with anti-actin (1/1000) in hybridization buffer with 0.5% non-fat milk for 2 hours at room temperature. Second antibody was incubated in hybridization buffer containing 0.5% non-fat milk for 1 hour. Detection of all western blots was performed by enhanced chemiluminescence (ECL; Amersham, UK).

**Statistics**
Unless otherwise noted, all data are expressed as the mean ± SE, and differences between values were compared for significance by the paired t-test. Differences between study groups were compared using the Mann-Whitney U test. The Wilcoxon signed rank test was used for comparisons within an individual between their stable phase and exacerbation. Statistical significance was defined as p<0.05.
Results

**Exacerbations of COPD are associated with down-regulation of cytokine inducible genes in peripheral blood neutrophils.**

Neutrophils isolated from patients with moderate to severe COPD appeared to have a markedly increased expression of several inflammatory genes, compared to cells obtained from normal control donors (see Chapter 2). These genes are typically induced by pro-inflammatory cytokines, such as TNFα. We tested the hypothesis that exacerbations of COPD would be reflected in a more pronounced expression of these genes, since increased levels of inflammatory cytokines have been found in the airways of patients with acute exacerbations of COPD as compared to a stable phase of their disease (1). We focused on a small set of regulated genes, including IL-1β, IL-1RA, IL-8, IL-8R1, MIP1β and CD83 to analyze their expression during stable phases and during exacerbations of COPD patients. cDNA was prepared from total RNA of cells from healthy volunteers and of above mentioned COPD patients, and was used for real time RT-PCR analysis of the indicated genes (Fig 1). Pro-inflammatory genes such as CD83, MIP-1β, and IL-1β were significantly increased in severe COPD patients compared to healthy volunteers (Fig 1, see also Chapter 2).

Interestingly, neutrophils isolated from these patients were characterized by a clear and significant decrease in CD83, MIP-1β, IL-1β, and IL-8 expression at the time of an exacerbation of their disease compared to the stable phase (Fig.1). The expression of IL-1RA and IL-1β returned to the level of healthy volunteers. Expression of CD83, MIP-1β and IL-8 was reduced to a level even lower than that of healthy volunteers. Not all analyzed genes were down-regulated in neutrophils from COPD patients with an exacerbation. For example the IL-8R1 gene was not significantly influenced.

**Kinetics of TNFα induced CD83 expression in neutrophils in vitro.**

The results obtained with CD83 were the most striking of all genes analyzed. To gain more insight into the mechanism underlying this phenomenon, we studied expression of CD83 under different conditions in vitro. We and others have shown that this gene is specifically induced by inflammatory mediators that activate the transcription factor NFκb, including TNFα and LPS (see Chapter 2,(20-24). We first set out experiments to study the characteristics of TNFα-induced activation of neutrophils in terms of expression of CD83 mRNA in these cells. Neutrophils were stimulated with TNFα for various periods of time to analyze the kinetics of TNFα-mediated upregulation of CD83 expression. CD83 expression was measured by real time RT-PCR as described in Materials and Methods. Specific primers were used to amplify only cDNA generated from CD83 mRNA and not genomic DNA. CD83 expression was normalized for β-actin
Exacerbations of COPD correlate with down-regulation of genes in neutrophils expression. Increased CD83 expression was already observed within 30 minutes of TNFα stimulation, and was further elevated over time (Fig. 2A). Maximal increase of CD83 expression was reached between 2-3 hours. Next, neutrophils were incubated with increasing concentrations of TNFα for 3 hours to determine the dose dependency of CD83 induction (Fig. 2B). A significant increase of CD83 expression of 7- and 14-fold was found at a concentration of 10 and 100 U/ml, respectively. These data demonstrate that the increase in CD83 mRNA expression by TNFα is time and concentration dependent. These findings are consistent with a role for cytokines such as TNFα in systemic inflammation in COPD, but do not shed light on the down-regulation of CD83 mRNA in neutrophils from COPD patients during an acute exacerbation of their disease.

Figure 1: Gene expression analysis in peripheral blood neutrophils of COPD patients. Peripheral blood neutrophils were isolated from healthy volunteers (Filled squares) and moderate to severe (class IIB and III) COPD patients during their stable phase (open circles) and at time of exacerbation (filled circles). A line connected the stable phase and exacerbation of the same patient. Expression of indicated genes were measured using real time RT-PCR and expressed as LOG2 fold-change compared to the group of healthy controls. Differences between the study groups were compared using the Mann-Whitney U test. Differences between stable phase and exacerbation were compared using the Wilcoxon signed rank test.
Figure 2: TNFα induced CD83 expression is time and dose dependent. Peripheral blood neutrophils were stimulated with TNFα (100U/ml) for several time points (A) or with several concentrations of TNFα for 3 hours in vitro (B). RNA was isolated and real time RT-PCR for CD83 was performed. Results were normalized for the housekeeping gene β-actin. Results were expressed as relative levels of CD83 ±S.E.M. (n=4). For statistical analysis the paired T-test was used (*p<0.05 versus 3 hrs no stimulation; **p<0.001 versus 3 hrs no stimulation).

GM-CSF down-regulates TNFα-induced CD83 mRNA in neutrophils
Besides TNF-α, a role for GM-CSF in the pathogenesis of COPD has been proposed (4, 25, 26). Therefore, we designed experiments to study the effect of co-stimulating neutrophils with GM-CSF and TNFα on the expression of CD83 mRNA. In Chapter 2 we demonstrated that TNFα but not GM-CSF could induce CD83 expression. Neutrophils were stimulated with increasing concentrations of TNFα in the presence or absence of GM-CSF (0.1 nM). RNA was isolated and CD83 expression was determined by real time RT-PCR (Fig 3A). Co-incubation with GM-CSF caused a dramatic inhibition of TNFα (100 U/ml) induced CD83 expression in human neutrophils. This inhibition was complete in neutrophils stimulated with 1 or 10 U/ml TNFα.

Neutrophils were subsequently stimulated with TNFα and increasing concentrations of GM-CSF to determine the dose dependency of the inhibition. As shown in Figure 3B, increasing concentrations of GM-CSF results in decreasing TNFα induced CD83 expression. Complete inhibition of 10 U/ml of TNFα induced CD83 expression was obtained at a concentration of 0.1 nM GM-CSF. These findings demonstrate that a combination of pro-inflammatory cytokines can result in down-regulation of a pro-inflammatory gene.
GM-CSF-induced down-regulation of CD83 mRNA is not associated by changes in NFκB signaling

As previously mentioned both activators of CD83, TNFα and LPS (see Chapter 2), are strong activators of the transcription factor NF-κB in neutrophils (27). In addition, TNFα induced CD83 expression in T-cells is NF-κB dependent (20, 21). Therefore, we investigated whether the effect of GM-CSF might be mediated through inhibition of NF-κB signaling. As a read out for this signaling pathway, the phosphorylation state of IκBα was studied. In an unphosphorylated state, IκB associates with NF-κB, retaining it in the cytoplasm and subsequently preventing translocation of NF-κB to the nucleus. TNFα but not GM-CSF stimulation was sufficient to induce phosphorylation of IκBα in neutrophils (and thus activation of NFκB) (Fig 3C). Stimulation of neutrophils with TNFα in the presence GM-CSF resulted in IκBα phosphorylation to a similar level as TNFα alone. Thus, the inhibitory effect of GM-CSF on TNFα-induced CD83 expression is independent of IκBα phosphorylation in human neutrophils.

Figure 3: TNFα induced CD83 expression in peripheral blood neutrophils is inhibited by GM-CSF. (A) Isolated neutrophils were incubated with increasing concentrations of TNFα (as indicated) and simultaneously without (black bars) or with GM-CSF (0.1 nM) (empty bars) for 3 hours in vitro. CD83 expression was measured by performing real time RT-PCR and results are expressed as relative levels of CD83 ±S.E.M. (n=4). For statistical analysis the paired T-test was used.*p<0.05. (B) Neutrophils were incubated with TNFα (10 U/ml) and simultaneously with increasing concentrations of GM-CSF for 3 hours in vitro. Results are expressed as relative levels of CD83 ± S.E.M. (n=4). For statistical analysis the paired T-test was used.*p<0.05 versus TNFα (10 U/ml). (C) Neutrophils were incubated with TNFα (10 U/ml) and/or GM-CSF (10^-10 M) for the times indicated. After stimulation, cytosolic extracts were prepared, normalized for protein content and analyzed by SDS-PAGE followed by Western blotting with anti-phospho-IκBα (ser 32) or anti-actin. Data is representative of three independent experiments.
Discussion

At chronic inflammatory loci, such as bronchial tissue in COPD, neutrophils migrate to the site of inflammation and can become activated even in the absence of microbial derived triggers. Several studies have monitored inflammatory diseases by measuring granulocyte activation at the site of inflammation (1-3). In contrast to local inflammatory sites, little is known about systemic inflammation associated with granulocyte activation in peripheral blood in COPD. Furthermore, it is not clear which inflammatory mediators are playing a role in the activation of granulocytes in the systemic compartment. The analysis of gene profiles of cytokine-induced genes in neutrophils (in vitro) can be used as an indication for the action of these cytokines in vivo. Indeed, we have found several differentially expressed genes in neutrophils from the blood of patients with moderate to severe COPD compared to normal donors (see Chapter 2).

Here we focused on a set of genes that were found to be regulated in vivo in COPD patients, and are also induced by cytokines in neutrophils in vitro (see Chapter 2). The expression of CD83, MIP-1β, and IL-1β were significantly enhanced in peripheral blood neutrophils from COPD patients in a stable phase of their disease (Fig. 1). However, to our surprise, the expression of CD83, MIP-1β, IL-1β, and IL-8 was dramatically down-regulated in peripheral neutrophils from COPD patients during an acute exacerbation of their disease (Fig. 1). This unanticipated result may have important consequences concerning the insight into the mechanisms of the chronic systemic inflammatory response in COPD. Apparently, either a quantitative (excess of inflammatory signals) and/or a qualitative (different inflammatory mediators) change reflects itself in a gene profile that fits more with a reduced inflammatory phenotype. It is tempting to speculate that neutrophils act as pro-inflammatory cells at the onset of an inflammatory reaction, but can have a more anti-inflammatory function at later stages of an acute exacerbation. This altered neutrophil phenotype might then be more involved in dampening the inflammatory reaction. Support for this hypothesis is the finding that peripheral blood neutrophils are found to be primed directly after post-injury multiple organ failure, but become unresponsive to several pro-inflammatory chemoattractants after 48 hours (28, 29).

TNFα is thought to be an important cytokine in the pathogenesis of COPD, has been implicated in many chronic inflammatory diseases (30) and is a strong activator of neutrophils (31). Besides TNFα, GM-CSF has also been implicated in the pathogenesis of COPD (25, 26). Interestingly, GM-CSF induces a distinct profile of expressed genes in neutrophils compared to TNFα (see Chapter 2). Not much is known regarding GM-CSF in systemic inflammation, but we did not find clear indications of specific GM-CSF induced genes in neutrophils isolated from the peripheral blood of COPD patients (see Chapter 2). Based on these findings we designed experiments to study the down-regulation of CD83 mRNA in an in vitro setting. We focused on TNFα-induced CD83
Exacerbations of COPD correlate with down-regulation of genes in neutrophils

expression in neutrophils in vitro as a model for processes occurring in vivo in patients with COPD.

Time course experiments showed that CD83 expression was significantly induced by TNFα after one hour and was maximal after three hours. Concentrations between 1 and 10 U/ml of TNFα were sufficient to induce CD83 mRNA expression. These concentrations are likely to be found near chronic inflammatory sites (4, 11, 32). The expression of CD83 induced by TNF is likely to be mediated by NFκB, since mediators such as LPS and TNFα, that are well known activators of NFκB, induce CD83 in neutrophils (26), whereas mediators that do not activate NFκB in neutrophils (like GM-CSF and IL-8) do not increase CD83 mRNA. In addition, a recent study provides evidence that NF-κB mediates TNFα induced CD83 expression in Jurkat T cells (20). The effect of a combination of pro-inflammatory cytokines, TNF and GM-CSF, revealed the surprising observation that GM-CSF inhibits TNFα induced CD83 expression. This finding is consistent with the hypothesis that cytokines such as GM-CSF can cross-modulate the expression of pro-inflammatory genes in neutrophils in vivo. Indeed, GM-CSF levels were markedly increased in serum and sputum from chronic bronchitic patients with an exacerbation, as compared with patients under baseline conditions (25). The precise molecular mechanism by which GM-CSF inhibits CD83 expression remains to be determined. However, our IκBα phosphorylation data demonstrated that GM-CSF is inhibiting CD83 expression independently of IκBα phosphorylation.

Another explanation for the down-regulation of genes at the time of exacerbation may be that the “hyper” activation of neutrophils causes enhanced homing to inflammatory loci. This hypothesis is supported by studies describing that circulating neutrophils are (pre)activated during the first 24 hours after major trauma and that a subsequent activation of these cells during this period can lead to enhanced sequestering in the organs and induction of an intense, cytotoxic, inflammatory response resulting in multiple organ injury (33, 34). Interestingly, the periods of exacerbation were characterized by neutrophilia rather than neutropenia (results not shown). Previous studies have also demonstrated an increase of neutrophils in the airways (25, 35-37). A consequence of this is that neutrophils obtained during an exacerbation in the periphery were likely recently released from the bone marrow. These neutrophils may not have encountered pro-inflammatory mediators such as TNFα. This might also explain why the expression of some genes decreases to below that of healthy volunteers. Most likely these processes occur simultaneously. The resulting phenotype of neutrophils obtained from patients during an exacerbation of COPD is the result of a very delicate balance between recruitment of neutrophils from the bone marrow, cross-modulation by multiple cytokines and enhanced homing to inflammatory sites.
In conclusion, our data indicate that combinations of cytokines *in vivo* can result in the production of specific gene profiles dependent on the combination and concentrations of cytokines. This suggests that cytokine/chemokine induced gene expression profiles may provide novel insights into the mechanisms of systemic inflammation in COPD in terms of type of inflammatory response, participation of unique combinations of cytokines and kinetics of the presence of different phenotypes of leukocytes in the peripheral blood. Thorough analysis of these gene profiles will allow characterization of different stages of COPD and possibly better timing of appropriate therapy.
Exacerbations of COPD correlate with down-regulation of genes in neutrophils

References


