Respiratory Medicine (2011) 105, 1222–1230

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Toll-like receptor expression in smokers with and without COPD☆

Ida von Scheele [a,*], Kjell Larsson [a], Barbro Dahlén [b], Bo Billing [b], Maria Skedinger [b], Ann-Sofie Lantz [b], Lena Palmberg [a]

[a] Lung and Allergy Research, The National Institute of Environmental Medicine, Karolinska Institutet, PO Box 287, SE-171 77 Stockholm, Sweden
[b] Dept of Respiratory Medicine and Allergology, Karolinska Institutet, Karolinska University Hospital, Huddinge, Sweden

Received 8 December 2010; accepted 11 February 2011
Available online 24 March 2011

KEYWORDS
Toll-like receptors;
COPD;
CD14;
Neutrophils

Summary
Introduction: Chronic obstructive pulmonary disease (COPD) is characterized by non-reversible airflow limitation and systemic engagement. Bacterial colonization in the lungs is common in COPD-patients and may be associated with frequent acute exacerbations. Pattern-recognition receptors (PRRs), like Toll-like receptor 2 (TLR2), TLR4 and CD14 are expressed on most immunologic active cell types and are most likely of importance in COPD patho-physiology.
Material and methods: Twenty smokers with and 20 without COPD and 20 healthy non-smokers participated in the study. At two visits, induced sputum was collected after spirometry, blood was sampled and bronchoscopy with bronchoalveolar lavage was performed. Expression of TLR2, TLR4 and CD14 on different cell types and soluble receptors were assessed in the different compartments.
Results: Expression of TLR2 was lower on sputum neutrophils and soluble TLR2 (sTLR2) was higher in the supernatant in the COPD group, indicating a down regulation of TLR2 at the transit from blood to sputum. Expression of CD14 on sputum neutrophils and gene expression of CD14 on alveolar macrophages was up-regulated in the two smoking groups compared with non-smokers. No differences between the groups were found regarding TLR4 expression.
Conclusions: Pattern-recognition receptors (PRRs), that are expected to make a first line of defense against invading micro-organisms, are differently regulated in smokers with COPD compared with smokers without airflow limitation and non-smokers. This is likely of importance in COPD patho-physiology, in particular for exacerbations, which often are caused by micro-organisms.

☆ This study was supported by grants from the Swedish Heart and Lung Foundation, King Gustaf V's and Queen Victoria's Foundation, MRC, Stockholm County Council and Karolinska Institutet, AstraZeneca, Sweden.
* Corresponding author. Tel.: +46 (0) 8 52482215; fax: +46 (0) 8 300619.
E-mail address: Ida.von.Scheele@ki.se (I. von Scheele).

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doi:10.1016/j.rmed.2011.02.012

Introduction

Chronic obstructive pulmonary disease (COPD) is a globally growing disease which is characterized by airway inflammation and tissue destruction with non-reversible airflow limitation. Today COPD is recognized as a systemic disease, thus not solely affecting the airways and lungs.[1] One of the main effector cells in COPD is the neutrophil granulocyte and during exacerbations, which are often caused by bacterial infections, neutrophils are rapidly recruited to the site of action and have a substantial role in tissue damaging and bacterial killing.[2,3] Bacterial colonization of the lungs is often reported in patients with COPD, and is associated with frequent acute exacerbations.[4] There are a number of reports demonstrating that COPD-patients have elevated numbers of neutrophils in sputum and bronchoalveolar lavage (BAL)-fluid.[5,6] Neutrophils together with other inflammatory active cells interact with pathogen- and/or damage associated molecular patterns (PAMPs and/or DAMPs). These are sensed through highly conserved pattern-recognition receptors (PRR), called Toll-like receptors (TLRs). Lipoproteins from Gram-positive bacteria are ligands for Toll-like receptor 2 (TLR2) and TLR4 recognizes, together with CD14, lipoproteins from Gram-negative bacteria. Signaling through TLRs leads to activation of the NF-κB pathway and formation and release of pro-inflammatory cytokines and chemokines. Both TLR2 and TLR4 have been extensively studied and seem to have both endogenous and exogenous ligands.[7] It has been shown that alveolar macrophages from smokers with and without COPD express less TLR2 than non-smokers[8] but the soluble forms of these receptors (sTLR2, sTLR4 and sCD14) have, however, been sparsely studied. Both sTLR2 and sTLR4 are believed to have a regulating role as decoy receptors in host innate immune response against microbial pathogens, resulting in a negative regulation of cytokines and chemokines. Soluble TLR2 is found in human plasma, breast milk and saliva and has the potential to modulate phagocytosis and superoxide production[9]; whereas sTLR4 has only been found as mRNA and not as protein in humans.[10] Soluble CD14 has a dualistic effect; on one hand sCD14 mediates the activation of membrane-negative CD14 cells, on the other hand it serves as a decoy receptor, by competing with mCD14 for LPS thereby reducing LPS-triggered activities. Soluble CD14 is also known to increase in serum during inflammatory conditions as an acute phase protein[11] and are elevated in bronchoalveolar lavage (BAL) fluid from smokers with and without COPD.[12,13]

The aim of this study was to find out whether the expression of innate immunity receptors on inflammatory cells are altered in smokers with and without COPD compared with non-smokers, and whether possible alterations are present already on circulating cells or if possible alterations occur when the cells enter the airways. Therefore, inflammatory cell expression of TLR2, TLR4 and CD14 and soluble TLR2 and CD14 were assessed in blood, bronchoalveolar lavage fluid (peripheral airways) and induced sputum (central airways) from smokers with and without COPD and in healthy non-smokers.

Material and methods

Subjects and study design

Twenty non-smoking healthy controls, 20 current smokers with normal spirometry and 20 current smokers with COPD were recruited from primary and secondary care at Karolinska University Hospital and by advertisement in the daily press. Smokers were included in the COPD group if post-bronchodilator FEV_1/FVC was <0.70, post-bronchodilator FEV_1 was 40–70% of predicted value and arterial oxygen saturation (SaO_2) $>$ 90%. Smokers were allocated to the non-COPD group if they had post-bronchodilator $FEV_1/FVC > 0.70$ and FEV_1 >80% of predicted value. The control group consisted of healthy non-smoking subjects with normal spirometry. Subject characteristics are displayed in Table 1. Subjects with a history of asthma or other

Table 1 Subject characteristics. Data are presented as mean and 95% confidence intervals (CI). FVC: Forced Vital Capacity, FEV_1: Forced Expiratory Volume in 1 s, % pred.: % of predicted value, DLCO: Carbon Monoxide Diffusing Capacity.

		Non-smoking controls $n = 20$	Smokers with COPD $n = 20$	Smokers without COPD $n = 20$
Subject characteristics	Age (years)	55 (51–59)	62 (59–65)	56 (53–59)
	Gender (♀/♂)	6/14	15/5	11/9
	Smoking (pack-years)	0	38 (34–41)	35 (28–41)
	Current smokers	$n = 0$	$n = 20$	$n = 20$
	Inhaled corticosteroids	$n = 0$	$n = 9$	$n = 0$
	FVC, %pred post-bonchodilatation	114 (107–121)	94 (85–103)	115 (108–121)
	FEV_1% predicted post-bonchodilatation	111 (106–117)	62 (54–70)	104 (98–109)
	FEV_1/FVC post-bonchodilatation	0.80 (0.77–0.83)	0.54 (0.50–0.58)	0.75 (0.72–0.78)
	DLCO (mmol/kPa/min)	25 (23–28)	11 (9–14)	20 (18–22)

pulmonary diseases or allergic diseases were excluded from the study, as were COPD-patients who have had an exacerbation within the last month prior to the study.

Spirometry was performed according to current guidelines (ATS/ERS)[14] and diffusion capacity (transfer factor, DLCO) was performed using carbon monoxide single breath technique.[15] Induced sputum and bronchoscopy including bronchoalveolar lavage (BAL) were performed on two separate days. Blood samples were drawn on the bronchoscopy day. All subjects gave their informed consent and the study was approved by the ethics committee at the Karolinska Institutet, Stockholm Sweden (Dnr 2005/733-31/1-4).

Sputum induction and processing

Sputum induction and processing were performed as previously described.[16] The cell pellet was re-suspended in 2 ml PBS and put on ice until antibody staining for the flow cytometry analysis. A total cell count and viability test with Trypan blue were performed. Cytocentrifuge-prepared slides were stained with May-Grünwald Giemsa stain and 300 cells (squamous cells excluded) were assessed for differential cell counts. The sputum samples were included in the analysis if they contained less than 30% squamous cells. The supernatant was dispensed into aliquots and kept in −70 °C until analysis.

BAL-fluid and alveolar macrophage isolation

Bronchoscopy was performed as previously described.[17] After pre-medication with scopolamine, analgesics and local anesthesia with xylocain the bronchoscope was wedged in a middle lobe segmental bronchus and 5 aliquots of 50 ml isotonic saline were instilled into the airway tree and gently sucked back. The lavage fluid was centrifuged and the supernatant was dispensed into aliquots and kept in −70 °C until analysis.

Approximately 2 million cells/well were then re-suspended in RPMI-cell medium supplemented with 10% serum. After 2 h the macrophages were presumed to be adhered. The supernatant was discarded and PBS was added to the macrophages in order to perform mRNA preparation.

Blood

Whole peripheral blood was collected in ethylene diamine-tetra-acetic acid (EDTA) vacutainer tubes (BD Bioscience, New Jersey, USA). The cells were stained for specific antibodies (TLR2, TLR4 and CD14) for further FACS analysis, but also analyzed in Attractors™ (BD Bioscience, San Jose, CA, USA) to perform a five-part white blood cell differential, as previously described.[18] The tests were performed within 2 h after sampling.

Flow cytometry (FACS)

To analyze the expression of TLR2, TLR4 and CD14 on sputum neutrophils and blood neutrophils and monocytes, 100,000 sputum cells or 100 μl of whole blood, were incubated with 10 μl monoclonal antibodies for 30 min (anti-TLR2-PE clone TL2.1, anti-TLR4-PE clone HTA125, anti-CD14-FITC clone 61D3, 10 μg/ml, eBioscience, San Diego, CA, USA). Anti-CD45 and SSC (Side Scatter) were used for gating cells both in blood and sputum (Fig. 1). Anti-CD66 was used to further identify neutrophils in sputum. Sputum cells were incubated on ice and blood cells in room temperature. As negative controls, isotype-matched antibodies were used. After incubation the cells were washed twice and re-suspended in PBS. Analyses were performed using FACSCalibur™ and median fluorescent intensity (MFI) was determined by CELLQuest™ (BD Bioscience Pharmingen) and calculated as relative median fluorescence intensity (rMFI = monoclonal antibody/corresponding isotype control).

mRNA preparation and real-time PCR

Preparation of mRNA from alveolar macrophages was performed in ten subjects from each group. The alveolar

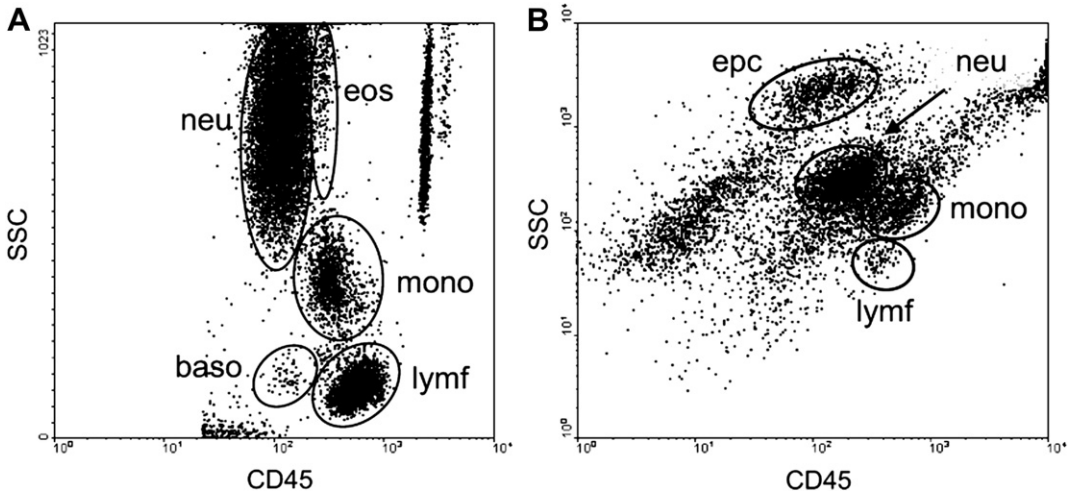

Figure 1 Leucocytes in blood and sputum analyzed by flow cytometry. Leucocytes stained with anti-CD45 in blood, to perform differential cell count (A). Induced sputum, double stained with anti-CD45 and anti-TLR2, or anti-TLR4 or anti-CD14 to measure cell surface expression on neutrophils of the receptor of interest (B).

Table 2 Differential cell count in blood, BAL-fluid and induced sputum. Data are presented as median (25–75th percentiles). *P*-values indicate differences compared with controls and significant differences are indicated in "bold". $P < 0.05$ are considered significant and n.s. indicates non significant.

		Non-smoking controls	Smokers with COPD	Smokers without COPD
Blood (10^3 cells/mL)	Neutrophils	2785 (2104–4283)	4072 (3618–5392)	3684 (3003–4312)
$n = 16–18$			**$P=0.012$**	n.s.
	Monocytes	465 (360–630)	666 (516–801)	620 (532–711)
			n.s.	n.s.
	Lymphocytes	1769 (1647–2404)	2212 (1942–2661)	2281 (1852–3161)
			$P=0.041$	**$P=0.027$**
	Basophils	56 (46–67)	91 (64–113)	74 (59–104)
			$P=0.018$	**$P=0.030$**
	Eosinophils	158 (111–247)	254 (236–334)	280 (135–369)
			n.s.	n.s.
BAL-fluid (10^6 cells/L)	Total cells	125 (87–147)	324 (173–455)	302 (246–457)
Cell viability 96% (95–97)			**$P=0.0002$**	**$P < 0.0001$**
$n = 13–18$	Neutrophils	3 (1–5)	7 (2–20)	8 (4–15)
			$P=0.045$	**$P=0.017$**
	Macrophages	108 (78–122)	266 (144–402)	272 (221–422)
			$P=0.0003$	**$P < 0.0001$**
	Lymphocytes	6 (4–14)	10 (3–20)	11 (8–18)
			n.s.	n.s.
	Basophils	0 (0–0)	0 (0–0)	0 (0–0)
			n.s.	n.s.
	Eosinophils	0 (0–0)	4 (1–12)	1 (0–2)
			$P=0.001$	n.s.
Sputum (cells/mg sputum)	Total cells	1265 (1011–2220)	1580 (1350–7383)	2190 (1610–3000)
Cell viability 92% (82–96)			n.s.	n.s.
$n = 10–13$	Neutrophils	220 (122–472)	530 (316–1430)	311 (132–592)
			$P=0.013$	n.s.
	Macrophages	286 (122–563)	195 (131–358)	186 (89–385)
			n.s.	n.s.
	Lymphocytes	17 (6–30)	15 (8–64)	8 (6–11)
			n.s.	n.s.
	Basophils	0 (0–0)	0 (0–0)	0 (0–0)
			n.s.	n.s.
	Eosinophils	0 (0–0)	8 (2–23)	2 (0–4)
			$P=0.006$	n.s.

macrophages were washed twice with PBS and total mRNA was isolated by PureLink™ Micro-to Midi Total RNA Purification System (Invitrogen). DNase I, amplification grade was used to remove genomic DNA (Invitrogen). First-strand cDNA was synthesized from 0.5 μg of total RNA, using QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). Real-time PCR was performed using an ABI Power SYBR Green Master mix (Applied Biosystems) with TLR2, TLR4 and CD14 primers, 1 μl of cDNA was amplified in 25 μl PCR reaction to identify the products of interest. Beta actin was used as an internal control gene. The primers were designed with the software Primer3 and purchased from Invitrogen.

Data were analyzed using 7500 Software v.2.0.1 (Applied Biosystem) and transformed using the ΔCt-method (ΔCt = the difference in threshold cycles for the test gene and beta actin). The results were then calculated and expressed as $2^{-\Delta Ct}$.

Soluble CD14 and TLR2

Soluble human CD14 (sCD14) and soluble TLR2 (sTLR2) were measured in the supernatants from BAL-fluid and sputum with purchased DuoSet ELISA CD14 kit and DuoSet ELISA sTLR2 kit (R&D Systems, Europe, Abingdon, UK). The analyses were performed according to manufacturer's protocol and the detection range was 62.5–4000 pg/ml and 125–8000 pg/ml for sCD14 and sTLR2, respectively. For duplicate samples an intra-assay coefficient of variation (CV) < 15% was accepted.

Endotoxin

The concentration of endotoxin was analyzed in BAL-fluid using a kinetic technique of *Limulus amebocyte* lysate assay according to the manufactures instructions, the

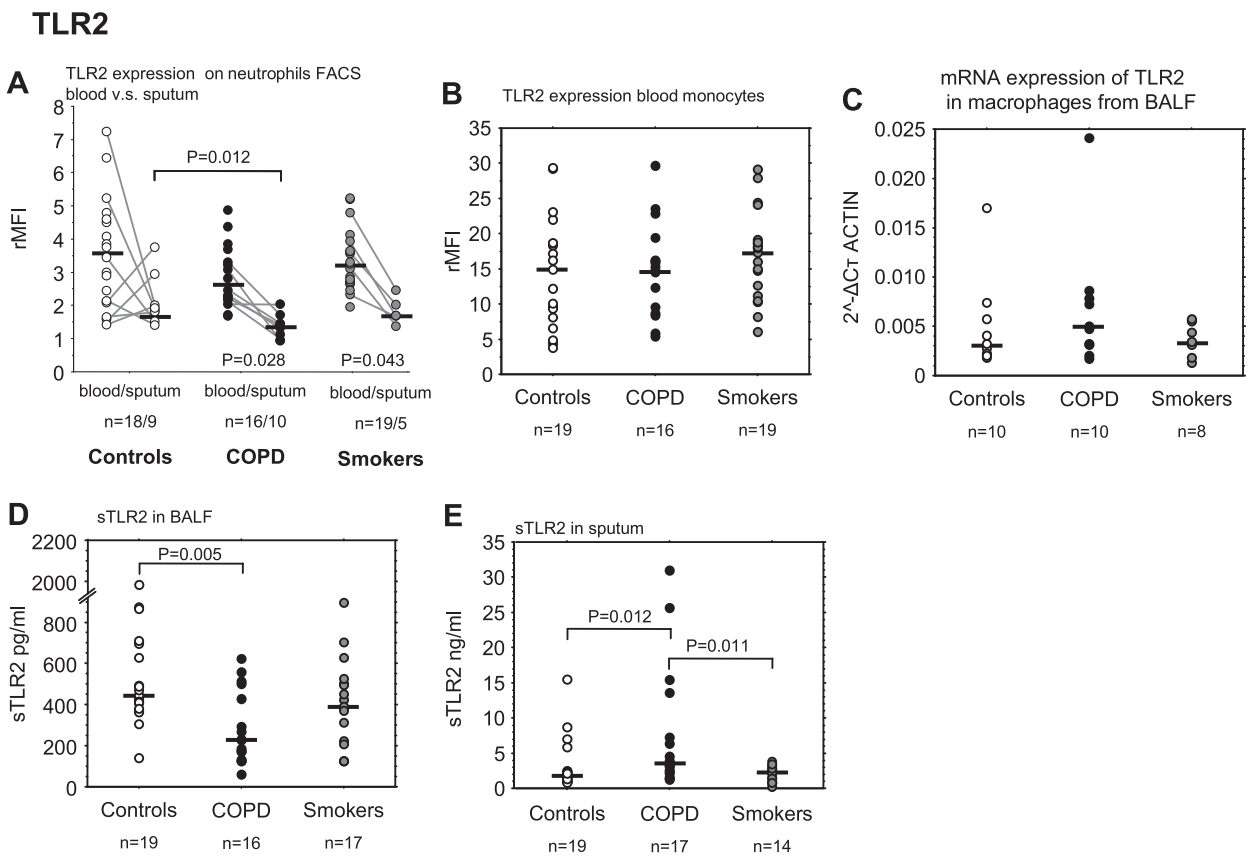

Figure 2 Surface expression of TLR2 on blood and sputum neutrophils, analyzed by flow cytometry. Data obtained from the same subject are connected with a line. Significant differences between compartments are stated with a *p*-value and between groups with a bracket (A). Toll-like receptor 2 expression on blood monocytes, measured by flow cytometry (B). Gene expression of TLR2 in alveolar macrophages, measured by real-time PCR (C). Soluble TLR2 in BAL-fluid supernatant, measured by ELISA (D). Soluble TLR2 in sputum supernatant measured by ELISA (E). $P < 0.05$ are considered significant.

samples were diluted in endotoxin specific buffer (Limulus Amebocyte lysate, Endosafe® Endochrome-K™ U.S. Lisence No. 1197, Coatech AB, Kungsbacka, Sweden) and *Escherichia coli* 0111:B4 was used as standard.

Statistics

Results are presented as scattergram, medians and interquartile ranges or as means and 95% confidence intervals. Comparisons between the groups were performed by Kruskal—Wallis (K—W) test followed by Mann—Whitney *U*-test if the $p < 0.05$ in the K—W test. Within group comparisons were performed using Wilcoxon signed rank test. All data were analyzed by StatView version 5.0.1 (SAS Institute Inc., Cary, NC). A *P*-value< 0.05 was considered significant.

Results

Due to technical problems with FACS-antibodies, difficulties in receiving adequate sputum or enough cells and problems related to bronchoscopy, datasets are not complete with regard to all outcome variables. Numbers of analyses are indicated in the figures.

Cell differential count in blood, BAL-fluid and sputum

The concentration of neutrophils was higher in the COPD group than in controls in blood, BAL-fluid and induced sputum (Table 2) and higher in BAL-fluid from smokers with normal spirometry compared with controls. The concentration of macrophages was elevated in BAL-fluid from the two smoking groups compared with the healthy controls. In blood, an increased concentration of both lymphocytes and basophiles was observed in the two groups of smokers compared with the control group. The concentration of eosinophils was increased in both BAL-fluid and sputum from COPD-patients compared to controls (Table 2) and was higher in BAL-fluid in the COPD group compared with smokers with normal spirometry ($p = 0.032$).

Toll-like receptor 2 (TLR2)

Toll-like receptor 2 expression on blood neutrophils did not differ between the three groups. Expression of TLR2 on sputum neutrophils was significantly lower in COPD-patients than in the control group (Kruskal—Wallis $p = 0.016$, Mann—Whitney $p = 0.012$). The expression of TLR2 on neutrophils was significantly lower in sputum than in blood in both groups of smokers ($p < 0.05$, Fig. 2A).

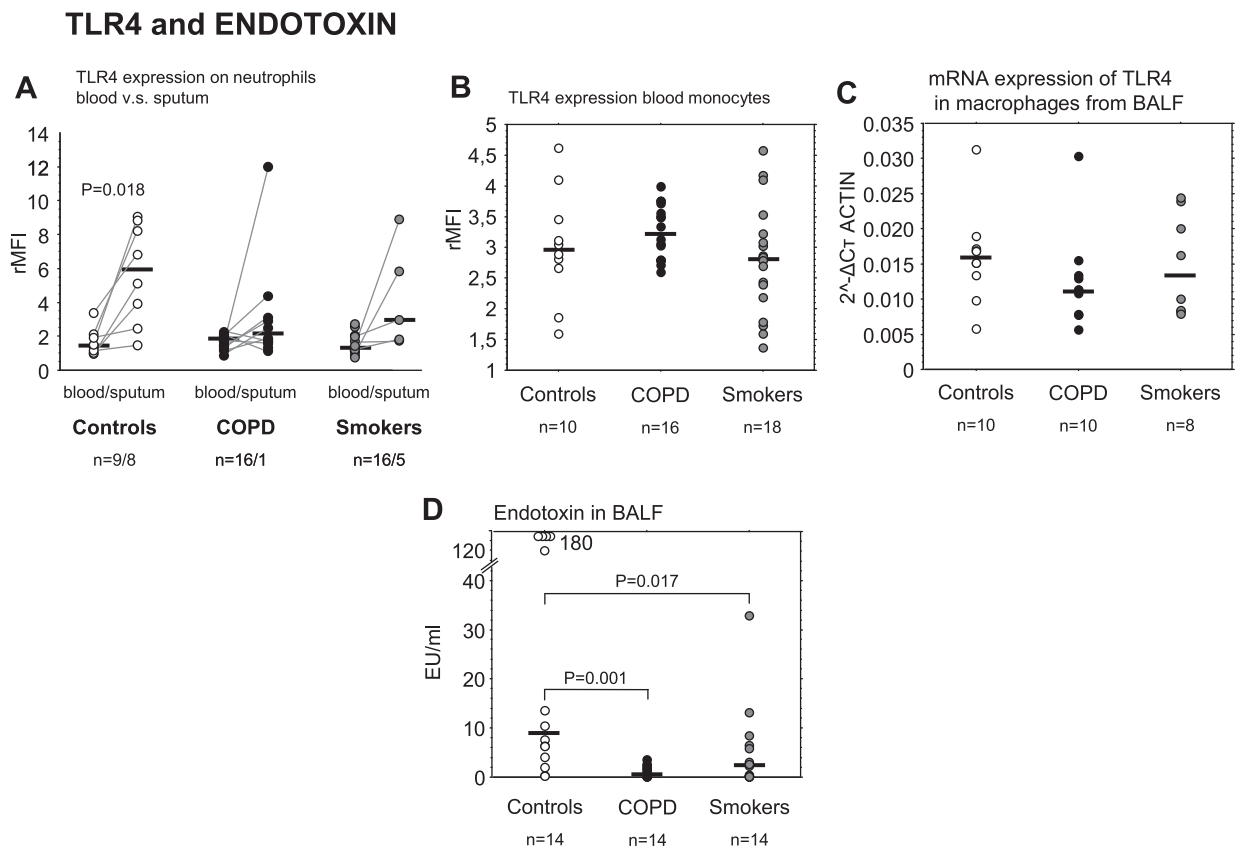

Figure 3 Surface expression of TLR4 on blood and sputum neutrophils, measured by flow cytometry. Data obtained from the same subject are connected with a line. Significant differences between compartments are stated with a *p*-value (A) and between groups with a bracket. Toll-like receptor 4 expression on blood monocytes, measured by flow cytometry (B). Gene expression of TLR4 in alveolar macrophages, measured by real-time PCR (C). Endotoxin in BAL-fluid supernatant, measured by kinetic-LAL assay (D).

Expression of TLR2 on blood monocytes and mRNA expression of TLR2 in macrophages obtained from BAL-fluid did not differ between the groups (Fig. 2B, C).

Soluble TLR2 (sTLR2) in the BAL-fluid was higher in the controls than in COPD (Kruskal–Wallis $p = 0.023$, Mann–Whitney $p = 0.005$, Fig. 2D). In sputum sTLR2 was higher in COPD than in controls (Kruskal–Wallis $p = 0.013$, Mann–Whitney $p = 0.012$) and smokers without COPD ($p = 0.011$ Fig. 2E).

Toll-like receptor 4 (TLR4) and endotoxin

The TLR4 expression on neutrophils was enhanced in sputum compared with blood in the control group. ($p = 0.018$, Fig. 3A). The TLR4 expression on neutrophils (blood and sputum) and monocytes (blood) and the gene expression of TLR4 in alveolar macrophages did not differ significantly between the three groups (Fig. 3A–C).

The endotoxin level in BAL-fluid supernatant was higher in the control group than in smokers without COPD (Kruskal–Wallis $p = 0.002$, Mann–Whitney $p = 0.017$) and COPD-patients ($p = 0.001$, Fig. 3D).

CD14

The CD14 expression on blood and sputum neutrophils did not differ between the three groups. There was a significant increase of CD14 expression on sputum neutrophils compared to blood neutrophils in both groups of smokers ($p < 0.05$, Fig. 4A) whereas no significant differences were found on blood monocytes (Fig. 4B). In BAL-fluid macrophages CD14 mRNA expression was elevated in both groups of smokers compared to the controls (Kruskal–Wallis $p = 0.004$, Mann–Whitney $p \leq 0.010$, Fig. 4C).

Soluble CD14 (sCD14) did not differ significantly between the groups, neither in BAL-fluid nor in sputum (Fig. 4D, E).

Discussion

In this report it was demonstrated that TLR2 expression is reduced on sputum neutrophils from patients with COPD compared with non-smokers, and that the expression of TLR2 on neutrophils is higher in blood than in sputum, irrespective of the presence of airway obstruction. Furthermore, for the first time it was shown that COPD was associated with higher levels of soluble TLR2 (sTLR2) in sputum and lower levels of sTLR2 in BAL-fluid compared with non-smokers. We also demonstrated that the expression of CD14 on neutrophils was enhanced in sputum compared with blood and that that the gene expression of CD14 on alveolar macrophages in BAL-fluid was increased in smokers compared with non-smokers. In addition, COPD-

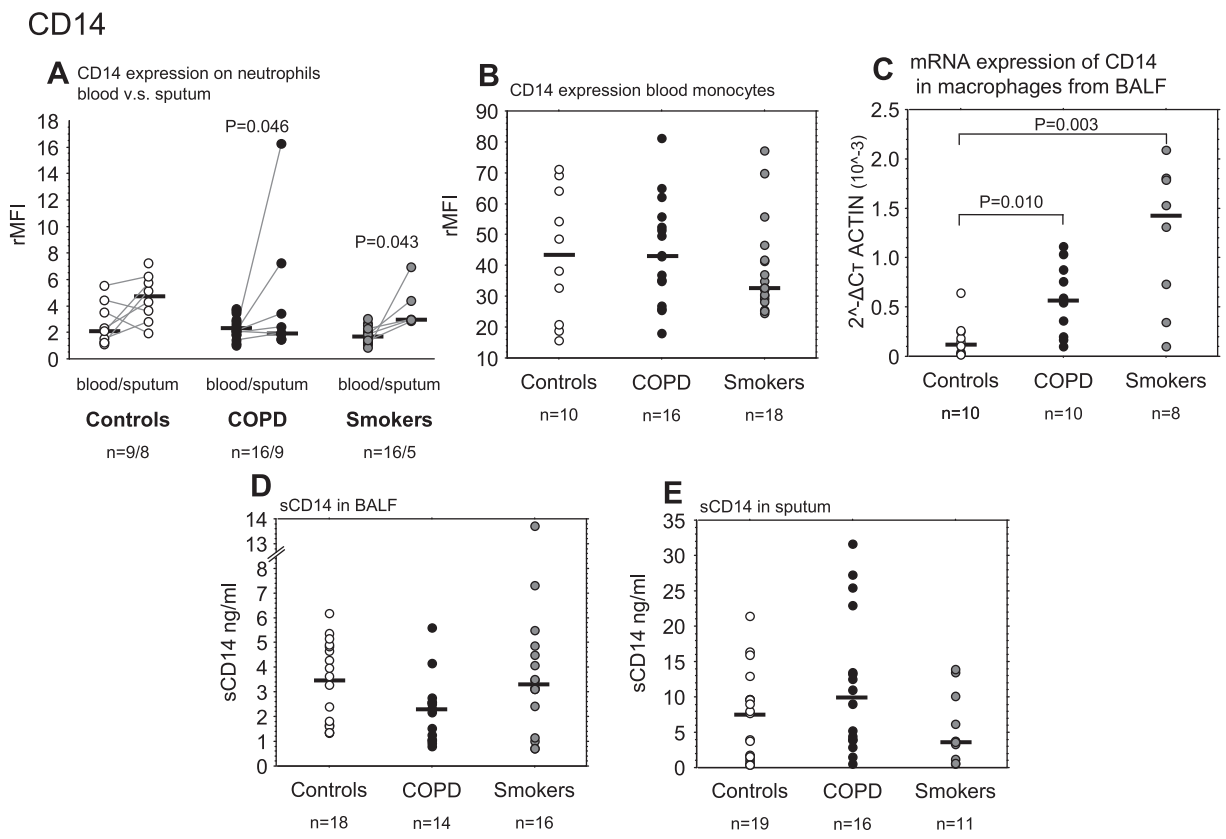

Figure 4 Surface expression of CD14 on blood and sputum neutrophils, measured by flow cytometry Data obtained from the same subject are connected with a line. Significant differences between compartments are stated with a *p*-value (A) and between groups with a bracket. Expression of CD14 on blood monocytes, measured by flow cytometry (B). Gene expression of CD14 in alveolar macrophages, measured by real-time PCR (C). Soluble CD14 in BAL-fluid supernatant, measured by ELISA (D). Soluble CD14 in sputum supernatant measured by ELISA (E).

patients exhibited neutrophilia compared with non-smokers in all three compartments, i e sputum (central airways), BAL-fluid (small airways) and in blood (systemically).

Down regulation of TLR2 has previously been demonstrated on alveolar macrophages from smokers[8] and on blood monocytes from non-smoking farmers[19]; who, like smokers, are continuously exposed to organic material. In our study we did not found expression of TLR2 on circulation monocytes to be altered, which is in agreement with the study by Droemann et al.[8] but in contrast to the study of Pons et al.[20] who found up regulation of TLR2 on circulation monocytes from COPD-patients. The explanations to these discrepancy findings are unclear.

From the present data it is clear that TLR2 receptors are down regulated on airway neutrophils in COPD and that this down regulation takes place during the transition from blood to the airways. In this respect, smokers without COPD hold a position in between, as the TLR2 expression on neutrophils was clearly elevated in blood compared with sputum whereas no difference in sputum neutrophil TLR2 expression was found between smokers with normal spirometry and non-smokers. The two smoking groups were matched with regard to cumulative exposure to tobacco smoke, which indicates that the susceptibility to tobacco smoke and the development of COPD seems to constitute a "constitutional" difference between smokers who do and do not develop COPD. A potential weakness of this finding is the problem of power; few observations of this parameter make firm conclusion uncertain.

Neutrophils participate in the first line of airway defense and TLR2 on the cell surface of the neutrophils are responsible for binding of microbial products, mainly from Gram-positive bacteria, in order to reduce and eliminate harmful influences of the exposure. The finding of decreased TLR2 expression on sputum neutrophils and increased levels of sTLR2 in sputum in COPD strongly suggest a shedding of the TLR2 in smokers who have developed COPD while no such shedding was observed in smokers with normal spirometry. Thus, receptor shedding does not seem to be a consequence of chronic exposure, as the cumulative exposure to tobacco smoke was similar in smokers with and without COPD. It might rather be an effect of bacterial colonization in patients with COPD causing an increased microbial burden, and possible lead to receptor shedding in order to counteract the chronic inflammation. The mechanisms of receptor down regulation and shedding are unclear as is the patho-physiological implications. The difference between the two groups of smokers, who had similar exposure to tobacco smoke but different clinical phenotypes, is of great interest and is likely of importance in the pathogenesis of COPD.

The novel finding that surface expression of TLR2 on neutrophils is attenuated on the transit from blood to sputum indicates that TLR2 expression is tightly regulated

and that a first line regulatory mechanism occurs. This finding is also in line with a previous report of a lower expression of TLR2 on alveolar macrophages than on blood monocytes.[8,21] On the other hand, in asthmatics, whose cellular phenotype is dominated by neutrophils, increased TLR2 gene expression was found on sputum cells[22]; which likely could be a consequence of treatment with inhaled steroids as previous *in vitro* experiments have demonstrated that the gene expression on TLR2 is up-regulated by steroids.[23]

Neutrophils are the dominating cells in sputum, thus the neutrophils are, most likely, the primary source of sTLR2 found in sputum of patients with COPD. In BAL-fluid, where the presence of neutrophils is less abundant and the number of macrophages is high, the amount of sTLR2 was lower in the COPD group than in the non-smokers. A possible explanation to this is that sTLR2 in BAL-fluid might origin from the macrophages, explaining the inversed pattern observed between sputum and BAL-fluid.

In the present study subjects with reversible airway obstruction or a history of asthma and allergy were excluded. Thus, the finding of increased concentration of eosinophils in BAL-fluid and sputum in the COPD group was not associated with co-existing asthma. From previous studies it is clear that eosinophilia is observed in some patients with COPD[24,25] and it is well documented that airway eosinophilia occurs in relation to acute exacerbations.[26]

The alteration of different innate immunity receptor expression during the transition from blood to the airways does not follow the same pattern, while TLR2 expression on neutrophils attenuates, TLR4 and mCD14 increase. This indicates different regulating mechanisms for different PRRs. The findings suggest an up regulation of TLR4 and CD14 as a result of stimulation when reaching the airways, and are in line with reported elevated expression of mCD14 on macrophages from induced sputum in COPD-patients.[27] The differences between PRR-regulation patterns upon pro-inflammatory stimulation may be a result of the receptors' ability to shed. The soluble form of TLR2 (sTLR2) is present in human plasma, saliva, BAL-fluid and sputum[9]; whereas sTLR4 only are found as mRNA in human and not as a protein.[10] This explanation might even account for the elevated expression of mCD14 in sputum neutrophils compared to the expression on blood neutrophils. Even though sCD14 is highly present in both central airways (sputum) and small airways (BAL-fluid) it is known to be even higher in serum. This might indicate less shedding of mCD14 locally (in the airways) than systemically (in the blood), resulting in an elevated mCD14 expression on sputum neutrophils compared to blood neutrophils.

In spite of the fact that we found an elevated mRNA expression of CD14 in alveolar macrophages obtained from BAL-fluid in smokers with and without airway obstruction, we were not able to detect higher amounts of sCD14 in BAL-fluid. As smokers are exposed to endotoxin through cigarette smoke[28] it is tempting to speculate that sCD14 had bind to its ligand, LPS (endotoxin), which also may explain the finding of lower levels of soluble endotoxin in supernatants from BAL-fluid in both groups of smokers compared with the non-smoking control group.

In summary our results support the hypothesis that pattern-recognition receptors are modulated in COPD. We suggest that a diminished TLR2 expression on airway inflammatory cells leads to reduced pathogen identification and removal which, in turn, facilitate bacterial colonization and increases the risk of exacerbations in COPD. Our findings also highlight how tightly regulated the expression of these receptors are, which may be crucial for the defense of the host.

Acknowledgments

The authors would like to thank Dr Anita Simhag and co-workers at Huddinge Hospital for excellent technical assistance.

Conflict of interest

None of the authors has any potential financial conflict of interest related to this manuscript.

References

1. Agusti A, Soriano JB. COPD as a systemic disease. *COPD* 2008;**5**:133—8.
2. Barnes PJ. New molecular targets for the treatment of neutrophilic diseases. *J Allergy Clin Immunol* 2007;**119**:1055—62. quiz 1063-1054.
3. Larsson K. Aspects on pathophysiological mechanisms in COPD. *J Intern Med* 2007;**262**:311—40.
4. Hansel TT, Barnes PJ. New drugs for exacerbations of chronic obstructive pulmonary disease. *Lancet* 2009;**374**:744—55.
5. Keatings VM, Barnes PJ. Granulocyte activation markers in induced sputum: comparison between chronic obstructive pulmonary disease, asthma, and normal subjects. *Am J Respir Crit Care Med* 1997;**155**:449—53.
6. Lacoste JY, Bousquet J, Chanez P, Van Vyve T, Simony-Lafontaine J, Lequeu N, Vic P, Enander I, Godard P, Michel FB. Eosinophilic and neutrophilic inflammation in asthma, chronic bronchitis, and chronic obstructive pulmonary disease. *J Allergy Clin Immunol* 1993;**92**:537—48.
7. Marshak-Rothstein A. Toll-like receptors in systemic autoimmune disease. *Nat Rev Immunol* 2006;**6**:823—35.
8. Droemann D, Goldmann T, Tiedje T, Zabel P, Dalhoff K, Schaaf B. Toll-like receptor 2 expression is decreased on alveolar macrophages in cigarette smokers and COPD patients. *Respir Res* 2005;**6**:68.
9. Raby AC, Le Bouder E, Colmont C, Davies J, Richards P, Coles B, George CH, Jones SA, Brennan P, Topley N, Labeta MO. Soluble TLR2 reduces inflammation without compromising bacterial clearance by disrupting TLR2 triggering. *J Immunol* 2009;**183**:506—17.
10. Liew FY, Xu D, Brint EK, O'Neill LA. Negative regulation of toll-like receptor-mediated immune responses. *Nat Rev Immunol* 2005;**5**:446—58.
11. Bas S, Gauthier BR, Spenato U, Stingelin S, Gabay C. CD14 is an acute-phase protein. *J Immunol* 2004;**172**:4470—9.
12. Martin TR, Rubenfeld GD, Ruzinski JT, Goodman RB, Steinberg KP, Leturcq DJ, Moriarty AM, Raghu G, Baughman RP, Hudson LD. Relationship between soluble CD14, lipopolysaccharide binding protein, and the alveolar inflammatory response in patients with acute respiratory distress syndrome. *Am J Respir Crit Care Med* 1997;**155**:937—44.
13. Regueiro V, Campos MA, Morey P, Sauleda J, Agusti AG, Garmendia J, Bengoechea JA. Lipopolysaccharide-binding

protein and CD14 are increased in the bronchoalveolar lavage fluid of smokers. *Eur Respir J* 2009;**33**:273—81.
14. *Standardization of Spirometry, 1994* Update. American Thoracic Society, 1995.
15. American Thoracic Society. Single-breath carbon monoxide diffusing capacity (transfer factor). Recommendations for a standard technique—1995 update. *Am J Respir Crit Care Med* 1995;**152**:2185—98.
16. Strandberg K, Ek A, Palmberg L, Larsson K. Fluticasone and ibuprofen do not add to the effect of salmeterol on organic dust-induced airway inflammation and bronchial hyperresponsiveness. *J Intern Med* 2008;**264**:83—94.
17. Larsson BM, Palmberg L, Malmberg PO, Larsson K. Effect of exposure to swine dust on levels of IL-8 in airway lavage fluid. *Thorax* 1997;**52**:638—42.
18. Sundblad BM, Sahlander K, Ek A, Kumlin M, Olsson M, Larsson K, Palmberg L. Effect of respirators equipped with particle or particle-and-gas filters during exposure in a pig confinement building. *Scand J Work Environ Health* 2006;**32**:145—53.
19. Sahlander K, Larsson K, Palmberg L. Altered innate immune response in farmers and smokers. *Innate Immun*; 2009.
20. Pons J, Sauleda J, Regueiro V, Santos C, Lopez M, Ferrer J, Agusti AG, Bengoechea JA. Expression of Toll-like receptor 2 is up-regulated in monocytes from patients with chronic obstructive pulmonary disease. *Respir Res* 2006;**7**:64.
21. Juarez E, Nunez C, Sada E, Ellner JJ, Schwander SK, and Torres M. Differential expression of Toll-like receptors on human alveolar macrophages and autologous peripheral monocytes. Respir Res;11(2).
22. Simpson JL, Grissell TV, Douwes J, Scott RJ, Boyle MJ, Gibson PG. Innate immune activation in neutrophilic asthma and bronchiectasis. *Thorax* 2007;**62**:211—8.
23. Homma T, Kato A, Hashimoto N, Batchelor J, Yoshikawa M, Imai S, Wakiguchi H, Saito H, Matsumoto K. Corticosteroid and cytokines synergistically enhance toll-like receptor 2 expression in respiratory epithelial cells. *Am J Respir Cell Mol Biol* 2004;**31**:463—9.
24. Chanez P, Vignola AM, O'Shaugnessy T, Enander I, Li D, Jeffery PK, Bousquet J. Corticosteroid reversibility in COPD is related to features of asthma. *Am J Respir Crit Care Med* 1997;**155**:1529—34.
25. Sarir H, Henricks PA, van Houwelingen AH, Nijkamp FP, Folkerts G. Cells, mediators and Toll-like receptors in COPD. *Eur J Pharmacol* 2008;**585**:346—53.
26. Saetta M, Di Stefano A, Maestrelli P, Turato G, Ruggieri MP, Roggeri A, Calcagni P, Mapp CE, Ciaccia A, Fabbri LM. Airway eosinophilia in chronic bronchitis during exacerbations. *Am J Respir Crit Care Med* 1994;**150**:1646—52.
27. Domagala-Kulawik J, Maskey-Warzechowska M, Hermanowicz-Salamon J, Chazan R. Expression of macrophage surface markers in induced sputum of patients with chronic obstructive pulmonary disease. *J Physiol Pharmacol* 2006;**57**(Suppl. 4):75—84.
28. Larsson L, Szponar B, Pehrson C. Tobacco smoking increases dramatically air concentrations of endotoxin. *Indoor Air* 2004; **14**:421—4.