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LBP and CD14 are increased in the bronchoalveolar lavage fluid of smokers.

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Abstract

Lipopolysaccharide-binding protein (LBP) and CD14 contribute to the recognition of pathogens by cells which triggers the activation of defence responses. Smoking is a risk factor for the development of chronic obstructive pulmonary disease (COPD) and infections. We theorized that levels of LBP and CD14 in the lungs from smokers would be higher than those in the lungs from never smokers. These elevated levels could affect host responses upon infection.

LBP, sCD14, IL-8 were detected by ELISA. NF-κB, p38 and IκBα were studied by immunoassays. Gene expression was assessed by RT-PCR.

Broncho-alveolar lavage levels of LBP and CD14 were significantly higher in smokers and COPD patients than in never smokers whereas levels of both proteins were not significantly different in smokers and COPD patients. IL-6 and IL-1β and cigarette smoke condensate induced the expression of LBP and CD14 by airway epithelial cells. LBP and sCD14 inhibited the nontypable *Haemophilus influenzae* (NTHi)-dependent secretion of IL-8 and the activation of NF-κB and p38 MAP kinase signalling pathways but they increased the internalisation of NTHi by airway epithelial cells.

Thus, in the inflamed airways of smokers both proteins could contribute to inhibit bacteria-dependent cellular activation without compromising the internalisation of pathogens by airway cells.

Introduction

One of the goals of the innate immune system is the rapid recognition of pathogens which triggers the activation of host defence responses. Lipopolysaccharide-binding protein (LBP) and CD14 (either membrane-bound or soluble) contribute to the recognition of lipopolysaccharide (LPS) [1, 2].

LBP catalyzes the transfer of LPS to membrane bound CD14 (mCD14) which greatly amplifies the cellular response to LPS [3]. LBP can also transfer LPS to serum lipoproteins which results in neutralization of LPS-induced cellular response [1, 2]. CD14 is also found in a soluble state (sCD14) [4] and two opposite functions have been described for. Upon transfer of LPS by LBP, sCD14 mediates the activation of membrane-negative CD14 cells [5]. But sCD14 can also compete with mCD14 for LPS thereby reducing LPS-triggered activities [1, 2]. Also, it has been shown that sCD14 strongly enhances the LBP-mediated transfer of LPS to serum lipoproteins [1, 2]. Studies using knock-out mice have shown that LBP and CD14 are important to clear infections [6, 7].

Several studies have reported that serum levels of LBP and sCD14 increase in inflammatory conditions and there is a correlation between severity of disease and the levels of these proteins [8-12]. Some of these studies suggest that these increased levels of both proteins might amplify the cellular response to LPS and Gram-negative bacteria. However, it may well be that these levels are a host mechanism to blunt an overwhelming inflammatory reaction [1, 2].

Cigarette smoke is the main risk factor for the development of lung cancer, chronic obstructive pulmonary disease (COPD) and it has been shown that exposure to cigarette smoke leads to lung inflammation [13]. Smoking is also one of the risk factors for respiratory infections [14]. In this context, the so-called “British hypothesis” states

that recurrent bronchial infections were the reason, at least in part, that some smokers developed progressive airway obstruction and other did not [15]. It has been hypothesized that cigarette smoking may impair lung immune function against respiratory infections by, for example, affecting the recognition of pathogens and hence the activation of host defence mechanisms. Given the positive correlations between smoking and inflammation and between the latter and the levels of LBP and sCD14 [8-12], we hypothesized that levels of these proteins in the lungs from smokers would be higher than those in lungs from never smokers.

We report that levels of LBP and sCD14 are increased in broncho-alveolar lavage fluid (BALF) from smokers and COPD patients. We show that cigarette smoke or known inducers of acute-phase proteins activate the production of LBP and sCD14 by airway epithelial cells. Finally we investigate whether LBP and sCD14 influence the cellular response to nontypable *Haemophilus influenzae* (NTHi) a typical respiratory pathogen.

Methods

Population and ethics

Participants (9 never smokers, 10 smokers with normal lung function and 10 COPD patients, all male subjects) were recruited consecutively from the endoscopy unit of Hospital Universitary Son Dureta (a tertiary referral hospital) from those requiring a bronchoscopy for the clinical evaluation of a solitary pulmonary nodule or haemoptysis. Participants gave their written consent after being fully informed of the nature, characteristics, risks and potential benefits of the study, which had been approved previously by the Ethics Committee of our institution. COPD patients (six current smokers and four ex-smokers) were clinically stable and had not had an episode of

exacerbation during the 3 months previous to the bronchoscopy. Six patients were being treated with inhaled steroids, but none was receiving oral steroid therapy. We excluded individuals with other chronic lung diseases (asthma, bronchiectasis and interstitial lung diseases), atopy, cardiac, hepatic or renal failure. To avoid any potential effect of acute smoking, active smokers refrained from smoking 12 h before bronchoscopy. We confirmed this by measuring the exhaled carbon monoxide concentration before bronchoscopy, and it was lower than 10 parts per million (p.p.m.) in all subjects.

Lung function

Forced spirometry was obtained in all participants. Spirometric reference values were those of a Mediterranean population [16].

Broncho-alveolar lavage

BALF was obtained as previously described [17]. Differential cells counts were determined as described [17]. To investigate whether airways were colonised with bacteria, bronchial secretions were obtained using a protected specimen brush [17] and were found to be culture negative in all cases.

LBP, sCD14 and IL-8 determinations

LBP, sCD14 levels were determined in the unconcentrated BALF after removing cells by centrifugation using ELISA kits (HyCult Biotecnology). The sensitivity of the ELISAs for LBP and sCD14 are 4.4 ng/ml and 2 ng/ml respectively. IL-8 levels were also determined by ELISA (Endogen) with a sensitivity < 2pg/ml.

Bacterial cultures.

A nontypable *H. influenzae* isolate, strain 157952, was donated by A. Oliver (Hospital Son Dureta). Bacteria were grown in agar chocolate plates (Biomerieux) at 37^{o}C and 5% CO_2. Bacteria were recovered in PBS and a cell suspension of ca 10^9 cfu/ml was prepared in PBS and used as inoculum.

Recombinant proteins

Purified human LBP and sCD14 were purchased from HyCult Biotecnology and R&D Systems respectively. Human IL-6, IL-1β and TNFα were obtained from Peprotech.

Cell cultures

Monolayers of human lung carcinoma cells (A549, ATCC CCL185) and primary human airway epithelial cells (NHBE, Cambrex) were grown as previously described [18]. 18 h before infection A549 cells were serum starved. Prior to the experiment, A549 and NHBE cells were washed three times with PBS. Cell viability was assessed by trypan blue dye exclusion and it was > 95% even after 8 h post infection (with and without LBP and sCD14).

Cytokine stimulation, adhesion and invasion assays

A549 cells were infected at a multiplicity of infection of 100 bacteria per cell. For cytokine stimulation, after 6 h of infection, cells were washed three times with PBS, and then incubated with fresh medium containing gentamicin (100 μg/ml) for 12 additional hours. Supernatants were carefully removed from the wells, cell debris were removed by centrifugation, and samples were frozen at –80^{o}C. For the adhesion assays, cells were washed five times with PBS after 2 h of infection. Cells were lysed with 1%-

saponin in PBS and tittered for viable counts of bacteria by plating in BHI agar plates supplemented with hemin (10 μg/ml; Sigma) and NAD (10 μg/ml; Sigma). For the invasion assays, after two hours of infection, monolayers were washed three with PBS and then incubated for one additional hour with fresh medium containing gentamicin (100 μg/ml) to kill extracellular bacteria. Monolayers were washed three times with PBS, cells were lysed as previously described and tittered for viable counts of intracellular bacteria. Experiments were carried out in triplicate in three independent occasions and results are expressed as per cent invasion or adhesion = 100 x (no. of bacteria recovered from well/initial no. of bacteria added).

NF-κB assay

NF-κB activation was measured using the TransAM NFκB kit (Active Motif). A549 cells seeded in 60 mm tissue culture dishes were infected for 1 h. Cytoplasmic and nuclear extracts were obtained exactly as described by the manufacturer of the kit (Active Motif) and presence of activated NF-κB was determined exactly as recommended by the manufacturer using 15 μg of protein (Active Motif). Results are the mean ± SEM from three independent experiments.

Immunoblots

Cytoplasmic proteins (15 μg) were separated by 10% SDS-PAGE, electrotransferred to nitrocellulose membrane and blocked with 3% skimmed milk in PBS. Immunostaining for IκBα was performed with polyclonal rabbit anti- IκBα antibody (Santa Cruz Biotecnology) whereas immunostainings for phosphorylated p38 was performed with polyclonal rabbit anti-phospho-p38 antibody (Cell Signaling). Immunoreactive bands were visualized by incubation with conjugated-horseradish

peroxidase swine anti-rabbit immunoglobulins (Dako P0217) using the SuperSignal West-dura system (Pierce). Blots were reprobed with polyclonal antibody anti human tubulin, or anti p38 to control that equal amounts of proteins were loaded in each lane.

RT-PCR

NHBE cells seeded in 60 mm tissue culture dishes were treated with the different stimuli for 24 h. After treatment, cells were washed with PBS and total RNA was purified using a Nucleospin RNAII kit (Macherey-Nagel) as recommended by the manufacturer. cDNA was obtained by incubating 1 μg of RNA with 1mM dNTPmix (Promega), 1 μl of Oligo $(dT)_{15}$ as primers (Promega), 20 units of RNasin (Promega), and 2.5 units of AMV reverse transcriptase (Promega) in a total volume of 20 μl in the manufacturer's buffer (Promega) for 1 h at 42°C followed by 5 min at 99°C to inactivate the enzyme. PCR was performed using 10 μl of cDNA as template and the following intron spanning primers: human LBP (sense, 5´- AGGGCCTGAGTCTCAGCATCT -3'; antisense, 5'- CAGGCTGGCCGTGTTGAAGAC -3'), human CD14 (sense, 5'-ACTTATCGACCATGGAGC-3'; antisense, 5'- AGGCATGGTGCCGGTTA-3'). Primers for human β-actin (sense, 5´-GGATGCCACAGGATTCCATACC-3´; antisense, 5´-GAGCAAGAGAGGTATCCTGACC-3´) were used as internal control. For the detection of LBP the thermocycling protocol was as follows: 40 cycles 94°C 30 seg, 57 °C 1 min, 72 °C 1 min; whereas for the detection of CD14 the protocol was 94°C for 2 min, 35 cycles of denaturation at 98°C for 30 sec, annealing at 60°C for 10 sec, and extension at 72°C for 30 min. For the detection of β-actin, only 30 cyles were applied, the temperature of annealing was 60°C for 1 min, and extension was 72°C for 1 min. Amplification products were resolved on a 1.5% agarose gel and recorded with a Gene Genius Bio imaging system (Syngene) as jpeg files. They were exported to a PC

for densitometry analysis using the ImageJ software (http://rsb.info.nih.gov/ij/download.html). Bands were selected in each lane and analyzed using the Histogram analysis tool recording the mean. Results were expressed as relative ratio of LBP or CD14 (mean of gen/mean β-actin x 100). cDNAs were obtained from three independent extractions of mRNA and each one amplified by RT-PCR in three independent occasions.

Production of cigarette smoke condensate (CSE)

CSE was prepared from commercial cigarettes (0.8 mg of nicotine, 10 mg of tar, 10 mg of CO; Phillip Morris, Spain) as previously described with modifications [19]. Briefly, one cigarette was combusted using a syringe modified apparatus that draws the smoke into a sterile glass containing 5-ml of tissue culture medium. A 10-ml of smoke was drawn within 10 s following a 30-s brake. This process was repeated five times per cigarette. The CSE was filtered through a 0.22-μM filter. The resulting solution was designated a 100% CSE solution and was used within 30 min.

Statistical analysis

Results are expressed as mean ± SEM. Differences between experimental groups were analyzed by non-parametric two sided Mann Whitney *U*-test or one-way analysis of variance using Kruskal-Wallis contrasts as appropriate (GraphPad Sotware Inc.). A p value lower than 0.05 was considered significant.

Results

Clinical data

Table 1 shows the clinical and functional data of subjects included in the study. COPD patients were slightly older than the other two groups. Cumulative smoking exposure was not significantly different between smokers with and without COPD. Lung function was normal in never smokers and smokers without COPD whereas, by definition, patients with COPD had airflow obstruction (which was moderate to severe) (Table 1). The volume of BALF recovered was significantly lower in COPD patients than in never smokers, but total cell count was significantly higher in patients with COPD and smokers with normal lung function than in never smokers (Table 1). Differential cell counts were not different between groups (Table 1).

Levels of LBP and sCD14 are increased in BALF from smokers and COPD patients

BALF levels of LBP (Fig. 1A) and sCD14 (Fig. 1B) were higher in smokers and COPD patients than in never smokers whereas levels of both proteins were not significantly different in smokers and COPD patients. The levels of LBP correlated with the levels of sCD14 ($r = 0.98$; $p < 0.05$). There was not significant correlation between the levels of LBP or sCD14 and BAL recovery and differential cell counts in any of the groups.

Airway epithelial cells produce LBP and sCD14

To investigate whether airway epithelial cells could be one of the sources LBP and sCD14 found in BALF of smokers and COPD patients, NHBE cells were incubated in the presence of IL-6 and IL-1β, known to stimulate the production of acute proteins [20, 21]. After 48 h, IL-6 alone or in combination with IL-1β increased the levels of LBP in culture supernatants (Fig. 2A), whereas both stimuli did not significantly increase the levels of sCD14 (Fig. 2B). After 72 h, the levels of LBP (Fig. 2A) and

sCD14 (Fig. 2B) were significantly higher in culture supernatants from stimulated cells than in those from non stimulated cells.

We sought to determine whether CSE could stimulate the production of LBP and sCD14 by NHBE cells. After 48 h, either 5% or 2.5% CSE increased significantly the levels of LBP but not of sCD14 (Fig. 2). After 72 h, both amounts of CSE increased the levels of LBP and sCD14 (Fig. 2). The levels of LBP and sCD14 induced by 5% CSE were significantly higher than those induced by 2.5% CSE ($p < 0.05$) (Fig. 2).

To determine whether the increased protein levels were paralleled by elevated levels of mRNA semi quantitative RT-PCR analysis were carried out. IL-6, alone and in combination with IL-1β, increased mRNA levels of *lbp* and *cd14* (Fig. 2C). CSE also increased the levels of *lbp* and *cd14* being the effect of 5% CSE higher than that of 2.5% CSE. Densitometric analysis confirmed these results (Fig. 2D).

LBP and sCD14 inhibit the cellular response to nontypable Haemophilus influenzae

NTHi is a pathogen frequently isolated from the airways of smokers and COPD patients and it induces the activation of an inflammatory program by airway epithelial cells [22]. We investigated the effect of LBP and sCD14 on the activation of NHBE and A549 cells by NTHi. In the presence of LBP, the amount of IL-8 secreted by infected NHBE cells was reduced (Fig. 3A). Likewise, NTHi-induced IL-8 secretion was also reduced in the presence of sCD14 (Fig. 3A). Combination of both proteins (LBP and sCD14) had the strongest effect (Fig. 3A). Similar results were obtained when A549 cells were used (Fig. 3B). Neither LBP nor sCD14 alone or both together induced the secretion of IL-8 by NHBE or A549 cells (data not shown). To rule out a nonspecific effect of LBP and sCD14 on the activation of NHBE cells, we investigated whether both proteins affect IL-8 production induced by TNFα. The amount of IL-8 secreted by

NHBE in response to TNFα (final concentration of 100 ng/ml) was independent of the presence of LBP and sCD14 (both used together at a final concentration of 100 μg/ml) [amount of IL-8 induced by TNFα in the absence (993 ± 25 pg/ml) or presence of both proteins (1084 ± 90 pg/ml); $p > 0.05$].

It has been shown that activation of NF-κB by NTHi is necessary for IL-8 secretion by epithelial cells [22]. We investigated whether LBP and sCD14 would affect NF-κB activation upon infection of A549 cells by NTHi. Cellular lysates prepared from infected cells in the presence or absence of LBP and sCD14 were analysed using an NF-κB ELISA. Lysates from infected cells in the presence of LBP and sCD14 yielded lower amounts of active NF-κB than lysates from infected cells, and this amount was similar to that of non infected cells (Fig. 4A). One of the major pathways for NF-κB activation involves the phosphorylation of IκBα followed by degradation of the protein thereby allowing the migration of NF-κB dimers to the nucleus. We analyzed the expression of IκBα in cytoplasmic extracts by immunoblot analysis. IκBα degradation was apparent in infected cells but absent if infection occurred in the presence of LBP and sCD14 (Fig. 4B).

p38 MAP kinase is also involved in NTHi-dependent secretion of IL-8 by epithelial cells [22]. We studied whether LBP and sCD14 would affect p38 MAP kinase activation upon infection of A549 cells by NTHi. Figure 4C shows that LBP and sCD14 inhibited the NTHi-induced phosphorylation of p38 MAP kinase.

Altogether, the data show that LBP and sCD14 decrease the ability of NTHi to stimulate a cell inflammatory response.

LBP and sCD14 increase the adhesion and internalisation of nontypable Haemophilus influenzae

We sought to determine whether both proteins modified the interaction between NTHi and airway epithelial cells. LBP or sCD14 alone did not alter the adhesion of NTHi to A549 cells (Fig. 5A) whereas both proteins (present at 100 ng/ml) increased the adhesion of NTHi to A549 cells. The effect of LBP and sCD14 on NTHi internalization by A549 cells was also studied. 100 ng/ml LBP plus sCD14 significantly increased the number of intracellular NTHi recovered (Fig. 5B).

Discussion

The main findings of this study are: (i) LBP and sCD14 levels are increased in BALF of smokers with and without COPD; (ii) airway epithelial cells in culture produce LBP and sCD14 in response to stimulation by cytokines and cigarette smoke condensate; and, (iii) LBP and sCD14 decrease the ability of NTHi to stimulate an inflammatory response while facilitating its adhesion and internalization by airway epithelial cells.

Previous studies have shown the presence of LBP and sCD14 in the airways of healthy subjects [12, 23]. Moreover, evidence indicate that these levels increase in patients with lung diseases [8, 12, 23] yet, to the best of our knowledge, this is the first study showing elevated levels of both molecules in smokers. The fact that the levels of LBP and sCD14 were not significantly different between smokers and COPD patients suggests that both proteins might not play an important role in the development of COPD. However, further studies should be carried out to rigorously rule out any participation of these molecules in the pathogenesis of COPD. Hepatocytes are considered the main source of circulating LBP and sCD14 [1]. In fact, the amount of LBP and sCD14 in human serum increases during acute response by several folds [1]. Therefore, accumulation of both proteins in the airway may be caused by extravasation

from vascular compartment or by local production; these explanations are not mutually exclusive. Results from this study support the latter because we showed that IL-6 and IL-1β, known inducers of acute phase proteins, [20, 21], up regulated the expression of both proteins. Similar results have been published for LBP [24] but, to our knowledge, ours is the first study describing that airway epithelial cells can express sCD14. Considering that IL-6 and IL-1β are increased in the airways of smokers [13], we propose that both cytokines may stimulate airway epithelial cells to produce LBP and sCD14 in vivo thereby accounting, at least in part, for the elevated levels of both proteins in the airways of smokers. Mechanistically, human promoter regions of LBP and CD14 contain binding sites for AP-1, C/EBP and APRE/STAT-3 [25, 26] and both cytokines are known to activate these signalling pathways [27, 28]. We showed that CSE also induced the expression of LBP and sCD14. The molecular mechanism responsible for this is currently unknown. It is known that CSE activates AP-1 and STAT-3 signalling pathways [29] which are involved in the expression of LBP and sCD14 [25, 26]. We cannot rigorously rule out a paracrine mechanism via induction of cytokine expression. However, neither IL-6 nor IL-1β were detected in the supernatants of CSE-treated cells (data not shown).

An important question is what could be the biological meaning of these high levels of LBP and sCD14 in the airways. The classical vision states that LBP will transfer pathogen-associated molecular patterns (PAMPs) to CD14 to facilitate cellular defence responses [1, 2]. In turn, high levels of both molecules would prone the host to develop an overwhelming inflammatory response leading to complications such as septic shock and acute respiratory distress syndrome [1, 2].

In contrast, high levels of LBP and sCD14 are associated with an inhibitory effect on cellular activation by bacterial components [1]. This is considered a host

defence mechanism to blunt an otherwise fierce inflammatory response [1, 2]. Currently discussed explanations are the ability of LBP to transfer bacterial PAMPs to serum lipoproteins which is greatly enhance by sCD14 resulting in the neutralization of the bioactivity of the bacterial molecules [30].The prevailing vision postulates that the balance of the cellular responses against PAMPs is modulated by the levels of LBP and sCD14.

We have shown that LBP and sCD14, alone and in combination, reduced the NTHi-dependent IL-8 secretion by airway epithelial cells. We chose to use intact live bacteria instead of killed bacteria or purified PAMPs, such as LPS, to reflect NTHi-airway epithelial cell interaction which is the likely in vivo scenario. In fact, it has been shown that purified LPS from NTHi does not recapitulate the cellular response induced by whole bacteria in human airway epithelial cells [31]. NTHi-induction of IL-8 secretion is dependent on the activation of NF-κB and MAP kinase p38 signalling pathways [22]. We hypothesized that LBP and sCD14 could downregulate the stimulation of these pathways by NTHi. Our results showed that both proteins inhibited the NTHi-dependent activation of NF-κB by affecting IκBα degradation. Both proteins also abrogated the NTHi-dependent phosphorylation of MAP kinase p38. Of note in our study the inhibition of the cellular activation was not caused by a reduction in the number of adherent or internalised bacteria. On the contrary, high levels of LBP and sCD14 increased the percentage of adherent and intracellular bacteria. Importantly, in the case of NTHi it has been clearly shown that there is no correlation between bacterial adhesion and the activation of airway epithelial cells [31, 32] and therefore it was not unexpected that the increased uptake of NTHi by airway cells was not associated with greater cell response.

One potential limitation of the current study deserves some discussion. It would be of interest to compare the production of LBP and sCD14 by primary airway epithelial cells from smokers versus never smokers. To this end, tissue samples should be obtained by a surgical procedure and expression of molecules analyzed by immunohistochemistry. However, we have shown that stimuli widely accepted to be present in the airways of smokers and COPD patients did stimulate the production of LBP and sCD14 by human primary airway epithelial cells.

Based on our data and on all the summarized evidence, we propose that the high levels of LBP and sCD14 found in the airways of smokers could be one of the host mechanisms contributing to prevent an overwhelming inflammatory response caused by smoking and the constant exposure of airways to bacteria and PAMPs. Thus, both proteins could contribute to reduce the bacteria and PAMPs-induced inflammatory response without compromising the internalisation of pathogens by airway cells. Future studies will aim to clarify all these issues.

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Table 1. Main clinical, functional and BAL fluid data (mean ± SEM)

	Never smokers (n=9)	Smokers with normal lung function (n=10)	COPD patients (n=10)
Age (years)	56±5	60±3	64±2*
Smoking history (pack-years)	0	37±5	45±3
FEV_1(% ref)	103±16	98±10	58±6*
FEV_1/FVC (%)	79±11	78±6	57±7*
Total cell count ($x10^3$/ml)	65.6±17.6	271±58*	182±33*
Macrophages (%)	92±1	92±2	85±3
Lymphocytes (%)	7 ± 1	7 ± 2	9 ± 2
Neutrophils (%)	1 ± 0.4	1 ± 0.2	3 ± 2
BALF recovery (millilitres)	105 ± 7	94 ± 4	70 ± 7*

FEV_1: forced expiratory volume in one second measured after bronchodilatation.

FVC: forced vital capacity.

* significant difference ($p < 0.05$) versus never smokers.

Figure 1. Analysis of the concentrations of LBP (panel A), and sCD14 (panel B) in unconcentrated BALF from never smokers (n=9), smokers (n=10), and COPD patients (n=10). The results were analyzed by one-way analysis of variance using Kruskal-Wallis contrasts.

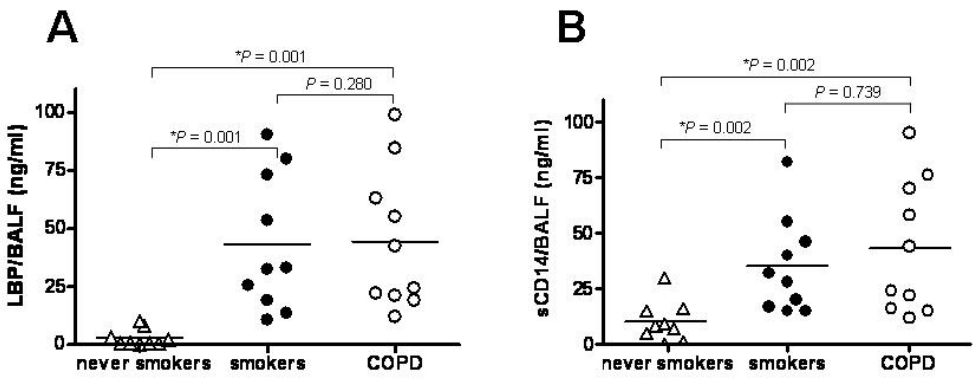


Figure 2. Analysis of the concentrations of LBP (panel A) and sCD14 (panel B) secreted by NBHE cells 48 h (white bars) and 72 h (gray bars) after stimulation with IL-6 (50 ng/ml); IL-6 and IL-1β (100 ng/ml and 5 ng/ml, respectively) and CSE (2.5 and 5% final concentrations). CON stands for non treated cells. The results were analyzed by one-way analysis of variance using Kruskal-Wallis contrasts.

Analysis by RT-PCR of mRNA levels (panel C) of *lbp* and *cd14* from NHBE treated with IL-6 (50 ng/ml); IL-6 and IL-1β (100 ng/ml and 5 ng/ml, respectively) and CSE (2.5 and 5% final concentrations) for 24 h. Densitometry analysis of gels (panel D)

(samples were obtained from three independent extractions of mRNA and each one was amplified by RT-PCR in three independent occasions).

- a significant difference ($p < 0.05$) from non-treated cells (CON). $^{\Delta}$ a significant difference ($p < 0.05$) from treated cells with 5% CSE.

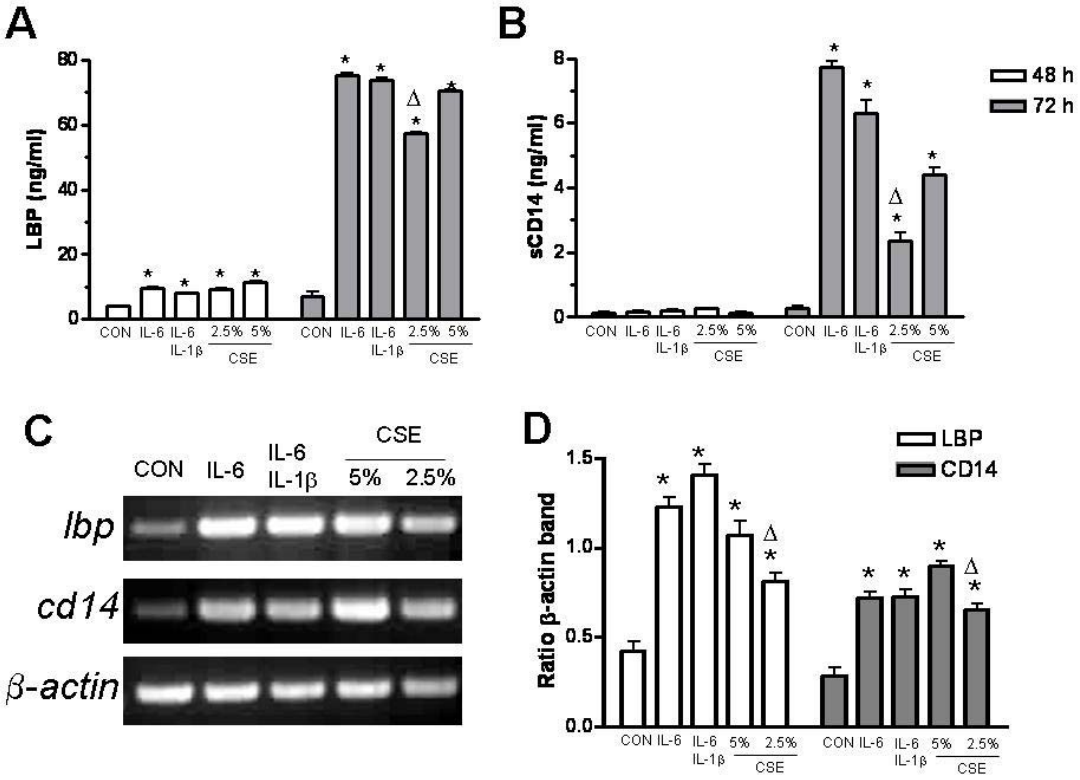


Figure 3. Levels of IL-8 secreted to the culture medium by NHBE cells (panel A) and A549 cells (panel B) infected with NTHi in the absence or presence of different concentrations of LBP and sCD14. The results were analyzed by one-way analysis of variance using Kruskal-Wallis contrasts.

∗ significant difference ($p<0.05$) from non infected cells (CON); Δ significant difference ($p<0.05$) from infected cells.

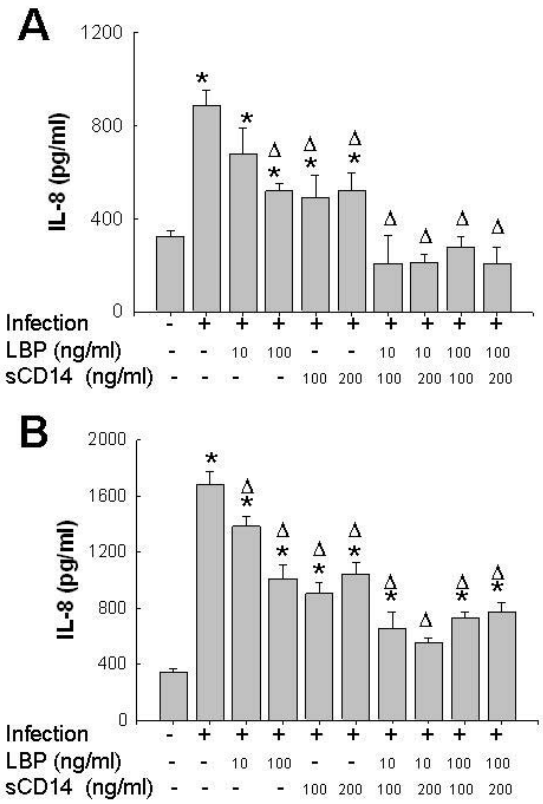


Figure 4. LBP (100 ng/ml) and sCD14 (100 ng/ml) reduce the NTHi-dependent activation of NF-κB and MAP kinase p38 pathways.

Panel A. LBP and sCD14 reduce the NTHi-dependent translocation of NF-κB to the nuclei of A549 cells. ∗ significant difference ($p<0.05$) from non infected cells; Δ significant difference ($p<0.05$) from infected cells.

Panel B. Upper panel, Immunoblot showing IκBα levels in cytoplasmic extracts of A549 cells infected with NTHi for 1 h in the absence or presence of LBP and sCD14. Lower panel, immunoblot showing tubullin levels under the same conditions. The results are representative of four independent experiments.

Panel C. Immunoblots showing phospho-p38, and total p38 levels in cytoplasmic extracts of A549 cells infected with NTHi for different time points (30 to 90 min) in the

absence or presence of LBP and sCD14. The results are representative of four independent experiments.

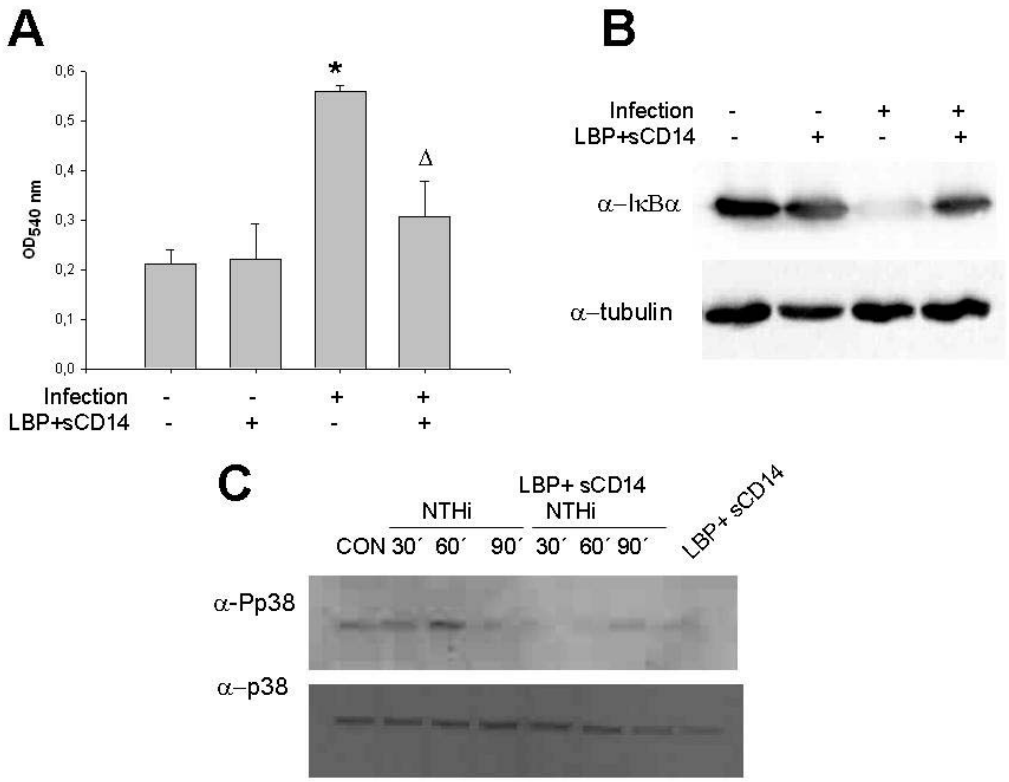
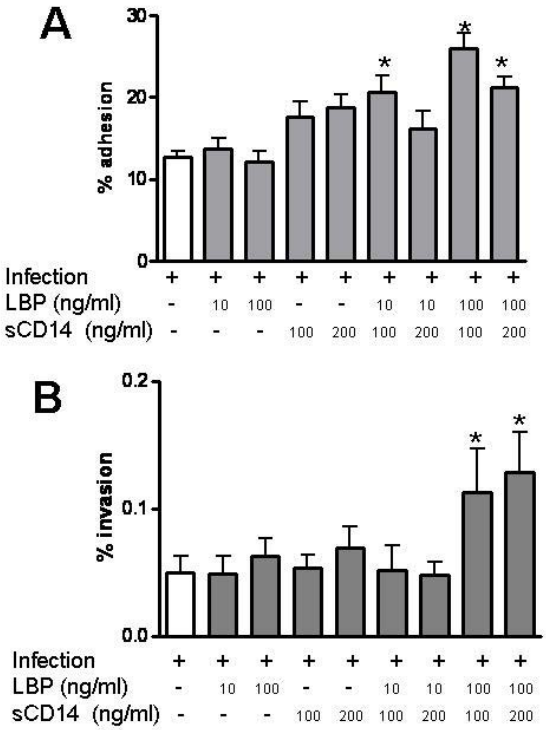


Figure 5. LBP and sCD14 enhance the adhesion (panel A) and internalisation (panel B) of NTHi by A549 cells. ∗ significant difference ($p<0.05$) from infected cells in the absence of LBP and sCD14.


A
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20
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0
*
*
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Infection + + + + + + + + +
LBP (ng/ml) - 10 100 - - 10 10 100 100
sCD14 (ng/ml) - - - 100 200 100 200 100 200
B
% invasion
0.2
0.1
0.0
*
*
Infection + + + + + + + + +
LBP (ng/ml) - 10 100 - - 10 10 100 100
sCD14 (ng/ml) - - - 100 200 100 200 100 200