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Soluble CD14 acts as a DAMP in human macrophages: origin and involvement in inflammatory cytokine/chemokine production

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Short title: sCD14 in human macrophages
ABBREVIATIONS

Abbreviations used in this article: sCD14, soluble form of CD14; CF, cystic fibrosis; DAMPs, danger-associated molecular patterns; ER, Endoplasmic Reticulum; FEV1, Force Expiratory Volume in one second; NLRs, NOD-like receptors; PAMPs, pathogen-associated molecular patterns; PI-PLC, phosphatidylinositol-phospholipase C.
The innate immune system is able to detect bacterial lipopolysaccaride (LPS) through the pattern recognition receptor CD14, which delivers LPS to various TLR signaling complexes that subsequently induce intracellular pro-inflammatory signaling cascades. In a previous study, we showed the overproduction of the soluble form of CD14 (sCD14) by macrophages from patients with cystic fibrosis (CF). CF is an autosomal recessive disorder that is caused by mutations in the gene that encodes the CFTR protein and characterized by persistent inflammation. Macrophages play a significant role in the initial stages of this disease due to their inability to act as suppressor cells leading to chronic inflammation in CF. In this work, we investigated the origin of sCD14 by human macrophages and studied the effect of sCD14 on the production of inflammatory cytokine/chemokine. Our data indicate that sCD14 stimulate pro-inflammatory cytokine/chemokine production in a manner that is independent of LPS but dependent on the TLR-4/CD14 membrane complex, NF-κB and the inflammasome. Therefore sCD14, overproduced by CF macrophage, originates primarily from the endocytosis/exocytosis process and should be considered to be a DAMP. In fact, this elucidation of the origin and inflammation-induced mechanisms associated with sCD14 contributes to our understanding of maintained tissue inflammation.

**Key words:** inflammation, cystic fibrosis, NF-κB, inflammasome
INTRODUCTION

CD14 was first characterized as a membrane-associated GPI-anchored protein and a cell surface differentiation marker that is present on the surface of monocytes, macrophages, dendritic cells and neutrophils (1–5). CD14 acts as a receptor for bacterial LPS in cooperation with TLR-4 and -2 (5, 6) and can bind various bacterial ligands and receptors on phagocytes, thereby mediating the phagocytosis of bacteria and the clearance of apoptotic cells (4, 7–9). CD14 also exists as two soluble forms: a form with a low molecular mass of 48-50 kDa and a form with a high molecular mass of 53-56 kDa. The mechanisms of CD14 production have been reported to be linked to neosynthesis, proteolytic or GPI-tail cleavage by phospholipases or endocytosis/exocytosis (2, 3, 10–18). In cells that express membrane CD14, including mononuclear cells, high concentrations of sCD14 are able to inhibit LPS-mediated functional responses (19, 20). In cells that lack membrane CD14, including most epithelial and endothelial cells, sCD14 enables these cells to respond to LPS (8, 21). At the local site of infection, pro-inflammatory signals that result from sCD14 can be protective, leading to local clearance of invading bacteria. However, widespread infection and activation of this pro-inflammatory signaling pathway can cause fatality through sepsis (22, 23). Furthermore, significant levels of sCD14 were detected in both serum and bronchoalveolar lavage fluid from patients with acute respiratory distress syndrome (24). In addition, it was demonstrated in a murine model that Streptococcus pneumoniae uses sCD14 in the bronchoalveolar space to cause invasive respiratory tract infections (25). Overall, sCD14 appears to act as a key component during pulmonary inflammation/infection.

Chronic bacterial airway infection and subsequent intense neutrophilic inflammation with the release of intracellular proteases are considered to be the main contributors to bronchiectasis and end-stage lung disease CF patients. Recent research highlighted the worsening role of inflammation and immune responses in CF airway disease (26). Macrophages are antigen-presenting phagocytes that secrete pro-inflammatory mediators and antimicrobial factors in response to challenge by extracellular pathogens. Recently, several studies showed that specific features of macrophage activation in CF patients play an important role during the CF disease process (27–29). In our previous work, we observed an increase in sCD14 secretion by peripheral monocyte-derived macrophages from stable adult CF patients (29). However, the origin and
involvement of sCD14 has not been studied. In this work, we examined the mechanisms involved in the release of sCD14 by peripheral monocyte-derived macrophages from adult with CF and the inflammatory signaling pathways that are induced by sCD14 in non CF cells.
MATERIALS AND METHODS

CF patients
The experiments were conducted according to the Good Clinical Practice guidelines (Kong, 1997) and approved by the Ethics Committee for human subjects of Rennes University Hospital (France, Ethics No. 11/38-827). All patients included in this study gave written informed consent. Sixty stable adult patients with CF were recruited at the ‘Centre de Ressources et de Compétences pour la Mucoviscidose’ of Rennes University Hospital (France). The CF patients considered for inclusion were Caucasian and included 27 males and 33 females, who were aged between 18 and 52 years (mean age: 30 ± 9). The CF diagnosis was based on typical clinical manifestations of the disease and confirmed by positive sweat tests and by CFTR gene mutation detection. Stable patients were defined by the absence of changes in symptoms in the 3 months prior to the study. All patients with CF received medication at the time of blood collection, including azithromycin (27%), aerosol DNase (48%), inhaled corticosteroids (72%) and azole therapy (47%). Oral corticosteroid therapy at the time of blood collection was an exclusion criterion, as this therapy may influence inflammatory phenotypes. Patients with the G551D mutation were not treated with ivacafor at the time of their participation in the study (except for patient 19, supplemental Table 1). The clinical features of the patients are reported in supplemental Table 1. According to the FEV1 values (% predicted), the majority of our patients (41/60) had mild to moderate lung disease (FEV1 values ≥ 55%). In their sputum samples, 32 patients had microbiological evidence of Pseudomonas aeruginosa, and Staphylococcus aureus and Aspergillus fumigatus were detected in 45 and 29 patients, respectively. The CF genotypes were representative of the French CF population, with 57% of participants having F508del/F508del mutations, 36% of patients having F508del/other mutations and 7% of patients having no F508del mutations (30). Blood monocyte counts were within the normal range, with a median number of 0.679x10^9/L (range 0.23-1.12x10^9/L).

Cell cultures and treatments
Leukocytes were isolated by Ficoll gradient centrifugation, as described previously (31). Peripheral blood mononuclear cells from healthy non-CF subjects (written consent for the use of
blood samples for the research protocol was obtained, according to the regulation for blood
transfusion of the French blood organization EFS, Rennes) were seeded according to the specific
blood count of each subject. Monocytes, which were selected via a 1-hour adhesion step, were
differentiated for 6 days using GM-CSF (400 UI/ml, Genzyme, Lyon, France) in RPMI 1640
medium supplemented with 2 mM glutamine, antibiotics and 10% FBS (Lonza, Levallois Perret,
France). Before treatment, the macrophages were placed in medium without serum for 24 hours.
To study proteolytic origin of sCD14, non-CF macrophages were treated by e64d (1 µM, Sigma-
Aldrich, Saint-Quentin Fallavier, France), pepstatin A (1 µM, Sigma-Aldrich, Saint-Quentin
Fallavier, France), EDTA (0.1 mM, Sigma-Aldrich, Saint-Quentin Fallavier, France) or aprotinin
(0.3 µg/ml, Sigma-Aldrich, Saint-Quentin Fallavier, France) for 24 hours. To study GPI-tail
cleavage by phospholipase, PI-PLC (1 U/ml, Life Technologies, ThermoFisher Scientific, Saint
Aubin, France) was added to non-CF macrophages for 2 hours in the absence or presence of the
PI-PLC inhibitor U-73122 (12.5 µM, 1-hour pretreatment, Bertin Pharma, Montigny le
Bretonneux, France). In parallel, macrophages from CF patients were treated with U-73122 for
24 hours. To study membrane trafficking pathways, the cells were supplemented with water-
soluble cholesterol (15 to 30 µg/ml, 1-hour pretreatment, followed by 24 hours, Sigma-Aldrich,
Saint-Quentin Fallavier, France) or with brefeldin A (100 ng/ml, 1-hour pretreatment, followed
by 2 h, Cell Signaling Technology, Ozyme, Saint Quentin en Yvelines, France). To study
inflammatory signaling pathways, human recombinant sCD14 protein (500 ng/ml, <0.1 EU/µg
endotoxin, Sigma-Aldrich, Saint-Quentin Fallavier, France) was added to non-CF macrophages
for 24 hours in the absence or presence of Bay 11-7082 (1 and 10 µM, 1-hour pretreatment,
Calbiochem, Merck Millipore, Molsheim, France) or the caspase inhibitor Z-WEHD-FMK (1 to
30 µM, 30-min pretreatment, Calbiochem, Merck Millipore, Molsheim, France). To study TLR
involvement in the inflammatory effect of sCD14, sCD14 was added to non-CF macrophages in
the presence of the TLR-4 antagonist LPS-RS (LPS from the photosynthetic bacterium
Rhodobacter sphaeroides, 2 µg/ml, InvivoGen, Toulouse, France), a CD14-neutralizing antibody
(anti-hCD14 IgA, 10 µg/ml, 1-hour pretreatment, InvivoGen, Toulouse, France), a TLR4-
neutralizing antibody (anti-hTLR4 IgA, 10 µg/ml, 1-hour pretreatment, Invitrogen, ThermoFisher
Scientific, Saint Aubin, France) or control IgG2a (10 µg/ml, 1-hour pretreatment, InvivoGen,
Toulouse, France). At the doses used, the treatments did not affect the viability of the
macrophages (data not shown). Viability was measured using the CellTiter 96® AQueous One
Solution Cell Proliferation Assay (Promega, charbonnières les bains, France) according to the instructions provided by the manufacturer.

**Gene expression**

RNA expression was analyzed using RT-qPCR assays. Briefly, total RNA was isolated from CF macrophages using NucleoSpin® RNA XS (Macherey Nagel, Hœrdt, France) and from non-CF macrophages using the PureLink RNA Mini Kit (Ambion®, ThermoFisher Scientific, Saint Aubin, France). The total RNA (1 μg) was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems ThermoFisher Scientific, Saint Aubin, France). Real-time PCR was performed using the Power SYBR Green PCR Master kit (Applied Biosystems ThermoFisher Scientific, Saint Aubin, France) according to the manufacturer's instructions and an ABI Prism 7300 detector (Applied Biosystem ThermoFisher Scientific, Saint Aubin, France). The gene-specific primers for 18S, CD14, IL-1β, IL-8 were Quantitect®Primer Assay primers obtained from Qiagen (Hilden, Germany). The gene-specific primers for 18S, TNF, MMP-9, MMP-12, Cathepsin B, Cathepsin D, Cathepsin F, Cathepsin K, Cathepsin S, ADAM-9, ADAM-10, ADAM-12, ADAM-17, and PLCγ were purchased from Eurogentec (Liège, Belgium). A list of the primer sequences used for the RT-qPCR analysis is reported in supplemental Table 2. The amplification curves of the PCR products were analyzed with the ABI Prism SDS software using the comparative cycle threshold (CT) method. Relative gene expression was calculated by comparing the number of thermal cycles that were necessary to generate threshold amounts of product (CT). The CT was calculated for the each gene and for the housekeeping gene 18S. For each cDNA sample, the 18S CT was subtracted from the CT for each gene to yield the ΔCT, thus normalizing the initial amount of RNA used. The amount of mRNA was calculated as 2^{−ΔΔCT}, where the ΔΔCT is the difference between the ΔCT of the two cDNA samples to be compared. The data from CF samples are expressed relative to the mRNA level found in non-CF samples. The data from the treatment samples are expressed relative to the mRNA level found in the controls.

**Cytokine/chemokine, sCD14 and NFκB p65 level quantification**
IL-1β, IL-8, TNF-α and sCD14 levels were measured in the supernatants of the cell cultures and in plasma from CF patients using a Duoset® ELISA kit (R&D system, Abingdon, United Kingdom), according to the instructions provided by the manufacturer. The NFκB p65 level was measured in the nuclear fraction of cell cultures using TransAM® NFκB p65 (Active Motif, La Hulpe, Belgium), according to the instructions provided by the manufacturer.

**Neosynthesis measurement**
Soluble CD14 neosynthesis was measured using Click-IT ® technology (Thermo-fischer scientific). Macrophages were placed in RPMI 1640 medium without methionine (Gibco, ThermoFisher Scientific, Saint Aubin, France). After 30 min incubation (37°C, 5% CO2), macrophages were pulse-chase labeled in the presence of Click-IT® L- Azidohomoalanin (50µM; Molecular Probes, ThermoFisher Scientific, Saint Aubin, France) in RPMI 1640 medium without methionine for 3 hours. Afterwards, medium was changed by RPMI 1640 with methionine and macrophages were incubated for 21 hours (37°C, 5% CO2). Then supernatants were recovered and analyzed for sCD14 level quantification using Duoset® ELISA kit (R&D system, Abingdon, United Kingdom). To measure neosynthesis, biotin-coupled detection antibody was substituted by Click-IT® Biotin DIBO Alkyne (Molecular Probes, ThermoFisher Scientific, Saint Aubin, France), for copper-free click chemistry.

**Cholesterol level quantification**
Total lipids were extracted with 200 µl CHCl₃/MeOH/NP-40 (7:11:0.1; v/v). The sample was centrifuged for 10 min at 14,000 rpm. The chloroformic layer was evaporated to dryness under a nitrogen stream. Then, the free cholesterol levels was determined using Cholesterol/Cholesteryl Ester Quantitation Kit (Merck Millipore, Molsheim, France), according to the instructions provided by the manufacturer. Cholesterol is oxidized by cholesterol oxidase to yield H₂O₂. The resulting H₂O₂ interacts with a sensitive cholesterol probe to produce resorufin, which can be detected by spectrophotometrically 570 nm. Total protein concentrations were determined using a BCA protein assay kit (Thermoscientific, Saint Aubin, France) to express cholesterol based on the protein concentration for each sample.
Zymography assay

The gelatinolytic activity of secreted MMP-9 was analyzed using gelatin zymography, as described previously (32). The samples were subjected to electrophoresis via 10% SDS-PAGE containing 1 mg/ml of gelatin (Sigma Aldrich, Saint-Quentin Fallavier, France) under non-reducing conditions. After electrophoresis, the gels were washed twice with 2.5% Triton X-100, rinsed with water, and incubated at 37°C overnight in reaction buffer (50 mM Tris, 5 mM CaCl₂, and 2.1 mM ZnCl₂, pH 8.0). The gels were stained with Coomassie brilliant blue (Sigma Aldrich, Saint-Quentin Fallavier, France) and de-stained in a solution of 25% ethanol and 10% acetic acid. Gelatinase activity appeared as clear bands against a blue background. The molecular weights of the gelatinolytic bands were estimated using a prestained protein ladder (10 - 250 kDa, Euromedex, Souffelweyersheim, France). Images of the zymograms were acquired using the Gel Doc 1000 Gel Documentation System (Bio-Rad laboratories, Marnes-La-Coquette, France), and both the surface and intensity of the lysis bands were quantified by densitometry using the MultiGauge software (Fujifilm, Tokyo, Japan).

Gene silencing by siRNA

ON-TARGETplus siRNAs against NLRC4 or NLRP3 were synthesized and annealed by Dharmacon RNAi Technologies (ThermoFisher Scientific, Saint Aubin, France). siRNA duplexes were transfected using the Lipofectamine RNAiMAX Reagent (Life Technologies, ThermoFisher Scientific, Saint Aubin, France). The transfection reagent and siRNAs were mixed, and complex formation was allowed to proceed for 5 min at room temperature before the complex was added to the macrophages. After 72 hours, the cells were treated or not treated with sCD14 (500 ng/ml, 24 hours) before being harvested for analysis.

Immunoblotting

Membrane-bound proteins were extracted from the macrophages by lysing the cells with RIPA lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton-X100, 12 mM deoxycholate, 2 mM NaF, 1 mM Na₃VO₄, 2 mM PMSF, cOmplete™ EDTA-free Protease Inhibitor Cocktail (Roche, Bâle, Switzerland) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche, Bâle, Switzerland). The macrophages were incubated at 4°C for 30 minutes in lysis buffer with vortexing for 30 seconds every 10 minutes. The supernatant containing proteins
was obtained by subsequent centrifugation at 10,000 g for 10 minutes at 4°C, and the protein concentration was determined using a BCA protein assay kit (ThermoFisher Scientific, Saint Aubin, France). Proteins were separated via SDS-PAGE and transferred to a nitrocellulose membrane. Then, the membrane was subjected to western blotting using a rabbit anti-IκBα, rabbit anti-P-IκBα, rabbit polyclonal anti-Caspase 1, mouse anti-IL-1β, rabbit anti-NLRP3, rabbit anti-NLRC4 (Cell Signaling Technology, Ozyme, Saint Quentin en Yvelines, France), rabbit anti-CD14 (Epitomics, Burlingame, CA, US) or mouse anti-HSC70 (Santa Cruz, Heidelberg, Germany) antibodies. Horseradish peroxidase-conjugated goat anti-rabbit (Cell Signaling Technology, Ozyme, Saint Quentin en Yvelines, France) or goat anti-mouse (Dako, Les Ulis, France) antibodies were used as secondary antibodies, and proteins were detected using enhanced chemiluminescence. The images were scanned with the Fujifilm LAS-3000 imager (Fujifilm, Tokyo, Japan) and analyzed with the MultiGauge software (Fujifilm, Tokyo, Japan) for densitometry. The intensity of the bands was normalized based on HSC-70.

**Statistical analysis**

The number of subjects and experiments used in each group is stated in the respective figures. Statistical significance was evaluated by using the GraphPad Prism software v.6.0 (GraphPad Software Inc., San Diego, CA, US). A non-parametric Mann-Whitney test was used to assess the statistical significance of differences between non-CF and CF groups. A parametric ratio Student’s t-test was used to assess statistical significance between treatments. For each analysis, a p-value <0.05 was considered to be significant.
RESULTS

**Origin of sCD14 in human CF macrophages**

We previously observed the overproduction of sCD14 in the supernatant of macrophages from stable adult patients with CF (29). However, plasma levels of sCD14 do not differ between stable adult CF patients and healthy subjects (Fig. 1A).

To elucidate the molecular mechanisms that underlie the production of the soluble form of CD14 in human CF macrophages, we studied several hypotheses based on the literature. It was reported that sCD14 could be generated by different mechanisms, including the endocytosis of mCD14, the cleavage of the GPI anchor by PI-PLC, direct proteolytic cleavage from the cell surface and neosynthesis.

To explore the neosynthesis hypothesis, we examined the regulation of CD14 production at the mRNA level in CF and non-CF macrophages. We found that CD14 mRNA levels were similar between CF and non-CF macrophages (Fig. 1B). Furthermore, pulse chase experiments with Click-IT® L- Azidohomoalanin showed no differences in neosynthesis of sCD14 between non-CF and CF macrophages whereas we demonstrated an increase of total sCD14 production in supernatant of CF macrophages (Fig. 1C). Thus, neosynthesis can be ruled out as a mechanism.

sCD14 may be formed by direct proteolytic cleavage from the cell surface. However, the inhibitors of metalloproteases (EDTA, Fig. 2A), cysteine proteases (e64d, Fig. 2D), aspartic proteases (pepstatin A, Fig. 2E) or serine proteases (aprotinin, Fig. 2F) did not influenced sCD14 production. Furthermore, the gene expression of the metalloproteases MMP-9 (Fig. 2B), MMP-12 (Supplemental Fig. 1A), ADAM-9, -10, -12, 17 (Supplemental Fig. 1B to 1E) and cathepsins B, D, F, K and S (Supplemental Fig. 1F to 1J) in CF macrophages is decreased. Moreover, the latent form of the MMP-9 protein and supernatant MMP-9 activity, as measured by zymography, are decreased in CF vs. non-CF macrophages (Fig. 2C). The protease cleavage hypothesis is excluded by these results.
The main hypothesis concerning sCD14 generation is the cleavage of the GPI tail by PI-PLC. We investigated this hypothesis by exposing non-CF macrophages to PI-PLC (1 U/ml) with or without its inhibitor, U-73122 (12.5 µM). We observed that PI-PLC significantly increased the production of sCD14 by non-CF macrophages and that this effect was inhibited by U-73122 (Fig. 3A). However, the treatment of CF macrophages with U-73122 did not inhibit the increased secretion of sCD14 into their supernatant (Fig. 3B). Furthermore, the expression of the PLC gene does not differ between non-CF and CF macrophages (Supplemental Fig. 1G). Indeed, PI-PLC participates in the production of sCD14 in non-CF macrophages but has no role in sCD14 overproduction from CF macrophages.

Cholesterol has been reported to increase sCD14 secretion through the endocytosis and subsequent cleavage of mCD14. Furthermore cholesterol is increased in CF macrophages (Fig. 4A). Thus, we investigated the effect of water-soluble cholesterol supplementation in non-CF macrophages. The results presented in Fig. 4B show that sCD14 production by non-CF macrophages is increased by supplementation with water-soluble cholesterol (15 µg/ml). By modulating membrane lipid composition, cholesterol could be involved in the production of sCD14 in CF macrophages.

The fungal metabolite brefeldin A blocks forward protein transport between the ER and the Golgi complex but not retrograde transport (33). Incubation with brefeldin A decreased sCD14 secretion in non-CF and CF macrophages, implicating the secretory ER/Golgi pathway in sCD14 secretion in CF (Fig. 4C).

**Function of human recombinant sCD14 in human macrophages**

To evaluate the inflammatory role of sCD14 in human non-CF macrophages, we used human recombinant sCD14 (hr-sCD14), which did not affect the viability of the cells (data not shown).

We had to check the effect of human recombinant sCD14 on the NFκB activation, which is required for cytokine/chemokine production. In fact, the mean concentration of sCD14 in CF macrophage supernatants was 14.68 ng/ml per 2x10^6 cells/well (Fig. 3B), which corresponds to 220 ng/ml per 30x10^6 cells, in comparison to the mean level of sCD14 observed in non-CF
macrophage supernatants, which was 2.35 ng/ml per 30x10^6 cells/well (Fig. 4B). Indeed, we used increasing concentrations of human recombinant sCD14 (1, 10, 100 and 500 ng/ml on 30x10^6 non-CF cells/well) to evaluate its inflammatory role in macrophages.

To determine whether sCD14 has an inflammatory effect, we investigated the production of pro-inflammatory cytokine/chemokine by non-CF macrophages exposed to sCD14. Treatment with sCD14 significantly increased IL-1β, IL-8 and TNF-α gene expressions at 6 hours and secreted levels at 24 hours in a dose-dependent manner (Fig. 5A and 5B). The effects on gene expression were maintained at 24 hours (Fig. 5A). These results show that sCD14 could participate in chronic inflammation by promoting pro-inflammatory cytokine/chemokine secretion. Furthermore, sCD14 potentiates LPS-induced cytokine/chemokine production by non-CF macrophages (Supplemental Fig. 2).

The NF-κB pathway is one of the pathways involved in cytokine expression. We studied NF-κB activation after sCD14 treatment in non-CF macrophages. The results showed the phosphorylation of IκBα at 10 min, concomitant with a decrease in the IκBα protein and a time-dependent translocation of the NFκB p65 subunit to the nucleus (Fig. 6A and 6B). Indeed, the NF-κB pathway is activated by sCD14 in non-CF macrophages. To determine whether this activation is related to the expression and secretion of inflammatory cytokines, we pretreated non-CF macrophages with an inhibitor of NF-κB, Bay 11-7082. Bay 11-7082, which is a potential anti-inflammatory agent, is an irreversible inhibitor of IKKα and cytokine-inducible IκBα phosphorylation that does not affect constitutive phosphorylation (34). A significant decrease in IL-1β, TNF-α and IL-8 gene expressions and secreted levels was observed with Bay 11-7082 (Fig. 6C and 6D). These results show that the NF-κB pathway participates in the pro-inflammatory cytokine/chemokine production induced by non-CF macrophages sCD14 treatment.

Unlike other pro-inflammatory cytokines, IL-1β production is tightly regulated by a unique two-signal mechanism. The primary signal induces the expression of pro-IL-1β and is mediated in part by NF-κB activation. The secondary signal activates the NLR-subset inflammasome intracellular signaling complexes, which are composed of NLRs, pro-caspase 1, and the adaptor
protein ASC. The formation of the inflammasome complex results in the proteolytic cleavage of pro-caspase 1 to yield active caspase 1, which in turn cleaves pro-IL-1β, producing mature and active IL-1β. To determine if caspase 1 could be involved in IL-1β processing in response to sCD14, we investigated caspase 1 expression and activation. Macrophage exposure to sCD14 induced early caspase 1 cleavage starting after 5 min through the early production of pro-caspase 1 and the appearance of cleaved caspase 1 (Fig. 7A). This cleavage is followed by the subsequent secretion of IL-1β by macrophages starting after 120 min (Fig. 7B). To investigate the involvement of caspase 1 in IL-1β secretion after sCD14 treatment, we pre-incubated non-CF macrophages with the caspase 1 specific inhibitor Z-WEHD-FMK. The caspase 1 inhibitor abolished IL-1β secretion by non-CF macrophages (Fig. 7C). All of these results showed that IL-1β production in response to sCD14 involves the caspase 1 pathway.

Caspase 1 pathway activation requires a specific platform called the inflammasome complex. However, various inflammasome complexes are formed depending on the stimulus. Concerning the inflammasome complex in macrophages, the most frequently involved stimuli are flagellin or LPS. We investigated the NLRC4 and NLRP3 inflammasome complexes by using RNA interference. The efficiency of transfection of NLRC4 siRNA and NLRP3 siRNA was assessed via western blotting of the total protein fraction from non-CF macrophages. Specific siRNAs directed against NLRC4 and NLRP3 turned off NLRC4 and NLRP3 protein expression in macrophages, respectively (Fig. 8A and 8B). Furthermore, while transfection with NLRP3 siRNA or NLRC4 siRNA had no significant effect on IL-1β secretion, in macrophages treated with sCD14, transfection with each siRNA significantly decreases IL-1β secretion (Fig. 8C). These results suggested that both inflammasome platforms (NLRC4 and NLRP3) participate in the induction of IL-1β secretion in response to sCD14 exposure.

In view of the mechanisms described above, the TLR-4 receptor and its signaling pathways should be involved in the effects induced by sCD14 in macrophages. To efficiently neutralize the biological activity of sCD14, non-CF macrophages were pre-incubated with a CD14-neutralizing antibody, resulting in decreased IL-8, TNF-α and IL-1β levels in the cell supernatants (Fig. 9). Moreover, the pretreatment of non-CF macrophages with an antagonist of TLR-4 (LPS-RS)
strongly inhibited the TNF-α, IL-8 and IL-1β production induced by sCD14 (Fig. 9). Finally to neutralize TLR4, non-CF macrophages were pre-incubated with a TLR4-neutralizing antibody, resulting in decreased IL-8 and TNF-α levels in the cell supernatants. Treatment with sCD14 did not modify the membrane expression of TLR-4, as measured by flow cytometry, showing that TLR-4 is not endocytosed after activation (data not shown). In this context, TLR-4 should be involved in the inflammatory effect of sCD14.
DISCUSSION

Macrophages coordinate inflammatory responses by secreting cytokines and chemokines that are responsible for diverse physiological effects that are important in the pathogenesis of CF and result in chronic infection/inflammation (35–37).

In a previous study, we observed an increase in cytokine/chemokine production in CF macrophage supernatants and particularly strong secretion of sCD14 (29). Elevated concentrations of sCD14 were also found in serum from patients with bacterial infections (38, 39) or pneumonia (40), as well as in other chronic inflammatory diseases, such as lupus erythematosus (41). However, we did not observe elevated sCD14 levels in plasma from stable adult CF patients, showing that sCD14 is not a systemic marker in CF, as previously observed in the plasma of children with CF (40).

It has been previously shown that sCD14 could be generated by several mechanisms, which include neosynthesis, direct proteolytic cleavage from the cell surface, cleavage of the GPI anchor by phospholipases and endocytosis/exocytosis (2, 3, 10–18). In our study, CD14 gene expression was the same in CF and non-CF macrophages. Moreover, no neosynthesis of sCD14 was observed in CF macrophage. Indeed, the elevated sCD14 levels observed in CF macrophage supernatants cannot be explained by an increase in gene expression and synthesis. Proteases, metalloproteases and/or cathepsins have been demonstrated to be present in large amounts in CF airways (42). However, the gene expression of MMPs, ADAMs as well as cathepsins is lower in CF macrophages than in non-CF macrophages. Furthermore, MMP-9 and its activity are also decreased in CF macrophage supernatants. Regarding these data, the origin of sCD14 in human CF macrophages was not direct proteolytic cleavage from the cell surface, as reported previously, explaining the increase in plasma sCD14 that occurs during sepsis (43). Cleavage of the GPI anchor by phospholipases is also suggested to explain sCD14 release. In our study, PI-PLC induced increased production of sCD14 in non-CF macrophage supernatants. The PI-PLC inhibitor reversed this effect but not the sCD14 overproduction observed in CF macrophages. Thus, PI-PLC participates in the production of sCD14 in non-CF macrophages but has no role in sCD14 overproduction in CF macrophages. Finally, the hypothesis that involves the
endocytosis/exocytosis of CD14 was considered. The depletion of cholesterol from the plasma membrane has been shown to affect endocytosis and protein sorting (44). As an example, cholesterol perturbation by filipin led to the redistribution of GPI-GFP from the cell surface to the Golgi complex (45), and the supplementation of the medium with cholesterol allowed the transport of secretory membrane proteins from the ER to the Golgi (46). In our study, cholesterol level is higher in CF than in non-CF macrophages and cholesterol supplementation in non-CF macrophages increased sCD14 secretion by macrophages. Furthermore, other work has shown that the inhibition of cholesterol synthesis by lovastatin decreased sCD14 production in RAW 264.7 macrophages (47). Endocytosis/exocytosis appears to be a suitable hypothesis because the use of brefeldin A to block forward transport between the ER and the Golgi complex decreases sCD14 secretion in CF macrophages.

It is well established that sCD14 alone is not sufficient to promote inflammation, except in cells that do not express mCD14, but the presence of sCD14 can enhance the sensitivity of monocytes and macrophages to LPS or lipopeptide by several orders of magnitude (48). In our study, we show that treatment with sCD14 alone enhances the induced inflammatory response and the effects of LPS by stimulating the secretion of IL-8, TNF-α and IL-1β in non-CF macrophages. However, one question remains to be answered: how can sCD14 induce the production of these inflammatory cytokines and chemokines?

Therefore, CD14 is assumed to function as a multi-ligand pattern recognition receptor by recognizing and binding to different PAMPs and DAMPs (49). In combination with the TLRs proteins, CD14 is an important mediator of innate immune responses to infection (5, 8). Membrane CD14 is a co-receptor for endotoxin through TLR-4 trans-membrane signaling, both at the cell surface and within the endosomal compartment. TLR-4 has been shown to contribute to the PAMP response of human cells, and its activation results in a pathway based on the activation of TIR-containing adaptor molecules: the rapid TIRAP/MyD88 pathway, which activates NF-κB and MAPK, resulting in the expression of pro-inflammatory cytokines and chemokines (50). In human monocyte-derived macrophages, sCD14 promoted the phosphorylation of IκBα, allowing the activation of NF-κB. Bay 11-7082, which is a potential anti-inflammatory agent and an irreversible inhibitor of IKKα and IκBα phosphorylation,
diminished sCD14-elicited IL-8, TNF-α and IL-1β expression and secretion, suggesting a role for NFκB signaling in sCD14-dependent macrophage inflammation. Furthermore, macrophage activation with sCD14 induces the activation of pro-caspase 1, leading to the cleaved and active form of caspase 1. Next, pro-IL-1β is cleaved by caspase 1, and IL-1β mature is secreted in macrophage supernatants; this effect is inhibited by Z-WEHD-FMK, which is a specific caspase 1 inhibitor. The activation of caspase 1 involves inflammasome complex formation, which requires NLRs. NLRs are pattern recognition receptors and play key roles in the regulation of the innate immune response in cooperation with TLRs. Among the NLRs, NLRC4 and NLRP3 appear to be the most involved in this response. The infection of macrophages with several Gram-negative bacteria, including *Pseudomonas aeruginosa*, which currently infects CF patients, activates caspase 1 through NLRC4 (51). The activation of caspase 1 through NLRP3 is induced by DAMPs, such as K⁺ efflux, ATP via P2XR7, ROS, and cathepsin D from lysosome damage (52). We turned off NRLC4 and NLRP3 protein expression using specific siRNAs directed against NLRC4 or NLRP3. The treatment of macrophages with NLRP3 siRNA or NLRC4 siRNA abolished IL-1β secretion, suggesting that both inflammasome platforms (NLRC4 and NLRP3) induce IL-1β secretion in response to sCD14 exposure. Altogether, our data suggest that sCD14 acts as a DAMP in macrophages. In fact, extracellular sCD14 can bind membrane proteins or phospholipids (53) that could be responsible for inflammatory cytokine production. An anti-CD14 neutralizing antibody is able to inhibit the sCD14-induced synthesis of TNF-α, IL-8 and IL-1β. To explore the action of sCD14 via the TLR-4 receptor, we used a potent antagonist of this receptor LPS-RS and an anti-TLR4 antibody, which decreases partly sCD14-induced cytokine production in macrophages. Thus, part of the effect of sCD14 appears to be mediated by the CD14/TLR-4 complex. However, further investigations are needed to completely understand the molecular mechanisms involved in sCD14 signaling pathway in macrophages.

In conclusion, numerous mechanisms concerning the origin of sCD14 were considered in this work, but in CF macrophages, the endocytosis/exocytosis process appears to be the main mechanism involved in sCD14 production. We propose that the overproduction of sCD14 previously observed in macrophages from CF patients (29) should be considered to be a DAMP that can induce pro-inflammatory cytokine/chemokine production independent of LPS.
Moreover, sCD14 can also enhance the LPS response. Both of these effects are dependent on the TLR-4/CD14 membrane complex, NF-κB and the inflammasome. The characterization of the origin and inflammatory function of sCD14 presented in this paper is an important step in understanding chronic infection/inflammation. Furthermore, this study provides a novel approach to the development of anti-inflammatory therapeutics in CF.
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AUTHOR CONTRIBUTIONS

M. Lévêque, K. Simonin-Le Jeune and C. Martin-Chouly designed research; M. Lévêque, K. Simonin-Le Jeune and C. Martin-Chouly performed the experiments; S. Moulis, C. Belleguic, G. Brinchault, B. Desrues, S. Le Trionnaire, J-P. Gangneux and S. Jouneau contributed new reagents or analytic tools; M. Lévêque and C. Martin-Chouly analyzed data; M. Lévêque, M-T. Dimanche-Boitrel and C. Martin-Chouly wrote the paper.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Figure 1. Soluble CD14 level in plasma and CD14 gene expression in macrophages. (A) sCD14 levels in plasma from healthy subjects (non-CF, n=8) and CF patients (n=12, patients 1-12, supplemental Table 1), as measured using an ELISA assay. Each symbol represents a single individual, and the line is the mean. (B) CD14 gene expression in non-CF macrophages (n=14) and CF macrophages (n=15, patients 6, 13-26; supplemental Table 1). mRNA levels were determined by RT-qPCR (supplemental Table 2). Each symbol represents a single individual, and the line is the mean. (C) Neosynthesis (O.D.) and total (pg/ml) sCD14 in macrophage supernatant from healthy subjects (non-CF, n=5) and CF patients (n=5, patients 6, 13-16, supplemental Table 1). The data are shown as the mean ± SEM. Mann-Whitney test: * p<0.05 vs. non-CF macrophages.

Figure 2. Altered CF macrophage phenotype is associated with markedly decreased expression and activity of proteases. (A) The inhibition of metalloproteases by EDTA (0.1 mM, 24 hours) did not influence sCD14 secretion in non-CF macrophages (n=8) (B) MMP-9 expression is reduced in CF macrophages (n=6, patients 6, 13-16 and 32, supplemental Table 1) vs. non-CF macrophages (n=9). Gene expression was determined by RT-qPCR (supplemental Table 2). (C) In a representative gelatin zymogram, latent MMP-9 (92 kDa) is observed based on proteolysis areas, which appeared as clear bands against a dark background. After scanning the gels, both the surface and intensity of the lysis bands were analyzed by densitometry (bar graph) and showed decreased expression of latent MMP-9 in CF macrophages (n=12, patients 33-44, supplemental Table 1) vs. non-CF macrophages (n=11). The results were expressed vs. the relative latent MMP-9 intensity observed in the supernatant of non-CF macrophages. (D, E, F) The inhibition of cysteine proteases by e64d (D, 1 µM, 24 hours, n=10), aspartic proteases by pepstatin A (E, 1 µM, 24 hours, n=4) and serine proteases by aprotinin (0.3 µg/ml, 24 hours, n=9) did not influence sCD14 secretion in non-CF macrophages. The data are shown as the mean ± SEM. Mann-Whitney test: * p<0.05 and ** p<0.01 vs. non-CF macrophages.

Figure 3. PI-PLC increases sCD14 production in non-CF macrophages. sCD14 levels were measured in supernatants from macrophages using an ELISA assay. (A) PI-PLC (1 U/ml, 2
hours) significantly increases sCD14 production by non-CF macrophages \((n=11)\). The inhibition of PI-PLC by U-73122 \((12.5 \, \mu M, 1 \, \text{hour pretreatment})\) inhibited PI-PLC-induced sCD14 secretion in non-CF macrophages \((n=7)\). \((B)\) U-73122 \((12.5 \, \mu M, 24 \, \text{hours})\) did not prevent spontaneous sCD14 production by CF macrophages \((n=6, \text{patients 43, 45-49; supplemental Table 1})\). The data are shown as the mean ± SEM. Ratio Student’s t-test: \# \# \text{p}<0.01 \text{ vs.} \text{untreated non-CF macrophages;} ** \text{p}<0.01 \text{ vs.} \text{PI-PLC-treated non-CF macrophages.}

**Figure 4. Impact of intracellular protein transport on sCD14 production by non-CF and CF macrophages.** \((A)\) Free cholesterol level is increased in CF \((n=8, \text{patients 43, 47, 50-55; supplemental Table 1})\) vs non-CF \((n=4)\) macrophages \((B)\) Water-soluble cholesterol \((15 \, \mu g/ml; 24 \, \text{hours})\) significantly increases sCD14 production by non-CF macrophages \((n=5)\). \((C)\) Brefeldin A \((\text{Bref A; 100 ng/ml; 3 h})\) significantly decreased sCD14 production by non-CF \((n=5)\) and CF \((n=5, \text{patients 56-60; supplemental Table 1})\) macrophages. Levels in the supernatants were measured using an ELISA assay. The data are shown as the mean ± SEM. Mann-Whitney test: ** \text{p}<0.01 \text{ vs.} \text{non-CF macrophages. Ratio Student’s t-test: # \text{p}<0.05 \text{ vs.} \text{untreated non-CF macrophages and § \text{p}<0.05 \text{ vs.} \text{untreated CF macrophages.}}

**Figure 5. Human recombinant sCD14 induces the expression and secretion of pro-inflammatory cytokines in non-CF macrophages.** \((A)\) IL-1β, IL-8 and TNF-α mRNA expression was quantified by RT-qPCR in non-CF macrophages treated with hr-sCD14 \((100 \text{ and } 500 \, \text{ng/ml, 6 hours and 24 hours})\). The data are shown as the mean ± SEM of four and five independent experiments for 6 hours and 24 hours, respectively. \((B)\) IL-1β, IL-8 and TNF-α release were measured by using an ELISA assay in supernatants from non-CF macrophages treated with hr-sCD14 \((1 \text{ to } 500 \, \text{ng/ml, 24 hours})\). The data are shown as the mean ± SEM \((n=6-11, \text{and } n=3-6 \text{ for IL-1β, IL-8 and TNF-α, respectively})\). Ratio Student’s t-test: * \text{p}<0.05; ** \text{p}<0.01; *** \text{p}<0.001 \text{ and} **** \text{p}<0.0001 \text{ vs. untreated macrophages.}

**Figure 6. NF-κB pathway is involved in the sCD14-induced production of pro-inflammatory cytokines in non-CF macrophages.** \((A)\) Cytosol P-IkBα and IkBα expression were determined
by Western blotting of the total protein fraction from non-CF macrophages treated with hr-sCD14 (500 ng/ml, 0 to 60 min, representative blot of three independent experiments). Equal protein loading was controlled via HSC-70 detection. (B) NFκB p65 activation was measured using an assay in non-CF macrophages that were treated or not treated with hr-sCD14 (n=5; 500 ng/ml, 24 hours). A positive control was used to establish the result. (C) IL-1β, IL-8 and TNF-α gene expression was analyzed by RT-qPCR in non-CF macrophages treated or not treated with hr-sCD14 (500 ng/ml, 6 hours) in the absence and presence of an NF-κB inhibitor (Bay 11-7082, 10 µM, 1 hour pretreatment, n=5) (D) IL-1β, IL-8 and TNF-α protein expression were quantified using an ELISA assay in non-CF macrophages that were treated or not treated with hr-sCD14 (500 ng/ml, 24 hours) in the absence or presence of Bay 11-7082 (1 and 10 µM, 1 hour pretreatment; n=4, n=4 and n=5 for IL-1β, IL-8 and TNF-α, respectively). The data are shown as the mean ± SEM. Ratio Student’s t-test: * p<0.05 and *** p<0.001 vs. untreated macrophages; # p<0.05 and ## p<0.01 vs. hr-sCD14-treated macrophages.

Figure 7. Caspase-1 is involved in the sCD14-induced production of IL-1β in non-CF macrophages. (A) Pro-caspase 1 and caspase 1 expression were determined by Western blotting analysis of the total protein fraction from non-CF macrophages treated with hr-sCD14 (500 ng/ml, 0 to 60 min, a representative blot of three independent experiments). (B) IL-1β secretion was measured using an ELISA assay in supernatants from non-CF macrophages treated with hr-sCD14 (500 ng/ml, 0 to 1440 min). The data are shown as the mean ± SEM (n=7). (C) IL-1β secretion was measured using an ELISA assay in supernatants from non-CF macrophages treated or not treated with hr-sCD14 (500 ng/ml, 24 hours) in the absence or presence of the caspase 1 inhibitor Z-WEHD-FMK (1 to 30 µM, 1-hour pretreatment). The data are shown as the mean ± SEM (n=4). Ratio Student’s t-test: * p<0.05 and ** p<0.01 vs. untreated macrophages; # p<0.05 vs. hr-sCD14-treated macrophages.

Figure 8. NLRC4 and NLRP3 inflammasome pathways are involved in IL-1β secretion in non-CF macrophages treated with hr-sCD14. (A and B) The efficiency of transfection with NLRC4 siRNA or NLRP3 siRNA was assessed by Western blotting of the total protein fraction from non-CF macrophages. The bar graph shows the results of densitometric analysis (n=6). The
results were expressed vs. the relative intensity observed in Control siRNA-treated non-CF macrophages. (C) IL-1β release was measured by ELISA in supernatants from non-CF transfected macrophages that were treated or not treated with hr-sCD14 (500 ng/ml, 24 hours; n=6). The data are shown as the mean ± SEM. Ratio Student’s t-test: ** p<0.01 vs. siControl-treated macrophages; ## p<0.01 vs. untreated macrophages; § p<0.05 and §§ p<0.01 vs. hr-sCD14-treated macrophages.

Figure 9. sCD14-induced cytokine release by non-CF macrophages is reversed by antagonists that target CD14 or TLR-4. IL-1β, IL-8 and TNF-α secretion was measured by ELISA in supernatants from non-CF macrophages that were treated or not treated with hr-sCD14 (500 ng/ml, 24 hours) in the absence or presence of an anti-CD14 neutralizing antibody (CD14-ab, 10 µg/ml, 1-hour pretreatment), a TLR-4 antagonist (LPS-RS, 2 µg/ml, 1-hour pretreatment), an anti-TLR4 neutralizing antibody (TLR4-ab, 10 µg/ml, 1-hour pretreatment) or the IgG2a control (10 µg/ml, 1 hour pretreatment). The data are shown as the mean ± SEM (n=3-6, n=3-6 and n=3-6 for IL-1β, IL-8 and TNF-α, respectively). Ratio Student’s t-test: * p<0.05 and ** p<0.01 vs. hr-sCD14-treated macrophages.
Figure 1

(A) sCD14 plasma level (ng/ml) for non-CF and CF individuals.

(B) Relative CD14 mRNA expression for non-CF and CF individuals.

(C) Comparison of neosynthesis and total sCD14 concentration (pg/ml) for non-CF and CF individuals.
Figure 2

A. Relative MMP-9 mRNA expression vs non-CF latent MMP-9

B. sCD14 concentration (pg/ml)

C. MW 92 kDa

D. Cysteine proteases

E. Aspartic proteases

F. Serine proteases

Fig 2
Figure 3

A

B

sCD14 concentration (pg/ml)

U-73122

PI-PLC

Non-CF

CF

Fig 3
Figure 4

A

Free cholesterol (µg/µg of proteins)

B

sCD14 concentration (pg/ml)

C

sCD14 concentration (pg/ml)

Figure 4
Figure 5

A

Relative IL-1β mRNA expression

B

Relative IL-8 mRNA expression

Control
sCD14 100 ng/ml
sCD14 500 ng/ml

6 h 24 h

Control
sCD14 100 ng/ml
sCD14 500 ng/ml

6 h 24 h

Control
sCD14 100 ng/ml
sCD14 500 ng/ml

6 h 24 h

(*0.0664)

IL-1β concentration (pg/ml)

IL-8 concentration (pg/ml)

TNF-α concentration (pg/ml)

(**0.0001)

(**0.0001)

(**0.0001)

(0.0001)

(*0.05)

(*0.05)

(*0.05)
Figure 6

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B

C

D

MW 40 kDa 39 kDa

MW 40 kDa 39 kDa

MW 40 kDa 39 kDa

MW 40 kDa 39 kDa
Figure 7

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MW: 48 kDa
MW: 20 kDa

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IL-1β concentration (pg/ml)

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IL-1β concentration (pg/ml)

Z-WEHD-FMK (μM) 0 1 10 30 0 1 10 30 sCD14

** **
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Figure 9

Fig 9
Table S1: Characteristics of CF patients

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Abbreviations: A.f.: Aspergillus fumigatus; P.a.: Pseudomonas aeruginosa; S.a.: Staphylococcus aureus
FEV1: Force Expiratory Volume in one second
Table S2: List of primer sequences used for RT-qPCR analysis in this study

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FIGURE S1. Proteases and phospholipase C-γ gene expressions in non-CF and CF macrophages. (A) MMP-12 (n=4, patients 6, 13, 15-16; supplemental Table 1), (B to E) Adam 9, Adam 10, Adam 12 (n= 6, patients 17-18, 20-22, 24; supplemental Table 1) and Adam 17 (n= 6, patients 17, 21-22, 24, 26, 41; supplemental Table 1), (F to J) cathepsin-B, cathepsin-D, cathepsin-F, cathepsin-K and cathepsin-S (n= 3-6, patients 15-16, 32; supplemental Table 1) and (K) phospholipase C-γ (PLCγ, n=3-6, patients 17, 18, 20, 22, 24; supplemental Table 1). Gene (mRNA) expression was quantified in CF and non-CF macrophages by RT-qPCR. The data are shown as the mean ± SEM. Mann-Whitney test: * p<0.05 vs. non-CF macrophages.
FIGURE S2. sCD14 potentiates LPS-induced inflammatory cytokines/chemokines production in non-CF macrophages. IL-1β, IL-8 and TNF-α levels were measured using an ELISA assay in non-CF macrophages incubated with LPS (from *P. aeruginosa*, 100 ng/ml) in the absence and presence of sCD14 (500 ng/ml) (n=8). Ratio Student’s t-test: * p< 0.05; ** p< 0.01 and **** p<0.0001 vs LPS-treated macrophages.