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

Manuella Leveque, Karin Simonin-Le Jeune, Stéphane Jouneau, Solenn Moulis, Benoit Desrues, Chantal Belleguic, Graziella Brinchault, Sophie Le Trionnaire, Jean-Pierre Gangneux, Marie-Thérèse Dimanche-Boitrel, et al.

► To cite this version:

Manuella Leveque, Karin Simonin-Le Jeune, Stéphane Jouneau, Solenn Moulis, Benoit Desrues, et al.. Soluble CD14 acts as a DAMP in human macrophages: origin and involvement in inflammatory cytokine/chemokine production. FASEB Journal, 2017, 31 (5), pp.1891-1902. 10.1096/fj.201600772R . hal-01526434

HAL Id: hal-01526434

<https://hal-univ-rennes1.archives-ouvertes.fr/hal-01526434>

Submitted on 12 Jul 2017

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

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

Manuella Lévêque^{*,‡}, Karin Simonin-Le Jeune^{*,‡}, Stéphane Jouneau^{†,‡,§}, Solenn Moulis^{*,‡},
Benoit Desrues^{§,‡,ϕ}, Chantal Belleguic[§], Graziella Brinchault[§], Sophie Le Trionnaire^{*,‡}, Jean-
Pierre Gangneux^{‡,§} Marie-Thérèse Dimanche-Boitrel^{*,‡} and Corinne Martin-Chouly^{*,‡,1}

^{*}Research Institute for Environnemental and Occupationnal Health (IRSET) INSERM U1085,
team ‘Stress Membrane and Signaling,’ F-35043 Rennes, France.

[†]Research Institute for Environnemental and Occupationnal Health (IRSET) INSERM U1085,
team ‘Chemical contaminant immunity and inflammation,’ F-35043 Rennes, France.

[‡]University of Rennes 1, UMS Biosit, F-35000 Rennes, France.

[§]Centre Hospitalier Universitaire de Rennes, Centre de Ressource et de Compétences de la
Mucoviscidose, F-35064 Rennes, France

^ϕURL440-COSS, Centre Eugène Marquis, F-35064 Rennes, France

¹Corresponding author:

Dr. Corinne Martin-Chouly

2, avenue du Pr Léon Bernard

35043 RENNES Cedex FRANCE

E-mail: corinne.chouly@univ-rennes1.fr

Tel: (+33) 223234904

Fax: (+33) 223235055

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.

ABSTRACT

The innate immune system is able to detect bacterial lipopolysaccharide (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- κ B 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- κ B, 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

91 involvement of sCD14 has not been studied. In this work, we examined the mechanisms involved
92 in the release of sCD14 by peripheral monocyte-derived macrophages from adult with CF and the
93 inflammatory signaling pathways that are induced by sCD14 in non CF cells.

94

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 $\geq 55\%$). 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 $0.679 \times 10^9/L$ (range $0.23-1.12 \times 10^9/L$).

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 μ M, Sigma-
 Aldrich, Saint-Quentin Fallavier, France), pepstatin A (1 μ M, Sigma-Aldrich, Saint-Quentin
 Fallavier, France), EDTA (0.1 mM, Sigma-Aldrich, Saint-Quentin Fallavier, France) or aprotinin
 (0.3 μ g/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 μ M, 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 μ g/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/ μ g
 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 μ M, 1-hour pretreatment,
 Calbiochem, Merck Millipore, Molsheim, France) or the caspase inhibitor Z-WEHD-FMK (1 to
 30 μ M, 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 μ g/ml, InvivoGen, Toulouse, France), a CD14-neutralizing antibody
 (anti-hCD14 IgA, 10 μ g/ml, 1-hour pretreatment, InvivoGen, Toulouse, France), a TLR4-
 neutralizing antibody (anti-hTLR4 IgA, 10 μ g/ml, 1-hour pretreatment, Invitrogen, ThermoFisher
 Scientific, Saint Aubin, France) or control IgG2a (10 μ g/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, Hoerd, 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 18 S 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 $2^{-\Delta\Delta CT}$, 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-1 β , 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 NF κ B p65 level was measured in the nuclear fraction of cell cultures using TransAM[®] NF κ B 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 (50 μ M; 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 μ l 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-I κ B α , rabbit anti-P-I κ B α , rabbit polyclonal anti-Caspase 1, mouse anti-IL-1 β , 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 μ M). 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 μ g/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 NF κ B 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 2×10^6 cells/well (Fig. 3B), which corresponds to 220 ng/ml per 30×10^6 cells, in comparison to the mean level of sCD14 observed in non-CF

macrophage supernatants, which was 2.35 ng/ml per 30×10^6 cells/well (Fig. 4B). Indeed, we used increasing concentrations of human recombinant sCD14 (1, 10, 100 and 500 ng/ml on 30×10^6 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-1 β , 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- κ B pathway is one of the pathways involved in cytokine expression. We studied NF- κ B activation after sCD14 treatment in non-CF macrophages. The results showed the phosphorylation of I κ B α at 10 min, concomitant with a decrease in the I κ B α protein and a time-dependent translocation of the NF κ B p65 subunit to the nucleus (Fig. 6A and 6B). Indeed, the NF- κ B 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- κ B, Bay 11-7082. Bay 11-7082, which is a potential anti-inflammatory agent, is an irreversible inhibitor of IKK α and cytokine-inducible I κ B α phosphorylation that does not affect constitutive phosphorylation (34). A significant decrease in IL-1 β , 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- κ B pathway participates in the pro-inflammatory cytokine/chemokine production induced by non-CF macrophages sCD14 treatment.

Unlike other pro-inflammatory cytokines, IL-1 β production is tightly regulated by a unique two-signal mechanism. The primary signal induces the expression of pro-IL-1 β and is mediated in part by NF- κ B 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-1 β , producing mature and active IL-1 β . To determine if caspase 1 could be involved in IL-1 β 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-1 β by macrophages starting after 120 min (Fig. 7B). To investigate the involvement of caspase 1 in IL-1 β 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-1 β secretion by non-CF macrophages (Fig. 7C). All of these results showed that IL-1 β 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 NLRP3 and NLRC4 protein expression in macrophages, respectively (Fig. 8A and 8B). Furthermore, while transfection with NLRP3 siRNA or NLRC4 siRNA had no significant effect on IL-1 β secretion, in macrophages treated with sCD14, transfection with each siRNA significantly decreases IL-1 β secretion (Fig. 8C). These results suggested that both inflammasome platforms (NLRC4 and NLRP3) participate in the induction of IL-1 β 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-1 β levels in the cell supernatants (Fig. 9). Moreover, the pretreatment of non-CF macrophages with an antagonist of TLR-4 (LPS-RS)

390 strongly inhibited the TNF- α , IL-8 and IL-1 β production induced by sCD14 (Fig. 9). Finally to
391 neutralize TLR4, non-CF macrophages were pre-incubated with a TLR4-neutralizing antibody,
392 resulting in decreased IL-8 and TNF- α levels in the cell supernatants. Treatment with sCD14 did
393 not modify the membrane expression of TLR-4, as measured by flow cytometry, showing that
394 TLR-4 is not endocytosed after activation (data not shown). In this context, TLR-4 should be
395 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-1 β 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- κ B and MAPK, resulting in the expression of pro-inflammatory cytokines and chemokines (50). In human monocyte-derived macrophages, sCD14 promoted the phosphorylation of I κ B α , allowing the activation of NF- κ B. Bay 11-7082, which is a potential anti-inflammatory agent and an irreversible inhibitor of IKK α and I κ B α phosphorylation,

diminished sCD14-elicited IL-8, TNF- α and IL-1 β expression and secretion, suggesting a role for NF κ B 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-1 β is cleaved by caspase 1, and IL-1 β 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 NLRC4 and NLRP3 protein expression using specific siRNAs directed against NLRC4 or NLRP3. The treatment of macrophages with NLRP3 siRNA or NLRC4 siRNA abolished IL-1 β secretion, suggesting that both inflammasome platforms (NLRC4 and NLRP3) induce IL-1 β 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-1 β . 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.

488 Moreover, sCD14 can also enhance the LPS response. Both of these effects are dependent on the
489 TLR-4/CD14 membrane complex, NF- κ B and the inflammasome. The characterization of the
490 origin and inflammatory function of sCD14 presented in this paper is an important step in
491 understanding chronic infection/inflammation. Furthermore, this study provides a novel approach
492 to the development of anti-inflammatory therapeutics in CF.

493

REFERENCES

1. Bazil, V., Horejshi, V., Baudys, M., Kristofova, H., Strominger, J. L., Kostka, W., and Hilgert, I. (1986) Biochemical characterization of a soluble form of the 53-kDa monocyte surface antigen. *Eur. J. Immunol.* **16**, 1583–1589
2. Haziot, A., Chen, S., Ferrero, E., Low, M. G., Silber, R., and Goyert, S. M. (1988) The monocyte differentiation antigen, CD14, is anchored to the cell membrane by a phosphatidylinositol linkage. *J. Immunol.* **141**, 547–552
3. Simmons, D. L., Tan, S., Tenen, D. G., Nicholson-Weller, A., and Seed, B. (1989) Monocyte antigen CD14 is a phospholipid anchored membrane protein. *Blood* **73**, 284–289
4. Wright, S. D. (1995) CD14 and innate recognition of bacteria. *J. Immunol.* **155**, 6–8
5. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science* **249**, 1431–1433
6. Schütt, C. (1999) CD14. *Int. J. Biochem. Cell Biol.* **31**, 545–549
7. Gegner, J. A., Ulevitch, R. J., and Tobias, P. S. (1995) Lipopolysaccharide (LPS) Signal Transduction and Clearance. Dual roles for LPS binding protein and membrane CD14. *J. Biol. Chem.* **270**, 5320–5325
8. Pugin, J., Heumann, D., Tomasz, A., Kravchenko, V. V., Akamatsu, Y., Nishijima, M., Glauser, M. P., Tobias, P. S., and Ulevitch, R. J. (1994) CD14 Is a pattern recognition receptor. *Immunity* **1**, 509–516
9. Schiff, D. E., Kline, L., Soldau, K., Lee, J. D., Pugin, J., Tobias, P. S., and Ulevitch, R. J. (1997) Phagocytosis of gram-negative bacteria by a unique CD14-dependent mechanism. *J. Leukoc. Biol.* **62**, 786–794
10. Bazil, V. and Strominger, J. L. (1991) Shedding as a mechanism of down-modulation of CD14 on stimulated human monocytes. *J. Immunol.* **147**, 1567–1574
11. Bufler, P., Stiegler, G., Schuchmann, M., Hess, S., Krüger, C., Stelter, F., Eckerskorn, C., Schütt, C., and Engelmann, H. (1995) Soluble lipopolysaccharide receptor (CD14) is released via two different mechanisms from human monocytes and CD14 transfectants. *Eur. J. Immunol.* **25**, 604–610

12. Durieux, J.-J., Vita, N., Popescu, O., Guette, F., Calzada-Wack, J., Munker, R., Schmidt, R. E., Lupker, J., Ferrara, P., Ziegler-Heitbrock, H. W. L., and Labeta, M. O. (1994) The two soluble forms of the lipopolysaccharide receptor, CD14: Characterization and release by normal human monocytes. *Eur. J. Immunol.* **24**, 2006–2012
13. Stelter, F., Pfister, M., Bernheiden, M., Jack, R. S., Bufler, P., Engelmann, H., and Schütt, C. (1996) The Myeloid Differentiation Antigen CD14 is N- and O-Glycosylated. *Eur. J. Biochem.* **236**, 457–464
14. Thieblemont, N. and Wright, S. D. (1999) Transport of bacterial lipopolysaccharide to the Golgi apparatus. *J. Exp. Med.* **190**, 523–534
15. Kirkland, T. N. and Viriyakosol, S. (1998) Structure-function analysis of soluble and membrane-bound CD14. *Prog. Clin. Biol. Res.* **397**, 79–87
16. Bazil, V., Baudys, M., Hilgert, I., Stefanová, I., Low, M. G., Zbrozek, J., and Horejsí, V. (1989) Structural relationship between the soluble and membrane-bound forms of human monocyte surface glycoprotein CD14. *Mol. Immunol.* **26**, 657–662
17. Coyne, C. P., Howell, T., Smoldaka, H., Willetto, C., Fenwick, B. W., and Chenney, E. (2002) Alterations in membrane-associated CD14 expression and the simultaneous liberation of soluble CD14 fragment in adherent macrophages mediated by a leukocyte carboxyl/aspartate protease. *J. Endotoxin Res.* **8**, 273–283
18. Labeta, M. O., Durieux, J.-J., Fernandez, N., Herrmann, R., and Ferrara, P. (1993) Release from a human monocyte-like cell line of two different soluble forms of the lipopolysaccharide receptor, CD14. *Eur. J. Immunol.* **23**, 2144–2151
19. Haziot, A., Rong, G. W., Bazil, V., Silver, J., and Goyert, S. M. (1994) Recombinant soluble CD14 inhibits LPS-induced tumor necrosis factor- α production by cells in whole blood. *J. Immunol.* **152**, 5868–5876
20. Schütt, C., Schilling, T., and Krüger, C. (1991) sCD14 prevents endotoxin inducible oxidative burst response of human monocytes. *Allerg. Immunol. (Leipz.)* **37**, 159–164
21. Tapping, R. I. and Tobias, P. S. (2000) Soluble CD14-mediated cellular responses to lipopolysaccharide. *Chem. Immunol.* **74**, 108–121
22. Opal, S. M., Scannon, P. J., Vincent, J.-L., White, M., Carroll, S. F., Palardy, J. E., Parejo, N. A., Pribble, J. P., and Lemke, J. H. (1999) Relationship between Plasma Levels of

- Lipopolysaccharide (LPS) and LPS-Binding Protein in Patients with Severe Sepsis and Septic Shock. *J. Infect. Dis.* **180**, 1584–1589
23. Salomao, R., Brunialti, M. K. C., Rapozo, M. M., Baggio-Zappia, G. L., Galanos, C., and Freudenberg, M. (2012) Bacterial sensing, cell signaling, and modulation of the immune response during sepsis. *Shock Augusta Ga* **38**, 227–242
 24. Martin, T. R., Ruhenfeld, G., Steinberg, K. P., Hudson, L. D., Raghu, G., Moriarty, A. M., Leturcq, D. J., Tobias, P. S., and Ulevitch, R. J. (1994) ENdotoxin, endotoxin-binding protein, and soluble cd14 are present in bronchoalveolar lavage fluid of patients with adult respiratory distress syndrome. *Chest* **105**, 55S – 56S
 25. Dessing, M. C., Knapp, S., Florquin, S., de Vos, A. F., and van der Poll, T. (2007) CD14 Facilitates Invasive Respiratory Tract Infection by *Streptococcus pneumoniae*. *Am. J. Respir. Crit. Care Med.* **175**, 604–611
 26. Ratner, D. and Mueller, C. (2012) Immune Responses in Cystic Fibrosis. *Am. J. Respir. Cell Mol. Biol.* **46**, 715–722
 27. Cifani, N., Pompili, B., Anile, M., Patella, M., Diso, D., Venuta, F., Cimino, G., Quattrucci, S., Di Domenico, E. G., Ascenzioni, F., and Porto, P. D. (2013) Reactive-Oxygen-Species-Mediated *P. aeruginosa* Killing Is Functional in Human Cystic Fibrosis Macrophages. *PLoS ONE* **8**, e71717
 28. Del Porto, P., Cifani, N., Guarnieri, S., Di Domenico, E. G., Mariggì, M. A., Spadaro, F., Guglietta, S., Anile, M., Venuta, F., Quattrucci, S., and Ascenzioni, F. (2011) Dysfunctional CFTR Alters the Bactericidal Activity of Human Macrophages against *Pseudomonas aeruginosa*. *PLoS ONE* **6**, e19970
 29. Simonin-Le Jeune, K., Le Jeune, A., Jouneau, S., Belleguic, C., Roux, P.-F., Jaguin, M., Dimanche-Boitre, M.-T., Lecureur, V., Leclercq, C., Desrues, B., Brinchault, G., Gangneux, J.-P., and Martin-Chouly, C. (2013) Impaired Functions of Macrophage from Cystic Fibrosis Patients: CD11b, TLR-5 Decrease and sCD14, Inflammatory Cytokines Increase. *PLoS ONE* **8**, e75667
 30. Bellis, G., Lemonnier, L., and Sponga, M. (2012) Registre Français de la Mucoviscidose.
 31. Grevenynghe, J. van, Rion, S., Ferrec, E. L., Vee, M. L., Amiot, L., Fauchet, R., and Fardel, O. (2003) Polycyclic Aromatic Hydrocarbons Inhibit Differentiation of Human Monocytes into Macrophages. *J. Immunol.* **170**, 2374–2381

32. Martin-Chouly, C. A. E., Astier, A., Jacob, C., Pruniaux, M.-P., Bertrand, C., and Lagente, V. (2004) Modulation of matrix metalloproteinase production from human lung fibroblasts by type 4 phosphodiesterase inhibitors. *Life Sci.* **75**, 823–840
33. Miller, S., G., Carnell, L., and Moore, H.-P., H. (1992) Post-Golgi membrane traffic: brefeldin A inhibits export from distal Golgi compartments to the cell surface but not recycling. *J. Cell Biol.* **118**, 267–283
34. Pierce, J. W., Schoenleber, R., Jesmok, G., Best, J., Moore, S. A., Collins, T., and Gerritsen, M. E. (1997) Novel Inhibitors of Cytokine-induced I B Phosphorylation and Endothelial Cell Adhesion Molecule Expression Show Anti-inflammatory Effects in Vivo. *J. Biol. Chem.* **272**, 21096–21103
35. Douglas, T. A., Brennan, S., Gard, S., Berry, L., Gangell, C., Stick, S. M., Clements, B. S., and Sly, P. D. (2009) Acquisition and eradication of *P. aeruginosa* in young children with cystic fibrosis. *Eur. Respir. J.* **33**, 305–311
36. Eickmeier, O., Huebner, M., Herrmann, E., Zissler, U., Rosewich, M., Baer, P. C., Buhl, R., Schmitt-Grohé, S., Zielen, S., and Schubert, R. (2010) Sputum biomarker profiles in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) and association between pulmonary function. *Cytokine* **50**, 152–157
37. Tang, A., Sharma, A., Jen, R., Hirschfeld, A. F., Chilvers, M. A., Lavoie, P. M., and Turvey, S. E. (2012) Inflammasome-Mediated IL-1 β Production in Humans with Cystic Fibrosis. *PLoS ONE* **7**, e37689
38. Lin, B., Noring, R., Steere, A. C., Klempner, M. S., and Hu, L. T. (2000) Soluble CD14 Levels in the Serum, Synovial Fluid, and Cerebrospinal Fluid of Patients with Various Stages of Lyme Disease. *J. Infect. Dis.* **181**, 1185–1188
39. Nockher, W. A., Wick, M., and Pfister, H.-W. (1999) Cerebrospinal fluid levels of soluble CD14 in inflammatory and non-inflammatory diseases of the CNS: upregulation during bacterial infections and viral meningitis. *J. Neuroimmunol.* **101**, 161–169
40. Marcos, V., Latzin, P., Hector, A., Sonanini, S., Hoffmann, F., Lacher, M., Koller, B., Bufler, P., Nicolai, T., Hartl, D., and Griese, M. (2010) Expression, regulation and clinical significance of soluble and membrane CD14 receptors in pediatric inflammatory lung diseases. *Respir. Res.* **11**, 32

41. Egerer, K., Feist, E., Rohr, U., Pruss, A., Burmester, G. R., and Dörner, T. (2000) Increased serum soluble CD14, ICAM-1 and E-selectin correlate with disease activity and prognosis in systemic lupus erythematosus. *Lupus* **9**, 614–621
42. Le-Barillec, K., Si-Tahar, M., Balloy, V., and Chignard, M. (1999) Proteolysis of monocyte CD14 by human leukocyte elastase inhibits lipopolysaccharide-mediated cell activation. *J. Clin. Invest.* **103**, 1039–1046
43. Arai, Y., Mizugishi, K., Nonomura, K., Naitoh, K., Takaori-Kondo, A., and Yamashita, K. (2015) Phagocytosis by human monocytes is required for the secretion of presepsin. *J. Infect. Chemother.* **21**, 564–569
44. Ikonen, E. (2001) Roles of lipid rafts in membrane transport. *Curr. Opin. Cell Biol.* **13**, 470–477
45. Nichols, B. J., Kenworthy, A. K., Polishchuk, R. S., Lodge, R., Roberts, T. H., Hirschberg, K., Phair, R. D., and Lippincott-Schwartz, J. (2001) Rapid Cycling of Lipid Raft Markers between the Cell Surface and Golgi Complex. *J. Cell Biol.* **153**, 529–542
46. Miwako, I., Yamamoto, A., Kitamura, T., Nagayama, K., and Ohashi, M. (2001) Cholesterol requirement for cation-independent mannose 6-phosphate receptor exit from multivesicular late endosomes to the Golgi. *J. Cell Sci.* **114**, 1765–1776
47. Frey, T. and De Maio, A. (2007) Increased Expression of CD14 in Macrophages after Inhibition of the Cholesterol Biosynthetic Pathway by Lovastatin. *Mol. Med.* **13**, 592–604
48. Jersmann, H. P. (2005) Time to abandon dogma: CD14 is expressed by non-myeloid lineage cells. *Immunol. Cell Biol.* **83**, 462–467
49. Schmitz, G. and Orsó, E. (2002) CD14 signalling in lipid rafts: new ligands and co-receptors. *Curr. Opin. Lipidol.* **13**, 513–521
50. Dentener, M. A., Bazil, V., Asmuth, E. J. V., Ceska, M., and Buurman, W. A. (1993) Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages. *J. Immunol.* **150**, 2885–2891
51. Franchi, L., Eigenbrod, T., Muñoz-Planillo, R., and Nuñez, G. (2009) The Inflammasome: A Caspase-1 Activation Platform Regulating Immune Responses and Disease Pathogenesis. *Nat. Immunol.* **10**, 241
52. Yu, H. B. and Finlay, B. B. (2008) The Caspase-1 Inflammasome: A Pilot of Innate Immune Responses. *Cell Host Microbe* **4**, 198–208

646 53. Yu, B., Hailman, E., and Wright, S. D. (1997) Lipopolysaccharide binding protein and
647 soluble CD14 catalyze exchange of phospholipids. *J. Clin. Invest.* **99**, 315–324
648
649

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

We would particularly like to thank Mrs. Laurence Hiard and the nurses Mrs. Marie-Thérèse Jousseau and Mrs. Isabelle Berhault at CRCM (*Centre de Ressources et de Compétences pour la Mucoviscidose*) from Rennes University Hospital. This work is supported by the Cystic Fibrosis French Foundation “Vaincre La Mucoviscidose” (RF20130500807).

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 I). 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 $\pm$ 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 influenced 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 μ M, 24 hours, $n=10$), aspartic proteases by pepstatin A (E, 1 μ M, 24 hours, $n=4$) and serine proteases by aprotinin (0.3 μ g/ml, 24 hours, $n=9$) did not influenced sCD14 secretion in non-CF macrophages. The data are shown as the mean $\pm$ 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 μ M, 1 hour pretreatment) inhibited PI-PLC-induced sCD14 secretion in non-CF macrophages ($n=7$). (B) U-73122 (12.5 μ M, 24 hours) did not prevent spontaneous sCD14 production by CF macrophages ($n=6$, patients 43, 45-49; supplemental Table 1). The data are shown as the mean $\pm$ SEM. Ratio Student's t-test: ## $p<0.01$ vs. untreated non-CF macrophages; ** $p<0.01$ vs. 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 ($n=8$, patients 43, 47, 50-55; supplemental Table 1) vs non-CF ($n=4$) macrophages (B) Water-soluble cholesterol (15 μ g/ml; 24 hours) significantly increases sCD14 production by non-CF macrophages ($n=5$). (C) Brefeldin A (Bref A; 100 ng/ml; 3 h) significantly decreased sCD14 production by non-CF ($n=5$) and CF ($n=5$, patients 56-60; supplemental Table 1) macrophages. Levels in the supernatants were measured using an ELISA assay. The data are shown as the mean $\pm$ SEM. Mann-Whitney test: ** $p<0.01$ vs. non-CF macrophages. Ratio Student's t-test: # $p<0.05$ vs. untreated non-CF macrophages and § $p<0.05$ vs. untreated CF macrophages.

Figure 5. Human recombinant sCD14 induces the expression and secretion of pro-inflammatory cytokines in non-CF macrophages. (A) IL-1 β , IL-8 and TNF- α mRNA expression was quantified by RT-qPCR in non-CF macrophages treated with hr-sCD14 (100 and 500 ng/ml, 6 hours and 24 hours). The data are shown as the mean $\pm$ SEM of four and five independent experiments for 6 hours and 24 hours, respectively. (B) IL-1 β , IL-8 and TNF- α release were measured by using an ELISA assay in supernatants from non-CF macrophages treated with hr-sCD14 (1 to 500 ng/ml, 24 hours). The data are shown as the mean $\pm$ SEM ($n=6-11$, $n=4$ and $n=3-6$ for IL-1 β , IL-8 and TNF- α , respectively). Ratio Student's t-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$ and **** $p<0.0001$ vs. untreated macrophages.

Figure 6. NF- κ B pathway is involved in the sCD14-induced production of pro-inflammatory cytokines in non-CF macrophages. (A) Cytosol P-I κ B α and I κ B α 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-1 β 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 $\pm$ 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-1 β , 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 μ g/ml, 1-hour pretreatment), a TLR-4 antagonist (LPS-RS, 2 μ g/ml, 1-hour pretreatment), an anti-TLR4 neutralizing antibody (TLR4-ab, 10 μ g/ml, 1-hour pretreatment) or the IgG2a control (10 μ g/ml, 1 hour pretreatment). The data are shown as the mean $\pm$ SEM ($n=3-6$, $n=3-6$ and $n=3-6$ for IL-1 β , 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

Fig 1

Figure 2

Fig 2

Figure 3

Fig 3

Figure 4

Fig 4

Figure 5

Fig 5

Figure 6

Fig 6

Figure 7

Fig 7

Figure 8

Fig 8

Figure 9

Fig 9

Table S1: Characteristics of CF patients

Patient	Age	Sex	Genotype	Microbiology	BMI	FEV1 % predicted
1	32	F	F508del / F508del	P.a / A.f / S.a	15	45
2	33	M	F508del / F508del	P.a / A.f / S.a	22,9	66
3	34	F	F508del / W486X	P.a / S.a	19,6	37
4	22	F	F508del / F508del	P.a / A.f / S.a	16,8	32
5	39	M	F508del / G551D	Af	22,1	40
6	32	M	F508del / F508del	P.a / S.a	20,1	82
7	20	M	F508del / F508del	P.a / A.f / S.a	19,9	66
8	20	M	F508del / F508del	S.a	23,2	98
9	39	F	F508del / 2789+5G→A	P.a / S.a	19,3	66
10	22	M	F508del / F508del	A.f / S.a	19,5	78
11	22	F	1248+1G→A	S.a	22,7	104
12	20	F	F508del / I507del	P.a / A.f	18,4	61
13	30	F	F508del / 1248+1G→A	P.a / S.a	18,3	33
14	31	F	F508del / N1303K	P.a / A.f / S.a	15,4	28
15	22	F	F508del / F508del	P.a / A.f / S.a	19,6	79
16	34	M	F508del / F508del	P.a / A.f / S.a	20,5	51
17	27	F	F508del / F508del	A.f / S.a	20,5	97,2
18	35	F	F508del / F508del	P.a / S.a	19,1	30,2
19	43	M	F508del / G551D	P.a	20,9	79,1
20	28	F	1248+1G→A	A.f / S.a	20,7	90,5
21	40	M	F508del / F508del	P.a / A.f	21,9	48,7
22	18	F	F508del / F508del	None	24,1	100,4
23	37	F	R600S / CFTRdup4-10	S.a	18,6	95
24	20	F	F508del / S945L	A.f / S.a	21,3	68,4
25	25	M	F508del / F508del	A.f / S.a	24,4	58,5
26	24	F	F508del / F508del	P.a / A.f / S.a	16,5	28,4
27	19	M	F508del / 2789+5G→A	S.a	22,23	94,7
28	40	F	F508del / S492F	P.a / S.a	27,82	55,3
29	53	F	F508del / 2789+5G→A	P.a / A.f	17,61	36
30	28	M	F508del / F508del	A.f	23,74	103
31	53	M	F508del / 2789+5G→A	S.a	22,14	96,2
32	20	M	F508del / F508del	A.f / S.a	22,3	62
33	19	M	F508del / F508del	P.a / A.f	20,4	91
34	26	M	F508del / F508del	S.a	20,2	58,7
35	37	M	F508del / F508del	S.a	21,1	77,4
36	23	F	F508del / F508del	S.a	17,1	54,5
37	49	F	F508del / 3272-26A→G	P.a / S.a	22,8	62,2
38	31	F	F508del / G91R	P.a / S.a	24,3	61,5
39	19	F	F508del / F508del	S.a	18,8	96
40	27	M	F508del / F508del	S.a	20,2	73,1
41	26	F	F508del / F508del	P.a / A.f / S.a	19,1	67
42	33	M	F508del / F508del	P.a	22,2	64,4
43	18	F	F508del / F508del	None	25,8	102,1
44	37	F	F508del / F508del	S.a	19,1	77,5
45	21	F	F508del / G551D	S.a	22,5	103,7
46	46	M	F508del / 2789+5G→A	A.f / S.a	22,2	96,6
47	35	F	F508del / F508del	P.a / S.a	19,3	35,2
48	25	M	F508del / F508del	A.f / S.a	23,5	55,3
49	39	M	F508del / F508del	P.a	21	49
50	18	F	F508del / F508del	P.a / A.f / S.a	19,1	62,3
51	23	M	F311L / N1303K	S.a	18,5	93,8
52	21	F	F508del / F508del	P.a / A.f / S.a	18,4	86,7
53	26	M	F508del / S1251N	P.a / S.a	18,81	41,1
54	23	F	F508del / F508del	P.a / S.a	16,86	33,7
55	38	F	F508del / F508del	None	18,2	41
56	30	M	F508del / 1749insTA	P.a / A.f / S.a	18,3	21,1
57	32	F	F508del / 4382delA	P.a / A.f / S.a	18	29,5
58	23	F	F508del / 1677delTA	P.a / A.f	18	49
59	52	M	F508del / 2789+5G→A	Af	22,5	107,9
60	38	M	F508del / 1078delT	P.a / A.f / S.a	21,7	63

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

Target genes	Primer	Sequence (5' to 3')
18S	Hs_RRN18S_1_SG	QT00199367
18S	Forward	CGCCGCTAGAGGTGAAATTC
	Reverse	TTGGCAAATGCTTTTCGCTC
CD14	Hs_CD14_1_SG	QT00208817
IL-1β	Hs_IL1B_1_SG	QT00021385
IL-8	Hs_CXCL8_1_SG	QT00000322
TNFα	Forward	AACCTCCTCTCTGCCATC
	Reverse	ATGTTTCGTCCTCCTCACA
MMP-9	Forward	TCTTCCCTGGAGACCTGAGA
	Reverse	ATTTCGACTCTCCACGCATC
MMP-12	Forward	ACACATTTTCGCCTCTCTGCT
	Reverse	ATTGTCAGGATTTGGCAAGC
Cathepsin B	Forward	AGAATGGCACACCCTACTGG
	Reverse	GCCACCACTTCTGATTGAT
Cathepsin D	Forward	GTACATGATCCCCTGTGAGAAGGT
	Reverse	GGGACAGCTTGTAGCCTTTGC
Cathepsin F	Forward	GCCTGTCCGTCTTTGTCAAT
	Reverse	TGGCTTGCTTCATCTTGTTG
Cathepsin K	Forward	CCGCAGTAATGACACCCTTT
	Reverse	GCACCCACAGAGCTAAAAGC
Cathepsin S	Forward	TGGGAGACATGACCAAGTGAA
	Reverse	AGCAAGCACCACAAGAACCT
Adam 9	Forward	TGCAAATGTCTTCAGGGAAC
	Reverse	TGCATTAACCTCACCGATGAC
Adam 10	Forward	CATTGCTGAATGGATTGTGG
	Reverse	GAGCCTGGAAGTGGTTTACG
Adam 12	Forward	AACCTCGCTGCAAAGAATGTG
	Reverse	CTCTGAAACTCTCGGTTGTCTG
Adam 17	Forward	ACCTGAAGAGCTTGTTTCATCGAG
	Reverse	CCATGAAGTGTTCCGATAGATGTC
PLCγ	Forward	AACCAGAAGTCCTTTGTCTTCATC
	Reverse	CTGGTTCTTCTCCAGTACTTCAT

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 (PLC γ , $n=5-6$, 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 $\pm$ 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-1 β , 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.