Research article

Innate Immunity

15(1) (2009) 53–62

ISSN 1753-4259 (print)
10.1177/1753425908100500

Synthetic peptides representing the N-terminal segment of surfactant protein C modulate LPS-stimulated TNF-α production by macrophages

Ignacio Garcia-Verdugo[1], Elvira Garcia de Paco[1], Quentin Espinassous[1], Azucena Gonzalez-Horta[2], Monique Synguelakis[1], Jean Kanellopoulos[1], Luis Rivas[3], Richard Chaby[1], Jesús Perez-Gil[2]

[1]*Institut de Biochimie et Biophysique Moléculaire et Cellulaire, UMR-8619 du CNRS, Université de Paris-Sud, Orsay, France*
[2]*Departamento de Bioquímica y Biología Molecular I, Universidad Complutense de Madrid, Madrid, Spain*
[3]*Centro de Investigaciones Biológicas, CSIC, Madrid, Spain*

Surfactant protein C (SP-C) consists of a hydrophobic α-helix inserted in pulmonary surfactant membranes, and a more polar N-terminal palmitoylated segment exposed to the aqueous phase. Previously, we showed that SP-C inserted in lipid vesicles interacts with bacterial lipopolysaccharide (LPS) and reduces LPS-elicited responses. As the N-terminal segment of SP-C was the most likely region responsible for these effects, a set of synthetic analogs of this stretch ($SPC_{(1-13)}$) were studied. Binding studies showed that $SPC_{(1-13)}$ binds LPS to the same extent as porcine SP-C under lipid-free conditions. In the absence of serum, both, palmitoylated and non-palmitoylated analogs enhanced the binding of tritiated LPS to macrophages as well as the LPS-induced production of TNF-α by these cells. These effects were reversed in the presence of serum; the analogs reduced the production of TNF-α in LPS-stimulated macrophages, probably by interfering with the formation of LPS/CD14/LBP complexes as suggested by analysis of the fluorescence emitted by a FITC derivative of Re-LPS. Our data indicate that water-soluble analogs of the N-terminal segment of SP-C can reduce LPS effects in the presence of serum, and thus might help in the design of new derivatives to fight endotoxic shock and pro-inflammatory events.

Keywords: Inflammation, LBP, LPS, SP-C synthetic peptides, TNF-α

Introduction

Surfactant protein C (SP-C) is a 4.2-kDa lipopeptide associated with pulmonary surfactant lipids that is synthesized and secreted specifically by alveolar epithelial type II cells.[1] Surfactant protein C which, on a molar basis, represents the major surfactant protein (65%) is one of the most hydrophobic peptides identified to date, and lacks homologues in the human genome. The protein consists of a regular hydrophobic α-helix comprising 23 residues and a more polar 10-residue N-terminal segment containing stoichiometrically palmitoylated vicinal cysteines.[2] The C-terminal α-helix adopts a transmembrane orientation in phospholipid bilayers,[3,4] with the N-terminal segment exposed to the aqueous phase.[5] Surfactant protein C catalyzes the transfer of surface-active phospholipids into the interface to form an operative surfactant film and modulates the behavior of surfactant membranes in the alveolar spaces (reviewed by Serrano and Perez-Gil[6]).

In addition to its important contribution to surfactant biophysical properties, SP-C is also involved in natural

Received 3 September 2008; Revised 3 November 2008; Accepted 4 November 2008

Correspondence to: Prof. Jesús Perez-Gil, Departamento de Bioquímica y Biología Molecular I, Universidad Complutense de Madrid, 28040 Madrid, Spain. Tel: +34 91 3944994; Fax: +34 91 3944672; E-mail: jpg@bbm1.ucm.es

defense mechanisms. When inserted in lipid vesicles, SP-C interacts with bacterial lipopolysaccharide (LPS).[7] A detailed analysis of LPS/SP-C interaction indicated that the N-terminal region of SP-C interacts with the lipid A region of LPS, whereas the C-terminal hydrophobic region of SP-C might provide an optimal conformation of the protein in the membranes.[8] Moreover, SP-C attenuates LPS-elicited responses. Surfactant protein C associated with liposomes inhibited the LPS-induced production of TNF-α by peritoneal and alveolar macrophages, and of nitric oxide by RAW 264.7 cells.[9] This is due to the capacity of SP-C to interact with LPS along with the ability of the protein to interact with CD14.[10]

CD14 acts as the LPS receptor for presentation to the LPS signaling receptor complex, TLR4/MD-2.[11] CD14 is expressed on the surface of myeloid and non-myeloid cells as a glycosylphosphatidylinositol-linked glycoprotein (mCD14) or in a soluble form in the serum (sCD14).[12] Another serum protein that mediates LPS signaling is the LPS binding protein (LBP). This protein is mainly synthesized in the liver and is present in large quantities in serum when induced as an acute phase protein. It catalyses the transfer of LPS to a binding site on CD14, thus increasing the binding affinity of CD14 to LPS.[13] By accelerating the binding of LPS to CD14, LBP enhances LPS-signaling through TLR4/MD-2, and the resulting cell responses. Although LBP and sCD14 play key roles in promoting sensitive responses to LPS, *in vitro* and *in vivo* experiments showed that both LBP and sCD14 can also inhibit responses to LPS by mechanisms that depend on their concentration and environment (reviewed by Kitchens and Thompson[14]).

We have recently described that peptides matching the N-terminal segment of SP-C interact with, and perturb, monolayers and liposomes made of surfactant lipids to the same extent that SP-C itself.[15–18] These data indicated that the N-terminal segment somehow mimics some of the biophysical features of whole SP-C molecule. In the present work, we were interested to determine whether the N-terminal segment of SP-C also carries out the LPS-binding and anti-endotoxin capacities described for SP-C in order to design soluble theraputic synthetic peptides to counteract lethal effects of LPS. As a first step to address this issue, we analyzed the interactions between the water-soluble analogs of the N-terminal segment of SP-C with radiolabeled or fluorescent derivatives of a rough-type LPS, and the capacity of these analogs to modulate the LPS-induced production of TNF-α by macrophages.

Materials and Methods

Reagents

Lipopolysaccharide from strain R595 of *Salmonella enterica* sv. Minnesota (Re-chemotype LPS; L-9764), bovine serum albumin with low endotoxin content (BSA), FITC isomer I, bovine brain L-α-phosphatidyl-L-serine (PS), egg L-α-phosphatidylcholine (PC), sphingomyelin (SM) from bovine brain, and L-α-phosphatidylethanolamine (PE) from *Escherichia coli* were purchased from Sigma Chemical Co. (St Louis, MO, USA). All lipids including Re-LPS were used without further purification. Organic solvents were from Merck (Darmstadt, Germany). Fetal calf serum (FCS) was from BioMedia (Boussens, France). All other cell culture reagents, the FITC-anti-mouse CD-11b (Mac-1) and the corresponding isotype control antibodies were from Invitrogen (Cergy Pontoise, France). Recombinant human soluble CD14 was obtained from R&D Systems (Abingdon, UK). Human recombinant LBP was from Biometec (Greifswald, Germany). Sephadex LH-20 and LH-60, and ^{3}H-labeled sodium borohydride (481 GBq/mmol) were from Amersham-Pharmacia Biotech (Buckinghamshire, UK). The liquid scintillation reagent Aqualyte was from Baker (Deventer, The Netherlands). Sandwich ELISA kit for TNF-α immunoassays was obtained from eBioscience (San Diego, CA, USA). Recombinant mouse macrophage colony-stimulating factor (M-CSF) was purchased from PeproTech (Neuilly-sur-Seine, France). The *Limulus* amebocyte lysate chromogenic test was from BioWhittaker (Walkersville, MD, USA).

Peptide synthesis and purification

Peptides used in this work (Table 1) were synthesized by Fmoc (fluoren-9-ylmethoxycarbonyl) chemistry in the laboratory of Prof. David Andreu at Universitat Pompeu Fabra in Barcelona, purified by HPLC and further analyzed by mass spectrometry and N-terminal Edman amino acid sequencing, as previously described.[16] In peptide pSP-$C_{O\text{-}O}$, the two cysteine residues were protected with acetoamidomethyl groups (*o*). In peptide pSP-C_{Palm}, cysteine residues were palmitoylated via thioester bonds. For cell culture assays, peptides from methanolic stock solutions were dried under vacuum and

Table 1. Structure of synthetic analogs of the N-terminal segment of porcine SP-C.

SP-C	LRIPCCPVNLKRL LVVVVVVVLVVVVIVGALLMGL *(Palm) (Palm)*
pSP-C_{Palm}	NH_2LRIPCCPVNLKRL$COONH_2$ *(Palm) (Palm)*
pSP-C	NH_2LRIPCCPVNLKRL$COONH_2$
pSP-$C_{\text{-O-O-}}$	NH_2LRIPOOPVNLKRL$COONH_2$ (O = acetoamidomethyl cysteine)

resuspended in pyrogen-free water. At concentrations used in this study, no endotoxin was detected in peptide samples as assayed with the *Limulus* amebocyte lysate chromogenic test. The concentration of peptides after solubilization in methanol or water was obtained upon acidic digestion of the peptides and further quantification of amino acid content as previously described.[19]

Isolation of SP-C

Porcine SP-C was prepared by an adaptation[19] of the method used by Cursted *et al*.[20] Briefly, minced lungs were washed with saline, and the mixture filtered. After removal of cells and debris, surfactant material was pelleted by centrifugation. The pellet was extracted following the Bligh and Dyer method and the lipid extract obtained was then purified by chromatography through a Sephadex LH-20 column to separate lipids from hydrophobic proteins. Surfactant protein C was further purified from the protein fraction by chromatography through a Sephadex LH-60 matrix, as described previously.[19]

Cell cultures

Macrophages were obtained from normal, uninfected Swiss outbred mice (6–12 weeks' old). All treatments were performed in accordance with the principles and procedures outlined in the European guidelines for care and use of experimental animals. Peritoneal macrophages were collected from peritoneal exudates harvested 5 days after intraperitoneal injection of thioglycollate broth as described previously.[9] Peritoneal cells were centrifuged, resuspended in culture media (Glutamax-RPMI 1640 supplemented with 1% streptomycin/penicillin) and immediately plated into 24-well plates. After incubation (3 h, 37°C, 5% CO_2), non-adherent cells were removed by washing with culture media. Bone marrow derived macrophages (BMDMs) were obtained from bone marrow cells collected by flushing femurs of mice as previously described.[10,21] After two washes in the culture media described above supplemented with 20% of FCS, bone marrow cells were resuspended in this culture media, containing M-CSF (20 ng/ml), plated in 24-well plates (800,000 cells/well) and maintained in culture for 6 days with one change of medium. At day 6, after removal of non-adherent cells, macrophage purity was >95% as estimated after analyzing the expression of CD-11b (Mac-1) by flow cytometry under standard procedures using a FACScan cytometer (Beckton Dickinson, San Diego, CA, USA).

Binding of [^{3}H]-Re-LPS to immobilized peptides

Lipopolysaccharide was tritium labeled ([^{3}H]-Re-LPS) by modification of the procedure of Watson and Riblet[22] as described previously.[7] Binding of [^{3}H]–Re-LPS to SP-C and peptides was performed as described previously[8] with slight modifications. Briefly, methanol solutions (100 µl) containing porcine SP-C or synthetic peptides were dispensed into wells of polypropylene microplates. Solvent was evaporated under vacuum and wells were incubated with [^{3}H]-Re-LPS (300,000 cpm/well) at room temperature, in the presence or absence of unlabeled LPS, in 100 µl of binding medium (5 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 0.05% (w:v) bovine serum albumin (BSA). After 3 h of incubation, the plates were washed with binding medium and the remaining bound radioactivity was recovered with 10% SDS and measured in a β-counter.

LPS stimulation and measurement of TNF-α in cell supernatants

Macrophages (10^6 cells/well) were washed with RPMI medium and then exposed to Re-LPS, peptides and combinations in the presence or absence of 2% FCS. Lipopolysaccharide and peptide were added at the same time or 1 h after pre-incubation at 20°C in glass tubes with shaking. In selected experiments, cells were first pre-incubated with the peptides alone for 1 h at 37°C in 5% CO_2 and, after washing to eliminate the remaining peptide, LPS was added to the cells. After incubation for 16 h, culture supernatants were collected and centrifuged to remove cell debris. Supernatants were stored at –80°C until quantification of TNF-α by specific sandwich enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. Cell viability was assayed with the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) method. None of the treatments used in this study (peptides, LPS and combinations) modified cell viability compared with control cells.

Binding of [^{3}H]-Re-LPS to macrophages

The binding of [^{3}H]-Re-LPS to macrophages was analyzed as described previously.[9] Peritoneal macrophages were seeded in 96-well plates (200,000 cells/well) in RPMI medium as described above. Tritiated LPS (200,000 cpm/well, final concentration) was resuspended either in RPMI alone, in RPMI containing serum (2%), or in RPMI with different concentrations of peptides, in glass tubes. Regardless of the sample composition, 0.05% (w:v) of BSA (final concentration)

was added. Mixtures were pre-incubated for 1 h at 20°C with shaking, and then added to the macrophages. After 1 h of incubation on ice, and three washes with cold PBS, the cell-bound radioactivity was recovered with 100 µl of 10% SDS and counted in a β-counter.

Interaction of FITC-Re-LPS with CD14 and LBP

Fluorescence assays with FITC-Re-LPS were performed as previously described[23] using a Cary Eclipse spectrofluorimeter (Varian, France) coupled to a peltier accessory. Fluorescent LPS was prepared as previously described.[23,24] The Re-LPS concentration of the fluorescent derivative was determined by quantification of 3-deoxy-D-*manno*-octulosonic acid (Kdo). Fluorescent LPS (20 ng/ml; 8×10^{-9} M) was added to a quartz cuvette (5×5 mm pathlength) warmed to 37°C containing PBS (assay buffer). Fluorescence of FITC-Re-LPS ($\lambda_{exc} = 490$ nm; $\lambda_{em} = 518$ nm) was recorded in kinetic mode with automatic stirring. Then, CD14 (10 µg/ml; 2×10^{-7} M), LBP (0.1 µg/ml; 1.6×10^{-9} M) and peptides (1 nmol/ml; 1×10^{-6} M) were sequentially added to the cuvette and fluorescence changes over time were monitored. For statistical analysis, we compared the increase in fluorescence emission after LBP addition in the presence versus absence of peptide from three independent experiments ($P < 0.05$, Student's *t*-test).

Interaction of peptide pSP-C_{L1W} with liposomes resembling the membrane of macrophages

Liposomes from a phospholipid mixture resembling the composition of the cell membrane of macrophages (PC:PE:PS:SM in a molar ratio of 1:0.4:0.7:0.5) were prepared as described by Schromm *et al.*[25] The N-terminal leucine residue in pSP-C was replaced by tryptophan, generating the pSP-C_{L1W} peptide. Tryptophan fluorescence emission spectra of the peptide was recorded ($\lambda_{exc} = 280$ nm) in a Cary Eclipse spectrofluorimeter after the addition of increasing amounts of liposomes as described previously.[16]

Statistical analysis

Statistical significance was assessed by analysis of variance (ANOVA) after confirmation of homogeneity of variances (Levene's test). Significance was considered when $P < 0.05$. Differences between groups were analyzed *post-hoc* by Student Newman-Keuls' multiple range test. Data presented in figures are representative of at least three independent preparations.

RESULTS

Binding of SP-C peptides and native SP-C to LPS

The capacity of several analogs of the N-terminal segment of porcine SP-C (Table 1) to bind Re-LPS was assayed using the solid phase LPS-binding assay previously described.[8] When increasing amounts of these peptides were attached to the bottom of the plate, we observed that the peptide containing the natural sequence, including double palmitoylation (pSP-C_{Palm}), bound to [^{3}H]-Re-LPS in a concentration-dependent manner and to the same extent as natural porcine SP-C (Fig. 1A). Non-palmitoylated peptide (pSP-C) also bound to [^{3}H]-Re-LPS but to a lesser extent than pSP-C_{Palm}. Binding of both peptides to [^{3}H]-Re-LPS was specific since unlabeled Re-LPS efficiently competed for the binding of the peptides to [^{3}H]-Re-LPS (Fig. 1B). Interestingly, blockade of cysteines

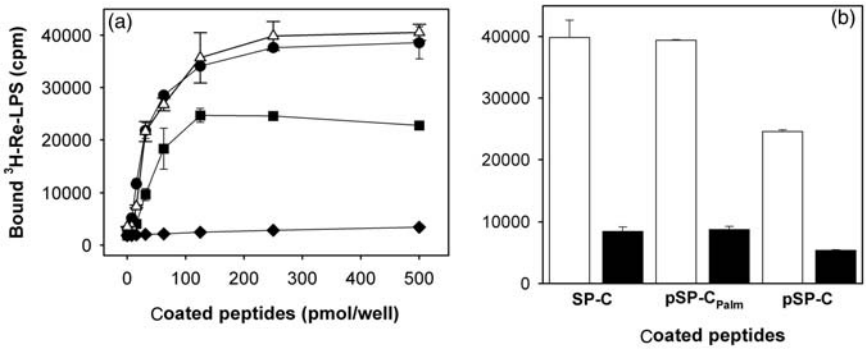

Fig. 1. Binding of SP-C and SP-C analogs to Re-LPS. Methanolic solutions of porcine SP-C or peptide analogs were added to polypropylene wells (0–500 pmol/well [A], 500 pmol/well [B]). After evaporation of the solvent, the peptide-coated wells were incubated for 3 h with [^{3}H]-Re-LPS (300,000 cpm) alone (A) or in the presence (closed bars) or absence (open bars) of unlabeled Re-LPS (100 µg; B). After washing, the remaining radioactivity was recovered in 10% SDS and measured in a β-counter. Results are the mean ± SD of triplicates. Symbols in (A) correspond to porcine SP-C (open triangles) and peptides pSP-C_{Palm} (filled circles), pSP-C (filled squares), and pSP-$C_{O\text{-}O}$ (filled diamonds).

($pSP\text{-}C_{O\text{-}O}$) abolished the interaction of the N-terminal segment of SP-C with LPS (Fig. 1A).

Modulation of LPS-induced TNF-α production by SP-C peptides in serum-free conditions

We analyzed the capacity of the peptides to modulate the production of TNF-α induced by Re-LPS in thioglycollate-elicited mouse peritoneal macrophages. Macrophages were stimulated in serum-free media with Re-LPS (25 ng/ml, 10 pmol/ml), pre-incubated with different amounts of peptide (0–1000 pmol/ml). After 16 h of culture, TNF-α was measured in supernatants with a specific sandwich ELISA. We observed that TNF-α production induced by Re-LPS pre-incubated in culture medium containing different amounts of $pSP\text{-}C_{Palm}$(Fig. 2A, open circles) was enhanced as compared to that induced by Re-LPS pre-incubated in culture medium in the absence of peptides (26 ± 1.11 ng/ml; Fig. 2A filled triangles). The effect of the peptide was concentration dependent. The same effect was observed when Re-LPS was pre-incubated with pSP-C (Fig. 2A, filled circles). However, pre-incubation of Re-LPS with peptide $pSP\text{-}C_{O\text{-}O}$ did not modify the secretion of TNF-α induced by Re-LPS alone (Fig. 2A, grey circles). Under our experimental conditions, none of the peptides used modified the basal secretion level of TNF-α, which was always lower than 10 pg/ml (data not shown). Moreover, LPS contaminants were not detected in the peptide stock solutions by the *Limulus* method. Interestingly, when peptides and Re-LPS were added at the same time (no pre-incubation step) or when cells were pre-incubated with peptides 1 h before the addition of Re-LPS, the peptides did not modify the TNF-α production induced by Re-LPS (data not shown).

To ensure that our results were not due to the inflammatory type of macrophages used (thioglycollate-elicited macrophages), the same type of experiments were performed with mouse bone marrow derived macrophages (BMDMs). As shown in Fig. 2B, here again pre-incubation of Re-LPS (25 ng/ml) with 1000 pmol/ml of $pSP\text{-}C_{Palm}$ or pSP-C enhanced the TNF-α release induced by Re-LPS (6.54 ± 0.16 ng/ml and 6.8 ± 0.4 ng/ml, respectively, versus 5.13 ± 0.12 ng/ml in the absence of peptide); again, $pSP\text{-}C_{O\text{-}O}$ did not show a significant effect.

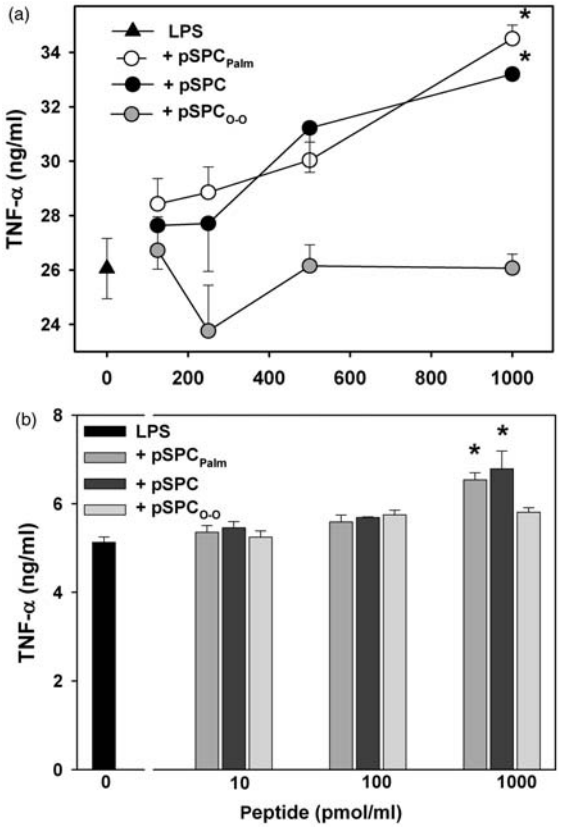

Fig. 2. Influence of peptides on LPS-induced production of TNF-α by macrophages. Thioglycollate-elicited mouse peritoneal macrophages (A) or bone marrow derived macrophages (BMDMs) (B), 1×10^6/well, were incubated for 16 h with Re-LPS (25 ng/ml) in RPMI medium without serum. Re-LPS solutions were previously pre-incubated with different amounts of SP-C peptides (circles in [A] and grey bars in [B]) or RPMI alone (closed triangle in [A] and closed bar in [B]). After incubation, supernatants were collected and TNF-α measured using an ELISA sandwich kit. Mean ± SD of triplicates from one representative experiment are shown (*different from LPS, $P < 0.05$, ANOVA). Peptides alone did not modify basal secretion (<10 pg/ml). In (A), error bars are represented only in one direction for clarity.

Influence of SP-C peptides on the binding of [^{3}H]-Re-LPS to macrophages

In order to study the mechanism by which peptides enhanced the LPS response, we first examined whether the peptides could promote the association of Re-LPS with macrophages. For this purpose, we incubated peritoneal macrophages with [^{3}H]-Re-LPS that was pre-incubated or not in the presence of $pSP\text{-}C_{Palm}$. As a positive control, Re-LPS was pre-incubated with 2% of serum. Incubation of [^{3}H]-Re-LPS with macrophages was performed at 4°C in order to avoid internalization of [^{3}H]-Re-LPS. As expected, serum increased the association of [^{3}H]-Re-LPS over 20% compared with exposure of macrophages to [^{3}H]-Re-LPS alone (Fig. 3). As shown in Figure 3, $pSP\text{-}C_{Palm}$ at 1000 pmol/ml also enhanced (by over 30%) the association of [^{3}H]-Re-LPS with macrophages. Similar results were obtained with the non-palmitoylated pSP-C peptide (data not shown).

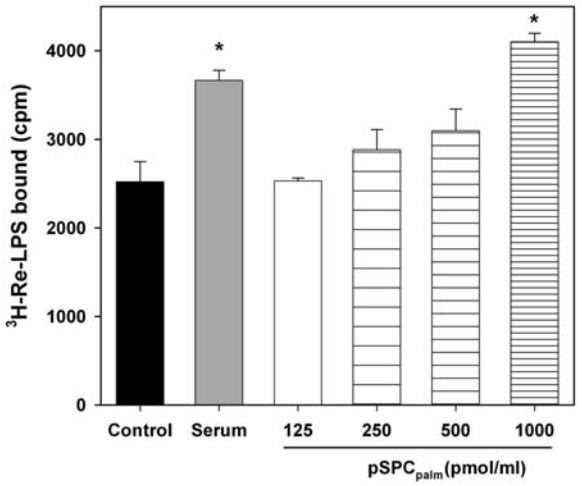

Fig. 3. Binding of [^{3}H]-Re-LPS to macrophages. Peritoneal macrophages were seeded in 96-well plates (200,000 cells/well). After washing with cold PBS, cells were incubated with [^{3}H]-Re-LPS (200,000 cpm/well) at 4°C (1 h). [^{3}H]-Re-LPS was previously pre-incubated with different amounts of pSP-C$_{Palm}$ peptide (open bars) or 2% fetal calf serum (grey bar) or RPMI alone (closed bar). (Mean ± SD; $n = 3$; *different from control, $P < 0.05$, ANOVA).

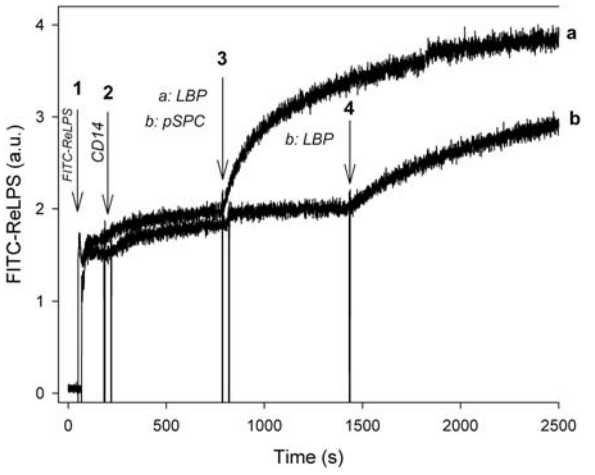

Fig. 4. Interaction of FITC-Re-LPS with CD14 and LBP. Fluorescence emission intensity in arbitrary units (at 518 nm) of FITC-Re-LPS (20 ng/ml; 8×10^{-9} M) was monitored over time at 37°C after addition of CD14 (10 μg/ml; 2×10^{-7} M), pSP-C peptide (1000 pmol/ml; 1 μM) and LBP (0.1 μg/ml; 1.6×10^{-9} M) at times indicated by the arrows. Tracing a is LPB only stimulus at time point 3 and tracing b is a sequential addition of pSP-C peptide followed at time 4 by LBP addition.

Influence of peptides on the interaction of FITC-LPS with CD14

We have previously reported that SP-C enhances the binding of Re-LPS to CD14.[10] This may explain the results presented above (Fig. 3). Indeed, an attractive possibility could be that SP-C peptides enhance the association of [^{3}H]-Re-LPS with macrophages by enhancing the interaction of Re-LPS with the membrane form of CD14, in a way similar to that described for LBP.[13,24] To test this hypothesis, we studied the capacity of the peptides to enhance Re-LPS/CD14 interactions by monitoring these interactions, as described previously,[23] by the changes in emitted fluorescence of a fluorescently labeled Re-LPS (FITC-Re-LPS). Indeed, interactions between LPS and proteins that bind LPS are reflected in this assay by an increase in fluorescence emission of FITC-Re-LPS.[26] We found that addition of CD14 (10 μg/ml; 2×10^{-7} M) to a PBS buffer containing FITC-Re-LPS (20 ng/ml; 8×10^{-9} M) modestly increased the fluorescence emission of FITC-Re-LPS (Fig. 4, trace a). As expected, further addition of catalytic concentrations of LBP (0.1 μg/ml; 1.6×10^{-9} M) markedly increased the fluorescence emission of FITC-Re-LPS, as previously described,[24] because LBP enhances CD14/LPS interaction. However, when pSP-C (1000 pmol/ml; 1×10^{-6} M) was added to PBS buffer containing FITC-LPS and CD14, we did not observe an increase in fluorescence emission (Fig. 4, trace b). Therefore, pSP-C is not able to enhance Re-LPS/CD14 interactions, or at least does not function as LBP. Interestingly, when we added LBP to the PBS buffer containing FITC-LPS, CD14 and pSP-C, the increase in fluorescence emission was lower than that observed when pSP-C was absent (compare traces a and b in Fig. 4), suggesting that pSP-C may interfere with the formation of sCD14–LPS–LBP complexes.[27] This effect of pSP-C was also observed with pSP-C$_{Palm}$ (data not shown).

Modulation of LPS-induced TNF-α release by SP-C peptides in the presence of serum

Stimulation experiments *in vitro* have shown that serum enhances LPS-responses due to the presence of LBP. Because the results presented in Figure 4 suggested that SP-C peptides could disturb the formation of sCD14–LPS–LBP complexes, we studied the effects of SP-C peptides on TNF-α secretion induced by LPS under serum-dependent LPS-response conditions. For this purpose, we stimulated peritoneal macrophages with Re-LPS at 2 ng/ml in the presence of serum. At that concentration of Re-LPS, the effects of serum in LPS-induced TNF-α release were much more pronounced than those observed at 25 ng/ml. As shown in Figure 5, in the presence of pSP-C$_{Palm}$ or pSP-C (1000 pmol/ml) the release of TNF-α induced by Re-LPS was reduced. In contrast, the peptide with blocked cysteines (pSP-C$_{O\text{-}O}$) did not modify the release of TNF-α under the same experimental conditions. Unlike the effect of the peptides in serum-free conditions, those obtained in the presence of serum were

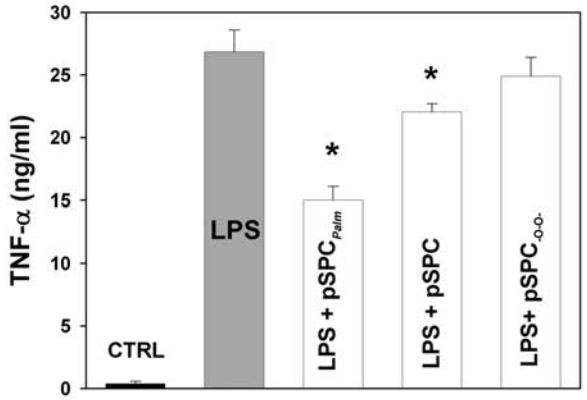

Fig. 5. Influence of peptides on LPS-induced production of TNF-α by macrophages in the presence of serum. Mouse peritoneal macrophages (1×10^6) were incubated for 16 h with Re-LPS (2 ng/ml) (grey bar) or Re-LPS and peptides (1000 pmol/ml; open bars) in RPMI media supplemented with 2% FCS. Afterwards, supernatants were collected and TNF-α measured using an ELISA sandwich kit. Mean ± SD of triplicates from one representative experiment are shown (*different from LPS, $P < 0.05$, ANOVA). Peptides alone did not modify basal secretion (closed bar).

observed not only when the peptides were pre-incubated with Re-LPS but also when peptides and Re-LPS were added simultaneously to the cells. Peptides alone did not modify the basal TNF-α secretion of macrophages (data not shown).

DISCUSSION

Surfactant protein C is a highly hydrophobic lipoprotein associated with lung surfactant membranes. Previously, it has been reported that SP-C inserted in phosphatidylcholine (PC) vesicles, binds to LPS[7] and reduces LPS-mediated cellular effects (*e.g.* splenocyte proliferation and release of pro-inflammatory mediators by macrophages),[9] thus suggesting an important role of SP-C in the first line of innate lung defense. The interaction of SP-C/PC with LPS requires the participation of the phosphate residue at position 1 of the reducing glucosamine unit of lipid A, and both the N-terminal hydrophilic region and the C-terminal hydrophobic region of SP-C.[8] In the light of these results, it was proposed that the N-terminal region of SP-C might be involved in peptide–LPS interactions, whereas the C-terminal non-polar region of the molecule will be important to achieve the optimal topology of the peptide in the phospholipid bilayer. Here, we confirm and strengthen this hypothesis insofar as synthetic analogs of the N-terminal segment of SP-C mimicked the total LPS binding capacity of natural SP-C in the absence of lipids (Fig. 1). In a previous study, it was shown that chemical deacylation of mouse SP-C slightly reduced the capacity of SP-C/PC to bind LPS, but further use of synthetic analogs led the authors to conclude that palmitoylation is not essential for SP-C-PC/LPS interactions.[8] Our present data in a lipid-free environment confirm the first observation: the non-palmitoylated peptide bound at a lesser extent to LPS as compared to the palmitoylated peptide, although binding was not abrogated in the absence of palmitoylation. Furthermore, we did not observe significant differences in terms of activity when we analyzed the capacity of both peptides to modulate LPS-induced TNF-α release in the absence of serum, an activity mostly related to the capacity of the peptides to bind to LPS. An intriguing result was that the blockade of cysteines (pSP-$C_{O\text{-}O}$ analog) abrogated LPS binding. To ensure that this absence of binding was not due to differences in the attachment of peptides to the plate, we performed an LPS binding test based on incorporation of the peptides to PC-vesicles and separation of unbound from vesicle-bound [^{3}H]-Re-LPS on a density gradient as described before.[7] This method confirmed that the peptide with blocked cysteines did not bind to LPS (results not shown). Thus, introduction of acetoamidomethyl groups in the cysteines might perturb the conformation of the LPS binding site in the N-terminal segment, resulting in the loss of peptide–LPS interactions. In general, interactions between LPS binding proteins and LPS are driven through electrostatic as well as hydrophobic forces (reviewed by Chaby[28]). We have previously proposed that the N-terminal segment of SP-C presents high intrinsic propensity to form β-turns, where the middle part of the turn would be occupied by hydrophobic residues (residues 3 to 8) while both ends would contain polar, positively charged amino acids.[15] This middle part contains the two cysteine residues and presents favorable free energy to interact with membranes.[16] Our data suggest that this hydrophobic segment within the N-terminal segment of SP-C might be important to ensure interactions between this segment and LPS in a lipid-free environment, because alteration of this region (*e.g.* introduction of acetoamidomethyl groups) perturbs such interactions with LPS.

In the absence of serum, the N-terminal segment of SP-C promoted the association of LPS with macrophages and enhanced LPS-elicited TNF-α release by an unknown mechanism. This mechanism involves peptide/LPS interactions because the absence of interaction of pSP-$C_{O\text{-}O}$ with LPS correlated with the inability to modify the LPS response. Furthermore, the effects of the two active peptides were observed only after pre-incubation with LPS, but not when peptides and LPS were added to the cells at the same time nor when cells were pre-incubated with the peptides before LPS challenge. To understand this unexpected enhancement of LPS response by SP-C peptides in the absence of

serum, we examined whether the N-terminal segment of SP-C could promote CD14/LPS interaction, as previously described for intact SP-C.[10] Our results indicated that the N-terminal segment did not enhance LPS–CD14 interactions, at least to the same extent as LBP. An inference derived from our results is that the enhancement of the LPS response (TNF-α release) induced by the N-terminal segment of SP-C in the absence of serum is due to a CD14-independent mechanism. This is in line with a number of studies suggesting that, in the absence of serum (and thus of LBP), the cellular responses of macrophages to LPS are CD14-independent and are likely triggered by the direct interaction of LPS with the MD-2 constituent of the TLR4/MD-2 receptosome.[29,30] Interestingly, another surfactant protein, SP-A, is also able to modulate LPS-induced TNF-α release in macrophages through a CD14-independent mechanism.[31] These CD14-independent mechanisms could include either other receptors involved (or not) in LPS signaling pathways or proceed under non-receptor pathways. In the latter context, LBP has been shown to mediate intercalation of LPS into phospholipid liposomes mimicking the macrophage cell membrane[25,32] through mechanisms that could explain the CD14-independent cell activation induced by LPS.

Therefore, it is conceivable that, in a similar way, the N-terminal segment of SP-C could enhance cell signaling by facilitating transfer of LPS to the membrane of macrophages. To strengthen this speculation, we studied the interaction of liposomes mimicking macrophage membrane composition with a modified pSP-C peptide, referred to as pSP-C_{L1W} where the N-terminal leucine residue is replaced by tryptophan in order to characterize lipid–peptide interactions by fluorescence spectroscopy as previously described.[16] This substitution did not modify the capacity of the peptide to interact with surfactant-like membranes[17] nor its capacity to enhance LPS-responses in the absence of serum (not shown). Emission fluorescence spectra of this modified peptide slightly increased and blue-shifted after addition of increasing amounts of liposomes (Fig. 6), suggesting that these peptides interact with liposomes mimicking macrophage membrane composition. This interaction is less pronounced than that observed for phosphatidylglycerol liposomes[16] or Re-LPS (not shown), which is consistent with the preference of the peptide for negatively charged membranes and with the low toxicity of the peptides in macrophage cultures. This putative capacity of the N-terminal segment to transfer LPS would explain the described effect of SP-C inserted in vesicles to reduce LPS-cellular responses.[9] When the N-terminal segment is associated with the C-terminal α-helix portion of SP-C inserted in the phospholipid bilayer, this N-terminal segment could transfer the LPS

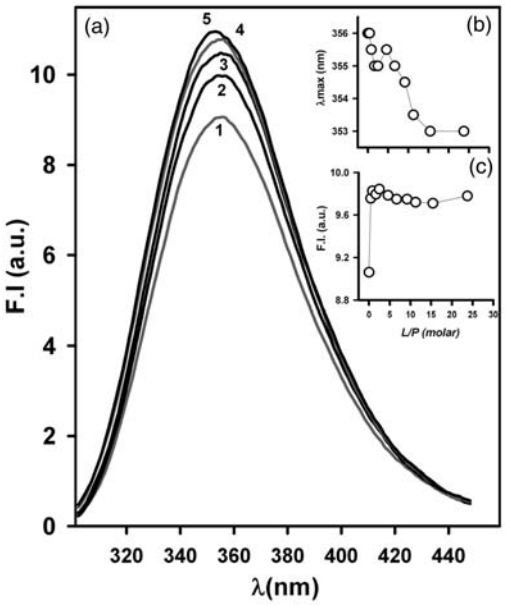

Fig. 6. Interaction of peptide pSP-C_{L1W} with liposomes resembling macrophage membranes, analyzed by fluorescence spectroscopy. Fluorescence emission spectra of peptide pSP-C_{L1W} (2 μg/ml) in the absence (spectrum 1) or presence of 0.5, 2, 11 and 14 μM liposomes with macrophage membrane-like composition (spectra 2–5), in 50 mM HEPES buffer, pH 7, containing 150 mM NaCl (A). Wavelength of maximum emission (inset B) and maximal fluorescence emission intensity (inset C) were plotted versus lipid to peptide molar ratio.

to the liposome,[7] thus reducing the accessibility of LPS to the cell membrane of the target cell. The demonstrated capacity of the N-terminal segment of SP-C to associate spontaneously with phospholipid membranes and to insert into and perturb phospholipid monolayers,[16,17] especially when negatively charged, reinforces this hypothesis. In addition, the N-terminal segment of SP-C can interact with LPS inserted into liposomes, and segregate LPS from these liposomes (Perez-Gil and Gonzalez-Horta, unpublished data), thus demonstrating a capacity to re-organize LPS in membranes.

However, in the presence of serum, the N-terminal segment of SP-C modulates the LPS response through a different mechanism. Under these conditions, the effect of the peptides was not producing an enhancement but an inhibition of the LPS-induced production of TNF-α. Furthermore, this inhibitory effect was observed not only when the peptides were pre-incubated with LPS, but also when peptides and LPS were added at the same time, in contrast to serum-free conditions where peptide effects required pre-incubation. Our experiments using an acellular system (FITC-Re-LPS) suggest that the N-terminal segment interferes with the formation of CD14/LBP/Re-LPS complexes.[27] Several hypotheses that may account for such effects are under study. One possibility is that N-terminal peptides perturb CD14/LPS

or LPS/LBP interaction or the stability of CD14/LBP/Re-LPS complexes. The action of the N-terminal segment on this complex could be the same as that described for SP-A. SP-A is able to compete with LBP for LPS binding[33] and to inhibit the LBP-mediated transfer of LPS to CD14.[23] An alternative mechanism to explain the reduction of LPS-induced TNF-α release in the presence of serum could be extrapolated from that proposed for LBP and sCD14. Both sCD14 and LBP, under particular physiological conditions, are able to transfer LPS to the high density lipoproteins (HDLs) present in serum, thus leading to reduced LPS responses.[14] In view of the lipid binding properties of the N-terminal segment of SP-C, the possible extension of this hypothesis can be of particular interest.

Conclusions

Using synthetic peptides mimicking the N-terminal segment of SP-C, the data show the intrinsic capacity of this segment to modulate the LPS response, as previously found for SP-C inserted into lipid vesicles. In the presence of serum, these water-soluble peptides reduce, like natural SP-C, the pro-inflammatory effects of LPS. *In vivo* studies analyzing the capacity of these peptides to block the pro-inflammatory effects of LPS after intravenous administration of the endotoxin are currently under study and will help elucidate the possible utility of soluble SP-C analogs as potential therapeutic agents against the lethal effects of septic shock.

Acknowledgements

Ignacio Garcia-Verdugo and Elvira Garcia de Paco contributed equally to this work.

This research was supported by grants from Centre National de la Recherche Scientifique and Paris-Sud University to IG–V, EGP, QE, MS, JK and RC, and grants from the Spanish Ministry of Science (BIO2006-03130, CONSOLIDER-INGENIO 2010 CSD2007-00010), and Community of Madrid (S0505/MAT/0283) to AG–H and JP–G, and from the Spanish Ministry of Health (FIS PI061125 and RD06/0021/0006) and Community of Madrid COMBACT program (S-BIO-0260/2006) to LR. Collaboration between the French and Spanish teams has been facilitated by a Marie Curie Research Training Network (MRTN-CT2004-512229).

References

1. Johansson J. Structure and properties of surfactant protein C. *Biochim Biophys Acta* 1998; **1408**: 161–172.
2. Curstedt T, Johansson J, Persson P. *et al*. Hydrophobic surfactant-associated polypeptides: SP-C is a lipopeptide with two palmitoylated cysteine residues, whereas SP-B lacks covalently linked fatty acyl groups. *Proc Natl Acad Sci USA* 1990; **87**: 2985–2989.
3. Pastrana B, Mautone AJ, Mendelsohn R. Fourier transform infrared studies of secondary structure and orientation of pulmonary surfactant SP-C and its effect on the dynamic surface properties of phospholipids. *Biochemistry* 1991; **30**: 10058–10064.
4. Vandenbussche G, Clercx A, Curstedt T *et al*. Structure and orientation of the surfactant-associated protein C in a lipid bilayer. *Eur J Biochem* 1992; **203**: 201–209.
5. Morrow MR, Taneva S, Simatos GA *et al*. NMR studies of the effect of pulmonary surfactant SP-C on the 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine headgroup: a model for transbilayer peptides in surfactant and biological membranes. *Biochemistry* 1993; **32**: 11338–11344.
6. Serrano AG, Perez-Gil J. Protein-lipid interactions and surface activity in the pulmonary surfactant system. *Chem Phys Lipids* 2006; **141**: 105–118.
7. Augusto L, Le Blay K, Auger G *et al*. Interaction of bacterial lipopolysaccharide with mouse surfactant protein C inserted into lipid vesicles. *Am J Physiol* 2001; **281**: L776–L785.
8. Augusto LA, Li J, Synguelakis M *et al*. Structural basis for interactions between lung surfactant protein C and bacterial lipopolysaccharide. *J Biol Chem* 2002; **277**: 23484–23492.
9. Augusto LA, Synguelakis M, Espinassous Q *et al*. Cellular antiendotoxin activities of lung surfactant protein C in lipid vesicles. *Am J Respir Crit Care Med* 2003; **168**: 335–341.
10. Augusto LA, Synguelakis M, Johansson J *et al*. Interaction of pulmonary surfactant protein C with CD14 and lipopolysaccharide. *Infect Immun* 2003; **71**: 61–67.
11. Miyake K. Innate recognition of lipopolysaccharide by CD14 and Toll-like receptor 4–MD-2: unique roles for MD-2. *Int Immunopharmacol* 2003; **3**: 119–128.
12. Ulevitch RJ, Tobias PS. Receptor-dependent mechanisms of cell stimulation by bacterial endotoxin. *Annu Rev Immunol* 1995; **13**: 437–457.
13. Hailman E, Lichenstein HS, Wurfel MM *et al*. Lipopolysaccharide (LPS)-binding protein accelerates the binding of LPS to CD14. *J Exp Med* 1994; **179**: 269–277.
14. Kitchens RL, Thompson PA. Modulatory effects of sCD14 and LBP on LPS-host cell interactions. *J Endotoxin Res* 2005; **11**: 225–229.
15. Plasencia I, Rivas L, Casals C *et al*. Intrinsic structural differences in the N-terminal segment of pulmonary surfactant protein SP-C from different species. *Comp Biochem Physiol A Mol Integr Physiol* 2001; **129**: 129–139.
16. Plasencia I, Rivas L, Keough KM *et al*. The N-terminal segment of pulmonary surfactant lipopeptide SP-C has intrinsic propensity to interact with and perturb phospholipid bilayers. *Biochem J* 2004; **377**: 183–193.
17. Plasencia I, Keough KM, Perez-Gil J. Interaction of the N-terminal segment of pulmonary surfactant protein SP-C with interfacial phospholipid films. *Biochim Biophys Acta* 2005; **1713**: 118–128.
18. Plasencia I, Baumgart F, Andreu D *et al*. Effect of acylation on the interaction of the N-terminal segment of pulmonary surfactant protein SP-C with phospholipid membranes. *Biochim Biophys Acta* 2008; **1778**: 1274–1282.
19. Perez-Gil J, Cruz A, Casals C. Solubility of hydrophobic surfactant proteins in organic solvent/water mixtures. Structural studies on SP-B and SP-C in aqueous organic solvents and lipids. *Biochim Biophys Acta* 1993; **1168**: 261–270.
20. Curstedt T, Jornvall H, Robertson B *et al*. Two hydrophobic low-molecular-mass protein fractions of pulmonary surfactant.

Characterization and biophysical activity. *Eur J Biochem* 1987; **168**: 255–262.
21. Girard R, Pedron T, Uematsu S *et al*. Lipopolysaccharides from Legionella and Rhizobium stimulate mouse bone marrow granulocytes via Toll-like receptor 2. *J Cell Sci* 2003; **116**: 293–302.
22. Watson J, Riblet R. Genetic control of responses to bacterial lipopolysaccharides in mice. II. A gene that influences a membrane component involved in the activation of bone marrow-derived lymphocytes by lipopolysaccharides. *J Immunol* 1975; **114**: 1462–1468.
23. Garcia-Verdugo I, Sanchez-Barbero F, Soldau K *et al*. Interaction of SP-A (surfactant protein A) with bacterial rough lipopolysaccharide (Re-LPS), and effects of SP-A on the binding of Re-LPS to CD14 and LPS-binding protein. *Biochem J* 2005; **391**: 115–124.
24. Tobias PS, Soldau K, Gegner JA *et al*. Lipopolysaccharide binding protein-mediated complexation of lipopolysaccharide with soluble CD14. *J Biol Chem* 1995; **270**: 10482–10488.
25. Schromm AB, Brandenburg K, Rietschel ET *et al*. Lipopolysaccharide-binding protein mediates CD14-independent intercalation of lipopolysaccharide into phospholipid membranes. *FEBS Lett* 1996; **399**: 267–271.
26. Tobias PS, Soldau K, Iovine NM *et al*. Lipopolysaccharide (LPS)-binding proteins BPI and LBP form different types of complexes with LPS. *J Biol Chem* 1997; **272**: 18682–18685.
27. Thomas CJ, Kapoor M, Sharma S *et al*. Evidence of a trimolecular complex involving LPS, LPS binding protein and soluble CD14 as an effector of LPS response. *FEBS Lett* 2002; **531**: 184–188.
28. Chaby R. Lipopolysaccharide-binding molecules: transporters, blockers and sensors. *Cell Mol Life Sci* 2004; **61**: 1697–1713.
29. Lynn WA, Liu Y, Golenbock DT. Neither CD14 nor serum is absolutely necessary for activation of mononuclear phagocytes by bacterial lipopolysaccharide. *Infect Immun* 1993; **61**: 4452–4461.
30. Viriyakosol S, Tobias PS, Kitchens RL *et al*. MD-2 binds to bacterial lipopolysaccharide. *J Biol Chem* 2001; **276**: 38044–38051.
31. Alcorn JF, Wright JR. Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway. *Am J Physiol* 2004; **286**: L129–L136.
32. Müeller M, Brandenburg K, Dedrick R *et al*. Phospholipids inhibit lipopolysaccharide (LPS)-induced cell activation: a role for LPS-binding protein. *J Immunol* 2005; **174**: 1091–1096.
33. Stamme C, Müller M, Hamann L *et al*. Surfactant protein a inhibits lipopolysaccharide-induced immune cell activation by preventing the interaction of lipopolysaccharide with lipopolysaccharide-binding protein. *Am J Respir Cell Mol Biol* 2002; **27**: 353–360.