Research article

Recombinant Factor C competes against LBP to bind lipopolysaccharide and neutralizes the endotoxicity

Peng Li[1], Bow Ho[2], Jeak Ling Ding[1]

Departments of [1]Biological Sciences and [2]Microbiology, National University of Singapore, Singapore

Endotoxin-mediated inflammation and septic shock remains a grave challenge to human healthcare management. It is, therefore, a worthwhile effort to develop anti-lipopolysaccharide (LPS) strategies to prevent downstream effects. Here, we demonstrate that purified recombinant Factor C (rFC) cloned from the horseshoe crab, binds LPS with high affinity, preventing it from binding a peptide derived from the human LPS-binding protein (LBP). Factor C is an innate immune defense protein present in the horseshoe crab hemocytes. The full-length rFC was found to be more efficacious in blocking LBP-mediated downstream effects than either of the individual LPS-binding peptides (Sushi 1 and Sushi 3) derived from rFC. When added to human macrophage culture, rFC blocks the LPS-induced phosphorylation of p38, which, in turn, inhibits the consequential overexpression of TNF-α and IL-8. The tandem arrangement of the LPS-binding Sushi domains in the Factor C molecule appears to be required for the synergy and amplification of LPS-binding *in vivo* to achieve such high affinity for LPS. Thus, rFC binds and neutralizes LPS to arrest signal transduction via the p38 pathway. The rFC does not show acute cytotoxicity and could be a potential lead for further development into an endotoxin-antagonist to inhibit inflammation and septic shock.

Keywords: Endotoxin/lipopolysaccharide (LPS), LPS binding, Factor C, LPS-binding protein (LBP), LPS neutralization, p38, TNF-α

INTRODUCTION

The best studied biological pyrogen is lipopolysaccharide (LPS, also known as endotoxin) found in the outer membrane of Gram-negative bacteria. During infection by Gram-negative bacteria, the host innate immune defense immediately responds to the pathogen invasion via LPS-induced signal transduction pathways. However, the excessive response will result in host inflammation and septic shock. The innate immune system initiates host defense against the endotoxin via specific recognition mechanisms. Upon systemic infection, LPS is released from the bacterial cell membrane into the blood stream and is captured by the LPS-binding protein (LBP) in the host.[1–4] The LBP–LPS complex is subsequently transferred to the host immune cells and interacts with CD14[5] and Toll-like receptor 4 (TLR4).[6] Then, the LPS-induced signal activates protein kinases such as p38[7] and transcription factors, which, in turn, triggers the transcription of numerous genes coding for pro-inflammatory cytokines and chemokines, tissue factor, adhesion molecules and inducible nitric oxide synthase. However, overproduction of these potent mediators initiates a series of events that can lead to clinical manifestations of sepsis in the host. Amongst the pro-inflammatory cytokines and chemokines, TNF-α and IL-8 play a critical role in the inflammatory response and are often regarded as a hallmark of LPS-induced inflammation.[8]

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Correspondence to: Jeak Ling Ding, Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, Singapore 117543
Tel: +65 6516 2776; Fax: +65 6779-2486; E-mail: dbsdjl@nus.edu.sg; URL: http://www.dbs.nus.edu.sg/Staff/ding.html

Abbreviations: ENC, endotoxin-neutralizing concentration; FITC, fluorescein-5-isothiocyanate; LPS, lipopolysaccharide; LBP, LPS binding protein; $LBP_{85–108}$, LBP peptide; PBS, phosphate-buffered saline; PMA, phorbol myristate acetate; rFC, recombinant Factor C protein.

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In the US, a multicenter observational cohort study has projected an estimated 751,000 cases of sepsis per annum.[9] Thus, LPS-neutralization drugs are urgently needed. Currently, studies around the world have focused on the intervention of LPS-induced inflammatory response with potential LPS-neutralizing drugs to compete against LBP binding and to inactivate the LPS signaling pathway. The perceived strategy is that binding of LPS to LPS-receptors is required to trigger an inflammatory response at the initial step. Numerous proteins and peptides in insects and mammals appear to have evolved and developed for the purpose of binding and neutralizing LPS during Gram-negative bacterial challenge. These include CAP (cationic antimicrobial protein) and LALF (*Limulus* anti-lipopolysaccharide factor).[10–15]

Factor C is a unique LPS-binding protein found in the horseshoe crab. Normally, Factor C exists as a serine protease zymogen. LPS is a ligand which activates Factor C. No homologue of Factor C has been found in mammals, although the C-terminal serine protease domain has substantial similarities with human α-thrombin (36.7%; unpublished data). As a 'living fossil' which has survived for several hundred million years, the evolutionary success of the horseshoe crab attests to its strong innate immune defense system, which it uses as a sole antimicrobial defense mechanism to thrive in microbiologically challenging habitats teeming with a wide diversity of pathogens. The horseshoe crab hemolymph contains mainly one type of hemocyte, which is extremely sensitive to LPS. The hemocytes contain two kinds of secretory granules – the large and small granules. Studies on these granules suggest that Factor C is localized in the large granules.[16] In the past decade, the function of the Factor C has been established.[17–19] As an ultrasensitive biosensor of LPS, it recognizes subnanogram levels of LPS[20] to trigger a coagulation cascade resulting in the entrapment of the invading Gram-negative bacteria.

Recombinant Factor C (rFC) has been produced showing extremely high sensitivity and avidity for LPS.[20–22] The endotoxin binds with a K_D of 10^{-10} M, to at least two LPS-binding domains, Sushi 1 and Sushi 3, within the Factor C molecule.[28] Thus, we reasoned that rFC could be applied as a candidate to prevent LPS from binding with other proteins. In this work, we used rFC as a potential LPS-neutralizing protein and examined its efficacy in preventing LPS-mediated host cell signal transduction. To reveal the biological significance of the LPS-neutralising potency, we examined the anti-LPS activity on the human macrophage culture. Our results demonstrate that rFC competes against human LBP peptide ($LBP_{85–108}$) for LPS and neutralizes the endotoxicity by down-regulating the LPS-induced signaling pathway in the macrophage. rFC neutralizes the LPS-induced phosphorylation of p38. Being non-cytotoxic, rFC and/or its derivatives could be developed into a potential LPS-antagonist.

Materials and Methods

Reagents

LPS from *Escherichia coli* O55:B5 was purchased from Sigma (purified by gel-filtration chromatography; St Louis, MO, USA). The specific activity of the LPS was calibrated into EU/ml against a known control standard endotoxin (CSE) from Cambrex Inc. (MD, USA). Low endotoxin-defined fetal bovine serum (FBS) was from Invitrogen (Carlsbad, CA, USA). Phorbol myristic acid (PMA) for transforming human monocytes, THP-1 cells, was obtained from Sigma-Aldrich (Fairlawn, NJ, USA). Immunoassays for TNF-α and IL-8 were purchased from PharMingen (San Diego, CA, USA). Cell Titer 96 AQueous for cytotoxicity assay was from Promega (Madison, WI, USA). Recombinant Factor C protein (rFC) and PyroGene, an rFC-based endotoxin detection kit was from Cambrex Inc. $LBP_{85–108}$ (IRVQGR-WKVRKSFFKLQGSFDVSV) peptide[23,35] used in this study was synthesized by Genemed Synthesis Inc. (San Francisco, CA, USA). Pyrogen-free water for making buffers was from Baxter (Morton Grove, IL, USA). Anti-p38 and anti-phospho-p38 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

Cell culture

Human monocytes, THP-1 were grown in RPMI 1640 medium supplemented with 5% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified incubator supplied with 5% CO_2. The cells were maintained at a density of 2.5 x $10^{5–6}$ cells/ml.

Molecular weight determination by SDS-PAGE

Proteins in reducing (with DTT) and non-reducing (without DTT) buffers were electrophoresed in 12% SDS-PAGE gel and detected by staining with Coomassie blue.

Competition between rFC and other LPS-binding peptides

The zymogenic form of rFC in the PyroGene kit can be activated by LPS to catalyze the hydrolysis of a synthetic substrate to release a fluorescent product which is quantified at excitation and emission wavelengths of 380 and 440 nm, respectively.[24] Thus, this assay can be used to detect the rFC bound to LPS. In this assay, different

concentrations of $LBP_{85–108}$ peptide were incubated for 1 h at 37°C with or without an equal volume of 50 EU/ml LPS in disposable, endotoxin-free borosilicate tubes.[25] Aliquots (50 µl) of each mixture were then dispensed into wells of a sterile microtiter plate (Nunclon™ surface, Nunc), followed by the addition of 50 µl of freshly reconstituted rFC reagent. The fluorescence at 440 nm of each well was monitored after 30 min.

Western analyses of p38 and anti-phospho-p38

In order to examine the effects of rFC on LPS-mediated intracellular signaling in the host cells, the status of p38 phosphorylation was examined.[26] Towards this, the THP-1 cells were treated with 2 EU/ml LPS with or without rFC at 37°C for 2 h. The total cellular proteins were resolved in 12% SDS-PAGE, and electrotransferred to polyvinylidene fluoride membranes. The membranes were blocked in Tris-buffered saline containing 0.05% Tween-20 and 5% skimmed milk for 1 h at room temperature, and incubated overnight at 4°C with appropriate primary antibody (anti-p38 or anti-phospho-p38). Immunoreactive proteins were detected by horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescence reagent.

Assay of TNF-α and IL-8 secreted from THP-1 cells

LPS induces THP-1 cells to secrete TNF-α. Thus, the potential suppression of LPS by rFC is measured by a reduction in TNF-α. THP-1 monocytes were transformed into macrophages by addition of a concentrated stock solution of phorbol myristic acid (0.3 mg/ml in dimethyl sulfoxide) to the cell suspension to give a final concentration of 30 ng/ml PMA and 0.01% dimethyl sulfoxide. PMA-treated cell suspensions were immediately plated into a 96-well microtiter plate at a density of 4 x 10^5 cells/ml and allowed to differentiate for 48 h at 37°C. Immediately before stimulation with 10 ng/ml LPS or LPS pre-incubated with various concentrations of $LBP_{85–108}$ peptide or rFC for 30 min, the culture medium was removed and the cells were washed twice with serum-free RPMI 1640 and incubated at 37°C. At indicated times, the culture medium was collected. Human TNF-α and IL-8 in the supernatants were assayed using ELISA. The means of values were obtained from at least three independent experiments.

Cytotoxicity test of rFC in eukaryotic cells

Aliquots (50 µl) of THP-1 (2 x 10^4 cells) were mixed with equal volumes of serial 2-fold dilutions of rFC ranging in concentration from 1.0 nM to 10 nM, in PBS and incubated for 24 h at 37°C. To determine the cytotoxicity induced by the rFC, 20 µl of CellTiter 96 AQueous One solution Reagent (Promega) was added into each well for 90 min at 37°C. MTS, [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] is normally bioreduced by metabolically active cells into a colored formazan product that is soluble in tissue culture medium, and is measurable at 490 nm. To determine the ratio level of cell lysis induced by rFC, two controls were included in the assay. Complete lysis, representing cells undergoing acute cytotoxicity, was assessed by incubating cells in PBS containing 0.2% Tween-20. This absorbance value should correspond to the background as the lysed cells do not metabolize MTS. The second control of 0% lysis representing viable cells was determined by incubating cells in medium only. The experiment was performed in triplicate.

RESULTS

The purity and biochemical status of rFC

Although many reports have documented the initiation of the blood coagulation cascade by LPS-activated Factor C,[17,20] so far no effort has been made to exploit the endotoxin-neutralization ability that is attributable to its high affinity for LPS. Here, we examined the efficacy of rFC in preventing LPS from activating the host cell. Figure 1 shows that under non-reducing buffer conditions, the molecular weight of the rFC is about 130 kDa. In contrast, in reducing buffer, the rFC displayed two

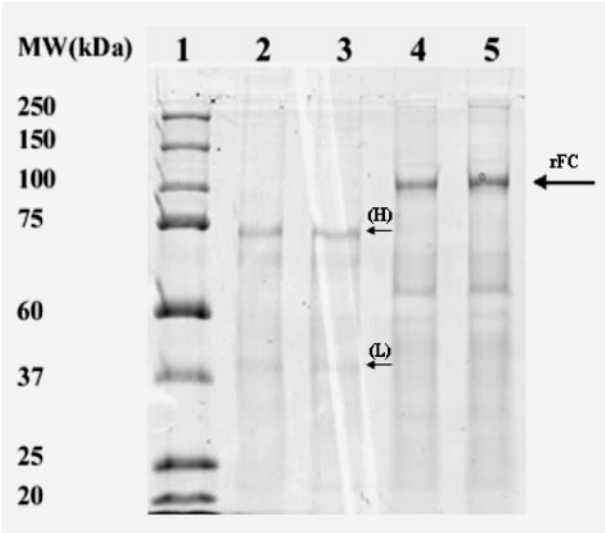

Fig. 1. SDS-PAGE shows the purity and state of rFC in reducing and non-reducing buffer conditions. Lanes: 1, protein marker; 2,3, rFC in reducing buffer (heavy, H and light, L chains of Factor C); 4,5, rFC in non-reducing buffer (intact Factor C).

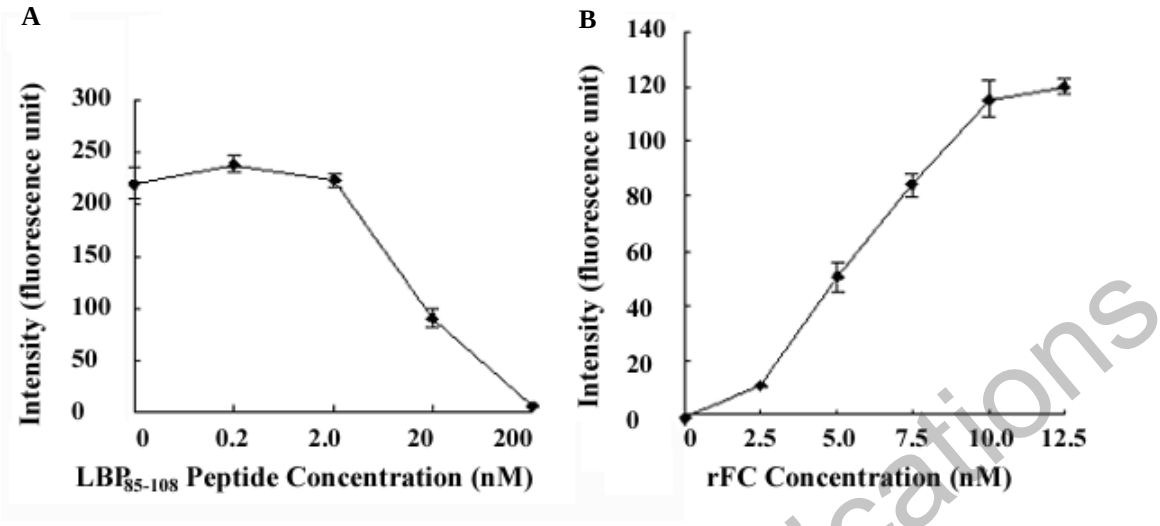

Fig. 2. The rFC and $LBP_{85–108}$ peptide compete with each other to bind LPS. (A) At a fixed rFC concentration of 5 nM, the LBP progressively suppresses rFC-mediated LPS-binding activity measured in fluorescence units of rFC enzyme activity. (B) 10 nM of rFC was sufficient to displace off 20 nM $LBP_{85–108}$ peptide from LPS and result in the recovery of LPS-binding by the rFC, upon which the rFC enzyme activity is restored.

protein bands of about 80 kDa (heavy chain) and 50 kDa (light chain). These molecular sizes agree well with the native, full-length Factor C and the cleaved/activated Factor C fragments from the horseshoe crab.[18]

rFC prevents $LBP_{85–108}$ peptide from binding LPS

At the initial phase of inflammation, the host LPS-binding protein (LBP) binds LPS to form a complex and activates the LPS receptors on the host cells. The activation of LPS receptors, in turn, activates the inflammation-responsive genes, which overexpress cytokines to induce endotoxity in the host.[26] Therefore, it was expected that if rFC binds LPS, it will negate the ability of $LBP_{85–108}$ peptide to complex with LPS, *i.e.* rFC neutralizes the endotoxicity of LPS via its competition against $LBP_{85–108}$ peptide. Here, we examined the efficacy of rFC in competing against LPS-induced inflammation. $LBP_{85–108}$ peptide, which is derived from the LPS binding motif of LBP protein,[23] was used here. To examine whether rFC and $LBP_{85–108}$ peptide compete with each other for LPS, a sensitive and precise rFC fluorimetric assay was performed to assess the ability of $LBP_{85–108}$ peptide to bind endotoxin in the presence of increasing doses of rFC or $LBP_{85–108}$ peptide. Figure 2A shows that increasing levels of $LBP_{85–108}$ peptide progressively inhibited the LPS-induced rFC activity. At 200 nM, the $LBP_{85–108}$ peptide completely inhibited rFC from binding with LPS. In contrast, only 10 nM rFC was sufficient to inhibit, effectively, 20 nM of $LBP_{85–108}$ peptide to bring about LPS inhibition. The resultant rFC enzyme activity triggered by LPS was registered as the intensity of fluorescence units measured in the PyroGene assay (Fig. 2B). As a control, we established that $LBP_{85–108}$ peptide itself does not affect Factor C activity. These results suggest that: (i) rFC and $LBP_{85–108}$ peptide are likely to compete with each other for LPS and they probably target the same motif of LPS; and (ii) rFC binds LPS to prevent it from binding LPS-effectors like LBP *in vivo*.

rFC inhibits LPS-mediated phosphorylation of p38

To understand better the biological significance of the rFC-mediated suppression of LPS activity, we used human macrophage cultures, which constitutively express the LPS receptors, CD14 and TLR4, on their membrane surface. Western analysis showed that over the time course of 2 h of LPS stimulation, p38, the major member of the MAPK family, was progressively phosphorylated. We next examined the effect of rFC on LBP–LPS complex driven p38 signaling. Figure 3A demonstrates that rFC down-regulated the phosphorylation of p38 by LPS. The degree of phosphorylation/unphosphorylation of p38 was determined from the intensity of the bands using a gel imaging software (Quantity One, Bio-Rad). Stimulation of the macrophage culture with 2 EU/ml LPS in the presence of $LBP_{85–108}$ peptide, caused a high level of phosphorylation of p38. But, in the presence of rFC, the phosphorylation of p38 was reduced (Fig. 3B), clearly indicating that rFC inhibited the p38 phosphorylation which would otherwise be induced by $LBP_{85–108}$ peptide–LPS complex. It is reasonable to attribute the reduction in p38 phosphorylation to rFC's ability to prevent $LBP_{85–108}$ peptide from binding LPS effectively (Fig. 2), hence blocking the LBP-induced intracellular signaling observed here.

rFC inhibits LPS-mediated TNF-α and IL-8 expression

The macrophages were stimulated with 2 EU/ml LPS with or without rFC (0–10 nM). The TNF-α level was quantified to determine the effect of rFC on the cytokine release. Figure 4A shows that rFC markedly inhibited the level of TNF-α induced by LPS. Only 10 nM of rFC was sufficient to block LPS-mediated TNF-α production

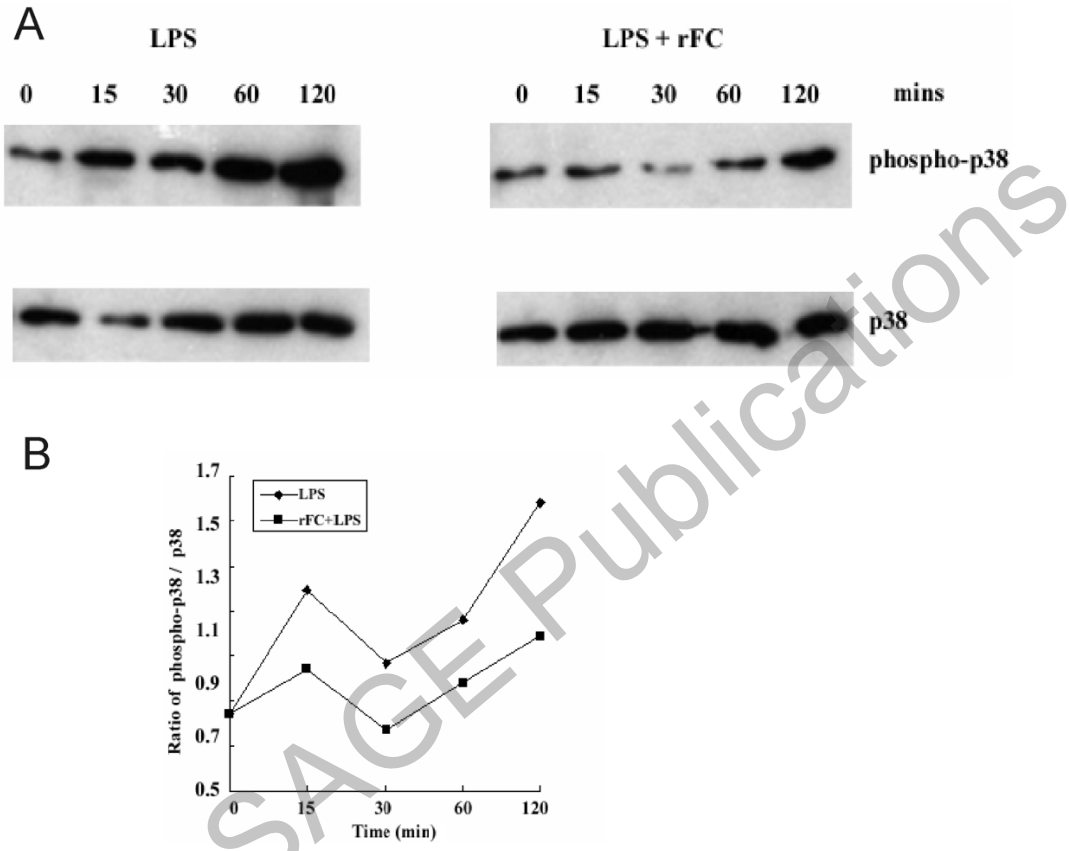

Fig. 3. rFC inhibits the p38 phosphorylation caused by LPS. (A) Western blot analysis of p38. The top panel shows LPS-induced p38 phosphorylation in human macrophages over 120 min. The lower panel shows that rFC effectively inhibited the LPS-mediated p38 phosphorylation. (B) Analysis of the intensity of each band on Western blot yielded a quantitative comparison of the ratio of phosphorylated verses unphosphorylated forms of p38.

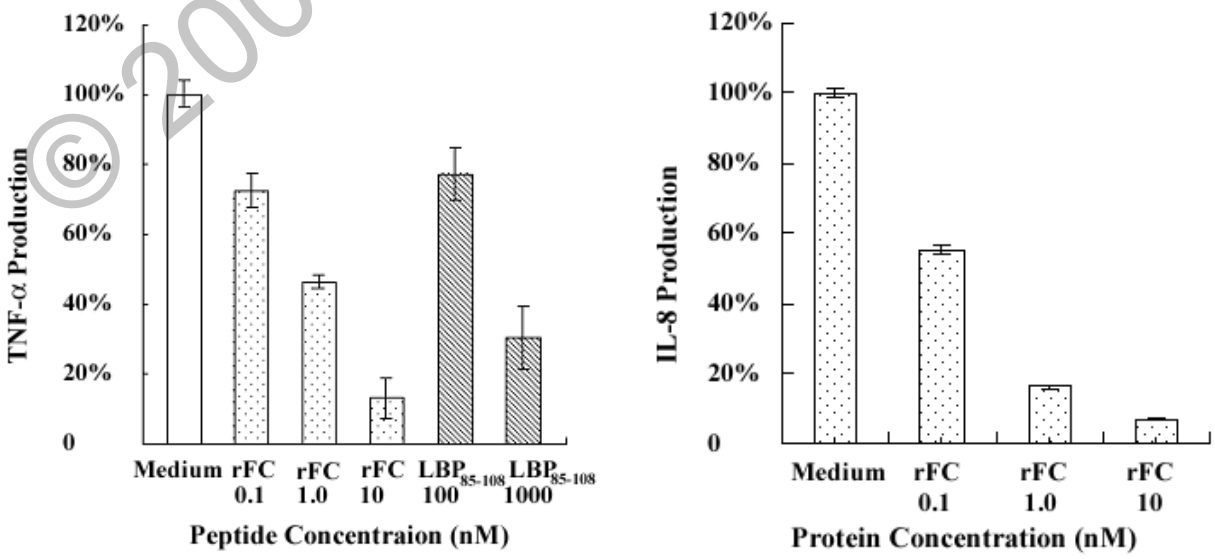

Fig. 4. rFC inhibits the LPS-induced TNF-α and IL-8 production. (A) rFC inhibits LPS-induced TNF-α production. (B) rFC inhibits LPS-induced IL-8 production. The LPS concentration is fixed at (2 EU/ml). The concentration of rFC ranges from 0.1–10 nM, and the concentrations of $LBP_{85–108}$ peptide were 100 nM and 1 μM. THP-1-derived macrophages were incubated with LPS and rFC for 6 h, and the production of TNF-α was detected by ELISA using anti-TNF-α.

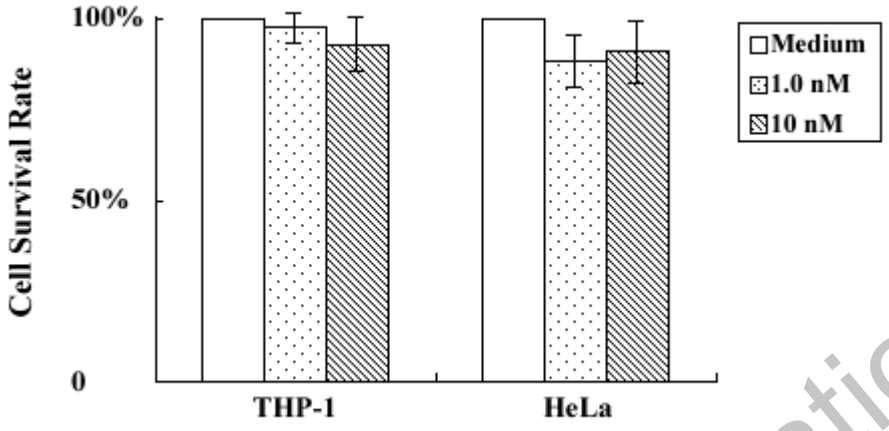

Fig. 5. rFC shows minimal toxicity to mammalian cells. THP-1 and HeLa cells were incubated with 1.0 or 10 nM rFC for 24 h. The cell survival rate was detected by MTS assay.

almost completely as compared to the negative control (medium alone without LPS or $LBP_{85–108}$ peptide) and the positive control (with LPS and 1 mM $LBP_{85–108}$ peptide). We measured the ability of rFC to inhibit IL-8 production by THP-1 cells when incubated with LPS. As shown in Figure 4B, 10 nM of rFC potently inhibited > 90% LPS-induced production of IL-8. Taken together, these results agree well with the potency of the rFC to inhibit the $LBP_{85–108}$ peptide–LPS complex mediated phosphorylation of p38 (Fig. 3).

rFC does not show acute cytotoxicity to human cells

The potential toxicity of rFC to mammalian cells was tested by incubating it with THP-1 and HeLa cells prior to the acute cytotoxicity test using MTS, a compound that could only be metabolized by healthy cells. At 10 nM, rFC showed minimal effect on cell permeabilization /cytotoxicity (Fig. 5). Therefore, rFC can effectively inhibit the LPS-induced sepsis without being toxic to the host cells.

DISCUSSION

World-wide, studies have focused much attention on the intervention of LPS-induced inflammatory response using LPS-neutralizing drug candidates to bind LPS and to inactivate the LPS signaling pathway. Numerous proteins and peptides from insects and mammals[10–13,27] have evolved for the purpose of binding and neutralizing LPS. Amongst these is Factor C, an LPS sensor protein found in the horseshoe crab. Many reports have documented the ability of LPS-activation of Factor C to trigger the coagulation cascade.[17–18,20–22] Owing to its extreme sensitivity to endotoxin, Factor C has played an important biotechnological role in pyrogen detection in the pharmaceutical and biomedical industries.[20] However, no effort has so far been made to exploit the high affinity of Factor C for LPS to the endotoxin-neutralization applications.

Here, we used rFC (Fig. 1), which is similar to the native Factor C purified from the blood of horseshoe crab,[17,18,20] to bind LPS in competition against the $LBP_{85–108}$ peptide. Our data clearly demonstrated that $LBP_{85–108}$ peptide can inhibit the LPS-induced rFC enzyme activity, and, conversely, rFC can effectively bind LPS to compete against the $LBP_{85–108}$ peptide (Fig. 2). It had been reported that the positively charged amino acids and hydrophobic amino acids are important for the LPS neutralizing proteins or peptides (*e.g.* LALF[14,15] and rFC[28,29]) found in horseshoe crab. These amino acids on the Sushi domains 1 and 3 of rFC are responsible for its high affinity for LPS,[28,29] thus offering multiple binding sites of LPS per molecule of rFC. On the other hand, the single LPS binding motif in the LBP protein spans only a short sequence of 24 amino acids.[12,23] Like rFC, the $LBP_{85–108}$ peptide interacts with LPS via: (i) electrostatic interactions between the positively charged amino acids of LBP and the negatively charged bisphosphate headgroups of LPS; and (ii) hydrophobic interactions between the hydrophobic amino acids of LBP and the acyl chains of LPS.[30] Our data clearly showed that the $LBP_{85–108}$ peptide can inhibit the LPS-induced rFC enzyme activity (Fig. 2A) and that, by increasing the concentration of rFC, the LPS-induced enzymatic activity of rFC was recovered (Fig. 2B). $LBP_{85–108}$ peptide was shown to bind the lipid A moiety of LPS.[30] Similarly, rFC has been shown to target the lipid A moiety. Thus, rFC and $LBP_{85–108}$ peptide compete with each

other for the same chemical moiety, lipid A, which is the bioactive pharmacophore of LPS. We envisage that, when rFC occupies the lipid A moiety of the LPS molecule, it deprives the $LBP_{85–108}$ peptide of its binding site on LPS *in vitro*. This direct competition could be responsible for rFC-mediated blockade in LPS signaling pathway. Thus, rFC could be used to bind LPS in a host cell environment to prevent it from activating the host.

Ariki *et al*.[32] reported that Factor C exhibits a K_D for LPS of 7.56 x 10^{-10} M as compared to that of LBP protein (3.5 x 10^{-9} M)[33] and CD14 (2.9 5 x 10^{-8} M).[33] LBP and CD14 are important proteins in LPS signaling pathway activation in the host cell.[2–4,34] Thus, we investigated the rFC-mediated intervention of the hallmark of LPS-induced signaling pathway in a human host cell. Our results demonstrated that the phosphorylation of p38 in the LPS signaling pathway was down-regulated by rFC (Fig. 3). Since p38 signaling occurs upstream of inflammatory response and septic shock,[26] inhibition of the phosphorylation of p38 indicates its likelihood of blocking overexpression of cytokines, hence preventing septic shock. Consistent with this suggestion, we observed that the LPS-induced production of TNF-α and IL-8 were effectively reduced by rFC (Fig. 4). These results suggest that rFC could mediate a blockade in LPS signaling pathway. Thus, rFC could be used to bind LPS in a host cell environment to prevent it from activating the host. Therefore, rFC could be a potential LPS-neutralizing agent. Furthermore, we showed that rFC is nontoxic to human monocytes and HeLa cells (Fig. 5).

In view of the potential development of rFC into an LPS-antagonist, we compared its anti-LPS activity against that of the Sushi 1 and Sushi 3 peptides, which have been derived from the LPS-binding domains of Factor C.[28] These 34-mer peptides have been shown to interact with LPS and LPS-analogues through electrostatic and hydrophobic interactions.[25,28,31] However, the suppression of LPS-induced TNF-α secretion in human macrophages by Sushi 1 and Sushi 3 peptides revealed that, even at > 10 μM,[28] these peptides only exerted 50% inhibition of LPS-induced TNF-α production.[23] In contrast, the full-length rFC from which the Sushi peptides were derived, was highly efficacious in inhibiting TNF-α production (Fig. 4) compared to that of Sushi 1 or Sushi 3 peptides. The co-operativity between the LPS-binding sites of Sushi 1 and Sushi 3 domains within the Factor C,[28] might be operational *in vivo*, in the macrophage cells. Such co-operativity between the two LPS-binding domains in rFC was reported[29] to lead to rapid amplification of the activation of Factor C, thus explaining the extreme sensitivity of rFC for trace levels of LPS in solution. Furthermore, Factor C is a large protein and contains other functional domains,[18] which might also enhance the overall interaction between rFC and LPS. Thus, future attempts may be made to engineer, strategically, hybrid multimers of tandem Sushi 1 and Sushi 3 flanked with only the essential juxtaposed domains. This hybrid will be smaller than rFC and, conceivably, just as effective as an LPS-binding and LBP-competitor molecule.

Acknowledgements

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