Pulmonary Surfactant Protein D Inhibits Lipopolysaccharide (LPS)-induced Inflammatory Cell Responses by Altering LPS Binding to Its Receptors*

Received for publication, September 19, 2008, and in revised form, October 25, 2008 Published, JBC Papers in Press, November 5, 2008 DOI 10.1074/jbc.M807268200

Pulmonary surfactant protein D (SP-D) is a member of the collectin family that plays an important role in regulating innate immunity of the lung. We examined the mechanisms by which SP-D modulates lipopolysaccharide (LPS)-elicited inflammatory cell responses. SP-D bound to a complex of recombinant SP-D modulates lipopolysaccharide (LPS)-elicited inflammation and inflammatory cell responses. SP-D down-regulates LPS-elicited responses and reveals the importance of the correct oligomeric structure of the protein in this process.

Pulmonary surfactant protein D (SP-D) is a member of the collectin family that also includes surfactant protein A (SP-A) and mannose binding lectin (1, 2). The structure of the collectins is characterized by four domains consisting of: 1) an extracellular TLR4 domain (sTLR4) and MD-2 (sMD-2) from extracellular TLR4/MD-2 complexes, 2) a collagenous domain containing carbohydrate recognition domain (CRD) (3). SP-A and mannose binding lectin contain collagenous domains consisting of 23 and 19 repeating Gly-X-Y triplets, respectively, with an interruption at the middle of the collagenous sequence (4, 5). In contrast, SP-D possesses a longer collagenous tail composed of 59 Gly-X-Y repeats without an interruption (6). These differences cause different oligomeric organization, with SP-A and mannose binding lectin exhibiting bouquet-like structures consisting of either six or four trimeric subunits (7) and SP-D exhibiting cruciform structures composed of four trimeric subunits (8).

Lipopolysaccharide (LPS) is a principal component of the outer membrane of Gram-negative bacteria that activates macrophages and induces a variety of inflammatory mediators, including TNF-α, IL-1, IL-6, IL-8, and interferon (9). LPS composed of O-antigen, core oligosaccharide, and lipid A is named smooth LPS, and LPS lacking O-antigen and a part of the core oligosaccharides is named rough LPS (10). Toll-like receptor 4 (TLR4) plays a critical role in recognition and signaling by LPS (11, 12). MD-2 binds directly to LPS and is required for TLR4-mediated signaling induced by LPS (13, 14). Structural examination of the TLR4-MD-2 complex revealed that MD-2 binds to the concave surface of the N-terminal and central domains of TLR4 (15). A study with recombinant soluble forms of the extracellular TLR4 domain (sTLR4) and MD-2 (sMD-2) from our laboratory indicates the importance of the N-terminal region of TLR4 in MD-2 binding (16).

Engineered genetic defects in the pulmonary collectins of mice have revealed their important functions in protecting the lung from microbial infection and inflammation. SP-D-null mice infected with group B Streptococcus or Haemophilus influenzae by intratracheal instillation show increased inflammation and inflammatory cell recruitment in the lung (17). Increased pulmonary inflammation in LPS (Escherichia coli O55:B5, smooth serotype)-instilled SP-D−/− mice and wild-type mice was decreased by intratracheal administration of SP-D and pulmonary surfactant (18). Intratracheal recombinant SP-D prevents endotoxin shock in the newborn preterm

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8 This work was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture, Japan and by Akiyama Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
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3 The abbreviations used are: SP-D, surfactant protein D; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; CRD, carbohydrate recognition domain; CRF, collagenase-resistant fragment; TNF, tumor necrosis factor; IL, interleukin; s, soluble; HEK, human embryonic kidney; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; HRP, horseradish peroxidase.
SP-D Inhibits TLR4/MD-2-mediated LPS Signaling

Chimeric Protein—We constructed a chimeric protein with SP-A and SP-D (A/D chimera) in which the N-terminal region and the collagenous domain of SP-D were replaced with those of SP-A, as represented schematically in Fig. 6A. The A/D chimera consists of Glu–Pro100 of SP-A and Asp203–Phe135 of SP-D. The cDNA for the chimera was constructed by using PCR and the overlap extension method (26) using the cDNAs for SP-A and SP-D. The two primers used at the SP-A/SP-D splicing junction were 5′-GGAGGTCCCGAAGTCTACAACG-AAGAAG-3′ and 5′-CCCTCAAGGCTTCAGATGTGCTTCTC-3′. An SP-A sense and an SP-D antisense primers were used as follows: 5′-AAGCTTATGTGGCTTGCCCCTG-G CCC-3′ and 5′-GAACACCAAGCCTCAAGACTAGTCT-3′, respectively. The BamHI and XbaI sites were incorporated into the flanking 5′ and 3′ primers, respectively. The construct was inserted into the pEE14 plasmid vector using the BamHI and XbaI sites. The organization of the recombinant plasmid and sequence of the cDNA insert was confirmed by a combination of restriction enzyme mapping and DNA sequencing. The recombinant A/D chimera was expressed using the glutamine synthetase gene amplification system (25), and the recombinant protein was produced and purified by affinity chromatography as described above for SP-D production.

Collagenase Treatment—Because the yield of the A/D chimera is greater than that of SP-D, the chimeric protein was used to obtain the collagenase-resistant fragment (CRF). The A/D chimera was digested with collagenase III from Clastrium histolyticum at 37 °C for 22 h. The CRF was isolated by gel-filtration chromatography using a Superose 610/300 GL (GE Healthcare Bio-Science AB). The N-terminal sequence of the isolated CRF was determined by using an Applied Biosystems (Foster City, CA) amino acid sequencer. The sequences obtained were GPPGLP or GLPDVA, corresponding to the end of the collagenous domain of human SP-A (Gly78–Pro100) or the SP-A/SP-D splice junction (Gly78–Lys–Pro100) of SP-A/Asp203–Val-Ala209 of SP-D), respectively. This indicates that the N-terminal region and the collagenous domain of SP-A were removed from the A/D chimera.

Endotoxin Removal—Endotoxin in the protein preparations was removed using polymixin-agarose and octyl-β-D-glucopyranoside as described elsewhere (27). The endotoxin level determined by Limulus amebocytes lysate assay was below 1.41 pg/μg of protein in the preparations of SP-D, the A/D chimera, and CRF.

Biotinylation—SP-D was biotinylated by using EZ-Linked sulfo-N-hydroxysuccinimide-LC-biotin (Pierce) according to the manufacturer’s instructions.

Gel-filtration Chromatography—SP-D, the A/D chimera, and CRF were analyzed by gel-filtration chromatography using 10 × 300 mm Superose 610/300 GL column. The protein was eluted at 0.5 ml/min in PBS at 4 °C, and the elution was monitored by measuring the absorbance at 280 nm. Threoglobulin (669 kDa), ferritin (440 kDa), aldolase (158 kDa), ovalbumin (43 kDa), and ribonuclease A (13.7 kDa) from Pharmacia Biotech were used as molecular mass standards.

Western Blot—A 100-ng sample of the protein was subjected to 13% SDS-PAGE under reducing and denaturing conditions, and transferred onto a polyvinylidene difluoride membrane.
The membrane was immunopробed with anti-human SP-D polyclonal antibody (5 μg/ml), or anti-human SP-A polyclonal antibody (5 μg/ml), or anti-human SP-D monoclonal antibody 7C6 or 7A10 (2 μg/ml), followed by incubation with HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-mouse IgG. The proteins that reacted with the antibodies were visualized by a chemiluminescence reagent (SuperSignal, Pierce) according to the manufacturer’s instructions.

Electron Microscopy—SP-D, the chimeric protein, and the CRF were diluted to 10 μg/ml in 50% glycerol and 20 mM ammonium bicarbonate, sprayed onto freshly cleaved mica, and rotary-shadowed molecules were observed under an H-7650 electron microscope (Hitachi Co. Ltd, Tokyo, Japan) operated at 75 kV.

Binding of SP-D to LPS—The binding of the biotinylated SP-D to Re595, Rc, or O26:B6 LPS (5 μg/well) coated onto microtiter wells was performed as described previously (22). To determine the effects of anti-SP-D monoclonal antibodies on the SP-D binding to LPS, the protein (2 μg/ml) was first incubated with anti-SP-D monoclonal antibody 7C6 or 7A10 at 37 °C for 1 h. The mixture of SP-D and antibody was then added to the LPS-coated wells (1 μg/well), and the mixture was incubated at 37 °C for 3 h.

Binding of SP-D to an sTLR4-sMD-2 Complex—A soluble form of recombinant extracellular TLR4 domain (sTLR4: Met1–Lys631) containing a His6 tag at the C-terminal end and a soluble form of recombinant MD-2 (sMD-2) containing the C-terminal fusion V5 tag and a His6 tag were expressed using a baculovirus-insect cell expression system as described previously (24). sTLR4 and sMD-2, co-expressed in insect cells, were purified from the medium using a nickel-chelating HisTrap column as described above and eluted with 35880 mM Hepes buffer (pH 7.4) containing 0.15M NaCl, 2 mM CaCl2, 35880% (w/v) bovine serum albumin, and 35880 mM ammonium bicarbonate, sprayed onto freshly cleaved mica, and rotary-shadowed molecules were observed under an H-7650 electron microscope (Hitachi Co. Ltd, Tokyo, Japan) operated at 75 kV.

Binding of LPS to TLR4/MD-2-expressing Cells—HEK293 cells, stably expressing TLR4 and MD-2 (293-hTLR4/MD-2-CD14) (InvivoGen, San Diego, CA), were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, in 50% glycerol and 20 mM ammonium bicarbonate, sprayed onto freshly cleaved mica, and rotary-shadowed molecules were observed under an H-7650 electron microscope (Hitachi Co. Ltd, Tokyo, Japan) operated at 75 kV.

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RESULTS

SP-D Binds to an sTLR4-sMD-2 Complex—We have previously shown that SP-D interacts with TLR4 (22) through the CRD of the collectin in a Ca\(^{2+}\)-dependent manner. We isolated a complex of sTLR4 and sMD-2 by gel-filtration chromatography and examined whether SP-D binds to an sTLR4-sMD-2 complex. When sMD-2 was immunoprecipitated with anti-V5 antibody-conjugated agarose, SP-D as well as sTLR4 was co-precipitated (Fig. 1A, WB: streptavidin and αTLR4). The biotinylated SP-D also co-sedimented with sTLR4 and sMD-2 by an affinity adsorption assay with streptavidin-conjugated agarose (Fig. 1B, WB: αTLR4 and αV5). These results indicate that SP-D can bind a complex of TLR4 and MD-2.

SP-D Binds to Re595 LPS and Rc LPS by Different Mechanisms—We have previously shown that SP-A binds to Re595 LPS and Rc LPS but not to O26:B6 LPS (20). SP-D has been reported to bind to Rc LPS and Rd LPS but not to Ra LPS and smooth LPS (28). In this study, we also examined the binding of SP-D to rough LPS (Rc LPS from E. coli and Re595 LPS from S. minnesota) and smooth LPS (O26:B6 LPS from E. coli) coated onto microtiter wells. SP-D bound to Re595 LPS and Rc LPS in the presence of Ca\(^{2+}\) in a concentration-dependent manner (Fig. 2, A and B). SP-D failed to bind solid-phase O26:B6 LPS. The SP-D binding to Re595 LPS was not significantly attenuated by the presence of EDTA (Fig. 2A), whereas EDTA completely blocked the binding of SP-D to Rc LPS (Fig. 2B). Therefore, the SP-D binding to Rc LPS but not to Re595 LPS was Ca\(^{2+}\)-dependent.

The effect of anti-SP-D monoclonal antibodies on the LPS binding was also investigated. Antibody 7C6 completely blocked the binding of SP-D to Re595 LPS, and antibody 7A10 partially inhibited this binding (Fig. 2C). In contrast, antibody 7A10 but not antibody 7C6 completely inhibited the SP-D binding to Rc LPS (Fig. 2D). Because the epitopes for antibodies 7A10 and 7C6 are located at the CRD and the neck region, respectively (22), the results suggest that the neck region and the CRD are involved in the binding of SP-D to Re595 LPS and Rc LPS, respectively. These results are consistent with those obtained from the experiments demonstrating Ca\(^{2+}\) dependence, or independence for binding different ligands. The data confirm that rough LPS but not smooth LPS is an SP-D ligand and indicate that SP-D binds to Re595 and Rc LPS by different mechanisms.

FIGURE 1. SP-D binds to an sTLR4-sMD-2 complex. A, immunoprecipitation with anti-V5 (MD-2) antibody. The isolated sTLR4-sMD-2 complex (100 ng) was incubated with biotinylated SP-D (100 ng) at 37 °C for 2 h. The sTLR4-sMD-2 complex was immunoprecipitated with anti-V5 antibody-conjugated agarose, and the immunoprecipitate was subjected to SDS-PAGE (7.5–15% polyacrylamide gel) under reducing conditions. As a control experiment, affinity-purified mouse IgG and protein G-Sepharose were used (normal IgG). The Western blot was then performed by using anti-V5 antibody for sTLR4 and HRP-streptavidin for biotinylated SP-D, respectively, as described under “Experimental Procedures.” B, affinity adsorption assay of SP-D. Biotinylated SP-D (800 ng) was incubated with an sTLR4-sMD-2 complex (100 ng) at 37 °C for 2 h. The biotinylated SP-D was pulled down by streptavidin-conjugated agarose, and the precipitates were subjected to SDS-PAGE (7.5–15% polyacrylamide gel) under reducing conditions. The Western blot was then performed as described above.
SP-D Attenuates LPS-induced TNF-α Secretion and NF-κB Activation

SP-D, an SP-A homologue, regulates LPS-induced inflammatory responses in a ligand-specific manner. SP-A binds to rough LPS and enhances its inflammatory response (20, 21). For comparison, we next investigated whether SP-D regulates TNF-α secretion elicited by smooth and rough LPS. SP-D significantly attenuated O26:B6 LPS-stimulated TNF-α secretion from alveolar macrophages in a concentration-dependent manner (Fig. 3A). Unlike SP-A, SP-D down-regulated TNF-α secretion elicited by Re595 LPS and Rc LPS (Fig. 3, B and C), both of which are ligands for SP-D (Fig. 2). The inhibitory effect of SP-D appeared more potent for antagonizing the stimulation elicited with Rc LPS, than that occurring with Re595 LPS. The IC50 for SP-D was 8.16 μg/ml with O26:B6 LPS, 19.51 μg/ml with Re595 LPS, and 5.77 μg/ml with Rc LPS, respectively. We also performed the experiments with SP-A as a control. SP-A down-regulated O26:B6 LPS-induced TNF-α secretion. The percentages of TNF-α secretion were 23 ± 10%, 15 ± 10%, and 15 ± 10% at 1.5, 7.5, and 15 μg/ml of SP-A, respectively. These results obtained from control experiments with SP-A are consistent with our previous studies (20, 21).

We next examined the effect of SP-D on LPS-elicited NF-κB activation in HEK293 cells transfected with TLR4 and MD-2. O26:B6 LPS, Re595 LPS, and Rc LPS induced robust NF-κB-dependent luciferase activities through TLR4 and MD-2 (Fig. 4). The presence of SP-D significantly inhibited NF-κB activation...
stimulated by both smooth (Fig. 4A) and rough serotypes (Fig. 4B and C) of LPS. The NF-kB activities in the presence of SP-D (10 μg/ml) were 30% (the mean of two experiments) and 119% of those in the absence of SP-D when the cells were stimulated with O26:B6 LPS and Re595 LPS, respectively. These results are consistent with those obtained by examining TNF-α secretion.

SP-D Inhibits Cell Surface Binding of LPS to TLR4/MD-2-expressing Cells and Alters MD-2-LPS Interaction—We next examined whether SP-D alters the binding of LPS to TLR4/MD-2-expressing HEK293 cells by using Alexa488-labeled E. coli O55:B5 LPS (smooth LPS) and Alexa488-labeled S. minnesota LPS (rough LPS). Flow cytometric analysis revealed significant LPS binding to the cell surface (Fig. 5, A and B, upper, gray shadow) when the cells were incubated with the fluorescent LPS at 4 °C. The binding of smooth and rough serotypes of Alexa488-conjugated LPS was inhibited by the presence of SP-D (Fig. 5, A and B, upper, dotted line). SP-D significantly
attenuated the mean fluorescence intensity of cell surface binding of both O55:B5 LPS and S. minnesota LPS (Fig. 5, A and B, lower).

Because MD-2 is critical for TLR4-mediated LPS signaling (13) and MD-2 directly interacts with LPS (14) and its principal core structural constituent, lipid A (29), we examined whether SP-D affects the binding of sMD-2 to LPS coated onto microtiter wells. SP-D significantly inhibited the binding of sMD-2 to different serotypes of LPS (Fig. 5C). The binding of sMD-2 to Re595 LPS and Rc LPS was reduced to near background levels by SP-D. The binding of sMD-2 to O26:B6 LPS and O111:B4 LPS was reduced to ~50% by SP-D. The results clearly demonstrate that SP-D alters MD-2-LPS interaction.

**Analysis of Chimeric Protein and CRF**—SP-A binds to Re595 LPS and Rc LPS and does not bind to O26:B6 LPS or O111:B4 LPS (20). In this study SP-D also binds to Re595 LPS and Rc LPS but not to O26:B6 LPS, and SP-D suppresses inflammatory responses elicited by both serotypes of LPS, unlike SP-A. We thus constructed a chimera composed of the N terminus plus the collagenous domain of SP-A and the neck plus the CRD of SP-D (A/D chimera) to determine whether the different responses between SP-A and SP-D are consequences of different oligomeric structures. Recombinant SP-D and the A/D chimera (Fig. 6A) were expressed in CHO-K1 cells and purified by affinity chromatography using mannose-Sepharose. CRF consisting of the neck region plus the CRD of SP-D was also purified by gel filtration after collagenase digestion. The main protein bands of SP-D and SP-A migrated at the positions of apparent molecular masses of 45 and 36 kDa, respectively, under reducing conditions when analyzed by electrophoresis (Fig. 6B, lanes a and b). The A/D chimera and CRF exhibited the expected sizes of apparent molecular masses of 32 and 18 kDa, respectively (Fig. 6B, lanes c and d). We also analyzed these proteins by Western blotting using anti-SP-A polyclonal antibody, anti-SP-D polyclonal antibody, and anti-SP-D monoclonal antibodies 7C6 and 7A10 whose epitopes are located at the neck region and the CRD of SP-D, respectively. SP-D or SP-A was recognized only by anti-SP-D antibody or anti-SP-A antibody, respectively (Fig. 6C, lanes a and b). The chimera was recognized by all four of the antibodies used (Fig. 6C, lane c) and CRF was detected by anti-SP-D polyclonal antibody and antibodies 7C6 and 7A10 (Fig. 6C, lane d). These results indicate that the chimera and the CRF are correctly expressed and secreted.

We further analyzed these proteins by gel-filtration chromatography and by electron microscopy to examine their oligomeric structure in solution. Gel-filtration analysis with Superose revealed that SP-D eluted at void fraction (Fig. 7A), indicating that SP-D is highly oligomeric (molecular mass of ~5 MDa). The elution profile of the A/D chimera showed two main peaks with apparent molecular masses of 220 and 620 kDa. CRF eluted at a fraction corresponding to the size of 68 kDa. The ultrastructures of the proteins were observed by electron microscopy using the rotary shadowing technique (Fig. 7B). In the preparation of SP-D, heterogenous particles, presumably consisting of two, or four trimers, or multimers were observed. One subpopulation of SP-D was a cruciform dodecamer. Another subpopulation includes multimerized oligomer con-
consisting of SP-D molecules associated at their N terminus. These observations are consistent with those of previous studies (8, 30). The preparation of the A/D chimera contained two, or four, or six trimers. Structures of four or six globular heads were seen and appeared to form a bouquet-like arrangement, which is typically observed for SP-A (7). The collagenous domain of SP-A consists of 23 Gly-YX-Y triplets without interruption (6).

The A/D Chimera and CRF Are Less Potent as Inhibitors for sMD-2 Binding to LPS, and Antagonists for LPS Signaling and TNF-α Secretion, by TLR4/MD-2-expressing Cells—The effects of the A/D chimera and CRF on the binding of sMD-2 to LPS coated onto microtiter wells were examined. SP-D and the chimera significantly attenuated the sMD-2 binding to various serotypes of LPS tested (Fig. 8), but the inhibitory effect of CRF on the sMD-2 binding to LPS was insignificant. Although active, the effect of the chimera as an inhibitor was generally less potent than that of SP-D. The A/D chimera was most different from SP-D at inhibiting sMD-2 binding to Re595 LPS and Rc LPS (Fig. 8, C and D). The activity of the A/D chimera was comparable to SP-D for inhibiting sMD-2 binding to O26:B6 LPS and O111:B4 LPS (Fig. 8, A and B).

LPS-elicited TNF-α secretion and NF-κB activity were also determined in the presence of SP-D, the chimera, and CRF. Because the oligomerization states are different, the findings are expressed as a function of monomer concentrations for the various recombinant proteins. The A/D chimera was less potent as an inhibitor of LPS-induced TNF-α secretion from alveolar macrophages than SP-D (Fig. 9, A–C). CRF was very weak at inhibiting TNF-α secretion. When normalized per mole of monomer, the IC50 was 181.3 ± 50.5 nM (mean ± S.D., n = 3) for SP-D; 239.9 ± 184.4 nM for the chimera, and 347.0 ± 87.4 nM for CRF in O26:B6 LPS-stimulation; 126.8 ± 45.8 nM for SP-D and 341.6 ± 122.9 nM for the chimera in Rc LPS-stimulation (p < 0.05, SP-D versus the chimera); 436.8 ± 226.2 nM for SP-D and 1201.6 ± 75.2 nM for the chimera in Re595 LPS stimulation (p < 0.01, SP-D versus the chimera). The luciferase assay for LPS-elicited NF-κB activation (Fig. 9, D–F) showed that SP-D was the most potent in attenuating LPS signaling and that the chimera and CRF were weak inhibitors in rank order of the chimera > CRF. These results obtained from cellular experiments are consistent with those obtained from the in vitro sMD-2 binding to LPS, indicating that the inhibitory effect of SP-D is dependent upon the formation of cruciform structure and its multimer.

DISCUSSION

Previous in vivo studies (17–19, 32) have provided compelling evidence that pulmonary collectins modulate pulmonary inflammation caused by microbes and their components. Several mechanisms of collectin-mediated modulation of inflammation have been proposed. Gardai et al. (33) have proposed that, in the absence of microbes, the direct binding of CRD of pulmonary collectins to signal inhibitory regulatory protein α induces the activation of tyrosine phosphatase SHP-1 and blocks the downstream signaling, resulting in inhibiting pulmonary inflammation. However, in the presence of microbes, the binding of the aggregated collagenous tail of SP-A to calreticulin/CD91 stimulates p38 phosphorylation, NF-κB activation, and cytokine production. They concluded that SP-A plays dual inflammatory roles by its interaction with signal inhibitory regulatory protein α and calreticulin/CD91. We have previously shown that SP-A inhibits inflammatory responses stimulated with O26:B6 LPS, peptidoglycan, and zymosan by direct interactions with TLR2 and TLR4 (21, 34, 35) and that SP-A does not attenuate but rather enhances Re595 LPS-induced signaling and TNF-α secretion (20). Because Re595 LPS but not O26:B6 LPS, peptidoglycan, or zymosan is a ligand for SP-A, this collectin exhibits dual functions in a ligand-specific manner. However, SP-D appears to exhibit anti-inflammatory effects regardless of ligand or non-ligand, because SP-D binds to Re595 LPS and Rc LPS but not to O26:B6 LPS and inhibits signaling and cytokine secretion stimulated with these different serotypes of LPS by altering LPS binding to its receptor (see Figs. 2–5). Gardai et al. (33) did not examine whether SP-D binds to calreticulin/CD91 via its collagenous tail. Because the collagenous tails and the globular domains of SP-A and C1q bind to the calreticulin/CD91 and the microbes, respectively, the orientation of the bouquet-like structure could be important. In contrast, the oligomeric structure of SP-D showing a cruciform is quite dif-
We have previously shown that SP-D directly binds to the extracellular TLR4 domain (22). In this study sMD-2 was immunoprecipitated together with sTLR4 and SP-D, and biotinylated SP-D also co-precipitated with sMD-2 and sTLR4; demonstrating that SP-D can bind an sTLR4-sMD-2 complex. Because the formation of a complex with TLR4 and MD-2 is critical for initiating LPS signaling, it is reasonable to assume that interaction of SP-D with a receptor complex may affect LPS signaling. Consistently, SP-D attenuates cell surface binding of Alexa-labeled LPS to TLR4/MD-2-expressing cells. We also examined the effect of SP-D on the binding of sMD-2 to LPS, because lipid A avidly binds to sMD-2 but not to sTLR4 (29). The results indicate that SP-D significantly decreases the sMD-2 binding to LPS. Taken together, these results support the conclusion that SP-D dampens LPS-induced inflammation by altering LPS-receptor interaction.

This study shows that SP-D binds *S. minnesota* Re595 LPS and *E. coli* Rc LPS, but not *E. coli* O26:B6 LPS. The experiments with anti-SP-D monoclonal antibodies indicate that the neck domain and the CRD are involved in the binding of SP-D to Re595 LPS and Rc LPS, respectively. A previous study (28) has shown by lectin blot analysis that SP-D interacts with Rc and Rd LPS, but not with Re LPS or smooth LPS. Although the differences between studies with Re LPS binding may be due to the methods used, this and our previous studies (20, 43) using microtiter well assays indicate that SP-A and SP-D exhibit significant binding to Re595 LPS, but not to O26:B6 LPS. Because SP-A inhibits inflammatory responses elicited by O26:B6 LPS but not by Re595 LPS (20), we tested whether SP-D modulates LPS signaling in a ligand-specific manner, as observed in SP-A. Unlike SP-A, SP-D inhibits inflammatory cell responses induced by rough LPS, an SP-D ligand, as well as smooth LPS, which is not a ligand for SP-D. The precise mechanism by which SP-D inhibits rough LPS-elicited signaling remains to be determined. The profiles of concentration-dependent inhibition by SP-D and the SP-A/SP-D chimera indicate that the difference between these molecules in the inhibitor activities on smooth LPS-induced responses appears small (see Fig. 9, A and D). In contrast, the difference of the inhibition of rough LPS-induced responses is more pronounced (see Fig. 9, B, C, E, and F). Because the chimera also binds to Re595 LPS and Rc LPS (data not shown) as well as SP-A and SP-D, the finding that

The inhibitory effects of A/D chimera and CRF on the LPS-induced cell responses. A–C, rat alveolar macrophages (10⁵ cells) were preincubated with the indicated concentrations of SP-D (C), A/D chimera (B), or CRF (A) for 30 min at 37 °C with 5% CO₂ and then stimulated with 100 ng/ml O26:B6 LPS (A), Rc LPS (B), or Re595 LPS (C) for 5 h. TNF-α secreted into the media was measured, and the results are expressed as percentages of LPS-induced TNF-α secretion in the absence of the protein. D–F, HEK293 cells transfected with TLR4 and MD-2 were incubated with the indicated concentration of SP-D (B), chimer (squiff), or CRF (A) for 1 h at 37 °C in an atmosphere of 5% CO₂ before adding LPS. The cells were stimulated with 100 ng/ml O26:B6 LPS (D), Rc LPS (E), or Re595 LPS (F) and further incubated for 5 h at 37 °C. LPS-induced NF-κB activation was determined as described under “Experimental Procedures.” The results are expressed as percentages of luciferase activity of LPS-induced NF-κB activation in the absence of the protein. The abscissa values are expressed as the concentration of monomeric collectin present in the reactions to normalize the differences in oligomerization among the recombinant proteins. The data shown are the means ± S.D. from three separate experiments.

It is difficult to determine the actual concentrations of pulmonary collectins in vivo, because alveolar macrophage (the epithelial lining fluid of the alveolus) cannot be directly measured. Nevertheless, their concentrations can be estimated based on the recovery of the proteins in the bronchoalveolar lavage fluids and the extrapolated hypophase volume (100–1000 μl/lung) (36, 37). The SP-D concentration in the alveolar hypophase can be calculated as ~63 μg/ml (38) when estimated from the average concentration (0.88 μg/ml) in the bronchoalveolar lavage fluids of human healthy volunteers (39). The calculated SP-A concentrations can be in the alveolar hypophase range from 180 μg/ml to 1.8 mg/ml (38, 40–42). Although the concentrations of pulmonary collectins in the hypophase under healthy and diseased states cannot be directly determined, the SP-D concentrations used in this study are within the best estimates of the physiological ranges.

FIGURE 9. The inhibitory effects of A/D chimera and CRF on the LPS-induced cell responses. A–C, rat alveolar macrophages (10⁵ cells) were preincubated with the indicated concentrations of SP-D (C), A/D chimera (B), or CRF (A) for 30 min at 37 °C with 5% CO₂ and then stimulated with 100 ng/ml O26:B6 LPS (A), Rc LPS (B), or Re595 LPS (C) for 5 h. TNF-α secreted into the media was measured, and the results are expressed as percentages of LPS-induced TNF-α secretion in the absence of the protein. D–F, HEK293 cells transfected with TLR4 and MD-2 were incubated with the indicated concentration of SP-D (B), chimer (squiff), or CRF (A) for 1 h at 37 °C in an atmosphere of 5% CO₂ before adding LPS. The cells were stimulated with 100 ng/ml O26:B6 LPS (D), Rc LPS (E), or Re595 LPS (F) and further incubated for 5 h at 37 °C. LPS-induced NF-κB activation was determined as described under “Experimental Procedures.” The results are expressed as percentages of luciferase activity of LPS-induced NF-κB activation in the absence of the protein. The abscissa values are expressed as the concentration of monomeric collectin present in the reactions to normalize the differences in oligomerization among the recombinant proteins. The data shown are the means ± S.D. from three separate experiments.
the inhibitory activity of the chimera on rough LPS-induced responses is less potent than that of SP-D may be a consequence of the orientation of the CRD and/or the length of the collagenous tail, when the proteins interact with rough LPS and an LPS receptor.

The recombinant SP-D prepared in this study is found to be highly oligomeric in solution by gel-filtration analysis and is organized as a cruciform dodecamer, or higher order multimers in electron micrographs. Disruption of interchain disulfide bond formation at the N terminus by Cys \( \rightarrow \) Ser mutations at Cys\(^{15}\) and Cys\(^{20}\) prevents the covalent oligomerization of trimeric subunits, resulting in loss of functions, such as inducing viral aggregation and enhancing interactions of influenza A virus with neutrophils (44). Expression of wild-type SP-D but not of SP-D\(^{C115S, C268S}\) in SP-D-null mice corrects the pulmonary phospholipid accumulation and emphysema phenotype of the SP-D\(^{−/−}\) mice (45). These studies indicate that the supratrimetric oligomerization of SP-D is required for native protein functions. In this study CRF is ineffective in modulating LPS signaling, although CRF possesses the neck plus the CRD, functions. In this study CRF is ineffective in modulating LPS-stimulated inflammatory responses.

In conclusion, SP-D can bind a complex of TLR4 and MD-2 in modulating LPS-stimulated inflammatory responses.

Acknowledgment—We thank Dr. Hitomi Sano (Dept. of Pediatrics, Sapporo City General Hospital) for helpful suggestions.

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