The purpose of the current study was to examine the binding of pulmonary surfactant protein A (SP-A) to TLR4 and MD-2, which are critical signaling receptors for lipopolysaccharides (LPSs). The direct binding of SP-A to the recombinant soluble form of extracellular TLR4 domain (sTLR4) and MD-2 was detected using solid-phase binding, immunoprecipitation, and Biacore. SP-A bound to sTLR4 and MD-2 in a Ca²⁺-dependent manner, and an anti-SP-A monoclonal antibody whose epitope lies in the region Thr¹⁸⁴–Gly¹⁹⁴ blocked the SP-A binding to sTLR4 and MD-2, indicating the involvement of the carbohydrate recognition domain (CRD) in the binding. SP-A avidly bound to the deglycosylated forms of sTLR4 and MD-2, suggesting a protein/protein interaction. In addition, SP-A attenuated cell surface binding of smooth LPS and smooth LPS-induced NF-κB activation in TLR4/MD-2-expressing cells. To know the role of oligomerization in the interaction of SP-A with TLR4 and MD-2, the collagenase-resistant fragment (CRF), which consisted of CRD plus neck domain of SP-A, was isolated. CRF assembled as a trimer, whereas SP-A assembled as a higher order oligomer. Although CRD was suggested to be involved in the binding, CRF exhibited approximately 600- and 155-fold higher KD for the binding to TLR4 and MD-2, respectively, when compared with SP-A. Consistently significantly higher molar concentrations of CRF were required to inhibit smooth LPS-induced NF-κB activation and tumor necrosis factor-α secretion. These results demonstrate for the first time the direct interaction between SP-A and TLR4/MD-2 and suggest the importance of supratrimeric oligomerization in the immunomodulatory function of SP-A.

In innate immune systems, toll-like receptors (TLRs)² are implicated in recognition and signaling of pathogen-associated molecular patterns (1). Stimulation of different TLRs induces distinct patterns of gene expression, which leads to the activation of innate immunity and instructs the development of antigen-specific acquired immunity (2). Among the TLR family, TLR4 plays a critical role in recognition and signaling of bacterial lipopolysaccharide (LPS) (3). TLR4 requires accessory protein MD-2 for an efficient response to LPS (4). We have recently demonstrated the direct interaction between MD-2 and extracellular TLR4 domain (5, 6). MD-2 binds LPS (7), but LPS has been demonstrated to be cross-linked with TLR4 and MD-2 only when coexpressed with CD14 (8), suggesting that LPS is in close proximity to the receptor complex.

The lung is constantly challenged by inhaled pathogens, pollutants, and particles that are present in the environment. Pulmonary surfactant, a mixture of lipids and proteins that serves to reduce the surface tension of the alveoli, is involved in the innate immune system of the lung. Recent studies demonstrate that the most abundant component of surfactant protein, surfactant protein A (SP-A), plays important roles in pathogen clearance and inflammatory responses (9–12). SP-A belongs to the collectin subgroup of the C-type lectin superfamily along with surfactant protein D (SP-D) and mannose-binding lectin. The primary structure of SP-A subunits are composed of a short amino-terminal segment, a collagen-like sequence characterized by Gly-Y repeats with an interruption near the mid-point of the domain, a neck domain, and a carbohydrate recognition domain (CRD) (13). Trimeric association occurs by the folding of collagenous domains into triple helices (14) and coiled-coil bundling of α-helices in the neck (15). Fully assembled SP-A is a bouquet-like octadecamer consisting of six trimmeric subunits that are stabilized by the amino-terminal sequences and disulfide bonds (16).

Recent studies from this and other laboratories have demonstrated that SP-A modulates inflammation by interacting with cell surface receptors including CD14 (17), TLR2 (18, 19), signal-inhibitory regulatory protein α, and calreticulin/CD91 (20). Although it has been suggested that SP-A activates cellular responses dependent on TLR4 (21), the interactions of SP-A with surfactant protein D (SP-D) and mannose-binding lectin. The primary structure of SP-A subunits are composed of a short amino-terminal segment, a collagen-like sequence characterized by Gly-Y repeats with an interruption near the mid-point of the domain, a neck domain, and a carbohydrate recognition domain (CRD) (13). Trimeric association occurs by the folding of collagenous domains into triple helices (14) and coiled-coil bundling of α-helices in the neck (15). Fully assembled SP-A is a bouquet-like octadecamer consisting of six trimmeric subunits that are stabilized by the amino-terminal sequences and disulfide bonds (16).

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SP-A Interacts with TLR4 and MD-2

with TLR4 or MD-2 have not been examined yet. The objectives of this study were to investigate the direct binding of SP-A to TLR4 and MD-2 and to clarify the structural requirement of SP-A for the interactions with TLR4/MD-2 and regulation of TLR4-dependent inflammatory responses. We show that SP-A binds to TLR4 and MD-2 via CRD and prevents LPS-induced signaling. The trimeric assembly consisting of CRD plus neck domain reveals specific but insufficient activities against TLR4 and MD-2, suggesting the importance of oligomerization for anti-inflammatory effects of SP-A.

EXPERIMENTAL PROCEDURES

Materials—The macrophage-like cell line U937 (JCRB9021) was obtained from the Health Science Research Resources Bank (Osaka, Japan) and maintained in RPMI 1640 medium with 10% heat-inactivated fetal calf serum (Invitrogen). Human embryonic kidney (HEK) 293 cells (CRL-1573) were obtained from American Type Culture Collection and grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. HEK 293 cells were transfected with plasmid DNA using the calcium phosphate precipitation method.

Experimental procedures—After transfection, cells were transferred to serum-free EX-VE10 medium (Invitrogen) for 3 days. The medium was collected, and four additional harvests were performed, allowing 24–48 h of culture between harvests. The medium was finally filtered with a 0.45-μm filter, and 50 μM CaCl₂ and 0.1% (v/v) Triton X-100 were added and incubated at 37 °C for 5 h. After washing the wells, the wells were incubated with anti-human SP-A polyclonal antibody followed by the incubation with HRP-labeled anti-rabbit IgG. The proteins that reacted with the antibodies were finally visualized by Coomassie Brilliant Blue staining. For native conditions, 15 μg of MD-2 were subjected to a native PAGE (Novex® 4–16% bis-Tris gel) and visualized by Coomassie Brilliant Blue staining.

Polyacrylamide Gel Electrophoresis—Four micrograms of recombinant MD-2 were resolved by 13% SDS-PAGE under non-reducing or reducing (1% β-mercaptoethanol) conditions and visualized by Coomassie Brilliant Blue staining. For native conditions, 15 μg of MD-2 were subjected to a NativePAGE™ gel and visualized by Coomassie Brilliant Blue staining.

Binding Assay in Solution—Endotoxin was removed from SP-A or CRF preparations using polymyxin B-agarose (Sigma) as described elsewhere (26). The endotoxin content in hSP-A or CRF preparations was below 2.7 pg/μg of protein as determined by Limulus amebocyte lysate assay.

TLR4 and MD-2—Expression and purification of a soluble form of recombinant extracellular domain of TLR4 (sTLR4) and recombinant MD-2 have been described recently (5). sTLR4 consists of the extracellular domain of Glu²⁴–Lys⁶³¹ and a His₉ tag at the carboxyl-terminal end. MD-2 contains the carboxyl-terminal fusion V5 tag and a His₉ tag. The proteins were expressed by a baculovirus-insect cell expression system and purified from the medium using a column of nickel-nitrotriacetic acid beads (Qiagen, Valencia, CA).

TLR4 peptide (TLR4E²⁴–K⁴⁷) and TLR2 peptide (TLR2E²¹–S⁴⁵) were purchased from Invitrogen. These peptides consist of amino-terminal regions of TLR4 or TLR2 and do not involve leucine-rich repeats. The sequence of TLR4E²⁴–K⁴⁷ and TLR2E²¹–S⁴⁵ is ESWEPCVEVPNITYQCMELNFYK and ESNQNQLSRGDNGICGSSGGLNS, respectively.

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visualized by using a chemiluminescence reagent (SuperSignal, Pierce).

**BIAcore Method**—BIAcore 3000 (BIAcore AB, Upptala, Sweden) was used to assess the interactions of hSP-A with sTLR4 or MD-2. Native hSP-A (50 μg/ml) in 10 mM sodium acetate (pH 4.0) was immobilized on a CM5 sensor chip using an amine coupling method. TLR4 or MD-2 in 5 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 5 mM CaCl2 was passed over the surface of the sensor chip, and the interactions were monitored for 2 min. The sensor surface was then washed with the same buffer to start the dissociation, and the chip was finally regenerated with 10 mM EDTA at the end of each experiment. The association rate constant (K_a) and the dissociation rate constant (K_d) were calculated according to the BIAevaluation software (Version 3.1, BIAcore AB) using a program named 1:1 (Langmuir) binding model. The dissociation constant (K_d) was determined by K_d/K_a.

**Ligand Blot Analysis**—For deglycosylation, sTLR4 and MD-2 (2 μg) were incubated with 1 unit of N-glycosidase F (Roche Diagnostics) at 37 °C for 2 h in 10 mM Tris buffer (pH 7.4) containing 10 mM EDTA, 2% (v/v) β-mercaptoethanol, 0.1% (w/v) SDS, and 1% (v/v) Nonidet P-40. The removal of oligosaccharides was confirmed by a carbohydrate detection kit (G. P. Sensor, J-Oil Mills, Yokohama, Japan) according to the manufacturer’s instructions.

For ligand blot analysis, sTLR4, MD-2, deglycosylated sTLR4, and deglycosylated MD-2 were electrophoresed under reducing conditions and transferred to polyvinylidene difluoride membrane. After nonspecific binding was blocked with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM CaCl2, 5 mg/ml BSA, and 1% (w/v) polyvinylpyrrolidone, the membranes were incubated with wtSP-A or BSA (2 μg/ml) for 17 h. The membranes were then incubated with anti-human SP-A polyclonal antibody followed by HRP-labeled anti-rabbit IgG. The binding of SP-A to sTLR4 or MD-2 was detected by using diaminobenzidine tetrahydrochloride as a substrate.

**Gel Filtration**—The molecular masses of hSP-A and CRF were determined by gel filtration chromatography. Analysis was performed using a 1.8 × 77-cm column of 8% agarose beads (Iberagar, Coina, Portugal) in 5 mM Tris buffer (pH 7.4) containing 2 mM CaCl2 at 4 °C. Elution was monitored at an absorbance of 280 nm. Blue dextran, BSA, and chymotrypsinogen A were used as molecular mass standards.

**LPS Binding to TLR4/MD-2**—HEK293 cells were plated at 1 × 10^5/well on 24-well plates on the day before transfection. The cells were transiently transfected by FuGENETM 6 transfection reagent (Roche Applied Science) with 50 ng of sTLR4 cDNA and 10 ng of MD-2 cDNA together with 30 ng of NF-kB reporter construct (pNF-kB-Luc, Stratagene) and 3.5 ng of a construct directing expression of Renilla luciferase (pRL-TK, Promega, Madison, WI). Forty-eight hours after transfection, the cells were stimulated with 100 ng/ml either smooth (O26:B6) or rough (Re595) LPS for 5 h in the absence or presence of hSP-A or CRF, which had been preincubated with the cells for 1 h before adding LPS to the wells. Luciferase activity was measured by dual luciferase reporter assay system (Promega) according to the manufacturer’s instructions.

**TNF-α Assay**—U937 cells (2.5 × 10^5/well) were placed on 24-well plates and induced to differentiate by incubation with 10 nM phorbol 12-myristate 13-acetate for 24 h. The cells were further incubated in the absence of phorbol 12-myristate 13-acetate for 24 h. After the cells were preincubated with the indicated concentrations of hSP-A or CRF for 1 h, the cells were stimulated with 10 ng/ml LPS for 5 h in RPMI 1640 medium containing 10% fetal calf serum. The culture medium was then collected, and TNF-α secretion was measured using a human TNF enzyme-linked immunosorbent assay set (BD Biosciences).

**Other Methods**—Polyclonal antibody against human SP-A was raised in rabbits against purified recombinant human SP-A. The biotinylation of hSP-A and CRF was performed as described previously (17). Significance was determined using two-tailed Student's t test.

**RESULTS**

Analysis of the State of Recombinant MD-2—MD-2 is a 20–30-kDa glycoprotein that contains a leader sequence but lacks a transmembrane domain. Although some of the MD-2 molecules remain attached to the extracellular domain of TLR4, the rest is secreted into the extracellular fluids as monomers and large disulfide-linked oligomers (27). To assess the state of recombinant MD-2 expressed by a baculovirus-insect
cell expression system, electrophoresis was performed under denaturing and non-denaturing conditions. As seen in Fig. 1A, recombinant MD-2 in the presence of SDS migrated mainly as monomers of 25 kDa under reducing and non-reducing conditions, although dimeric forms of an apparent molecular mass of 45 kDa were also detected in the unreduced state. When analyzed by non-denaturing native PAGE, recombinant MD-2 consisted of larger oligomers (band between 66 and 360 kDa) and species of ~59 and 28 kDa, corresponding to dimers and monomers (Fig. 1B). These results indicate that our preparation of MD-2 contained oligomers and monomers under the native conditions. Previous studies demonstrated larger oligomers of recombinant MD-2 resolved by SDS-PAGE under non-reducing conditions. Previous studies demonstrated larger oligomers of MD-2 for LPS signaling is still controversial (28, 29), but the inconsistency may be due to the different expression system. Although the relevance of large oligomers of MD-2 for LPS signaling is still controversial (28, 29), we have tested that our preparation actively confers LPS responsiveness to TLR4-transfected cells (5).

Binding of SP-A to TLR4 and MD-2—We first investigated direct interactions of SP-A with purified sTLR4 or MD-2 coated onto microtiter wells. In the presence of Ca$^{2+}$, wtSP-A clearly bound to solid-phase sTLR4 in a concentration-dependent manner (Fig. 2A), indicating that SP-A interacted with the extracellular domain of TLR4. Likewise when MD-2 was coated onto the wells, wtSP-A avidly bound to MD-2 in the presence of Ca$^{2+}$ (Fig. 2B). The interactions of SP-A with sTLR4 and MD-2 were also examined by a solution-phase assay. When wtSP-A and sTLR4 were coincubated, wtSP-A was associated with sTLR4 that was immunoprecipitated with anti-sTLR4 antibody (Fig. 2C, upper panel). Similarly wtSP-A was clearly coprecipitated with MD-2 (Fig. 2C, lower panel). The results demonstrate the direct binding of SP-A to sTLR4 and MD-2 in the solution phase. We further determined the binding parameters by surface plasmon resonance analysis (Fig. 3). When various concentrations of sTLR4 were overlaid to hSP-A immobilized on a sensor chip, an association rate constant of $K_a = 1.89 \times 10^4$ M$^{-1}$ s$^{-1}$ and a dissociation rate constant of $K_d = 3.7 \times 10^{-4}$ s$^{-1}$ were disclosed. The passage of MD-2 over immobilized hSP-A yielded an association rate constant of $K_a = 9.58 \times 10^3$ M$^{-1}$ s$^{-1}$ and a dissociation rate constant of $K_d = 3.13 \times 10^{-4}$ s$^{-1}$. The consequent dissociation constant of $K_d / K_a = 1.96 \times 10^{-8}$ M or $3.28 \times 10^{-8}$ M for wtSP-A binding to sTLR4 or MD-2, indicate the high affinity interactions of SP-A with sTLR4 and MD-2. Taking into account the presence of larger oligomers of MD-2 (Fig. 1B), the actual $K_d$ for SP-A/MD-2 interaction may be much lower than the calculated $K_d$, suggesting the higher binding affinity between the proteins.

Properties of SP-A Binding to TLR4 and MD-2—Because SP-A belongs to a C-type lectin family, it recognizes various ligands via CRD in a Ca$^{2+}$-dependent manner. For the purpose of clarifying the binding properties of SP-A with the pattern recognition receptors, we next examined the binding of SP-A to TLR4 and MD-2 in the presence of EDTA or excess mannose (Fig. 2, A and B). Inclusion of 5 mM EDTA instead of CaCl$_2$ in the binding buffer almost completely abolished the binding of wtSP-A to solid-phase sTLR4 or MD-2. On the other hand, the association of wtSP-A...
with sTLR4 or MD-2 was partially attenuated by the presence of 0.2 M mannose. As much as 0.2 M mannose induced a 50–60% reduction in the binding of wtSP-A to sTLR4 or MD-2. These results suggest that SP-A associates with sTLR4 and MD-2 in a Ca\textsuperscript{2+}/H\textsuperscript{11001}-dependent manner and raised a possibility that CRD is involved in the interactions of SP-A with these pattern recognition receptors.

To determine the role of SP-A CRD in the binding to TLR4 and MD-2, a solid-phase binding assay was performed in the presence of anti-SP-A monoclonal antibody, whose epitopes are located in SP-A CRD. When wtSP-A was preincubated with monoclonal antibody PE10 before adding to the wells, wtSP-A failed to recognize sTLR4 (Fig. 4A) or MD-2 (Fig. 4B) coated onto the wells. In contrast, control antibody 6B2 did not alter the binding ability of wtSP-A to either sTLR4 or MD-2. Because monoclonal antibody PE10 recognizes Thr\textsuperscript{184}–Gly\textsuperscript{194} regions of SP-A CRD (30), Thr\textsuperscript{184}–Gly\textsuperscript{194} of SP-A may be the critical region required for the binding of SP-A to sTLR4 and MD-2.

The replacement of Glu\textsuperscript{195} by Gln and Arg\textsuperscript{197} by Asp in rat SP-A results in altered carbohydrate binding specificity of the CRD of SP-A (31). We generated hSP-A mutant SP-A\textsubscript{E195Q,A197D} in which substitutions of Gln for Glu\textsuperscript{195} and Asp for Ala\textsuperscript{197} were introduced based upon the corresponding regions of rat SP-A. The binding of SP-A\textsubscript{E195Q,A197D} to sTLR4 or MD-2 was then examined, and it was found that SP-A\textsubscript{E195Q,A197D} avidly bound to sTLR4 (Fig. 4C) and MD-2 (Fig. 4D) to the same extent as wtSP-A. The results suggest that the carbohydrate binding specificity of SP-A may not be necessary for sTLR4 or MD-2 recognition. Taking all these results into account, we conclude that SP-A interacts with sTLR4 and MD-2 via CRD in a Ca\textsuperscript{2+}-dependent manner, but the lectin property of SP-A may not be involved in the recognition of sTLR4 and MD-2.

**Binding Properties of TLR4 and MD-2 to SP-A**—Since the lectin property of SP-A seemed to be unnecessary for binding to TLR4 and MD-2, we next investigated whether carbohydrate moieties linked with TLR4 or MD-2 were involved in SP-A binding. Both TLR4 and MD-2 possess N-linked carbohydrates (32). After sTLR4 and MD-2 were treated with N-glycosidase F, the molecular mass of sTLR4 was reduced from 80 to 70 kDa,
SP-A Interacts with TLR4 and MD-2

FIGURE 6. TLR4 synthetic peptide corresponding to the amino-terminal regions of TLR4 fails to compete with solid-phase sTLR4 for SP-A binding. Biotinylated hSP-A (1 μg/ml) was preincubated in the absence or presence of various concentrations of TLR2 peptide (△), TLR4 peptide (●), or sTLR4 (□) at 37 °C for 1 h. The binding of the preincubated hSP-A to sTLR4 coated onto microtiter wells was examined as described under “Experimental Procedures.” The results are expressed as percentages of hSP-A binding to solid-phase sTLR4 in the absence of competitors. The data shown are the means ± S.E. of three experiments. *, p < 0.02 when compared with the binding without competitors.

and the migration of MD-2 was altered from 25 to ~22 kDa (Fig. 5, Coomassie stain). Since no glycans could be detected with the deglycosylated proteins, the carbohydrates were considered to have been effectively removed by the enzyme treatment (Fig. 5, Glycoanalysis). Ligand blot analysis was performed to examine the binding of wtSP-A to sTLR4 or MD-2, and it was clearly demonstrated that wtSP-A interacted with both glycosylated and deglycosylated forms of sTLR4 and MD-2 (Fig. 5, Ligand binding). Furthermore, SP-A bindings appeared to be stronger against deglycosylated sTLR4 and MD-2 than the glycosylated proteins. Therefore, we conclude that SP-A directly interacted with the peptide portions of sTLR4 and MD-2. These results are quite consistent with the results indicating that carbohydrate binding specificity is not required for SP-A to bind to sTLR4 and MD-2.

The extracellular domain of TLR4 contains the amino-terminal region (Met1–Phe54) and leucine-rich repeat motifs (Ser55–Phe567). The amino-terminal Glu54–Pro54 region of TLR4 is critical for interaction with MD-2 (6). We therefore investigated whether the amino-terminal region of TLR4 was involved in the binding to SP-A. The binding of hSP-A to solid-phase sTLR4 was examined in the absence or presence of sTLR4, TLR4 synthetic peptide (TLR4E24–K47), or TLR2 synthetic peptide (TLR2E21–S45). These TLR peptides correspond to the amino-terminal sequences of TLR4 or TLR2. Although sTLR4 in binding buffer apparently competed with solid-phase sTLR4 for SP-A binding, neither sTLR4 peptide nor TLR2 peptide reduced the binding between SP-A and sTLR4 (Fig. 6). We next examined the binding of MD-2 to solid-phase sTLR4 (250 ng/well) that had been preincubated in the absence or presence of SP-A (10–40 μg/ml). The results demonstrated that the MD-2 binding to solid-phase sTLR4 was not significantly altered by SP-A (data not shown). These results suggest that sTLR4 may associate with MD-2 independently of SP-A binding.

Effects of SP-A on LPS Binding to TLR4/MD-2-expressing Cells—We next examined whether SP-A alters the cell surface binding of Alexa-conjugated LPS on HEK293 cells stably expressing TLR4 and MD-2. When the cells were incubated at 4 °C with the labeled smooth LPS (Fig. 7A) or rough LPS (Fig. 7B), significant LPS binding was observed on the cell surface (Fig. 7, black line). After the cells were preincubated with hSP-A (10 μg/ml), the binding of the labeled smooth LPS on the cell surface was significantly attenuated, whereas the binding of rough LPS to the cells was not altered (Fig. 7, gray line). Inclusion of anti-hSP-A antibody during the preincubation of the cells with hSP-A restored the smooth LPS binding to the cell surface (data not shown), indicating the specific inhibitory effect of hSP-A on the interaction between smooth LPS and the cell surface. These results suggested that hSP-A inhibited the binding of smooth LPS to the cell surface receptor complex of TLR4 and MD-2. Because SP-A can bind to rough LPS but not to smooth LPS (33), it is considered that TLR4/MD-2 complex failed to recognize smooth LPS after SP-A had bound to TLR4/MD-2. The results are consistent with the previous studies demonstrating that SP-A inhibited the binding of TLR2 to peptidoglycan or zymosan, which are non-ligands of SP-A (18, 19). In contrast, the SP-A ligand, rough LPS, may associate with SP-A on TLR4/MD-2 complex, resulting in the sufficient binding to the cell surface in the presence of SP-A.

Effects of SP-A on LPS-induced NF-κB Activation—The next question was whether SP-A modulated LPS-induced signaling as a consequence of interacting with TLR4 and MD-2. We therefore investigated the effects of hSP-A on LPS-induced NF-κB activation in TLR4/MD-2-transfected HEK293 cells. As shown in Fig. 8, both smooth and rough LPS (100 ng/ml) induced profound luciferase activities mediated by TLR4 and MD-2. When the transfected HEK293 cells were preincubated with hSP-A (10 μg/ml) for 1 h, smooth LPS-induced NF-κB activation was significantly attenuated. In contrast, hSP-A failed to reduce rough LPS-induced NF-κB activation. The
results correlated well to the findings indicating that SP-A reduced the binding of smooth LPS but not rough LPS to TLR4/MD-2-expressing cells (Fig. 7). Furthermore, these results were consistent with a previous study indicating that SP-A inhibited smooth but not rough LPS-induced TNF-α secretion in rat alveolar macrophages and macrophage cell line U937 (33).

Assembly of CRF—Although the CRD of SP-A was suggested to involve the critical regions for TLR4 and MD-2 binding, the role of the oligomeric structure of SP-A remained to be clarified. We therefore sought to examine whether CRD plus neck domain was sufficient for TLR4 and MD-2 binding. Human SP-A was digested with collagenase, and collagenase-resistant fragment was isolated by gel filtration. The amino-terminal sequence of the isolated CRF as determined by an Applied Biosystems (Foster City, CA) protein sequencer was Gly-Leu-Pro-Ala-His, which corresponded to the sequence of hSP-A beginning at Gly78. The results indicated that the collagenous region of hSP-A was completely removed from the SP-A molecule.

When gel filtration chromatography was performed under 5 mM Tris buffer (pH 7.4) containing 2 mM CaCl2, hSP-A was eluted with an apparent molecular mass greater than 1.5 MDa (Fig. 9A), and CRF was recovered at an apparent molecular mass of 60 kDa (Fig. 9B). Electrophoretic analysis revealed that CRF migrated as a monomer of ~20 kDa in the presence of SDS (Fig. 9C). These results suggested that CRF assembled as trimers under a non-denaturing condition. Our observation seemed to be consistent with a previous report demonstrating that CRF was eluted as a trimer under a low ionic condition but was partially dissociated as a monomer in 0.5 M NaCl (34). The trimeric assembly of CRF was also consistent with the studies using collagen-depleted mutant of SP-A in which the neck domain plus CRD was suggested to be sufficient for trimeric assembly, whereas the amino-terminal segment was required for the association of trimeric subunits into higher oligomers (35).

Binding Activities of SP-A and CRF to TLR4 and MD-2—We then investigated whether CRF could interact with sTLR4 and MD-2. Biotinylated CRF revealed binding to sTLR4 (Fig. 10A) and MD-2 (Fig. 10B) coated onto microtiter wells, and these bindings were significant because inclusion of 5 mM EDTA instead of CaCl2 abolished the bindings (the binding of 40 μg/ml CRF to sTLR4 or MD-2 in the presence of EDTA versus CaCl2 was 6.0 ± 4.0 versus 60.2 ± 3.8 ng or 4.2 ± 1.2 versus 26.0 ± 2.6 ng, respectively; p < 0.02). However, these binding activities were significantly weaker than those of biotinylated hSP-A. The apparent Kd in each binding was calculated by the Scatchard plot analysis, assuming the molecular mass of hSP-A to be 1.5 MDa and that of CRF to be 60 kDa as observed by gel filtration analysis (Fig. 9). The estimated Kd in sTLR4 binding was 2.89 nM or 1.77 μM for hSP-A or CRF, respectively. The binding of hSP-A or CRF to MD-2 had an apparent Kd of 5.26 nM or 0.82 μM, respectively. These data indicated that the trimeric CRF exhibited significantly lower binding affinity to sTLR4 and MD-2, although CRD was suggested to be a critical region for TLR4 and MD-2 binding (Fig. 4, A and B). From these results, it is suggested that the amino-terminal regions containing the collagenous domain of SP-A play an important role in recognition of sTLR4 and MD-2.

Effects of SP-A and CRF on LPS-induced Cellular Response—Because CRF exhibited significantly lower binding affinities for sTLR4 and MD-2, the effect of CRF on LPS-induced cellular response was compared with that of hSP-A. HEK293 cells were transfected with TLR4 and MD-2 and preincubated with various concentrations of hSP-A or CRF for 1 h before stimulating with smooth LPS (10 ng/ml) for 5 h. When the NF-κB activation was determined (Fig. 11A), it was found that although CRF, like hSP-A, showed the inhibitory activity specifically on smooth LPS-induced NF-κB activation, significantly
higher molar concentrations of CRF were required to induce the sufficient reduction compared with hSP-A. The IC\textsubscript{50} of hSP-A required to inhibit smooth LPS (10 ng/ml)-induced NF-κB activation was 4.9 nM, whereas that of CRF was ~37 nM. The results clearly suggested that CRF was not as effective as hSP-A in modulating LPS-stimulated intracellular signaling. Cellular experiments and also in vitro sTLR4 or MD-2 binding experiments were performed in the presence of 2–5 mM CaCl\textsubscript{2}. Therefore, these results are consistent with the data indicating that the binding affinities of CRF for sTLR4 and MD-2 were significantly lower than those of hSP-A.

We finally compared the effects of hSP-A and CRF on LPS-induced TNF-α secretion in differentiated U937 cells. The cells were induced to be differentiated by phorbol 12-myristate 13-acetate, and the indicated concentrations of hSP-A or CRF were added to the cells 1 h prior to smooth LPS stimulation (10 ng/ml). After a 5-h incubation, the amounts of TNF-α in culture media were determined. As shown in Fig. 11A, as little as 0.5 nM hSP-A induced a 55% reduction in TNF-α secretion stimulated by 10 ng/ml smooth LPS, and TNF-α levels were inhibited by ~80% in the presence of 2 nM hSP-A. On the other hand, as much as 12.5 nM CRF was necessary to inhibit the smooth LPS-induced TNF-α secretion by only 24%. Furthermore, only a 33% reduction in TNF-α secretion was observed even after the amount of CRF was increased to 50 nM (data not shown). Compared with hSP-A, the immunomodulatory activity of CRF was therefore considered to be insufficient.

**DISCUSSION**

In addition to the interactions of SP-A with CD14 (33) or TLR2 (18), the current study demonstrated for the first time the direct binding of SP-A to TLR4 and MD-2. TLR2, TLR4, and CD14 contain 19, 24, and 10 leucine-rich motifs, respectively. The structure of these motifs comprises a β-strand and an α-helix connected by loops, which appear to be involved in protein/protein interaction (36). The extracellular domain of TLR4 consists of the amino-terminal region Glu\textsuperscript{24}–Phe\textsuperscript{54} and leucine-rich repeats containing the region Ser\textsuperscript{55}–Phe\textsuperscript{567}. The current study indicates that the TLR4 peptide corresponding to the amino-terminal Glu\textsuperscript{24}–Lys\textsuperscript{57} of TLR4 failed to compete with sTLR4 for SP-A binding. Likewise the TLR2 peptide corresponding to the amino-terminal Glu\textsuperscript{21}–Ser\textsuperscript{45} of TLR2 did not compete with sTLR2 for SP-A binding (data not shown). From these results, we speculate that SP-A may be able to interact with leucine-rich motifs of various pattern recognition receptors.

We have demonstrated previously that the direct interaction of SP-A with TLR2 inhibits the binding of peptidoglycan to TLR2 and attenuates peptidoglycan-induced signaling (18). We consider that SP-A competes TLR2 binding with peptidoglycan, which interacts with the leucine-rich repeats of TLR2 (37). Likewise SP-A prevented the interaction between zymosan and TLR2 and inhibited zymosan-induced cellular response mediated by TLR2 (19). Consistently it is considered that the direct binding of SP-A to MD-2 competes with the interaction of smooth LPS with MD-2. Because peptidoglycan and zymosan as well as smooth LPS are non-ligands of SP-A, the presence of SP-A on pattern recognition receptors interferes with the receptor recognition of these ligands. In contrast, SP-A did not inhibit the binding of rough LPS to TLR4- and MD-2 complex and failed to attenuate rough LPS-induced signaling mediated by TLR4/MD-2. Because rough LPS is a ligand of SP-A, we consider that rough LPS must be able to associate with SP-A, which had bound to TLR4- and MD-2 complex, and may be recognized to induce a TLR4/MD-2-dependent cellular response.

From these interpretations, it is considered that the physiological relevance of the interaction of SP-A with MD-2 is to alter the direct interaction of MD-2 with its ligands. This idea seems to be consistent with the effects of SP-A on TLR2. However, the effect of the binding of SP-A to TLR4 may be controversial because TLR4 by itself does not interact with LPS in the absence of MD-2, and thus the binding of SP-A to MD-2 alone but not to TLR4 may be sufficient in attenuating LPS-induced signaling. However, we have recently observed that the amounts of lipid A binding to MD-2 increased when co-incubation...
bated with sTLR4, indicating the important role of sTLR4 in ligand recognition by TLR4-MD-2 complex. Although further experiments are required, we speculate that the physiological meaning of the direct binding of SP-A to TLR4 may be to modulate the subsequent structural changes of TLR4 initiated by LPS binding to MD-2. For instance, the binding of SP-A to TLR4 might alter the cell surface clustering of TLR4 into receptosomes triggered by LPS binding to MD-2 (38).

The current study suggested that SP-A interacted with TLR4 and MD-2 via CRD, but the collagenase-resistant fragment of SP-A, which formed noncovalent trimers composed of CRD and the neck domain, did not act as effectively as highly oligomerized SP-A. Significantly higher molar concentrations of SP-A were required for interacting with sTLR4 and MD-2 and to alter LPS-induced cellular responses. These results indicate that the collagen-like domain and/or amino terminus are required for sufficient interactions between pattern recognition receptors and SP-A, although CRD is a critical binding domain. The results highlight the importance of a supratrimeric assembly of SP-A in the immunomodulatory effects by SP-A. A recent study from another laboratory demonstrated that the trimeric human SP-A mutant SP-A1ΔAVC,C6S inhibited LPS-induced TNF-α production by macrophage-like U937 cells (39). Their mutant SP-A1ΔAVC,C6S lacked the amino-terminal signal peptides and had substitution of Ser for Cysε but possessed the intact collagen-like domain of SP-A. In their studies, molar concentrations of SP-A1ΔAVC,C6S higher than that of wild type SP-A1 seemed to be required to inhibit the cellular response. Taking these results together with ours, it is considered that the collagenous domain plays an important role in SP-A functions, and the degree of oligomerization contributes to the immunomodulatory property of SP-A.

Although various interactions of lung collectins with their ligands including lipids, microorganisms, and cellular receptors depend on CRD, the roles of the collagen-like domain and amino-terminal domain have also been emphasized. SP-A aggregates dipalmitoylphosphatidylcholine-containing phospholipid vesicles by interacting with dipalmitoylphosphatidylcholine via CRD and inhibits surfactant secretion from alveolar type II cells (40, 41). However, the amino-terminal domain is required for phospholipid aggregation (35, 39), and both amino terminus and collagen-like domain are required to inhibit surfactant secretion (35, 42). The importance of the collagen-like domain of SP-A in animal models has also been described clearly (43). Pulmonary surfactant isolated from SP-A knock-out mice was deficient in the surfactant aggregate tubular myelin and had surface tension lowering activity that was easily inhibited by serum proteins (44, 45). These abnormalities were restored by introducing wild type SP-A but not by SP-A containing a deletion of collagen-like domain (ΔAGB–P80) (43). Furthermore, the amino terminus of SP-D could replace the amino terminus of SP-A for tubular myelin formation but not for the surface tension lowering properties of SP-A, suggesting the important role of the amino-terminal domain of SP-A in its in vivo functions (46). In the case of SP-D, two amino-terminal cysteine residues at positions 15 and 20 are critical for stabilizing the dodecameric structure, and the SP-D mutant (RrSP-Dser15/20) in which these cysteine residues were mutated was assembled as trimers (47). Although RrSP-Dser15/20 bound to the hemagglutinin of influenza A, it did not induce viral aggregation and failed to enhance interactions between influenza A and neutrophils, which were mediated by wild type SP-D (47). In addition, expression of RrSP-Dser15/20 in SP-D knock-out mice failed to correct the pulmonary phospholipid accumulation, emphysema, and foamy macrophages characteristic of SP-D-null mice (48). Taking all these results into account, supratrimeric oligomerization is considered to be essential in various in vivo and in vitro activities of pulmonary collectins.

Another collectin, mannosese-binding lectin, formed a bouquet-like oligomer like SP-A. Recently it has been demonstrated that human mannose-binding lectin mutations associated with increased risk of infection compromise higher oligomerization, resulting in reduced ligand binding capacity and impaired capability to activate complements (49). Another study also suggested that the oligomerization state of mannosese-binding lectin present in human serum had a direct effect on its carbohydrate binding properties (50). Although human mutations in lung collectins associated with increased risk of infection have not been identified yet, it seems plausible that some mutations in lung collectins compromise oligomerization and induce susceptibility to lung infection. It is therefore considered important to clarify the role of the state of oligomerization in host defense functions of lung collectins.

It has been demonstrated that the interactions of SP-A with CD14 or TLR2 result in regulation of microbial component-induced cellular responses (17–19, 33). We have proposed that SP-A prevents the association of microbial components with CD14 or TLR2 by its direct binding to these receptors. Although CD14 enhances LPS-induced cellular response, LPS can transmit its signal in a TLR4/MD-2-dependent manner in the absence of CD14 (7). The present results suggest that the interaction between SP-A and TLR4/MD-2 complex may also be involved in the mechanism by which SP-A regulates LPS-induced inflammation. Interestingly Alcorn and Wright (51) recently showed that SP-A could attenuate smooth LPS-induced TNF-α production by CD14-null mouse alveolar macrophages. They also reported that SP-A inhibited phorbol 12-myristate 13-acetate-induced TNF-α secretion in the absence of functional TLR4 (C3H/HeJ macrophages). These results suggest the possibility of CD14- or TLR4-independent regulation of the cellular response by SP-A. Other mechanisms of SP-A-mediated down-regulation of inflammation have also been suggested elsewhere. Stamme et al. (52) reported that SP-A prevented binding between LPS and LPS-binding protein, whereas others recently reported that SP-A attenuated the binding of LPS to CD14 but not to LPS-binding protein (53). In contrast to these studies suggesting the down-regulatory functions of SP-A in microbial stimulation, Guillot et al. (21) described that SP-A by itself activated inflammatory cytokine secretion. We cannot definitively interpret these discrepancies, but their SP-A preparations appeared to contain high amounts of endotoxin (140 pg/μg), which might have affected some experimental results.
Finally we believe that SP-A modulates LPS-induced cellular responses by various mechanisms involving interactions with pattern recognition molecules including CD14, TLRs, MD-2, and LPS-binding protein.

In conclusion, SP-A directly binds to TLR4 and MD-2. SP-A modulates LPS binding to a surface complex of TLR4 and MD-2 and LPS-binding protein.

Finally we believe that SP-A modulates LPS-induced cellular responses. We suggest the trimeric form consisting of CRD plus neck domain is not sufficient as an immunomodulatory molecule. We suggest the important role of supratricular oligomerization in the host defense function of SP-A.

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