Human Pulmonary Surfactant Protein D Binds the Extracellular Domains of Toll-like Receptors 2 and 4 through the Carbohydrate Recognition Domain by a Mechanism Different from Its Binding to Phosphatidylinositol and Lipopolysaccharide†

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ABSTRACT: Pulmonary surfactant protein D (SP-D), a member of the collectin group of innate immune proteins, plays important roles in lipopolysaccharide (LPS) recognition. We have previously shown that surfactant protein A (SP-A), a homologous collectin, interacts with Toll-like receptor (TLR) 2, resulting in alteration of TLR2-mediated signaling. In this study, we found that natural and recombinant SP-Ds exhibited specific binding to the extracellular domains of soluble forms of recombinant TLR2 (sTLR2) and TLR4 (sTLR4). Binding was concentration- and Ca^{2+}-dependent, and SP-D bound to *N*-glycosidase F-treated sTLRs on ligand blots. Anti-SP-D monoclonal antibody 7A10 blocked binding of SP-D to sTLR2 and sTLR4, but there was no inhibitory effect of monoclonal 7C6. Epitope mapping with recombinant proteins consisting of the carbohydrate recognition domain (CRD) and the neck domain plus CRD (NCRD) localized binding sites for 7A10 and 7C6 to sequential epitopes associated with the CRD and the neck domain, respectively. Interactions with 7A10 but not 7C6 were blocked by prior binding of the NCRD to sTLRs. Although antibody 7A10 significantly inhibited the binding of SP-D to its major surfactant-associated ligand, phosphatidylinositol (PI), and *Escherichia coli* Rc LPS, 7C6 enhanced binding to both molecules. An $SP\text{-}D^{E321Q,\ N323D}$ mutant with altered carbohydrate specificity exhibited attenuated PI binding but showed an increased level of binding to sTLRs. Thus, human SP-D binds the extracellular domains of TLR2 and TLR4 through its CRD by a mechanism different from its binding to PI and LPS.

Pulmonary surfactant protein D (SP-D)[1] plays important roles in the innate immunity of the lung against microorganisms (*1*, *2*). This protein is a member of the collectin subgroup of the C-type lectin superfamily along with surfactant protein A (SP-A) and mannose-binding lectin (*3*, *4*). The structure of the collectin is characterized by four distinct domains: (1) an amino terminus involved in interchain disulfide formation, (2) a collagen-like domain, (3) a neck domain, and (4) a carbohydrate recognition domain (CRD) (*5*). Electron microscopic observation reveals that SP-D exists as a dodecamer, which is assembled into a cruciform structure composed of four trimers (*6*).

SP-D binds phosphatidylinositol (PI), a component of surfactant (*7*, *8*), and the CRD mediates this interaction (*9*−*11*). SP-D also exhibits lectin-dependent binding to microorganisms and to cell wall components, including bacterial lipopolysaccharide (LPS) (*12*, *13*). We have previously shown that rat SP-A and SP-D bind to CD14 but through different mechanisms (*14*, *15*). Rat SP-A recognizes the peptide portion of CD14 through the neck domain, while rat SP-D recognizes the carbohydrate moieties of CD14 through the CRD. We have also shown that SP-A modulates peptidoglycan- and zymosan-induced cell responses by interaction with Toll-like receptor (TLR) 2 (*16*, *17*). Human SP-A binds to TLR2 through the CRD (*16*). Extrapolating from these observations, we find it is probable that SP-D, an SP-A homologue, interacts with TLRs.

TLRs play central roles in the innate immune system, protecting against invasion of microorganisms and stimulat-

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[1] Abbreviations: SP-D, surfactant protein D; SP-A, surfactant protein A; CRD, carbohydrate recognition domain; NCRD, recombinant protein consisting of the neck domain plus CRD; wt, wild type; mAb, monoclonal antibody; TLR, Toll-like receptor; sTLR, soluble form of the recombinant extracellular TLR domain; HRP, horseradish peroxidase; PI, phosphatidylinositol; LPS, lipopolysaccharide; SE, standard error.

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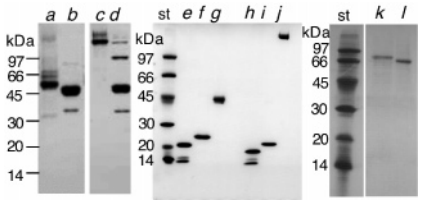

FIGURE 1: Electrophoretic analysis of native and recombinant human SP-Ds, CRD, NCRD, sTLR2, and sTLR4. Natural human SP-D (lanes a and c), recombinant human SP-D expressed in insect cells (lanes b and d), the carbohydrate recognition domain (CRD) (lanes e and h), the neck plus carbohydrate recognition domain (NCRD) (lanes f and i), recombinant human SP-D expressed in CHO-K1 cells (lanes g and j), sTLR4 (lane k), and sTLR2 (lane l) were subjected to SDS−polyacrylamide gel electrophoresis under reducing (lanes a, b, e−g, k, and l) and nonreducing (lanes c, d, and h−j) conditions. The proteins were visualized by Coomassie Brilliant Blue staining.

ing the clonal responses of adaptive immunity (*18*). TLR2 and TLR4 have been shown to function as sensors to elicit signaling of pathogen-associated molecular patterns, including LPS, peptidoglycan, and zymosan (*19*−*21*). We have constructed soluble forms of recombinant TLR2 and TLR4 proteins (sTLR2 and sTLR4, respectively) consisting of the putative extracellular domains and have shown that sTLR2 directly binds to peptidoglycan and zymosan and that sTLR4 is capable of interacting with MD-2 (*17*, *22*, *23*).

The purpose of this study was to determine (1) whether human SP-D, a homologous collectin, binds to TLR2 and TLR4, (2) the structural determinants that participate in SP-D−TLR recognition, and (3) the differences in the recognition of other ligands, including PI and LPS. This study provides evidence that human SP-D binds to the extracellular domains of TLR2 and TLR4 by a mechanism different from its binding to PI and LPS.

EXPERIMENTAL PROCEDURES

SP-D. Natural human SP-D derived from bronchoalveolar lavage fluids of patients with alveolar proteinosis was purified by affinity chromatography on mannose-Sepharose, as previously described for the purification of rat SP-D (*24*). The 1.181 kb cDNA for human SP-D was inserted into the pEE14 vector, and recombinant SP-D was expressed in CHO-K1 cells using the glutamine synthetase amplification system (*25*). Recombinant human SP-D was purified from serum-free media with the mannose-Sepharose column, as previously described for recombinant rat SP-D (*10*). The cDNA for human SP-D was also inserted into the pVL1392 plasmid vector and was expressed in the baculovirus−insect cell system using the methods described by O'Reilly et al. (*26*). The recombinant viruses were used to infect cell monolayers of *Tni* cells in serum-free medium at a multiplicity of infection of 1−5, and recombinant human SP-D was purified with a column of mannose-Sepharose as described previously (*11*).

Natural and recombinant human SP-Ds were analyzed by SDS−polyacrylamide gel electrophoresis. The major forms of natural SP-D (Figure 1, lanes a and c) and recombinant SP-D (Figure 1, lanes g and j) expressed in CHO-K1 cells migrated at 45−50 kDa under reducing and denaturing conditions and migrated at approximately ~120 kDa under nonreducing and denaturing conditions. The major form of recombinant SP-D expressed in insect cells appeared at 40−45 kDa under reducing conditions (Figure 1, lane b) and exhibited various degrees of oligomers under nonreducing conditions (Figure 1, lane d). This difference in molecular masses can be attributed to differences in glycosylation between mammalian and insect cells (*27*). The difference could also reflect some combination of underhydroxylation of proline and lysine and a decreased level of hydroxyllysine glycosides. Most collagenous proteins are undermodified because of low levels of prolyl and lysyl hydroxylase in the insect cells and show more rapid mobility on SDS−PAGE. These SP-Ds were purified with affinity columns of mannose-Sepharose, indicating that the recombinant SP-Ds expressed in mammalian and insect cells retain carbohydrate binding activity.

We also expressed, in the baculovirus−insect cell system, a mutant protein of human SP-D with $Glu^{321} \rightarrow Gln$ and $Asn^{323} \rightarrow Asp$ substitutions (SP-$D^{E321Q,N323D}$) whose cDNA was constructed by a site-directed mutagenesis kit (QuickChange mutagenesis kit, Stratagene, Cedar Creek, TX). For isolation of SP-$D^{E321Q, N323D}$, which failed to bind to mannose-Sepharose, the medium containing expressed protein was directly applied to a protein G-Sepharose affinity column covalently coupled with polyclonal anti-human SP-D IgG (*28*).

Recombinant Fusion Proteins Containing the Neck plus CRD or CRD. A recombinant trimeric protein consisting of the human neck domain plus CRD (NCRD) was expressed in a bacterial expression system, purified, and characterized as previously described (*29*). Endotoxin concentrations were measured with a chromogenic assay and ranged from 0.1 to 3 pg/μg of protein.

Limited modifications of these procedures were used to generate a protein consisting of the human CRD without the contiguous neck domain. Briefly, the human CRD sequence, as defined by the normal exon boundries, was cloned into the BamHI and HindIII sites in pET-30a+, to encode a fusion protein with an N-terminal His tag. The fusion protein was expressed in RosettaBlue competent cells and recovered as a soluble protein from the CelLytic B lysate. Following centrifugation, the supernatant was dialyzed versus 50 mM Tris buffer (pH 7.5) containing 500 mM NaCl, 10% (w/v) imidazole, and 0.05% (v/v) Emulphogene, and the fusion protein was purified by sequential nickel chelation and gel filtration chromatography (*29*). The protein migrated with the predicted size and showed a decrease in mobility following sulfhydryl reduction, consistent with the formation of normal intrachain disulfide bonds (data not shown). Endotoxin levels were approximately 37 pg/μg of protein.

Recombinant SP-D proteins consisting of the NCRD and the CRD exhibited apparent molecular masses of 23 and 19 kDa, respectively, under reducing and nonreducing conditions (Figure 1, lanes e, f, h, and i), as described previously (*29*). The NCRD forms a trimer under nondenaturing conditions (*29*).

sTLR2 and sTLR4. A soluble form of recombinant TLR2 (sTLR2) consisting of the putative extracellular domain (Met^1−Arg^{587}) and a six-His tag at the C-terminal end and a soluble form of recombinant TLR4 (sTLR4) consisting of the putative extracellular domain (Met^1−Lys^{631}) and a six-His tag at the C-terminal end were expressed by the baculovirus−insect cell system, and recombinant proteins

were purified with a column of nickel−nitrilotriacetic acid beads as described previously (*22*, *23*). The purified proteins of sTLR4 and sTLR2 exhibited apparent molecular masses of 80 and 70 kDa (Figure 1, lanes e and f), respectively, under reducing conditions, as described previously (*22*, *23*).

Anti-SP-D Monoclonal Antibodies. Hybridomas producing anti-SP-D monoclonal antibodies (mAbs) were established, and mAbs were prepared as described previously (*30*).

Biotinylation of SP-D. Biotinylation of native and recombinant human SP-Ds was performed using sulfo-NHS-biotin (Pierce) according to the manufacturer's instructions.

Binding of Biotinylated SP-D to sTLR2, sTLR4, PI, and Rc LPS. sTLR2 or sTLR4 (250 ng/well) was coated onto microtiter wells. PI (4 μg/well, Sigma) or Rc LPS from *E. coli* (5 μg/well, Sigma) in 20 μL of ethanol was also coated onto the microtiter wells as described previously (*7*, *14*). Nonspecific binding was blocked with 10 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA for the binding to sTLR2 and sTLR4. For the binding to PI and Rc LPS, 5 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA was used. The indicated concentrations of biotinylated proteins of natural or recombinant human SP-Ds were then incubated with the solid-phase ligands at room temperature for 5 h or at 37 °C for 3 h. The wells were washed with PBS containing 0.1% (v/v) Triton X-100 and 3% (w/v) skim milk and were further incubated with HRP-conjugated streptavidin (1000-fold dilution, Vector Laboratories) at 37 °C for 30 min. After the wells were washed, the peroxidase reaction was performed using *o*-phenylenediamine as a substrate, and the binding of SP-D to the solid-phase ligand was assessed by measuring the absorbance at 492 nm. In some experiments, biotinylated SP-D (1 or 5 μg/mL) was preincubated at 37 °C for 1 h with mAb (20 or 50 μg/mL) before reacting with the solid-phase ligands.

Deglycosylation of sTLR2 and sTLR4. sTLR2 or sTLR4 (2 μg) was incubated with 1 unit of *N*-glycosidase F (Roche Diagnostic GmbH, Mannheim, Germany) at 37 °C for 2 h in 10 mM Tris buffer (pH 7.4) containing 10 mM EDTA, 2% (v/v) β-mercaptoethanol, 1% (v/v) Nonidet P-40, and 0.1% (w/v) SDS. 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.

Ligand Blot. sTLR2, sTLR4, and their deglycosylated forms (2 μg/lane) were resolved by SDS−PAGE under reducing conditions and transferred to a PVDF membrane. After nonspecific binding was blocked with 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, 0.5% (w/v) BSA, and 1% (w/v) polyvinylpyrolidone, the membrane was incubated with recombinant SP-D (1 μg/mL) expressed in CHO cells or BSA at room temperature overnight. It was further incubated with anti-human SP-D polyclonal antibody, followed by incubation with HRP-labeled anti-rabbit IgG. The binding of SP-D to sTLR2 or sTLR4 on the membrane was detected by using diaminobenzidine tetrahydrochloride as a substrate.

Binding of NCRD to sTLR2 and sTLR4. NCRD (2 μg/mL, 50 μL/well) was incubated with sTLR2 (250 ng/well) or sTLR4 coated onto microtiter wells at room temperature for 5 h. Nonspecific binding was blocked with 10 mM Hepes buffer (pH 7.4) containing 0.15 M NaCl, 5 mM $CaCl_2$, and 5 mg/mL BSA. The wells were then incubated with 10 μg/mL antibody 7C6, 7A10, or control mAb at room temperature for 1 h, followed by an incubation with HRP-labeled anti-mouse IgG. The binding of NCRD to the solid-phase ligand was finally detected by measuring the absorbance at 492 nm.

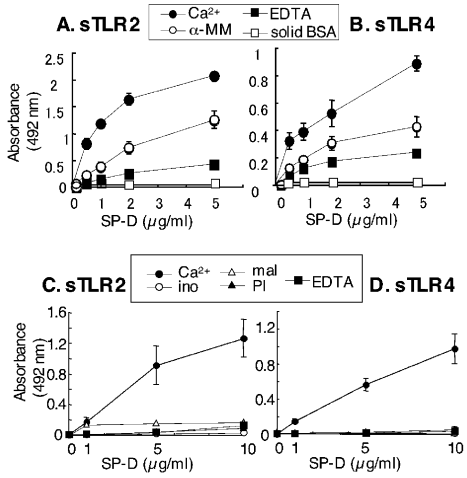

FIGURE 2: SP-D binds to sTLR2 and sTLR4. (A and B) The indicated concentrations of biotinylated natural human SP-D were incubated with sTLR2 (A) or sTLR4 (B) (250 ng/well) coated onto microtiter wells at 37 °C for 3 h in the presence of 5 mM $CaCl_2$ (●) or 5 mM EDTA (■). After the incubation, the wells were washed and further incubated with HRP-conjugated streptavidin. The binding of SP-D to sTLR2 or sTLR4 was finally detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The binding experiments were also performed in the presence of 0.2 M α-methyl mannoside (○) in a buffer containing 5 mM $CaCl_2$. The binding of human SP-D to BSA (□) coated onto microtiter wells was carried out as a control in the presence of 5 mM $CaCl_2$. The data shown are the means ± SE from three separate experiments. (C and D) The binding experiments with biotinylated recombinant SP-D expressed in insect cells were also performed in the presence of 0.2 M *myo*-inositol (○, ino) or maltose (△, mal) or 100 μg/mL phosphatidylinositol (▲, PI) in a buffer containing 5 mM $CaCl_2$. The data are means ± SE from three separate experiments.

RESULTS

SP-D Binds to the Extracellular Domains of TLR2 and TLR4. We examined whether SP-D binds to sTLR2 and sTLR4 coated onto microtiter wells. When the indicated concentrations of natural human SP-D were incubated with solid-phase sTLR2 or sTLR4 (250 ng/well), SP-D exhibited a concentration-dependent binding to sTLR2 (Figure 2A) and sTLR4 (Figure 2B) in the presence of Ca^{2+}, showing that SP-D binds to the extracellular domains of TLR2 and TLR4. Human SP-D showed no binding to BSA coated onto microtiter wells. Inclusion of excess α-methyl mannoside in the binding buffer attenuated the binding of SP-D to sTLR2 and sTLR4 by approximately 40−50%. When EDTA was used instead of $CaCl_2$, the level of binding of SP-D to solid-phase sTLRs was reduced by 75−80%, indicating that most of the binding of SP-D to sTLR2 and sTLR4 is Ca^{2+}-dependent. We attempted to determine the apparent amounts of the SP-D protein that had been binding to the solid-phase TLRs by measuring the absorbance of the avidin−HRP form binding to the known amounts of biotinylated SP-D coated onto microtiter wells. Then, the apparent K_d in the binding of SP-D to sTLR2 or sTLR4 was calculated by Scatchard

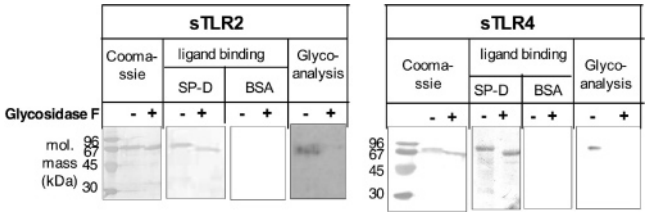

FIGURE 3: N-Linked oligosaccharide moieties of sTLR2 and sTLR4 were not required for the recognition by SP-D. sTLR2 or sTLR4 (2 μg) was treated with *N*-glycosidase F at 37 °C for 2 h. *N*-Glycosidase F-treated and untreated proteins of sTLR2 and sTLR4 were electrophoresed and transferred onto a PVDF membrane. The membrane was either stained with Coomassie Brilliant Blue (Coomassie stain) or incubated with recombinant SP-D (1 μg/mL) expressed in CHO-K1 cells or BSA (ligand binding). The binding of SP-D to sTLR2 or sTLR4 was detected by using an anti-SP-D polyclonal antibody raised against CHO-K1 cell-derived SP-D. The oligosaccharide moieties of sTLR2 and sTLR4 were visualized using a carbohydrate detection kit (Glycoanalysis).

plot analysis, assuming the oligomeric structure of natural human SP-D to be a dodecamer. The estimated K_d for sTLR2 or sTLR4 binding was 10.8 or 15.8 nM, respectively. Since SP-D preferentially interacts with maltose and *myo*-inositol with a higher affinity than mannose (*31*) and PI is an endogenous ligand for SP-D (*7*, *8*), we performed the competition experiments with *myo*-insoitol, maltose, and PI (Figure 2C,D). PI abrogated the binding of SP-D to the sTLR proteins at a level comparable to that of the binding in the presence of EDTA.

Because SP-D binds carbohydrates and sTLR4 is N-linked glycosylated (*32*), we next examined whether SP-D binds to the oligosaccharide moieties of sTLR2 and sTLR4. After sTLR2 and sTLR4 were resolved by SDS−PAGE and transferred onto PVDF membranes, the blot was overlaid with SP-D, and bound SP-D was detected using an anti-SP-D antibody. SP-D reacted with bands corresponding to sTLR2 and sTLR4 on the membrane [Figure 3, ligand binding, glycosidase F (−)], indicating the direct binding of SP-D to sTLR2 and sTLR4. When sTLR2 and sTLR4 were treated with *N*-glycosidase F, both proteins exhibited reduced molecular masses (Figure 3, Coomassie stain) and did not contain any glycan when analyzed by glycoanalysis (Figure 3, Glycoanalysis). Ligand blot analysis revealed that SP-D bound to deglycosylated forms of sTLR2 and sTLR4 [Figure 3, ligand binding, glycosidase F (+)].

Epitope Mapping. We mapped the epitopes for antibodies 7C6 and 7A10. Immunoblotting analysis revealed that both antibodies recognized SP-D and the recombinant protein consisting of the neck domain plus CRD (NCRD) and that antibody 7A10 but not antibody 7C6 bound to the recombinant CRD protein (CRD) (Figure 4A). The results were further confirmed with an ELISA (Figure 4B). Antibody 7A10 exhibited strong binding to both NCRD and CRD coated onto microtiter wells. No binding of antibody 7C6 to CRD was detected. Antibody 7C6 bound weakly to NCRD, but its binding was reproducible and significant when compared with that of the control antibody. The reason for the weak reactivity of 7C6 toward NCRD may be that antibody 7C6 reacts with the N-terminal end of the neck near the splice junction or that NCRD contains a partial epitope for antibody 7C6, since NCRD and SP-D both contain the entire neck and the CRD. These results indicate that the epitopes for antibodies 7C6 and 7A10 are located at

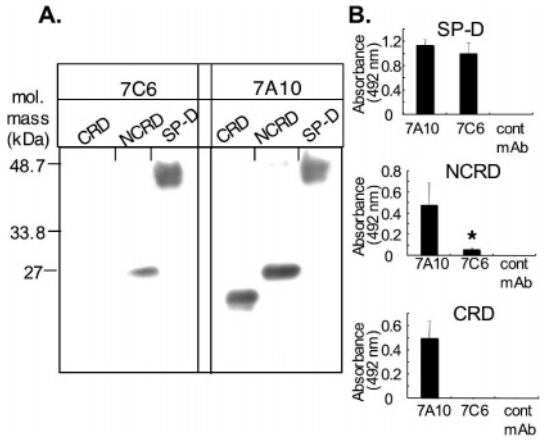

FIGURE 4: Epitope mapping for antibodies 7A10 and 7C6. (A) Western blot analysis of antibodies 7A10 and 7C6. Recombinant proteins (0.1 μg/lane) of SP-D, the neck plus carbohydrate recognition domain (NCRD), and the carbohydrate recognition domain (CRD) were electrophoresed under reducing conditions, and the proteins were transferred onto a PVDF membranes. The membrane was incubated with antibody 7C6 or 7A10 (10 μg/mL), and the binding of antibody to the proteins was detected by HRP-labeled anti-mouse IgG, as described in Experimental Procedures. (B) ELISA. SP-D, NCRD, or CRD (100 ng/well) was coated onto microtiter wells. Antibody 7A10 or 7C6 or control monoclonal antibody (cont mAb) (10 μg/mL) was incubated with the solid-phase protein at 37 °C for 1 h, followed by the incubation with HRP-labeled anti-mouse IgG. The binding of antibody to SP-D, NCRD, or CRD was finally detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The data are means ± SE from three separate experiments. The asterisk indicates $p < 0.05$, when compared with control mAb. Recombinant human SP-D was derived from CHO-K1 cells, and the NCRD and CRD were derived from *E. coli*.

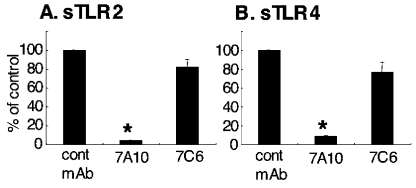

FIGURE 5: Antibody 7A10 but not 7C6 completely blocks the binding of SP-D to sTLR2 and sTLR4. The monoclonal antibody (20 μg/mL) was preincubated with biotinylated recombinant SP-D (1 μg/mL) expressed in insect cells at 37 °C for 1 h, and the mixture of the antibody and SP-D was further incubated with sTLR2 (A) or sTLR4 (B) (250 ng/well) coated onto microtiter wells at 37 °C for 3 h, followed by the incubation with HRP-conjugated streptavidin. The binding of SP-D to sTLR2 or sTLR4 was finally detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The relative absorbance is expressed as the percent of control (absorbance at 492 nm for the binding of SP-D to sTLR2 or sTLR4 in the presence of control mAb). The data are means ± SE from four separate experiments. Asterisks indicate $p < 0.001$, when compared with control mAb. The mean absorbances for the binding of SP-D to sTLR2 and sTLR4 in the presence of control mAb were 0.285 and 0.398, respectively.

the neck domain and the CRD, respectively. There is also a possibility that the antibody recognizes the region bridging the neck and the CRD or a cross-reacting epitope.

Anti-SP-D Monoclonal Antibody 7A10 Blocks the Binding of SP-D to sTLR2 and sTLR4. We next examined the effects of anti-SP-D monoclonal antibodies (mAbs) on the binding of SP-D to sTLR2 and sTLR4. Antibody 7A10 blocked the binding of SP-D to sTLR2 and sTLR4 (Figure 5AB). In contrast, antibody 7C6 failed to inhibit the binding of SP-D to sTLR proteins. The results indicate different effects of

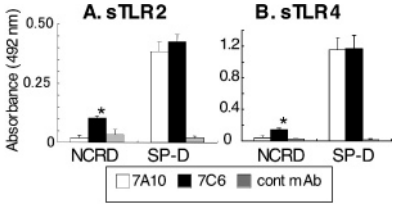

FIGURE 6: Interactions with antibody 7A10 but not 7C6 are blocked by prior binding of NCRD to sTLRs. SP-D or the NCRD (2 μg/mL) was incubated with sTLR2 (A) or sTLR4 (B) coated onto microtiter wells (250 ng/well) at room temperature for 5 h. After the wells had been washed, 10 μg/mL antibody 7A10, 7C6, or control monoclonal antibody (cont mAb) was then added to the wells and the mixture incubated at room temperature for 1 h, followed by the incubation with HRP-conjugated anti-mouse IgG. The binding of antibody to NCRD or SP-D that had been binding to solid-phase sTLR2 or sTLR4 was finally detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The data are means ± SE from three separate experiments. Asterisks indicate $p < 0.05$, when compared with antibody 7A10 and cont mAb. SP-D and NCRD were derived from CHO-K1 cells and *E. coli*, respectively.

anti-SP-D mAbs on the binding of SP-D to the sTLR proteins and suggest that the domain containing the epitope for antibody 7A10 is involved in the binding of SP-D to sTLR2 and sTLR4.

Antibody 7C6 but Not Antibody 7A10 Recognizes NCRD Bound to sTLR2 and sTLR4. We next examined whether antibodies 7A10 and 7C6 recognize SP-D and NCRD when they are bound to sTLR2 and sTLR4 coated onto microtiter wells. Both anti-SP-D mAbs, but not a control mAb, avidly bound to full-length SP-D when bound to solid-phase sTLR proteins (Figure 6A,B). There was no significant binding of antibody 7A10 to the trimeric NCRD (*29*) when bound to the solid-phase sTLR proteins. By contrast, antibody 7C6 exhibited weak but detectable binding. These results support the conclusion that SP-D binds to sTLR2 and sTLR4 through the CRD.

SP-D Binds to sTLR2 and sTLR4 by a Mechanism Different from Its Binding to Phosphatidylinositol and Lipopolysaccharide. A previous study (*10*) demonstrates that introduction of mutations at the primary carbohydrate binding site in rat SP-D ($Glu^{321} \rightarrow Gln$ and $Asn^{323} \rightarrow Asp$) significantly attenuated the binding of rat SP-D to PI. These mutations were shown to change the carbohydrate binding specificity of SP-A and MBL from mannose > galactose to the converse (*33*, *34*). Here, we produced a mutant protein of human SP-D with $Glu^{321} \rightarrow Gln$ and $Asn^{323} \rightarrow Asp$ substitutions ($SP\text{-}D^{E321Q,N323D}$) and examined whether introduction of these mutations into human SP-D alters its ability to interact with its ligands. Because the binding of wild-type (wt) SP-D and $SP\text{-}D^{E321Q,N323D}$ to the ligands was compared using the biotinylated proteins, we first examined whether the biotinylated proteins coated onto microtiter wells are equally detected using our streptavidin−peroxidase conjugate. Almost equivalent dose−response curves were obtained when biotinylated wt and mutant SP-Ds were detected with a streptavidin conjugate (Figure 7A), indicating that the biotinylated proteins can be used to compare the ligand binding properties of wt SP-D and $SP\text{-}D^{E321Q,N323D}$.

Consistent with the previous study with rat SP-D (*10*), recombinant human SP-D avidly bound to PI coated onto microtiter wells, but introduction of the mutations attenuated

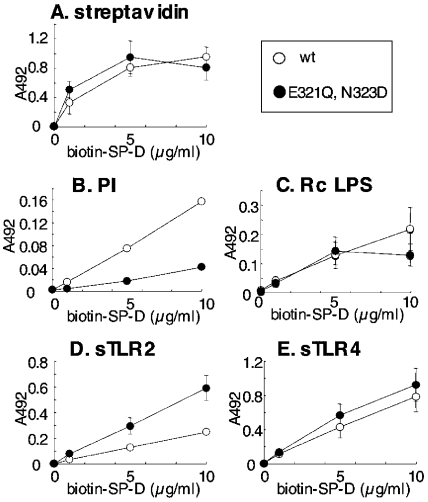

FIGURE 7: Binding of $SP\text{-}D^{E321Q,N323D}$ to PI, Rc LPS, and sTLR proteins. (A) Fifty microliters of the indicated concentrations of biotinylated wt SP-D or biotinylated $SP\text{-}D^{E321Q,N323D}$, which was expressed in insect cells, at the indicated concentrations was coated onto microtiter wells, and HRP-conjugated streptavidin was then reacted with the solid-phase biotinylated proteins at 37 °C for 30 min. After the wells had been washed, the reaction of biotin with strepavidin was detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The data are means ± SE from three separate experiments. (B−E) The indicated concentrations of biotinylated wt SP-D or biotinylated $SP\text{-}D^{E321Q,N323D}$ were incubated at room temperature for 5 h with PI or Rc LPS (4 μg/well) or sTLR2 or sTLR4 (250 ng/well) coated onto microtiter wells, followed by the incubation with HRP-conjugated streptavidin. The binding of SP-D to the solid-phase ligand was finally detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The data are means ± SE from three separate experiments for Rc LPS, sTLR2, and sTLR4 (C−E, respectively). The mean absorbances from two experiments are presented for PI binding (B): (○) wt SP-D (wt) and (●) $SP\text{-}D^{E321Q,N323D}$ (E321Q, N323D).

PI binding (Figure 7B). In contrast, $Glu^{321} \rightarrow Gln$ and $Asn^{323} \rightarrow Asp$ substitutions failed to attenuate the binding of SP-D to sTLR2 and sTLR4 (Figure 7D,E). The binding of $SP\text{-}D^{E321Q,N323D}$ to the sTLR proteins was somewhat stronger than that of wt SP-D. These results are consistent with the observation that SP-D retains the ability to bind to the deglycosylated sTLR proteins (Figure 3). Because SP-D has been shown to bind *E. coli* Rc LPS containing core terminal glucose (*13*), we also sought to determine whether $SP\text{-}D^{E321Q,N323D}$ retains Rc LPS binding activity. Unexpectedly, when wt SP-D and $SP\text{-}D^{E321Q,N323D}$ were compared for Rc LPS binding, no significant difference in binding was observed (Figure 7C).

We also examined the effects of mAbs on the binding of SP-D to PI and Rc LPS. Antibody 7A10 significantly inhibited the binding of SP-D to PI and Rc LPS when compared with control mAb (Figure 8). However, antibody 7C6 exhibited no inhibitory effect. In fact, this antibody enhanced the binding of SP-D to PI and Rc LPS by factors of 16.3 and 4.9, respectively. Control experiments without SP-D revealed that antibody did not bind to the solid-phase lipids. Although it remains unclear why the level of binding of SP-D to PI and Rc LPS is increased in the presence of antibody 7C6, the results indicate that the CRD, which contains the epitope for antibody 7A10, is involved in the binding of SP-D to PI and Rc LPS,.

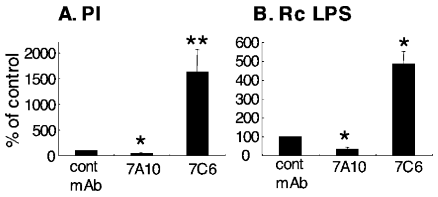

FIGURE 8: Antibody 7C6 enhances the binding of SP-D to PI and Rc LPS. The monoclonal antibody (50 μg/mL) was preincubated with biotinylated recombinant SP-D (5 μg/mL) expressed in insect cells at 37 °C for 1 h, and the mixture of antibody and SP-D was further incubated with PI (A) and Rc LPS (B) (5 μg/well) coated onto microtiter wells at room temperature for 5 h, followed by the incubation with HRP-conjugated streptavidin. The binding of SP-D to PI and Rc LPS was finally detected by measuring the absorbance at 492 nm, as described in Experimental Procedures. The relative absorbance is expressed as the percent of control (absorbance at 492 nm for the binding of SP-D to PI or Rc LPS in the presence of control mAb). The data are means ± SE from three (A) or four (B) separate experiments. One asterisk indicates $p < 0.001$, when compared with control mAb, and two asterisks indicate $p < 0.05$, when compared with control mAb. The mean absorbances for the binding of SP-D to PI and Rc LPS in the presence of control mAb were 0.056 and 0.290, respectively.

Taken together, these results indicate that SP-D binds to sTLR2 and sTLR4 through the CRD, as is the case in its binding to PI and Rc LPS, but that the mechanism of binding to sTLR2 and sTLR4 is different.

DISCUSSION

We have demonstrated that SP-D, an important innate immune effector implicated in LPS recognition, binds to TLR2 and TLR4. We have focused on the mechanism of binding of SP-D to the sTLR proteins in this study and conclude that the SP-D CRD binds to a sequential determinant associated with the extracellular domains of TLR2 and TLR4.

In previous studies, we have shown that the binding of SP-A, a related lung collectin, to TLR2 results in alteration of TLR2 signaling elicited by peptidoglycan and zymosan (*16*, *17*). SP-A downregulates petidoglycan- and zymosan-stimulated TNF-α secretion and NF-κB activation by interacting with TLR2. It is presumed that the interaction of SP-A with TLR2 prevents the ligands from binding to TLR2. Whether SP-D similarly alters TLR2- or TLR4-mediated signaling elicited by pathogen-associated molecular patterns is under investigation.

We have previously shown that SP-A binds to the peptide portion of CD14 but that SP-D binds to the oligosaccharide moieties of CD14 (*15*). However, the mechanisms of binding to TLRs appear to be similar for SP-A and SP-D. Human SP-A binds to sTLR2 in a Ca^{2+}-dependent manner, and this binding occurs through the SP-A CRD. For example, an anti-SP-A mAb that recognizes the Thr^{184}−Gly^{194} region in the SP-A CRD abrogated binding of SP-A to sTLR2 (*16*). However, excess carbohydrates did not abrogate binding of SP-A to sTLR2.

Antibody 7C6 failed to abrogate the binding of SP-D to sTLRs (see Figure 5). This may suggest that the binding of SP-D to sTLR is not mediated by the neck domain. However, there is also a possibility that the affinity of antibody 7C6 for the neck domain is too low for the antibody to block the binding of SP-D to sTLR proteins. Antibody 7A10 blocked the binding of SP-D to the sTLR proteins. Thus, the binding of SP-D to sTLR2 and sTLR4 occurs through the CRD, probably to a sequential epitope exposed near the ligand binding surface of the folded CRD. As discussed below, binding to TLRs blocks interactions of the CRD with 7A10, an antibody that blocks interaction with PI and LPS. Although excess α-methyl mannoside partially inhibited binding of SP-D to the sTLRs, SP-D bound to deglycosylated sTLR proteins. Thus, the binding of SP-D to sTLR2 and sTLR4 does not require binding of the SP-D lectin domain to carbohydrates on the TLRs. The capacity of TLRs to interact with $SP\text{-}D^{E321Q,N323D}$ (see Figure 7D,E), a mutant that shows altered saccharide binding preferences and attenuated binding to PI (*10*), is consistent with this conclusion. However, it is possible that local conformational changes associated with saccharide binding can influence interactions with the TLRs.

SP-D shows calcium-dependent, carbohydrate-sensitive binding to Rc and Rd LPS, but not to Re LPS and lipid A (*13*). These and other observations suggested that carbohydrates associated with the core oligosaccharide mediate binding to the SP-D CRD. It has generally been assumed that the mechanism of binding is similar to that of PI, which involves calcium-dependent, carbohydrate-sensitive interactions with the inositol moiety. However, as shown in Figure 7C, the binding of $SP\text{-}D^{E321Q,N323D}$ to Rc LPS was similar to that of wt SP-D. The molecular basis for the differential effects of the mutation on LPS and PI recognition merits further investigation.

TLR is a type I membrane protein which consists of an extracellular domain containing the repeats of a leucine-rich motif, a transmembrane domain, and a cytoplasmic signaling domain. sTLR2 and sTLR4 which we constructed contain the extracellular domains, in which the structure of leucine-rich repeats is common to sTLR2 and sTLR4 and in which the amino-terminal structure is unique. MD-2, which is an accessory protein and is essential for TLR4-mediated signaling, binds to sTLR4 but not to sTLR2 (*22*). Recent studies (*35*, *36*) indicate that the amino-terminal TLR4 region, which contains a structure different from TLR2, is responsible for the binding to MD-2. For these reasons, we infer that SP-D specifically recognizes the extracellular leucine-rich repeat domains, which are common to sTLR2 and sTLR4.

We measured the absorbance at 492 nm to determine the binding of SP-D to sTLR proteins coated onto microtiter wells. However, the values of the absorbance varied in the experiments. Different preparations of sTLR proteins may cause different nonspecific adsorption of sTLR2 and sTLR4 onto the wells. In addition, we used natural SP-D and recombinant SP-Ds expressed in CHO-K1 cells and in insect cells. These proteins exhibit slightly different folding of the proteins, which may affect the binding. However, the estimated K_d values obtained from panels A and B of Figure 2 appear close: 10.8 or 15.8 nM for sTLR2 or sTLR4 binding, respectively.

Although the NCRD migrated at the position corresponding to a monomer when analyzed by SDS−PAGE (see Figure 1, lanes f and i), analysis by gel filtration indicates that the NCRD is a trimer (*29*). The intact CRD fusion protein elutes as a dimer via gel filtration. A previous study (*37*) has shown that a collagen domain deletion mutant (CDM) and a collagenase-resistant fragment (CRF) of rat SP-D migrate at 67 and 18 kDa, respectively, by gel filtration

chromatography, and the ability of CRF to bind mannose-Sepharose, PI, and glucosylceramide is impaired compared with that of CDM. Thus, it is possible to infer that the oligomer formation of SP-D, NCRD, and CRD could affect the SP-D−ligand interactions and their affinities.

Antibody 7A10 significantly inhibited the binding of SP-D to sTLRs, PI, and Rc LPS (see Figures 5 and 8). By contrast, antibody 7C6 failed to inhibit the binding of SP-D to sTLR2 and sTLR4 (see Figure 5) but strengthened the binding of SP-D to PI and Rc LPS (see Figure 8). When NCRD containing the S-protein binding sequence (*29*) that had been preincubated with antibody 7C6 was examined for the binding to PI or Rc LPS using the S-protein−HRP conjugate, antibody 7C6 strengthened the binding of NCRD to PI or Rc LPS by a factor of 56.4 or 37.1, respectively, although control experiments with an unrelated monoclonal antibody showed very weak binding of NCRD: A_{492} (absorbance at 492 nm) = 0.014 ± 0.005 (the mean ± standard error, n = 4) for PI and A_{492} = 0.016 ± 0.004 (the mean ± standard error, n = 3) for Rc LPS. In addition, when the binding of biotinylated 7A10 to SP-D that had been binding to the solid-phase glycolipids was examined after SP-D had been preincubated with antibody 7C6, the level of binding of 7A10 to SP-D that had been binding to PI or Rc LPS was 278 or 94%, respectively, of the control binding obtained from preinicubation with an unrelated antibody: A_{492} = 0.25 ± 0.031 (the mean ± standard error, n = 3) for PI and A_{492} = 1.127 ± 0.169 (the mean ± standard error, n = 3) for Rc LPS. This may suggest that antibody 7C6 does not appear to interfere with the CRD function of SP-D due to blocking of ligand access. Although the mechanism of the antibody-enhanced binding of SP-D to the lipids remains to be determined, it is possible to infer that antibody 7C6 may act to cross-link SP-D molecules and thereby increase the valency, apparent binding affinity, and total level of binding of SP-D to PI and LPS. The differences further support our conclusion that the interaction of SP-D with sTLRs is mechanistically different from that of SP-D with PI and Rc LPS.

As indicated above, there is no significant binding of antibody 7A10 to the trimeric NCRD when it is bound to solid-phase sTLR proteins, but antibody 7C6 shows detectable binding. We infer that antibody 7A10 fails to recognize its epitope, because the epitope is masked by binding to sTLR proteins. The epitope for antibody 7C6, the neck domain, is still available for antibody binding even after the NCRD binds to sTLR proteins. Because full-length native SP-D forms a cruciform dodecamer assembled from four trimers, 7A10 can recognize at least one of four trimers that are not interacting with the sTLR proteins. These results support the conclusion that the CRD is a functional domain for interaction of SP-D with sTLR2 and sTLR4.

Because antibody 7A10 inhibits the binding of SP-D to sTLRs, as well as PI and Rc LPS, the epitope for 7A10 in the CRD is probably located in a region critical for interaction with all three molecules, at or near the site of ligand binding. To further localize the binding site for the TLRs, we examined the binding properties of human NCRDs with mutations targeting selected highly exposed residues that flank the carbohydrate binding site at the known ligand binding surface. In particular, we examined the effects of substitutions at Asp^{325}, Arg^{343}, and Lys^{348} ($NCRD^{D325N}$, $NCRD^{R343A}$, and $NCRD^{K348Q}$) on binding to TLRs and 7A10 (data not shown). The trimeric mutants bound to the sTLRs, and antibody 7A10 recognized these mutants at a level comparable to that of the wt NCRD, indicating that Asp^{325}, Arg^{343}, and Lys^{348} are not required for interactions with sTLRs or 7A10. Given the large size of the antibody, other exposed residues on or near the ligand binding surface could be involved. Alternatively, conformational changes associated with ligand binding might alter the accessibility of an epitope more remote from the ligand binding surface.

In summary, our results demonstrate that human SP-D, an innate immune effector with LPS binding activity, interacts with the extracellular domains of TLR2 and TLR4. The spatial proximity of distinct but interacting LPS and TLR binding sites on the SP-D CRD suggests relatively direct roles of SP-D in modulating TLR-dependent cellular responses to microbial cell wall glycoconjugates, consistent with known modulatory effects of SP-D on LPS-dependent macrophage activation.

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