Pulmonary Surfactant Protein D Binds MD-2 through the Carbohydrate Recognition Domain[†]

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ABSTRACT: Pulmonary surfactant protein D (SP-D) is a member of the collectin family and plays crucial roles in the innate immunity of the lung. We have previously shown that surfactant protein A (SP-A), a homologous collectin, interacts with MD-2 and alters lipopolysaccharide signaling. In this study, we examined and characterized the binding of SP-D to MD-2 using a soluble form of recombinant MD-2 (sMD-2). SP-D bound in a concentration- and Ca^{2+}-dependent manner to sMD-2 coated onto microtiter wells. Excess mannose abolished the binding of SP-D to sMD-2. In solution, SP-D cosedimented with sMD-2 in the presence of Ca^{2+}. The direct binding of SP-D to sMD-2 was confirmed by BIAcore analysis. Anti-SP-D monoclonal antibody that recognizes the carbohydrate recognition domain (CRD) of SP-D significantly inhibited the binding of SP-D to sMD-2, indicating the involvement of the CRD for the binding to sMD-2. Ligand blot analysis revealed that SP-D bound to N-glycopeptidase F-treated sMD-2. In addition, the biotinylated SP-D pulled down the mutant sMD-2 with $Asn^{26} \rightarrow$ Ala and $Asn^{114} \rightarrow$ Ala substitutions, which lacks the consensus for N-glycosylation. Furthermore, the sMD-2 mutant cosedimented SP-D. These results demonstrate that SP-D directly interacts with MD-2 through the CRD.

Pulmonary surfactant protein D (SP-D)[1] is a member of the collectin family along with surfactant protein A (SP-A) and mannose binding lectin (MBL) (*1, 2*). The structure of the collectin is composed of four characteristic domains: an amino terminus involved in interchain disulfide bonding, a collagen-like domain, a neck domain, and a C-type lectin domain [a carbohydrate recognition domain (CRD)]. Pulmonary collectins, SP-A and SP-D, are believed to function as the first-line defense of the lung. Studies (*1*−*5*) with genetic defects in mice have demonstrated that SP-A and SP-D modulate inflammation caused by pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS) in the lung, and that they contribute to the clearance of microbes from the lung.

Several endogenous ligands for SP-D have been identified. SP-D binds to phosphatidylinositol (*6, 7*), a component of surfactant. This collectin also directly interacts with various microorganisms and their cell wall components, including LPS (*8*−*10*). We have shown that SP-D binds to CD14 and Toll-like receptors (TLRs) 2 and 4 (*11, 12*), which are implicated in recognizing and signaling PAMPs (*13*−*15*). TLR4 has been shown to be responsible for recognition and signaling of LPS (*13*). TLR4 is a type I membrane protein containing a leucine-rich motif structure in its extracellular domain. MD-2 is critical for LPS-induced TLR4 signaling (*16*). TLR4 does not respond efficiently to LPS without MD-2. MD-2 has been shown to bind the concave surface of the amino-terminal and central domains of TLR4 (*17*). The TLR4−MD-2 heterodimer is believed to form the complete recognition site for LPS (*18*−*21*). In addition, MD-2 possesses properties that are common to a family of extracellular lipid-binding proteins (*22*). Thus, MD-2 might play a more direct and essential role in LPS binding, probably as a coreceptor for TLR4 (*23*). The reduced form of MD-2 is a 20−25 kDa monomeric protein with seven cysteine residues. Secreted MD-2 exists as heterogeneous collections of disulfide-linked oligomers, which can confer LPS responsiveness directly to TLR4 on the cell surface (*24*). We have also found that SP-A modulates inflammatory responses elicited by

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[1] Abbreviations: SP-D, surfactant protein D; SP-A, surfactant protein A; TLR, Toll-like receptor; LPS, lipopolysaccharide; sMD-2, soluble form of recombinant MD-2; CRD, carbohydrate recognition domain; CRF, collagenase-resistant fragment.

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peptidoglycan, zymosan, and LPS by direct interaction with TLRs and MD-2 (*25*−*27*). Because the formation of the TLR4−MD-2 complex is critical for the initiation of LPS signaling, it is important to determine whether SP-D directly interacts with MD-2 in addition to TLR4. In this study, we show that SP-D, an SP-A homologue, interacts with MD-2 through the CRD.

EXPERIMENTAL PROCEDURES

Recombinant Human SP-D. The cDNA for human SP-D, used in this study, encoded the Thr^{11} polymorphic form. Recombinant human SP-D was expressed in CHO-K1 cells using the glutamine synthetase amplification system, as described previously (*11*). For protein purification, the cells were incubated in serum-free EXCELL 302 medium (SAF Bioscience, Lenexa, KS) for 3 days. The medium was then collected and filtered with a 0.45 μm filter. $CaCl_2$ was added at a final concentration of 2 mM. The medium was finally applied to a mannose-Sepharose column (*28*). The SP-D protein was eluted with 5 mM Tris buffer (pH 7.4) containing 150 mM NaCl and 10 mM EDTA and dialyzed against 5 mM Tris buffer (pH 7.4) containing 150 mM NaCl. Purification of recombinant SP-D by the affinity matrix on mannose-Sepharose indicated that recombinant SP-D used in this study retained carbohydrate binding activity. In some experiments, LPS-stripped SP-D was used for sMD-2 binding. Endotoxin was removed from SP-D using polymyxin B-agarose (Sigma) as described previously (*27*). The endotoxin content in the SP-D preparation was <.33 pg/μg of protein when determined by *Limulus* amembocyte assay.

Biotinylation of SP-D. Recombinant SP-D was biotinylated using EZ-link sulfo-NHS-biotin (Pierce, Rockford, IL) according to the manufacturer's instructions.

MD-2. The 0.48 kb cDNA for human MD-2 containing a V5 tag and a six-His tag at the C-terminal end was inserted into pVL1392 vector. A soluble form of recombinant human MD-2 (sMD-2) was expressed by the baculovirus−insect cell system, and sMD-2 was purified with a column on nickel-nitrilotriacetic acid beads as described previously (*29, 30*). In some experiments, sMD-2 was purified from the medium using a 5 mL Ni-chelating HisTrap affinity column (GE Healthcare) pre-equilibrated with 100 mM Hepes buffer (pH 7.4) containing 500 mM NaCl and 20 mM imidazole. The bound proteins were then eluted with 100 mM Hepes buffer (pH 7.4) containing 500 mM NaCl and an imidazole gradient (from 20 to 500 mM). We also generated a mutant sMD-2 with $Asn^{26} \rightarrow$ Ala and $Asn^{114} \rightarrow$ Ala substitutions (sMD-$2^{N26A/N114A}$) to eliminate the consensus for N-linked glycosylation. The mutant cDNA was constructed with the QuickChange II XL site-directed mutagenesis kit (Stratagene, Cedar Creek, TX). sMD-$2^{N26A/N114A}$ was isolated using a 5 mL Ni-chelating HisTrap affinity column as described above.

Binding of SP-D to sMD-2. sMD-2 (100 ng/well) was coated onto microtiter wells, and nonspecific binding was blocked with 10 mM Hepes buffer (pH 7.4) containing 150 mM NaCl, 2 mM $CaCl_2$, and 5% (w/v) BSA (buffer A). The indicated concentrations (0, 1, and 5 μg/mL) of SP-D in buffer A were added and incubated at 37 °C for 3 h. After the incubation, the wells were washed with PBS containing 0.1% (v/v) Triton X-100 and 3% (w/v) skim milk and further incubated with anti-SP-D polyclonal IgG (10 μg/mL) followed by the incubation with HRP-labeled anti-rabbit IgG. The peroxidase reaction was finally conducted by using *o*-phenylenediamine as a substrate, and the absorbance at 492 nm was measured. In some experiments, SP-D was preincubated with anti-SP-D monoclonal antibody 7A10, 7C6, or 6B2 (*31*) at 37 °C for 1 h before the incubation with the solid phase sMD-2. To examine the effect of Ca^{2+} on binding, we included 5 mM EDTA instead of Ca^{2+}.

The binding study was also performed in solution. The biotinylated SP-D (200 ng) was incubated with sMD-2 (50 ng) at 37 °C for 2 h in buffer A. The mixture was further incubated at 4 °C with anti-V5 antibody-conjugated agarose (Sigma, St. Louis, MO) for 2 h. When streptavidin-conjugated agarose (Invitrogen, Carlsbad, CA) was incubated for 30 min, 2 μg of the biotinylated SP-D and 0.5 μg of sMD-2 were used. The beads were sedimented by centrifugation at 2000*g* for 10 s, washed three times with buffer A containing 0.1% (v/v) Triton X-100, and suspended in SDS sample buffer. The samples were subjected to SDS−PAGE under reducing conditions, and the proteins on the gel were transferred onto a polyvinylidene difluoride (PVDF) membrane. The biotinylated SP-D and sMD-2 on the membrane were detected by using HRP-bound streptavidin and anti-V5 polyclonal antibody, respectively. The proteins were finally visualized by a chemiluminescence reagent (Super-Signal, Pierce).

Because the expression level of a mutant sMD-2 was quite low and the sMD-$2^{N26A/N114A}$ protein was somewhat unstable, sMD-2 and sMD-$2^{N26A/N114A}$ were partially purified from the Sf900II medium (Invitrogen) culturing *Tni* cells (kind gift of Dr. Voelker) using a 5 mL Ni-chelating HisTrap affinity column as described above. The same volumes of partially purified sMD-2 proteins were used for the pull-down assay with biotinylated SP-D. Biotinylated SP-D (250 ng) was incubated with sMD-2 (250 μL) at 37 °C for 2 h in buffer A. The mixture was further incubated with streptavidin-conjugated agarose at 4 °C for 30 min or Ni-NTA agarose (Qiagen, Valencia, CA) at 4 °C for 2 h. The beads were finally sedimented by centrifugation, washed three times with buffer A containing 0.1% (v/v) Triton X-100 instead of BSA, and suspended in SDS sample buffer. The sedimented proteins were visualized as described above.

BIAcore Analysis. BIAcore 3000 (BIAcore AB, Uppsala, Sweden) was used to evaluate the interaction of SP-D with sMD-2. SP-D in 10 mM sodium acetate (pH 5.0) was immobilized on a C1 sensor chip (BIAcore) using an amine coupling method according to the manufacturer's instructions. sMD-2 in 5 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 5 mM $CaCl_2$, and 0.2 mg/mL BSA was passed over the surface of the sensor chip, and the interaction was 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. Interaction of sMD-2 with SP-D was also analyzed with immobilized sMD-2. To examine the effect of the oligomeric structure of SP-D, SP-D was digested with collagenase and the collagenase-resistant fragment (CRF), which consists of the neck and the CRD, was obtained as described previously (*27*). For the immobilization of the protein on a C1 sensor chip, 40 μL of 25 μg/mL SP-D or CRF or 50 μL of 50 μg/mL sMD-2 was injected.

Recombinant Fusion Proteins of the Neck with the CRD and the CRD. A recombinant trimeric protein consisting of the neck with the carbohydrate recognition domain (NCRD) and the CRD was expressed in a bacterial expression system and purified as described previously (*32*).

Analysis of Epitopes for Anti-SP-D Monoclonal Antibodies. The epitope location was determined by immunoblotting. Briefly, recombinant proteins (100 ng/lane) of SP-D, NCRD, and CRD were electrophoresed under reducing conditions, and the proteins on the gel were transferred onto PVDF membranes. The membranes were probed with anti-SP-D monoclonal antibody (10 μg/mL) 7A10, 7C6, or 6B2. Antibody binding was detected by using HRP-labeled anti-mouse IgG antibody.

To determine whether monoclonal antibodies recognize the same epitope, competition experiments with biotinylated antibody were performed. SP-D (10 ng/lane) was electrophoresed under reducing conditions and transferred onto PVDF membranes. The membranes were first incubated with monoclonal antibody (50 μg/mL) and then incubated with biotinylated antibody 6B2 (2 μg/mL). The binding of the biotinylated 6B2 to reduced SP-D was finally detected by HRP-labeled streptavidin.

Treatment of sMD-2 with N-Glycopeptidase F. sMD-2 (500 ng) was treated with N-glycopeptidase F (0.5 milliunit/μg of protein) (Takara, Japan) at 37 °C for 17 h according to the manufacturer's instructions.

Lectin Blot and Ligand Blot. sMD-2 treated with N-glycopeptidase F was electrophoresed under reducing conditions and transferred onto PVDF membranes. The membranes were blocked with 5% (w/v) BSA and then incubated with Tris-buffered saline containing 0.1% (v/v) Tween 20 and 5% (w/v) BSA. The membranes were then incubated with biotinylated concanavalin A (J-Oil Millz, Tokyo, Japan) (4 μg/mL) at room temperature for 30 min followed by the incubation with HRP-labeled streptavidin. The binding of concanavalin A to the oligosaccharide moieties was detected by using a chemiluminescence reagent.

For ligand blot experiments, the PVDF membranes were incubated with SP-D (10 μg/mL) at 4 °C overnight after nonspecific binding was blocked with 20 mM Tris buffer (pH 7.4) containing 150 mM NaCl, 5 mM $CaCl_2$, 0.5% (w/v) BSA, and 1% (w/v) polyvinylpyrrolidone (Sigma). SP-D binding to the membrane was detected by anti-SP-D antibody and HRP-labeled anti-rabbit IgG antibody. The peroxidase reaction was assessed by using a chemiluminescence reagent.

SDS−PAGE. SP-D and sMD-2 were subjected to SDS−PAGE under reducing and nonreducing conditions.

Antibody. Anti-SP-D polyclonal antibody was raised against purified recombinant human SP-D. Anti-SP-D monoclonal antibodies (6B2, 7A10, and 7C6) were prepared and characterized as described previously (*24*).

RESULTS

Electrophoretic Analysis of Recombinant Proteins. Recombinant human SP-D was analyzed by SDS−polyacrylamide gel electrophoresis. The major form of SP-D expressed in CHO cells appeared at 45 kDa under reducing conditions (Figure 1, lane a), and it exhibited various degrees of oligomerization under nonreducing conditions (Figure 1, lane b). The preparation of recombinant SP-D used in this study

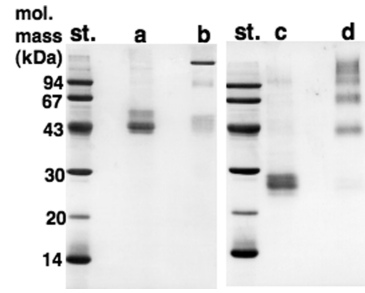

FIGURE 1: Electrophoretic analysis of SP-D and sMD-2. Recombinant SP-D (lanes a and b) and recombinant sMD-2 (lanes c and d) were subjected to SDS−PAGE (5 μg/lane) under reducing (lanes a and c) and nonreducing (lanes b and d) conditions. The proteins were visualized by Coomassie Brilliant Blue staining. Lane st. contained the molecular mass standard.

contained a minor upper band at approximately 50 kDa, which was not seen in the previous study (*11*). This may be because the cDNA used in this study encoded the Thr^{11} polymorphic form which is presumably O-glycosylated. Human SP-D is reported to contain the 50 kDa variant which is detected in approximately half of the samples of bronchoalveolar lavage fluids from normal subjects and is produced by post-translational glycosylation (*33*). The recombinant proteins were purified by affinity chromatography using a column of mannose-Sepharose, indicating that the recombinant proteins retain lectin activity. We also expressed a soluble form of recombinant MD-2 (sMD-2) by using the baculovirus−insect cell system. sMD-2 migrated as bands with molecular masses of 25−28 kDa under reducing conditions (Figure 1, lane c) and formed various degrees of oligomers under nonreducing conditions (Figure 1, lane d). sMD-2 was biologically active since the additions of sMD-2 and LPS elicited NF-κB activation in HEK293 cells expressing TLR4 (*30*).

SP-D Binds to sMD-2. We first examined whether SP-D bound to sMD-2 coated onto microtiter wells. SP-D exhibited a concentration-dependent binding to sMD-2 in the presence of 2 mM $CaCl_2$ (Figure 2A). Inclusion of 5 mM EDTA instead of Ca^{2+} abolished the binding of SP-D to the solid phase sMD-2, indicating that the binding of SP-D to sMD-2 is Ca^{2+}-dependent. The effect of carbohydrate on sMD-2 binding was examined (Figure 2B). Excess mannose blocked the binding of SP-D to solid phase sMD-2.

Because SP-D (*9*) and MD-2 (*34*) are LPS-binding proteins, we examined the sMD-2 binding by ligand botting with LPS-depleted SP-D. Endotoxin was separated from sMD-2 by SDS−PAGE, and sMD-2 on the membrane was incubated with SP-D. SP-D colocalized with sMD-2 on the membrane (Figure 2C), indicating the direct binding of SP-D to sMD-2. This result rules against the required formation of a ternary complex in which SP-D and sMD-2 interact through bound LPS.

The interaction of SP-D with sMD-2 was also examined in solution. Biotinylated SP-D (b-SP-D) and V5-tagged sMD-2 were incubated, and sMD-2 was immunoprecipitated with anti-V5 antibody (Figure 3A). sMD-2 coprecipitated SP-D in the presence of Ca^{2+}, indicating the direct binding of sMD-2 to SP-D. SP-D was not coprecipitated with sMD-2 in the presence of EDTA. When b-SP-D was pulled down with streptavidin-conjugated agarose in the presence of Ca^{2+}, sMD-2 was cosedimented (Figure 3B). However, sMD-2 was

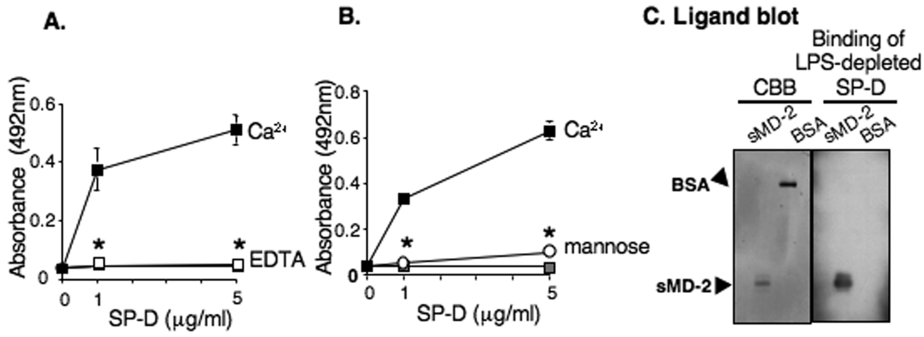

FIGURE 2: SP-D exhibits Ca^{2+}-dependent binding to sMD-2 coated onto microtiter wells. (A) The indicated concentrations (0, 1, and 5 μg/mL) of SP-D were incubated with sMD-2 [100 ng/well (black squares and white squares)] or BSA (gray squares) coated onto microtiter wells in the buffer containing 2 mM Ca^{2+} (black squares and gray squares) or 5 mM EDTA (white squares) at 37 °C for 3 h. After the incubation, the wells were washed and further incubated with 10 μg/mL anti-SP-D polyclonal antibody, followed by the incubation with HRP-conjugated anti-rabbit IgG. The binding of SP-D to the solid phase protein was finally detected by measuring the absorbance at 492 nm as described in Experimental Procedures. The data shown are means ± the standard deviation from three separate experiments. An asterisk denotes $p < 0.01$ when compared with the binding in the presence of 2 mM Ca^{2+}. (B) Effect of mannose on the binding of SP-D to sMD-2. SP-D was incubated with sMD-2 (black squares and white circles) or BSA (gray squares) coated onto microtiter wells in the buffer containing 2 mM Ca^{2+} (black squares and gray squares) or 2 mM Ca^{2+} and 200 mM mannose (white circles). An asterisk denotes $p < 0.01$ when compared with the binding in the presence of 2 mM Ca^{2+}. (C) Binding of LPS-depleted SP-D to sMD-2 by assessed with a ligand blot. sMD-2 (500 ng) and BSA were electrophoresed and transferred onto a PVDF membrane. The binding of LPS-depleted SP-D (10 μg/mL) to sMD-2 was performed. Binding of SP-D to the membrane was detected by anti-SP-D polyclonal antibody as described in Experimental Procedures. The PVDF membrane was also stained with Coomassie Brilliant Blue (CBB).

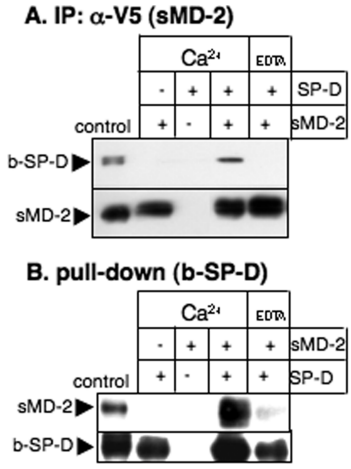

FIGURE 3: SP-D binds sMD-2 in solution. (A) Immunoprecipitation with sMD2. V5-tagged sMD-2 (50 ng) was incubated with biotinylated SP-D (200 ng) in the buffer containing 2 mM $CaCl_2$ (Ca^{2+}) or 5 mM EDTA (EDTA) at 37 °C for 2 h. sMD-2 was immunoprecipitated with anti-V5 antibody-conjugated agarose, and the immunoprecipitates were subjected to SDS−PAGE under reducing conditions. The Western blot was then performed by using anti-V5 antibody for sMD-2 and HRP-conjugated streptavidin for b-SP-D, as described in Experimental Procedures. (B) Pull-down assay of SP-D. Biotinylated SP-D (2 μg) was incubated with sMD-2 (500 ng) in the buffer containing 2 mM $CaCl_2$ (Ca^{2+}) or 5 mM EDTA (EDTA) at 37 °C for 2 h. Biotinylated SP-D was pulled down with streptavidin-conjugated agarose, and the precipitates were subjected to SDS−PAGE under reducing conditions. The Western blot was then performed by using HRP-conjugated streptavidin for b-SP-D and anti-V5 antibody for sMD-2, as described in Experimental Procedures.

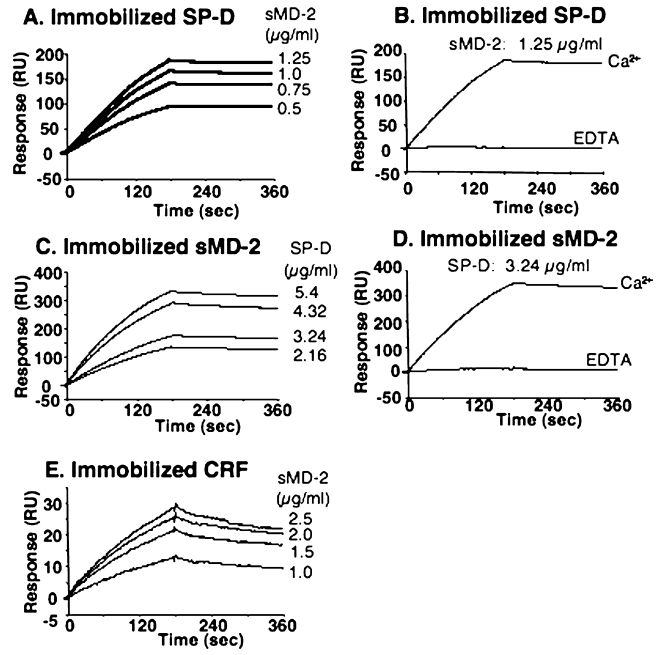

FIGURE 4: BIAcore analysis. The interactions between sMD-2 and SP-D were assessed by surface plasmon resonance analysis (BIAcore) as described in Experimental Procedures. Sensorgrams for the bindings of various concentrations of sMD-2 to SP-D or CRF immobilized on a C1 sensor chip (ABE) or of SP-D to sMD-2 immobilized on a C1 sensor chip (CD) are shown. For the immobilization of the protein on a C1 sensor chip, 40 μL of 25 μg/mL SP-D or CRF or 50 μL of 50 μg/mL sMD-2 was injected. In panels B and D, 5 mM Tris buffer (pH 7.4) containing 5 mM EDTA was used instead of the buffer containing 2 mM Ca^{2+} to monitor the interaction. RU, resonance units.

barely detected when EDTA was included. These results indicate that SP-D binds sMD-2 in solution.

We further analyzed the binding of SP-D to sMD-2 by surface plasmon resonance analysis (BIAcore). When various concentrations of sMD-2 were applied to SP-D immobilized on a sensor chip, concentration-dependent sensorgrams for association and dissociation were obtained. sMD-2 did not dissociate well once it bound to SP-D (Figure 4A), indicating high-affinity interactions between sMD-2 and SP-D. Because secreted MD-2 forms various degrees of large oligomers (50−400 kDa) in solution (*35*), it is inappropriate to calculate parameters for association and dissociation. When various concentrations of SP-D were applied to immobilized sMD-2, high-affinity interactions were also observed (Figure 4C). When 5 mM EDTA was used instead of 2 mM Ca^{2+}, there was no detectable interaction (Figure 4B,D). Since SP-D exhibits cruciform structures composed of four trimeric

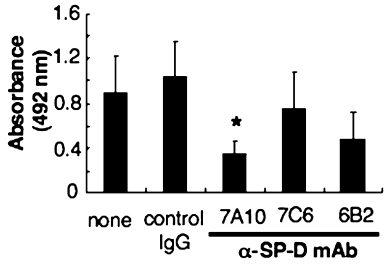

FIGURE 5: Effects of anti-SP-D monoclonal antibodies on the binding of SP-D to sMD2. Anti-SP-D monoclonal antibody 7A10, 7C6, or 6B2 or control mouse IgG (20 μg/mL) was incubated with biotinylated SP-D (2 μg/mL) at 37 °C for 1 h, and the mixture of antibody and SP-D was further incubated with sMD-2 (250 ng/well) coated onto microtiter wells at 37 °C for 5 h. The binding of the biotinylated SP-D was finally detected by using HRP-conjugated streptavidin, as described in Experimental Procedures.

subunits (*36*), CRF that lacks the amino terminus and the collagenous domain of SP-D and does not form oligomeric structures was also immobilized and examined for sMD-2 interaction (Figure 4E). There were significant interactions between CRF and sMD-2. However, the dissociation of sMD-2 from immobilized CRF appeared faster than that from immobilized SP-D, suggesting that oligomer formation affects the binding affinity.

Taken together, the findings demonstrate that SP-D binds MD-2 in a Ca^{2+}-dependent manner.

Anti-SP-D Monoclonal Antibody Blocks the Binding of SP-D to sMD-2. We next investigated the effect of anti-SP-D monoclonal antibodies on the binding of SP-D to sMD-2. Antibody 7A10, but not 7C6, significantly attenuated the binding of SP-D to sMD-2 coated onto microtiter wells (Figure 5). Antibody 6B2 decreased the level of binding by approximately 45%, but this was not significant.

These results suggest that the binding to sMD-2 is epitope-specific for anti-SP-D monoclonal antibodies, promoting a further analysis of the epitopes for these antibodies. Antibody 7A10 recognized the recombinant CRD and NCRD (the neck with the CRD), and antibody 7C6 bound to the NCRD but not the CRD (Figure 6A). The data indicate that the epitopes for antibodies 7A10 and 7C6 are associated with the CRD and the neck region, respectively, confirming our previous study (*11*). Antibody 6B2 also recognized the CRD and the NCRD, indicating that the epitope for antibody 6B2 is within the CRD. We next examined whether antibodies 7A10 and 6B2 recognized the same CRD epitope. When biotinylated 6B2 was used for the binding to reduced SP-D, unlabeled 6B2 completely blocked the binding of biotinylated 6B2 to SP-D (Figure 6B). Antibody 7C6 did not alter the binding of biotinylated 6B2 to SP-D, consistent with the results that antibody 7C6 recognizes the neck region. Antibody 7A10 attenuated but did not prevent binding of biotinylated 6B2 to SP-D. The results suggest that antibodies 7A10 and 6B2 recognize different epitopes within the CRD. The combined results demonstrate that SP-D binds to sMD-2 through the CRD and suggest that the epitope for 7A10 approximates the region of SP-D required for interaction with sMD-2.

Binding of SP-D to N-Glycopeptidase-Treated sMD-2 and sMD-2$^{N26A/N114A}$. Since MD-2 has N-linked oligosaccharide moieties, we investigated whether the sugar chains on sMD-2 were involved in SP-D binding. We first performed ligand

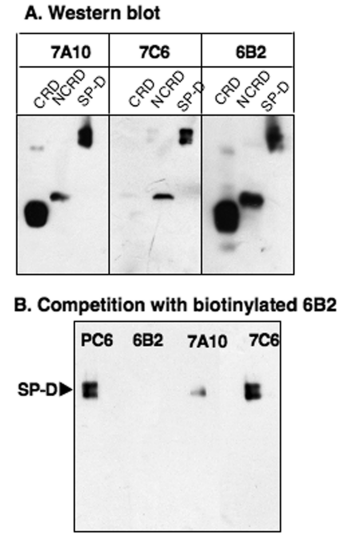

FIGURE 6: Epitope analysis of anti-SP-D monoclonal antibodies. (A) Western blot. Recombinant proteins (100 ng/lane) of SP-D, CRD, and NCRD (neck with the CRD) were electrophoresed under reducing conditions, and the proteins were transferred onto PVDF membranes. The membranes were incubated with anti-SP-D monoclonal antibody (10 μg/mL) 7A10, 7C6, or 6B2, and the binding of the antibody was detected by HRP-labeled anti-mouse IgG, as described in Experimental Procedures. (B) Competition with biotinylated 6B2 for binding to SP-D. SP-D (10 ng/lane) was electrophoresed under reducing conditions, and the protein was transferred onto the PVDF membrane. The membrane was incubated with anti-SP-D monoclonal antibody (50 μg/mL) 6B2, 7A10, or 7C6, or control monoclonal antibody PC6, and was further incubated with biotinylated antibody 6B2 (2 μg/mL). The binding of biotinylated antibody 6B2 to SP-D was finally detected by HRP-conjugated streptavidin, as described in Experimental Procedures.

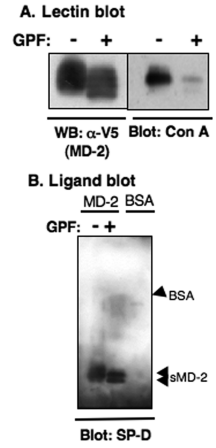

FIGURE 7: Binding of SP-D to glycopeptidase F-treated sMD-2. sMD-2 was treated with glycopeptidase F (GPF) at 37 °C for 17 h. Untreated and treated sMD-2 were electrophoresed and transferred onto PVDF membranes. (A) Analysis of glycopeptidase F-treated sMD-2. The Western blot (WB) for sMD-2 was performed by using anti-V5 antibody. The oligosaccharide moieties of sMD-2 were visualized by lectin blot using biotin-conjugated concanavalin A (Blot: Con A). (B) Binding of SP-D to sMD-2 (GPF: + and −) or BSA was also detected by using anti-SP-D polyclonal antibody (Blot: SP-D), as described in Experimental Procedures. Arrowheads indicate the positions of sMD-2 and BSA.

blot analysis of sMD-2 treated with N-glycopeptidase F (Figure 7). Glycopeptidase-treated sMD-2 exhibited a lower molecular mass of the protein and was faintly detected by concanavalin A (Figure 7A). Concanavalin A evidently bound to the upper band which is presumed to be undigested

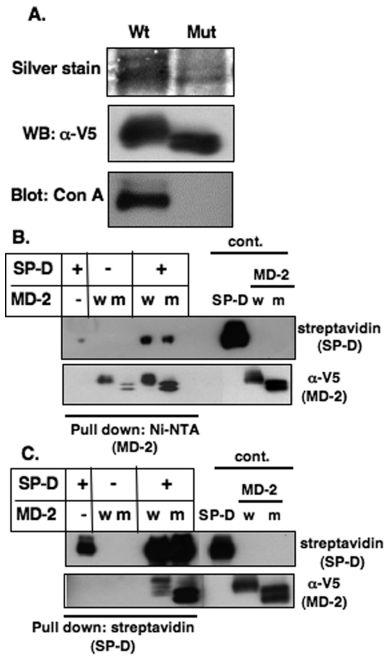

FIGURE 8: SP-D binds to sMD-$2^{N26A/N114A}$. (A) Analysis of partially purified sMD-2. Partially purified wild-type (Wt) sMD-2 and sMD-$2^{N26A/N114A}$ (Mut) (15 μL/lane) were analyzed by silver staining, Western blotting using anti-V5 antibody to detect sMD-2, and lectin blot using concanavalin A (Con A). (B and C) Pull-down assays of biotinylated SP-D and sMD-2. Biotinylated SP-D (250 ng) was incubated with partially purified sMD-2 (250 μL) at 37 °C for 2 h. sMD-2 or biotinylated SP-D was pulled down with Ni-NTA agarose (B) or streptavidin-conjugated agarose (C), respectively. The precipitates were finally analyzed by Western blot by using anti-V5 antibody and HRP-labeled streptavidin as described in Experimental Procedures. Letters w and m denote wild-type sMD-2 and mutant sMD-2, respectively.

sMD-2. Ligand blot analysis showed that SP-D bound to the lower band of glycopeptidase-treated sMD-2 in addition to the upper band (Figure 7B). However, the presence of carbohydrate moieties in two bands of glycopeptidase-treated sMD-2 cannot be excluded since the double bands seen in binding of SP-D to glycopeptidase-treated sMD-2 (Figure 7B) appear to be similar to those in concanavalin A binding (Figure 7A). Thus, we constructed an sMD-2 mutant in which consensus sequences for N-linked glycosylation sites were mutated.

The interaction of SP-D with unglycosylated sMD-2 was further determined by using the sMD-2 mutant (sMD-$2^{N26A/N114A}$) in which the only potential sites for N-linked glycosylation at Asn^{26} and Asn^{114} were replaced with Ala. sMD-$2^{N26A/N114A}$ was expressed in a baculovirus−insect cell system. Because the expression level of the mutant protein was quite low and sMD-$2^{N26A/N114A}$ was somewhat unstable, sMD-2 and sMD-$2^{N26A/N114A}$ were partially purified from the medium and 250 μL of the partially purified protein preparation was used for the experiments. sMD-2 and sMD-$2^{N26A/N114A}$ were electrophoresed (Figure 8A). Silver stain and Western blot revealed that the mutant protein exhibited a lower molecular mass than wild-type sMD-2. Mutant sMD-2 was not detected with concanavalin A. When SP-D and sMD-2 were incubated and pulled down with Ni-NTA agarose, both wild-type sMD-2 and mutant sMD-2 cosedimented with SP-D (Figure 8B). Furthermore, biotinylated SP-D pulled down sMD-$2^{N26A/N114A}$ as well as wild-type sMD-2 (Figure 8C). sMD-$2^{N26A/N114A}$ protein exhibited two bands on the membrane, although concanavalin A did not recognize either of these proteins. The double bands are attributed to limited degradation of the sMD-$2^{N26A/N114A}$ protein.

DISCUSSION

In this study, we demonstrated the direct binding of SP-D to sMD-2 by several methods. SP-D bound to sMD-2 coated onto microtiter wells. Ligand blotting assays were used to demonstrate that endotoxin-depleted SP-D binds endotoxin-free sMD-2. sMD-2 and biotinylated SP-D cosedimented each other in bead pull-down assays. BIAcore analysis demonstrated high-affinity interactions between these proteins. We subsequently focused on the mechanism of binding of SP-D to sMD-2. Binding requires the SP-D CRD and Ca^{2+}. The binding of SP-D to sMD-2 was blocked in the presence of excess mannose. SP-D bound to the sMD-2 mutant in which consensus sequences for N-linked glycosylation sites were mutated.

SP-D binds to sMD-2 in the presence of Ca^{2+}. Chelation of Ca^{2+} weakened binding of SP-D to sMD-2. In addition, excess mannose blocked the binding of SP-D to solid phase sMD-2. Because SP-D possesses a C-type lectin domain, this finding might suggest that SP-D binds to sMD-2 by its lectin property. sMD-2 used in this study is produced by insect cells. Although proteins that are N-glycosylated in mammalian cells are generally also glycosylated in insect cells, the carbohydrate moieties of proteins expressed by insect cells are different. The pentasaccharide core common to N-glycoproteins such as Man3GlcNAc2 is synthesized in insect cells, but terminal modifications that lead to the formation of more complex oligosaccharides do not occur in insect cells (*37*). Thus, we examined whether SP-D interacts with N-glycan attached on the sMD-2 protein or with the peptide portion of the protein. The glycopeptidase-treated sMD-2 was examined for SP-D binding. SP-D binds the glycopeptidase-treated sMD-2, which concanavalin A faintly recognizes (see Figure 7). This may suggest that the presence of carbohydrate moieties on the sMD-2 protein cannot be excluded. In addition, the complete inhibition of binding of SP-D to the native sMD-2 coated onto microtiter wells (see Figure 2B) raises the possibility that SP-D binds to the glycan attached to the sMD-2 protein. We showed that SP-D binds to sMD-$2^{N26A/N114A}$ (see Figure 8) that lacks the consensus for N-glycosylation, indicating that the N-glycan on the mutant protein is not required for SP-D binding. This may suggest that SP-D binds to the mutant protein by a protein−protein interaction since MD-2 does not contain an O-linked glycosylation site (*38*). However, this finding does not necessarily mean the direct binding of SP-D to the peptide portion of wild-type sMD-2. It remains a possibility that SP-D interacts with wild-type MD-2 through the N-glycan.

Alternatively, the binding of SP-D to sMD-$2^{N26A/N114A}$ may raise another possibility that SP-D binds to the peptide portion of wild-type sMD-2. Inhibition of the binding of SP-D to sMD-2 by mannose could be interpreted as follows; the binding with excess mannose may alter the structure of SP-D, which could prevent SP-D from interacting with the MD-2 protein. It is possible that a region near or adjacent to the carbohydrate recognition site of SP-D may be involved

in the recognition of MD-2. A previous study (*11*) has also shown that N-linked oligosaccharide moieties of TLR2 and TLR4 are not required for the recognition by SP-D, although excess carbohydrates significantly attenuate the binding of SP-D to TLRs. In addition, the binding of SP-D to phosphatidylinositol is abolished in the presence of myoinositol, but SP-D fails to bind to lysophosphatidylinositol (*6*). SP-D binds to fatty acids in a Ca^{2+}-dependent manner, and its binding is inhibited by 30 mM glucose (*39*).

Anti-SP-D monoclonal antibodies 7A10 and 6B2 attenuate the binding of SP-D to sMD-2, although the inhibition by antibody 6B2 is not significant. Since the epitopes for antibodies 7A10 and 6B2 are located at the CRD, the functional domain for the interaction with MD-2 is considered to be the CRD. The competition experiments with biotinylated 6B2 indicate that the epitopes for these antibodies are within the CRD but distinct from each other. This may explain the different inhibitory effects caused by antibodies 7A10 and 6B2. Antibody 7A10 inhibited the binding of SP-D to sMD-2 more strongly than antibody 6B2. Ca^{2+}-dependent binding of SP-D to sMD-2 supports the conclusion that the CRD is a functional domain for sMD-2 binding.

MD-2 is an essential accessory protein for TLR4 (*16*). MD-2 directly binds to LPS (*34, 38*). The $Cys^{95} \rightarrow$ Tyr mutation of MD-2 abolishes endotoxin-induced signaling (*40*), indicating the importance of Cys^{95} in LPS signaling. It is unknown how MD-2 interacts with LPS. Eritoran, an endotoxin antagonist, binds the hydrophobic pocket in human MD-2 (*17*). Because MD-2 belongs to a novel family of proteins, the MD-2-related lipid recognition (ML) family (*19, 22*), MD-2 is thought to recognize lipid A, the minimal structure of LPS. Lipid A is composed of six C_{12-14} fatty acids linked to a phosphorylated N-acetylglucosamine dimer. SP-D is also a lipid binding protein, recognizing both phosphatidylinositol and glucosylceramide (*6, 41*). Both a hydrophobic acyl chain and a polar headgroup are required for lipid recognition by SP-D. SP-D recognizes Rc and Rd LPS but does not appear to bind lipid A (*9*). A recent report indicates that interactions with the side chain of inner core heptoses provide a potential mechanism for the recognition of diverse types of LPS by SP-D (*42*). Although it is possible that SP-D and MD-2 interact with each other through LPS associated with each protein, ligand blot analysis reveals that the LPS-depleted SP-D binds to the electrophoresed sMD-2 on the membrane. Under this condition, LPS is separated from the sMD-2 protein. Thus, the SP-D protein recognizes the sMD-2 protein, although the region in the sMD-2 molecule that is involved in interaction with SP-D remains to be elucidated.

In a previous study, we showed that SP-A downregulates smooth LPS-induced TNF-α secretion and NF-κB activation by direct interaction with TLR4 and MD-2 (*27*). We have also reported that SP-D binds to TLR4 (*11*), and this study demonstrates that SP-D interacts with MD-2. Because the formation of the TLR4−MD-2 complex is crucial for triggering LPS signaling (*16*), the binding of SP-D to TLR4 and MD-2 could alter LPS-elicited cellular responses. Whether SP-D similarly alters TLR4−MD-2 complex-mediated signaling stimulated with LPS is under investigation.

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REFERENCES

1. Day, A. J. (1994) The C-type carbohydrate recognition domain (CRD) superfamily. *Biochem. Soc. Trans. 22*, 83–88.
2. Kuroki, Y., and Voelker, D. R. (1994) Pulmonary surfactant proteins. *J. Biol. Chem. 269*, 25943–25946.
3. LeVine, A. M., Bruno, M. D., Huelsman, K. M., Ross, G. F., Whitsett, J. A., and Korfhagen, T. R. (1997) Surfactant protein A-deficient mice are susceptible to group B streptococcal infection. *J. Immunol. 158*, 4336–4340.
4. LeVine, A. M., Whitsett, J., Hartshorn, K. L., Crouch, E. C., and Korfhagen, T. R. (2001) Surfactant protein D enhances clearance of influenza A virus from the lung in vivo. *J. Immunol. 167*, 5868–5873.
5. LeVine, A. M., Whitsett, J. A., Gwozdz, J. A., Richradson, T. R., Fisher, J. H., Burhans, M. S., and Korfhagen, T. R. (2000) Distinct effects of surfactant protein A or D deficiency during bacterial infection on the lung. *J. Immunol. 165*, 3934–3940.
6. Ogasawara, Y., Kuroki, Y., and Akino, T. (1992) Pulmonary surfactant protein D specifically binds to phosphatidylinositol. *J. Biol. Chem. 267*, 21244–21249.
7. Persson, A. V., Gibbons, B. J., Shoemaker, J. D., Moxley, M. A., and Longmore, W. J. (1992) The major glycolipid recognized by SP-D in surfactant is phosphatidylinositol. *Biochemistry 31*, 12183–12189.
8. Hartshorn, K. L., Crouch, E. C., White, M. R., Eggleton, P., Tauber, A. I., Chang, D., and Sastry, K. (1994) Evidence for a protective role of pulmonary surfactant protein D (SP-D) against influenza A viruses. *J. Clin. Invest. 94*, 311–319.
9. Kuan, S.-F., Rust, K., and Crouch, E. (1992) Interaction of surfactant protein D with bacterial lipopolysaccharides. Surfactant protein D is an *Escherichia coli*-binding protein in bronchoalveolar lavage. *J. Clin. Invest. 90*, 97–106.
10. Kuroki, Y., Takahashi, M., and Nishitani, C. (2007) Pulmonary collectins in innate immunity of the lung. *Cell. Microbiol. 9*, 1871–1879.
11. Ohya, M., Nishitani, C., Sano, H., Yamada, C., Mitsuzawa, H., Shimizu, T., Saito, T., Smith, K., Crouch, E., and Kuroki, Y. (2006) 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. *Biochemistry 45*, 8657–8664.
12. Sano, H., Chiba, H., Iwaki, D., Sohma, H., Voelker, D. R., and Kuroki, Y. (2000) Surfactant proteins A and D bind CD14 by different mechanisms. *J. Biol. Chem. 275*, 22442–22451.
13. Akira, S., and Takeda, H. (2004) Toll-like receptor signaling. *Nat. Rev. Immunol. 4*, 499–511.
14. Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. B. (1999) Phylogenetic perspectives in innate immunity. *Science 284*, 1313–1318.
15. Wright, S. D., Ramos, R. A., Tobias, P. S., Ulevitch, R. J., and Mathison, J. C. (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. *Science 249*, 1431–1433.
16. Shimazu, R., Akashi, S., Ogata, H., Nagai, Y., Fukudome, K., Miyake, K., and Kimoto, M. (1999) MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med. 189*, 1777–1782.
17. Kim, H. M., Park, B. S., Kim, J.-I., Kim, S. E., Lee, J., Oh, S. C., Enknbayar, P., Matsushima, N., Lee, H., Yoo, O. J., and Lee, J.-O. (2007) Crystal structure of the TLR4-MD-2 complex with bound endotoxin antagonist Eritorian. *Cell 130*, 906–917.
18. Akashi, S., Saitoh, S., Wakabayashi, Y., Kikuchi, T., Takamura, N., Nagai, Y., Kusumoto, Y., Fukase, K., Kusumoto, S., Adachi, Y., Kusugi, A., and Miyake, K. (2003) Lipopolysaccharide

interaction with cell surface Toll-like receptor 4-MD-2: Higher affinity than that with MD-2 or CD14. *J. Exp. Med. 198*, 1035–1042.
19. Gangloff, M., and Gay, N. J. (2004) MD-2: The Toll 'gatekeeper' in endotoxin signaling. *Trends Biochem. Sci. 29*, 294–300.
20. Nagai, Y., Akashi, S., Nagafuku, M., Ogata, M., Iwakura, Y., Akira, S., Kitamura, T., Kosugi, A., Kimoto, M., and Miyake, K. (2002) Essential role of MD-2 in LPS responsiveness and TLR4 distribution. *Nat. Immunol. 3*, 667–672.
21. Poltorak, A., Ricciardi-Castagnoli, P., Citterio, S., and Beutler, B. (2000) Physical contact between lipopolysaccharide and toll-like receptor 4 revealed by genetic complementation. *Proc. Natl. Acad. Sci. U.S.A. 97*, 2163–2167.
22. Inohara, N., and Nunez, G. (2002) ML: A conserved domain involved in innate immunity and lipid metabolism. *Trends Biochem. Sci. 27*, 219–221.
23. Visintin, A., Latz, E., Monks, B. G., Espevik, T., and Golenbock, D. T. (2003) Lysines 128 and 132 enable lipopolysaccharide binding to MD-2, leading to Toll-like receptor-4 aggregation and signal transduction. *J. Biol. Chem. 278*, 48313–48320.
24. Mullen, G. E. D., Kennedy, M. N., Visintin, A., Mazzoni, A., Leifer, C. A., Davies, D. R., and Segal, D. M. (2003) The role of disulfide binds in the assembly and function of MD-2. *Proc. Natl. Acad. Sci. U.S.A. 100*, 3929–3924.
25. Murakami, S., Iwaki, D., Mitsuzawa, H., Sano, H., Takahashi, H., Voelker, D. R., Akino, T., and Kuroki, Y. (2002) Surfactant protein A inhibits peptidoglycan-induced tumor necrosis factor-α secretion in U937 cells and alveolar macrophages by direct interaction with Toll-like receptor 2. *J. Biol. Chem. 277*, 6830–6837.
26. Sato, M., Sano, H., Iwaki, D., Kudo, K., Konishi, M., Takahashi, H., Takahashi, T., Imaizumi, H., Asai, Y., and Kuroki, Y. (2003) Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF-κB activation and TNF-α secretion are down-regulated by lung collectin surfactant protein A. *J. Immunol. 171*, 417–425.
27. Yamada, C., Sano, H., Shimizu, T., Mitsuzawa, H., Nishitani, C., Himi, T., and Kuroki, Y. (2006) Surfactant protein A directly interacts with TLR4 and MD-2 and regulates inflammatory cellular response: Importance of supratrimeric oligomerization. *J. Biol. Chem. 281*, 21771–21780.
28. Kuroki, Y., Tsutahara, S., Shijubo, N., Takahashi, H., Shiratori, M., Hattori, A., Honda, Y., Abe, S., and Akino, T. (1993) Elevated levels of lung surfactant protein A in sera from patients with idiopathic pulmonary fibrosis and pulmonary alveolar proteinosis. *Am. Rev. Respir. Dis. 147*, 723–729.
29. Hyakushima, N., Mitsuzawa, H., Nishitani, C., Sano, H., Kuronuma, K., Konishi, M., Himi, T., Miyake, K., and Kuroki, Y. (2004) Interaction of soluble form of recombinant extracellular TLR4 domain with MD-2 enables lipopolysaccharide binding and attenuates TLR4-mediated signaling. *J. Immunol. 173*, 6949–6954.
30. Mitsuzawa, H., Nishitani, C., Hyakushima, N., Shimizu, T., Sano, H., Matsushima, N., Fukase, K., and Kuroki, Y. (2006) Recombinant soluble forms of extracellular Toll-like receptor 4 domain and MD-2 inhibit LPS binding on cell surafce and dampen LPS-induced pulmonary inflammation in mice. *J. Immunol. 177*, 8133–8139.
31. Nagae, H., Takahashi, H., Kuroki, Y., Honda, Y., Nagata, A., Ogasawara, Y., Abe, S., and Akino, T. (1997) Enzyme-linked immunosorbent assay using F(ab′)2 fragment for the detection of human pulmonary surfactant protein D in sera. *Clin. Chim. Acta 266*, 157–171.
32. Crouch, E., Tu, E., Briner, D., McDonald, B., Smith, K., Holmskov, U., and Hartshorn, K. (2005) Ligand specificity of human surfactant protein D: Expression of a mutant trimeric collectin that shows enhanced interactions with influenza virus. *J. Biol. Chem. 280*, 17046–17056.
33. Mason, R. J., Nielsen, L. D., Kuroki, Y., Matsuura, E., Freed, J. H., and Shannon, J. M. (1998) A 50-kDa variant form of human surfactant protein D. *Eur. Respir. J. 12*, 1147–1155.
34. Viriyakosol, S., Tobias, P. S., Kitchens, R. L., and Kirkland, T. N. (2001) MD-2 binds to bacterial lipopolysaccharide. *J. Biol. Chem. 276*, 38044–38051.
35. Visintin, A., Mazzoni, A., Spitzer, J. A., and Segal, D. M. (2001) Secreted MD-2 is a large polymeric protein that efficiently confers lipopolysaccharide sensitivity to Toll-like receptor 4. *Proc. Natl. Acad. Sci. U.S.A. 98*, 12156–12161.
36. Crouch, E., Persson, A., Chang, D., and Heuser, J. (1994) Molecular structure of pulmonary surfactant protein D (SP-D). *J. Biol. Chem. 269*, 17311–17319.
37. O'Reilly, D. R., Miller, L. K., and Luckow, V. A. (1992) *Baculovirus Expression Vector. A Laboratory Manual*, W. H. Freeman and Co., New York.
38. da Silva Correia, J., and Ulevitch, R. J. (2002) MD-2 and TLR4 N-linked glycosylations are important for a functional lipopolysaccharide receptor. *J. Biol. Chem. 277*, 1845–1854.
39. DeSilva, N. S., Ofek, I., and Crouch, E. C. (2003) Interactions of surfactant protein D with fatty acids. *Am. J. Respir. Cell Mol. Biol. 29*, 757–770.
40. Schromm, A. B., Lien, E., Henneke, P., Chow, J. C., Yoshimura, A., Heine, H., Latz, E., Monks, B. G., Schwartz, D. A., Miyake, K., and Golenbock, D. T. (2001) Molecular genetic analysis of an endotoxin nonresponder mutant cell line: A point mutation in a conserved region of MD-2 abolishes endotoxin-induced signaling. *J. Exp. Med. 194*, 79–88.
41. Kuroki, Y., Gasa, S., Ogasawara, Y., Shiratori, M., Makita, A., and Akino, T. (1992) Binding specificity of lung surfactant protein SP-D for glucosylceramide. *Biochem. Biophys. Res. Commun. 187*, 963–969.
42. Wang, H., Head, J., Kosma, P., Brade, H., Müller-Loennies, S., Sheikh, S., McDonald, B., Smith, K., Cafarella, T., Seaton, B., and Crouch, E. (2008) Recognition of heptoses and the inner core of bacterial lipopolysaccharides by surfactant protein D. *Biochemistry 47*, 710–720.

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