The Journal of Immunology

RESEARCH ARTICLE | JULY 01 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[1]

Morihito Sato; ... et. al

J Immunol (2003) 171 (1): 417–425.

https://doi.org/10.4049/jimmunol.171.1.417

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[1]

Morihito Sato,*† Hitomi Sano,* Daisuke Iwaki,* Kazumi Kudo,* Masanori Konishi,* Hiroki Takahashi,‡ Toru Takahashi,* Hitoshi Imaizumi,† Yasufumi Asai,† and Yoshio Kuroki[2]*

The lung collectin surfactant protein A (SP-A) has been implicated in the regulation of pulmonary host defense and inflammation. Zymosan induces proinflammatory cytokines in immune cells. Toll-like receptor (TLR)2 has been shown to be involved in zymosan-induced signaling. We first investigated the interaction of TLR2 with zymosan. Zymosan cosedimented the soluble form of rTLR2 possessing the putative extracellular domain (sTLR2). sTLR2 directly bound to zymosan with an apparent binding constant of 48 nM. We next examined whether SP-A modulated zymosan-induced cellular responses. SP-A significantly attenuated zymosan-induced TNF-α secretion in RAW264.7 cells and alveolar macrophages in a concentration-dependent manner. Although zymosan failed to cosediment SP-A, SP-A significantly reduced zymosan-elicited NF-κB activation in TLR2-transfected human embryonic kidney 293 cells. Because we have shown that SP-A binds to sTLR2, we also examined whether SP-A affected the binding of sTLR2 to zymosan. SP-A significantly attenuated the direct binding of sTLR2 to zymosan in a concentration-dependent fashion. From these results, we conclude that 1) TLR2 directly binds zymosan, 2) SP-A can alter zymosan-TLR2 interaction, and 3) SP-A down-regulates TLR2-mediated signaling and TNF-α secretion stimulated by zymosan. This study supports an important role of SP-A in controlling pulmonary inflammation caused by microbial pathogens. ***The Journal of Immunology,*** **2003, 171: 417–425.**

Inhalation of fungi or yeast causes organic dust toxic syndrome, which is characterized by lung cell reactions involving inflammation and immune responses (1, 2). Zymosan is a yeast cell wall particle containing mainly polysaccharides, of which β-glucan and mannan are the major constituents (3). Zymosan can activate macrophages, monocytes, and leukocytes (4–6), resulting in the stimulated secretion of inflammatory products including TNF-α, IL-8, hydrogen peroxide, and arachidonic acid (7–9). Intratracheal challenge of zymosan has been shown to induce pulmonary inflammation in animal models (10). Intrapulmonary TNF-α production causes lung tissue injury and severe pulmonary inflammation in disseminated candidemia under immunosuppressive conditions (11).

Toll-like receptors (TLRs)[3] have been identified to be functional receptors for microbial ligands to trigger inflammatory responses (12–14). The studies with transgenic mice harboring null alleles for TLR4 demonstrate that TLR4 is a signaling receptor for LPS (15, 16). MD-2 has been shown to be required for TLR4-mediated LPS signaling (17). Macrophages from TLR2-deficient mice were unable to produce proinflammatory cytokines in response to peptidoglycan (PGN) and Gram-positive bacteria (16), indicating that TLR2 is responsible for the recognition of Gram-positive bacteria. Although the ligands for TLRs have been identified (18–27), the mechanisms by which TLRs recognize their ligands are still not understood. One recent report has shown that LPS is cross-linked specifically to TLR4 and MD-2 only when coexpressed with CD14 (28), indicating that LPS directly binds to the LPS receptor complex consisting of TLR4, MD-2, and CD14. In contrast to LPS signaling, PGN can elicit NF-κB activation in the absence of CD14 in TLR2-trasfected cells, although CD14 enhances PGN signaling (29, 30), suggesting that TLR2 alone can interact with PGN regardless of whether CD14 is coexpressed. The study from this laboratory demonstrates that the extracellular TLR2 domain directly binds PGN (31). Zymosan has also been demonstrated to induce NF-κB activation in TLR2-transfected Chinese hamster ovary cells, but this activation was inhibited when a dominant-negative mutant of TLR2, in which Pro^{681} was replaced with His (TLR2-P681H), was coexpressed (5). The report also shows that transfection of the dominant-negative mutant of TLR2 blocks zymosan-stimulated TNF-α production in RAW-TT10 cells. Taken together, these results indicate that zymosan induces TLR2-mediated NF-κB activation and TNF-α production. However, the mechanism by which TLR2 recognizes zymosan remains unknown.

Pulmonary surfactant is a mixture of lipids and proteins that covers the peripheral airway and functions to keep alveoli from collapsing at expiration (32). Surfactant protein A (SP-A) is the major constituent of the surfactant and belongs to the collectin subgroup of the C-type lectin superfamily along with surfactant protein D, mannose-binding protein, and conglutinin (33, 34). SP-A is now thought to be an important component of the innate immune system of the lung. Proinflammatory cytokine secretion is

Departments of *Biochemistry and †Traumatology and Critical Care Medicine, and ‡Third Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan

Received for publication November 8, 2002. Accepted for publication April 25, 2003.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This study was supported in part by a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan and a grant from the Akiyama Foundation.

[2] Address correspondence and reprint requests to Dr. Yoshio Kuroki, Department of Biochemistry, Sapporo Medical University School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan. E-mail address: kurokiy@sapmed.ac.jp

[3] Abbreviations used in this paper: TLR, Toll-like receptor; PGN, peptidoglycan; SP-A, surfactant protein A; HEK, human embryonic kidney; sTLR2, soluble form of extracellular TLR2 domain; ^{125}I-sTLR2, ^{125}I-labeled sTLR2; ^{125}I-SP-A, ^{125}I-labeled SP-A.

0022-1767/03/$02.00

significantly elevated in mice homozygous for null alleles of SP-A after intratracheal challenge with smooth LPS (35). In vitro studies have also revealed that SP-A attenuates TNF-α secretion elicited by smooth LPS, which is not a ligand for SP-A, in alveolar macrophages (36, 37). In contrast, the protein rather increases TNF-α secretion stimulated with rough LPS lacking *O*-Ag, an SP-A ligand (37). PGN derived from *Staphylococcus aureus*, which is not an SP-A ligand, also induces proinflammatory cytokines in leukocytes, but PGN-elicited TNF-α secretion is inhibited by SP-A (38). These in vivo and in vitro studies provide compelling evidence that SP-A has been implicated in the regulation of pulmonary inflammation caused by bacterial ligands. Interactions of SP-A with the pattern recognition receptors, CD14 and/or TLR2, constitute likely mechanisms by which SP-A modulates cellular responses caused by LPS and PGN (37–39).

In this study, we focused on cellular responses elicited by zymosan as a constituent of fungi or yeast that may cause organic dust toxic syndrome and fungal infection under immunosuppressive conditions. The purposes of this study were to determine 1) the interaction of TLR2 with zymosan, 2) the role of SP-A in modulating zymosan-induced signaling and proinflammatory cytokine release, 3) the interaction of SP-A with zymosan, and 4) the role of SP-A in altering the interaction of TLR2 with zymosan. This is the first report demonstrating that TLR2 directly binds zymosan. Our findings also demonstrate that SP-A down-regulates zymosan-induced signaling and proinflammatory cytokine secretion by attenuating the binding of TLR2 to zymosan. Our findings provide molecular bases for the recognition of zymosan by TLR2 and for a dampening role of SP-A in controlling pulmonary inflammation caused by fungi.

Materials and Methods

Materials

Zymosan A derived from *Saccharomyces cerevisiae*, polymyxin B, and polymyxin B-agarose were purchased from Sigma-Aldrich (St. Louis, MO). The murine macrophage cell line RAW264.7 (TIB-71) and human embryonic kidney (HEK)293 cells (CRL-1573) were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in endotoxin-free DMEM containing 10% heat-inactivated FCS.

TNF-α secretion in alveolar macrophages and RAW264.7 cells

Alveolar macrophages were isolated from Sprague Dawley rats by lavaging the lungs with pyrogen-free normal saline (Otsuka Pharmaceutical, Tokyo, Japan), as described previously (38).

RAW264.7 cells or rat alveolar macrophages were seeded into 24-well plates (5×10^5/well) in 0.5 ml of DMEM containing 10% FCS. After incubating the cells for 2 h, the medium was replaced, and the indicated concentrations of zymosan were added into the wells and incubated in the presence or the absence of SP-A for 24 h. In some experiments, the cells were preincubated with SP-A for the indicated periods, washed once with 0.5 ml of PBS, and then incubated with the medium containing zymosan. The culture medium was finally collected.

TNF-α secretion was measured using an L929 cell bioassay (37) based on the method described by Flick et al. (40). The L929 cells were seeded into 96-well plates (6×10^4/well) in 100 μl/well RPMI 1640 containing 10% FCS and 2 μg/ml actinomycin D (Sigma-Aldrich). Dilutions of standard rTNF-α (1–2000 pg/ml; Sigma-Aldrich) or samples (1/100 to 1/150) in a volume of 100 μl/well were added, and the cells were incubated at room temperature for 15 min followed by incubation overnight at 37°C with 5% CO_2. On the next day, the medium was removed, and the cells were stained with 0.2% (w/v) crystal violet for 10 min. The wells were then washed with water, and 100 μl/well 33% (v/v) acetic acid was added to extract the retained crystal violet. The absorbance at 570 nm was finally measured.

NF-κB reporter gene assay

A 2.6-kb human TLR2 cDNA was subcloned into pcDNA3.1(+) (Invitrogen, San Diego, CA), and activation of NF-κB was measured as described previously (29). cDNAs for human TLR4 and human MD-2 were generous gifts from Dr. K. Miyake (University of Tokyo, Tokyo, Japan). HEK293 cells were plated at 2×10^5 cells/well in 24-well plates on the day before transfection. The cells were transiently transfected by FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Basel Switzerland) with 30 ng of an NF-κB reporter construct (pNF-κB-Luc; Stratagene, La Jolla, CA) and 3 ng of a construct that directed expression of *Renilla* luciferase under the control of the constitutively active thymidine kinase promoter (pRL-TK; Promega, Madison, WI), together with 166 ng of TLR2 cDNA in pcDNA3.1(+) vector, or with 83 ng of TLR4 cDNA in pcDNA3.1(+) vector and 83 ng of MD-2 cDNA in pEFBOS vector. Forty-eight hours after transfection, the cells were stimulated with the indicated concentrations of zymosan for 18 h, and luciferase activity was measured by the Dual Luciferase reporter assay system (Promega) according to the manufacturer's instructions. To examine the effect of SP-A on zymosan-induced NF-κB activation, SP-A (20 μg/ml) was included in the incubation medium containing zymosan.

A soluble form of recombinant extracellular TLR2 domain (sTLR2)

The recombinant soluble form of TLR2 consisting of the putative extracellular domain (Met^1-Arg^{587}) and a His_6 tag at the C-terminal end was constructed, and the sTLR2 protein was expressed in the baculovirus-insect cell expression system using the methods described by O'Reilly et al. (41), as described previously (31). The protein was purified from the medium using a column of nickel-nitrilotriacetic acid beads (Qiagen, Valencia, CA) by a method described previously (39). Polyclonal Ab against sTLR2 was prepared as described previously (31).

Binding of sTLR2 to zymosan

The binding study of sTLR2 to zymosan by sedimentation was performed by modification of the method previously described for PGN binding (31). We first tested whether zymosan was localized in the pellet after sedimentation. One hundred micrograms of zymosan was centrifuged at $2000 \times g$ for 5 min and separated into a supernatant and a pellet. The ability of each fraction to induce TNF-α secretion from RAW264.7 cells was examined by measuring secreted TNF-α using an L929 cell bioassay. The pellet fraction contained >99% TNF-α-inducing activity, indicating that zymosan is sedimentable by centrifugation.

The sTLR2 protein was iodinated by the method of Bolton and Hunter (42) using Bolton-Hunter reagent (Amersham Biosciences, Arlington Heights, IL). The specific radioactivity ranged from 561 to 711 cpm/ng, and >80% of the radioactivity was precipitated by treatment with 10% (w/v) TCA. Glass tubes that had been coated with silicone (SIL-COAT5; Iwaki Glass, Tokyo, Japan) were used in the experiments. To visualize the binding of sTLR2 to zymosan, the preparation of ^{125}I-labeled sTLR2 (^{125}I-sTLR2; 5 μg/100 μl) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 5% (w/v) BSA (buffer A) was centrifuged at $2000 \times g$ for 5 min, and the supernatant obtained was mixed with 5 μg of zymosan in a fresh silicone-coated tube. The mixture of the protein and the zymosan was incubated at 37°C for 6 h with gentle shaking. After the incubation, the mixture was centrifuged at $2000 \times g$ for 5 min, and the supernatant was removed. The pellet was suspended with 200 μl of PBS containing 0.1% (v/v) Triton X-100 (buffer B) and centrifuged again at $2000 \times g$ for 5 min, and the supernatant was removed. This washing step was repeated three times. The final pellet obtained was suspended in 30 μl of the sampling buffer for SDS-PAGE and electrophoresed. After SDS-PAGE, the gel was dried and exposed to Kodak X-OMAT film (Eastman Kodak, Rochester, NY) at −80°C overnight for autoradiography.

The binding characteristics were also analyzed by sedimentation assay using ^{125}I-sTLR2. The indicated concentrations of ^{125}I-sTLR2 were incubated with 1 μg of zymosan in 100 μl of buffer A in silicone-coated glass tubes at 37°C for various periods with gentle shaking. After the incubation, the reaction mixture in the glass tube was transferred into a polypropylene tube. PBS (200 μl) was added into the glass tube and used to wash the tube. The recovered PBS was combined with the reaction mixture in the polypropylene tube. The mixture was centrifuged at $10{,}000 \times g$ for 15 min, and the supernatant was removed. The resultant pellet was washed three times with 200 μl of buffer B. The final pellet was resuspended with the buffer B and transferred into a new tube. The radioactivity of ^{125}I-sTLR2 binding to the zymosan pellet was measured using a gamma radiation counter. Specific binding was determined by subtracting the binding obtained in the absence of zymosan from that in the presence of zymosan. In some experiments, ^{125}I-sTLR2 (2 μg/ml) was incubated with zymosan in the presence of excess unlabeled sTLR2 to examine the specificity of the sTLR2 binding.

To examine the effect of SP-A on the binding of sTLR2 to zymosan, the indicated concentration of SP-A was preincubated with 2 μg/ml ^{125}I-sTLR2 for 1 h. After preincubation, 2 μg of zymosan was added, and the

mixture of the proteins and the zymosan was further incubated for 6 h. The amount of sTLR2 binding to the zymosan pellet was determined as described above.

Surfactant protein A

Human SP-A was purified from the delipidated surfactant that had been isolated from bronchoalveolar lavage fluids of patients with alveolar proteinosis, in the manner described previously (43, 44). Endotoxin in SP-A preparation was removed by polymyxin B-agarose in the presence of octyl-β-D-glucoside (Sigma-Aldrich) by the method described by McIntosh et al. (36). The endotoxin level in the preparations of polymyxin-treated SP-A was <0.02 pg/μg SP-A, when determined by chromogenic assay using *Limulus* amebocyte lysate system (Endospecy, Seikagaku Kogyo, Tokyo). Polymyxin-treated SP-A was used for the experiments in this study.

Binding of SP-A to zymosan

SP-A was iodinated using Bolton-Hunter reagent as described above for the iodination of sTLR2. The specific activity ranged from 300 to 342 cpm/ng, and >94% of the radioactivity was precipitated by treatment with 10% (w/v) TCA. The preparation of ^{125}I-labeled SP-A (^{125}I-SP-A; 250 ng/50 μl) in 20 mM Tris buffer (pH 7.4) containing 0.15 M NaCl and 3% (w/v) BSA (buffer C) and zymosan (25 μg/100 μl) in the buffer C were separately centrifuged at 10,000 × *g* at 4°C for 10 min, because zymosan can be sedimentable by centrifugation as described above. The supernatant from the SP-A preparation was added to the zymosan pellet. A mixture of the protein and the zymosan was suspended and incubated in the presence of 5 mM $CaCl_2$ at 37°C for 1 h. The mixture was then centrifuged at 10,000 × *g* at 4°C for 10 min. The supernatant was stored, and washing of the resultant pellet with 50 μl of the buffer C was repeated three times. In some experiments, the binding assay was performed in the absence of zymosan. The radioactivities of the combined supernatant and the pellet were finally measured using a gamma radiation counter. The localization of ^{125}I-SP-A was expressed as percentages of radioactivities in the supernatant and the pellet. In some experiments, various concentrations (5–50 μg/ml) of ^{125}I-SP-A were incubated with zymosan, and the amount of ^{125}I-SP-A cosedimented with the zymosan pellet was determined.

Other methods

Protein concentrations were estimated by the BCA assay (Pierce, Rockford, IL) using BSA as a standard. SDS-PAGE was performed by the method of Laemmli (45).

Results

The extracellular TLR2 domain binds zymosan

We first examined whether zymosan induced TNF-α secretion in RAW264.7 cells and whether zymosan initiated TLR2-mediated signaling. Coincubation of zymosan with the cells significantly increased the amounts of TNF-α secreted. The secretion of TNF-α was dependent on the zymosan concentration (Fig. 1*A*). After HEK293 cells had been transiently transfected with TLR2 cDNA or cDNAs of TLR4 and MD-2 and incubated with 25–200 μg/ml zymosan, the cell responsiveness was measured using the NF-κB reporter gene assay. Increased NF-κB activation was observed with the increased concentrations of zymosan in TLR2-transfected cells (Fig. 1*B*). In contrast, the cells transfected with TLR4 and MD-2 did not respond to any concentrations of zymosan. These results confirmed the previously published work (5) showing that zymosan induces TNF-α release and NF-κB activation in immune cells and that TLR2 but not TLR4 is responsible for the recognition of zymosan.

We thus investigated whether TLR2 directly recognized zymosan. sTLR2 was constructed, and the sTLR2 protein was expressed in the baculovirus-insect cell system. The protein exhibited a single band with an apparent molecular mass of 75 kDa, as shown in Fig. 2*A* (control sTLR2). After the mixture of ^{125}I-sTLR2 and zymosan suspension was incubated and centrifuged, the protein that had been cosedimented with the zymosan was analyzed by SDS-PAGE. Zymosan cosedimented ^{125}I-sTLR2 (Fig. 2*A*). The sTLR2 protein was not sedimentable in the absence of zymosan. When zymosan was incubated with ^{125}I-SP-A and centrifuged as a control experiment, zymosan failed to cosediment SP-A (see Fig. 6*A*).

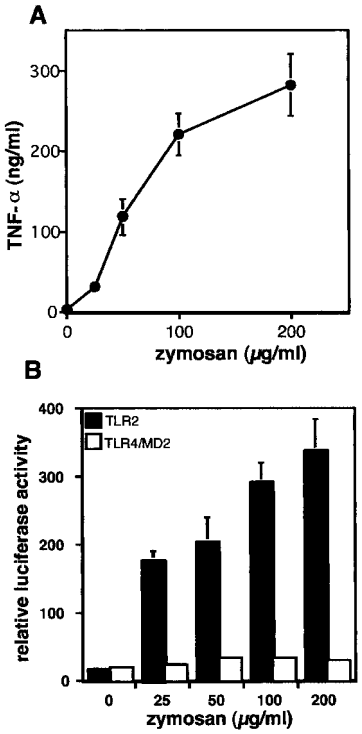

FIGURE 1. Zymosan-induced TNF-α secretion and NF-κB activation. *A*, RAW264.7 cells (5 × 10^5) were incubated with zymosan (25–200 μg/ml) for 24 h. The culture medium was collected, and TNF-α secreted was determined by L929 cell bioassay, as described in *Materials and Methods*. The data shown are the means ± SE of three experiments. *B*, HEK293 cells (2 × 10^5) were transfected with 166 ng of TLR2 cDNA in pcDNA3.1(+) vector (■), or with 83 ng of TLR4 cDNA in pcDNA3.1(+) and 83 ng of MD-2 cDNA in pEFBOS vector (□), together with 30 ng of an NF-κB reporter construct (pNF-κB-Luc) and 3 ng of *Renilla* luciferase control reporter plasmid (pRL-TK). Forty-eight hours after transfection, the cells were stimulated with 25–200 μg/ml zymosan for 18 h. Luciferase activities were determined as described in *Materials and Methods*. The data shown are the means + SE of three experiments for TLR2-transfected cells and are the means of two experiments for TLR4/MD-2-transfected cells.

A binding study with ^{125}I-sTLR2 was also conducted by sedimentation assay. ^{125}I-sTLR2 exhibited a time-dependent binding to zymosan (Fig. 2*B*). The binding reached a plateau at 6 h. Excess unlabeled sTLR2 competed well with ^{125}I-sTLR2 for zymosan binding (Fig. 2*C*), indicating that the binding of the labeled sTLR2 is specific for the sTLR2 molecule. ^{125}I-sTLR2 was found to exhibit a concentration-dependent and a saturable binding to zymosan (Fig. 2*D*). An analysis of the binding as described by Klotz (46) (Fig. 2*D*, *inset*) revealed that half-maximal binding occurred at 48 × 10^{-9} M, when the molecular mass of sTLR2 was estimated as 75 kDa. A maximum of ~14 ng (0.19 pmol) of sTLR2 was assumed to bind to 1 μg of zymosan. These results demonstrate that the extracellular TLR2 domain directly binds to zymosan.

We next examined whether the Ab against the extracellular TLR2 domain affected zymosan-elicited cellular responses. Ab raised against sTLR2 blocked the binding of ^{125}I-sTLR2 to zymosan (Fig. 3*A*). This Ab also significantly inhibited TNF-α secretion stimulated with zymosan (Fig. 3*B*). The inhibition of TNF-α secretion by anti-sTLR2 IgG was concentration dependent. Taken together, these results support the concept that the direct binding of TLR2 to zymosan initiates TLR2-mediated signaling and TNF-α secretion.

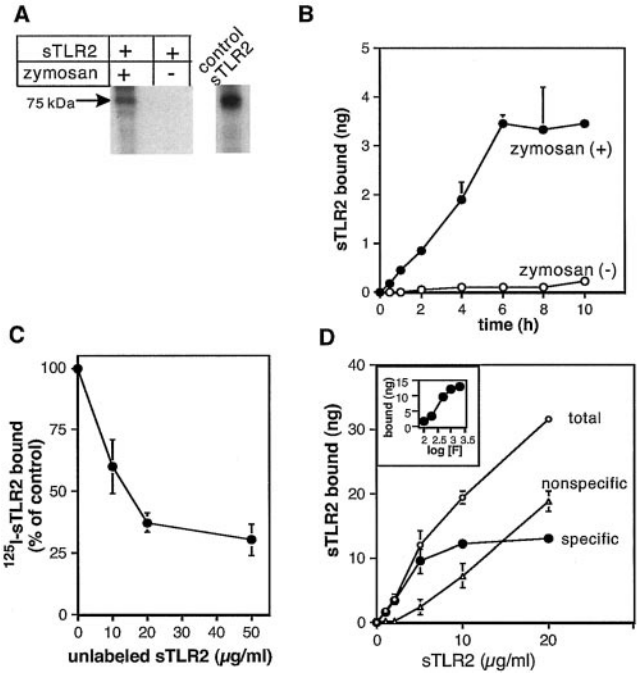

FIGURE 2. The direct binding of sTLR2 to zymosan. *A*, ^{125}I-sTLR2 (500 ng) was incubated with or without 5 μg of zymosan (100 μl/tube) at 37°C for 6 h. The mixture was centrifuged, the pellet obtained was electrophoresed, and the protein cosedimenting with zymosan was visualized by autoradiography as described in *Materials and Methods*. *B*, ^{125}I-sTLR2 (2 μg/ml) was incubated in the presence (●) or the absence (○) of zymosan (1 μg) at 37°C for various periods. sTLR2 binding to zymosan was separated by centrifugation at 10,000 × *g*. After the zymosan pellet was washed, the amount of ^{125}I-sTLR2 binding to the zymosan pellet was determined as described in *Materials and Methods*. The data shown are the means + SE of three experiments. *C*, ^{125}I-sTLR2 (2 μg/ml) was incubated with zymosan (1 μg) in the presence of various concentrations of unlabeled sTLR2 at 37°C for 6 h. The amount of sTLR2 binding to zymosan was determined as described above. The results are expressed as percentages of ^{125}I-sTLR2 binding to zymosan in the absence of unlabeled protein. The data shown are the means ± SE of three experiments. *D*, The indicated concentrations of ^{125}I-sTLR2 (100 μl/tube) were incubated in the presence (total; ○) or the absence (nonspecific; △) of zymosan (1 μg) at 37°C for 6 h. The amount of sTLR2 binding to zymosan was determined as described above. Specific binding (●) was calculated by subtracting nonspecific binding (△) from total binding (○). The *inset* shows the Klotz plot of the binding data. The data shown are the means ± SE of three experiments.

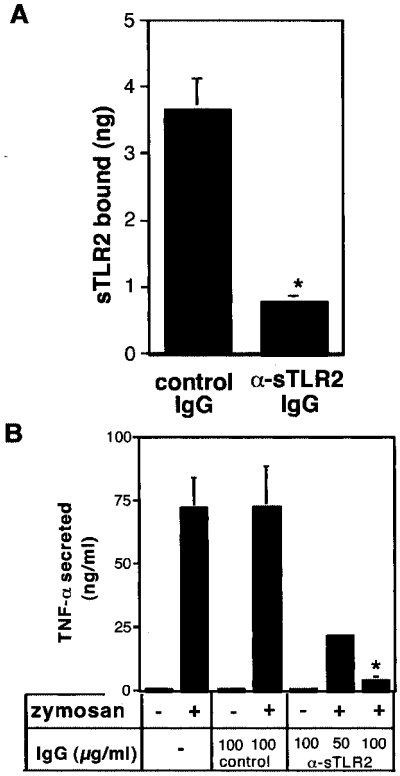

FIGURE 3. Anti-sTLR2 Ab that blocks the binding of sTLR2 to zymosan inhibits zymosan-elicited TNF-α secretion in RAW264.7 cells. *A*, ^{125}I-sTLR2 (2 μg/ml) was preincubated with anti-sTLR2 IgG (100 μg/ml) or control IgG (100 μg/ml) for 1 h. Zymosan (1 μg) was then added into the incubation tube, and the mixture of ^{125}I-sTLR2, zymosan, and the Ab was further incubated at 37°C for 6 h. sTLR2 binding to zymosan was separated by centrifugation at 10,000 × *g*. After the zymosan pellet was washed, the amount of ^{125}I-sTLR2 binding to the zymosan pellet was determined as described in *Materials and Methods*. The data shown are the means + SE of three experiments. *, $p < 0.01$, when compared with control IgG. *B*, RAW264.7 cells (5×10^5) were preincubated with the indicated concentrations of anti-sTLR2 IgG or control IgG for 1 h. Zymosan (25 μg/ml) was then added into the medium, and the cells were further incubated for 24 h. The culture medium was collected, and TNF-α secreted was determined by L929 cell bioassay as described in *Materials and Methods*. The data shown are the means + SE of three experiments, except the experiments with 50 μg/ml anti-sTLR2 IgG (the mean of two experiments). *, $p < 0.01$, when compared with zymosan (+) plus control IgG.

SP-A decreases zymosan-induced TNF-α secretion

We next examined the effect of SP-A on zymosan-induced TNF-α secretion. When 25 μg/ml zymosan was incubated with RAW264.7 cells, TNF-α secretion was stimulated (Fig. 4*A*). Inclusion of 25 μg/ml polymyxin B in the culture medium containing zymosan did not diminish the zymosan-elicited TNF-α secretion, indicating that the stimulatory effect is not due to the endotoxin contamination in the zymosan preparation. Coincubation of 20 μg/ml SP-A with zymosan significantly inhibited the zymosan-induced TNF-α secretion. SP-A alone did not affect the basal secretion of TNF-α. When RAW264.7 cells were incubated with 25–200 μg/ml zymosan in the presence of 20 or 40 μg/ml SP-A, the inhibition of TNF-α secretion by SP-A was significant ($p < 0.01$) at all concentrations of zymosan tested when compared with the experiments in the absence of SP-A (Fig. 4*B*). SP-A at 40 μg/ml inhibited the zymosan (25 μg/ml)-elicited TNF-α secretion by ~80%. Although the increased concentrations of zymosan weakened the inhibitory effect of SP-A, 20 μg/ml protein still attenuated the zymosan (200 μg/ml)-stimulated cytokine release by ~34%.

Concentration-dependent inhibition of TNF-α secretion by SP-A was also examined in RAW264.7 cells and rat alveolar macrophages. When various concentrations of SP-A were incubated with RAW264.7 cells stimulated with 25 or 100 μg/ml zymosan, the protein significantly decreased TNF-α secretion (Fig. 5*A*). Half-maximal inhibition was observed at ~10 μg/ml human SP-A. The inhibitory effect of SP-A on zymosan-induced TNF-α secretion was also confirmed by using rat alveolar macrophages (Fig. 5*B*). The increasing concentrations of SP-A exhibited greater inhibition of TNF-α secretion. Half-maximal inhibition occurred at ~15 μg/ml SP-A in alveolar macrophages.

These results clearly demonstrate that SP-A down-regulates zymosan-induced TNF-α secretion in immune cells.

Zymosan is not a ligand for SP-A

The binding study was performed by a sedimentation assay, because zymosan localizes in the pellet fraction after centrifugation,

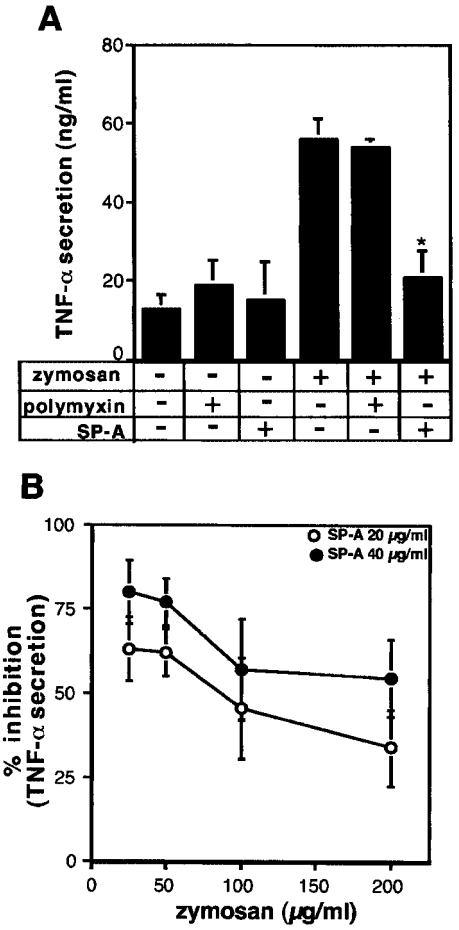

FIGURE 4. SP-A attenuates zymosan-stimulated TNF-α secretion in RAW264.7 cells. *A*, RAW264.7 cells (5×10^5) were incubated with 25 μg/ml zymosan in the absence or the presence of 20 μg/ml SP-A or 25 μg/ml polymyxin B for 24 h. The culture medium was collected, and TNF-α secreted was determined by L929 cell bioassay as described in *Materials and Methods*. The data shown are the means + SE of three experiments. *, $p < 0.01$, when compared with the incubation of zymosan (+). *B*, the inhibitory effects of SP-A on TNF-α secretion induced by various concentrations of zymosan. RAW264.7 cells (5×10^5) were incubated with 25–200 μg/ml zymosan in the absence or the presence of 20 μg/ml (○) or 40 μg/ml (●) of SP-A for 24 h. The culture medium was collected, and TNF-α secreted was determined by L929 cell bioassay as described in *Materials and Methods*. The results are expressed as percent inhibition ((TNF-α secretion without SP-A − TNF-α secretion with SP-A)/TNF-α secretion without SP-A × 100). The data shown are the means ± SE of three experiments. All values obtained are significant ($p < 0.01$), when compared with the incubation without SP-A at each concentration of zymosan.

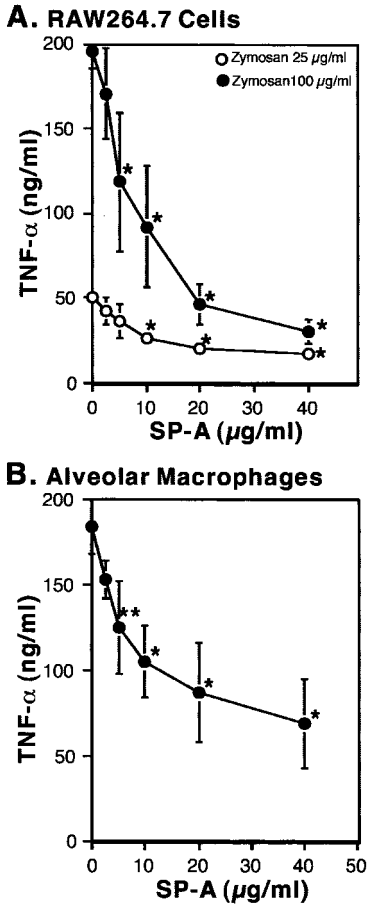

FIGURE 5. Concentration-dependent inhibition of zymosan-induced TNF-α secretion by SP-A. RAW264.7 cells (*A*) or rat alveolar macrophages (*B*) (5×10^5) were incubated with 25 μg/ml (○) or 100 μg/ml (●) zymosan in the presence of 0–40 μg/ml SP-A for 24 h. The culture medium was collected, and TNF-α secreted was determined by L929 cell bioassay as described in *Materials and Methods*. The data shown are the means ± SE of three experiments. *, $p < 0.01$ and **, $p < 0.05$, when compared with the values obtained without SP-A.

as described in *Materials and Methods*. Autoradiography revealed that admixture of ^{125}I-SP-A (250 ng) and zymosan (5 μg) followed by centrifugation resulted in no protein band being recovered from the pellet fraction (Fig. 6*A*). More than 99% of the labeled SP-A localized in the supernatant regardless of whether zymosan was present (Fig. 6*B*), indicating that zymosan does not cosediment SP-A. In addition, no significant increase of ^{125}I-SP-A recovered in the zymosan pellet was observed when increasing concentrations (5–50 μg/ml) of ^{125}I-SP-A were incubated with zymosan (Fig. 6*C*). These results demonstrate that zymosan is not a ligand for human SP-A and rule out the possibility that the binding of SP-A to zymosan interferes with the zymosan-cell interaction.

Inhibitory effect of SP-A persists even after preincubation with the cells and its removal

We next investigated whether the interaction of SP-A with the cells affected the subsequent cellular responses to zymosan. RAW264.7 cells were preincubated with 20 μg/ml SP-A for 0.5–4 h before zymosan treatment. The medium containing SP-A was removed, and the cells were subsequently washed once with PBS to remove unbound SP-A. A fresh medium was added and incubated for 24 h in the presence of 25 μg/ml zymosan. Preincubation of SP-A with the cells and its removal from the medium failed to abolish the inhibitory effect of the protein on TNF-α secretion. The longer the period of preincubation of SP-A, the greater was the inhibition of TNF-α secretion (Fig. 7). This finding demonstrates that the inhibitory effect of SP-A persists even after preincubation with the cells and its removal from the medium, and supports the idea that the interaction of SP-A with the cells alters the subsequent cellular responses to zymosan.

SP-A attenuates zymosan-induced NF-κB activation in TLR2-transfected HEK293 cells

Because zymosan-elicited signaling is mediated by TLR2 (Fig. 1*B*), we examined the effect of SP-A on zymosan-induced NF-κB activation in TLR2-transfected HEK293 cells. SP-A alone did not

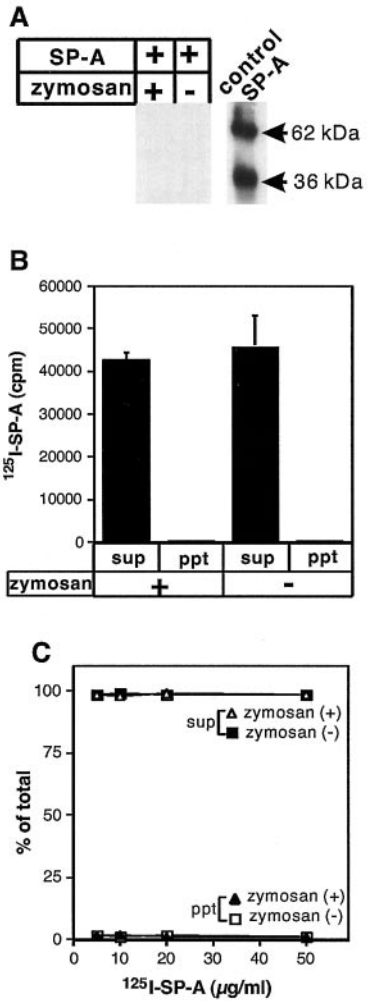

FIGURE 6. Zymosan is not a ligand for SP-A. *A*, ^{125}I-SP-A (250 ng) was incubated with or without 5 μg of zymosan (100 μl/tube) at 37°C for 1 h. The mixture was centrifuged, the pellet obtained was electrophoresed, and the protein cosedimenting with zymosan was visualized by autoradiography as described in *Materials and Methods*. *B*, ^{125}I-SP-A (5 μg/ml) was incubated in the presence or the absence of 5 μg of zymosan at 37°C for 1 h. The mixture of the protein and zymosan was centrifuged, and the radioactivities of the supernatant and the pellet obtained were measured using a gamma radiation counter as described in *Materials and Methods*. The data shown are the means + SE of three experiments. *C*, The indicated concentrations of ^{125}I-SP-A were incubated in the presence (△, ▲) or the absence (□, ■) of 5 μg of zymosan at 37°C for 1 h. The radioactivities of ^{125}I-SP-A recovered in the pellet (▲, □) and the supernatant (△, ■) were measured after centrifugation as described in *Materials and Methods*. The results are expressed as percentages of radioactivities in the supernatant and the pellet. The data shown are the means ± SE of three experiments.

induce NF-κB activation, whereas 25 μg/ml zymosan stimulated NF-κB activation (Fig. 8*A*). Coincubation of 20 μg/ml SP-A with zymosan significantly decreased NF-κB activation. We also tested whether SP-A affected NF-κB activation stimulated with higher concentrations of zymosan. Although the inhibitory effects of SP-A appeared weaker compared with that at 25 μg/ml zymosan, the protein still exhibited significant inhibition even when the cells were stimulated in combination with 50–200 μg/ml zymosan (Fig. 8*B*). These data clearly demonstrate that SP-A can alter TLR2-mediated zymosan signaling.

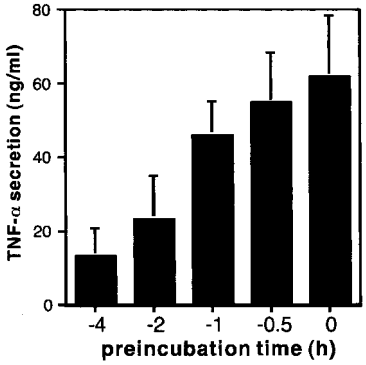

FIGURE 7. The longer preincubation of SP-A with RAW264.7 cells produces a greater inhibition on TNF-α secretion. RAW264.7 cells (5×10^5) were preincubated with 20 μg/ml SP-A for the indicated periods, washed once with PBS to remove unbound SP-A, and then further incubated with the medium containing 25 μg/ml zymosan for 24 h. The culture medium was finally collected, and TNF-α secreted was determined by L929 cell bioassay as described in *Materials and Methods*. The data shown are the means + SE of three experiments.

SP-A alters zymosan-TLR2 interaction

Because we have recently reported that SP-A binds sTLR2 (38) and have shown in this study that sTLR2 directly binds zymosan (Fig. 2), we investigated whether SP-A affected the binding of sTLR2 to zymosan. ^{125}I-sTLR2 (2 μg/ml) was preincubated with SP-A (1–20 μg/ml) for 1 h, and this mixture was further incubated with 2 μg of zymosan for 6 h. The amount of sTLR2 binding to zymosan in the presence of various concentrations of SP-A was compared with that in the absence of SP-A. SP-A significantly reduced the binding of sTLR2 to zymosan in an SP-A concentration-dependent manner (Fig. 9). SP-A at 20 μg/ml decreased the binding of sTLR2 to zymosan to the level of ~19% of that without SP-A. These results are essentially consistent with those disclosed by the inhibitory effect of SP-A on NF-κB activation in TLR2-transfected cells and on TNF-α secretion in immune cells. Taken together, these results demonstrate that SP-A alters zymosan-TLR2 interaction and down-regulates TLR2-mediated zymosan signaling and TNF-α secretion.

Discussion

This study demonstrates that the extracellular TLR2 domain directly binds zymosan and that SP-A down-regulates zymosan-induced TNF-α secretion in RAW264.7 cells and alveolar macrophages and zymosan-elicited NF-κB activation in TLR2-transfected HEK293 cells. These results are consistent with the relationship between NF-κB and TNF-α expression, because zymosan-induced TNF-α production is accompanied by activation of NF-κB (6). Inhibition of NF-κB activation by caffeic acid phenethyl ester led to a dramatic reduction in TNF-α production in response to zymosan. In addition, competition experiments provide clear evidence that SP-A can alter the interaction of TLR2 with zymosan. Because SP-A binds to TLR2 (38), alteration of zymosan-TLR2 interaction and its signaling by SP-A constitutes a likely mechanism by which SP-A down-regulates proinflammatory cytokine release. Because the recognition of zymosan by TLR2 is coupled with NF-κB activation and TNF-α production (5), our findings are in line with the concept that the binding of zymosan to the receptor triggers its signaling and cytokine release. This study strongly supports the concept of the important role of SP-A in controlling pulmonary inflammation resulting from organic dust toxic syndrome and fungal infection.

sTLR2 exhibited a concentration-dependent and a saturable binding to zymosan (see Fig. 2*D*). The apparent binding constant

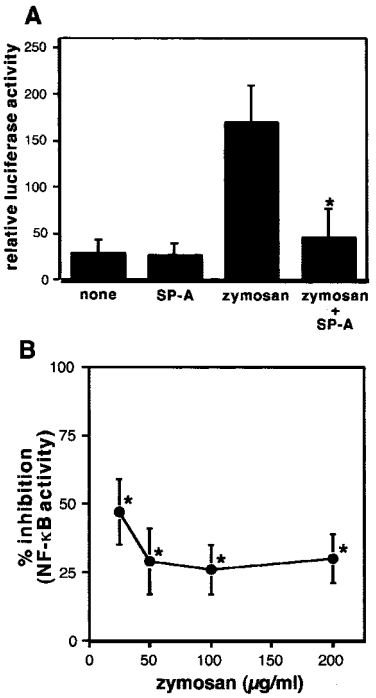

FIGURE 8. SP-A attenuates zymosan-induced NF-κB activation in TLR2-transfected HEK293 cells. *A*, HEK293 cells (2×10^5) were transfected with 166 ng of TLR2 cDNA in pcDNA3.1(+) vector together with 30 ng of an NF-κB reporter construct (pNF-κB-Luc) and 3 ng of *Renilla* luciferase control reporter plasmid (pRL-TK). Forty-eight hours after transfection, the cells were stimulated with 25 μg/ml zymosan for 18 h in the absence or the presence of 20 μg/ml SP-A. Luciferase activities were determined as described in *Materials and Methods*. The data shown are the means + SE of three experiments. *, $p < 0.01$, when compared with zymosan treatment without SP-A. *B*, HEK293 cells were transfected with TLR2 cDNA, an NF-κB reporter construct, and *Renilla* luciferase control reporter plasmid, as described above. Forty-eight hours after transfection, the cells were stimulated with 25–200 μg/ml zymosan for 18 h in the absence or the presence of 20 μg/ml SP-A. Luciferase activities were determined as described in *Materials and Methods*. The results are expressed as percent inhibition ((luciferase activity without SP-A − luciferase activity with SP-A)/luciferase activity without SP-A × 100). The data shown are the means ± SE of three experiments. *, $p < 0.01$, when compared with the incubation without SP-A at each concentration of zymosan.

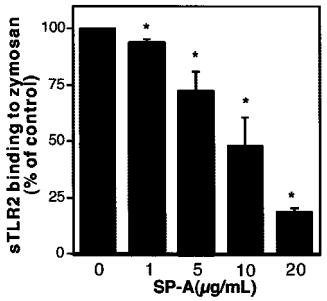

FIGURE 9. SP-A attenuates the binding of sTLR2 to zymosan. SP-A (0–20 μg/ml) was preincubated with 2 μg/ml ^{125}I-sTLR2 for 1 h. After the preincubation, the mixture of SP-A and sTLR2 was further incubated with 2 μg of zymosan for 6 h. The incubation mixture was then centrifuged at 10,000 × *g* for 15 min, and the resultant pellet was washed. The amount of ^{125}I-sTLR2 cosedimenting with the zymosan pellet was finally determined as described in *Materials and Methods*. The results are expressed as percentage of sTLR2 binding to zymosan in the absence of SP-A. The data shown are the means + SE of three experiments. *, $p < 0.01$, when compared with control binding without SP-A. The amount of sTLR2 binding to zymosan in the absence of SP-A was 2.8 ± 0.3 ng (mean ± SE; $n = 3$).

calculated was 48 nM, which was not as high as we expected, but this value is comparable with that obtained for sTLR2 binding to PGN (31). Because we used the soluble form of rTLR2 possessing only the extracellular domain, the membrane-spanning and the cytoplasmic domains, and/or other molecules including CD14 and other classes of TLR may be required to bind zymosan with higher affinity. Coordination of TLR2 with TLR6 has been reported to be important in activating macrophages in response to zymosan (47). Zymosan has been shown to bind macrophages via glucan receptors (4, 48). Although macrophage mannose receptor and complement receptor 3 have been considered to be the major receptors in the recognition of zymosan (49–51), the real identity of the receptor is still controversial. Dectin-1 has recently been reported to be a major receptor on macrophages (52). These receptors function to phagocytose zymosan and β-glucans, and TLRs trigger inflammatory responses. Although TLR2 is recruited to macrophage phagosomes (5), the intermolecular relationship between the phagocytic receptors and the signaling receptor remains unknown.

We have previously shown that SP-A binds to rough LPS but not to smooth LPS and that SP-A modulates LPS-induced cellular responses (37). The protein inhibits smooth LPS-elicited TNF-α secretion in alveolar macrophages, whereas it somewhat increases rough LPS-stimulated cytokine release. SP-A also inhibits proinflammatory cytokine release elicited by PGN, which is also not an SP-A ligand (38). This study shows that zymosan is not a ligand for SP-A and that SP-A down-regulates zymosan-induced TNF-α secretion. Taken together, these studies raise the possibility that SP-A may exhibit anti-inflammatory function against pulmonary inflammation that, we can say at least, is caused by microbial components that are not SP-A ligands.

Mannose-binding protein attenuates TNF-α secretion caused by streptococcal cell wall components by its direct binding to streptococcal rhamnose glucose polymer (53). Thus, one can hypothesize that the binding of SP-A to zymosan might shield zymosan from interacting with the cell surface receptor. However, the failure of the SP-A's binding to zymosan rules out the possibility that its direct interaction with zymosan inhibits zymosan signaling. In this study, we have shown that SP-A inhibits the binding of sTLR2 to zymosan in the cell-free system. Although we were unable to determine the effect of SP-A on the binding of zymosan to membrane-bound TLR2 on the cells, it is possible to conclude that the interaction of SP-A with the cells is involved in the modulation of TNF-α secretion by SP-A, because the inhibitory effect of SP-A persists even after preincubation of the protein with the cells and its removal (see Fig. 7). This may suggest that the interaction of SP-A with the cells alters the cellular responses to zymosan. Because SP-A binds sTLR2 (38) and zymosan is not a ligand for SP-A (see Fig. 6), it is reasonable to propose that the binding of SP-A to TLR2 may prevent zymosan from binding to TLR2, and consequently, the zymosan-induced signaling and TNF-α secretion are inhibited. The idea is clearly supported by the present results demonstrating that the binding of sTLR2 to zymosan is significantly decreased in the presence of SP-A, because the experiments with anti-sTLR2 Ab have revealed that the in vitro binding of sTLR2 to zymosan correlates well with the zymosan-elicited cellular responses (see Fig. 3). In addition, this study is also consistent with our previous studies (37–39) proposing that SP-A modulates inflammatory responses against the bacterial components by interaction with pattern-recognition receptors. Another possible mechanism may be that the interactions of SP-A with serum components in medium affect the binding of zymosan to cell surface

receptors. However, this is not a primary mechanism, because the inhibitory effect of SP-A on zymosan-induced TNF-α secretion persists even after SP-A is removed from the medium. One can also postulate another possible mechanism, that the signaling mediated through the interactions of SP-A with the cell surface molecules other than TLRs may affect the TLR2-mediated signaling stimulated with zymosan, resulting in the inhibition of TNF-α secretion.

A previous study has shown that SP-A attenuates proinflammatory cytokine release evoked by both viable and nonviable *Candida albicans* in alveolar macrophages and monocytes (54). The inhibitory effect of SP-A is independent of fungal SP-A binding but is related to the SP-A-cell interaction, because preincubation of SP-A with the cells and its removal before macrophage-*Candida* coincubation sufficed to suppress the cytokine response. Although the study did not elucidate the mechanism of SP-A's impact, it is possible to assume that the binding of SP-A to TLR may alter the interaction of *Candida* to the cell surface receptor, resulting in the down-regulation of proinflammatory cytokine release. Intrapulmonary TNF-α production causes local inflammatory tissue injury and lethal septic shock in *Candida* sepsis during immunosuppression (11). Taken together with this study, these works give credence to the important physiological role of SP-A in regulating pulmonary inflammation elicited by fungi.

In conclusion, this study demonstrates that the extracellular TLR2 domain directly binds to zymosan, and that SP-A alters the interaction of TLR2 with zymosan, and attenuates downstream signaling and TNF-α release. Our findings provide molecular bases for zymosan's recognition by TLR2 and for a dampening role of SP-A in controlling pulmonary inflammation in organic dust toxic syndrome and fungal infection.

Acknowledgments

We thank Dr. Kensuke Miyake (University of Tokyo) for providing cDNAs for TLR4 and MD-2.

References

1. Emanuel, D. A., F. J. Wenzel, and B. R. Lawton. 1975. Pulmonary mycotoxicosis. *Chest 67:293.*
2. Rylander, R. 1986. Lung diseases caused by organic dusts in the farm environment. *Am. J. Ind. Med. 10:221.*
3. DiCarlo, F. J., and J. V. Fiore. 1957. On the composition of zymosan. *Science 127:756.*
4. Sanguedolce, M. V., C. Capo, P. Bongrand, and J.-L. Mege. 1992. Zymosan-stimulated tumor necrosis factor-α production by human monocytes: down-modulation by phorbol ester. *J. Immunol. 148:2229.*
5. Underhill, D. M., A. Ozinsky, A. M. Hajjar, A. Stevens, C. B. Wilson, M. Bassetti, and A. Aderem. 1999. The Toll-like receptor 2 is recruited to macrophage phagosomes and discriminates between pathogens. *Nature 401:811.*
6. Young, S.-H., J. Ye, D. G. Frazer, X. Shi, and V. Castranova. 2001. Molecular mechanism of tumor necrosis-α production in 1→3-β-glucan (zymosan)-activated macrophages. *J. Biol. Chem. 276:20781.*
7. Daum, T., and M. S. Rohrbach. 1992. Zymosan induces selective release of arachidonic acid from rabbit alveolar macrophages via stimulation of a β-glucan receptor. *FEBS Lett. 309:119.*
8. Nobel, P. W., P. M. Henson, C. Lucas, M. Mora-Worms, P. C. Carre, and D. W. H. Riches. 1993. Transforming growth factor-β primes macrophages to express inflammatory gene products in response to particulate stimuli by an autocrine/paracrine mechanism. *J. Immunol. 151:979.*
9. Okazaki, M., N. Chiba, Y. Adachi, N. Ohno, and T. Yadomae. 1996. Signal transduction pathway on β-glucans-triggered hydrogen peroxide production by murine peritoneal macrophages in vitro. *Biol. Pharm. Bull. 19:18.*
10. Barrios, R., G. G. Santos, J. Figueroa, and P. A. Reyes. 1980. Zymosan-induced experimental hypersensitivity pneumonitis in rabbits. *Am. J. Pathol. 99:731.*
11. Lechner, A. J., T. L. Tredway, D. S. Brinks, C. A. Klein, and G. M. Matuschak. 1992. Differential systemic and intrapulmonary TNF-α production in *Candida* sepsis during immunosuppression. *Am. J. Physiol. 263:L526.*
12. Aderem, A., and R. Ulevitch, J. 2000. Toll-like receptors in the induction of the innate-immune response. *Nature 406:782.*
13. Medzhitov, R., P. Preston-Hurlburt, and J. C. A. Janeway. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature 388:394.*
14. Medzhitov, R., and J. C. A. Janeway. 1997. Innate immunity: the virtues of a nonclonal system of recognition. *Cell 91:295.*
15. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J. Immunol. 162:3749.*
16. Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda, and S. Akira. 1999. Differential roles of TLR2 and TLR4 in recognition of Gram-negative and Gram-positive bacterial cell wall components. *Immunity 11:443.*
17. Shimazu, R., S. Akahsi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med. 189:1777.*
18. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanism triggered by microbial lipoproteins through Toll-like receptors. *Science 285:732.*
19. Chow, J. C., D. W. Young, D. T. Golenbock, W. J. Christ, and F. Gusovsky. 1999. Toll-like receptor-4 mediates lipopolysaccharide-induced signal transduction. *J. Biol. Chem. 274:10689.*
20. Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill, and A. Aderem. 2001. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. *Nature 410: 1099.*
21. Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda, and S. Akira. 2000. A Toll-like receptor recognizes bacterial DNA. *Nature 408:740.*
22. Hirschfeld, M., C. J. Kirschning, R. Schwandner, H. Wesche, J. H. Weis, R. M. Wooten, and J. J. Weis. 1999. Inflammatory signaling *by Borrelia burgdorferi* lipoproteins is mediated by Toll-like receptor 2. *J. Immunol. 163:2382.*
23. Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogek, and J. J. Weis. 2000. Repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like recptor 2. *J. Immunol. 165:618.*
24. Kirschning, C. J., H. Wesche, T. M. Ayres, and M. Rothe. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med. 188:2091.*
25. Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern-recognition receptor for diverse bacterial products. *J. Biol. Chem. 274:33419.*
26. Means, T. K., S. Wang, E. Lien, A. Yoshimura, D. T. Golenbock, and M. J. Fenton. 1999. Human Toll-like receptors mediate cellular activation by *Mycobacterium tuberculosis*. *J. Immunol. 163:3920.*
27. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. 163:1.*
28. da Silva Correia, J., K. Soldau, U. Christen, P. S. Tobias, and R. J. Ulevitch. 2001. Lipopolysaccharide is in close proximity to each of the proteins in its membrane receptor complex. *J. Biol. Chem. 276:21129.*
29. Mitsuzawa, H., I. Wada, H. Sano, D. Iwaki, S. Murakami, T. Himi, N. Matsushima, and Y. Kuroki. 2001. Extracellular Toll-like receptor 2 region containing Ser^{40}-Ile^{64} but not Cys^{30}-Ser^{39} is critical for the recognition of *Staphylococcus aureus* peptidoglycan. *J. Biol. Chem. 276:41350.*
30. Schwandner, R., R. Dziarski, H. Wesche, M. Rothe, and C. J. Kirschning. 1999. Peptidoglycan- and lipoteichoic acid-induced cell activation is mediated by Toll-like receptor 2. *J. Biol. Chem. 274:17406.*
31. Iwaki, D., H. Mitsuzawa, S. Murakami, H. Sano, M. Konishi, T. Akino, and Y. Kuroki. 2002. The extracellular Toll-like receptor 2 domain directly binds peptidoglycan derived from *Staphylocuccus aureus*. *J. Biol. Chem. 277:24315.*
32. King, R. J., and J. A. Clements. 1972. Surface active materials from dog lung. I. Method of isolation. *Am. J. Physiol. 223:707.*
33. Day, A. J. 1994. The C-type carbohydrate recognition domain (CRD) superfamily. *Biochem. Soc. Trans. 22:83.*
34. Kuroki, Y., and D. R. Voelker. 1994. Pulmonary surfactant proteins. *J. Biol. Chem. 269:25943.*
35. Borron, P., J. C. McIntosh, T. R. Korfhagen, J. A. Whitsett, J. Taylor, and J. R. Wright. 2000. Surfactant associated protein-A inhibits LPS-induced cytokine and nitric oxide production in vivo. *Am. J. Physiol. 278:L840.*
36. McIntosh, J. C., S. Mervin-Blake, E. Conner, and J. R. Wright. 1996. Surfactant protein A protects growing cells and reduces TNF-α activity from LPS-stimulated macrophages. *Am. J. Physiol. 271:L310.*
37. Sano, H., H. Sohma, T. Muta, S. Nomura, D. R. Voelker, and Y. Kuroki. 1999. Pulmonary surfactant protein A modulates the cellular responses to smooth and rough lipopolysaccharides by interactions with CD14. *J. Immunol. 163:387.*
38. Murakami, S., D. Iwaki, H. Mitsuzawa, H. Sano, H. Takahashi, D. R. Voelker, T. Akino, and Y. Kuroki. 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.*
39. Sano, H., H. Chiba, D. Iwaki, H. Sohma, D. R. Voelker, and Y. Kuroki. 2000. Surfactant proteins A and D bind CD14 by different mechanisms. *J. Biol. Chem. 275:22442.*
40. Flick, D. A., and G. E. Gifford. 1984. Comparison of in vitro cell cytotoxic assays for tumor necrosis factor. *J. Immunol. Methods 68:167.*
41. O'Reilly, D. R., L. K. Miller, and V. A. Luckow. 1992. *Baculovirus Expression Vector: A Laboratory Manual.* Freeman, New York.
42. Bolton, A. E., and W. M. Hunter. 1973. The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent. *Biochem. J. 133:529.*

43. Kuroki, Y., R. J. Mason, and D. R. Voelker. 1988. Alveolar type II cells express a high-affinity receptor for pulmonary surfactant protein A. *Proc. Natl. Acad. Sci. USA 85:5566.*
44. Kuroki, Y., S. Tsutahara, N. Shijubo, H. Takahashi, M. Shiratori, A. Hattori, Y. Honda, S. Abe, and T. Akino. 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.*
45. Laemmli, U. K. 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature 227:680.*
46. Klotz, I. M. 1982. Numbers of receptor sites from Scatchard graphs: facts and fantasies. *Science 217:1247.*
47. Ozinsky, A., D. M. Underhill, J. D. Fontenot, A. M. Hajjar, K. D. Smith, C. B. Wilson, L. Schroeder, and A. Aderem. 2000. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between Toll-like receptors. *Proc. Natl. Acad. Sci. USA 97:13766.*
48. Tapper, H., and R. Sundler. 1995. Glucan receptor and zymosan-induced lysosomal enzyme secretion in macrophages. *Biochem. J. 306:829.*
49. Czop, J. K., and K. F. Austen. 1985. A β-glucan inhibitable receptor on human monocytes: its identity with the phagocytic receptor for particulate activators of the alternative complement pathway. *J. Immunol. 134:2588.*
50. Giaimis, J., P. Lombard, P. Fonteneau, C. D. Muller, R. Levy, M. Makaya-Kumba, J. Lazdin, and P. Poindron. 1993. Both mannose and β-glucan receptors are involved in phagocytosis of unopsonized, heat-killed *Saccharomyces cerevisiae* by murine macrophages. *J. Leukocyte Biol. 54:564.*
51. Sung, S. S., R. S. Nelson, and S. C. Silverstein. 1983. Yeast mannans inhibit binding and phagocytosis of zymosan by mouse peritoneal macrophages. *J. Cell Biol. 96:160.*
52. Brown, G. D., P. R. Taylor, D. M. Reid, J. A. Willment, D. L. Williams, L. Martinez-Pomares, S. Y. C. Wong, and S. Gordon. 2002. Dectin-1 is a major β-glucan receptor on macrophages. *J. Exp. Med. 196:407.*
53. Soell, M., E. Lett, F. Holveck, M. Scholler, D. Wachsmann, and J.-P. Klein. 1995. Activation of human monocytes by streptococcal rhamnose glucose polymers is mediated by CD14 antigen, and mannan binding protein inhibits TNF-α release. *J. Immunol. 154:851.*
54. Rosseau, S., P. Hammerl, U. Maus, A. Günther, W. Seeger, F. Grimminger, and J. Lohmeyer. 1999. Surfactant protein A down-regulates proinflammatory cytokine production evoked by *Candida albicans* in human alveolar macrophages and monocytes. *J. Immunol. 163:4495.*