Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway

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Alcorn, John F., and Jo Rae Wright. Surfactant protein A inhibits alveolar macrophage cytokine production by CD14-independent pathway. *Am J Physiol Lung Cell Mol Physiol* 286: L129–L136, 2004. First published September 5, 2003; 10.1152/ajplung.00427.2002.—The lung collectin surfactant protein A (SP-A) has both anti-inflammatory and prophagocytic activities. We and others previously showed that SP-A inhibits the macrophage production of tumor necrosis factor (TNF-α) stimulated by the gram-negative bacterial component LPS. We propose that SP-A decreases the production of proinflammatory cytokines by alveolar macrophages via a CD14-independent mechanism. SP-A inhibited LPS-stimulated TNF-α production in rat and mouse macrophages in the presence and absence of serum (72% and 42% inhibition, respectively). In addition, SP-A inhibited LPS-induced mRNA levels for TNF-α, IL-1α, and IL-1β as well as NF-κB DNA binding activity. SP-A also diminished ultrapure LPS-stimulated TNF-α produced by wild-type and CD14-null mouse alveolar macrophages by 58% and 88%, respectively. Additionally, SP-A inhibited TNF-α stimulated by PMA in both wild-type and TLR4-mutant macrophages. These data suggest that SP-A inhibits inflammatory cytokine production in a CD14-independent manner and also by mechanisms independent of the LPS signaling pathway.

**THE PULMONARY EPITHELIUM** is a fine, delicate structure ideally suited for its primary function of gas exchange. The same properties that promote gas exchange also make the lung susceptible to infection and inflammation by the pathogens, allergens, and irritants that are inhaled in vast quantities with every breath. Fortunately, the lung has several innate immune systems that help protect the epithelium. One of these systems is pulmonary surfactant, a mixture of lipids and proteins that reduces surface tension at the air–liquid interface of the lung and participates in host defense.

The most abundant protein in surfactant is surfactant protein A (SP-A) (44). SP-A is a member of the collectin family of proteins, all of which have COOH-terminal lectin domains and collagen-like NH2-terminal regions. SP-A binds to alveolar macrophages in a calcium-dependent manner that is partially inhibited by competing sugars (19, 26). SP-A regulates a variety of immune cell functions both in vivo and in vitro. For example, SP-A increases alveolar macrophage phagocytosis of *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Mycoplasma pneumoniae*, *Escherichia coli*, and *Mycobacterium tuberculosis* by opsonin-dependent mechanisms in vitro (6, 9, 10, 20, 28, 40). In addition, SP-A-null mice fail to clear *P. aeruginosa*, group B *Streptococcus*, and *Haemophilus influenzae* as effectively as wild-type animals (15, 16, 17). These studies show that SP-A plays a critical role in promoting resolution of lung infections by facilitating pathogen clearance.

SP-A has also been demonstrated to alter alveolar macrophage cytokine production induced by variety of stimuli. For example, SP-A reduces macrophage tumor necrosis factor (TNF)-α secretion stimulated by gram-negative bacterial endotoxin, LPS, both in vitro and in vivo (3, 21). Furthermore, SP-A inhibits TNF-α production induced by *Candida albicans* and in vivo viral challenge (13, 30). SP-A also dose-dependently inhibits nitric oxide (NO) production and inducible nitric oxide synthase protein stimulated by LPS in alveolar macrophages (34). Conflicting reports have been published showing that SP-A enhances inflammatory cytokine production; however, the method by which SP-A was purified and the assay by which TNF-α was measured are different from those utilized in our studies (12). Additionally, several of these studies were conducted in the macrophage-like THP-1 cell line (42), and we speculate that SP-A may have cell-specific effects.

The LPS signaling pathway in immune cells has recently been elucidated. LPS activates alveolar macrophages via the cell membrane receptor CD14 (37). This interaction is facilitated by the serum protein LPS-binding protein (LBP). CD14 lacks a transmembrane domain, but LPS binding results in activation of a variety of cellular signaling kinases including MAPK, PKC, and PLC (22, 38). The recently identified Toll-like receptor (TLR) family is a critical component of LPS-mediated cellular activation (11). C3H/HeJ mice, which have a mutation in the TLR4 gene, are hyporesponsive to LPS (1, 29). It was recently shown that CD14 and TLR4 form a receptor complex in the presence of LPS (5). LPS activation of alveolar macrophages results in increased production of TNF-α mRNA and protein. The TNF-α gene is primarily regulated positively by two nuclear transcription factors, activator protein (AP)-1 and NF-κB (2, 25). Nuclear translocation of NF-κB has been shown to be a key step in LPS-induced TNF-α production (4).

Recent work has identified some of the mechanisms by which SP-A inhibits LPS activation of alveolar macrophages. It has been proposed that binding of SP-A to LPS may mediate some of its effects. However, SP-A inhibits stimulation of TNF-α release by both rough LPS, to which it binds, and smooth LPS, to which it does not bind (39), suggesting that binding is not the only mechanism of inhibition. In addition, it has been shown that SP-A interacts with CD14 in vitro, thereby altering binding of LPS to CD14 (31). In these studies, SP-A inhibited TNF-α release induced by a smooth LPS serotype to which SP-A does not bind, presumably via blocking LPS-CD14 binding. In contrast, SP-A enhanced rough LPS activa-

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tion of TNF-α by increasing association of LPS with CD14. However, in our laboratory (35), SP-A was shown to increase rough LPS binding to alveolar macrophages while still inhibiting rough LPS-induced production of NO metabolites. Furthermore, it was shown recently that SP-A can inhibit both TNF-α protein and NF-κB DNA binding activity in response to a rough serotype of LPS (33), and it was proposed that SP-A inhibits cytokine production by blocking the interaction of LPS with LBP. Differences in these studies may be explained by the specific serotype of LPS utilized and the cell type analyzed. It is likely that SP-A has additional functional interactions with alveolar macrophages that alter responsiveness to LPS.

In this study we set out to test the hypothesis that the effect of SP-A on LPS-induced cytokine production is independent of CD14. Furthermore, to define the mechanism of SP-A action on LPS signaling, we examined SP-A effects on alveolar macrophages from C3H/HeJ (TLR4 mutant) and CD14-null mice. These experiments were conducted with both commercial LPS preparations and ultrapure LPS, free of contaminating lipoproteins. Additionally, to bypass membrane receptor-associated effects of SP-A, we examined the effects of SP-A on PMN-induced TNF-α release by alveolar macrophages. We propose that SP-A inhibits LPS-induced TNF-α production and secretion in the presence and absence of serum by CD14-independent mechanisms. Furthermore, we propose that SP-A has anti-inflammatory effects on alveolar macrophages independent of LPS signaling pathways.

MATERIALS AND METHODS

Animals and reagents. All experiments were conducted with pathogen-free male Sprague-Dawley rats (Tacconi, Germantown, NY) or male 129F, CD14-null, or C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME) as indicated. Animals ranged from 2 to 4 mo of age when killed by intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, IL) followed by exsanguination. LPS of the O26:B6 strain used in these studies was obtained from Sigma (St. Louis, MO) and resuspended in water from the Picosystem water purification system (Hydro Water Systems, Research Triangle Park, NC). Water from this system contains <0.25 endotoxin U/ml. Ultrapure LPS of the O111:B4 strain was obtained from List Biological Laboratories (Campbell, CA) and resuspended in Picopure water. Contaminating lipoproteins were removed by the manufacturer by the method of Manthey and Vogel (18). PMA was purchased from Sigma, resuspended in 100% EtOH, and stored at −70°C. PMA solutions contained <0.20 endotoxin U/ml. L929 cells used in TNF-α assays were obtained from the American Type Culture Collection (Rockville, MD). These cells were maintained in minimum essential medium Eagle (Sigma) containing 10% horse serum (HyClone, Logan, UT). CD14-null mice. Heterozygous CD14 gene-targeted mice (C57BL/6J background) were purchased from Jackson Laboratories (Jackson, CA) containing 0.2% BSA, low endotoxin (Sigma). Average cell yields were ~4–6 × 10⁶ cells/rat and 3–5 × 10⁶ cells/mouse and were >95% macrophages. Alveolar macrophages were then diluted to a final concentration of 5 × 10⁵ cells/ml, and 100 µl of cells (5 × 10⁴ cells) per well were plated in 96-well plates. Macrophages were then allowed to adhere to the plate for 1 h at 37°C and were then washed and changed to fresh medium. Alveolar macrophage supernatants were collected at indicated time points after treatment and centrifuged at 500 g to remove cellular debris. The supernatants were then divided into aliquots and frozen at −70°C before use in TNF-α bioassays.

Purification of SP-A. SP-A was isolated from the lavage of alveolar proteinosis proteins as previously described (45). Briefly, the surfactant pellet from the lavage fluid was extracted with butanol. Butanolic-insoluble proteins were then resuspended in octylglucopyranoside (OGP) in 0.15 M NaCl. SP-A, which is insoluble in OGP and salt, was then suspended in Tris-buffered water, pH 7.4. Remaining OGP was then removed by dialysis against 5 mM Tris-buffered water, pH 7.4. SP-A was then treated to remove phospholipids by washing with polyethylene beads as previously described (46). SP-A preparations were tested for endotoxin contamination with the Limulus amoebocyte lysate assay (BioWhittaker, Rockland, ME). All SP-A preparations contained <0.01 endotoxin U/ml.

Measurement of TNF-α protein. TNF-α levels were measured with a L929 bioassay that detects bioactive protein concentrations. L929 cells were grown to confluence and collected by trypsinization. Cells were then pelleted at 230 g and resuspended at a concentration of 3 × 10⁶ cells/ml. The L929 cells were then plated at a density of 3 × 10⁴ cells/well in 100 µl of medium and incubated overnight at 37°C. On the following day, cells were changed to minimum essential medium containing 0.2% BSA and actinomycin D at 1 µg/ml. Alveolar macrophage supernatant samples were added to the L929 cells typically at dilutions ranging from 1:10 to 1:1,000. A TNF-α standard curve was generated with recombinant human TNF-α of known concentrations ranging from 0 to 250 pg/ml. Supernatant samples were incubated with L929 cells for 21–24 h at 37°C. After incubation, cells were fixed with 1% formaldehyde in PBS and then stained with 1% crystal violet. The optical density (OD) was then measured by resuspending the dried crystal violet stain in 33% acetic acid and reading absorbance at 570 nm. The absorbance measured correlates directly to the number of viable L929 cells remaining in the wells. TNF-α activity was then determined by the relative amount of killing of the L929 cells, which is inversely proportional to the retained crystal violet in each well.

Purification of alveolar macrophage RNA. Rat alveolar macrophages were plated at a density of 2.5 × 10⁶ cells/well in 24-well plates and allowed to adhere for 1 h at 37°C. Cells were then changed to fresh medium and treated with or without SP-A for 24 h at 37°C. After this period, cells were stimulated with 100 ng/ml of LPS for 1 h at 37°C. Cells were then lysed, and the total cell RNA was isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA). RNA was purified from pooled wells containing 1 × 10⁶ cells per treatment group. Purified RNA quantity and quality were then assessed by measuring absorbance at 260 and 280 nm. Average RNA yield was ~6–10 µg per 1 × 10⁶ cells with a OD260/280 of 1.8. RNA samples were frozen at −20°C before analysis.

Quantification of cytokine mRNA. Alveolar macrophage RNA samples were analyzed for cytokine mRNA levels with the Riboquant Multi-Probe RNase Protection Assay System (PharMingen, San Diego, CA). Briefly, radiolabeled antisense RNA probes were transcribed from a provided template designed from known cytokine mRNA sequences. In these experiments the rCK-1 (PharMingen)
template set was used, which contains templates for rat IL-1α, IL-1β, TNF-α, IL-3, IL-4, IL-5, IL-6, IL-10, IL-2, IFN-γ, and two housekeeping genes, GAPDH and L32. The radiolabeled probes were then hybridized with the sample RNA pools overnight. After hybridization, RNase A was added to the samples to digest any remaining single-stranded RNA molecules; target cytokine mRNAs were protected from digestion because of the formation of double-stranded RNA hybrids with the probe sequences. Cytokine mRNAs were then electrophoresed on polyacrylamide gels and visualized by phosphoimaging. mRNA species were identified on the basis of migration distance in the gel and probe base pair size. Densitometry of the resolved bands was determined with the MacBas 2.0 software package (Fuji Medical Systems, Stamford, CT). Cytokine mRNA was then quantified by correcting for load with the housekeeping gene expression levels.

**Isolation of alveolar macrophage nuclear extracts.** Alveolar macrophages were isolated from lavage and plated onto 100-mm tissue culture dishes at a density of 1 × 10⁵ cells/plate. Cells were adhered to the plate for 1 h and then treated with SP-A and LPS for 1 h. After incubation, alveolar macrophages were washed with calcium-free PBS and scraped off of the dishes into fresh calcium-free PBS. Cells were then pelleted at 16,000 g at 4°C. Cells were next resuspended in buffer A containing (in mM) 10 HEPES, 10 KCl, 2 MgCl₂, 1 DTT, 0.1 EDTA, 0.4 PMSF, 0.2 NaF, and 0.2 NaVO₃ with 0.3 mg/ml leupeptin for 15 min on ice. Cell membranes were then lysed with buffer B, which contained 10% NP-40 in water. Nuclei were then pelleted at 16,000 g at 4°C and resuspended in hypertonic buffer C containing (in mM) 50 HEPES, 50 KCl, 300 NaCl, 0.1 EDTA, 1 DTT, 0.4 PMSF, 0.2 NaF, and 0.2 NaVO₃ with 10% glycerol for 20 min on a rotator at 4°C. The nuclei were then pelleted at 16,000 g at 4°C, and the supernatant was collected and stored at −80°C to be used as the nuclear extract. Total protein in extracts was determined by bicinchoninic acid and used to normalize gel load.

**Measurement of transcription factor DNA binding activity.** DNA binding activity of nuclear transcription factors was measured with the Promega Gel Shift Assay System (Madison, WI). Briefly, consensus oligonucleotides for the DNA region recognized by nuclear transcription factors were end-labeled with [γ-³²P]ATP via T4 polynucleotide kinase incubation at 37°C for 10 min. The oligonucleotides used were as follows: AP-1 (c-jun): 5’-CCGTTGATGATCGCGAGA-3’, 3’-CCGAGACTACTGATCCTGCTTT-5’; NF-kB: 5’-AGTTGAGGGAGCTTTCCTCCAGGC-3’, 3’-TCAATCTCCTCTGGAGGTCC-5’. The radiolabeled probe sequences were then incubated with nuclear extracts from alveolar macrophages at 20 min at room temperature in the presence or absence of unlabeled competitor and noncompetitor oligonucleotides. DNA binding by transcription factors was then measured by gel electrophoresis and autoradiography. Transcription factor binding activity is relative to the shifted band produced by the change in consensus oligonucleotide mass on transcription factor binding. Band image density was then measured with NIH Image software.

**Data analysis.** All of the data presented in Figs. 1–3 and 5–8 were subjected to unpaired Student’s t-test analysis assuming unequal variances. All statistic analyses were performed with the Microsoft Excel software package. Analyses with a resultant P < 0.05 were determined significant.

**RESULTS**

**SP-A inhibits release of TNF-α by alveolar macrophages stimulated by LPS.** We sought to confirm our previously reported finding (21) that SP-A inhibits TNF-α production in vitro and to determine the time dependence of inhibition. Therefore, we compared the effects of SP-A (20 µg/ml) when added simultaneously with LPS (100 ng/ml) versus when added 24 h before LPS (Fig. 1); in both cases, serum was not included in the medium. SP-A alone had no effect on TNF-α production by resting alveolar macrophages at 24 or 48 h after addition. SP-A inhibited LPS-induced TNF-α production to a level of 28 ± 3% of LPS alone. When SP-A was added 24 h before LPS, SP-A inhibited LPS-induced TNF-α to 25 ± 14% of LPS alone. These data show that SP-A inhibits LPS activation of alveolar macrophages similarly when added at the same time as LPS or when added 24 h before addition of LPS. Western blot analysis of culture supernatants confirmed that SP-A is still present in the medium of the SP-A-pretreated macrophages at 24 h and at 48 h after addition to the medium (data not shown).

The finding described above that SP-A inhibits TNF-α production stimulated by LPS in the absence of serum suggests that the serum protein LBP is not required for SP-A’s inhibitory effect. LBP is an LPS-binding protein that is found in normal serum and enhances the effects of LPS in activating macrophages (37). Recent work showed that SP-A inhibits the binding of LPS to LBP, and this interaction was proposed as a mechanism for the SP-A inhibitory effect (33). To clarify the role of LBP in the SP-A inhibitory pathway, we stimulated macrophages pretreated with SP-A for 24 h with LPS (100 ng/ml) in the presence or absence of 10% fetal bovine serum. LPS stimulated an ~70-fold increase in TNF-α in the presence of serum compared with a 10-fold increase in the absence of serum (data not shown). Consistent with our previous findings (21), SP-A inhibited LPS-induced TNF-α production to a level of 58 ± 5% of LPS alone in the presence of serum. Although the magnitude of inhibition produced by SP-A was less in the presence of serum than in serum-free medium, SP-A still significantly inhibited LPS-induced production of bioactive TNF-α by alveolar macrophages (Fig. 1). The lower level of inhibition by SP-A seen in the serum condition may be due to the large increase in TNF-α protein levels above those in serum-free conditions. These data show that SP-A inhibits activation of alveolar macrophages by LPS when added simultaneously with or 24 h before stimulation, both in the absence and presence of the serum protein LBP.

Recent work identified proinflammatory lipoprotein contamination in commercial LPS preparations (13). These lipoproteins act as agonists for additional TLRs and stimulate NF-κB activation in macrophages. To demonstrate that the SP-A
inhibition of LPS activation is not due to these contaminants, we used ultrapure LPS to stimulate alveolar macrophages. Ultrapure LPS (10 µg/ml) in the absence of serum was incubated with alveolar macrophages from 129J mice for 24 h with SP-A (20 µg/ml), and TNF-α was then measured in the supernatant (Fig. 2). SP-A inhibited LPS-induced TNF-α production to a level of 42 ± 6% of LPS alone.

**SP-A inhibits transcription of inflammatory cytokine mRNAs by alveolar macrophages stimulated by LPS.** To determine the point in the signaling pathway at which SP-A has its inhibitory action, we measured LPS-induced inflammatory cytokine transcriptional activation. If SP-A interferes with LPS activation of macrophage signaling pathways, we would expect SP-A to inhibit cytokine mRNA levels. Previous work showed that inflammatory cytokine mRNA levels are elevated within 1 h of LPS stimulation of alveolar macrophages (36). In this study mRNA levels peaked at 1 h, remained elevated at 3 h after stimuli, and then quickly returned to basal levels of expression. We incubated alveolar macrophages with SP-A (20 µg/ml) for 24 h at 37°C. After this period we stimulated the cells with LPS (100 ng/ml) for 1 h. The macrophages were then lysed, and total RNA was isolated as described in MATERIALS AND METHODS. Cytokine mRNA levels were then measured by ribonuclease protection assay, and the data were quantified by phosphorimaging densitometry (Fig. 3). Cytokine mRNA band densities were corrected for load with the expression levels of the two housekeeping genes, GAPDH and L32. LPS stimulated a two- to threefold increase in mRNA for the inflammatory cytokines TNF-α, IL-1α, and IL-1β. SP-A alone had no effect on cytokine mRNA levels. SP-A inhibited LPS-induced increases in mRNA to 42 ± 8%, 29 ± 18%, and 76 ± 1% of LPS-alone expression levels for TNF-α, IL-1α, and IL-1β, respectively. These data demonstrate that SP-A inhibits cytokine transcription stimulated by LPS, in addition to inhibiting TNF-α protein secretion.

**SP-A inhibits DNA binding activity of NF-κB from LPS-stimulated alveolar macrophage nuclear extracts.** LPS stimulation of alveolar macrophages results in increased DNA binding activity of the nuclear transcription factors NF-κB and AP-1. Both the TNF-α and IL-1β genes are positively regulated by the nuclear transcription factor NF-κB. The TNF-α gene is also regulated positively by the AP-1 (c-jun) region in THP-1 cells and rat liver macrophages (25). To test the hypothesis that SP-A could inhibit transcription by decreasing activity of either NF-κB or AP-1 in the nucleus, we treated alveolar macrophages with SP-A (20 µg/ml) simultaneous with LPS (100 ng/ml) for 1 h at 37°C in the presence of serum. After this incubation, nuclear extracts were prepared from the macrophages as described in MATERIALS AND METHODS. DNA-binding activity was analyzed by gel shift assay and densitometry (Fig. 4). SP-A alone had no effect on NF-κB activity in the nuclear extracts. In the presence of serum, LPS induced an increase in NF-κB activity to a level of 74 arbitrary units above control conditions. SP-A inhibited the LPS-induced NF-κB DNA-binding activity by 25%. Neither LPS nor SP-A stimulated a change in the DNA-binding activity of AP-1. These data show that SP-A inhibits only the NF-κB pathway initiated by LPS stimulation of alveolar macrophages. These findings suggest that SP-A inhibits an early point in the LPS signaling pathway, likely at an early point in initiation of LPS signaling.

**SP-A inhibits TNF-α secretion stimulated by LPS in CD14-deficient alveolar macrophages.** To address further the mechanism of SP-A inhibition of LPS proinflammatory cytokine induction, we examined the effects of SP-A on alveolar macrophages isolated from CD14-null mice. SP-A has been shown to bind to CD14 in vitro, and this has been proposed as a mechanism for SP-A’s abrogation of LPS cell activation (31). If SP-A’s anti-inflammatory action is solely dependent on CD14 binding, then we would expect that LPS stimulation of TNF-α in the absence of CD14 would not be inhibited by SP-A. Because CD14-null mice are hyporesponsive to LPS, it was necessary to use 100-fold more LPS to stimulate TNF-α levels similar to those in wild-type mice. We incubated alveolar macrophages from CD14-null mice with SP-A (20 µg/ml) and LPS (10 µg/ml) for 24 h at 37°C in the absence of serum (Fig. 5A). SP-A inhibited LPS-induced TNF-α to 28 ± 2% of LPS alone. Stimulation of TNF-α in these macrophages was possibly due, at least in part, to contaminating lipoproteins in our LPS preparation. To examine this possibility, we tested ultrapure LPS at the same concentration (10 µg/ml) with these macrophages (Fig. 5B). Ultrapure LPS stimulated 52.1 µg/ml
of TNF-α compared with 43.3 μg/ml of TNF-α induced by the Sigma LPS. SP-A inhibited TNF-α production to 12 ± 4% of ultrapure LPS alone. These data show that contaminating lipoproteins in LPS have no effect on the CD14-independent action of SP-A.

**SP-A inhibits release of TNF-α by alveolar macrophages induced by phorbol ester PMA.** To determine whether the inhibitory effect of SP-A on alveolar macrophages was specific to the stimuli, LPS, we examined whether SP-A could inhibit TNF-α stimulated by an alternative pathway. The cell-permeant phorbol ester PMA induces TNF-α production by alveolar macrophages by directly stimulating PKC (43). PMA stimulated a three- to fivefold increase in TNF-α at 24 h after treatment. Mouse (129J) alveolar macrophages were stimulated with PMA at a concentration of 100 ng/ml (162 nM) for 24 h at 37°C in the presence or absence of SP-A (20 μg/ml). SP-A inhibited PMA-stimulated TNF-α production to control levels (Fig. 6). Similarly, SP-A inhibited PMA-stimulated TNF-α when added 24 h before PMA treatment in rat alveolar macrophages.

Next, we investigated the time dependence of the SP-A inhibitory effect on PMA-induced TNF-α production. Rat alveolar macrophages were preincubated with SP-A (20 μg/ml) for 24 h before addition of PMA (100 ng/ml). PMA stimulated an increase in TNF-α at the earliest time point measured, 2 h after treatment, with sustained increases throughout the 24-h period. SP-A caused significant inhibition of TNF-α at 8 and 16 h after PMA treatment (Fig. 7). These data show that, similar to its effects on LPS, SP-A inhibits both the initial activation of alveolar macrophages by PMA and the prolonged stimulation over a 24-h time course.

**SP-A inhibits PMA-induced TNF-α produced by C3H/HeJ alveolar macrophages.** The data presented with PMA as the stimulus show that SP-A can inhibit alveolar macrophage cytokine production by LPS-independent mechanisms. To confirm that SP-A can inhibit TNF-α in the absence of an intact LPS signaling pathway, we tested the ability of SP-A to inhibit
PMA-induced TNF-α from TLR4-mutant C3H/HeJ macrophages. We incubated C3H/HeJ alveolar macrophages with SP-A (20 μg/ml) and PMA at a concentration of 100 ng/ml (162 nM) for 24 h at 37°C (Fig. 8). SP-A inhibited TNF-α levels to 60 ± 12% of PMA-induced levels. These data show that SP-A can still exert anti-inflammatory effects on C3H/HeJ macrophages by TLR4-independent mechanisms.

**DISCUSSION**

The results from this study show that SP-A inhibits production of inflammatory cytokines by a variety of mechanisms. Inhibition of LPS-induced cytokine production by SP-A can occur independent of CD14 and of serum, which contains the LPS-binding protein LBP. SP-A inhibits cytokine transcription as well as release of bioactive protein. Furthermore, SP-A inhibits release of TNF-α by pathways that are independent of LPS because SP-A inhibited PMA-induced release of this cytokine.

Although our data show that SP-A inhibits LPS-induced TNF-α release by pathways independent of serum and CD14, other studies show that effects of SP-A may also be mediated via an interaction of SP-A with CD14 and LBP. For example, studies by Sano and coworkers (31) suggest that interactions between SP-A and CD14 regulate LPS-mediated release of cytokines. They reported that SP-A binds to rough but not smooth LPS and that SP-A binds to CD14 (31). Furthermore, incubation of soluble recombinant CD14 with SP-A reduced the binding of CD14 to smooth LPS but enhanced the binding to rough LPS. We also observed that inhibition does not appear to be dependent on SP-A binding to LPS because we used a smooth serotype of LPS to which SP-A does not bind effectively. Furthermore, Stamme and coworkers (33) recently reported that SP-A inhibition is dependent on SP-A interacting with LBP. These authors propose that SP-A competes with LPS for binding of LBP and decreases the ability of LBP to form immunostimulatory complexes with LPS. However, in our studies SP-A had similar effects on TNF-α production in the presence and absence of serum, which indicates that LPS-LBP interaction is not obligatory for the SP-A effects measured herein. Together, these studies show that there are multiple mechanisms by which SP-A inhibits cytokine release.

We originally sought to determine the role of TLR4 in SP-A-mediated inhibition of LPS-induced TNF-α production. Our original studies were conducted with commercial LPS, which was subsequently shown to be contaminated with lipoproteins (13). Stimulation of C3H/HeJ macrophages with this LPS resulted in enhanced TNF-α production that was not inhibited by SP-A. However, stimulation of these macrophages, containing a TLR4 mutation, with ultrapure LPS did not stimulate production of TNF-α, suggesting that the stimulation observed with non-ultrapure LPS is due to contaminants other than LPS. Therefore, our data, in aggregate, suggest that SP-A does not inhibit TNF-α production stimulated by contaminants in our LPS preparation. We are unsure of the identity of these contaminants. Interestingly, Murakami et al. (24) showed that SP-A inhibits TLR2-stimulated production of inflammatory mediators. Therefore, we conclude that either the contaminated LPS does not contain TLR2 agonists or SP-A does not inhibit TLR2-mediated cytokine production in our experimental system.

Interestingly, Guillot and coworkers (7) recently showed that SP-A stimulates cytokine production through TLR4. In their studies purified SP-A stimulated TNF-α production by cells transfected with TLR4. In contrast, we do not see stim-
ulation of TNF-α production by SP-A alone with SP-A preparations that have been treated to remove contaminating endotoxin. The SP-A preparation used by Guillot and coworkers contained detectable endotoxin (0.14 ng/μg), but the stimulatory effect was not abrogated by addition of polymyxin B but was inhibited by heat treatment of the protein. We cannot definitively explain the reasons for these discrepancies, although differences in cell types tested and other experimental variables may be important.

We do not know the mechanism by which LPS stimulates release of TNF-α in CD14-null macrophages. The most well-characterized LPS signaling pathway on macrophages involves interactions between LPS and LBP and the receptor complex of CD14, TLR4, and MD2. Signaling is initiated by binding of LPS to membrane-bound CD14. CD14 and TLR4 coassociate with LPS and a third protein, termed MD2, to form the intact LPS to membrane-bound CD14, TLR4, and MD2. Signaling is initiated by binding of LPS to CD14-independent mechanisms. Additional work will be necessary to determine the interactions of SP-A with alveolar macrophages required for SP-A’s anti-inflammatory action.

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REFERENCES


