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EFFECT OF SURFACTANT PROTEIN A (SP-A) ON THE PRODUCTION OF CYTOKINES BY HUMAN PULMONARY MACROPHAGES

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ABSTRACT—Surfactant protein A (SP-A) is thought to play a role in the modulation of lung inflammation during acute respiratory distress syndrome (ARDS). However, SP-A has been reported both to stimulate and to inhibit the proinflammatory activity of pulmonary macrophages (Mϕ). Because of the interspecies differences and heterogeneity of Mϕ subpopulations used may have influenced previous controversial results, in this study, we investigated the effect of human SP-A on the production of cytokines and other inflammatory mediators by two well-defined subpopulations of human pulmonary Mϕ. Surfactant and both alveolar (aMϕ) and interstitial (iMϕ) macrophages were obtained from multiple organ donor lungs by bronchoalveolar lavage and enzymatic digestion. Donors with either recent history of tobacco smoking, more than 72 h on mechanical ventilation, or any radiological pulmonary infiltrate were discarded. SP-A was purified from isolated surfactant using sequential butanol and octyl glucoside extractions. After 24-h preculture, purified Mϕ were cultured for 24 h in the presence or absence of LPS (10 μg/mL), SP-A (50 μg/mL), and combinations. Nitric oxide and carbon monoxide (CO) generation (pmol/μg protein), cell cGMP content (pmol/μg protein), and tumor necrosis factor alpha (TNFα), interleukin (IL)-1, and IL-6 release to the medium (pg/μg protein) were determined. SP-A inhibited the lipopolysaccharide (LPS)-induced TNFα response of both interstitial and alveolar human Mϕ, as well as the IL-1 response in iMϕ. The SP-A effect on TNFα production could be mediated by a suppression in the LPS-induced increase in intracellular cGMP. In iMϕ but not in aMϕ, SP-A also inhibited the LPS-induced IL-1 secretion and CO generation. These data lend further credit to a physiological function of SP-A in regulating alveolar host defense and inflammation by suggesting a fundamental role of this apoprotein in limiting excessive proinflammatory cytokine release in pulmonary Mϕ during ARDS.

KEYWORDS—Nitric oxide, carbon monoxide, cyclic guanosin 3′5′-monophosphate, pulmonary surfactants; bronchoalveolar lavage fluid

INTRODUCTION

Cytokines seem to act predominantly in a paracrine way when producing their deleterious effects during sepsis. Therefore, the local release of cytokines by pulmonary Mϕ would be of importance in the pathogenesis of the adult respiratory distress syndrome (ARDS). Recently, it has been found that alveolar type II cells and some of their secretory products, including surfactant-associated protein A (SP-A) and D, have nonsurfactant-related functions and are actively associated with various defense functions in the lung (1). SP-A is the most abundant of the surfactant proteins. It is a calcium-dependent lectin structurally similar to the complement component, C1q, and the mannose-binding protein, an acute-phase protein with opsonic function. Some of its nonsurfactant-related functions include augmentation of alveolar macrophage migration, enhancement of macrophage phagocytosis of opsonized or nonopsonized bacteria or viruses (1), and regulation of reactive oxygen species production (2).

SP-A seems also to influence the proinflammatory activity of aMϕ, and it may participate this way in the modulation of lung inflammation during the ARDS; however, it has been reported both to stimulate (3) and to inhibit (4) the production of cytokines by pulmonary Mϕ.

Alveoli are lined with surfactant that consists of about 90% lipids and 5–10% surfactant-specific proteins. The major function of this material is to reduce the surface tension in the alveoli, thereby preventing alveolar collapse and edema. Alveolar Mϕ but not interstitial Mϕ reside in this surfactant-rich microenvironment and contain surfactant, being more exposed to a potential influence of surfactant constituents. However, during lung inflammation, alveolar epithelium is disrupted and cells in the interstitium can also contact surfactant. Furthermore, there have been several studies that have detected circulating antibodies to the surfactant proteins or the surfactant proteins themselves in the serum (5), suggesting that these proteins may be introduced into the circulation in some conditions and be able to interact with interstitial and even circulating immune cells.

Besides different localizations, it is reasonable to assume that the iMϕ and aMϕ populations exert distinct functions that are altered upon exposure to inflammatory agents and that the intensity and nature of these events have an impact on the net

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outcome of the inflammatory response. Accordingly, the question arises of whether SP-A affects differently both Mϕ populations in humans. To explore this possibility we studied the lipopolysaccharide (LPS)-induced production of tumor necrosis factor alpha (TNFα), interleukin (IL)-1β, and IL-6 by human interstitial and alveolar Mϕ. To gain further insight into the mechanisms underlying the SP-A effect, we also investigated the cyclic guanosine 3′5′-monophosphate (cGMP) pathway of LPS-induced cytokine secretion (6).

MATERIALS AND METHODS

Human lung tissue procurement

This study was approved by the review board and the ethic committee of the Hospital San Carlos. Human lung tissue was obtained from male multiple organ donors. A written informed consent was obtained from the next-of-kin of each donor. Ages ranged from 22 to 46 years, and cranial trauma or spontaneous intracranial hemorrhage was the cause of death in all donors. Donors with either recent history of tobacco smoking, more than 72 h receiving mechanical ventilation or any radiological pulmonary infiltrate were excluded from this study. Immediately after obtaining the organs that were going to be used for transplantation, the left lung was excised. A bronchoalveolar lavage (BAL) was performed through the main bronchus using a total of 500 mL of 0.9% normal saline at 4°C, and the lung was then placed in cold saline solution. Cold ischemia period was less than 3 h in all cases.

Isolation of SP-A

SP-A was purified from the fluid obtained at lung lavage as described previously in detail (7). Briefly, the whole surfactant pellet from lung lavage fluids was extracted with butanol; butanol-insoluble proteins were resolubilized with octylglucopyranoside (OGP); and subsequently, SP-A was solubilized in 5 mM Tris-buffered water, pH 7.4. The purity of SP-A was checked by one-dimensional SDS/PAGE in 12% acrylamide under reducing conditions (50 mM dithiothreitol). Quantification of SP-A was carried out by amino acid analysis in a Beckman System 6300 High Performance analyzer (7). The protein hydrolysis was performed with 0.2 mL of 6 M HCl, containing 0.1% (w/v) phenol in evacuated and sealed tubes at 108°C for 24 h. Norleucine was added to each sample as internal standard.

Cell isolation and culture

Bronchoalveolar cells were separated from lavage fluid by centrifugation. The sedimented cells were washed twice with Hank's balanced salt solution (HBSS) and then centrifuged (250 g, 10 min). To isolate iMϕ (6), pulmonary tissue was washed with a calcium-free solution and then minced into small fragments (1 to 2 mm). After copious washing with the same solution, to remove most blood cells and remaining airway Mϕ, tissue fragments underwent an enzymatic digestion with elastase (27 orcein-elastin U/mL elastase) in a 37°C shaking bath. Digestion was stopped by addition of 4°C fetal calf serum, the tissue was filtered through nylon mesh (200 and 20 μm), and the cell suspension was washed twice with HBSS and centrifuged (250 g, 10 min). The cell pellets were resuspended in RPMI-1640 medium (10% heat-inactivated fetal calf serum, 100 IU/mL penicillin G, 50 μg/mL gentamycin), and poured into 75 cm^2 culture flasks. After 90 min of incubation (37°C, under O_2/CO_2 atmosphere), the supernatants were removed and the cells were washed four times with phosphate-buffered saline (PBS) to remove contaminating non-adherent cells. Adherent cells were found to be 99.5 ± 0.3% viable (Trypan blue exclusion test), and to be composed of 92.5 ± 3.1% Mϕ, as judged by Wright–Giemsa stained cytocentrifuge preparations, being most of the remaining cells neutrophils or fibroblasts. The cells were gently scraped, plated onto collagen-coated 96-well plastic dishes (5×10^5 cells per well) and precultured for 24 h. Under these conditions, approximately 95% of the cell were attached; cell viability was higher than 97% and macrophage purity was always greater than 98%. Cells were cultured for another 24 h in the presence or absence of LPS (*Escherichia Coli* 055:B5, 10 μg/mL), 8-Br-cGMP (1 mmol/L), methylene blue (MB, 10^{-5} mol/L), hemin (1 μmol/L), SP-A (50 μg/mL), and combinations. Macrophage cultures from each individual donor were plated in duplicate wells and each series of experiments was repeated with a minimum of at least six donors.

Biochemical determinations

Supernatants and cells were recovered, and the cGMP content of the cells and TNFα, IL-1, IL-6, carbon monoxide (CO), and nitric oxide (NO) release to the medium were determined. Cell cGMP content was measured with specific RIA (^{125}I-RIA Kit, Radiochemical Centre, Amersham, Bucks, UK). The cytokines measurements were performed by specific ELISA kits. An aliquot of the cell suspension was used for protein quantitation, which was performed spectrophotometrically by the Coomassie brilliant blue dye method.

Release of NO was measured by the Griess reaction as nitrite (NO_2^-) concentration after nitrate (NO_3^-) reduction to NO_2^-. Briefly, samples were deproteinized by the addition of sulfosalicylic acid. They were then incubated for 30 min at 4°C, and subsequently centrifuged for 20 min at 12000 g. After incubation of the supernatants with *E. coli* NO_3^- reductase (37°C, 30 min), 1 mL of Griess reagent (0.5% naphthylethylenediamine dihydrochloride, 5% sulfonilamide, 25% H_3PO_4) was added. The reaction was performed at 22°C for 20 min, and the absorbance at 546 nm was measured, using $NaNO_2$ solution as standard.

To quantify the amount of CO formed, hemoglobin (Hb) was added to bind CO as carboxyhemoglobin (CO-Hb) and the proportion of CO-Hb was estimated (6). For that, Hb (4 μmol/L) was mixed gently into the sample and 1 min was allowed to ensure maximum CO binding. Then, samples were diluted with buffer phosphate (0.01 mol/L KH_2PO_4/K_2HPO_4, pH 6.85) containing 2 mg/mL sodium hydrosulfite, gently mixed in stoppered cuvettes and allowed to stand at room temperature for 10 min. Absorbance was read at 420 and 432 nm against a matched cuvette containing only buffer.

Statistical analyses

N represents the number of separate macrophage preparations employed (each from a different donor lung). The different assays were performed in at least duplicates, and the means were calculated. The results are presented as the means [± standard error of the mean (SEM)], obtained by combining the results from each cell preparation. Mean comparison was done by the Friedman's analysis of variance of ranks, followed by a two-tailed Wilcoxon's rank sum test for paired data to identify the source of the found differences; a confidence level 95% or greater ($P < 0.05$) was considered significant.

RESULTS

LPS significantly increased the release of TNFα (Fig. 1A), IL-1 (Fig. 2A), and IL-6 (Fig. 3A) by both iMϕ and aMϕ. The increase in TNFα production was more pronounced in iMϕ than in aMϕ.

The baseline release of TNFα, IL-1, and IL-6 by unstimulated Mϕ was not affected by 50 μg/mL SP-A, which, however, significantly decreased the LPS-induced TNFα release by both iMϕ and aMϕ (Fig. 1A). SP-A also decreased the LPS-induced IL-1 release by iMϕ. However, it did not modify the IL-1 release by aMϕ (Fig. 2A). SP-A did not modify the LPS-induced IL-6 release by aMϕ and iMϕ (Fig. 3A).

LPS also increased the content of cGMP on aMϕ as well as on iMϕ (Fig. 4). SP-A significantly reduced the increase on cGMP content induced by LPS (Fig. 4A).

MB, a guanylate cyclase (GC) inhibitor, was effective in suppressing the LPS-induced increase in cGMP content (Fig. 4A). This inhibitory effect was accompanied by an inhibition in the TNFα secretion (Fig. 1A) without modifying IL-6 release (Fig. 3A). Neither LPS nor SP-A showed any detectable effect on the NO release to the medium (Fig. 5). In contrast, LPS significantly increased CO release to the medium by both iMϕ an aMϕ. In iMϕ, this increase was significantly

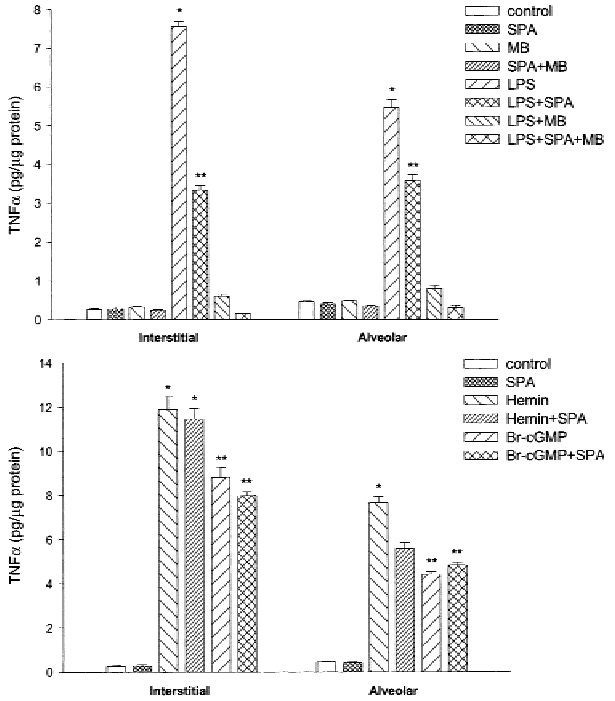

FIG. 1. **Secretion of TNFα by human pulmonary macrophages in the presence or absence of LPS (10 μg/mL), SP-A (50 μg/mL), methylene blue (MB, 10^{-5} mol/L), hemin (1 μmol/L), 8-Br-cGMP (1 mmol/L), and combinations.** Each column represents the mean ± SE of duplicate samples from 6 different experiments. *P < 0.01 vs. all other groups, **P < 0.01 vs. the rest (upper panel); *P < 0.01 vs. control and SP-A, **P < 0.05 vs. Br-cGMP groups (lower panel).

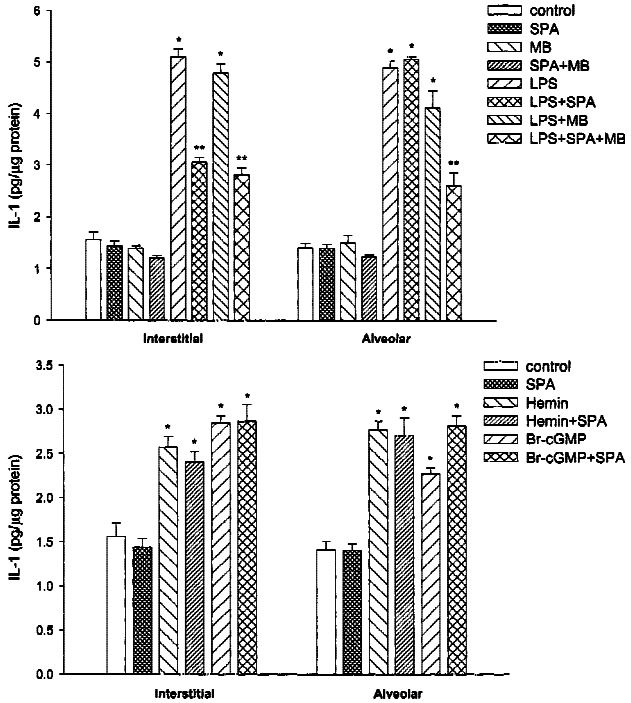

FIG. 2. **Secretion of IL-1 by human pulmonary macrophages in the presence or absence of LPS (10 μg/mL), SP-A (50 μg/mL), methylene blue (MB, 10^{-5} mol/L), hemin (1 μmol/L), 8-Br-cGMP (1 mmol/L), and combinations.** Each column represents the mean ± SE of duplicate samples from 6 different experiments. *P < 0.01 vs. all other groups, **P < 0.01 vs. the rest (upper panel); *P < 0.01 vs. control and SP-A (lower panel).

reduced in the presence of SP-A, which, however, did not modify the LPS-induced CO production on aMϕ (Fig. 6A).

The addition of 8-Br-cGMP to the medium increased TNFα (Fig. 1B) and IL-1 (Fig. 2B) release by both aMϕ and iMϕ but did not modify IL-6 release (Fig. 3b). The addition of hemin, the substrate for heme oxygenase, induced similar changes. The hemin effects on TNFα and IL-1 release were accompanied by an increase in cGMP content (Fig. 4B) and CO release to the medium (Fig. 6B). SP-A did not modify hemin nor cGMP effects.

In additional experiments, we studied the dose-dependent activity of SP-A by using a lower concentration (10 μg/mL) and another one higher (100 μg/mL) than 50 μg/mL. Even 100 μg/mL SP-A did not affect either the baseline concentrations of cytokines, CO and cGMP, or the LPS-stimulated release of IL-6 by Mϕ. In iMϕ, SP-A dose-dependently inhibited the the LPS-stimulated increases in TNFα (to 6.54 ± 0.05 at 10 μg/mL SP-A, and 2.94 ± 0.01 at 100 μg/mL SP-A; pg/μg protein; N = 3), IL-1 (to 4.25 ± 0.08 at 10 μg/mL SP-A, and 2.87 ± 0.04 at 100 μg/mL SP-A; pg/μg protein; N = 3), CO (to 5.15 ± 0.03 at 10 μg/mL SP-A and 2.68 ± 0.09 at 100 μg/mL SP-A; pmol/μg protein; N = 3), and cGMP (to 0.26 ± 0.02 at 10 μg/mL SP-A, and 0.11 ± 0.01 at 100 μg/mL SP-A; pmol/μg protein; N = 3). In aMϕ, SP-A did not affect the LPS-stimulated production of either IL-1 or CO even at the maximal concentration used. The effect on LPS-stimulated TNFα and cGMP increases was related to the SP-A concentration also in aMϕ (to 4.34 ± 0.28 at 10 μg/mL SP-A, and 3.02 ± 0.11 at 100 μg/mL SP-A; pg/μg protein; for TNFα; and to 0.17 ± 0.01 at 10 μ/mL SP-A, and 0.13 ± 0.01 at 100 μg/mL SP-A; pmol/μg protein; for cGMP; N = 3) . There were no significant differences between the effects of 50 μg/mL and 100 μg/mL SP-A in any case.

DISCUSSION

The main findings of the present study indicate that SP-A can modulate lung inflammation in humans by decreasing the LPS-induced production of proinflammatory cytokines by both iMϕ and aMϕ. They also show some differences in the response to SP-A between both Mϕ populations.

Pulmonary Mϕ are considered to be present in at least two anatomically different compartments (8, 9). Alveolar Mϕ are found in the bronchoalveolar lumen, where they act as a primary defensive line phagocytosing inhaled material. Interstitial Mϕ, similar in number, reside within the interstitial space and are thought to be precursors of the former. To our knowledge, all previous studies involving interactions between SP-A and pulmonary Mϕ were carried out using exclusively the alveolar variety, easily accessible by BAL. The method used by us permits also the recovery of iMϕ, which, in fact, seem to be more active from the immunological viewpoint (10). As a source of lung tissue, we used multiple organ donors because pulmonary Mϕ from patients with lung cancer, whose surgical resection specimen would have been a more accessible source of pulmonary tissue, have shown a higher TNFα and

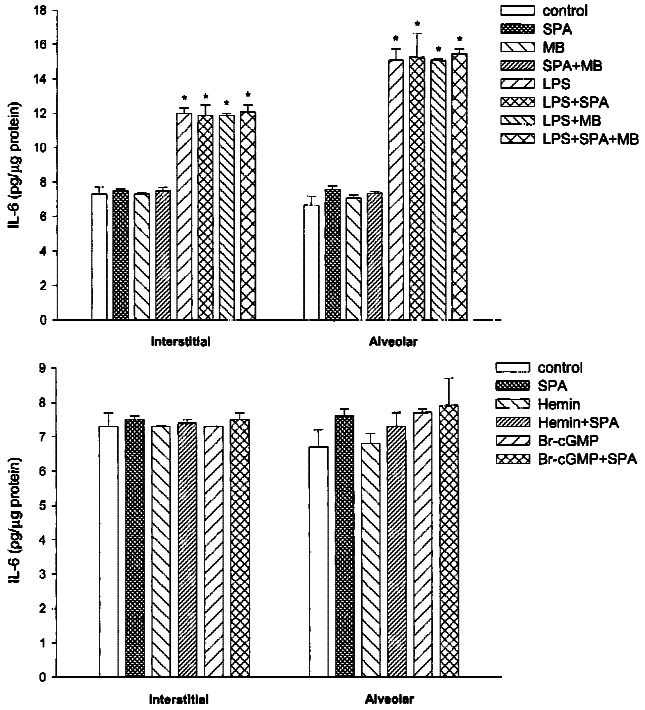

FIG. 3. **Secretion of IL-6 by human pulmonary macrophages in the presence or absence of LPS (10 µg/mL), SP-A (50 µg/mL), methylene blue (MB, 10^{-5} mol/L), hemin (1 µmol/L), 8-Br-cGMP (1 mmol/L), and combinations.** Each column represents the mean±SE of duplicate samples from 6 different experiments. *P < 0.01 vs. non-LPS groups.

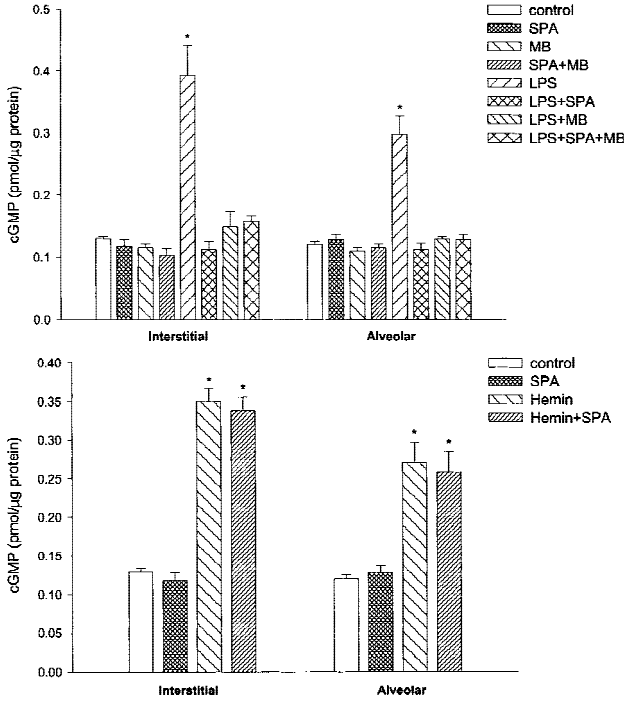

FIG. 4. **Levels of cGMP in human pulmonary macrophages in the presence or absence of LPS (10 µg/mL), SP-A (50 µg/mL), methylene blue (MB, 10^{-5} mol/L), hemin (1 µmol/L), and combinations.** Each column represents the mean ± SE of duplicate samples from 6 different experiments. *P < 0.01 vs. all other groups (upper panel); *P < 0.01 vs. control and SP-A (lower panel).

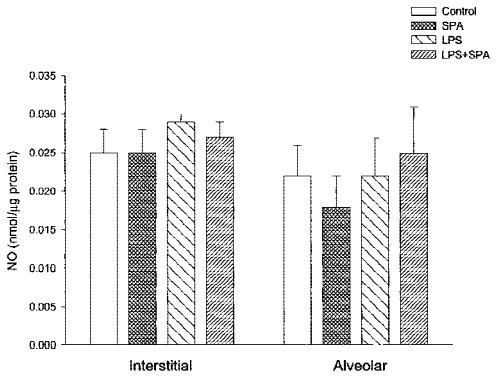

FIG. 5. **Production of nitric oxide by human pulmonary macrophages in the presence or absence of LPS (10 µg/mL) ± SP-A (50 µg/mL).** Each column represents the mean ± SE of duplicate samples from 6 different experiments.

IL-1 secretion *in vitro* than healthy controls (11). Similarly, we discarded the donors with recent history of tobacco smoking in view that functional changes have been reported in aMϕ from smokers (12). In bronchoalveolar fluid, the concentration of SP-A is normally 1–2 μg/mL (13). However, dilution due to the lavage procedure probably causes a 10- to 100-fold reduction in the concentrations normally present in the alveolar hypophase. Therefore, we believe that the SP-A concentration we have used is in the physiological range. On the other hand, the concentration of LPS used as a stimulus was selected according to previous dose-response studies in human pulmonary Mϕ (14).

Leukocytes obtained from the alveolar compartment by lavage techniques repeatedly have been shown to be hyporesponsive to inflammatory stimuli as compared with leukocytes isolated from peripheral blood. This relative "dampening" of leukocyte activity within the alveolar space is thought to protect the host from persistent immune cell activation via inhaled antigens. Surfactant has since long been implicated in this suppression, since surfactant lipid mixtures and individual lipid components were noted to inhibit lymphocyte proliferation and Ig secretion, as well as phagocyte oxygen radical production. Recently, immunosuppressive activity has also been demonstrated for SP-A, since this major surfactant component inhibited lymphocyte proliferation and IL-2 production (15), and reduced TNFα generation in Mϕ (4, 16). However, others have reported that SP-A per se stimulated proinflammatory cytokine production in mononuclear cells, secretion of Igs by splenocytes, and proliferation of lymphocytes (3, 17).

Our observation that SP-A decreased TNFα secretion from LPS-stimulated Mϕ appears to contradict the data from Kremlev and Phelps (3), who showed that SP-A could stimulate the production of TNFα by rat aMϕ and TNFα, IL-1, and IL-6 by human monocytes. Although our study focused on examining the effects of SP-A on LPS-activated Mϕ, we did test the ability of SP-A to induce production of TNFα, IL-1, or IL-6 by unstimulated cells and we could not detect any significant increase in the baseline liberation of these cytokines. We currently have no unequivocal explanation for these contrasting results, although there were some differences in experi-

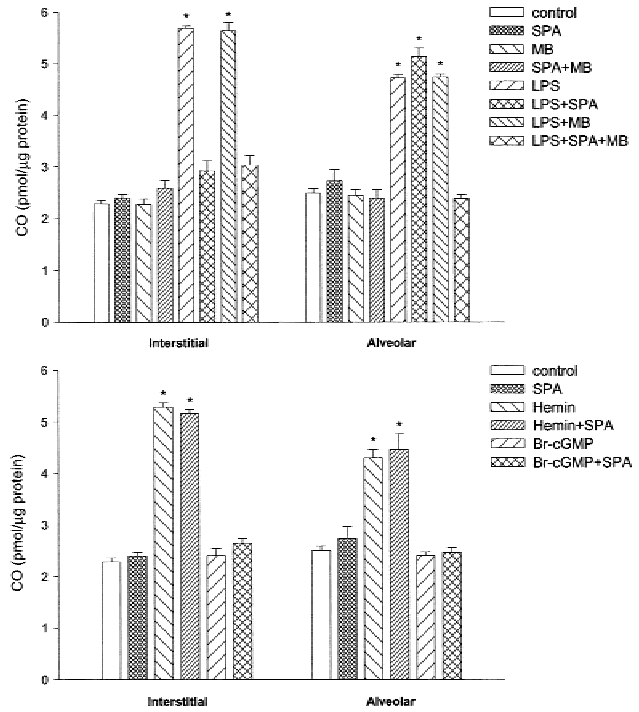

FIG. 6. **Production of carbon monoxide by human pulmonary macrophages in the presence or absence of LPS (10 µg/mL), SP-A (50 µg/mL), methylene blue (MB, 10^{-5} mol/L), hemin (1 µmol/L), 8-Br-cGMP (1 mmol/L), and combinations.** Each column represents the mean ± SE of duplicate samples from 6 different experiments. *$P <$ 0.01 vs. all other groups.

mental design. For example, the cells were isolated and plated under slightly different conditions. It is possible that these subtle differences in design induced differences in the responsiveness of the aMϕ. Major differences in the two studies are the different origin of pulmonary Mϕ, the different source of human SP-A and the different methods of SP-A isolation. Various cell types might respond differentially to SP-A, as well as the cells from different animals, and it is well known that interspecies variation in the mediator-induced behavior potentially is a problem when extrapolating from animal studies to humans. In an attempt to address this issue, we have employed Mϕ from human origin and we tested two different subpopulations of pulmonary Mϕ. The origin and chemical features of the SP-A batches employed in these studies must also be taken into consideration. We isolated SP-A from previously healthy organ donors. The SP-A used in the Kremlev and Phelps study (3) was from patients with alveolar proteinosis, whose SP-A are known to differ from normal subjects (18), and was isolated by a different technique. Also, SP-A appears to avidly bind LPS, and it even may enhance presentation of LPS to aMϕ (19); thus, even a trace contamination of endotoxin with SP-A might alter the Mϕ response. In agreement with our results, Rosseau et al. (4) have found that SP-A strongly suppress the proinflammatory cytokine response of human aMϕ and monocytes to *Candida albicans*, effecting down-regulation of proinflammatory cytokine synthesis at the transcriptional level. In this study, internalization of SP-A by Mϕ seemed necessary for the interference with the cytokine response. *In vivo*, aMϕ are continuously in contact with surfactant and SP-A and yet are not permanently activated. However, it is also recognized that SP-A likely functions differently *in vitro* than in the complex lipid-rich milieu of the alveolar spaces where any potential proinflammatory effect could be overcome by an antagonistic effect of another surfactant component. In fact, the previous *in vivo* surfactant exposure could also influence the *in vitro* behavior of aMϕ after isolation. This potential influence of previous surfactant exposure should be negligible in the iMϕ subpopulation.

Our findings, that exogenously added cGMP increases the release of TNFα and IL-1 and that LPS-stimulated TNFα and IL-1 release can be abolished by the GC inhibitor MB, are in agreement with the hypothesis of cGMP playing a role in the regulation of both TNFα and IL-1 production by macrophages (20). In contrast, a different regulatory mechanism seems to be present for IL-6. Cyclic GMP is generated by the enzyme GC, and the best known activator of the soluble isoform of this enzyme is NO (21). Nevertheless, a high output, inducible NO-generating system similar to that reported in rats and mice has not been found in human Mϕ under a variety of conditions (22, 23). In fact, we did not find any increase in nitrite concentration in the supernatants of LPS-stimulated macrophages despite their showing an increased production of cGMP and cytokines, confirming previous findings of our group (6) as well as others (22, 23). Since GC can be activated also by CO (21) and we have found this gas can participate in the LPS-induced cytokine production in human Mϕ (6), we chose to examine the production of CO in our system. We found an LPS-induced increase in CO generation by both iMϕ and aMϕ. Hemin, a precursor of CO elicited the same effect serving as a control. SP-A was able to suppress the LPS-induced production of CO on iMϕ but not on aMϕ.

At least two sources of CO are available, one of which is the metabolism of heme (24), catalysed by heme oxygenase. A second source is lipid peroxidation. Induction of heme oxygenase has been observed in the presence of several oxidant stresses, especially agents that deplete glutathione (25), and LPS has been found to induce heme oxygenase activity in rat macrophages and Kupffer cells (26). Our finding that hemin increases CO, TNFα, and IL-1 production and cGMP content indicates that endogenous heme oxygenase-dependent CO production is effective in triggering the production of both TNFα and IL-1 by macrophages, and SP-A seems not to interfere with this pathway.

On the other hand, an increase in the generation of oxygen free radicals is a well-known component in the pathophysiology of sepsis. Several specific intracellular signaling mechanism have been found to be influenced by redox changes, including calcium shifts, tyrosine kinase activation, and the nuclear transcription factor NF-κB. It has been shown that the LPS stimulatory pathway in the alveolar macrophage possesses a signal transduction mechanism that is sensitive to redox changes (27). Given that SP-A did not interfere with the effect of exogenously added cGMP or hemin, the inhibitory effect of SP-A on LPS-induced CO production in iMϕ might be explained by an SP-A-mediated attenuation of the LPS-induced oxygen radical production in iMϕ. Although SP-A does not have a scavenger effect for superoxide anion (28),

several studies suggest that SP-A alters oxygen radical production (28). Alveolar Mϕ incubated with SP-A have a decrease in superoxide production, indicating a dampening of the respiratory burst (15, 28) and suggesting that SP-A has a protective role against the oxidant injury caused by aMϕ in the lung. In contrast with these results, van Iwaarden et al. (2) found an increase in the oxygen radical production by rat aMϕ induced by human SP-A.

Collectively, our findings suggest functional differences between iMϕ and aMϕ. The LPS-induced increase in IL-1 release and CO generation was inhibited by SP-A in iMϕ, but not in aMϕ. These findings are also in agreement with earlier reports of the functional differences between aMϕ and Mϕ from digested lung tissue (9). Nevertheless, we cannot exclude a possible influence of the previous *in vivo* surfactant exposition of the aMϕ subpopulation. In addition, there exists the possibility that the enzyme treatment per se could have influence the phenotype of the cells, although Johansson et al. (8) showed that enzyme treatment did not modify the receptor expression of rat iMϕ.

The LPS-elicited synthesis of IL-1 in aMϕ, and IL-6 in both types of Mϕ was not suppressed by SP-A. This selective effect together with the finding that SP-A did not affect the baseline levels of cytokine generation in our Mϕ suggest a distinct immunomodulatory rather than a general inhibitory effect of SP-A on Mϕ inflammatory responsiveness.

In conclusion, the major surfactant protein SP-A was found to inhibit the LPS-induced TNFα response of both interstitial and alveolar human Mϕ, as well as the IL-1 response in iMϕ. The SP-A effect on TNFα production could be mediated by a suppression in the LPS-induced increase in intracellular cGMP. In iMϕ, but not in aMϕ, SP-A also inhibited the LPS-induced IL-1 secretion and CO generation. These data lend further credit to a physiological function of SP-A in regulating alveolar host defense and inflammation and suggest a fundamental role of this apoprotein in limiting excessive proinflammatory cytokine release in pulmonary Mϕ during the ARDS.

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DISCUSSION

DR. MAIER: I appreciate the opportunity to discuss this well-performed, extensive study. As the authors have shown, there is an impact of human SPA on pulmonary macrophage function in the lung with a selective effect on the intra-alveolar versus the interstitial populations. A significant advantage to their studies is that they used human tissues as a source for cells and awesome products. They used normal tissue and, as such, avoided many of the potential artifacts and complicating factors that have been reported in the literature. They show that SPA inhibits the production of both macrophage population's production of TNF and a selective inhibition of IL-1 in the interstitial macrophage, while there is no effect on IL-6 or nitric oxide production.

As published previously, the authors have demonstrated a carbon monoxide-dependent induction in cGMP, which appears to be the mechanism for upregulating TNF in the alveolar macrophage. In the current study SPA blocks cGMP and TNF in the alveolar macrophage population, yet does not block carbon monoxide production. Therefore inhibition of CO production in the alveolar macrophage is not the cause of the decreased GMP. Do the authors have any suggestions or data to help us understand at what point SPA is working in the alveolar macrophage that inhibits TNF production but not carbon monoxide.

I would also ask the authors to extend their comments on the LPS interactions in their experiments. The amount of LPS used was extremely high, at 15 mcg/ml. We and others have shown maximal production after 10 to 100 ng of LPS in human cells rather than these enormous doses. There are recent data showing that at extremely high doses, LPS may signal the macrophage through both the CD-14-dependent pathway and also a Toll protein-independent pathway. Do the authors have data at lower doses of LPS which would avoid this complicating dual pathway signaling to confirm SPA has an effect on the CD-14-dependent pathway? In addition, in their experimental methods I could not tell if they provided serum and therefore a source for lipopolysaccharide-binding protein (LPB). Was there serum present in their experimental conditions? Without LBP, it is highly likely that LPS was not signaling through CD-14.

Lastly, while SPA appears to have an effect, SPA, as they pointed out, constitutes only 5 to 10% of total surfactant in vivo. It is well known that the other phospholipids in surfactant also have significant LPS-binding and immunomodulatory effects. Have the authors added back increasing doses of SPA to depleted surfactant to establish the relative contribution of SPA to intact surfactant? This would establish a clinical relevance to SPA separate from the effect of surfactant overall?

I would like to thank the Society for the opportunity to discuss this paper.

DR. ARIAS-DIAZ: Thank you, Dr. Maier, for your interesting comments. In the first place, previous studies of our group about carbon monoxide regulating TNF production were performed exclusively using the interstitial variety of macrophages. So the different behavior of carbon monoxide in alveolar macrophages was an unexpected finding. SP-A was able to suppress the LPS-induced production of carbon monoxide on interstitial macrophages, but not on alveolar macrophages. However, SP-A antagonized the LPS-induced increase in cGMP and TNF in both types of cells. In our opinion, other guanylate-cyclase-stimulating molecule, different from nitric oxide and carbon monoxide, could be the responsible for the cGMP increase in alveolar macrophages. We currently haven't any other explanation for this finding.

With respect to the LPS concentration used by us, I agree it is a high LPS concentration, but we think it may be closer to the one present in pathological conditions. In the purulent exudate of a gram-negative pneumonia, LPS concentration is known to be very high. At 10 micrograms per mL, LPS might bind to membranes and different receptors than CD-14. Nevertheless, it continues to be a valid conclusion that SP-A is able to overcome some of the LPS effects either CD-14-specific or unspecific.

With regard to the presence of serum in the cultures, we add 10% fetal calf serum to the RPMI-1640 media.

Finally, regarding whether we have studied the effect of SP-A in combination with the lipid component of surfactant, yes we did. We have repeated some of these experiments with SP-A with and without either phosphatidylcholine or dipalmitoylphosphatidylcholine. We found that neither phosphatidylcholine nor dipalmitoylphosphatidylcholine had any effect on TNF production in the absence of SP-A. Interestingly, they did not interfere with the effect of SP-A on the production of cytokines by LPS-stimulated pulmonary macrophages, in spite that we had previously found that SP-A binds and aggregates phospholipid vesicles in the RPMI-1640 medium.