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Intercellular Adhesion Molecule-1 Mediates Cellular Cross-Talk between Parenchymal and Immune Cells after Lipopolysaccharide Neutralization

Jin-Hwa Lee,* Lorenzo Del Sorbo,* Stefan Uhlig,† Giuliana A. Porro,* Thomas Whitehead,* Stefanos Voglis,* Mingyao Liu,† Arthur S. Slutsky,* and Haibo Zhang2*

The mechanisms by which parenchymal cells interact with immune cells, particularly after removal of LPS, remain unknown. Lung explants from rats, mice deficient in the TNF gene, or human lung epithelial A549 cells were treated with LPS and washed, before naive alveolar macrophages, bone marrow monocytes, or PBMC, respectively, were added to the cultures. When the immune cells were cocultured with LPS-challenged explants or A549 cells, TNF production was greatly enhanced. This was not affected by neutralization of LPS with polymixin B. The LPS-induced increase in the expression of ICAM-1 on A549 cells correlated with TNF production by PBMC. The cellular cross talk leading to the TNF response was blunted by an anti-ICAM-1 Ab and an ICAM-1 antisense oligonucleotide. In A549 cells, a persistent decrease in the concentration of intracellular cAMP was associated with colocalization of LPS into Toll-like receptor 4 and the Golgi apparatus, resulting in increased ICAM-1 expression. Inhibition of LPS internalization by cytochalasin D or treatment with dibutyryl cAMP attenuated ICAM-1 expression and TNF production by PBMC. In conclusion, lung epithelial cells are not bystanders, but possess memory of LPS through the expression of ICAM-1 that interacts with and activates leukocytes. This may provide an explanation for the failure of anti-LPS therapies in sepsis trials. The Journal of Immunology, 2004, 172: 608–616.

Lipopolysaccharides are components of the outer membrane of Gram-negative bacteria and causative agents of sepsis. To study the interaction between LPS-stimulated parenchymal cells and immune cells, previous studies have used coculture systems of epithelial cells with leukocytes (1–3). Although such coculture models have shown increased cytokine responses compared with cell culture with each cell type individually, the chosen experimental design did not allow specific identification of the role of the parenchymal cells for two reasons: first, the two cell types were in contact throughout the experiment, and second, LPS was present during the entire study period. To further define the role of parenchymal cells in their interaction with leukocytes, the activation of the parenchymal cells must be experimentally separated from that of the leukocytes. To investigate this, we first exposed parenchymal cells to LPS, and then removed the LPS before the addition of immune cells. We theorized that the activation of parenchymal cells might enable them to cross talk with immune cells even after LPS is removed. If the parenchymal cells remembered LPS, this could explain why many septic studies have observed a continuation of the inflammatory process even after neutralization of LPS with specific Abs (4).

We focused on lung cells, because the lung is frequently the first organ to fail during Gram-negative sepsis (5, 6). As coculture systems, we used either primary rat lung explants together with rat alveolar macrophages (AM),7 primary mouse lung explants lacking TNF gene expression together with bone marrow monocytes isolated from wild-type control mice, or human lung epithelial A549 cells together with human PBMC. We chose these model systems for three reasons. First, LPS induces changes in the expression of inflammatory mediators in lung explants and human A549 cells (3, 7–10). Second, LPS-treated A549 cells do not produce TNF, the primary end point of leukocyte activation in our study (11). Third, AM and PBMC are part of the innate immune system and interact with the pulmonary epithelium (3). Moreover, these immune cells release a wide variety of inflammatory mediators that may cause acute lung injury and worsen the symptoms of sepsis (4).

A particularly important mediator of macrophages or monocytes is TNF. TNF plays a pivotal role in organizing the host defense response, and as such may also participate in the overshooting immune response that is thought to contribute to acute lung injury (12). In this study, measurement of TNF release into the culture medium served as a marker of leukocyte activation.

Materials and Methods

Reagents

Monoclonal anti-human ICAM-1 (CD54), mouse IgG1 isotype control, and recombinant soluble human CD14 (sCD14) were purchased from R&D Systems (Minneapolis, MN). BODIPY fluorescent conjugate LPS from Escherichia coli serotype 055:B5 and BODIPY Texas Red (TR) ceramide were obtained from Molecular Probes (Eugene, OR). PE anti-human Toll-like receptor 4 (TLR4) mAb (isotype: mouse IgG2a, k) and PE mouse IgG2a isotype control were purchased from eBioscience (San Diego, CA). LPS from E. coli serotype 055:B5, polymyxin B sulfate, and dibutyryl

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3 Abbreviations used in this paper: AM, alveolar macrophage; sCD14, soluble CD14; TLR, Toll-like receptor; TR, Texas Red.
The cells were centrifuged at 1500 g and layered over a three-step gradient (52, 65, and 75% Percoll). Cell purity and viability were measured from the monocyte-enriched fraction at the interface of the 52 and 65% Percoll layers. DNA oligonucleotides were synthesized on an automated DNA synthesizer (Abbott, North Chicago, IL; Model ABLSG-113, 313, 513). The antisense oligonucleotide was chosen based on the published data in which ISIS 1012 was shown to inhibit ICAM-1 mRNA in A549 cells (18). A concentration of 1 μM ISIS 1939 has been shown to decrease ICAM-1 synthesis to near baseline level in A549 cells (18). The control ICAM-1 sense sequence was 5’-GAGAGGGAAAGTGGTGGGGG-3’. LPS uptake and colocalization with TLR4 and Golgi
A549 cells were seeded at 2.5 × 10^5 cells/100-mm dish in chamber slides (Nunc, Naperville, IL), and treated with 1 μg/ml BODIPY fluorescent conjugate LPS for 0, 4, and 8 h. This concentration of LPS was based on the amount of LPS initially added to prepare the LPS-sCD14 complex. Some cells were incubated for 30 min with 10 μM cyclohexatin D before the LPS treatment. Mice were dissected, and their AM were collected for isolation. The slides were visualized using a Zeiss510 (Oberkochen, Germany) confocal scanning laser microscope.

ICAM-1 expression on A549 cells by flow cytometry
A549 cells were treated with LPS for 4 h, trypsinized, and washed in PBS. After 30-min incubation with mouse anti-human ICAM-1 mAb, cells were washed and incubated for 30 min with FITC-labeled goat anti-mouse IgG. Cells were washed in PBS and fixed. Fluorescence from FITC-labeled anti-ICAM-1 Abs was collected through a 530-nm pass band filter with a total of 10^6 cells assessed.

Intracellular cAMP assay
Lung explants and A549 cells were collected after incubation with LPS. The supernatants of homogenates were assayed for cAMP by using a cAMP enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI).

LiquiChip multiple cytokine assay
Eleven cytokines (human and mouse IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12(p70), TNF, GM-CSF, and IFN-γ, and human IL-8 and mouse IL-5) in culture medium were simultaneously measured using LiquiChip cytokine kits (Qiagen, Valencia, CA).

Assay for LPS
The LPS content of the culture medium was determined by the Limulus amebocyte lysate assay (Pyrochrome, Cape Cod, Falmouth, MA), as previously described (10).

Statistical analysis
A two-way ANOVA, followed by the Tukey-Kramer test, was used for statistical analysis of the data. Differences were considered statistically significant at p < 0.05. Data are presented as means ± SE.

Results

TNF production after LPS stimulation of cocultures of lung explants and AM
Rat lung explants alone (Fig. 1A), AM alone (Fig. 1B), or in combination (Fig. 1A) released spontaneously only small amounts of TNF in culture supernatant. LPS (100 ng/ml) exposure only mildly stimulated TNF release from the explants, in contrast to its strong effect on AM (Fig. 1B). When lung explants were cocultured with AM, LPS stimulation resulted in a comparable TNF release as observed with AM alone (Fig. 1A). As in vivo, this coculture remains in a quiescent state until stimulated.

After the initial 4-h incubation (Fig. 2A), lung explants were washed and transferred to a new culture dish containing fresh medium. Following medium replacement, 2 μg/ml polymyxin B, a cationic polypeptide that binds to the lipid A portion of LPS, resulting in inhibition of LPS activity (19), was added to the lung explant culture. This concentration of polymyxin B has been shown to inhibit the calcium phosphate degradation induced by 100–500 ng/ml LPS in human AM (19), block CD14 expression in human macrophages, and attenuate LPS-induced production of cytokines in mouse AM (20). It is also noteworthy that in addition to its inhibition of LPS activity, polymyxin B is a nonspecific antagonist of protein kinase C (21).

The transferred, polymyxin B-treated lung explants were then incubated for additional 4 h with naive rat AM (0.5 × 10^6 and 1 ×
The addition of AM elicited an 8-fold greater release of TNF (Fig. 2B). This increased TNF response was not due to LPS contamination because the addition of polymyxin B did not affect the TNF levels (Fig. 2B). The ability of polymyxin B to prevent LPS-induced TNF release is illustrated in Fig. 2C.

LPS-primed lung explants produce elevated levels of tissue TNF

After treatment with LPS for 8 h, lung explants produced a much higher concentration of TNF than control explants (Fig. 3A). After a 4-h stimulation with LPS, lung explants that were washed, treated with polymyxin B, and incubated for another 4 h still had higher TNF levels than controls (Fig. 3B).

cAMP levels in LPS-treated lung explants

In many cases, a proinflammatory state of cells is associated with a down-regulation of cAMP (22). cAMP can modulate TNF production in leukocytes; its role in the inflammatory response of parenchymal cells is less understood. We find a persistent decrease in tissue cAMP concentration over 8 h with LPS stimulation (Fig. 3).

To further clarify the source of TNF during the cellular cross talk, the above experiments were repeated using lung explants obtained from TNF−/− mice. A similar TNF response was observed when naive bone marrow monocytes obtained from TNF−/− mice were incubated with the LPS-challenged lung explants from TNF−/− mice (Fig. 4A). This result confirmed that the immune cells were the major source of TNF in the coculture models. Identical coculture experiments showed that the immune cells were a major source of GM-CSF as well (Fig. 4B).

TNF production after LPS stimulation in cocultures of A549 cells and PBMC

It is difficult to know with lung explants which cell type is responsible for the interaction with the AM and monocytes. To specifically define the role of alveolar epithelium in the cellular cross talk, we used human lung A549 cells instead of lung explants and repeated the experiments. We chose human lung epithelial A549 cells, because these cells do not produce TNF, but do produce a wide variety of other mediators in response to LPS (3, 9, 23, 24). This also allowed us to avoid using anti-TNF Abs, which can affect the production of other inflammatory mediators as well.

We repeated the experiments shown in Figs. 1, 2, and 4 using A549 cells and human PBMC. Cocultures of A549 cells and PBMC released only small amounts of TNF in the absence of LPS, but high amounts in its presence (Fig. 5A). To investigate whether A549 cells retained a similar memory of LPS exposure, as did the lung explants, A549 cells were treated with LPS, followed by vigorous washing and treatment with polymyxin B. Then addition of naive PBMC (0.5 × 10^6 and 1 × 10^6) to the LPS-challenged A549 cells resulted in a 32-fold increase in TNF production (Fig. 5B).

As was seen in lung explants, LPS treatment decreased cAMP levels in A549 cells, and this decrease persisted for 8 h after the LPS was removed (Fig. 5C).

LPS concentration in cocultures of A549 cells and PBMC after addition of polymyxin B to fresh medium

It is critical to demonstrate the LPS concentration in the cultures. After washing and the addition of polymyxin B, the LPS level in A549 cell culture medium was 63.8 ± 11.3 pg/ml. This concentration of LPS does not induce any significant TNF response by fresh PBMC, as was demonstrated in a separate experiment in
which human PBMC were incubated for 4 h with LPS at 0, 10, 100, and 1000 pg/ml producing TNF concentrations of 28.2 ± 2.9, 15.8 ± 3.9, 26.2 ± 6.2, and 103.6 ± 8.7 pg/ml, respectively. These data further confirm that LPS-exposed lung tissue or epithelial cells deliver signals to immune cells to produce TNF, even after LPS has been removed or neutralized.

Mechanisms of cellular cross talk

We examined two potential mechanisms by which cellular cross talk could take place after LPS removal: 1) mediator release from LPS-challenged A549 cells into the culture medium, or 2) direct cell-cell (i.e., A549 cells and PBMC) contact.

A549 cells were incubated with LPS for 4 h, washed, and incubated for an additional 4 h in the presence of polymyxin B. The supernatant was then used as a conditioned medium for PBMC for 4 h. The concentration of TNF released by PBMC was 27.0 ± 0.59 pg/ml in the control DMEM culture medium, and 30.0 ± 1.7 pg/ml in the conditioned medium, as compared with 9.6 ± 2.1 pg/ml in culture supernatant after 4-h stimulation of A549 cells with LPS (p = NS). This result indicates that the levels of mediators in the conditioned medium were too low to induce cellular cross talk.

Mediation of cell cross talk through ICAM-1 that depends on intracellular cAMP

One of the possible inflammatory responses that are associated with decreased cAMP levels is an increased expression of ICAM-1 (25, 26). Further reasons to focus on ICAM-1 were the following: 1) ICAM-1 is found on both type I and type II alveolar epithelial cells (9, 24, 27); 2) LPS stimulation increases expression of ICAM-1 on type II cells in vitro (9, 24, 27); 3) the ligand for ICAM-1, i.e., Mac-1 (CD11a/CD18), is localized on leukocytes (28); and 4) ICAM-1 plays a central role in cell-cell contact-mediated immune responses (29) and the adherence of leukocytes to epithelial cells (27). We therefore tested the hypothesis that direct cell-cell contact provided by ICAM-1 plays a crucial role in mediating the cellular cross talk following removal of LPS.

ICAM-1 expression on A549 cells increased at 4 h, peaked at 8 h, and declined to near baseline levels at 24 h after LPS stimulation (Fig. 6A). Between 4 and 8 h, when surface expression of ICAM-1 was high, addition of PBMC increased TNF production (Fig. 6B), while at 24 h, when ICAM-1 expression was low, the TNF response of the added PBMC was also low (Fig. 6C).

Additional A549 cells were pretreated with 200 μg/ml anti-human ICAM-1 mAb for 1 h and were then treated with LPS and incubated for 4 h. After washing, followed by addition of polymyxin B, the cells were incubated for an additional 4 h with 1 × 10^6 human PBMC that were treated with anti-human ICAM-1 mAb for 1 h before the addition to the coculture. Treatment with an anti-ICAM-1 Ab decreased TNF production following addition of PBMC at both 4 and 24 h (Fig. 6, B and C).

To confirm the role of ICAM-1 in mediating cellular cross talk, A549 cells were pretreated with 1 μM ICAM-1 sense or antisense oligonucleotide in the presence of serum-free DMEM, 100 ng/ml LPS, and 20 μg/ml lipofectamine for 4 h. The A549 cell culture...
medium was replaced with fresh DMEM, plus the antisense oligonucleotide, and the cells were incubated with PBMC for an additional 4 h.

Treatment of A549 cells with the oligonucleotide ISIS 1939 blocked the cellular cross talk, as shown by the complete inhibition of TNF production by the PBMC added later (Fig. 7A). This finding suggested that the interaction between LPS-treated A549 cells and PBMC depended on a direct interaction mediated by ICAM-1. The use of antisense also significantly attenuated other cytokine responses, including IL-1β, IL-6, IL-8, and GM-CSF (Fig. 7, B and C). These results indicate that the ICAM-1-mediated cellular cross talk contributes to a generalized inflammatory response.

ICAM-1 expression and cell cross talk after LPS treatment were cAMP dependent

We next examined the effect of cAMP on the LPS-elicited expression of ICAM-1 on A549 cells by the addition of dibutyryl cAMP, a membrane-permeable cAMP analog that activates cAMP-dependent protein kinases. Dibutyryl cAMP (100 μg/ml) was added 30 min before LPS challenge of A549 cells and together with PBMC following replacement of the medium of the LPS-challenged A549 cells. This concentration of dibutyryl cAMP has been previously reported to block ICAM-1 expression on PBMC in the presence of LPS (29). Dibutyryl cAMP inhibited LPS-induced ICAM-1 expression on A549 cells (Fig. 8A). This was accompanied by a reduction in the production of TNF when PBMC were subsequently added (Fig. 8B). These data suggest that cellular cross talk is mediated by ICAM-1, whose expression is modulated by the intracellular cAMP concentration.

LPS internalization by A549 cells

The sustained intracellular signaling alterations caused by LPS led us to examine the possibility of LPS uptake by A549 cells, as has been described for several other cell types (30, 31). Cells were incubated with 1 μg/ml BODIPY fluorescent LPS-sCD14 complex for various lengths of time (Fig. 9). Subsequently, fixed A549 cells were stained for actin filaments (Fig. 9A; red label). A diffuse distribution of LPS (Fig. 9B; green label) was apparent in A549 cells by 4 h, with more intense perinuclear staining after 8 h.

**FIGURE 5.** TNF production by A549 cells alone, PBMC alone, and their coculture 4 and 8 h with and without LPS stimulation (A). *p < 0.05 vs A549 cells alone at the identical conditions. Priming of A549 cells with LPS increased TNF production by PBMC added later (B). A549 cells were incubated with or without LPS for 4 h. After replacement of medium, extensive washing, followed by treatment with polymyxin B, the A549 cells were incubated for an additional 4 h in the presence of resting PBMC (n = 12 samples from at least four subjects in each group). *p < 0.05 vs PBMC + polymyxin B without LPS pretreatment under identical conditions. †p < 0.05 vs PBMC (0.5 × 10⁶ cells) at the identical conditions. Concentrations of intracellular cAMP were measured in A549 cells 4 and 8 h after LPS stimulation (C). #p < 0.05 vs control A549 cells at the identical time points.

**FIGURE 6.** Effect of LPS on surface expression of ICAM-1 in A549 cells at 4, 8, and 24 h (A). *p < 0.05 vs controls (□) at the identical time points, respectively. Effect of anti-human ICAM-1 mAb on TNF production by PBMC later added to LPS-primed A549 cells at 4 h (B) and 24 h (C). A549 cells were pretreated with 200 μg/ml anti-human ICAM-1 mAb for 1 h, and were then randomized to either serve as a control or receive LPS (100 ng/ml) and incubated for 4 h. After washing, followed by addition of polymyxin B, the cells from the control and LPS-treated groups were then incubated for an additional 4 h with 1 × 10⁶ human PBMC that were pretreated with or without 200 μg/ml anti-human ICAM-1 mAb for 1 h, respectively. Experiments were repeated nine times. *p < 0.05 vs control A549 cells at the identical conditions and time points; †p < 0.05 vs LPS-primed A549 cell without ICAM-1 mAb or with control IgG at the identical time points, respectively.
LPS is transported to the Golgi complex

The trafficking of LPS was investigated in A549 cells. After 4-h incubation with BODIPY fluorescent LPS, the cells were exposed to three different stains (BODIPY TR C5-ceramide, selective for the Golgi apparatus; PE anti-human TLR4 mAb; and mouse isotype control) and visualized with a confocal microscope (Fig. 10). There is a considerable overlap between LPS and Golgi- or TLR4-specific strains, which were absent in experiments with control IgG Abs (Fig. 10D, right column).

ICAM-1 expression and cellular cross talk are dependent on uptake of LPS

To examine whether LPS internalization is required for mediating the cellular cross talk, A549 cells were pretreated for 30 min with 10 μM cytochalasin D to inhibit endocytosis of LPS. Fig. 9, C and D, demonstrates that cytochalasin D completely blocked the internalization of LPS, which was apparent in control A549 cells 4 h after LPS stimulation (Fig. 9, A and B). At the same time, cytochalasin D decreased ICAM-1 expression on A549 cells (Fig. 9E) and the production of TNF by PBMC (Fig. 9F).

Discussion

To study the interaction between LPS-stimulated parenchymal cells and immune cells, we first stimulated parenchymal cells with LPS, and then removed and neutralized the LPS before immune cells were added. Our model is different from other models of cellular cross talk, in which two cell types are cocultured from the very beginning, and LPS is present during the entire study period (1–3, 32). We demonstrated that LPS-primed lung parenchymal cells were able to deliver signals to leukocytes to produce TNF. These findings indicate that parenchymal lung cells possess a memory of LPS that is not affected by subsequent neutralization of LPS. These data further indicate that LPS continues to exert its inflammatory effect in lung epithelial cells by initiating a cell signaling cascade in leukocytes, which, once triggered, no longer requires the extracellular presence of LPS. The results obtained from coculturing human A549 cells and PBMC were similar to those obtained in coculturing rat lung explants and AM, and mouse lung explants and monocytes.

We wanted to determine whether A549 cells increased PBMC production of TNF or whether the PBMC allowed the epithelial
cells to produce TNF. We addressed this concern in three ways: 1) in line with previous findings (33), by using microarray technology, we found that TNF gene expression is absent in A549 cells after stimulation with either LPS, TNF, mechanical stress, or combined TNF and mechanical stress, suggesting that A549 cells were not the source of TNF; 2) a similar cellular cross talk was observed after stimulation with either LPS, TNF, mechanical stress, or combined TNF and mechanical stress, suggesting that A549 cells were not the source of TNF; 3) a similar cellular cross talk was observed

FIGURE 9. LPS uptake by A549 cells at various times. A549 cells were incubated with 1 mg/ml BODIPY fluorescent conjugate LPS-sCD14 complex for the indicated time points, fixed, and stained for actin filaments (F-actin) in red. A diffuse distribution of LPS in green was apparent in A549 cells by 4 h, with more intense staining located around nuclei at 8 h (A). Pretreatment with cytochalasin D at 10 μM completely blocked the endocytosis of LPS (B), attenuated ICAM-1 expression on A549 cells, and decreased TNF production when PBMC were added later (C). *, p < 0.05 vs control A549 cells at the identical time point; †, p < 0.05 vs LPS + A549 cells at the identical time point.

FIGURE 10. Colocalization of BODIPY fluorescent LPS with Golgi- and TLR4-specific fluorophores. A549 cells were treated with fluorescently labeled LPS-sCD14 complex (green) for 4 h, followed by immune staining with BODIPY TR C2-ceramide (red in A), PE anti-human TLR4 mAb (red in B), or PE mouse IgG2a isotype control (C). Colocalization of green and red pixels appears yellow in overlay. Experiments were repeated six times.
when TNF−/− mice bone marrow monocytes were added to the LPS-challenged lung explants obtained from TNF−/− mice; and 3) we further demonstrated that the TNF production was dependent on the number of leukocytes subsequently added. We conclude that the immune cells were the major source of TNF. However, lung alveolar epithelial cells are not mere bystanders, but are actively participating in the cellular cross talk after LPS removal.

Our findings may explain the discrepancies observed between previous animal and clinical studies in which anti-LPS Abs were successful when given before endotoxin injection in animals (34–36), but not in already endotoxemic patients (4, 5). The present study provides an explanation for the failure of anti-LPS Abs in sepsis trials.

In our attempt to understand the mechanism of how parenchymal cells remembered LPS, we focused on ICAM-1. The magnitude of the response was dependent on the level of ICAM-1 expression following LPS stimulation. The inhibition of this response by the anti-ICAM-1 Abs or the antisense oligonucleotide ISIS 1939 clearly showed the involvement of ICAM-1 in the LPS-initiated cellular cross talk. We also demonstrated that the ICAM-1 antisense oligonucleotide was more effective than anti-ICAM-1 Abs, at the doses used, in blocking the cellular cross talk. The differences of efficacy observed may be due to the mechanisms of action in which anti-ICAM-1 Abs neutralize only membrane-bound and soluble proteins, whereas the antisense ISIS 1939 prevents ICAM-1 synthesis by destabilizing the ICAM-1 mRNA through RNase H-mediated hydrolysis (18).

ICAM-1 is located on the apical side of lung epithelial cells in vitro (27) and on the surface of alveolar epithelial cells in vivo (37, 38). ICAM-1 expression in alveolar type II cells is stimulated by LPS through NF-κB-dependent and independent mechanisms (9, 39). ICAM-1 not only enhances the adhesion of leukocytes to LPS-treated pulmonary epithelial cells (24), but also stimulates cell signaling cascades, resulting in specific immune and inflammatory responses (40). Although these processes can serve as a protective mechanism against microbial pathogens, ICAM-1-mediated stimulation of leukocytes may also cause lung injury through the release of inflammatory mediators such as TNF.

A number of studies have addressed the importance of pulmonary ICAM-1 expression by interacting with leukocytes in relation to lung inflammation under in vivo conditions (41, 42). In a murine pneumonia model, pretreatment with ICAM-1 Abs and antisense oligonucleotides largely reduced lung injury (43). In Candida albicans-infected rats, administration of anti-ICAM-1 Abs led to increased survival, decreased lung wet/dry weight ratios and lung neutrophil counts, reduced microscopic lung injury, and bacterial load in the lung (44). Also, rats treated with the ICAM-1 antisense oligonucleotide ISIS 17470 showed an attenuated ileitis as a result of down-regulation of leukocyte adherence and inflammation (45). Thus, ICAM-1 inhibition is a potential candidate for anti-inflammatory treatment in inflammatory diseases.

Intracellular cAMP levels orchestrate various cellular signaling cascades among the proinflammatory responses (22, 46). Therefore, any change in intracellular cAMP concentrations is expected to have extensive ramifications within the cell. The persistently low cAMP levels in the LPS-treated explants or A549 cells are consistent with observations in other cell types (47, 48).

The effect of dibutyryl cAMP mimicked that of the anti-ICAM-1 Abs or antisense oligonucleotides. The mechanism by which dibutyryl cAMP inhibits ICAM-1 may be due to inactivation of NF-κB because the promoter region of the ICAM-1 genes contains binding sites for NF-κB (49), and cAMP inhibits the activation of NF-κB in a variety of human cells (26, 50). In support of our results, Morichika et al. (26) have recently demonstrated that dibutyryl cAMP in a concentration-dependent manner inhibited LPS-induced ICAM-1 expression on monocytes. These results indicate that the intracellular cAMP levels are a determinant of ICAM-1 expression.

LPS is internalized in several cell types (15, 30, 31, 51–53), but this has never been examined previously in lung epithelial cells. In this study, we demonstrate that LPS was internalized in A549 cells as early as 4 h after LPS challenge. In support of our finding, Risco et al. (54) demonstrated that in rat type II epithelial cell microvilli, LPS initially enters the cytoplasm forming discrete patches, and is later localized in condensed chromatin-free areas around the nuclei, a finding similar to that reported by others (30, 31). We demonstrated that the LPS was colocalized to the Golgi apparatus and TLR4 in A549 cells. Several investigators have previously demonstrated that LPS is localized to the Golgi complex of cardiomyocytes (30) and m-ICcl2, a murine small intestinal epithelial cell line (31). More recently, it has been demonstrated that LPS, TLR4, MD-2, and CD14 complexes recycle between plasma membrane and Golgi complex, resulting in intracellular trafficking of LPS (55, 56).

To ascertain whether LPS internalization by A549 cells was required in mediating the cellular cross talk, we inhibited endocytosis with cytochalasin D. Administration of cytochalasin D blocked the LPS internalization in A549 cells and attenuated ICAM-1 expression and TNF production by PBMC subsequently added. Because cytochalasin D does not affect ICAM-1 expression (57), these data indicate that LPS internalization plays an important role in mediating cellular cross talk. We did not examine directly whether and how intracellular trafficking of LPS affects cellular cross talk. However, Lutz et al. (56) have demonstrated that blocking LPS trafficking by brezafibron A to disrupt Golgi-associated TLR4 is not associated with signal transduction in human embryonic kidney 293 (HEK293) cells.

In conclusion, LPS-treated lung epithelial cells are not bystanders and express ICAM-1 under the control of intracellular cAMP levels even after LPS is removed or neutralized. ICAM-1-mediated adhesion is a sufficient stimulus for leukocytes to produce inflammatory cytokines. Our study presents evidence of a viable interaction between parenchymal cells, leukocytes, and LPS that may also provide an explanation for the failure of anti-LPS Abs in sepsis trials.

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