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Differential Regulation of Cytokine Release and Leukocyte Migration by Lipopolysaccharide-Stimulated Primary Human Lung Alveolar Type II Epithelial Cells and Macrophages

Andrew J. Thorley,* Paul A. Ford,* Mark A. Giembycz, ‡ Peter Goldstraw, ‡ Alan Young, § and Teresa D. Tetley²*

Bacterial colonization is a secondary feature of many lung disorders associated with elevated cytokine levels and increased leukocyte recruitment. We hypothesized that macrophages, the epithelium would be an important source of these mediators. We investigated the effect of LPS (0, 10, 100, and 1000 ng/ml LPS, up to 24 h) on primary human lung macrophages and alveolar type II epithelial cells (ATII; isolated from resected lung tissue). Although macrophages produced higher levels of the cytokines TNF-α and IL-1β (p < 0.0001), ATII cells produced higher levels of chemokines MCP-1, IL-8, and growth-related oncogene α (p < 0.001), in a time- and concentration-dependent manner. Macrophage (but not ATII cell) responses to LPS required activation of ERK1/2 and p38 MAPK signaling cascades; phosphorylated ERK1/2 was constitutively up-regulated in ATII cells. Blocking Abs to TNF-α and IL-1β during LPS exposure showed that ATII cell (not macrophage) MCP-1 release depended on the autocrine effects of IL-1β and TNF-α (p < 0.003, 24 h). ATII cell release of IL-6 depended on autocrine effects of TNF-α (p < 0.006, 24 h). Macrophage IL-6 release was most effectively inhibited when both TNF-α and IL-1β were blocked (p < 0.03, 24 h). Conditioned media from ATII cells stimulated more leukocyte migration in vitro than conditioned media from macrophages (p < 0.0002). These results show differential activation of cytokine and chemokine release by ATII cells and macrophages following LPS exposure. Activated alveolar epithelium is an important source of chemokines that orchestrate leukocyte migration to the peripheral lung; early release of TNF-α and IL-1β by stimulated macrophages may contribute to alveolar epithelial cell activation and chemokine production. The Journal of Immunology, 2007, 178: 463–473.

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3 Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; ATII, alveolar epithelial type II; GRO, growth-related oncogene; PSG, penicillin/streptomycin/glutamine; KO, knockout.

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the alveolar epithelium is a rich source of chemokines (15, 16). Indeed, ATIIs cell chemokine release increases dramatically following exposure to LPS, reflecting the cell surface expression of TLR4 (17).

There have been numerous investigations that focused on the functional contribution to pulmonary homeostasis of a single cell type (e.g., the macrophage, epithelial cell). However, to our knowledge, there are no comparative studies of human lung macrophages and epithelial cells from the same subjects; yet, it is important to understand how these cells complement each other in maintaining homeostasis of the alveolar unit. Thus, we isolated primary human alveolar macrophages and ATIIs cells from the specimens of lung tissue obtained from sequential subjects to compare the response of these cells to LPS and to determine how this may influence leukocyte recruitment. We hypothesized that these cells would differentially release cytokines (IL-1β, TNF-α, and IL-6) and chemokines (IL-8, MCP-1, and growth-related oncogene α (GROα)) following LPS exposure and this, in turn, would govern peripheral blood leukocyte migration in an in vitro model.

Materials and Methods

Isolation of primary human alveolar macrophages and type II epithelial cells

ATII cells and macrophages were isolated from lungs of grossly normal appearance following resection for lung carcinoma, with the approval of the Royal Brompton and Harefield Ethical Committee, as previously described (n = 16 consecutive subject samples; Ref. 15). Briefly, lung sections were perfused by injection of sterile saline until the cell count was <1 × 10⁶ cells/ml. The draining lavage was then collected and centrifuged (290 × g, 10 min, 20°C). The cell pellet was resuspended in serum-free DCCM-1 (React Scientific) containing 1% penicillin/streptomycin/H11003 glutamine (PSG; Invitrogen Life Technologies) and plated in 24-well tissue culture plates (0.5 × 10⁶/ml) for 40 min in DCCM-1 containing 1% PSG at a concentration of 1 × 10¹⁰ cells/ml, washed to remove any residual LPS. Fresh serum-free medium was then added and the cells were incubated for 40 min in DCCM-1 containing 1% PSG. The mixture was passed through a 300-μm filter, followed by a 40-μm filter to remove large tissue debris. The cell suspension was then centrifuged at 2000 rpm for 10 min at 20°C and the resulting pellet was resuspended in DCCM-1 medium containing 50 μg/ml DNase. These cells were then used to wash the cell culture flask for 2 h at 37°C in a humidified incubator to allow differential adherence of contaminating mononuclear cells.

After 2 h, the nonadherent ATII cells were removed and the cell suspension was centrifuged as before. The cell pellet was then resuspended in DCCM-1 containing 10% newborn calf serum and 1% PSG at a concentration of 1 × 10⁶ cells/ml. Cells were then seeded at 1 × 10⁶ ATIIs cells/well. Cells reached confluence by 48 h. These cells have been thoroughly characterized using electron microscopy, staining for alkaline phosphatase, and cells were incubated with 100 ng/ml LPS in the presence or absence of 10 and 20 μg/ml Ab and/or TNF-α. As an appropriate control, cells were also exposed to the relevant carrier protein, in this case mouse IgG (R&D Systems).

Effect of Ab blockade of IL-1β and TNF-α on LPS stimulation of alveolar macrophages and ATII cells

Cells from three further subjects were cultured as described above and serum-starved for 24 h before experiments. The medium was then removed and the cells were incubated with LPS at concentrations of 10, 100, or 1000 ng/ml, in serum-free DCCM-1. Each treatment was performed in triplicate. The resulting conditioned medium was aspirated and the secreted cytokines and chemokines were measured by ELISA.

Conditioned medium was also generated from ATII epithelial cells and macrophages from three further subjects, to use in the monocye and neutrophil migration assays. Following stimulation for 24 h with 100 ng/ml LPS, the medium was removed and the cells were washed thoroughly to remove any residual LPS. Fresh serum-free medium was then added and the cells were cultured for a further 24 h. The LPS-free conditioned medium from this 24-h time period was then used for leukocyte migration assays.

MAPK expression

Cells from a further four subjects were stimulated with LPS at a concentration of 100 ng/ml. The cells were harvested at regular intervals for up to 4 h. The cells were washed and harvested by scraping into lysis buffer (0.05% deoxycholate, 0.1% Nonidet P-40, 0.1% Triton X-100, and 0.025% w/v SDS; Sigma-Aldrich) containing the phosphatase inhibitors sodium pyrophosphate (1 mM) and sodium orthovanadate (2 mM) plus the protease inhibitors PMSF (1 mM), aprotonin (25 μg/ml), and leupeptin (10 μg/ml; Sigma-Aldrich). The cells were lysed in vitro in a lysis buffer at 80°C for 10 min to remove cellular debris, samples were stored at −70°C until analysis. Phospho-ERK 1/2 and p38 MAPK levels in the cell lysates were determined by immunoblotting.
FIGURE 1. LPS-induced time- and dose-dependent release of IL-1β (A), TNF-α (B), and IL-6 (C) by primary human alveolar macrophages and ATII cells. Cells were cultured with increasing doses of LPS for up to 24 h. Conditioned media were aspirated at each time interval and assayed for cytokine release by ELISA. Data are expressed as picograms per milliliter of conditioned media, mean ± SE (n = 6 subjects). The asterisks denote significant differences between the untreated and LPS-treated cells at each time interval: *, p < 0.05; **, p < 0.005; ***, p < 0.0005.

SDS-PAGE and immunoblot analysis

Following the quantification of total protein (Bio-Rad protein assay), the samples were diluted 1/6 with a 4% loading buffer (containing 2-ME, glycerol, SDS, and bromophenol blue in Tris buffer; Invitrogen Life Technologies) and boiled for 5 min. Rainbow m.w. markers (Invitrogen Life Technologies) were used to determine the m.w. of the immunoreactive bands. Forty micrograms of protein was loaded per lane on a 4–12% bis-acrylamide gel (Invitrogen Life Technologies) and then electrophoresed continuously at 80 mA for ~50 min in running buffer (Invitrogen Life Technologies) using a Bio-Rad Mini Protein II Electrophoresis System. After separation, the protein bands were electrophoretically transferred, at 400 mA per gel for 1 h to a nitrocellulose membrane (Invitrogen Life Technologies) using a transfer buffer (Invitrogen Life Technologies). Nitrocellulose blots were agitated with TBST (20 mM Tris, 150 mM NaCl, and 0.1% Tween 20; Sigma-Aldrich) plus 5% milk for 1 h to block nonspecific binding sites. Nitrocellulose blots were washed three times for 10 min with TBST and incubated overnight at 4°C with specific mAbs (Santa Cruz Biotechnology) to total ERK 1/2, to phosphorylated ERK 1/2, to total p38 and to phosphorylated p38 (in TBST plus 5% milk); isotype Abs (Santa Cruz Biotechnology) to total ERK 1/2, to phosphorylated ERK 1/2, to total p38 and to phosphorylated p38 (in TBST plus 5% milk); isotype Abs (Santa Cruz Biotechnology) were used as control to detect nonspecific binding. Following washing, the nitrocellulose blots were incubated with HRP-conjugated secondary Abs (in TBST plus 5% milk; Santa Cruz Biotechnology) to the primary Ab. The blots were then washed again as described above and developed using the ECL detection system (Upstate Technology). Equal sample loading was confirmed by probing for the housekeeping protein GAPDH (data not shown). The degree of phosphorylation was determined by densitometry using Labworks software.

Measurement of cytokines and chemokines by ELISA

DuoSet (R&D Systems) kits consisting of paired Abs were used to measure cytokine and chemokine release from ATII cells and alveolar macrophages following LPS stimulation. The threshold limit of detection of the assays is 15.6 pg/ml for TNF-α and MCP-1, 4.7 pg/ml for IL-6, 3.9 pg/ml for IL-1β, and 31.25 pg/ml for IL-8 and GROα. The interassay coefficient of variance was <5% for all assays conducted.

Leukocyte migration assays

Neutrophils and monocytes were isolated as described above and resuspended in DCCM-1 medium at a concentration of 1 × 10⁶/ml. A total of 150 µl of the cell suspension (either neutrophils or monocytes) was then placed into the upper chamber of a Transwell insert (8-µm pore membrane) and 300 µl of conditioned medium was placed directly into the 24-well plates (i.e., in the lower chamber). Plates were then incubated overnight in a humidified incubator at 37°C.

Following incubation, the cell culture insert was removed and the nonmigratory cells in the upper chamber were aspirated and wiped away from the membrane. The underside of the membrane was then examined for adherent migratory cells; this was always negative. Migratory leukocytes in the lower chamber were counted under a light microscope. Migration was expressed as cells per field of vision, n = 5 fields/well.

The possible effect of chemokinesis (i.e., nonspecific migration) was determined by preincubating the leukocytes with conditioned medium in a tissue culture insert for 15 min, before addition of the same conditioned medium to the lower chamber. The assay was then conducted as described above.

Anti-chemokine Ab blockade of leukocyte migration

The effect of supermaximal levels of blocking mouse mAbs to GROα (50 µg/ml), IL-8 (25 µg/ml), RANTES (5 µg/ml), and MCP-1 (10 µg/ml) was determined by addition of Abs (R&D Systems) to conditioned medium in the lower chamber of a parallel set of experiments. The supermaximal Ab levels that were used were based on the concentration of chemokine in the conditioned medium as recommended by the supplier, to completely neutralize chemokine activity. As the appropriate negative controls, the effect of addition of the relevant IgG isotypes (IgG1 and IgG2A; R&D Systems) to the conditioned media was also investigated.
Statistical analyses

Data are presented as mean ± SE. A one-way ANOVA was used to analyze the time- and concentration-dependent effects of LPS on cytokine and chemokine release from ATII cells and alveolar macrophages. Paired t-tests were used to determine significant differences in LPS-induced mediator release following addition of neutralizing Abs to IL-1β and TNF-α. This statistical test was also used to evaluate the significance of addition of chemokine neutralizing Abs in migration assays. A p value < 0.05 was considered to be statistically significant.

Results

Cytokine release from primary human alveolar macrophages and type II epithelial cells

IL-1β (Fig. 1A). Following 1000 ng/ml LPS exposure for 24 h, macrophages released significantly more (over twice as much) IL-1β than ATII cells (p < 0.0001). Release by macrophages increased significantly in a concentration-dependent manner at each time point, but was most marked at 12 h (p < 0.0001). In contrast, ATII cell production of IL-1β plateaued at 6 and 12 h, but showed the most marked concentration-dependent response at 24 h (p < 0.02). Macrophages also released IL-1β basally whereas ATII cells did not.

TNF-α (Fig. 1B). Similarly, macrophages released significantly more (approximately twice as much) TNF-α as ATII cells in response to 1000 ng/ml LPS for 24 h (p < 0.0001); production increased steadily over the 24 h of the study. Although ATII cell release of TNF-α increased steadily over 24 h when exposed to 1000 ng/ml LPS, lower concentrations of LPS induced maximal TNF-α release by 12 h, in a concentration-dependent manner (p < 0.0004). ATII cells did not produce TNF-α basally, unlike macrophages, and did not release TNF-α in response to 1 ng/ml LPS.

IL-6 (Fig. 1C). ATII cells released significantly more (approximately four times as much) IL-6 as macrophages (p < 0.0001), and release increased in a time- and concentration-dependent manner over 24 h (p < 0.0002). In macrophages, however, release of IL-6, while also concentration-dependent (p < 0.001), began to plateau after 6 h. Both cell types produced IL-6 basally.

Chemokine release from primary human alveolar macrophages and type II epithelial cells

IL-8 (Fig. 2A). LPS-stimulated ATII epithelial cells released significantly more (approximately three times as much) IL-8 as macrophages (LPS: 1000 ng/ml, 24 h, p < 0.0001). Release of IL-8 from ATII cells was concentration dependent (p < 0.0008) and continued to increase over the 24 h, with a sharp increase between 6 and 12 h, whereas in macrophages it started to plateau after 6 h. Although the response to LPS by macrophages was much flatter, with very little difference between control and LPS-stimulated cells, there was a significant concentration-dependent response (p < 0.0001). IL-8 was released basally by both cell types but in greater amounts by macrophages.

MCP-1 (Fig. 2B). ATII epithelial cells released significantly more (approximately six times as much) MCP-1 than alveolar macrophages (LPS 1000 ng/ml, 24 h, p < 0.0001). Release of MCP-1 from ATII cells was concentration dependent (p < 0.0008) and continued to increase over the 24 h, with a sharp increase between 6 and 12 h, whereas in macrophages it started to plateau after 6 h. Although the response to LPS by macrophages was much flatter, with very little difference between control and LPS-stimulated cells, there was a significant concentration-dependent response (p < 0.0001). MCP-1 was released basally by both cell types but in greater amounts by macrophages.

GRO-α (Fig. 2C). ATII epithelial cells released significantly more (approximately three times as much) GRO-α than macrophages (LPS 1000 ng/ml, 24 h, p < 0.001). Release of GRO-α from ATII cells was concentration dependent (p < 0.0008) and continued to increase over the 24 h, with a sharp increase between 6 and 12 h, whereas in macrophages it started to plateau after 6 h. Although the response to LPS by macrophages was much flatter, with very little difference between control and LPS-stimulated cells, there was a significant concentration-dependent response (p < 0.0001). GRO-α was released basally by both cell types but in greater amounts by macrophages.
Macrophage minus LPS control vs LPS plus anti-TNF-α treated and neutralizing Ab-untreated cells at each time interval. To investigate this possibility in these experiments, neutralizing Abs to IL-1β and TNF-α were added to the media along with 100 ng/ml LPS. Macrophages only released ~1000 pg/ml after 24 h. Both cell types released MCP-1 basally and in response to LPS stimulation in a time- and concentration-dependent manner (ATII, p < 0.003; alveolar macrophages, p < 0.002). ATII cell release was steady over the full time course whereas, for macrophages, there was a marked increase in release between 12 and 24 h. GROα (Fig. 2C). ATII cells released significantly more GROα (approximately four times more) than macrophages (1000 ng/ml LPS, 24 h, p < 0.001). Release from both cell types was time- and concentration-dependent (p < 0.0001 for both cell types). Release of GROα from macrophages appeared to be steady and constant over the 24 h, whereas in ATII cells there was a dramatic increase in release after 6 h (~3.5-fold, 1000 ng/ml LPS) which continued for up to 24 h. Both cell types released GROα basally.

Ab blockade of cytokine and chemokine release from alveolar macrophages and ATII cells

Previous studies have implied that many of the proinflammatory effects of LPS are mediated indirectly through release of IL-1β and TNF-α which then feed back to stimulate the cells in an autocrine manner. To investigate this possibility in these experiments, neutralizing Abs to IL-1β and TNF-α were added to the media along with 100 ng/ml LPS.

Neutralizing Abs to TNF-α inhibited IL-6 release from alveolar macrophages at 12 h (p < 0.03) and neutralization of both cytokines significantly inhibited release at 12 and 24 h (p < 0.03 and p < 0.003, respectively). Neutralization of IL-1β alone had no effect (Fig. 3A), although at 24 h the inhibition of IL-6 release following IL-1β blockade was significant at the p < 0.1 level (p = 0.09). In ATII cells, neutralization of IL-1β had no effect on IL-6 release, whereas neutralization of TNF-α significantly inhibited release at 24 h (p < 0.006). Neutralization of both cytokines concomitantly had no increased effect over neutralization of TNF-α alone (Fig. 3B). When supernatants were assayed for MCP-1 release, results showed that neutralization of IL-1β and TNF-α alone or in combination had no effect on release of MCP-1 from alveolar macrophages (Fig. 4A). However, ATII cell release of MCP-1 was inhibited by neutralization of TNF-α at 12 and 24 h (p < 0.03 and p < 0.01, respectively), while neutralization of IL-1β inhibited MCP-1 release at 24 h (p < 0.04). Neutralization of both cytokines in tandem inhibited MCP-1 release at 12 and 24 h (p < 0.004 and p < 0.0002, respectively) and appeared to have a small additive effect (p < 0.006; Fig. 4B), bringing MCP-1 levels back down to those observed at 3 h. Addition of the Ab carrier protein alone to the media had no effect on mediator release and thus ruled...
out the possibility of any nonspecific inhibitory effects it may have (data not shown).

**LPS activation of MAPK**

Under resting conditions phospho-ERK-2 was not detected in alveolar macrophages; however ERK-1 was modestly phosphorylated relative to the total ERK-1 pool. Exposure of alveolar macrophages to LPS (100 ng/ml) resulted in a time-dependent phosphorylation of ERK-1 and ERK-2 (Fig. 5). This effect was detectable as early as 5 min after stimulation, was maximal at 30 min, and was still detectable at 120 min (pERK-2/total ERK 30 min vs 120 min; \( p < 0.036 \), Fig. 5). p38 MAPK, which was not activated in resting cells, was also phosphorylated by LPS. Again, this effect was detected 5 min after stimulation, peaked at 30 min, and then declined over the remaining 210 min of the experiment (Fig. 5). Due to the low levels of p38 phosphorylation, densitometry was not accurate enough to calculate the phospho-p38/p38 ratios. In contrast to the findings with macrophages, there was high constitutive phosphorylation of ERK-1 and ERK-2 by ATII cells and LPS did not induce a measurable change at this high basal level of activation (Fig. 6). Phosphorylated p38 MAPK was difficult to detect in ATII cells and did not alter following stimulation with LPS (Fig. 6).

Leukocyte migration in response to conditioned media from LPS-stimulated primary human alveolar macrophages and type II epithelial cells

**Chemokinesis.** To validate our assay system and assess the contribution of chemokinesis to the cell migration observed, neutrophils and monocytes were preincubated with conditioned medium for 30 min before their introduction to the invasion chamber containing conditioned medium in the lower chamber.

Following preincubation of monocytes with conditioned medium, there was a small but significant increase in migration (\( p < 0.007 \); data not shown); however, this was significantly less than that of naive monocytes that had not been preincubated with conditioned medium (\( p < 0.0001 \)). Similarly, in the neutrophil migration assay, following preincubation with conditioned medium from both alveolar macrophages and ATII cells, there was a small but significant increase in neutrophil migration (\( p < 0.001, p < 0.0001 \), respectively; data not shown). Again, migration of naive neutrophils toward conditioned medium was significantly greater (~4-fold greater, \( p < 0.001 \)). This suggests that a small proportion of both monocytes and neutrophils migrate across the membrane independently of chemotactic mediators in conditioned medium. Consequently, the data have been corrected by subtraction of nonspecific migration (i.e., migration following preincubation of cells in conditioned media) from that induced by conditioned media (i.e., naive cell migration toward conditioned media) to provide migration due to factors released into the media by macrophages and ATII cells. Thus, migration of both monocytes and neutrophils toward conditioned medium from alveolar macrophages and ATII cells was significantly increased (\( p < 0.0001 \) monocytes, \( p < 0.001 \) neutrophils).

**Ab blockade of leukocyte migration.** Following validation of the migration assay system, Abs to chemokines detected in the conditioned media (IL-8, MCP-1, GROα, and RANTES) were added alone or in combination to the lower chamber in an attempt to inhibit both monocyte and neutrophil migration. To exclude any effect of the Ab carrier protein on leukocyte migration, leukocytes were also exposed to the conditioned medium in the presence and absence of the relevant nonimmune IgGs. There was no effect of
IgG alone as no significant difference in migration was observed (data not shown).

Monocyte migration. As observed in the preliminary investigation of chemokinesis, conditioned medium from LPS-stimulated alveolar macrophages (Fig. 7A) and ATII cells (Fig. 7B) caused a significant increase in monocyte migration. Conditioned medium from ATII cells caused a significantly higher number of monocytes to migrate which correlated with the total amount of chemokine detected ($p < 0.0002$; $r^2 > 0.98$). Addition of RANTES neutralizing Abs had no significant effect on monocyte migration in response to conditioned media from either cell type. Neutralizing Abs to IL-8 caused significant inhibition of monocyte migration toward conditioned media from both alveolar macrophages and ATII cells (29%, $p < 0.01$; 38.6%, $p < 0.006$, respectively). Inhibition of GROα was similar to IL-8; however, inhibition was slightly greater with ATII cell-conditioned media compared with that from alveolar macrophages (51.3%, $p < 0.0009$; 35.5%, $p < 0.002$, respectively). The greatest inhibition of monocyte migration was observed with Abs to MCP-1. As observed with the previous Abs, inhibition of monocyte migration in response to ATII cell-conditioned medium was greater than the inhibition of migration toward alveolar macrophage-conditioned medium (82.8%, $p < 0.0001$; 70.1%, $p < 0.0001$). The addition of all Abs to the conditioned media from either ATII cells or alveolar macrophages caused a small but nonsignificant increase in inhibition over that of produced by Abs to the major chemoattractive chemokine.

Neutrophil migration. Conditioned medium from LPS-stimulated primary human alveolar macrophages (Fig. 8A) and ATII cells (Fig. 8B) caused a significant increase in neutrophil migration as observed in the preliminary chemotaxis assays ($p < 0.0001$). Conditioned medium from ATII cells, however, induced significantly greater numbers of neutrophils to migrate compared with that of alveolar macrophages ($p < 0.01$). The increase in migration observed significantly correlated with the total chemokine levels measured in the conditioned media ($r^2 > 0.92$).

In observations similar to that seen in the monocyte migration assays, addition of neutralizing Abs to RANTES had no significant effect on neutrophil migration. Neutralizing Abs to MCP-1 significantly inhibited neutrophil migration toward alveolar macrophage and ATII cell-conditioned media (24.4%, $p < 0.05$; 37.3%, $p < 0.002$, respectively). Abs to IL-8 and GROα significantly inhibited neutrophil migration. However, when neutralizing Abs to GROα were added to macrophage-conditioned medium, inhibition was greater than when Abs to IL-8 were used (53.8%, $p < 0.008$; 40.9%, $p < 0.001$, respectively). The opposite was true for ATII cells; Abs to IL-8 caused greater inhibition than those to GROα (61.3%, $p < 0.0001$; 52%, $p < 0.0001$, respectively). Addition of all Abs to conditioned medium from either macrophages or ATII cells caused a further small, but significant, decrease in neutrophil migration over that observed with Abs against the major chemoattractive chemokine.

Discussion

These studies show distinct differences between human alveolar macrophages and human ATII epithelial cells in their response to LPS stimulation; this impacted on the magnitude of leukocyte migration toward conditioned media from these cells, which differed,
reflecting the profile of chemokines released by each cell type. Thus, under these experimental conditions, ATII cells produced higher levels of the chemokines and leukocyte migration than alveolar macrophages, whereas macrophages were a rich source of IL-1β and TNF-α. Ab blockade of IL-1β and TNF-α showed that LPS induction of these cytokines, and their autocrine effects, were important for ATII cell, but less so for macrophage, cytokine, and chemokine release. In contrast, phosphorylation of components of the MAPK pathways, ERK1/2 and p38, following LPS occurred in macrophages but not ATII cells, suggesting differential activation of signaling pathways by LPS. This work is unique in its use of primary human cells obtained from the same subjects to give a true comparative study of alveolar macrophages and ATII cells, and clearly and unequivocally demonstrates significant differences between these two cell types in their response to LPS and regulation of cytokine and chemokine release.

LPS triggers intracellular signaling via TLR4 activation. We have shown previously that human ATII cells express both TLR4 and TLR2 (17), while others have shown expression of these receptors by human alveolar macrophages (20). Alveolar macrophages from smokers and COPD patients express normal TLR4 levels, although TLR2 expression is decreased (20). The MAPK signal transduction pathways are an important component of TLR signaling which regulate cellular functions such as gene transcription, programmed cell death, and inflammation. Here, we have shown that LPS induces rapid phosphorylation of p38 and ERK1/2, by alveolar macrophages together with a marked time- and concentration-related increase in release of IL-1β, TNF-α, and IL-6 and a less marked, but similar, pattern of increased release of IL-8, MCP-1, and GROα. Previous investigations show that both p38 MAPK and ERK1/2 are essential for primary human alveolar macrophage and THP-1 cell TNF-α synthesis (21, 22). Inhibition of activation of one or other of these MAPKs leads to partial reduction in TNF-α levels, while inhibition of both MAPKs completely prevents TNF-α synthesis (22, 23). However, JNK and NF-κB, not studied here, are also important for LPS-induced human alveolar macrophage release of TNF-α (24, 25). LPS induction of chemokine release from human macrophages, including IL-8, GROα, MIP-1α, and MCP-1 has previously been demonstrated to involve p38 MAPK (23, 26). When THP-1 cells were stimulated with shed factors from Helicobacter pylori, which contain LPS, IL-8 release was found to be dependent upon phosphorylation of p38 MAPK, ERK1/2, and JNK, as well as NF-κB (27). Its effects were attributed to LPS as they were inhibited by polymyxin and anti-CD14 Abs. Other compounds, including 8-isoprostan E, cigarette smoke, Bacillus anthracis, secretory phospholipase A2, and rhinovirus, induce release of a range of chemokines, including IL-8, MCP-1, MIP-1α, MIP-1β, and IFN-γ-inducible protein 10 via differential activation of p38 MAPK, ERK1/2, and JNK (25, 26, 28–30).

In direct contrast to our findings in macrophages, there was constitutively high phosphorylation ERK1/2 in ATII cells, and there was no detectable difference in phosphorylation of either ERK or p38 MAPK after LPS treatment. Such fundamental differences between alveolar macrophages and ATII cells in MAPK expression may contribute to their differential response to LPS stimulation. To our knowledge, there are no other published studies of the effects of LPS and activation of MAPKs on cytokine and chemokine release by primary human ATII cells. High basal levels of activated ERK1/2 have been shown in primary rat alveolar type II cells in vitro (31), as well as the adenocarcinoma A549 cell line, which is often used as a surrogate for alveolar type II cells (32–34). In contrast to the current study, stimulation of IL-8 release by A549 cells following cigarette smoke condensate, Burkholderia cepacia, and crystalline silica resulted in increased activation of either ERK, p38, or both MAPKs, respectively (31, 33, 34). This may be due to differences in cell signaling processes between primary ATII cells and the A549 adenocarcinoma cell line; indeed, we have previously demonstrated morphological and biochemical differences (19) between these cell types in vitro. However, the apparent lack of increased phosphorylation of ATII cell p38 MAPK and ERK1/2 following LPS stimulation is surprising in the face of the striking increases of cytokine, and particularly chemokine, release. It is possible that LPS triggers p38 MAPK and/or ERK1/2 activation in primary ATII cells but at such a low level that it cannot be detected by immunoblotting. Furthermore, other signaling pathways seem likely to be involved, for example the JNK MAPK pathway, NF-κB, or other downstream pathways. Alternatively, there may be differences in the relative sensitivity of MyD88-sensitive and -insensitive pathways; the profile of cytokine release and rapid responses support the involvement of the MyD88-sensitive pathway, but it is unclear whether the MyD88-insensitive pathway is also involved.

ERK1/2 is important in controlling the cell cycle and this may involve sustained signaling (35–37). Some studies suggest that ERK1/2 and p38 MAPK signaling may have opposing actions to modify or fine tune the effects of external stimuli/conditions (38, 39). Basal, activated, phosphorylated ERK is high in human alveolar macrophages cultured for up to 6 h after isolation from bronchoalveolar lavage and its constitutive activity is required for prolonged survival of these cells in the face of adversity in situ (40). In the present study, we also detected constitutive activated ERK expression by human lung macrophages, even after many hours in vitro (over 28 h) although the levels were much lower than total levels. We hypothesize that constitutively active ERK may also contribute to the basal release of low levels of TNF-α, IL-1β, and higher levels of IL-8 by macrophages. In contrast, as noted earlier, there was no further phosphorylation of p38 MAPK or ERK1/2 after LPS stimulation of ATII cells, suggesting either that these MAPKs play a minor role in LPS-induced cytokine and chemokine production by these cells or that only small increases, not detectable by immunoblotting, are involved. ATII cells are confluent at the time of investigation (3–4 days; Ref. 19), forming a single cell monolayer (15, 19), with all of the characteristics of ATII cells in situ. These cells differentiate into alveolar type-I-like epithelial cells in longer term culture (7–8 days following plating). Thus, high basal phosphorylated ERK1/2 may be related to ATII cell differentiation in this model; low basal levels of phosphorylated p38 MAPK described in the present study might also be required, as p38 MAPK can counteract the action of ERK (38, 39). These possibilities have not been examined in primary human lung alveolar epithelial cells.

There was a remarkable difference between cell types in response to IL-1β and TNF-α Ab blockade, where ATII cell release of MCP-1 and IL-6 was extremely sensitive to this treatment. It is important to note that, even in the presence of relatively high autocrine levels of TNF-α and IL-1β, macrophages produced relatively low levels of IL-6 and MCP-1, and use of super maximal levels of anti-TNF-α and anti-IL-1β Ab had little effect on MCP-1, with lesser effects on IL-6 production compared with ATII cells over a 24 h period. One might argue that this is unimportant with such small levels of LPS-induced IL-6 and MCP-1 release. However, the LPS-stimulated release of these mediators by macrophages is highly significant, from virtually nothing to hundreds of picograms per milliliter of media; the fact that there was little change in these levels, particularly for MCP-1, even after 24 h of incubation with blocking Abs to TNF-α and IL-1β suggests that there are no substantial autocrine effects of these mediators on
macrophages and that LPS induction of these mediators is largely independent of either TNFR- or IL-1R-signaling pathways. Alternatively, macrophages may corelease soluble TNF-α receptor and IL-1R antagonist, in the same way as neutrophils (41), to auto-regulate cell signaling. The macrophage response was in direct contrast to the effects of the same treatment, under identical conditions, on ATII cell release of MCP-1 and IL-6, where MCP-1 release was completely inhibited by anti-TNF-α, and partially inhibited (~50%) by anti-IL-1β; IL-6 release was also significantly inhibited (~60%) by anti-TNF-α. This inhibition occurred even in the face of high levels of MCP-1 and IL-6 following treatment of ATII cells with LPS, although there were relatively lower levels of extracellular TNF-α and IL-1β in the media. These findings imply that only low levels of TNF-α and/or IL-1β are required to induce synthesis of high levels of MCP-1 and moderately high levels of IL-6. Thus, it is probable that both the TNF-α receptor- and the IL-1βR-mediated signaling pathways are important mechanisms in the prolonged effects of LPS on ATII cell release of IL-6 and MCP-1.

Two recent articles (42, 43) using mouse embryonic fibroblasts highlight the significance of LPS-induced TNF-α secretion and positive TNF-α-feedback mechanisms in the induction of a chronic response (with respect to NF-κB expression), as compared with negative feedback mechanisms involved in the acute effects of TNF-α stimulation when used in the absence of LPS, which help explain differential responses to TNF-α and LPS. It is suggested that both the MyD88-dependent and -independent pathways of TLR4 activation contribute to TNF-α synthesis and secretion and up-regulation of NF-κB activity. Of particular relevance to the present study is the evidence of autocrine feedback of LPS-induced TNF-α release and subsequent activation of the TNF pathway via the TNFR, to elicit the chronic responses and prolonged chemokine and cytokine expression. In an earlier study of fetal rat alveolar epithelium, IL-1βR antagonism was demonstrated to ameliorate LPS-induced NF-κB translocation and activation, illustrating a similar positive feedback effect of LPS-induced IL-1β (44). Furthermore, the feedback control of human blood neutrophil LPS-induced IL-8 secretion has been shown to be autoregulated by corelease of the soluble TNF-α receptor and IL-1R antagonist. When the activity of soluble TNF-α receptor and IL-1R antagonist are blocked with neutralizing Abs, IL-1β was found to play a significant role in LPS-induced feedback control of IL-8 production (41). Thus, these studies support the concept of positive feedback control of LPS-induced mediator expression by coinduction of TNF-α and IL-1β release; however, it is clear that the exact cell-derived cytokine and signaling pathways vary according to the stimulus and nature of the cell source, e.g., myeloid or nonmyeloid in origin.

There is much debate in the literature as to the role of IL-1β and TNF-α in the initiation of the inflammatory response following endotoxin exposure. Studies in rats showed that following LPS exposure there was a concentration-dependent increase in neutrophil numbers in the lung which was partly abolished following treatment with neutralizing Abs to TNF-α (45). Abs to TNF-α have also proven effective, with reduced circulating IL-1, IL-6, and IL-8, and increased survival, in primate and rabbit models of endotoxemia (46, 47), suggesting a key role for TNF-α in the inflammatory response. In wild-type and knockout (KO) TNFR I and II mice, i.p. LPS caused a rapid increase in pulmonary TNF-α, neutrophils, and chemokines in wild-type and KO TNFRII, but not KO TNFR I, mice, suggesting that LPS induction of TNF and positive feedback via the TNFRI receptor is an important mechanism in the pulmonary response (48). Studies of mice with a knockout for the p55 TNFR showed that these mice had increased resistance to LPS due to the lack of TNF-α signaling in the absence of the p55 TNFR (49). Similar results have also been found in animal models of septic shock using an IL-1R antagonist (50). Although these studies in animal models highlight the role of TNF-α and IL-1β in LPS-induced pulmonary inflammation, they do not identify which cells are responsible.

The differences in macrophage and ATII cell signaling under identical experimental conditions might be explained by differences in absolute cell numbers. However, in this study, there is no difference in the absolute numbers of cells per unit area of tissue culture well. Indeed, by 24 h, macrophages release over twice as much TNF-α and IL-1β, but at the same time release only about a third of the IL-8, MCP-1, and GROα, compared with that of ATII cells, clearly illustrating that this is not a cell number phenomenon. From the evidence in the present investigation, we hypothesize that, in vivo, LPS-stimulated macrophage release of TNF-α and IL-1β, which in turn, or in concert with the stimulating factor, activates alveolar epithelial cells to release chemokines and trigger leukocyte migration. In healthy nonsmokers, the ratio of ATII cells to macrophages is ~5:1, illustrating the significance of LPS-stimulated and cytokine-stimulated chemokine release by ATII cells (51). Subsequent to augmented macrophage recruitment, further release of TNF-α and IL-1β and stimulation of ATII cells may then amplify the inflammatory response due to increased ATII cell chemokine secretion. Of relevance to this hypothesis is the recent in vivo study by Jayaseelan et al. (52). LPS-induced inflammation in mice resulted in increased CXCL5, a mouse chemokine homologous to human epithelial neutrophil-activating peptide 78 and granulocyte chemotactic peptide 2, being crucial in the inflammatory response; ATII cells were the major source of the CXCL5 and LPS induction of CXCL5 operated via the signaling pathways p38 and JNK MAPKs. The present unique comparative study of relevant human alveolar macrophages and ATII cells from the same subjects also strongly supports the concept that the resident ATII cell is likely to be an important modulator of the inflammatory response, not only via direct interaction with inflammatory mediators, but also via cross-talk with myeloid and other inflammatory and resident cells.

The pattern of LPS-induced chemokine secretion by each cell type was vastly different and of particular note was IL-8 release. Macrophages released high levels of IL-8 basally which only increased by ~50% following 1000 ng/ml LPS. In contrast, ATII cells released low levels basally which increased 700% following 1000 ng/ml LPS exposure for 24 h. This is a novel finding which partly reflects the differential responses to LPS discussed earlier. Previous studies using peripheral blood monocytes have shown that IL-8 can work in an autocrine manner via the CXCR1 receptor to cause further release of IL-8 (53). This mechanism may be present in ATII cells, but is down-regulated in alveolar macrophages. Because alveolar macrophages used in this study release high basal levels of IL-8, it is likely that autoregulation of IL-8 synthesis is imperative, possibly involving corelease of soluble TNF and IL-1 receptors and/or agonists (41) to prevent inappropriate inflammation. In contrast to IL-8, GROα was released in both a time- and concentration-dependent manner by both cell types and release did not plateau; nevertheless, ATII cells secreted significantly higher levels. GROα exhibits broadly similar effects on leukocytes as IL-8 and binds the same receptors. This may be of clinical importance in diseases such as COPD because GROα is elevated in COPD sputum and has been shown to elicit increased migration of monocytes from COPD subjects compared with healthy nonsmokers (54).

Conditioned media from both cell types stimulated leukocyte migration and the magnitude of the response was related to the...
IL-1 and positive feedback control of chemokine release by TNF-α—the demonstration that ATII cells are likely to play a central role in that they are likely to act in concert to regulate the inflammatory elicitation due to the presence of other factors present investigation were from healthy humans, suggesting that under inflammatory conditions (60), the neutrophils used in the less, but significant, inhibition of neutrophil migration. Although a finding and suggests that epithelial cell-derived MCP-1 is likely to be a critical factor in monocyte recruitment to the lung (58). One might hypothesize that in the peripheral lung, where the alveolar epithelium is in close contact with the pulmonary microvasculature, epithelial-derived MCP-1 mediates effects within the capillary network to further increase recruitment of monocytes. Interestingly, the present study suggests that GROα and IL-8 also induce monocyte migration, but not to the same magnitude as MCP-1. Although classically thought of as neutrophil chemokines, other studies have shown the potential importance of GROα and IL-8 in regulating monocyte migration, adhesion to (54), and migration through (59) the microvascular cell wall.

In the leukocyte migration model used for these experiments, significantly more neutrophils than monocytes migrated toward the conditioned media dependent upon the concentration of GROα or IL-8, which bind to the same cell receptors. Blockade of MCP-1, traditionally thought to be a monocyte-specific chemokine, caused less, but significant, inhibition of neutrophil migration. Although a recent rodent study showed that neutrophil sensitivity to, and migration in response to, MCP-1 was increased by up to 100-fold under inflammatory conditions (60), the neutrophils used in the present investigation were from healthy humans, suggesting that MCP-1 is a chemoattractant for normal human neutrophils. However, we cannot exclude the possibility that exposure of healthy neutrophils to conditioned medium from LPS-treated cells may elicit an inflammatory response due to the presence of other factors that induce sensitivity to MCP-1.

In conclusion, this unique investigation of primary human alveolar macrophages and ATII cells from the same subjects shows that they are likely to act in concert to regulate the inflammatory response, via differential release of primary cytokines by macrophages and chemokines by ATII cells. Of particular relevance is the demonstration that ATII cells are likely to play a central role in the initiation of LPS-induced inflammation, via activation of TLR4 and positive feedback control of chemokine release by TNF-α and IL-1β. In addition, we hypothesize that, in vivo, LPS-stimulated release of IL-1β and TNF-α by macrophages exacerbates chemokine release from ATII cells. Thus, therapeutic strategies should take account of the cellular targets as well as the mechanisms of mediator action.

Disclosures
The authors have no financial conflict of interest.

References


