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Involvement of Phosphoinositide 3-Kinases in Neutrophil Activation and the Development of Acute Lung Injury

Ho-Kee Yum,* John Arcaroli,* John Kupfner,* Robert Shenkar,* Josef M. Penninger, † Takehiko Sasaki, † Kuang-Yao Yang,* Jong Sung Park,* and Edward Abraham2*†

Activated neutrophils contribute to the development and severity of acute lung injury (ALI). Phosphoinositide 3-kinases (PI3-K) and the downstream serine/threonine kinase Akt/protein kinase B have a central role in modulating neutrophil function, including respiratory burst, chemotaxis, and apoptosis. In the present study, we found that exposure of neutrophils to endotoxin resulted in phosphorylation of Akt, activation of NF-κB, and expression of the proinflammatory cytokines IL-1β and TNF-α through PI3-K-dependent pathways. In vivo, endotoxin administration to mice resulted in activation of PI3-K and Akt in neutrophils that accumulated in the lungs. The severity of endotoxemia-induced ALI was significantly diminished in mice lacking the p110 catalytic subunit of PI3-K. In PI3-K−/− mice, lung edema, neutrophil recruitment, nuclear translocation of NF-κB, and pulmonary levels of IL-1β and TNF-α were significantly lower after endotoxemia as compared with PI3-K+/- controls. Among neutrophils that did accumulate in the lungs of the PI3-K−/− mice after endotoxin administration, activation of NF-κB and expression of proinflammatory cytokines was diminished compared with levels present in lung neutrophils from PI3-K+/- mice. These results show that PI3-K, and particularly PI3-Kγ, occupies a central position in regulating endotoxin-induced neutrophil activation, including that involved in ALI. The Journal of Immunology, 2001, 167: 6601–6608.

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cute lung injury (ALI) is characterized by neutrophil accumulation in the lungs, increased expression of proinflammatory cytokines, loss of epithelial and endothelial integrity, widening of the alveolar-arterial O2 gradient, and the development of interstitial pulmonary edema (1–4). The neutrophils present in the lungs during ALI produce cytokines, including IL-1β and TNF-α, and play a major role in the development of ALI (5, 6). Induced neutropenia followed by endotoxin challenge or other pathophysiologic insults, such as blood loss, associated with the development of ALI attenuates increases in lung vascular permeability and other indices of lung injury (6).

Increased activation of the transcriptional regulatory factor NF-κB is present among pulmonary cell populations, including neutrophils, in ALI (6–12). Blockade of NF-κB activation decreases endotoxin-induced edema, neutrophil infiltration, and proinflammatory cytokine expression in the lungs (8, 9). Neutrophils appear to be important in endotoxemia-associated activation of NF-κB in the lungs, because neutrophil depletion markedly reduces pulmonary levels of activated NF-κB (6).

Phosphoinositide 3-kinases (PI3-K) and the downstream serine/threonine kinase Akt/protein kinase B have a central role in modulating neutrophil activation, chemotaxis, and apoptosis (13–17). PI3-K is a heterodimeric complex, comprising a p110 catalytic subunit, of which there are four characterized isoforms (α, β, γ, and δ). The type IA PI3-Ks, p110α, p110β, and p110δ, associate with the p85 family of regulatory subunits, but type IB p110γ binds to a p101 adaptor molecule. Whereas type IA PI3-Ks are activated by interaction with tyrosine-phosphorylated molecules, p110γ is activated by engagement of G-protein coupled receptors. The serine/threonine kinase Akt/protein kinase B is the best characterized target of the PI3-K-generated phosphoinositides PtdIns-3,4-P2 and PtdIns-3,4,5-P3. In vivo, activation of Akt is dependent on phosphorylation of threonine 308 and serine 473, events modulated by phosphatidylinositol-dependent kinases 1 and 2, following binding of phosphoinositides to the Akt pleckstrin-homology domain (14, 18).

PI3-K and Akt have been shown to participate in signaling pathways that lead to NF-κB activation and increased NF-κB-dependent transcription (19–23). Although there are several proposed mechanisms by which PI3-K and Akt can enhance NF-κB activity, a consistently demonstrated interaction between Akt and enhanced nuclear translocation of NF-κB centers on the ability of Akt to activate the regulatory IκB kinase, IκBα, through phosphorylation at an Akt phosphorylation-consensus sequence at Thr325 (24). Akt-dependent activation of IκBα leads to accelerated degradation of IκBα and enhanced translocation of NF-κB to the nucleus.

Although activated neutrophils contribute to the development and severity of ALI, the role that signaling through PI3-K plays in this process has not been well delineated. Activation of PI3-K and Akt occurs in monocytes or macrophages cultured with LPS (20, 25), but has not been examined in neutrophils. Similarly, involvement of PI3-K or Akt in affecting NF-κB activation or the expression of proinflammatory cytokines in neutrophils has not been described. Transgenic mice lacking the γ isoform of PI3-K demonstrate reduced migration of neutrophils toward chemokines...
as well as impaired clearance of *Escherichia coli* or *Staphylococcus* aureus from the peritoneum (26–28). However, the in vivo role that PI3-K plays in activating NF-κB, modulating proinflammatory cytokine expression, or participating in acute inflammatory responses, such as ALI, has not been examined. In the present experiments, we explored these issues and demonstrate that PI3-K occupies a central position in regulating endotoxin-induced neutrophil activation, including that involved in ALI.

**Materials and Methods**

**Mice**

Male C57/BL6 mice, 8–12 wk old, were purchased from Harlan Sprague-Dawley (Indianapolis, IN). PI3-K−/− mice (28) were bred in the homologous state for the PI3-K−/− mice was confirmed by PCR analysis of mouse tail-derived DNAs. The mice were kept on a 12-h light/dark cycle with free access to food and water. All experiments were conducted in accordance with institutional review board-approved protocols.

**Materials**

Isolfraneous was obtained from Abbott Laboratories (Chicago, IL). *E. coli* 0111:B4 endotoxin, collagenase, DNase, and l-α-phosphatidylinositol were obtained from Sigma-Aldrich (St. Louis, MO). RPMI 1640, 25 mM HEPES, l-glutamine were obtained from BioWhittaker (Walkersville, MD), while PBS and penicillin/streptomycin were purchased from Gemini Bio-Products (Calabassas, CA). Percoll was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The Cosmogene-Plus Protein Assay Reagent and BCA Protein Assay Reagent were purchased from Pierce (Rockford, IL). Sequence DNA polymerase was obtained from United States Biochemical (Cleveland, OH). LY294002 and wortmannin were purchased from Calbiochem (La Jolla, CA). Anti-phosphorylated-Akt (Ser473) and anti-total Akt were purchased from Cell Signaling (Danvers, MA). Anti-phosphoinositide 3-kinase agoniste conjugate was purchased from Upstate Biotechnology (Lake Placid, NY). Silica gel plates for thin-layer chromatography were purchased from J. T. Baker (Phillipsburg, NJ). The Abs for neutrophil isolation were obtained from StemCell Technologies (Vancouver, British Columbia, Canada).

**Models of endotoxemia**

The model of endotoxemia was used as reported previously (5, 6, 29). Mice received a i.p. injection of LPS at a dose of 1 mg/kg in 0.2 ml of PBS. This dose produces acute neutrophilic alveolitis and interstitial edema, historically consistent with ALI in mice (6, 30, 31).

**Isolation and culture of neutrophils**

Lung or peripheral neutrophils were purified from intraparenchymal pulmonary or bone marrow cell suspensions. To obtain the bone marrow cell suspension, the femur and tibia of a mouse were flushed with 3 ml of chilled (4°C) PBS. The excised lungs were minced in RPMI 1640/penicillin/streptomycin and the cells passed through a glass wool column. Lungs neutrophils were isolated from intraparenchymal pulmonary cell suspensions, prepared as previously described by our laboratory (5, 6, 29). In brief, the chest of the mouse was opened and the lung vascular bed flushed with 3 ml of chilled (4°C) PBS injected into the right ventricle. Lungs were then excised, avoiding the paratracheal lymph nodes and thymus, and washed twice in RPMI 1640, 25 mM HEPES, l-glutamine supplemented with penicillin/streptomycin. The excised lungs were minced finely, filtered (40 μm) medium containing 5% FBS, 20 U/ml collagenase, and 1 μg/ml DNase. Following incubation for 60 min at 37°C, any remaining intact tissue was disrupted by passage through a 21-gauge needle. Tissue fragments and the majority of dead cells were removed by rapid filtration through a glass wool column and cells collected by centrifugation.

The cell pellet from the intraparenchymal pulmonary or bone marrow cell suspensions were resuspended in RPMI 1640, 5% FCS and then incubated with 10 μl of primary Abs specific for cell surface markers F4/80, CD4, CD45R, CD5, and TER119 for 15 min at 4°C. This mixture composed (StemCell Technologies) is specific for T and B cells, RBC, monocytes, and macrophages. After a 15-min incubation, 100 μl of antibiotin tetrameric Ab complexes were added and the cells incubated for 15 min at 4°C. Following this, 60 μl of colloidal magnetic iron particles were added to the suspension and incubated for 15 min at 4°C. The entire cell suspension was then placed into a column surrounded by a magnet. The T cells, B cells, RBC, monocytes, and macrophages were captured in the column, allowing the neutrophils to pass through by negative-selection methods. The neutrophil suspension was then layered on 50% Percoll, centrifuged at 3000 rpm for 15 min and the neutrophil layer was collected. Finally, as determined by trypan blue exclusion, was consistently greater than 98%. Neutrophil purity, as determined by Wright-stained cytospin preparations, was greater than 97%.

Bone marrow neutrophils (1 × 10^6/ml) were cultured in RPMI 1640, 10% FCS, penicillin/streptomycin with or without LPS (100 ng/ml). The PI3-K inhibitors, LY294002 (100 μM) and wortmannin (200 μM), were added to the neutrophil cultures for 1 h before LPS stimulation.

**Wet-to-dry lung weight ratios**

All mice used for lung wet-to-dry weight ratios were of identical ages. Lungs were excised, rinsed briefly in PBS, blotted, and weighed to obtain the “wet” weight. Lungs were then dried in an oven at 80°C for 7 days to obtain the “dry” weight.

**Myeloperoxidase (MPO) assay**

MPO activity was assayed as reported previously (6). Excised lungs from three to four mice per treatment group were frozen in liquid nitrogen, weighed, and stored at −86°C. Lungs were homogenized for 30 s in 1.5 ml of 20% sucrose, 1 mM sodium pyrophosphate, 10 mM sodium fluoride, 10 mM sodium ascorbate, 10 mM sodium selenite, 10 mM potassium pyrophosphate, 10 mM NaF, 300 μM p-nitrophenyl phosphate, 1 μM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin (pH 7.3) for 15 min. The protein concentration of each sample was assayed using a BCA protein assay kit standardized to BSA, according to manufacturer’s protocol. For electrophoresis, 50 μg of protein were loaded on a 10% Tris-HCI SDS polyacrylamide gel. Protein was electrotransferred to a nitrocellulose membrane and then blocked with 5% nonfat dry milk in 20 mM of TBS with 0.1% Tween. After blocking, the membrane was incubated overnight at 4°C with rabbit polyclonal phosphorylated Akt (p-Akt)-specific primary Ab using a dilution of 1:1000 followed by anti-rabbit Ig, HRP-coupled secondary Ab at a dilution of 1:2000. After washing three times, bands were detected using ECL Western blotting detection reagents (Amersham Pharmacia Biotech). The membranes were then stripped using Immuno Pure IgG Elution Buffer (Pierce), and reprobed with Abs specific for total Akt (t-Akt). Densitometry was performed using a chemiluminescence system and analysis software (Bio-Rad, Hercules, CA) to determine the ratio between phosphorylated and total kinase.

**Quantitative PCR**

Groups of five mice, with results obtained from individual mice, were used for each experimental condition. RNA was isolated using the RNAspin kit (Qiagen, Valencia, CA) following the manufacturer’s protocol. Primers and probes for IL-1β and TNF-α were designed using Primer Express software supplied by PerkinElmer (Foster City, CA).

The IL-1β primer and probe sequence consisted of the following: forward primer, 5'-CTGGTAAAGCCTCCTCCCATTCA-3'; reverse primer, 5'-TGTGTGGTTGTTGTTCTGTTG-3'; probe, 5'-GAGTATCAGACACCCACACTG-3'; the TNF-α primer and probe sequence consisted of the following: forward primer, 5'-TCTGGTGGTTGTTGTTCTGTTG-3'; reverse primer, 5'-CTCCTGTGTTGAGTTGCACC-3'; probe, 5'-TGCCCGCGACTAAGTGAGC-3'.

To optimize the primer sets, a primer optimization experiment was performed as described in the manufacturer’s protocol. Based on the primer optimization, the concentration of primers and probe for IL-1β and TNF-α contained 200 nM for the probe, the forward primer, and the reverse primer, and a 10-fold excess of each experimental cDNA template above the forward primer, and a reverse primer (PerkinElmer), at concentrations of 50 nM, were used to normalize the amount of RNA in each sample.

All reagents used in the one-step RT-PCR were purchased from PerkinElmer. Each one-step RT-PCR contained a total volume of 50 μl. The reverse transcription reaction was performed for 30 min at 48°C using MultiScribe Reverse Transcriptase with a final concentration of 0.25 U/μl. After the reverse transcription step, AmpliTaq Gold polymerase, with a final concentration of 0.025 U/μl, was activated by an increase in temperature to 95°C for 10 min followed by 40 cycles of amplification (95°C for
15a and 60°C for 1 min) with a Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA). The quantity of cytochrome mRNA was determined from a standard curve with 10-fold dilutions of known amounts of target RNA with each primer and probe set. RNA amounts were determined using software provided with the Gene Amp 5700 Sequence Detection System. Quantification was determined by dividing the amount of 18s ribosomal RNA by the target amount for each cytokine sample.

Cytokine ELISA

After the lung vascular bed had been flushed by injecting 5 ml of chilled (4°C) PBS into the right ventricle, lungs were collected and then homogenized for 30 s in a lysis buffer containing 10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.6% ipegal, 5 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml aprotinin, 10 µg/ml soybean trypsin inhibitor, and 1 µg/ml pepstatin. The homogenates were centrifuged at 10,000 rpm at 4°C for 10 min and supernatants were collected. Protein content of the supernatants was determined using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Immune complexes were formed by incubation with a 500-fold excess of unlabeled oligonucleotide, as described previously (32). Briefly, protein (150 µg) from neutrophil lysates was incubated with 10 µl of anti-PI3K p85 Ab coupled to protein A-Sepharose (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. Immunoprecipitates were washed three times with buffer containing 137 mM NaCl, 20 mM Tris-HCl, 1 mM CaCl2, 1 mM MgCl2, 0.1 mM Na3VO4, and 1% Nonidet P-40 (pH 7.4); then three times with buffer containing 0.1 M Tris-HCl, 5 mM LiCl, and 0.1 mM Na2VO4; and finally twice with buffer containing 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, and 0.1 mM Na3VO4. Assays were then performed in a reaction mixture containing the washed beads in 0.88 mM ATP, 100 mM MgCl2, 30 µCl [γ-32P]ATP, and 20 µg of phosphatidylinositol, incubated with agitation for 15 min at 37°C. The reactions were stopped with 20 µl of 6 M HCl. The organic layer was extracted with 160 µl of H2O–MeOH (1:1) and separated on a silica gel thin-layer chromatography plate (J. T. Baker). Thin-layer chromatography plates were developed in CHCl3–CH3OH–H2O–NH4OH (60:47:11:3.2) and dried. Radiolabeled phosphatidylinositol phosphates were visualized by autoradiography on X-Omat film (Eastman Kodak, Rochester, NY) and PI3-phosphate was quantified using a gel analysis system and software (Bio-Rad).

EMS A

EMSAs were tested as described previously (6, 10, 11, 29). Isolated neutrophils or homogenates from a whole lung were incubated for 15 min in buffer A (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl (pH 7.9)). After cytoplasm was removed from the nuclei by 15 passages through a 25-gauge needle, the nuclei were collected by centrifugation at 4°C for 6 min at 4°C. The nuclear pellet was incubated on ice for 15 min in buffer C (20 mM HEPES (pH 7.9), 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 25% glycerol), after which the extract was centrifuged at 4°C for 10 min at 12,000 × g. The supernatant was collected, divided into aliquots, and stored at −86°C. Protein concentration was determined by using the Coomassie-Plus Protein Assay Reagent standardized to BSA, according to the manufacturer’s protocol.

Activation of NF-κB was determined as described previously by our laboratory (6, 10, 11, 29, 32). The κB-DNA sequence of the Ig gene was used. Synthetic double-stranded sequences (with enhancer motifs underlined) were filled in and labeled with [α-32P]dATP using Sequenase DNA polymerase as follows: κB, 5′-TGTCTGGACGTCTCGACCTCCGAGC-3′ and 3′-GCTTCGACCTCGAAGCCTCTGT-5′.

DNA binding-reaction mixtures of 20 µl contained 10 µg of nuclear extract, 10 mM Tris-HCl (pH 7.5), 50 mM EDTA, 0.5 mM DTT, 1 mM MgCl2, 4% glycerol, 0.08 µg of poly(dI-dC)poly(dI-dC), and 0.7 fmol of [32P]-labeled double-stranded oligonucleotide. The samples were incubated at room temperature for 20 min, they were loaded onto a 4% polyacrylamide gel (acylamide-bisacrylamide 80:1, 2.5% glycerol) in Tris-borate-EDTA and run at 10 V/cm. Each gel was then dried and subjected to autoradiography. The specificity of κB-oligonucleotide binding was demonstrated using supershift studies with anti-p50 or anti-p65 antisemur (Santa Cruz Biotechnology, Santa Cruz, CA) and ablation of the κB band through incubation with a 500-fold excess of unlabeled oligonucleotide, as previously described (6, 10, 11, 29).

PI3-K assay

Neutrophil extracts were collected using a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl2, 1 mM EGTA, 1 mM trisodium vanadate, 10 mM sodium pyrophosphate, 10 mM NaF, 300 µM p-nitrophenyl phosphate, 1 mM PMSF, 10 µg/ml leupeptin, 10 µg/ml aprotinin (pH 7.3). Cellular debris was removed by centrifugation at 16,000 × g for 15 min at 4°C. PI3-K activity was measured as described previously (32). Briefly, protein (150 µg) from neutrophil lysates was incubated with 10 µl of anti-PI3K p85 Ab coupled to protein A-Sepharose (Upstate Biotechnology, Lake Placid, NY) overnight at 4°C. Immunoprecipitates were washed three times with buffer containing 137 mM NaCl, 20 mM Tris-HCl, 1 mM CaCl2, 1 mM MgCl2, 0.1 mM Na3VO4, and 1% Nonidet P-40 (pH 7.4); then three times with buffer containing 0.1 M Tris-HCl, 5 mM LiCl, and 0.1 mM Na2VO4; and finally twice with buffer containing 150 mM NaCl, 10 mM Tris-HCl, 5 mM EDTA, and 0.1 mM Na3VO4. Assays were then performed in a reaction mixture containing the washed beads in 0.88 mM ATP, 100 mM MgCl2, 30 µCl [γ-32P]ATP, and 20 µg of phosphatidylinositol, incubated with agitation for 15 min at 37°C. The reactions were stopped with 20 µl of 6 M HCl. The organic layer was extracted with 160 µl of H2O–MeOH (1:1) and separated on a silica gel thin-layer chromatography plate (J. T. Baker). Thin-layer chromatography plates were developed in CHCl3–CH3OH–H2O–NH4OH (60:47:11:3.2) and dried. Radiolabeled phosphatidylinositol phosphates were visualized by autoradiography on X-Omat film (Eastman Kodak, Rochester, NY) and PI3-phosphate was quantified using a gel analysis system and software (Bio-Rad).

Statistical analysis

To limit variability and provide appropriate controls, for each experimental condition the entire group of animals was prepared and studied at the same time. For each experimental condition, mice in all groups had the same birth date and had been housed together. Separate groups of mice (n = 3–9 per group) were used for wet-to-dry lung weight ratios, MPO assay, Western blotting, PI3-K assays, ELISA, and EMSA. Data are presented as mean ± SEM for each experimental group. One-way analysis of variance and the Tukey-Kramer multiple comparisons test (for multiple groups) or Student’s t test (for comparisons between two groups) were used. Values of p < 0.05 were considered significant.

Results

Role of PI3-K in LPS-induced neutrophil activation

Incubation of bone marrow neutrophils with LPS resulted in phosphorylation of Akt (Fig. 1), enhanced nuclear translocation of NF-κB (Fig. 2A), and increased expression of the proinflammatory cytokines IL-1β and TNF-α (Fig. 2, B and C). All of these endotoxin-induced events were dependent on PI3-K. Addition of the isofrom nonspecific PI3-K inhibitors wortmannin or LY294002 to the neutrophil cultures prevented LPS-associated Akt phosphorylation (Fig. 1) and significantly inhibited endotoxin-induced nuclear translocation of NF-κB (Fig. 2A). Similarly, blockade of PI3-K diminished LPS-induced elevations in mRNA levels and protein release of IL-1β and TNF-α in neutrophils (Fig. 2, B and C).

The PI3-K γ isofrom has been shown to be important in affecting G-protein-associated neutrophil activation under both in vitro and in vivo conditions (26–28). However, the role of PI3-Kγ in LPS-induced neutrophil signaling has not previously been examined. To address this issue, we used transgenic PI3-Kγ−/− mice in which...
the P110γ catalytic subunit of PI3-K was absent (28). Neutrophils from these animals are unable to produce PtdIns-3,4,5-P3 and activate Akt when stimulated with G-protein-coupled receptor agonists, such as fMLP, C5a, or IL-8. Phosphorylation of Akt and nuclear translocation of NF-κB after LPS exposure was reduced in PI3-K−/− neutrophils as compared with that found in PI3-K+/+ neutrophils, showing that PI3-K is directly involved in LPS-induced neutrophil activation (Fig. 3).

Endotoxemia increases PI3-K and Akt activation in lung neutrophils in vivo

In the above in vitro experiments, LPS stimulation of neutrophils resulted in increased levels of phosphorylated Akt through a PI3-K-dependent pathway. To determine whether endotoxin had similar effects in vivo, we administered LPS to mice and then examined PI3-K and Akt activation in neutrophils that accumulated in the lungs.

In these in vivo experiments, PI3-K activation was significantly increased in lung neutrophils starting within 1 h after LPS administration (Fig. 4A). Further increases in PI3-K activity in lung neutrophils occurred over the 24 h after initiation of endotoxemia (Fig. 4A). Activation of Akt, with a similar time course to that seen with PI3-K, was present in lung neutrophils after administration of endotoxin (Fig. 4B).

Activation of PI3-Kγ contributes to the development of endotoxin-induced ALI

In our initial in vitro experiments (Fig. 3), we found that PI3-Kγ was directly involved in phosphorylation of Akt in LPS-stimulated neutrophils. To examine the in vivo role of PI3-Kγ in modulating the development of endotoxin-induced ALI, we administered LPS to transgenic PI3-Kγ−/− and control PI3-Kγ+/+ mice and then measured activation of Akt and NF-κB, as well as parameters of lung inflammation and injury.

Administration of LPS to control PI3-Kγ+/+ mice resulted in activation of Akt in lung neutrophils (Figs. 5A). In contrast, endotoxin-induced activation of Akt was significantly decreased, compared with levels found in PI3-Kγ+/+ mice, in lung neutrophils from PI3-Kγ−/− mice. Similarly, whereas endotoxemia was associated with increased nuclear translocation of NF-κB in lung neutrophils from PI3-Kγ−/− mice, such NF-κB activation was significantly diminished in the PI3-Kγ−/− mice (Fig. 5B).

Increased lung edema, coupled with neutrophil infiltration and elevations in proinflammatory cytokine production, is indicative of
the development of ALI after endotoxin administration. Endotoxemia-induced elevations in mRNA levels for IL-1 and TNF-α in pulmonary neutrophils were reduced in PI3-K mice compared with levels present in lung neutrophils from PI3-K mice (Fig. 5C). Pulmonary protein levels for these proinflammatory cytokines after LPS administration were also significantly lower in PI3-K mice, than in PI3-K mice (Fig. 5D).

Endotoxemia resulted in significantly fewer neutrophils being recruited to the lungs of PI3-K mice as compared with PI3-K controls (Fig. 6A). Although MPO levels in the lungs of PI3-K mice were approximately half of those present in PI3-K animals after endotoxin administration, lung MPO levels in the LPS-treated PI3-K mice were still significantly elevated compared with those in control, unmanipulated animals. Endotoxemia-induced lung edema also was reduced in PI3-K mice compared with that present in PI3-K controls (Fig. 6B).

Discussion
PI3-K and Akt occupy a major role in cellular functions regulating host defense and immune response. Activation of PI3-K delays apoptosis of neutrophils and lymphocytes (13, 19, 20, 24, 33). Neutrophil chemotaxis in response to G-protein receptor agonists, such as IL-8 or fMLP, is dependent on PI3-K (15, 16, 26, 27, 34, 35). In vivo, the γ isoform of PI3-K appears to be particularly important in migration of both neutrophils and macrophages toward chemotactic stimuli, including those associated with bacterial infection (26–28). Cells deficient in the p110 subunit of PI3-K show reduction in movement toward chemoattractants that signal through G-protein coupled receptors, such as fMLP, and a reduced ability to migrate to the peritoneum in bacteria induced peritonitis (26–28). PI3-K and Akt are important in regulating NF-κB activation in multiple cell types (19–25). However, almost all of the studies reported have used cell lines or isolated cell populations to demonstrate dependence of NF-κB activation on PI3-K/Akt and no studies have examined the dependence of NF-κB activation on PI3-K/Akt in neutrophils. Both the stimulus and cell type appear to be important in defining the relationship between PI3-K and NF-κB activation. For example, whereas inhibition of PI3-K blocked NF-κB activation by LPS, IL-1, platelet-derived growth factor (PDGF), or phospho ester in nonmyeloid cell lines, such treatment had minimal effect on the activation of NF-κB by hydrogen peroxide, ceramide, or okadaic acid (25, 34, 36, 37).
Endotoxin is an important mediator of organ system dysfunction and death associated with severe Gram-negative infections. We and others have shown that endotoxemia results in the development of ALI (6, 8, 9, 30, 31). After endotoxin administration, neutrophils that accumulate in the lungs show increased activation of NF-κB and produce proinflammatory cytokines, such as TNF-α, whose transcription is dependent on NF-κB (5, 6, 38). In the present experiments, we demonstrate that PI3-K has an important role in regulating endotoxin-induced activation of NF-κB and proinflammatory cytokine expression in neutrophils both in vitro and in transgenic mice lacking the catalytic subunit of PI3-K (PI3-K−/−). In lung neutrophils 1 h after endotoxin administration as compared with that found in control mice (PI3-K+/+). Similarly, amounts of mRNA (C) and protein (D) for IL-1β and TNF-α were significantly diminished in lung neutrophils (C) and lung homogenates (D) collected from PI3-K−/− mice compared with PI3-K+/+ mice after endotoxemia. Representative gels are shown in A and B and the histograms show combined data from three experiments, using separate groups of mice. Akt activation is shown as the relative ratio of p-Akt to t-Akt. Each experimental group included five mice; similar results were obtained in a second experiment using separate groups of mice. *p < 0.05; **p < 0.01 vs control (CON); †p < 0.05 for PI3-K−/− (LPS PI3-K−/−) vs PI3-K+/+ (LPS PI3-K+/+) after exposure to endotoxin.
and in vivo. Although previous studies (19–25) found that PI3-K occupied a central position in modulating NF-κB activation and cytokine production in various cell populations, including fibroblasts, epithelial cells, and mast cells, such a role had not been shown in neutrophils.

The present studies show that activation of PI3-K, and particularly of the PI3-K isoform, plays a major role in endotoxin-induced neutrophil activation as well as the development of endotoxemia-associated ALI. Lung edema, proinflammatory cytokine production, and neutrophil accumulation after endotoxin administration were reduced in transgenic mice lacking the catalytic subunit of PI3-K. In addition, endotoxemia-elicited lung neutrophils demonstrated decreased activation in PI3-K−/− mice with diminished NF-κB activation and expression of proinflammatory cytokines compared with that found in lung neutrophils from control PI3-K+/+ animals.

There are several potential mechanisms by which PI3-K may contribute to the development of neutrophil-driven acute inflammatory processes, such as ALI (Fig. 7). Decreased numbers of neutrophils were present after endotoxin administration in the lungs of PI3-K−/− mice. Neutrophil chemotaxis to CXC chemokines, such as IL-8, is dependent on PI3-K, apparently through mechanisms involving cytoskeletal reorganization (15, 24). In mice exposed to endotoxin, pulmonary levels of the CXC chemokines macrophage-inflammatory protein-2 and KC are increased and contribute to the development of neutrophilic alveolitis (6, 8, 38). Inhibition of PI3-K activation may therefore diminish endotoxin-induced accumulation of neutrophils in the lungs through decreasing chemotaxis toward chemokines and other chemotactic molecules, such as C5a, that signal through G-protein coupled receptors and that are known to be involved in the initiation and development of ALI (39). However, a direct role for PI3-K in modulating endotoxin-associated neutrophil activation is also likely, because LPS-induced phosphorylation of Akt and nuclear translocation of NF-κB were both decreased in PI3-K−/− neutrophils.

In addition to there being fewer neutrophils present after endotoxin administration in the lungs of PI3-K−/− mice, the neutrophils that were present showed decreased activation of NF-κB and diminished expression of proinflammatory cytokines, including IL-1β and TNF-α, compared with lung neutrophils in control PI3-K−/− mice. Neutrophils are a major source of IL-1β and TNF-α in the lungs after endotoxemia (5, 6, 38) and both of these cytokines are important early mediators of the development of ALI (1–4). PI3-K, via activation of Akt, can lead to increased nuclear translocation and transcriptional activity of NF-κB (19–25), which has a central role in regulating the expression of cytokines, including TNF-α and IL-1β, as well as other proinflammatory mediators involved in ALI.

An additional mechanism by which inhibition of PI3-K may ameliorate the development of ALI is through enhancing neutrophil apoptosis, thereby increasing the removal of activated neutrophils from the lungs. The percentage of apoptotic neutrophils is decreased in patients with adult respiratory distress syndrome and in animal models of ALI (40, 41). Activation of Akt through PI3-K dependent pathways has been shown to decrease apoptosis by several mechanisms. Activated Akt maintains integrity of the outer mitochondrial membrane in a caspase-independent manner, preventing release of cytochrome c (42). Akt can phosphorylate Bad, a proapoptotic member of the Bcl-2 family, at Ser155, thereby preventing Bad from binding and inhibiting the antiapoptotic protein Bcl-xL (24). Additionally, Akt-induced phosphorylation of caspase 9 at Ser196 decreases apoptosis by inhibiting this death protease (13, 24). Phosphorylation of the transcription factor Forkhead prevents its nuclear translocation and activation of proapoptotic genes (43). Finally, recent studies have shown that activation of NF-κB is potentiated by Akt, leading to the transcription of antiapoptotic genes, such as mcl-1, A1, A20, and Bcl-xL (19, 24, 44, 45).

FIGURE 6. Endotoxemia-induced increases in lung MPO activity (A) and lung edema (B) were significantly decreased in mice lacking the catalytic subunit of PI3-K (PI3-K−/−) compared with controls (PI3-K+/+) (n = 6 in each group). Lung MPO levels were measured 1 h after endotoxin administration and were significantly increased in both PI3-K−/− and PI3-K+/+ mice compared with controls (CON). The wet-to-dry weight ratio of each endotoxin-treated animal was corrected by subtracting the mean value of the endotoxin-treated groups was plotted. *, p < 0.05; **, p < 0.01 vs unmanipulated PI3-K+/+ or PI3-K−/− animals and the increased mean of the endotoxin-treated groups was plotted; †, p < 0.05 vs LPS-treated PI3-K−/−.

FIGURE 7. Neutrophil-associated mechanisms through which PI3-K may contribute to the development of LPS-induced ALI. In the present experiments, exposure of neutrophils to LPS resulted in activation of Akt through a PI3-K-dependent step. Activation of Akt can lead to 1) enhanced nuclear translocation of NF-κB and expression of proinflammatory cytokines, such as IL-1β and TNF-α, 2) increased neutrophil chemotaxis into the lungs, and 3) diminished apoptosis of activated neutrophils in the lungs. All of these Akt-dependent events may contribute to the development and perpetuation of neutrophil-driven inflammatory processes, such as ALI.
In the present studies, in vitro inhibition of PI3-K decreased LPS-induced activation of NF-κB and expression of IL-1β and TNF-α in neutrophils. A similar decrease in NF-κB activation was present in LPS-stimulated PI3-K−/− neutrophils. Findings in lung neutrophils from PI3-K−/− mice indicate that PI3-K, and in particular PI3-K, has an important in vivo role in activating NF-κB and in inducing expression of NF-κB-dependent pro-inflammatory mediators after endotoxin exposure. The severity of endotoxin-induced ALI was reduced in PI3-K−/− mice, consistent with the decreases in numbers and activation of lung neutrophils in these animals. These results suggest that PI3-K may be a useful pharmacologic target in the prevention or treatment of ALI as well as other acute inflammatory conditions where neutrophils play an important pathophysiologic role.

References