Blocking p21-activated Kinase Reduces Lipopolysaccharide-induced Acute Lung Injury by Preventing Polymorphonuclear Leukocyte Infiltration

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***Rationale*: Excessive recruitment of polymorphonuclear leukocytes (PMNs) to the lung promotes acute lung injury (ALI). Chemokine receptors and adhesion molecules initiate leukocyte–endothelial interactions, but mediators of PMN migration through the alveolo-capillary membrane remain to be identified. p21-Activated kinase (PAK) is an effector of small GTPases and has been implicated in cell migration.**

***Objectives*: To test the role of PAK in ALI.**

***Methods*: An inhibitory PAK peptide was used to determine the role of PAK in cytoskeletal actin polymerization, cell adhesion, and oxidative burst. PMN migration was investigated *in vitro* and in a murine model of lipopolysaccharide-induced lung injury.**

***Measurements and Main Results*: PMN migration into lung interstitium and alveolar space was suppressed by an inhibitory PAK peptide. Neutrophils that had taken up the inhibitory PAK peptide were unable to enter the alveolar space. CXCL2/3, an important PMN chemoattractant in murine lung injury, induced PAK phosphorylation in PMNs. Blocking PAK function inhibited chemotaxis, chemokine-induced cytoskeletal actin polymerization, and adhesion-induced oxidative burst.**

***Conclusions*: We conclude that neutrophil PAK is a critical mediator of PMN migration and may be an attractive target in ALI.**

Keywords: acute respiratory distress syndrome; polymorphonuclear leukocytes; inflammation; migration

> **AT A GLANCE COMMENTARY**
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> **Scientific Knowledge on the Subject**
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> Chemokine receptors and adhesion molecules initiate leukocyte–endothelial interactions, but mediators of polymorphonuclear leukocyte migration through the alveolo-capillary membrane remain to be identified.
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> **What This Study Adds to the Field**
>
> p21-Activated kinase is a critical mediator in acute lung injury.

Excessive recruitment of polymorphonuclear leukocytes (PMNs) to the lung is one of the hallmarks of the early phase of acute lung injury (ALI) and acute respiratory distress syndrome (ARDS). In experimental studies, depletion of PMNs has been demonstrated to curb lung damage (1). Clinical observations suggest that lung function in patients with ARDS negatively correlates with neutrophil counts in the blood (2).

The molecular requirements for PMN trafficking into inflamed lungs differ fundamentally from those in other tissues (3, 4). Although PMN activation might be sufficient to allow PMN adhesion to the pulmonary endothelium (5), neutrophil transmigration into the lung interstitium and alveolar airspace requires molecules on PMNs and endothelium, including adhesion molecules and chemokine receptors (6, 7). After adhesion to the endothelium is established, transmigration is initiated by stimulus-induced cytoskeletal reorganization of PMNs and endothelial cells. Small GTPases, such as Rac and Cdc42, play a key role in the directed migration of PMNs to sites of inflammation (8, 9).

p21-Activated kinases (PAKs) 1, 2, and 3 constitute a family of serine/threonine kinases activated by Rac and Cdc42 (10). Because our inhibitory peptide does not distinguish between PAK isoforms, we use PAK to indicate any of the three isoforms throughout this article. Under resting conditions, PAK forms a homodimer in which catalytic activity is blocked by binding an inhibitory domain in the N-terminus of the opposing catalytic subunit (11). Activation of PAK involves phosphorylation of the thr423 and ser141 residues, dissociation of the dimer, and release of the catalytic domains (12). PAK binds to SH3-containing adaptor proteins, including Nck and PAK-associated guanine nucleotide exchange factor (PIX), which mediate translocation of PAK to targets at the cell membrane and cell–cell junctions (13, 14), ultimately resulting in cytoskeletal remodeling and cell contractility (15). Interfering with PAK interaction with Nck blocks endothelial migration and angiogenesis (16). In neutrophils, PAK is activated by chemoattractants such as formylmethionylleucylphenylalanine (fMLP) (17, 18) and is implicated in the directional movement of PMNs toward a chemotactic gradient *in vitro* (19). It is unclear whether PAK is involved in PMN recruitment to sites of inflammation.

This study was designed to investigate the role of PAK in a murine model of ALI induced by aerosolized lipopolysaccharide (LPS). In this model, PMN activation is indirect through mediators produced by alveolar macrophages and other lung-resident cells (20, 21). PAK activity was inhibited *in vivo* using an N-terminal Nck-blocking peptide (16), and effects on PMN migration into the

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different compartments of the lung and PMN-dependent lung damage were studied.

METHODS

Inhibitory PAK Peptide

To block PAK function *in vitro* and *in vivo*, we used an inhibitory PAK peptide as previously described (16). A peptide in which two prolines critical for SH3 binding were mutated to alanines was used as a control. To detect entry into cells, a peptide was synthesized with an N-terminal fluorescein isothiocyanate (FITC) moiety (16). To characterize the kinetic of this peptide, we incubated mouse blood with the fluorescent peptide and determined its uptake into PMNs at different time points (1 h, 3 h, and 6 h).

F-Actin Formation

Human PMNs were purified as described (22) and incubated with inhibitory PAK or control peptide (20 μg/ml) for 1 hour. PMNs were plated on fibronectin-coated glass slides, and some were stimulated with CXCL1 (100 ng/ml) for 10 minutes. Cells were fixed and permeabilized, and F-actin was stained as described (7). In separate experiments, F-actin content was measured in PMNs in suspension using flow cytometry (7, 23). In all experiments, the viability of cells was determined by Trypan blue exclusion and was always greater than 95%.

PMN Adhesion Assay

Human PMNs were pretreated with PAK or control peptide (20 μg/ml) for 1 hour and labeled with calcein acetoxymethyl (AM) (Molecular Probes, Carlsbad, CA), and adhesion to fibrinogen-coated (2 μg/ml) plates was measured in a plate-reader.

PMN Oxidative Burst

Oxidative burst of adherent PMNs was quantified by measuring the superoxide dismutase–inhibitable reduction of cytochrome c as described (22). Some PMNs were treated with dihydrocytochalasin B (1 μg/ml) to disrupt cell spreading on the surface (24).

Western Blotting

PMNs from C57Bl/6 mice were stimulated with CXCL2/3 (100 ng/ml) for the indicated times and prepared for Western blotting as described previously (25). Blots were probed with anti-PAK2 (1:1,000) (Santa Cruz Biotechnology, Santa Cruz, CA) or pS141PAK (1:2,000) (Biosource, Carlsbad, CA). Lysates of whole lung tissue were probed for PAK2, pS141 PAK, and pS536 p65, a marker for activation of nuclear factor-κB.

In Vitro Transmigration Assay

Murine pulmonary endothelial cells (PECs) were isolated using a positive immunomagnetic selection with CD31 (EasySep Biotin Selection Kit; StemCell Technologies, Vancouver, BC, Canada), cultured in Dulbecco's modified Eagle's medium with 10% of fetal calf serum, 20 mM *N*-2-hydroxyethylpiperazine-*N′*-ethane sulfonic acid, 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA), and 50 μg/ml endothelial cell growth supplement (Sigma, St. Louis, MO), and grown on fibronectin-coated filters in a Transwell system (6.5-mm diameter, 3.0-μm pore size) (Corning, Inc., Corning, NJ).

PMNs from C57Bl/6 mice were isolated as described (7). PMNs, PECs, or both were incubated with the inhibitory or control PAK peptide (20 μg/ml) for 1 hour. Filters were moved to wells containing 400 μl of phenol red-free Dulbecco's modified Eagle medium with or without CXCL2/3 (250 ng/ml) (PeproTech, Inc., Rocky Hill, NJ). A total of 2.5×10^5 calcein-labeled (5 μM) PMNs were plated on filters with or without endothelial cells. Filters were incubated for 2 hours at 37°C, and fluorescence was measured in the bottom wells.

In Vivo Distribution of the Inhibitory PAK Peptide

A FITC-tagged inhibitory PAK peptide (16) was injected intraperitoneally. Six hours after injection, PAK-positive cells were identified by flow cytometry, and their expression of CD31 and CD45 was determined. Some mice inhaled LPS after an intraperitoneal injection of the fluorescent inhibitory PAK peptide. Twelve hours later, PMNs were identified in blood, lungs, and bronchoalveolar lavage fluid (BALF) and investigated for their peptide uptake. In some experiments, lungs from these mice were fixed for confocal microscopy.

Murine Model of ALI

LPS inhalation was used to induce ALI in wild-type male C57Bl/6 mice as described previously (26). All animal experiments were approved by the Animal Care and Use Committee of the University of Virginia.

PMN Trafficking in the Lung

PMN recruitment into the different compartments of the lung was assessed as described (26).

pPAK-expressing Cells in the Lung

To determine pPAK expression of neutrophils recruited to the lung, lungs were homogenized 3 hours after LPS exposure. Cells were permeabilized (Cytofix/Cytoperm; BD, Franklin Lakes, NJ) and probed with fluorescently labeled (Zenon Rabbit IgG Kit; Molecular Probes) anti–phospho-Ser141 PAK antibody (Biosource). pPAK expression was analyzed in all leukocytes ($CD45^+$), PMNs ($CD45^+$, GR-1^{high}), and lymphocytes ($CD45^+$, GR-1^-).

Statistical Analysis

Statistical analysis was performed with JMP Statistical Software 5.1 (SAS Institute, Inc., Cary, NC). Differences between the groups were evaluated by one-way analysis of variance followed by a *post hoc* Tukey test. Data were presented as mean ± SEM, and $p < 0.05$ was considered statistically significant.

RESULTS

PAK Regulates Cytoskeletal Reorganization in Human PMNs

Remodeling of the cytoskeleton in response to an inflammatory stimulus is critical for the migratory activity of PMNs. We therefore investigated the role of PAK in actin polymerization in CXCL1-stimulated human PMNs. CXCL1 activation resulted in a marked increase in F-actin in a typical semilunar shape (Figure 1A). Inhibition of PAK function by addition of the inhibitory peptide reduced actin polymerization substantially and prevented F-actin localization to the leading edge of the lamellipod. A control peptide in which two key prolines were mutated had no detectable effect. Quantification by flow cytometry showed that the inhibitory PAK peptide caused an approximately 30-fold decrease in F-actin relative to control cells (Figure 1B).

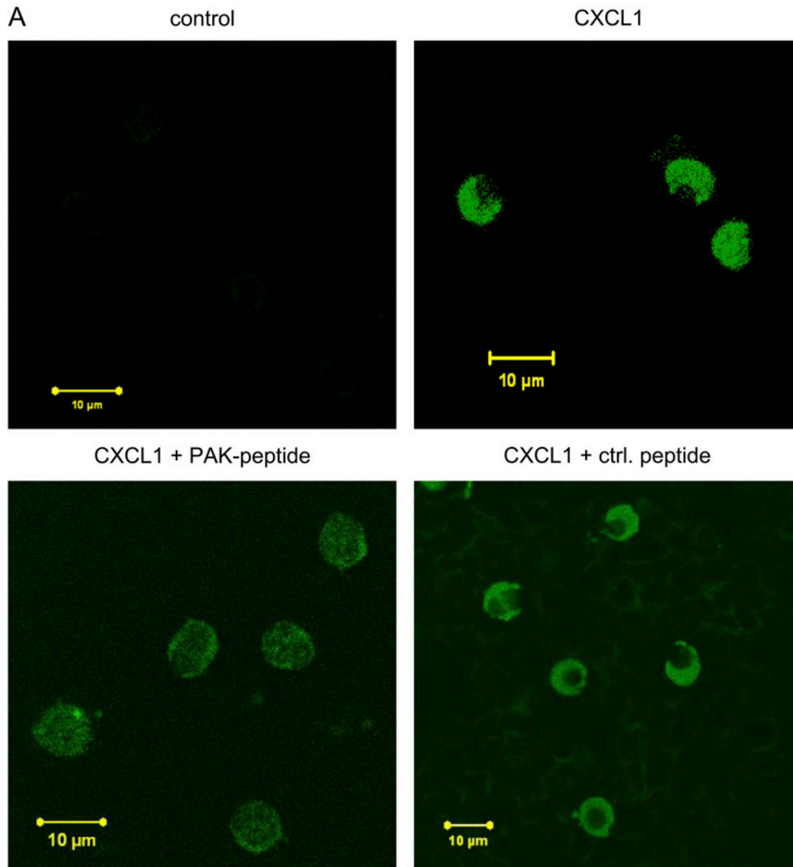
PMN Adhesion to Fibrinogen Is PAK Mediated

To test whether the inhibitory PAK peptide impaired cell adhesion to a biological surface, we performed a static adhesion assay. Human PMNs were allowed to adhere to fibrinogen-coated wells with or without CXCL1. CXCL1 induced a significant increase of adhesion ($p < 0.05$) (Figure 2A). The inhibitory PAK peptide reduced cell adhesion to baseline levels ($p < 0.05$), whereas the control peptide had no effect.

Oxidative Burst in Adherent PMNs Is PAK Dependent

Unregulated release of reactive oxygen species (ROS) from PMNs can be detrimental in the setting of ALI (27). ROS formation occurs upon neutrophil activation and involves integrin-dependent cytoskeletal reorganization in PMNs adherent to a surface (28, 29). We therefore investigated the role of PAK in the formation of ROS in adherent PMNs.

Oxidative burst in response to adhesion to biological surfaces was investigated as superoxide dismutase–inhibitable reduction of cytochrome c. Adhesion-induced oxidative burst was markedly reduced when tumor necrosis factor-α–primed PMNs were pretreated with the inhibitory PAK peptide (Figures 2B and



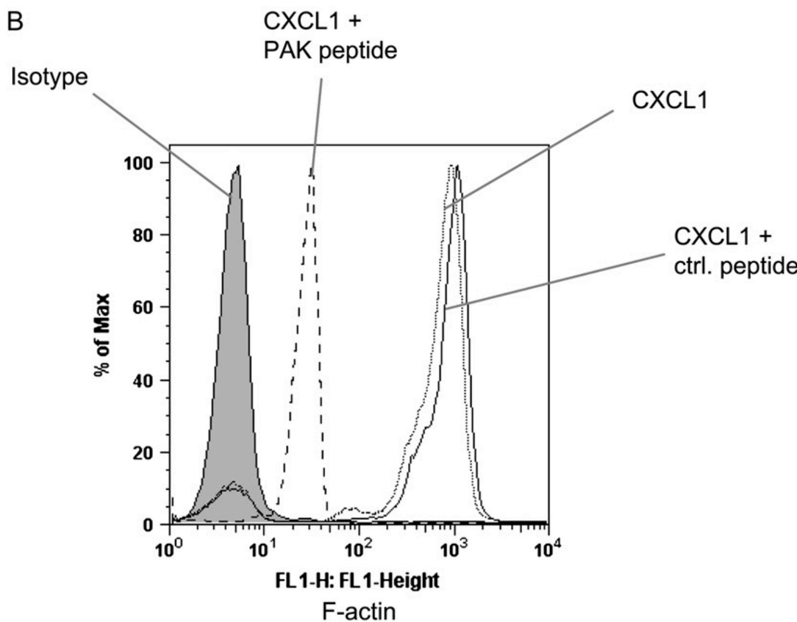


Figure 1. p21-Activated kinase (PAK) activity is required for CXCL1-induced cytoskeletal remodeling. Human polymorphonuclear leukocytes (PMNs) were plated on fibronectin-coated glass slides, treated without (control) or with CXCL1 alone, treated with PAK or with control peptide, fixed, and stained for F-actin (*A*). F-actin content in suspended PMNs was analyzed by flow cytometry (*B*).

2C). Similar inhibition was observed when cytoskeletal actin polymerization was disrupted by dihydrocytochalsin B. These data suggest that the stimulation of actin polymerization by PAK may mediate these effects, although direct effects on the NADP-reduced oxidase activity have also been reported (18) and could be involved. PAK activity did not affect the release of ROS from PMNs in suspension (data not shown).

CXCR2-dependent PAK Activation

CXCL2/3 (macrophage inflammatory protein-2) is a critical CXCR2 ligand in lung injury (30). To directly test whether PAK can be phosphorylated after CXCR2 ligation, murine PMNs were stimulated with CXCL2/3, which induced a transient phosphorylation of PAK on ser141 in PMNs with a peak between 15 and 30 minutes (Figure 3A), consistent with a possible role for PAK in CXCR2-dependent models of ALI.

A Role for Neutrophil PAK for *In Vitro* Transmigration

We investigated the role of PAK in PMNs for transmigration. Baseline and CXCL2/3-stimulated migration of PMNs through a Transwell filter were reduced when PMNs were pretreated

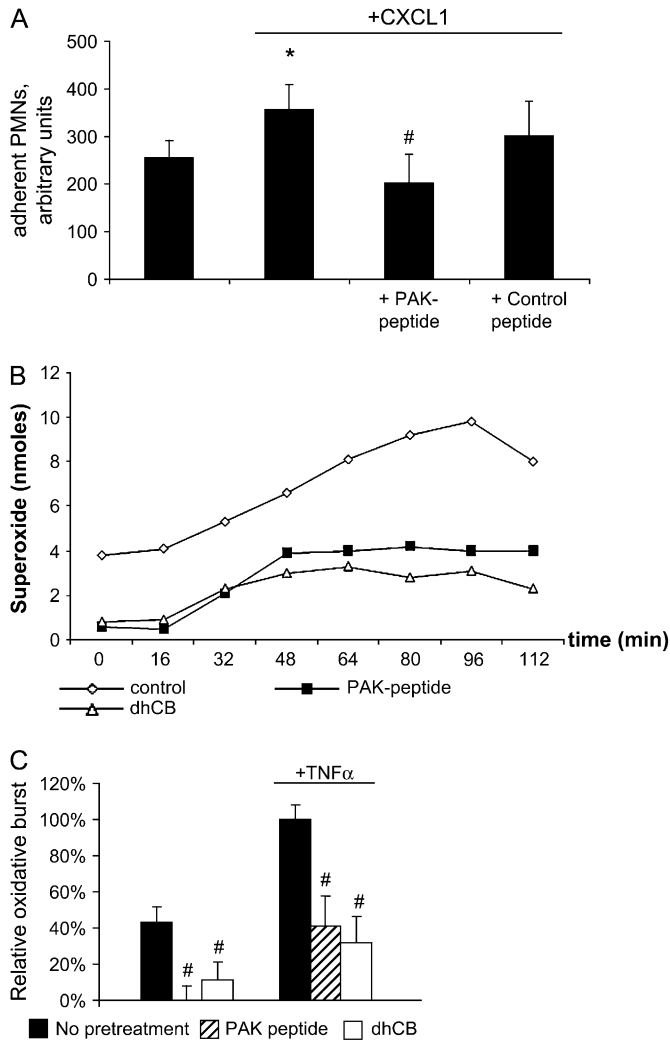


Figure 2. Role of p21-activated kinase (PAK) in adhesion and oxidative burst in human polymorphonuclear leukocytes (PMNs). Human PMNs were calcein labeled and incubated in fibrinogen-coated wells for 2 hours with or without CXCL1. CXCL1 induced a significant ($p < 0.05$) increase in cell adhesion. The inhibitory PAK peptide, but not the control peptide, significantly reduced adhesion ($p < 0.05$) (*A*). Adhesion-induced superoxide production was measured as superoxide dismutase–inhibitable reduction of cytochrome c. The inhibitory PAK peptide and dihydrocytochalasin B (dhCB) reduced oxidative activity of tumor necrosis factor (TNF)-α–primed adherent PMNs similarly, indicating a critical role of PAK in superoxide production (*B*). Respiratory burst at maximal response without (*left*) or with (*right*) TNF-α. Superoxide production by adherent PMNs treated with TNF-α was set equal to 100% (*C*). *$p < 0.05$ versus negative control; #$p < 0.05$ versus positive control. The inhibitory PAK peptide had no effect on oxidative burst in cell suspension (data not shown).

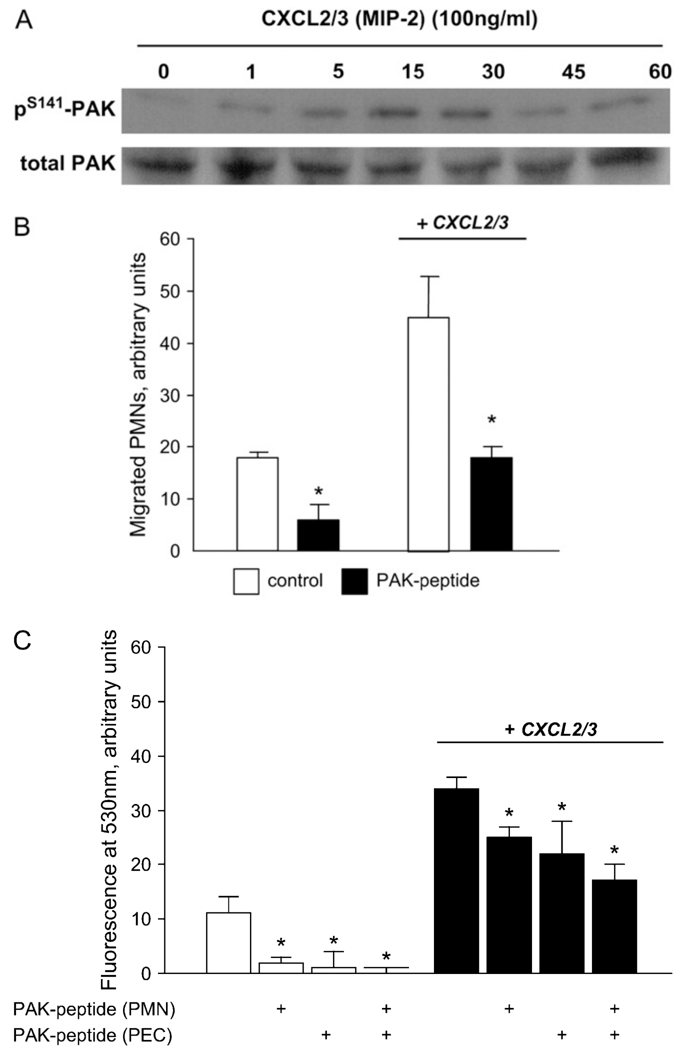


Figure 3. CXCR2-dependent p21-activated kinase (PAK) phosphorylation and *in vitro* transmigration. The CXCR2 ligand CXCL2/3 (100 ng/ml) induced PAK-phosphorylation in murine polymorphonuclear leukocytes (PMNs) with a peak between 15 and 30 minutes (*A*). Calcein-labeled PMNs from C57Bl/6 mice were allowed to migrate through 3-μm Transwell filters with or without CXCL2/3 added to the lower well. Migration was reduced when PMNs were pretreated with the inhibitory PAK peptide (*solid bars*) (*B*). PMN migration across a layer of pulmonary endothelial cells (PECs) was investigated using a similar protocol, except that PMNs, endothelial cells, or both were pretreated with the inhibitory PAK peptide and washed before adding media without (*open bars*) or with CXCl2/3 (*solid bars*, 250 ng/ml) to the lower wells (*C*). Mean fluorescence was corrected for baseline fluorescence (endothelial cells only). Mean ± SEM of three experiments. *$p < 0.05$ versus positive control.

with the inhibitory PAK peptide ($p < 0.05$ vs. untreated control) (Figure 3B), consistent with a critical role for PAK in PMN migration. To investigate the role of PAK in transendothelial migration, pulmonary endothelial cell monolayers were grown on Transwell filters, and PMNs were allowed to migrate to the lower well. To distinguish between effects on PMNs and endothelial cells, each cell type was pretreated with the inhibitory PAK peptide for 1 hour and washed before beginning the assay. Migration was reduced when PMNs or endothelial cells were pretreated with the inhibitory PAK peptide (Figure 3C). Treatment of both populations inhibited more efficiently ($p < 0.05$ vs. untreated control). Significant inhibition was observed with spontaneous and CXCL2/3-induced migration.

In Vivo Distribution of the Inhibitory PAK Peptide

To investigate the cellular targets of the inhibitory PAK peptide *in vivo*, we injected mice with FITC-tagged inhibitory PAK peptide. Lungs were harvested and digested, and cells positive for

the inhibitory PAK peptide were gated and investigated for their expression of CD31, an endothelial cell marker, and CD45, a marker for leukocytes (Figure 4A). About 10% of PAK-positive cells were found to be CD31 positive (not shown), consistent with a previously described role of PAK in endothelial cells (25). However, the majority of PAK-positive cells (80%) were CD45-positive leukocytes. Uptake of the peptide in the lung was confirmed by confocal microscopy (Figure 4B). The observed pattern was consistent with inhibitory PAK peptide in endothelial cells and neutrophils. LPS inhalation did not change the distribution of PAK in the lung significantly (Figure 4B, *right bottom panel*). To assess the kinetics of uptake of the inhibitory PAK peptide in PMNs, whole mouse blood was incubated with the fluorescent inhibitory PAK peptide, and its uptake into PMNs was determined by flow cytometry. Mean fluorescence in PMNs was stable for at least 6 hours (data not shown).

PAK Is Required for LPS-induced PMN Migration into the Lung

To investigate the role of PAK in a murine model of ALI, mice were exposed to aerosolized LPS. LPS exposure resulted in phosphorylation of PAK2 in total lung extracts as shown by Western blot, suggesting that PAK is involved in LPS-induced lung injury (Figure 5A). We also observed phosphorylation of nuclear factor-κB p65 subunit, indicating activation of inflammatory pathways. When mice were pretreated with the inhibitory PAK peptide, PMN accumulation in the vascular space was only weakly affected (Figure 5B). However, PMN migration into the lung interstitium and alveolar space were reduced by 60 and 70%, respectively ($p < 0.05$) (Figures 5C and 5D). The inactive control peptide did not affect PMN migration.

To investigate whether PMNs that had taken up the inhibitory PAK peptide retained their LPS-induced migratory activity into the lung, mice were injected with FITC-labeled inhibitory PAK peptide and exposed to LPS. Twelve hours after LPS inhalation, blood, lung tissue, and BALF were investigated for PMN content of the inhibitory PAK peptide (Figure 6). In the blood, the majority of PMNs had taken up FITC-PAK peptide. In contrast, fewer FITC-positive PMNs were found in lung tissue, and virtually none were found in the BALF. This progressive loss of FITC-positive cells suggests that uptake of the inhibitory PAK peptide prevents PMN entry into the lung interstitial and alveolar space, implying a role for PAK in transendothelial (from blood to interstitium) and transepithelial (from interstitium to alveolar space) migration.

LPS Induces Recruitment of phospho-PAK (pPAK)-expressing PMNs and Macrophages

To determine whether PAK in neutrophils was phosphorylated during LPS-induced lung injury, we analyzed lymphocytes ($CD45^+GR\text{-}1^-$) and PMNs ($CD45^+GR\text{-}1^{high}$) by flow cytometry. Staining for pPAK was detected in PMNs but not in lymphocytes. Three hours after LPS inhalation (Figure 7B), the majority of leukocytes were pPAK-positive PMNs, whereas lymphocytes represented a minor fraction. Most PMNs, but only a minority of lymphocytes, stained for pPAK.

DISCUSSION

In the present study, we identified neutrophil PAK as a critical signaling molecule in LPS-induced lung inflammation. PAK is activated by cytokines implicated in LPS-induced inflammation *in vivo*. PAK contributes to neutrophil adhesion and migration across the endothelium through effects on neutrophils and endothelial cells. In a murine model of ALI/ARDS, inhibiting PAK substantially reduced PMN migration into lung interstitium and alveolar space. Most importantly, PMNs recruited to inflamed lung express pPAK, and PMNs that take up the inhibitory PAK peptide cannot be recruited to the inflamed lung. We also show

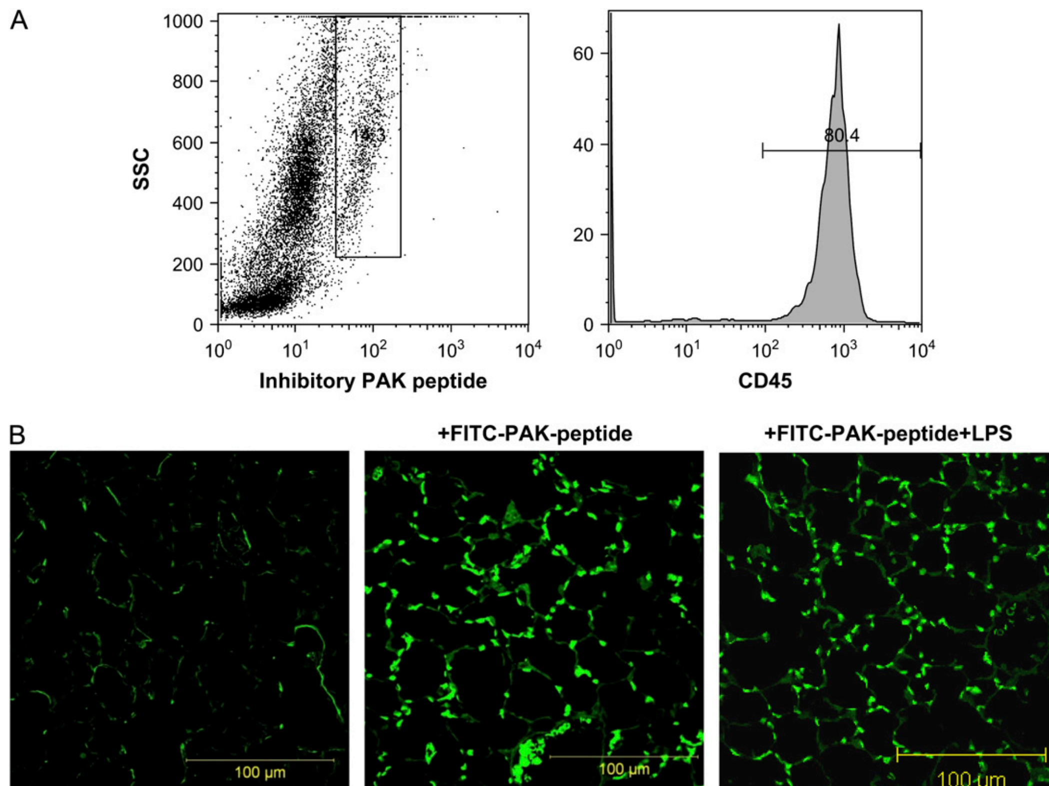


Figure 4. *In vivo* distribution of the inhibitory p21-activated kinase (PAK) peptide. A fluorescein isothiocyanate (FITC)–tagged inhibitory PAK peptide was injected intraperitoneally. Six hours later, a single-cell suspension from the lungs was prepared for flow cytometry. FITC-positive cells were gated by side scatter (SSC) and FITC. Cells that had taken up the inhibitory PAK peptide were found to be 80% $CD45^+$ (*A*). Confocal microscopy confirmed uptake of the peptide in the lung (*B*). Data are representative of three experiments.

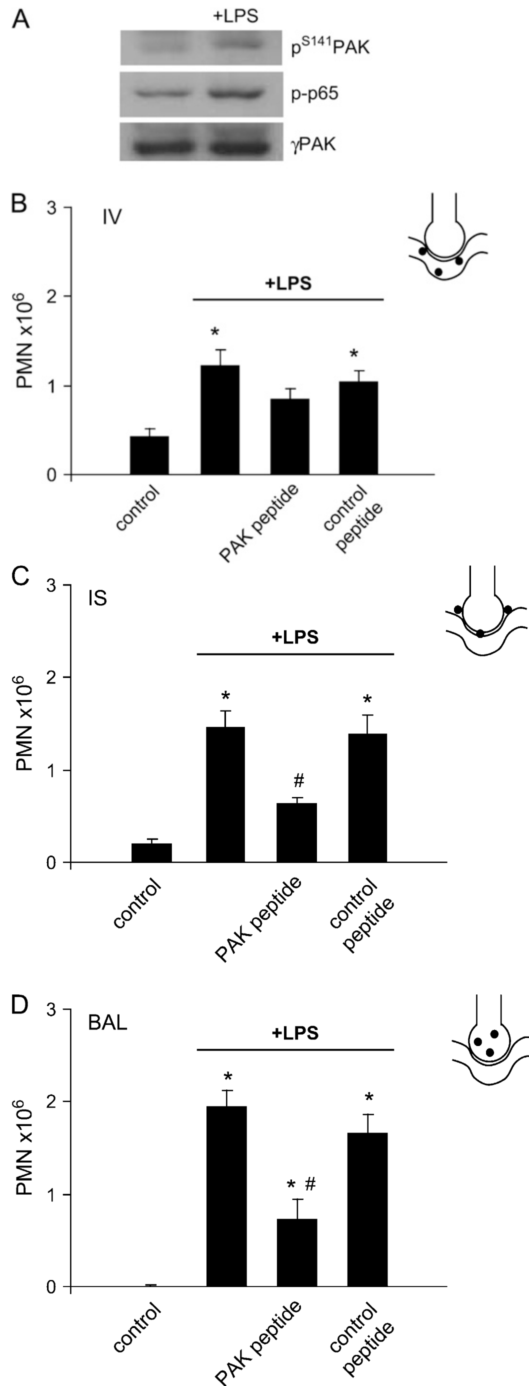


Figure 5. Role of p21-activated kinase (PAK) in lipopolysaccharide (LPS)-induced migration of polymorphonuclear leukocytes (PMNs) into the lung. Mice received an intraperitoneal injection of the inhibitory PAK peptide before LPS inhalation. Lung extracts were analyzed by Western blotting for nuclear factor-κB p65 phosphorylation on ser536 (p-p65), for PAK phosphorylation on ser141 (p^{S141}PAK), or for total PAK2 (γPAK) as a loading control (*A*). Accumulation of PMNs in the vasculature (IV) (*B*), the lung interstitium (IS) (*C*), and the bronchoalveolar space (by bronchoalveolar lavage [BAL]) (*D*) were analyzed. Values are means ± SEM; n = 4. *$p < 0.05$ versus negative control without LPS; $^{\#}p < 0.05$ versus positive control with LPS.

that PAK is involved in human neutrophil activation, suggesting a potential role for PAK in regulating leukocyte-dependent inflammatory responses in inflammatory diseases.

Effects of PAK have been demonstrated in endothelial cells and other nonleukocyte cells (17, 31). In addition, neutrophil PAK has been implicated in mediating chemotaxis *in vitro* (19). PAK2 in PMNs is rapidly phosphorylated in response to fMLP (32) and other chemoattractants (33), but a role for PAK in neutrophil transmigration *in vivo* has not been demonstrated. PAK phosphorylation occurs at several sites and follows distinct kinetics in response to various stimuli (32). PAK has been implicated in NADPH oxidase-dependent superoxide release from PMNs (18) and phagocytic activity (34). PAK-dependent cytoskeletal remodeling has been demonstrated in murine PMNs where C5a failed to induce polarization of F-actin in cells lacking the PAK-associated protein PIX (19). Here, we show similar results by disrupting the interaction between PAK and Nck. The inability to polarize may be a cause for impaired neutrophil adhesion and migration *in vivo*. It might also explain why transmigration *in vitro* and *in vivo* was reduced in PMNs that had taken up the inhibitory PAK peptide. Furthermore, we show that adhesion-dependent but not adhesion-independent oxidative burst of PMNs required PAK. Adhesion-dependent oxygen radical production is highly relevant to neutrophil-dependent tissue injury (35). Our observation that PAK did not reduce the release of ROS from PMNs in suspension indicates that the neutrophil oxidase is not directly affected by PAK.

PAK is activated by various stimuli, including growth factors, thrombin, histamine, and bacterial compounds such as LPS and fMLP (25, 32). In addition, chemoattractants can induce PAK activation, among them CXCL1 and CXCL8 (33, 36). Both chemokines have been shown to be critically involved in ALI (7, 37). Whether the protective effects observed with anti-CXCR2 strategies (7, 38, 39) can be in part attributed to reduced PAK activation remains speculative at this time but encourages further investigation.

Despite considerable progress in understanding PAK regulation at the molecular level and compelling evidence for the role of PAK for cell motility and migration *in vitro*, only a few studies have addressed its function in disease models. A recent study suggested PAK's involvement in Alzheimer disease (40). We chose a model of ALI because ALI and ARDS cause significant morbidity and mortality. Recent epidemiologic studies indicate that the incidence of ARDS is much higher than suggested in earlier reports (41). Neutrophils are critical to the development, progression, and prognosis of the disease (2, 42), but, despite advances in our understanding of the pathophysiology in ALI/ARDS, molecular mechanisms underlying neutrophil migration into the lung remain incompletely characterized (43). There are no therapeutic strategies to reduce PMN migration in ALI/ARDS. Nonspecific antiinflammatory approaches have failed to show efficacy (44).

The present data show that neutrophils recruited to inflamed lung tissue contained PAK phosphorylated at a critical activation site. When PAK activity is inhibited by an inhibitory PAK peptide, neutrophils that take up the peptide are underrepresented in the population recruited to the lung tissue. The PAK requirement is even more stringent for neutrophils that reach the BAL, suggesting that PAK is involved in transendothelial and transepithelial migration. Alternatively, PMNs that reach lung interstitium or alveolar airspace might have lost the inhibitory PAK peptide during their transmigration through the alveolo-capillary wall, but we found no evidence for the loss of inhibitory PAK peptide during at least 6 hours. In conclusion, the present studies suggest that targeting PAK may be useful to control lung injury

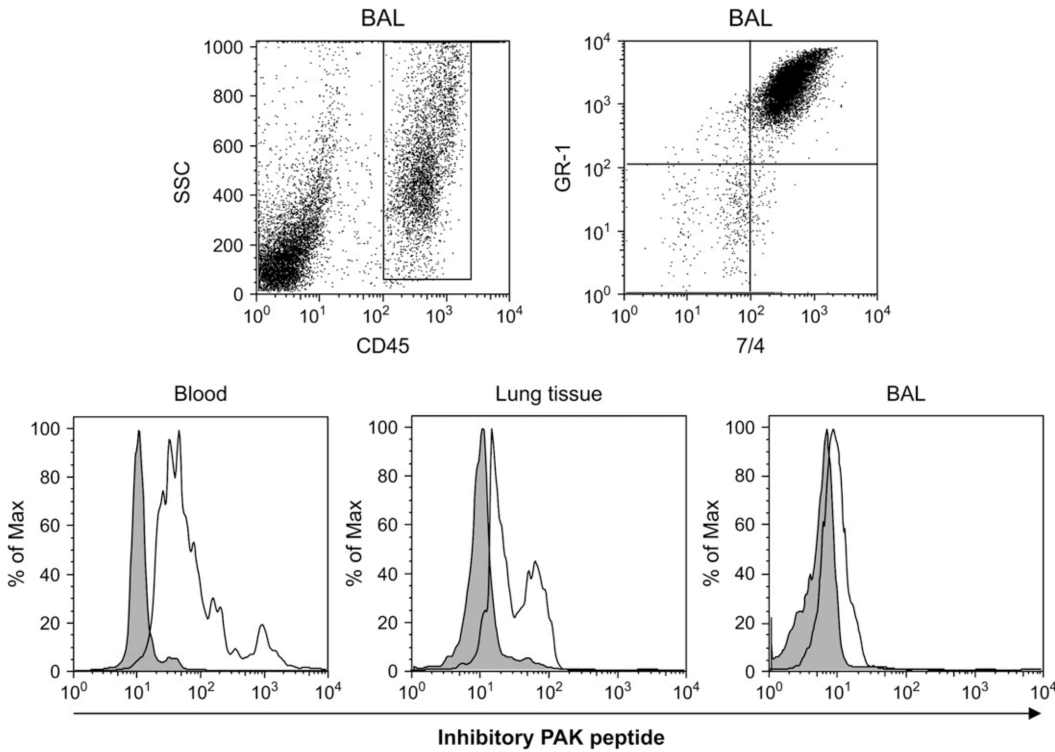


Figure 6. To investigate whether polymorphonuclear leukocytes (PMNs) that had taken up the inhibitory p21-activated kinase (PAK) peptide retained their LPS-induced migratory activity into the lung, mice were injected with fluorescein isothiocyanate–labeled PAK peptide and exposed to LPS. After 12 hours, PMNs (identified as CD45^{+} GR-1^{high} 7/4^{high}; *top panels*) in blood, lung tissue, and bronchoalveolar lavage (BAL) fluid were analyzed for their uptake of the inhibitory PAK peptide (*bottom panels*, *open histograms*). Data are representative of three experiments. *Shaded histograms* indicate background fluorescence in uninjected mice.

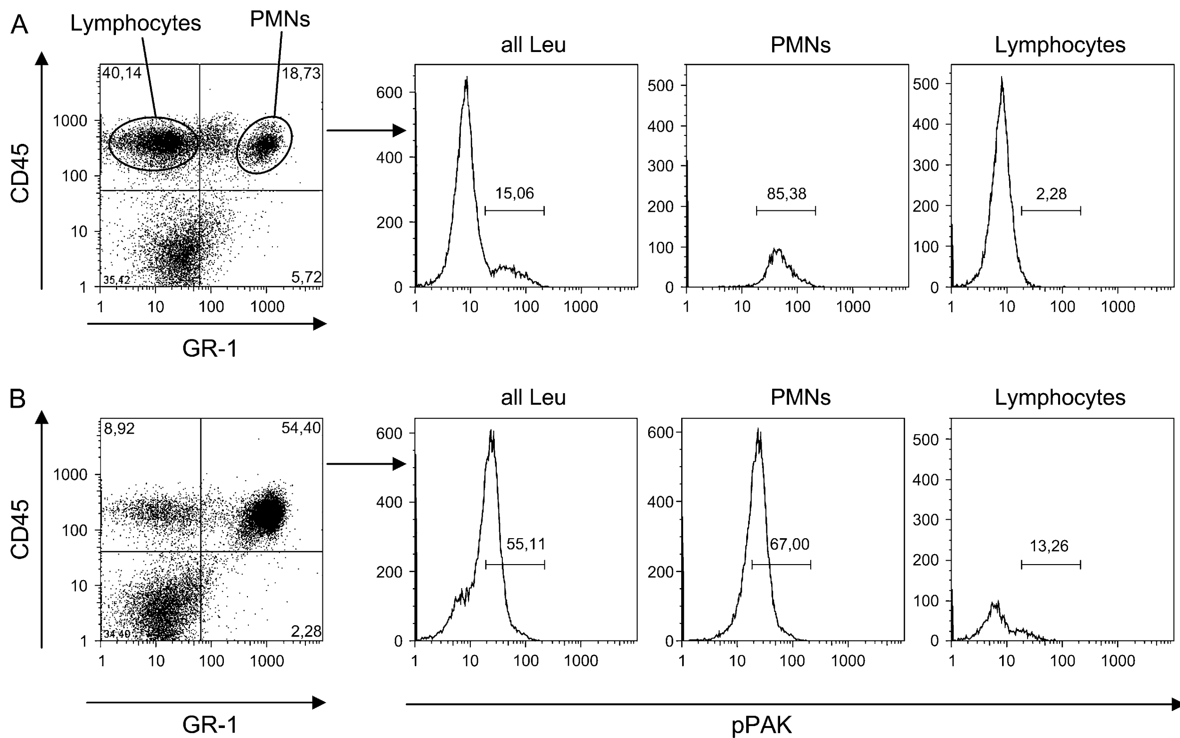


Figure 7. Lung homogenate was analyzed for phospho-PAK (pPAK)-expressing cells. In the resting lung (*A*), the majority of all leukocytes (all Leu, CD45^{+}) were pPAK negative. Characterization of the cell types revealed that lymphocytes (CD45^{+}, GR-1^{-}) did not express pPAK. Some pPAK-positive polymorphonuclear leukocytes (PMNs) (CD45^{+}, GR-1^{high}) were present in the resting lung. LPS inhalation (*B*) led to a marked increase in pPAK-positive PMNs, whereas most lymphocytes remained pPAK negative. Data are representative of four experiments in each group.

by reducing excessive PMN infiltration and PMN-dependent lung damage.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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References

1. Abraham E, Carmody A, Shenkar R, Arcaroli J. Neutrophils as early immunologic effectors in hemorrhage- or endotoxemia-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1137–L1145.
2. Azoulay E, Darmon M, Delclaux C, Fieux F, Bornstain C, Moreau D, Attalah H, Le Gall JR, Schlemmer B. Deterioration of previous acute lung injury during neutropenia recovery. *Crit Care Med* 2002;30:781–786.
3. Doerschuk CM, Beyers N, Coxson HO, Wiggs B, Hogg JC. Comparison of neutrophil and capillary diameters and their relation to neutrophil sequestration in the lung. *J Appl Physiol* 1993;74:3040–3045.
4. Forlow SB, Rose CE, Ley K. Leukocyte adhesion and emigration in the lung. In: Weir EK, Reeve JN, Refsnes M, editors. Interactions of blood and the pulmonary circulation. Armonk, NY: Futura Publishing Company; 2002. pp. 255–275.
5. Worthen GS, Schwab B III, Elson EL, Downey GP. Mechanics of stimulated neutrophils: cell stiffening induces retention in capillaries. *Science* 1989;245:183–186.
6. Basit A, Reutershan J, Morris MA, Solga M, Rose Jr CE, Ley K. ICAM-1 and LFA-1 play critical roles in LPS-induced neutrophil recruitment into the alveolar space. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L200–L207.
7. Reutershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS, Ley K. Critical role of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. *J Clin Invest* 2006;116:695–702.
8. Glogauer M, Marchal CC, Zhu F, Worku A, Clausen BE, Foerster I, Marks P, Downey GP, Dinauer M, Kwiatkowski DJ. Rac1 deletion in mouse neutrophils has selective effects on neutrophil functions. *J Immunol* 2003;170:5652–5657.
9. Srinivasan S, Wang F, Glavas S, Ott A, Hofmann F, Aktories K, Kalman D, Bourne HR. Rac and Cdc42 play distinct roles in regulating PI(3,4,5)P3 and polarity during neutrophil chemotaxis. *J Cell Biol* 2003;160:375–385.
10. Bokoch GM. Biology of the P21-activated kinases. *Annu Rev Biochem* 2003;72:743–781.
11. Parrini MC, Lei M, Harrison SC, Mayer BJ. Pak1 kinase homodimers are autoinhibited in trans and dissociated upon activation by Cdc42 and Rac1. *Mol Cell* 2002;9:73–83.
12. Lei M, Lu W, Meng W, Parrini MC, Eck MJ, Mayer BJ, Harrison SC. Structure of PAK1 in an autoinhibited conformation reveals a multistage activation switch. *Cell* 2000;102:387–397.
13. Bokoch GM, Wang Y, Bohl BP, Sells MA, Quilliam LA, Knaus UG. Interaction of the Nck adapter protein with P21-activated kinase (PAK1). *J Biol Chem* 1996;271:25746–25749.
14. Manser E, Loo TH, Koh CG, Zhao ZS, Chen XQ, Tan L, Tan I, Leung T, Lim L. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell* 1998;1:183–192.
15. Sells MA, Knaus UG, Bagrodia S, Ambrose DM, Bokoch GM, Chernoff J. Human P21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr Biol* 1997;7:202–210.
16. Kiosses WB, Hood J, Yang S, Gerritsen ME, Cheresh DA, Alderson N, Schwartz MA. Dominant-negative P65 PAK peptide inhibits angiogenesis. *Circ Res* 2002;90:697–702.
17. Knaus UG, Morris S, Dong HJ, Chernoff J, Bokoch GM. Regulation of human leukocyte P21-activated kinases through G protein–coupled receptors. *Science* 1995;269:221–223.
18. Martyn KD, Kim MJ, Quinn MT, Dinauer MC, Knaus UG. P21-activated kinase (Pak) regulates NADPH oxidase activation in human neutrophils. *Blood* 2005;106:3962–3969.
19. Li Z, Hannigan M, Mo Z, Liu B, Lu W, Wu Y, Smrcka AV, Wu G, Li L, Liu M, *et al.* Directional sensing requires G beta gamma-mediated PAK1 and PIX alpha-dependent activation of Cdc42. *Cell* 2003;114:215–227.
20. Andonegui G, Bonder CS, Green F, Mullaly SC, Zbytnuik L, Raharjo E, Kubes P. Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs. *J Clin Invest* 2003;111:1011–1020.
21. Maus UA, Koay MA, Delbeck T, Mack M, Ermert M, Ermert L, Blackwell TS, Christman JW, Schlondorff D, Seeger W, *et al.* Role of resident alveolar macrophages in leukocyte traffic into the alveolar air space of intact mice. *Am J Physiol Lung Cell Mol Physiol* 2002;282:L1245–L1252.
22. Sullivan GW, Carper HT, Mandell GL. The specific type IV phosphodiesterase inhibitor rolipram combined with adenosine reduces tumor necrosis factor-alpha-primed neutrophil oxidative activity. *Int J Immunopharmacol* 1995;17:793–803.
23. Walzog B, Seifert R, Zakrzewicz A, Gaehtgens P, Ley K. Cross-linking of CD18 in human neutrophils induces an increase of intracellular free Ca2+, exocytosis of azurophilic granules, quantitative up-regulation of CD18, shedding of L-selectin, and actin polymerization. *J Leukoc Biol* 1994;56:625–635.
24. Fuortes M, Jin WW, Nathan C. Adhesion-dependent protein tyrosine phosphorylation in neutrophils treated with tumor necrosis factor. *J Cell Biol* 1993;120:777–784.
25. Stockton RA, Schaefer E, Schwartz MA. P21-activated kinase regulates endothelial permeability through modulation of contractility. *J Biol Chem* 2004;279:46621–46630.
26. Reutershan J, Basit A, Galkina EV, Ley K. Sequential recruitment of neutrophils into lung and bronchoalveolar lavage fluid in LPS-induced acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L807–L815.
27. Moraes TJ, Zurawska JH, Downey GP. Neutrophil granule contents in the pathogenesis of lung injury. *Curr Opin Hematol* 2006;13:21–27.
28. Wang Q, Pfeiffer GR, Stevens T, Doerschuk CM. Lung microvascular and arterial endothelial cells differ in their responses to intercellular adhesion molecule-1 ligation. *Am J Respir Crit Care Med* 2002;166:872–877.
29. Nathan CF. Neutrophil activation on biological surfaces: massive secretion of hydrogen peroxide in response to products of macrophages and lymphocytes. *J Clin Invest* 1987;80:1550–1560.
30. Sue RD, Belperio JA, Burdick MD, Murray LA, Xue YY, Dy MC, Kwon JJ, Keane MP, Strieter RM. CXCR2 is critical to hyperoxia-induced lung injury. *J Immunol* 2004;172:3860–3868.
31. Ding J, Knaus UG, Lian JP, Bokoch GM, Badwey JA. The renaturable 69- and 63-KDa protein kinases that undergo rapid activation in chemoattractant-stimulated guinea pig neutrophils are P21-activated kinases. *J Biol Chem* 1996;271:24869–24873.
32. Zhan Q, Ge Q, Ohira T, Van DT, Badwey JA. P21-activated kinase 2 in neutrophils can be regulated by phosphorylation at multiple sites and by a variety of protein phosphatases. *J Immunol* 2003;171:3785–3793.
33. Huang R, Lian JP, Robinson D, Badwey JA. Neutrophils stimulated with a variety of chemoattractants exhibit rapid activation of P21-activated kinases (PAKS): Separate signals are required for activation and inactivation of PAKS. *Mol Cell Biol* 1998;18:7130–7138.
34. Dharmawardhane S, Brownson D, Lennartz M, Bokoch GM. Localization of P21-activated kinase 1 (PAK1) to pseudopodia, membrane ruffles, and phagocytic cups in activated human neutrophils. *J Leukoc Biol* 1999;66:521–527.
35. Usatyuk PV, Natarajan V. Regulation of reactive oxygen species-induced endothelial cell-cell and cell-matrix contacts by focal adhesion kinase and adherens junction proteins. *Am J Physiol Lung Cell Mol Physiol* 2005;289:L999–1010.
36. Wang D, Sai J, Carter G, Sachpatzidis A, Lolis E, Richmond A. PAK1 kinase is required for CXCL1-induced chemotaxis. *Biochemistry* 2002;41:7100–7107.
37. Krupa A, Kato H, Matthay MA, Kurdowska AK. Proinflammatory activity of anti-IL-8 autoantibody: IL-8 complexes in alveolar edema fluid from patients with acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2004;286:L1105–L1113.
38. Belperio JA, Keane MP, Burdick MD, Londhe V, Xue YY, Li K, Phillips RJ, Strieter RM. Critical role for CXCR2 and CXCR2 ligands during the pathogenesis of ventilator-induced lung injury. *J Clin Invest* 2002;110:1703–1716.
39. Kaneider NC, Agarwal A, Leger AJ, Kuliopulos A. Reversing systemic inflammatory response syndrome with chemokine receptor pepducins. *Nat Med* 2005;11:661–665.
40. Zhao L, Ma QL, Calon F, Harris-White ME, Yang F, Lim GP, Morihara T, Ubeda OJ, Ambegaokar S, Hansen JE, *et al.* Role of P21-activated kinase pathway defects in the cognitive deficits of alzheimer disease. *Nat Neurosci* 2006;9:234–242.

41. Rubenfeld GD, Caldwell E, Peabody E, Weaver J, Martin DP, Neff M, Stern EJ, Hudson LD. Incidence and outcomes of acute lung injury. *N Engl J Med* 2005;353:1685–1693.
42. Kurdowska A, Noble JM, Grant IS, Robertson CR, Haslett C, Donnelly SC. Anti-interleukin-8 autoantibodies in patients at risk for acute respiratory distress syndrome. *Crit Care Med* 2002;30:2335–2337.
43. Matthay MA, Zimmerman GA, Esmon C, Bhattacharya J, Coller B, Doerschuk CM, Floros J, Gimbrone MA Jr, Hoffman E, Hubmayr RD, *et al.* Future research directions in acute lung injury: summary of a National Heart, Lung, and Blood Institute Working Group. *Am J Respir Crit Care Med* 2003;167:1027–1035.
44. Adhikari N, Burns KE, Meade MO. Pharmacologic treatments for acute respiratory distress syndrome and acute lung injury: systematic review and meta-analysis. *Treat Respir Med* 2004;3:307–328.