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Polymorphonuclear Cell Transmigration Induced by 
*Pseudomonas aeruginosa* Requires the Eicosanoid Hepoxilin A3

Bryan P. Hurley,*† Dario Siccardi,*†‡ Randall J. Mrsny,† and Beth A. McCormick2*†

Lung inflammation resulting from bacterial infection of the respiratory mucosal surface in diseases such as cystic fibrosis and pneumonia contributes significantly to the pathology. A major consequence of the inflammatory response is the recruitment and accumulation of polymorphonuclear cells (PMNs) at the infection site. It is currently unclear what bacterial factors trigger this response and exactly how PMNs are directed across the epithelial barrier to the airway lumen. An in vitro model consisting of human PMNs and alveolar epithelial cells (A549) grown on inverted Transwell filters was used to determine whether bacteria are capable of inducing PMN migration across these epithelial barriers. A variety of lung pathogenic bacteria, including *Klebsiella pneumoniae*, *Escherichia coli*, and *Pseudomonas aeruginosa* are indeed capable of inducing PMN migration across A549 monolayers. This phenomenon is not mediated by LPS, but requires live bacteria infecting the apical surface. Bacterial interaction with the apical surface of A549 monolayers results in activation of epithelial responses, including the phosphorylation of ERK1/2 and secretion of the PMN chemokine IL-8. However, secretion of IL-8 in response to bacterial infection is neither necessary nor sufficient to mediate PMN transepithelial migration. Instead, PMN transepithelial migration is mediated by the eicosanoid hepoxilin A3, which is a PMN chemoattractant secreted by A549 cells in response to bacterial infection in a protein kinase C-dependent manner. These data suggest that bacterial-induced hepoxilin A3 secretion may represent a previously unrecognized inflammatory mechanism occurring within the lung epithelium during bacterial infections. *The Journal of Immunology*, 2004, 173: 5712–5720.

Diseases involving bacterial-induced lung inflammation have been described, including bacterial-associated bronchitis, pneumonia, and chronic conditions of patients having a mutation in the cystic fibrosis transmembrane conductance regulator (1–3). Several bacterial species have been implicated as causes of pneumonia, including *Streptococcus pneumoniae*, *Chlamydia pneumoniae*, *Haemophilus influenzae*, *Klebsiella pneumoniae*, *Legionella pneumophila*, *Escherichia coli*, and *Pseudomonas aeruginosa* (1, 3–5). *Pseudomonas aeruginosa* (PA) is the bacterial pathogen most associated with lung damage due to in inflammation in individuals with cystic fibrosis (2).

One of the pathogenic hallmarks of both cystic fibrosis and pneumonia is the accumulation of large numbers of neutrophils or polymorphonuclear cells (PMNs) in the lumen of the lower airway (1, 2, 6). PMNs present in the lumen can aid in eradicating offending bacteria via potent killing mechanisms, including release of proteases and reactive oxygen species. However, these mechanisms are nonspecific and can lead to lung tissue damage, which if excessive, contributes to the pathology of the disease (7).

To reach the airway lumen, PMNs are required to travel through several distinct tissue compartments within the alveolar wall (8, 9). PMNs must first escape the alveolar capillary, which involves the termination of their flow through the vessel followed by adherence to the lumen surface of the endothelium. The majority of PMNs migrate across the junctions that connect the endothelial cells. Once across the endothelial barrier, PMNs navigate the endothelial basement membrane through pre-existing holes allowing PMNs to gain access to the interstitial space. PMNs then must travel through the extracellular matrix that encompasses the interstitial space, where fibroblasts are thought to provide further directional guidance. PMNs can then interact with the epithelial basement membrane and basolateral surface of the epithelium. Finally, PMNs migrate between the epithelial cells to the apical surface of the epithelial barrier where they gain access to the airway lumen (8, 9). This process involves the integrated actions of cytokines, adhesion molecules with specificity for particular ligands as well as highly timed and compartmentalized secretion of various PMN-specific chemokines such as IL-8 (8–10). Despite significant progress in this area of study, the specific molecular mechanism governing PMN migration through each compartment to the lumen remains to be defined.

Although there are numerous reports of various bacterial products capable of inducing IL-8 secretion in several different primary and transformed airway epithelial cell lines (5, 6, 11, 12), it is unclear at present whether epithelial IL-8 production by lung epithelial cells is sufficient for mediating transepithelial PMN migration. Thus, the objective of this study was to specifically investigate the molecular mechanisms that are responsible for the final step of PMN recruitment during bacterial infection, namely, PMN transepithelial transmigration using a reductionistic in vitro model. Our approach to investigate the molecular mechanisms responsible
for a distinct phase of bacterial-mediated inflammation in the lung has revealed new insight into how bacteria interact with the lung epithelium. We have uncovered a previously unrecognized inflammatory pathway in the lung epithelium involving the secretion of the eicosanoid hepoxilin A₃ (HXA₃). HXA₃ is an arachidonic acid metabolite synthesized mainly through the actions of 12-lipoxygenases, which can induce the release of calcium from intracellular stores of various cell types, including; the stimulation of insulin secretion from pancreatic islets of Langerhans, the regulation of cell volume in platelets, and the stimulation of PMN chemotaxis (13–16). Our studies herein suggest a role for HXA₃, in the directed migration of PMNs across lung epithelial monolayers infected with pathogenic bacteria.

Materials and Methods

Growth and maintenance of epithelial cells

The A549 cell line was derived through explant culture of lung carcinomaous tissue from a 58-year-old Caucasian male. These cells display properties of type II alveolar epithelial cells and also form polarized barriers when grown on permeable filters (10, 17–19). A549 cells were maintained in Ham’s F-12K medium with 2 mM L-glutamine 1.5 g/L NaHCO₃, 10% FBS, and 100 U of penicillin/streptomycin. Polarized monolayers of A549 cells were grown and maintained on the underside of 0.33-cm² collagen-coated Transwell filters to study PMN migration in the physiological basolateral to apical direction.

PMN transmigration assay

PMNs were isolated from whole blood anticoagulated with acid citrate/dextrose obtained from healthy human volunteers. The buffy coat was obtained by a 400 × g spin at room temperature. Plasma and mononuclear cells were removed by aspiration, and the majority of the RBCs were removed using a 2% gelatin sedimentation technique. Residual RBCs were removed by lysis in cold NH₄Cl lysis buffer. This technique allows for rapid isolation of functionally active PMNs (>98%) at 90% purity (20).

Bacterial strains

The strains used in this study were all grown overnight at 37°C under aerobic conditions to a stationary growth phase. PA (PA01, PA14, PA103), the enteric commensal E. coli (F-18), and the E. coli K12 strain (MC1000) were grown in L broth. K. pneumoniae (ATCC 9590) was grown in nutrient broth. Bacteria were resuspended to a concentration of ~6 × 10⁹ bacteria/ml HBSS and diluted accordingly. LPS from E. coli and PA (100 μg/ml) was obtained from Sigma-Aldrich (St. Louis, MO).

PMN transmigration assay

The PMN transmigration assay using inverted cell culture monolayers of polarized cells has been described previously (20). Briefly, inverted monolayers were washed and equilibrated in HBSS for ~30 min. Inverted monolayers were then flipped over and treated with 25 μl of various concentrations of bacteria. Alternatively, monolayers were treated with LPS or with HBSS alone. After 1 h, all monolayers were washed and flipped back over into 24-well plates. For a positive control of the ability of PMNs to migrate, 10⁻⁸ μM fMLP was added to the bottom (apical) chamber of uninfected monolayers for each experiment. PMNs (1 × 10⁶) were then added to the top (basolateral) chamber and the plate was placed at 37°C for 2 h. PMNs that fully migrated into the apical chamber were quantified by the myeloperoxidase assay (20). Data are displayed as mean (SD) of at least three independent monolayers/condition.

Assay to assess barrier integrity

To determine whether the bacterial infection resulted in disruption of the A549 barriers, movement of the protein HRP across infected vs uninfected monolayers was compared (21). As a positive control for barrier disruption, uninfected monolayers were treated with trypsin-EDETA (T-EDETA) followed by washing and addition of HRP. As with the PMN transmigration assay, inverted monolayers were washed and equilibrated in HBSS for ~30 min. Monolayers were then flipped to expose the apical surface and treated for 1 h with HBSS alone, bacteria, or with T-EDETA. Monolayers were then washed and flipped back over into 24-well plates. HRP (0.5 μg/ml) in HBSS (100 μl) was added to the apical chamber (top) and 600 μl of HBSS was added to the basolateral chamber (bottom). After 2 h, the amount of HRP that translocated to the basolateral chamber was quantified by a HRP activity assay.

Cell viability assay

To determine whether the bacterial infection resulted in toxicity to the A549 monolayers, release of the enzyme lactate dehydrogenase (LDH) into the supernatant of monolayers under infected conditions was compared with uninfected monolayers. As a positive control for cell death, Triton X-100 was added to monolayers. As with the PMN transmigration assay, inverted monolayers were washed and equilibrated in HBSS for ~30 min. Monolayers were then flipped to expose the apical surface and treated for 1 h with HBSS alone or bacteria, washed, and flipped back over into 24-well plates containing either HBSS or Triton X-100. After 2 h, the amount of LDH that has been released into the apical and basolateral chambers was quantified using the LDH assay (Sigma-Aldrich).

IL-8 assay

Inverted monolayers were washed and equilibrated in HBSS for ~30 min, flipped over, and treated with either 2 × 10⁶ PA01/monolayer (low concentration) or 2 × 10⁹ E. coli/monolayer (high concentration) or with HBSS alone. After 1 h, all monolayers were washed and flipped back over into 24-well plates and 100 μl of HBSS was added to the top (basolateral) chamber and 600 μl of HBSS was added to the bottom (apical) chamber. After 2 h, supernatants in the apical and basolateral chambers were collected and filtered (0.2 μM) before performing the IL-8 ELISA (Pierce/Endogen, Rockford, IL) (22).

Detection of ERK1/2 activation

A549 monolayers seeded on 1.5-cm² permeable filters were infected with PA01 [low] or E. coli F-18 [high] on the apical surface for various times. Cells were treated with lysis buffer which includes 1% Triton X-100, 100 mM NaCl, 10 mM HEPES (pH 7.6), 2 mM EDTA, 200 mM PMSF, 4 mM Na₃VO₄, 40 mM NaF, and 1 Complete Mini protease inhibitor mixture tablet/10 ml lysis buffer (Roche Diagnostics, Mannheim, Germany). Cells were then scraped from the permeable filter and passed through a 25-gauge needle, followed by centrifugation (14,000 rpm at 4°C for 30 min). The supernatant represents the Triton X-100-soluble fraction and protein concentration of each supernatant was measured by the D. Protein Assay (Bio-Rad, Hercules, CA). Lysates were normalized for protein concentration and ~30 μg was run on an 8–16% gradient polyacrylamide gel and transferred to nitrocellulose. Blots were probed with either an anti-ERK1 Ab or anti-phospho-ERK1/2 Ab (Cell Signaling, Beverly, MA) followed by incubation with the appropriate HRP-conjugated goat anti-species Ab and detection with ECL reagent (Pierce/Endogen, Rockford, IL) (23).

Detection of protein kinase C (PKC) activation

A cell fractionation protocol was used to obtain membrane-soluble proteins, as PKC accumulates in the membrane upon activation (24, 25). A549 monolayers seeded on 1.5-cm² permeable filters were infected with PA01 [low] or E. coli F-18 [high] on the apical surface for various times. Cells were scraped off of permeable filters in buffer containing 150 mM Tris (pH 8.0), 15 mM EDTA, 6 mM EGTA, 200 mM PMSF, 4 mM Na₃VO₄, 40 mM NaF, and 1 Complete Mini protease inhibitor mixture tablet/10 ml buffer. Scraped cells were subjected to sonication followed by centrifugation at 55,000 rpm for 1 h. Cell pellet was resuspended in a lysis buffer containing 0.1% Triton X-100, 0.2% SDS, 50 mM Tris (pH 8.0), 5 mM EDTA, 2 mM EGTA, 200 mM PMSF, 4 mM Na₃VO₄, 40 mM NaF, and 1 Complete Mini protease inhibitor mixture tablet/10 ml buffer. Lysates were again subjected to sonication followed by centrifugation at 30,000 rpm for 10 min. Supernatant was collected and concentrated using Centricon filters with a Mₘ cut-off of 30,000. Lysates were normalized for protein concentration and run on an 8–16% gradient polyacrylamide gel followed by transference to nitrocellulose. Blots were probed with an anti-phospho-pan PKC Ab (Cell Signaling) followed by incubation with HRP-conjugated goat anti-rabbit Ab and detection with ECL reagent (Pierce/Endogen) (24, 25).

Drug treatments

For drug treatment experiments, cells were pretreated for either 1 h with chelerythrine chloride (CCL), U0126, caffeic acid, or cinnamyl-3,4-dihydroxy-a-cyanocinnamate (CDC; BIOMOL, Plymouth Meeting, PA) or for 18 h with cycloheximide (Sigma-Aldrich) before infection. The drug concentration was maintained during infection, but was removed by washing three times before addition of PMNs. Each drug at the doses presented in this study had minimal effect on cell viability both in the presence or
absence of bacterial infection, as assessed by the barrier integrity assay described above. Also, none of these drugs had any major effect on the amount of bacteria adhering to the A549 monolayers.

Detection of HXA₃
A549 monolayers seeded on 1.5-cm² permeable filters were infected with PA01 in the presence or absence of 50 μM CDC. After 1 h, A549 cells were washed three times and HBSS was added. After 2 h, conditioned HBSS was collected and placed at −80°C. A method for the detection and quantification of HXA₃ in cell supernatants has been recently described (15).

Pathogenic bacteria induce PMN transepithelial migration
As a model for the airway epithelium, we chose the A549 cell line. A549 cells are alveolar epithelial cells that are capable of forming polarized barriers and are widely used as a model of the airway epithelial surface. In addition, these cells have been used extensively for PMN transepithelial migration studies (10). A549 cells are grown on the underside of Transwell permeable supports (Millipore, Bedford, MA) fitted with a 2000-Da cutoff filtration apparatus. Filtrate components were size fractionated by hydrophobic surface chromatography using methanol elution. The methanol fraction was dried under vacuum and injected onto a Vydac C18 (10 μm; 300 Å) semi-preparative column (10 × 250 cm). A methanol gradient of 1–10% over 10 min, then 10–60% over 25 min, followed by 60–100% over 45 min was used to isolate HXA₃ fractions having no detectable absorbance at 280 nm and weak absorbance at 214 nm. Samples were analyzed using a Genesis C18 (4 mm, 120 Å) analytical HPLC column (4.6 × 150 mm) equilibrated with 5 mM triethylamine/acetic acid (pH 7.2). Samples were chromatographed using a linear methanol gradient of 0–100% over 60 min and analyzed using a ThermoFinnigan LCQDeca HPLC/electrospray mass spectrometer set in the negative ion mode. Compounds were characterized for retention time, UV spectra (UV6000LP photodiode array detector), and m/z signals (15).

Results
Pathogenic bacteria induce PMN transepithelial migration
As a model for the airway epithelium, we chose the A549 cell line. A549 cells are alveolar epithelial cells that are capable of forming polarized barriers and are widely used as a model of the airway epithelial surface. In addition, these cells have been used extensively for PMN transepithelial migration studies (10). A549 cells are grown on the underside of Transwell permeable supports where they form cellular barriers as described in Materials and Methods. To investigate whether respiratory pathogens can induce PMN transepithelial migration across model lung epithelium, we selected bacterial strains that have been associated with lung disease involving inflammation, including, PA as well as two species of Gram-negative commensal enteric bacteria, E. coli (F-18) and K. pneumoniae (1–3). We also selected a laboratory K-12 E. coli strain (MC1000), which has distinct properties from natural human colonizing bacteria and might potentially provide some insight into the specificity of the bacterial-induced transepithelial migration response. We infected A549 monolayers with [high] (2 × 10⁸ bacteria/monolayer) and observed a potent PMN transmigration response to both species of Gram-negative enteric bacteria tested (Fig. 1A). The response to MC1000 was minimal and similar to uninfected monolayers (HBSS). Surprisingly, the PA strains (PA01, PA14, and PA103) had a weak response. To determine the concentration where E. coli F-18 and K. pneumoniae lose the ability to induce PMN transmigration, we performed dose-response studies. As shown in Fig. 1B, E. coli F-18 promoted PMN transmigration only at [high], as F-18 was unable to induce PMN transmigration at or below a concentration of 1 × 10⁷ bacteria/monolayer. In contrast, K. pneumoniae had a much larger concentration range of effective response, with an equally potent PMN transmigration response occurring over several orders of magnitude. The K12 E. coli MC1000 had little or no response at all concentrations tested (Fig. 1B). Despite the fact that the PA strains were incapable of inducing PMN transmigration at [high], we observed that these strains did, in fact, stimulate a robust response at lower concentrations (2 × 10⁶–1 × 10⁷ bacteria/monolayer; Fig. 1, B and C). The lack of a PMN transmigration response at [high] of PA strains did not appear to be the consequence of PA toxicity to the monolayer. We assessed A549 barrier integrity as well as A549 cell viability and even at the [high] (2 × 10⁸ bacteria/monolayer) of infection, none of the strains resulted in any significant barrier disruption as measured by HRP flux across the monolayers or cell death as assessed by LDH release (Table I). Although, it is currently unclear why [high] of PA strains fail to induce the PMN transmigration response, such data suggest that several bacterial species capable of causing lung inflammation and disease induce PMN transmigration across lung epithelial cells in this in vitro model.

We next sought to determine the host-pathogen interactions critical for the PMN transmigration response. Induction of the PMN transmigration response required live bacteria interacting with the apical surface, since there was no detectable response to treatment of the apical surface of A549 monolayers with heat-killed bacteria (Fig. 2A). We also observed that purified LPS from PA or E. coli was incapable of inducing any degree of PMN transmigration. Although bacterial fMLP is a potent PMN chemoattractant, it does not appear to be involved in the bacterial-induced PMN transmigration response reported herein, since a specific antagonist of fMLP, tBOC, was unable to prevent bacterial-induced migration, while effectively blocking transmigration (96% inhibition) in response to artificially imposed fMLP gradients across A549 monolayers. Furthermore, A549 cells pretreated with the eukaryotic protein synthesis inhibitor cycloheximide were markedly reduced (50%) in facilitating PMN transmigration in response to bacterial infection, suggesting that A549 epithelial monolayers are playing
Pathogenic bacteria induce IL-8 secretion

IL-8 is a well-known PMN chemoattractant that is secreted by the epithelial cells of several mucosal surfaces upon interaction with bacteria (5, 11, 20, 21). The signaling cascade leading to IL-8 secretion has been well studied and thought to represent an innate response by mucosal surfaces upon the sensing of infection (2, 26, 27). The signaling cascade is initiated by epithelial cell recognition of pattern motifs presented by ubiquitous bacterial surface products such as LPS or the bacterial protein flagellin through TLRs (12, 26–28). Such specific recognition is followed by signaling through mitogen-activated protein (MAP) kinases and NFκB leading to the epithelial secretion of IL-8 (2, 26–28). As shown in Fig. 2, B and D, PA01 induces a significant increase in IL-8 secretion compared with untreated A549 monolayers (HBSS). IL-8 depicted in Fig. 2, B and D, represents the quantity of IL-8 recovered in the basolateral compartment, since the quantity of IL-8 in the apical compartment was undetectable. Unlike results observed with the PMN transmigration response (Fig. 2A), heat-killed PA01 displayed a comparable, if not greater, IL-8 secretion response compared with infection with live bacteria (Fig. 2B). This indicates that the bacterial stimuli inducing the IL-8 response differ from the stimuli responsible for inducing the PMN transmigration response. In addition, the IL-8 secretion response occurred to a similar degree with either [high] or [low] PA01 infection (Fig. 2D), whereas the PMN transmigration response only occurred with [low] PA01 infection (Fig. 2C). Taken together, these data indicate that bacterial-induced secretion of IL-8 from A549 cells is not sufficient to mediate PMN transmigration.

Pathogenic bacteria induce ERK1/2 phosphorylation

To further understand signaling events involved in bacterial interaction with the apical surface of A549 monolayers that may lead to IL-8 production, we investigated whether PA01 and/or *E. coli* F-18 activates the MAP/ERK pathway. The MAP/ERK pathway is comprised of several serine/threonine kinases involved in the regulation of a wide range of cellular responses including the production of proinflammatory molecules such as IL-8 (29). ERK1 and 2 kinases are phosphorylated by activated MEK1/2 as a consequence of the phosphorylation cascade of the active MAP/ERK pathway (29). For these studies, we selected concentrations of the respective bacteria that are capable of inducing PMN transmigration, i.e., [low] PA01 and [high] *E. coli* F-18. PA01 (2 × 10^6 bacteria/monolayer) and F-18 (2 × 10^6 bacteria/monolayer) infection of A549 monolayers both result in an increase in the phosphorylated form of ERK1 and 2 within A549 cells compared with untreated (HBSS) A549 monolayers (Fig. 3), suggesting that the MAP/ERK pathway is activated upon infection (29). The amount of total ERK1 protein is unchanged in response to infection.

### Table I. Effect of bacterial infection of A549 monolayers on barrier integrity and cell viability

<table>
<thead>
<tr>
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<th>Barrier Integrity^a</th>
<th>Cell Viability^b</th>
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<tbody>
<tr>
<td>HBSS</td>
<td>30 (1)</td>
<td>ND^g</td>
</tr>
<tr>
<td>T-EDTA</td>
<td>210 (75)</td>
<td>ND</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>24 (12)</td>
<td>34 (8)</td>
</tr>
<tr>
<td>PA01</td>
<td>24 (8)</td>
<td>30 (9)</td>
</tr>
<tr>
<td>Klebsiella</td>
<td>30 (6)</td>
<td>25 (6)</td>
</tr>
<tr>
<td>F-18</td>
<td>30 (26)</td>
<td>26 (7)</td>
</tr>
</tbody>
</table>

^a Barrier integrity is assessed by the amount (nanograms per milliliter) of HRP (MW 45 kDa) that crosses the A549 monolayers in 2 h following treatment with the conditions listed above. Monolayers were treated with T-EDTA as a positive control to intentionally disrupt the barrier integrity of the monolayer. There is no significant difference among uninfected, untreated monolayers (HBSS), and all infected monolayers. All conditions were performed in triplicate with SDs reported in parentheses. Each experiment was performed at least twice.

^b Cell viability was assessed using the LDH assay. Values are reported as percent release of LDH into the supernatant as a percentage of total LDH (cell associated plus released in supernatant). Monolayers were treated with Triton X-100 as a positive control to intentionally disrupt A549 cell viability. There is no significant difference among uninfected, untreated monolayers (HBSS), and all infected monolayers. All conditions were performed in triplicate with SDs reported in parentheses. Each experiment was performed at least twice.

^c [high] refers to 2 × 10^8 bacterial/monolayer.

^d [low] refers to 2 × 10^6 bacterial/monolayer.

^e ND, Not done.

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**FIGURE 2.** PMN transmigration vs IL-8 secretion. A549 monolayers are treated with buffer alone (HBSS), 2 × 10^6 bacteria/monolayer [low] of PA01, or an equivalent concentration of PA01 that has been heat killed (20 min. at 70°C), and monolayers are assessed for either PMN transmigration (A) or IL-8 secretion (B). A549 monolayers are treated with buffer alone (HBSS), 2 × 10^6 bacteria/monolayer [high], or 2 × 10^6 bacteria/monolayer [low] of PA01 and monolayers are assessed for either PMN transmigration (C) or IL-8 secretion (D). Each condition was performed in triplicate and data presented are a representative figure of an experiment performed at least twice.
MAP/ERK inhibitors do not block bacterial-induced PMN transmigration

Since the evidence above suggests that the MAP/ERK pathway is activated in response to bacterial infection, we next sought to determine whether this signaling pathway contributes to either the IL-8 response or the PMN transmigration response described above. We treated A549 monolayers with the MEK1/2-specific inhibitor U0126 (29) before infection with either F-18 or PA01: one set of experiments was assayed for IL-8 secretion and another was assayed for PMN transmigration. U0126 (20–40 μM) efficiently reduces the PA01-and F-18-stimulated IL-8 secretion by 50–75% (Fig. 4A). This result was not surprising because IL-8 secretion from many cell types has been shown to be dependent on the MAP/ERK pathway (29). However, at doses of U0126, which efficiently blocked the IL-8 response, there was no significant effect on PMN transmigration (Fig. 4B). The MEK1-specific inhibitor PD98059 was also capable of blocking IL-8 production while having no significant effect on the PMN transmigration response (data not shown). These results suggest that bacterial-induced IL-8 secretion appears to be an unnecessary event for the bacterial-induced PMN transepithelial migration in vitro. This notion is further substantiated by the fact that the addition of neutralizing IL-8 Abs failed to inhibit bacterial-induced PMN transmigration (data not shown). In addition, the majority of the IL-8 secreted by A549 monolayers is recovered in the basolateral chamber, which is counter to the direction of the PMN migration. Taking together all of the above data, we hypothesize that although IL-8 is produced in response to bacterial infection, IL-8 is not responsible for the specific step of transepithelial migration of PMNs to the airway lumen. Rather IL-8 is more likely required for the recruitment of PMNs from the blood stream through the interstitial space (5, 8, 20, 22). Hence, PMN transepithelial migration must involve a distinct PMN chemoattractant.

PKC inhibitors block bacterial-induced PMN transmigration

Previous studies from our laboratory have reported that PKC activity is required for bacterial-induced PMN transmigration across intestinal epithelial cells (25). Using the PKC inhibitor CCL (30), we show in Fig. 5A that CCL pretreatment of A549 cells nearly completely ablates PA01 and significantly reduces F-18-induced PMN transmigration. Furthermore, PMN transmigration in response to an imposed fMLP gradient is unaffected by CCL pretreatment of A549 cells, demonstrating that the drug pretreatment of A549 cells does not nonspecifically interfere with the ability of PMNs to migrate across the monolayer (data not shown). Also, CCL treatment at the same doses had no effect on the ability of PA01 or F-18 to induce ERK1/2 phosphorylation or IL-8 secretion from A549 cells (data not shown and Fig. 5B). These data suggest that PKC activity within A549 cells is necessary for the bacterial-induced PMN transmigration response.

Since PKC activity appeared to play a significant role in bacterial-induced PMN transmigration, we next sought to determine whether PKC is activated in A549 cells in response to bacterial infection. We treated A549 monolayers with either buffer alone (HBSS), 1 μM PMA as a positive control, or with PA01 for 15–45
min. After treatment, cells are lysed and separated into cytoplasmic and membrane fractions (20). Previous studies have shown that upon activation, PKC becomes phosphorylated and relocates to the cell membrane (20, 21). Fig. 6 depicts the amount of phosphorylated PKC in the cellular membrane fraction of A549 cells treated under various conditions and clearly demonstrates that infection with PA01 results in an increase in the amount of phosphorylated PKC in the membrane fraction compared with uninfected monolayers.

**Bacterial-induced PMN transmigration involves HXA₃**

Since PKC is activated within A549 cells upon infection with PA01 and since PKC activity appears to be involved in the process of PA01-induced PMN transmigration, the mechanism mediating bacterial-induced PMN transmigration across lung epithelial cells displays similarities to bacterial-induced PMN transmigration across intestinal epithelial cells (15, 25). A recent report from our group has identified a PKC-dependent PMN chemoattractant produced by intestinal epithelial cells in response to Salmonella typhimurium infection that is responsible for mediating PMN transepithelial migration (15). This molecule, previously termed pathogen-elicited epithelial chemoattractant, was identified as HXA₃ (15). HXA₃ is an eicosanoid metabolized from arachidonic acid (13). The enzyme that is largely responsible for the synthesis of HXA₃ from arachidonic acid is 12-lipoxygenase (12-LO). 12-LO converts arachidonic acid to 12-hydroxyperoxyeicosatetraenoic acid (12-HpETE), which can then be converted to HXA₃ (13). We therefore investigated whether bacterial-induced PMN transmigration across the lung epithelial cells requires 12-LO activity. Pretreatment of A549 monolayers with CDC, a 12-LO-specific inhibitor (31), significantly blocked PMN transmigration induced by bacteria, but did not significantly interfere with PMN migration in response to a fMLP gradient (Fig. 7A and data not shown). An inhibitor of the 5-lipoxygenase (5-LO) pathway, caffeic acid (32), which converts arachidonic acid into leukotrienes rather than hepoxilins is unable to block bacterial-induced PMN transmigration (Fig. 7B). These data suggest that the 12-LO enzymatic pathway, which is the major synthetic pathway of HXA₃, is involved in the process of bacterial-induced PMN transmigration across lung epithelial cells.

To determine whether A549 cells secrete HXA₃, we analyzed supernatants from the apical surface of polarized monolayers of A549 cells for the presence of HXA₃ using HPLC separation followed by mass spectrometry as described in Materials and Methods (15). A549 monolayers incubated in buffer alone did not produce any detectable HXA₃ (<1.0 pmol). In response to infection with PA01, however, A549 cells produced ~210 pmol HXA₃. Secretion of HXA₃ was prevented by pretreating A549 cells with the 12-LO inhibitor CDC (<1.0 pmol).

We next verified that HXA₃ acts as a PMN chemoattractant across the A549 barrier; we added commercially available HXA₃ (50 ng/ml; Calbiochem, San Diego, CA) to the apical chamber of A549 monolayers with PMNs simultaneously placed in the basolateral chamber. Of the total number of PMNs added to the apical surface, 18.4 ± 5.1% migrated to the apical chamber in response to a gradient of HXA₃. A different arachidonic acid metabolite lipoxin A₄ (50 ng/ml) placed apically is incapable of directing PMNs across the epithelium as the amount of PMNs migrating to the apical chamber (1.6 ± 0.3%) is different from the number of spontaneously migrating PMNs, where no eicosanoid gradient is added (1.2 ± 0.1). Based on this data, we hypothesize that A549 cells release HXA₃ in response to bacterial infection and this molecule is capable of directing PMN transepithelial migration.

**Discussion**

Bacterial species such as *E. coli*, *K. pneumoniae*, and PA present in the airway are capable of causing severe inflammation in diseases such as pneumonia and cystic fibrosis (1–3). One aspect of the inflammatory process involves the infiltration of lung tissue by neutrophils (2, 5, 6, 8, 33). Neutrophils are capable of crossing both the endothelial and epithelial barriers in response to an infection in the airspace (8). In this study, we have modeled the directed migration of neutrophils across lung epithelial monolayers; a distinct phase of the complex neutrophil recruitment process that occurs during lung inflammation. We have shown that several bacterial species stimulate PMN transmigration across lung epithelial cell monolayers. Each bacterial strain we investigated in this study displays a distinct profile in terms of concentration required for induction of transmigration. For example, the intestinal commensal *E. coli* (F-18) induces a PMN transmigration response only at [high] infection (2 × 10⁸ bacteria/monolayer), whereas the intestinal commensal *K. pneumoniae* results in a robust PMN transmigration not only at 2 × 10⁸ bacteria/monolayer, but also at lower concentrations over several orders of magnitude. A large response to *K. pneumoniae* occurs with as little as 100,000 bacteria/monolayer. The K12 *E. coli* strain (MC1000), which is less likely to be pathogenic in the lung, is not capable of inducing substantial PMN transmigration at any concentration tested.
In contrast to *E. coli* and *K. pneumoniae*, PA strains display a unique feature in which a productive PMN transmigration response occurs with a bell curve distribution over a range of infection concentrations. At higher concentrations (2 × 10^9 bacteria/monolayer), the PMN transmigration response is minimal. In contrast, lower concentrations of ~1 × 10^5–1 × 10^6 bacteria/monolayer results in substantial PMN transmigration. Below a concentration of ~1 × 10^6 bacteria/monolayer, the PMN transmigration response is diminished. These data imply that there is something unique about the interaction of higher concentrations of PA with the epithelial monolayer that renders them incapable of facilitating PMN transepithelial migration. This result cannot be explained simply by destruction of A549 cell barriers due to infection with these [high] PA, since barrier properties and cell viability remained intact under these infection conditions. Several possibilities might explain this result. For example, PA can participate in quorum sensing, a process in which the density of organisms in a given population can alter the behavior of individual organisms within the population (34). Also, *Pseudomonas* species are capable of forming biofilms when enough bacteria are present, and these structures can interact with various surfaces in a unique manner when compared with interactions by unorganized populations of bacteria (35). Another possibility is that PA, in contrast to *E. coli* or *K. pneumoniae*, has the capacity to constitutively produce a substance (i.e., inhibitor) that interferes with induction of the PMN migration process and is only effective if enough PA is present to allow the accumulation of the appropriate amount of that substance. Future experiments will determine the mechanism underlying the inability of [high] PA to promote PMN transmigration.

Irrespective of the unique profiles of individual bacterial strains and species with respect to the concentration required to induce PMN transmigration, it is clear that several different species that are relevant to lung inflammation demonstrate the ability to induce PMN transmigration across lung epithelial cells. This observation led to the question of how this process is orchestrated mechanistically. Several reports have been published demonstrating that PA and/or products of PA are capable of initiating the secretion of the PMN chemokine IL-8 (2, 6, 11, 12). This phenomenon is believed to be important in the PMN recruitment process. Mechanistic studies of bacterial-induced PMN transepithelial migration response described herein have revealed that although the A549 monolayers produce IL-8 in response to bacterial infection, it does not appear to be responsible for mediating the transepithelial migration of the PMNs. This conclusion is based on several observations. First, the majority of IL-8 produced in response to PA01 is secreted into the basolateral chamber of the A549 monolayers, counter to the direction of PMN migration. Second, neutralizing Abs to IL-8 have no effect on bacterial-induced PMN transmigration. This result is consistent with a report of the inability of IL-8-neutralizing Abs to prevent *C. pneumoniae*-induced PMN transmigration across primary human airway epithelial cells (5). Also calling into question the role for IL-8 in mediating PMN transepithelial migration is the fact that heat killing PA before the addition to A549 monolayers results in an IL-8 response that is equal to, if not greater than, live PA infection, whereas heat-killed PA are completely unable to induce PMN transepithelial migration. In addition, PA stimulates significant IL-8 production at [high] despite the fact that [high] PA are not capable of facilitating PMN transepithelial migration. The IL-8 response due to infection with PA is mediated in part by the MAPK pathway ERK1/2. Inhibitors that sufficiently block the ERK1/2 pathway result in a 50–75% reduction in the amount of IL-8 produced in response to PA when A549 monolayers are pretreated with such inhibitors. ERK1/2 pathway inhibitors do not interfere with the PMN transmigration response. Finally, we have shown that inhibitors of PKC ablate bacterial-induced PMN transmigration, but have no effect on IL-8 production. Based on this evidence, IL-8 does not appear to contribute to PMN migration across infected lung epithelial monolayers. Since IL-8 does contribute to overall PMN recruitment during lung infection and inflammation (8, 9, 10, 33), we hypothesize that the primary role played by IL-8 during PMN infiltration of lung tissue may be to guide PMNs through the interstitium up to the epithelium, rather than guiding PMNs across the epithelium into the airspace (15, 20). This assertion is based on the fact that epithelial cells produce IL-8 in response to bacterial infection predominantly from the basolateral surface of the A549 monolayers.

Previous studies from our group have demonstrated that PMN migration across intestinal epithelial cell monolayers infected with *S. typhimurium* requires PKC (25). Furthermore, we have recently demonstrated that the chemottractant responsible for *S. typhimurium*-induced PMN migration across intestinal epithelial monolayers is the eicosanoid HXA₃, and this molecule is produced and/or secreted in a PKC-dependent manner (15). This arachidonic acid metabolite is one of the products of the 12-LO pathway and has been previously shown to stimulate Ca²⁺ release within PMNs as well as to possess PMN chemotactic activity (13, 16). Likewise, we demonstrate in this report that PA infection of A549 cells also results in activation of this novel signaling pathway PKC is activated within A549 monolayers in response to PA. An inhibitor of the PKC pathway completely blocked bacterial-induced PMN transmigration, suggesting that PKC is involved in mediating this process. Moreover, analogous to infected intestinal epithelial cells, A549 lung epithelial cells are capable of producing HXA₃ in response to bacterial infection. Pretreatment of A549 cells with a specific inhibitor of the 12-LO pathway before infection with bacteria resulted in both a reduction in PMN transmigration as well as a reduction in the amount of HXA₃ secreted by the A549 monolayers. In addition, HXA₃ acts as a chemottractant for PMNs when a gradient is established across A549 monolayers. Thus, lung epithelial cells produce HXA₃ in response to bacterial infection and this PMN chemottractant is responsible for guiding PMNs across the lung epithelium. At present it is unclear whether signaling through PKC is important to initiate production of HXA₃ and/or in orchestrating secretion of HXA₃ from the apical surface of A549 monolayers. Thus, future experiments will further delineate the role of PKC as well as other details of HXA₃ production and secretion. In this regard, it will be important to determine whether any of the 12-LO enzymes are specifically activated by infection. There are at least four distinct genes that encode 12-LO, some of which are expressed in epithelial cells (36, 37). These include platelet-type 12-LO, leukocyte-type 12-LO, epidermal-type 12-LO, and 12-(R)-LO (36). In addition, 15-lipoxygenase 1 is capable of synthesizing 12-HETE as a minor product and has been shown to be expressed in A549 cells in response to IL-4 and IL-13 (38). Alternatively, bacterial infection could simply stimulate the production of increased amounts of HXA₃ precursors such as arachidonic acid, thus providing more substrate for a constitutively active 12-LO enzyme. It is also plausible that both an increase in 12-LO activity and an increase in the amount of available 12-LO substrate may occur in conjunction.

It is noteworthy to mention that this novel inflammatory pathway involving PKC, 12-LO, and the production and secretion of HXA₃ appears to occur at multiple mucosal sites (i.e., intestine and lung) (15). Inhibition of the 12-LO pathway during *S. typhimurium* infection of human intestinal xenografts results in a dramatic reduction in inflammation and subsequent tissue destruction demonstrating the importance of this pathway in a more complex disease.
model (15). This pathway may represent a conserved innate immune mechanism for the detection and eradication of pathogens interfacing with host mucosal surfaces. Intestinal epithelial cells are fairly restrictive in terms of the bacterial stimulus needed to trigger HXA3 production. *S. typhimurium*, through its effector molecule, SipA, activates HXA3 secretion whereas commensal *E. coli* strains such as F-18 (which does not possess SipA) are incapable of stimulating PMN transmigration across intestinal monolayers (39). Initiation of PMN transmigration across lung epithelial cells appears to be more promiscuous as several bacterial species (i.e., *E. coli*, *K. pneumoniae*, and PA) all are capable of inducing this phenomenon. PMN transmigration across lung epithelial cells has also been shown to occur in response to *C. pneumoniae* (5). The intestinal mucosa is colonized by a plethora of commensal bacterial organisms, yet does not exist in a constant state of inflammation, whereas the lower airway epithelium is not generally considered to be chronically colonized with bacteria except under pathological conditions (1, 40). Thus, lung epithelial cells may react to the presence of bacteria in a more indiscernible manner or there could be dampening responses that occur in the gut (26). Future studies will determine whether *E. coli*, *K. pneumoniae*, and PA share a bacterial trigger for HXA3 production or whether they possess discrete factor(s) which harness this or perhaps other pathways leading to PMN transepithelial migration. Our data thus far suggest that at least the PA strain PA01 and the commensal *E. coli* strain F-18, albeit at different concentrations of infection, initiate a common host cell pathway to promote PMN transepithelial migration.

A recent review by Burns et al. (8) provides a very thorough and informative breakdown of issues governing the overall PMN recruitment process from the capillaries to the airway lumen, which involves transendothelial transmigration, transmural migration, migration through the interstitium adjacent to fibroblasts, and transepithelial transmigration. Our approach to construct a reductionist model system for a distinct phase in the PMN recruitment process (transepithelial migration) has resulted in the identification of a novel inflammatory pathway, which now provides a testable hypothesis in unraveling the complex multifactorial mechanism that occurs during bacterial-induced PMN recruitment to the airway. Additional experiments using alternative in vitro cell culture models and more complex in vivo models will be performed to address both the prevalence of the HXA3 secretion process as well as to ascertain the specific anatomic location of action for both IL-8 and HXA3 in mediating PMN recruitment during the disease process.

In summary, we have presented evidence for a novel inflammatory pathway that occurs within lung epithelial cells. Bacterial interaction with airway epithelial cells activates PKC and results in the production and secretion of HXA3, likely through the action of the 12-LO enzymatic pathway. Future studies will be geared to construct a reductionist model system and more complex in vivo models will be performed to further elucidate the complex multifactored mechanism that occurs during bacterial-induced PMN recruitment to the airway epithelial monolayers. Our approach to construct a reductionist model system for a distinct phase in the PMN recruitment process (transepithelial migration) has resulted in the identification of a novel inflammatory pathway, which now provides a testable hypothesis in unraveling the complex multifactored mechanism that occurs during bacterial-induced PMN recruitment to the airway.

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