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Hepoxilin A_3 Facilitates Neutrophilic Breach of Lipoxygenase-expressing Airway Epithelial Barriers[1]

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Abstract

A feature shared by many inflammatory lung diseases is excessive neutrophilic infiltration. Neutrophil homing to airspaces involve multiple factors produced by several distinct cell types. Hepoxilin A_3 is a neutrophil chemo-attractant produced by pathogen infected epithelial cells hypothesized to facilitate neutrophil breach of mucosal barriers. Using a Transwell model of lung epithelial barriers infected with *P. aeruginosa*, we explored the role of hepoxilin A_3 in neutrophil trans-epithelial migration. Pharmacological inhibitors of enzymatic pathways necessary to generate hepoxilin A_3, including phospholipase A_2 and 12-lipoxygenase, potently interfere with *P. aeruginosa*-induced neutrophil trans-epithelial migration. Both transformed and primary human lung epithelial cells infected with *P. aeruginosa* generate hepoxilin A_3 precursor arachidonic acid. All four known lipoxygenase enzymes capable of synthesizing hepoxilin A_3 are expressed in lung epithelial cell lines, primary small airway epithelial cells, and human bronchial epithelial cells. Lung epithelial cells produce increased hepoxilin A_3 and lipid derived neutrophil chemotactic activity in response to *P. aeruginosa* infection. Lipid derived chemotactic activity is soluble epoxide hydrolase sensitive, consistent with hepoxilin A_3 serving a chemotactic role. Stable inhibitory structural analogues of hepoxilin A_3 are capable of impeding *P. aeruginosa*-induced neutrophil trans-epithelial migration. Finally, intranasal infection of mice with *P. aeruginosa* promotes enhanced cellular infiltrate into the airspace as well as increased concentration of the 12-lipoxygenase metabolites hepoxilin A_3 and 12-HETE. Data generated from multiple models herein

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provide further evidence that hepoxilin A_3 is produced in response to lung pathogenic bacteria and functions to drive neutrophils across epithelial barriers.

Keywords

Neutrophils; Bacterial; Lipid Mediators; Chemotaxis; Inflammation; & Lung

Introduction

Infectious lung disease is a significant global health problem, and represents a particularly difficult challenge as antibiotic resistant pathogens emerge (1–4). During acute pneumonia, there is a rapid influx of innate immune cells to the lungs where they release caustic molecules that result in gross tissue damage (3, 5). Neutrophils are among the first responders in innate immunity, rapidly deployed to sites of infection to confront a variety of pathogens (5–7). Without appropriate resolution, however, neutrophils can exacerbate pathology during infectious and idiopathic inflammatory processes, such as the case in both acute and chronic lung diseases including pneumonia, cystic fibrosis, and severe bouts of asthma (3, 6–8).

To reach the airspace during infection, neutrophils must exit the circulatory system of the lung, navigate through the extracellular milieu, and ultimately cross the mucosal epithelial barrier. Evidence points to a tiered signaling hierarchy that mediates neutrophil adhesion, initial migration, deep tissue homing, and entry into organ spaces (9, 10). In this report, we examine the process by which neutrophils migrate across lung epithelial barriers. Our group previously identified the eicosanoid hepoxilin A_3 (HXA_3) as a potent chemoattractant that drives neutrophil migration across gut and lung epithelial barriers (11–15). HXA_3 likely interacts directly with neutrophil receptors and induces chemotaxis without granule secretion or superoxide production (15–17).

A diverse array of biological function are exhibited by the group of lipid mediators known as eicosanoids, particularly during inflammatory processes (18). Eicosanoids such as HXA_3 are generated through the liberation of arachidonic acid (ARA) from membrane phospholipids catalyzed by phospholipase A_2 (PLA_2) (16). Lipoxygenases represent an enzyme family that converts ARA into a discrete subset of bioactive lipid eicosanoids (19). The 12-lipoxygenase enzymatic activity (as defined by the site of oxygen addition to ARA) is a prerequisite for HXA_3 synthesis, and is known to be exhibited by at least three lipoxygenase proteins identified in humans (16, 20, 21). The catalytic efficiency, however, varies between each enzyme and potentially between tissues where enzymes are expressed. Three human genes that encode enzymes with 12-lipoxygenase activity include *alox12*, *alox15*, and *alox12B* encoding 12-LO, 15-LO, and 12(R)-LO respectively (19, 22–25).

In the current study, we examined the prevalence of pathogen-induced, HXA_3-mediated PMN trans-epithelial migration in a variety of lung models. We employed multiple human lung epithelial carcinoma cell lines, normal bronchial epithelial cells transformed by adenovirus, primary lung epithelial cells, and an *in vivo* mouse model of lung infection to explore the role of HXA_3 in mediating PMN trans-epithelial migration. We analyzed lung epithelial cell models for expression of 12-lipoxygenases and evaluated the efficacy with which multiple distinct strategies to target HXA_3 impact pathogen induced PMN trans-epithelial migration. We also explored whether mice produce 12-lipoxygenase products in the lung when infected with pathogen. Our studies herein provide further evidence for the hypothesis that HXA_3 mediates PMN trans-epithelial migration in response to infection and likely contributes to PMN infiltration of the airspace.

Materials and Methods

Bacterial Strains

P. aeruginosa strain PAO1 and non-pathogenic *E. coli* K12 strain MC1000 were grown aerobically in LB-broth overnight at 37°C. For infection of epithelial cells, overnight cultures were washed once in HBSS and resuspended at a concentration of 6×10^7 bacteria/ml of HBSS.

Cell Culture

Source and description of lung epithelial cell lines maintained in various media with antibiotics are described in Table I. Polarized monolayers were grown on the underside of 0.33 cm^2 collagen coated Transwell filters to study PMN migration in the physiological basolateral to apical direction as previously described (11–14). Epithelial barriers derived from each cell line were restrictive to the movement of small proteins as determined by measurement of horse radish peroxidase (HRP) flux across each epithelial barrier (12).

PMN Isolation

PMNs (polymorphonuclear leukocytes or neutrophils) were isolated from human blood treated with acid citrate/dextrose (MGH IRB protocol #: 1999-P-007782). The buffy coat was recovered by centrifugation. Plasma and mononuclear cells were removed by aspiration, and the majority of the red blood cells (RBCs) were removed using 2% gelatin sedimentation. Residual RBCs were removed by lysis in cold NH_4Cl lysis buffer. After lysis, cells were washed, counted, and resuspended in HBSS(−) at a concentration of 5×10^7 cells/ml. (11–14).

PMN Trans-epithelial Migration Assay

Transwell inserts containing lung epithelial cell monolayers seeded on the underside were exposed to 25 μl of 6×10^7 bacteria/ml for 1 hr (11–14). After infection, PMNs (1×10^6) were added to the top (basolateral) chamber and incubated at 37°C for 2 hr. PMNs that fully migrated across the cell monolayer reaching the bottom (apical) chamber were quantified by the myeloperoxidase (MPO) assay. Uninfected lung epithelial monolayers or monolayers infected with non-pathogenic *E. coli* strain MC1000 serve as negative controls for PMN transmigration. Establishment of a concentration gradient of PMN chemo-attractant fMLP (100 nM added to apical chamber of uninfected monolayers at the same time that PMNs are added to the basolateral chamber) serves as a positive control for the ability of freshly isolated PMNs to migrate (26).

Cell Viability/Barrier Integrity Assays

The amount of lactic dehydrogenase (LDH) released into the supernatant with and without infection of PAO1 in the presence or absence of each of the inhibitors employed was quantified using the LDH based In Vitro Toxicology Assay Kit (Sigma, St. Louis MO). Barrier integrity of lung epithelial monolayers grown on Transwells was assayed by the horse radish peroxidase (HRP) flux assay as previously described (12).

Inhibitors

Lung epithelial cells were pre-treated 1 to 2 hours with each inhibitor followed by washing prior to infection and/or addition of PMNs for each assay. PLA_2 inhibitor (ONO-RS-082), lipoxygenase inhibitor (CDC), DAG lipase inhibitor (RHC-80267) and cyclo-oxygenase inhibitor (NS-398) were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY). For experiments involving stable HXA_3 analogues (PN-II-218-36 & MV-I-237-20), analogues were added at various concentrations to the apical well of the infected Transwells at the

same time that PMNs were added to the basolateral well and PMNs that migrated across the monolayers in the presence or absence of apically applied analogues were quantified by myeloperoxidase. Stable HXA_3 ether analogues were synthesized in the Falck laboratory (manuscript in preparation). The inhibitors presented in this study did not affect cell viability either in the presence or absence of bacterial infection as assessed by both the LDH release assay and the barrier integrity assay (HRP flux). None of the compounds had any major effect on the amount of bacteria adhering to the lung epithelial monolayers.

PGE_2 E.I.A.: lung epithelial cells were grown to confluence in 24-well plates and used 5 to 7 days after seeding. Lung epithelial cells were pre-treated with inhibitors for 1 to 2 hrs, washed, and infected with 6×10^7 bacteria/ml for 1 hr at 37°C. Each well was washed three times in HBSS followed by incubation at 37°C for 2 hrs. Supernatents were collected and the amount of PGE_2 in each well was quantified using the Prostaglandin E_2 Express EIA Kit from Caymen Chemical (Ann Arbor, MI) (11).

Arachidonic Acid Release Assay

Lung epithelial cells were grown to confluence in 24-well plates and used 5 to 7 days after seeding (14). Cells were washed 3 times with PBS(−) and treated with media containing 0.2 μC/ml ^{3}H-arachidonic acid (ARA), and incubated for 18–24 hrs. Cells were then washed 3 times to remove unincorporated ^{3}H-ARA and treated with 0.5 ml of bacteria (6×10^7 bacteria/ml). Following infection, cells were incubated at 37°C for up to 6 hrs. Supernatants (100μl) were collected at 2, 4, and 6 hrs and radioactivity was measured by scintillation counting. After collection of supernatant, cells were lysed with 500 μl/well of 1% SDS, 1% Triton-X-100 and sampled (250μl) for measurement by scintillation counting. Data are displayed as the percentage of cpms measured in the supernatant at each time point of the total (% ARA release = [released/released + cell associated] × 100). Lung epithelial cell viability was maintained during the 6 hr infection as determined by the LDH release assay.

RT-PCR

Lung epithelial cells were grown in 6-well plates to confluence. Cells were lysed and RNA was purified using the Aurum total RNA mini kit (Biorad). The RNA concentration was standardized to 0.1 ug/ml. Before use, samples were treated with RQ1 DNAse (Promega) according to manufacture's protocols. cDNA was generated from 2.5 μl clean RNA using the iScript kit (Invitrogen). cDNA (2 μl) was amplified using the iTaq PCR kit (Biorad) and primers specific to the genes *gapdh*, *alox12*, *alox15*, *alox12b*, and *elox3* (Table II). Primers were synthesized at the MGH DNA Core facility. The amplified product was run on a 1.5% agarose gel containing 100ug/ml ethidium bromide (Biorad) and imaged under a UV light. The expected product sizes listed in Table II were confirmed using EZ Load 100 bp PCR Molecular Ruler (Biorad).

Extraction of Lipids from Supernatants

Lung epithelial cells were seeded on 25 cm^2 (SAEC) or 162 cm^2 (H292 & BEAS-2B) flasks and grown to confluence. Confluent cell monolayers were treated with or without 6×10^7 bacteria/ml HBSS for 1 hr at 37°C, washed three times with HBSS, and incubated an additional 2 hrs at 37°C. Supernatants were collected acidified to pH 4.0 and, in experiments involving eicosanoid quantification, mixed with lipid extraction standards (LTB4-d_4/15(S)-HETE-d_8 obtained from CaymenChemical) (27, 28). Acidified supernatants were poured through a Supelco Discovery®; DSC-18 SPE Column and eluted with methanol. The lipid fraction suspended in methanol was dried under a stream of nitrogen to 100 μl methanol and stored at −80°C until processed further (11).

Measurement of lipid associated PMN chemo-attractant activity

Each extracted lipid sample for H292 lung epithelial cells with or without PAO1 infection (prepared in triplicate and stored at −80°C) was dried under a stream of nitrogen and re-suspended in 1.7 ml HBSS. One ml of re-suspended extracted lipid supernatants (referred to as straight) was added to the apical well of a Transwell containing H292 lung epithelial barrier. A volume of 0.6 ml re-suspended lipids was diluted in 1.2 ml HBSS (referred to as Diluted 1:3). One ml of diluted 1:3 resuspended lipids was added to the apical well of a Transwell containing H292 lung epithelial barrier. The remaining 0.8 ml diluted 1:3 re-suspended lipids were further diluted by adding 0.8 ml HBSS (referred to as Diluted 1:6). One ml of diluted 1:6 resuspended lipids was added to the apical well of a Transwell containing H292 lung epithelial barrier. The remaining 0.6 ml diluted 1:6 re-suspended lipids were further diluted by adding 0.6 ml HBSS (referred to as Diluted 1:12). One ml of diluted 1:12 resuspended lipids was added to the apical well of a Transwell containing H292 lung epithelial barrier. Lipids extracted from BEAS-2B and SAEC supernatants that have been treated either with or without PAO1 (prepared in triplicate and stored at −80°C) were re-suspended in 1 ml HBSS after drying remaining methanol with a stream of nitrogen. Re-suspended lipid extracts were then added to the apical well of a Transwell containing H292 lung epithelial barrier. PMNs (1×10^6) were added to the top (basolateral) chamber in a volume of 120 μl for all diluted re-suspended lipid samples that have been added to the bottom (apical) chamber. Transwells were then incubated at 37°C for 2 hr and PMNs that have fully migrated across the cell monolayer reaching the bottom (apical) chamber were quantified by the myeloperoxidase assay (11).

Assessment of soluble epoxide hydrolase sensitivity

Lipid extracts from the supernatant of lung epithelial cells in the presence or absence of PAO1 infection (prepared in triplicate and stored at −80°C) were re-suspended in 1 ml HBSS after removing methanol by drying under a stream of nitrogen. Each sample was split into two 0.5 ml fractions. To one of the two fractions, 3.5 μl of soluble epoxide hydrolase (sEH – 10 mg/ml) was added (29, 30). Fractions with and without sEH were mixed at 100 rpm for 2 hrs at 30°C. After incubation, 0.7 ml HBSS was added to each fraction, mixed and 1 ml mixed fraction was added to the apical well of a Transwell containing H292 lung epithelial barrier. PMNs (1×10^6) were added to the top (basolateral) chamber in a volume of 120 μl for all diluted re-suspended lipid samples with or without sEH treatment that have been added to the bottom (apical) chamber. Transwells were then incubated at 37°C for 2 hr and PMNs that have fully migrated across the cell monolayer reaching the bottom (apical) chamber were quantified by the myeloperoxidase assay.

Measurement and Quantification of Eicosanoids

HXA_3 and 12-HETE were quantified after lipid extraction from either human lung epithelial cell supernatants or bronchoalveolar lavage (BAL) fluid of mice by LCMS/MS based lipidomics (11). In brief, extracted samples were analyzed by a triple quadruple linear ion trap LC/MS/MS system (MDS SCIEX 3200 QTRAP) equipped with a LUNA C18-2 mini-bore column using a mobile phase gradient of water/acetonitrile/acetic acid (72:28:0.01, v:v:v) and isopropanol/acetonitrile (60:40, v:v) with a 0.50 ml/flow rate (31). MS/MS analyses were carried out in negative ion mode and prominent fatty acid metabolites were quantified by multiple reaction monitoring (MRM mode) using established transitions for HXA_3 (335→127 *m/z*), 12-HETE (319→179 *m/z*), LTB_4-d_4 (339→197 *m/z*) and 15-HETE-d_8 (327→182 *m/z*). Calibration curves (1–1000 pg) and specific LC retention times for each compound were established with synthetic standards (Cayman Chemical, Ann Arbor, MI). Structures were confirmed for selected autacoids by MS/MS analyses using enhanced product ion mode with appropriate selection of the parent ion in quadrupole 1.

Mouse Model of Acute Pneumonia

C3H/HeN mice (6–8 weeks old) purchased from Harlan Laboratories were injected i.p. with anesthesia (Ketamine + Xylazine) prior to intra-nasal challenge with 4×10^8 CFU *Pseudomonas aeruginosa* (PAO1) or PBS control (10μl into each nostril) (32, 33). Five mice per treatment for each individual experiment were used. After 18 hrs, mice were euthanized by CO_2 asphyxiation in accordance with an IACUC approved protocol.

Collection of BAL fluid

Broncholveolar lavage was performed at 18 hr post infection using 1 ml of 0.5 mM EDTA in PBS without calcium or magnesium and repeated three times. Fluid was centrifuged (220 × g) for 5 minutes and harvested cells were washed with ammonium chloride lysis buffer to remove RBCs. Total cell counts were determined by hemocytometer diluted in trypan blue containing buffer (32, 33). Myeloperoxidase activity of BAL fluid cells was assessed as previously described (12). For measurement of eicosanoids 12-HETE and HXA_3, supernatants from centrifuged BAL fluid was subjected to the lipid extraction protocol with standards and quantified by LCMS/MS as described above.

Statistics

Data displayed for each figure are presented as a representative experiment with a mean (standard deviation) of at least three independent data points/condition. Each experiment was repeated multiple times yielding similar results. Statistical analysis was performed by a two-tailed unpaired Student's t test for each internally controlled experiment and considered significant when p values are less than 0.05.

Results

A diversity of human lung epithelial cell models were examined to determine whether they were capable of forming barriers on Transwells and whether they facilitate pathogen induced PMN trans-epithelial migration. The lung epithelial models include both carcinoma cell lines as well as transformed normal bronchial epithelial cells (Table 1). All cell lines form functional barriers, confirmed by evidence for the restriction of HRP movement across each monolayer of A549, Calu-3, H292, and BEAS-2B cells grown on Transwells (data not shown) (12). To investigate whether *P. aeruginosa* (PAO1) is capable of instigating PMN trans-epithelial migration across each of these barriers, the apical surface of each monolayer was treated with HBSS or infected with either pathogenic PAO1 or non-pathogenic *E. coli* (MC1000). After 1 hr, non-adherent bacteria were washed away and PMNs were added to the basolateral side (12, 26). The number of PMNs that migrate from the basolateral to the apical well after incubation for 2 hrs was determined by quantification of MPO activity. We found a consistent trend in PMN trans-epithelial migration amongst all of the lung epithelial barriers tested (Fig. 1A). Few PMNs migrated across uninfected monolayers or monolayers infected with MC1000. Infection with PAO1, on the other hand, resulted in a robust and highly significant increase in the numbers of PMNs that migrated across lung epithelial barriers. We also observed that a significant number of PMNs migrated across uninfected monolayers derived from each of the cell lines in response to an imposed chemo-tactic gradient of fMLP. This served as a positive control for the ability of isolated PMNs to migrate towards a chemo-tactic gradient in each assay with each distinct cell line monolayer (26). These data are consistent with observations previously reported for A549 and Calu-3 cells, suggesting that PAO1 induced PMN trans-epithelial migration is a highly reproducible phenomena observed using a range of distinct lung epithelial cell barrier models (12, 14).

Inhibitors of 12-lipoxygenases and phospholipase A_2 (PLA_2) were previously observed to potently interfere with PAO1 induced PMN migration across A549 barriers (11, 12, 14). To

expand investigation of this phenomenon in alternative lung epithelial cell models, we established monolayers of a distinct carcinoma lung epithelial cell line (H292) and a transformed normal bronchial epithelial cell line (BEAS-2B) (Table 1). Prior to treatment with buffer or infection with bacteria, monolayers were treated for 1 to 2 hrs with pharmacological inhibitors of eicosanoid metabolic enzymes. ONO-RS-082 and CDC inhibit PLA_2 and 12-lipoxygenases, respectively. Both enzymatic activities are crucial for the generation of the PMN chemo-attractant HXA_3. The cyclo-oxygenase inhibitor NS-398 interferes with synthesis of a group of eicosanoids unrelated to the synthesis of HXA_3 known as prostaglandins, which are bio-active, but do not serve as PMN chemo-attractants (34, 35). The diacyl glycerol (DAG) lipase inhibitor RHC-80267 was previously shown not to impact PAO1 induced epithelial migration across A549 monolayers (14). DAG lipase is an alternative means of generating the eicosanoid precursor ARA from DAG rather than from membrane phospholipids as is the case with PLA_2 (36).

Pretreatment of H292 and BEAS-2B lung epithelial monolayers with either a 12-lipoxygnease inhibitor (CDC) (37) or a PLA_2 inhibitor (ONO-RS-082) (38) potently blocked PAO1 induced PMN trans-epithelial migration, indicating a key role for these epithelial enzymatic activities in facilitating pathogen induced PMN trans-epithelial migration (fig 1B & 1C). Neither inhibitor interfered with migration across uninfected monolayers in response to an imposed fMLP gradient indicating that the general ability of PMNs to move across epithelial monolayers is not impacted by pre-treatment with either inhibitor. These studies are consistent with previous observations using A549 lung epithelial monolayers (12, 14). Also consistent with previous studies involving A549 monolayers, the DAG lipase inhibitor has no significant affect on PAO1 induced PMN migration across H292 and BEAS-2B monolayers (fig 1B & 1C) (14). The inhibitor of the cyclo-oxygenase pathway (NS-398), an eicosanoid metabolic pathway unrelated to HXA_3 synthesis, exerted no significant impact on PAO1 induced PMN trans-epithelial migration (fig 1B & 1C). We have previously demonstrated that PAO1 infection of lung epithelial cells results in a significant increase in the release of prostaglandins, specifically prostaglandin E_2 (PGE_2) (11). Importantly, the concentration of NS-398 employed in the PMN migration experiments (fig. 1B & 1C) was sufficient to prevent PAO1 induced PGE_2 release (fig 1D), confirming the effectiveness of the pharmacological inhibitor in our assay. Pre-treatment of H292 cells with the 12-lipoxygenase inhibitor CDC has no effect on PGE_2 release as would be expected. CDC at an equivalent concentration is, however, capable of interfering with PAO1 induced HXA_3 release as previously reported (12). Migration in response to HBSS alone or infection with MC1000 was minimal for all conditions tested (fig. 1B & 1C).

The rate limiting step in eicosanoid synthesis is often attributed to the generation of ARA (39, 40). We have previously demonstrated that infection of A549 lung epithelial cells with PAO1 result in a significant release of ARA (11, 14). We examined ARA release from a distinct transformed human lung epithelium cell line (H292) as well as from primary small airway epithelial cells (SAEC) after 2 hrs, 4 hrs, and 6 hrs stimulation with PAO1 or MC1000 (fig. 2A & 2B). The results indicated a significant elevation in ARA release by both H292 and SAEC epithelium treated with pathogenic PAO1 over time relative to untreated epithelial cells or epithelium treated with non-pathogenic MC1000 (Fig. 2A & 2B). These results are consistent with the hypothesis that PAO1 infection induces increased cellular generation of eicosanoids.

Our studies suggest that 12-lipoxygenase activity is critical for PAO1 induced PMN trans-epithelial migration. There are three human lipoxygenases that exhibit 12-lipoxygenase activity including 12-LO (gene *alox12*), 12(R)-LO (gene *alox12B*), and 15-LO (gene *alox15*) (19, 21, 25). The 12-lipoxygenases convert ARA to HXA_3 via the short-lived intermediate 12-HpETE (20). In the case of 12(R)-LO, an additional enzyme eLOX3 (gene

elox3), serving as a hepoxilin synthase, is required to convert 12-HpETE to HXA_3 (19, 22). A full panel of lung epithelial models described in Table I including multiple cell lines, primary normal human bronchial epithelial cells (NHBE), and primary small airway epithelial cells (SAECs) were evaluated for expression of all known lipoxygenase genes involved in direct synthesis of HXA_3. Specific primers to amplify *alox12, alox15, alox12B,* and *elox3* were designed and utilized to analyze gene expression by reverse transcription (RT)-PCR (Table II). We found that all four 12-lipoxygenase genes were expressed in each and every lung epithelial model investigated, suggesting that lung epithelial cells indeed express the genes necessary to synthesize the PMN chemo-attractant HXA_3 (Fig. 2C).

Our hypothesis predicts that lung epithelial cells synthesize and release HXA_3 in response to infection with PAO1 leading to directed PMN migration across infected epithelial barriers. Since HXA_3 is an eicosanoid, its chemotactic potential would be expected to be enriched in the lipid extracted fraction of supernatants from lung epithelial cells infected with PAO1. To determine whether H292 lung epithelial cells produce increased lipid associated chemotactic activity in response to PAO1 infection, H292 cells were treated with PAO1 after which supernatants were collected, acidified, and passed through a C18 column followed by elution with methanol. Methanol fractions were dried under a stream of nitrogen and resuspended in HBSS for assessment of PMN chemotactic potential by establishing a gradient of the fraction at various dilutions across epithelial monolayers grown on Transwells (fig. 3A & B). PAO1 clearly induced significantly more lipid associated chemotactic activity when compared to either uninfected H292 cells (Fig. 3A) or H292 cells infected with the non-pathogenic *E. coli* strain MC1000 (Fig. 3B). The observation that PAO1 is capable of significantly increasing PMN chemotactic activity in the lipid fraction of lung epithelial cell supernatant was not restricted to the H292 cell line. The normal bronchial transformed cell line BEAS-2B (Fig. 3C) as well as the primary small airway epithelial cells (Fig. 3D) also released significantly greater lipid associated PMN chemotactic activity when cells were infected with PAO1 as assessed by quantifying the number of PMNs migrating across an H292 barrier in response to gradients of supernatant derived lipid enriched fractions from infected and uninfected BEAS-2B and SAEC cells.

Our previous studies have demonstrated that the lung epithelial cell line A549 releases the PMN chemotactic eicosanoid HXA_3 in response to infection with PAO1 (12). We observed herein that PAO1 infection of H292 cells results in a greater than 5-fold increase in the amount of HXA_3 released as quantified by LCMS (2.6 pg +/− 1.9 uninfected versus 14.4 pg +/− 0.9 PAO1 infected). This observation is consistent with our finding of increased lipid associated chemotactic activity following PAO1 exposure (fig. 3A). To determine if HXA_3 contributes to the increased lipid associated chemotactic activity described above, we employed the enzyme soluble epoxide hydrolase (sEH) (29). A unique structural feature of HXA_3 amongst PMN lipid chemotactic factors is that HXA_3 possesses an epoxide group (16). The enzyme sEH is capable of hydrolyzing the epoxide group and converting hepoxilins into trioxillins, which lack PMN chemotactic activity (41). Lipid associated fractions from conditioned supernatant of PAO1 treated and untreated H292 cells were subjected to treatment with sEH prior to analysis of chemotactic potential. As expected, lipid extracted conditioned supernatants from PAO1 treated H292 cells exhibited substantial chemotactic activity, whereas very little chemotactic activity was observed from lipid extracted conditioned supernatants from uninfected H292 cells (Fig. 3E). The amount of chemotactic activity derived from lipid extracted conditioned supernatants of PAO1 infected H292 cells was significantly reduced when pretreated with sEH, suggesting that chemotactic factor responsible for directing PMNs to move across epithelial monolayers was sensitive to sEH treatment (Fig 3E). Such results are consistent with the hypothesis that HXA_3 is a lipid associated chemotactic factor responsible for PAO1 induced PMN trans-epithelial migration (12).

Our evidence thus far suggests that lung epithelial cells produce HXA_3 in response to infection with *P. aeruginosa* and this molecule serves as a PMN chemo-attractant hypothesized to guide PMNs across epithelial monolayers. We next employed structural analogues of HXA_3 whereby the epoxide was replaced with ether, designed to be stable yet non-functional. Elimination of the epoxide abolishes chemotactic activity based on observations that trioxilins, HXA_3 metabolites lacking the epoxide, no longer possess PMN chemotactic activity (15). Further, it is believed that the epoxide group contributes to the relative instability of HXA_3 and removal generates a more stable molecule (16). One of the analogues termed PN-II-218-36 was designed to competitively inhibit HXA_3 chemotactic activity, as it is structurally identical to HXA_3 with the exception of the epoxide replaced with ether. In light of the well known ω-hydroxylation of HXA_3, a second compound MV-I-237-20 bearing an additional hydroxyl group on the C(20)-position was also prepared to address this contingency (42, 43). A gradient of HXA_3 added exclusively to the apical well of lung epithelial monolayers drove greater than 30% PMNs added to the basolateral well across the monolayer (Fig. 4A), consistent with previous reports (12, 13). At the same concentration, neither HXA_3 analogue exhibits any PMN chemotactic activity (Fig. 4A). When the compound PN-II-218-36 was added to the apical side of PAO1 infected lung epithelial monolayers, PMN trans-epithelial migration was impeded in a dose dependant manner with complete inhibition at the addition of 156 μg/ml PN-II-218-36 (Fig. 4B, black bars). The structurally less similar analogue MV-I-237-20 was not as effective at interfering with PMN trans-epithelial migration (Fig. 4B, gray bars). The effect of PN-II-218-36 appears to be selective to PAO1 induced migration as PMNs migrating towards an fMLP gradient were significantly less impacted by the presence of analogue PN-II-218-36 (Fig. 4C).

P. aeruginosa induced acute pneumonia models have been widely employed to investigate various aspects of PMN recruitment into the airspace (44–53). Our *in vitro* system models migration of PMNs across the airway epithelial barrier, an event representing the final step in PMN emigration from circulation to the airspace during acute infectious pneumonia. Since we hypothesize that HXA_3 is the key chemo-attractant for directing PMNs across the lung epithelial barrier during infection with PAO1, we next investigated whether HXA_3 is produced in the airspace during *in vivo* infection. C3H/HeN mice were challenged with 4×10^8 CFU PAO1 or mock infection (PBS) (10μl into each nostril) and sacrificed 18 hrs post infection. Bronchoalveolar lavage (BAL) fluid was collected to determine the number of cells and the amount of MPO activity present. In addition, the quantity of 12-LO synthesized eicosanoids including 12-HETE and HXA_3 was measured in the BAL fluid. There was a significant increase in the number of cells and MPO activity in PAO1 infected mice suggesting substantial PMN recruitment to the airspace consistent with previous studies (figure 5A & B) (32, 33). This observation correlated with a highly significant increase in 12-LO eicosanoids in the BAL fluid of infected mice, both PMN chemo-attractant HXA_3, and the more stable 12-LO metabolite 12-HETE (figure 5C & D).

Discussion

PMN infiltration of the airway mucosa and accumulation in the airspace, if excessive and prolonged, can be severely detrimental to health during airway inflammatory disease states (5–7). Although recruitment of PMNs to mucosal surfaces is critical during infection to provide pathogen clearance, pathology from such infections often reflects indiscriminant and potent actions of PMNs on host tissue (52). Moreover, in the case of airway inflammation not directly associated with microbial threats, any potential benefits derived from PMN presence in the airspace would appear to be outweighed by their propensity to cause host tissue damage. Therefore, a thorough understanding of the mechanism by which PMNs not only exit the microvasculature, but also specifically how they cross mucosal epithelial

barriers during disease may lead to the development of therapies geared towards reducing the numbers of PMNs that breach the mucosal barrier and collect in the airspace (6, 10).

A plethora of host derived chemo-attractants are described that engage PMNs in an effort to relay them to the airspace (54). These include protein CXC chemokines (CXCL1-3, 5, & 8), CXC-like extracellular matrix breakdown product N-acetyl Pro-Gly-Pro (PGP), protein fragment complement component C5a, and lipid mediators leukotriene B_4 (LTB_4) & platelet activating factor (PAF) (55–58). Despite the wealth of knowledge regarding these chemo-attractants and their receptors, a clear understanding of their respective roles in PMN recruitment following infection *in vivo* is unclear. CXC chemokines are released basolaterally from infected mucosal epithelial cells where they imprint underlying extracellular matrix enabling directed PMN movement through sub-epithelial space (10, 26, 59, 60). Evidence from *in vivo* modeling notes a requirement for CXC chemokines such as CXCL5 and CXC-like PGP in recruitment of PMNs to the airspace, likely by directing PMNs across the endothelial barrier and through the tissue space leading up to the epithelial barrier (55–58, 60, 61). Chemo-attractants LTB_4, PAF, and C5a are found in the infected lung, but a discrete role in the orchestration of PMN movement from blood to airspace has not been elucidated (62, 63). The bacterial tri-peptide fMLP triggers anti-bacterial responses once primed PMNs arrive at the site of infection, but is unlikely involved in recruitment (26, 57).

Using a multifaceted *in vitro* model, we previously identified the eicosanoid HXA_3 as the key chemotactic signal that mediates directed migration of PMNs across epithelial barriers (12, 15, 60). Identification of HXA_3 as mediator of PMN trans-epithelial migration thus reveals an additional, but uniquely important chemo-attractant hypothesized to facilitate PMN recruitment to the airspace during infection. Studies reported herein provide further support both *in vitro* and *in vivo* for the involvement of HXA_3 in facilitating trans-epithelial migration of PMNs in the lung.

As described above, we demonstrate that the phenomena of *P. aeruginosa* induced PMN migration across lung epithelial barriers is shared amongst a range of distinct lung epithelial cell barrier models. *P. aeruginosa* induced PMN trans-epithelial migration represents both a phospholipase A_2 and 12-lipoxygenase dependant event as evidenced by failure of PMNs to migrate in response to epithelial infection following pretreatment with specific inhibitors of each of these enzymatic pathways. PLA_2 and 12-lipoxygenase enzymatic pathways are responsible for mediating the synthesis of HXA_3 (16, 60). At present, it is unclear which specific PLA_2 or 12-lipoxygenase isoform is critical for lung epithelial cells to synthesize HXA_3 in response to *P. aeruginosa* infection. Our previous studies suggest that the calcium dependant PLA_2 isoform cytosolic PLA_2-α (c PLA_2-α), generally thought to be the major PLA_2 isoform responsible for eicosanoid generation, is not involved in HXA_3 synthesis despite being activated in lung epithelial cells upon infection with *P. aeruginosa* and serving a key role in the generation of the eicosanoid prostaglandin E_2 (11).

Since 12-lipoxygenase activity has been described to be present in more than a one enzyme, multiple enzymatic sources for HXA_3 generation can be considered (19, 64). In the context of human lipoxygenases, there are three known enzymes with 12-lipoxygenase activity: 12-LO, 12(R)-LO, and 15-LO encoded by the genes *alox12*, *alox12B*, and *alox15*, respectively (19, 21, 25). 12(R)-LO additionally requires the hepoxilin synthetic enzyme eLOX3 encoded by the gene *elox3* to generate HXA_3 (22). We observed herein that all lung epithelial models, including primary small airway epithelial cells and primary bronchial epithelial cells, express all four known human lipoxygenase genes that encode enzymes with the capacity for HXA_3 synthesis. While these different lipoxygenase enzymes catalyze HXA_3 formation at different rates, each of these expressed genes has the potential to encode

enzymes involved in the production of HXA_3 by lung epithelial cells in response to treatment with *P. aeruginosa*. Dissecting the lipoxygenase family in an inflammatory context to determine which lipoxygenase enzyme(s) are critical contributors to the generation of HXA_3 represents an important future pursuit (19, 21, 64).

We have previously demonstrated that A549 lung epithelial cells produce HXA_3 in response to *P. aeruginosa* and gradients of HXA_3 established across A549 barriers facilitate PMN trans-epithelial migration (12, 13). We found in the current investigation that infection of the H292 cell line also resulted in the release of HXA_3 and gradients of HXA_3 across H292 monolayers drive PMN trans-epithelial migration. In addition to multiple lung epithelial cell lines, we observed that primary lung epithelial cells release the universal eicosanoid precursor ARA in response to infection with *P. aeruginosa* in a time dependent manner. Increased release of ARA is supportive of the hypothesis that enhanced production of eicosanoids such as HXA_3 occurs in response to pathogen infection. Since our hypothesis assumes that HXA_3 is released in response to infection and this PMN chemo-attractant drives trans-epithelial migration, we would predict that the lipid enriched fraction of the supernatant from *P. aeruginosa* infected lung epithelial cells would possess abundant chemotactic activity. This was indeed the case as multiple lung epithelial cell lines, including primary small airway epithelial cells, exhibited a significant increase in lipid fraction associated PMN chemotactic activity in response to infection with *P. aeruginosa*. Furthermore, this lipid associated PMN chemotactic activity was diminished when exposed to soluble epoxide hydrolase (sEH). HXA_3 is distinguishable from other lipid chemo-attractants such as LTB_4 and PAF in that HXA_3 possesses an epoxide within its structure that is critical for activity and vulnerable to the actions of sEH (16, 29, 41). Clearly, *P. aeruginosa* infection of lung epithelial cells results in the increased production of lipid associated PMN chemotactic activity that is sensitive to sEH and this observation is consistent with an increased presence of functional HXA_3 in the supernatants of infected lung epithelial cells.

To further probe the hypothesis that HXA_3 mediates *P. aeruginosa* induced PMN trans-epithelial migration, we employed structural analogues of HXA_3 that lack the critical epoxide. Both HXA_3 structural analogues, whereby the epoxide is replaced with an ether, exhibit no PMN chemotactic activity, which is in direct contrast to native HXA_3 that exhibits abundant PMN chemotactic activity. Each analogue does however display antagonistic activity. Addition of the epoxide deficient HXA_3 structural analogues significantly impedes *P. aeruginosa* induced PMN trans-epithelial migration. It is unclear how exactly the analogues interfere with *P. aeruginosa* induced PMN trans-epithelial migration. We speculate that such analogues may serve as competitive inhibitors of HXA_3. In the *P. aeruginosa* induced PMN trans-epithelial migration assays, we hypothesize that HXA_3 is produced by the lung epithelial cells in response to infection and this drives PMNs across the epithelial monolayer. Addition of inhibitory analogues might interfere with the ability of native HXA_3 to interact with PMNs and promote chemotaxis. Consistent with the notion is the observation that HXA_3 structural analogues were more effective at interfering with *P. aeruginosa* induced PMN trans-epithelial migration than with PMN migration in response to a gradient of the peptide chemo-attractant fMLP. Little is currently known regarding the receptor that HXA_3 interacts with on PMNs to evoke a chemotactic response. Future studies using native HXA_3 and the inhibitory analogues described above will greatly assist in addressing this important issue.

Investigators have explored many aspects of PMN airspace recruitment using *P. aeruginosa* infected mice. Such studies have established roles for key adhesion molecules, cytokines, chemo-attractants, toll-like receptors, signaling components, and transcription factors in the multifaceted process of emigration of PMNs from bloodstream to airway (44–53). Multiple

studies have now convincingly established HXA_3 as a PMN chemo-attractant (12, 13, 15, 17, 60). We have shown that airway epithelial cells secrete HXA_3 in response to infection and hypothesize that HXA_3 is the key chemo-attractant required to facilitate PMN breach of the mucosal barrier. To extend our *in vitro* observations into the context of an *in vivo* model, we examined whether an increase in the concentration of HXA_3 occurs in the airspace of mice in association with PMN accumulation subsequent to infection. Using a well established model of acute bacterial pneumonia in mice, we observed that PMNs accumulate in the airspace following intra-nasal exposure to *P. aeruginosa*, consistent with previous observations. Further, we observed a significant increase in the presence of eicosanoids derived from the 12-lipoxygenase enzymatic pathway including 12-HETE and HXA_3. Therefore, our results suggest that in addition to an increase in the number of PMNs in the airspace in response to *P. aeruginosa* infection, there is also an increase in the concentration of HXA_3 measured in the BAL fluid. Although increased presence of HXA_3 located within the airspace and associated with greater numbers of transmigrated PMNs suggests the potential for HXA_3 in serving a role in driving PMNs across the mucosa into the airspace, such an observation is insufficient to demonstrate a cause and effect relationship. However the novel finding that *in vivo* infection of the lung with *P. aeruginosa* results in release of HXA_3 in the airspace in combination with the substantial evidence from *in vitro* modeling demonstrating the role of HXA_3 in driving PMN trans-epithelial migration encourages further investigation of this potentially important inflammatory mechanism (11–15, 17, 60). Future studies will examine if reducing HXA_3 synthesis and/or activity *in vivo* will have an impact on the numbers of PMNs that accumulate in the airspace following infection.

In conclusion, our study herein provides further evidence for the role of HXA_3 in PMN recruitment to the airspace, particularly with respect to its chemotactic activity in guiding PMNs across the mucosal barrier. Based on our studies to date, interfering with the synthesis and/or function of the eicosanoid chemo-attractant HXA_3 represents a potentially compelling mechanistic target for developing and exploring a novel class of pharmaceutical compounds with significant untapped therapeutic potential towards alleviating PMN mediated mucosal surface injury during lung disease. Eicosanoid subsets including leukotrienes and prostaglandins have been exploited as therapeutic targets to alleviate overzealous inflammation (65). Multiple leukotriene inhibitors are available for treatment of allergies and asthma including 5-LO inhibitor Zileuton (66). Ibuprofen, a cyclo-oxygenase inhibitor, is an anti-inflammatory therapy used for many ailments including cystic fibrosis (67). There are few, if any, studies exploring the therapeutic benefits of 12-LO inhibition or HXA_3 neutralization in lung disease. There are many circumstances where controlling PMN infiltration into the airspace could alleviate inflammatory damage and improve lung function. For disease processes as diverse as pneumonia, cystic fibrosis and asthma, such a targeted therapeutic strategy could prove effective. Further exploration of the role of HXA_3 in PMN recruitment is warranted in order to realize this novel potential therapeutic benefit.

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Abbreviations used in this paper

PMNs	polymorphonuclear cells (i.e. neutrophils)
HXA_3	hepoxilin A_3

LO	lipoxygenase
CDC	cinnamyl 3,4-dihydroxy-α-cyanocinnamate
PGE_2	prostaglandin E_2
ARA	Arachidonic acid
sEH	soluble epoxide hydrolase
Ana	analogue
BAL fluid	bronchial alveolar lavage fluid
MPO	myloperoxidase
12-HETE	12-Hydroxyeicosa-5Z,8Z,10E,14Z-tetraenoic acid

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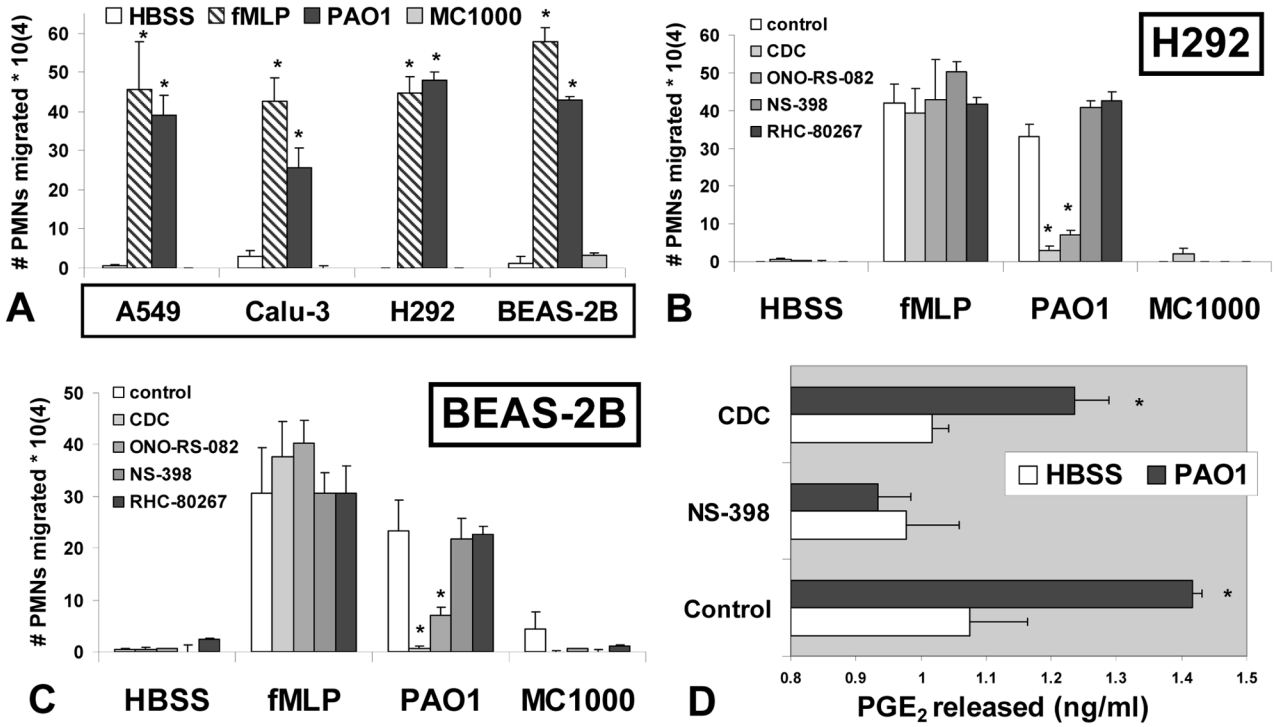

Figure 1. PMN migration across *P. aeruginosa* treated lung epithelial barriers is diminished following inhibition of the HXA_3 synthetic pathway

(A) Four distinct lung epithelial cell lines (Table I: A549, Calu-3, H292, and BEAS-2B) grown on Transwells to form epithelial barriers were treated with either *P. aeruginosa* strain PAO1 (black bars) or K12 *E. coli* strain MC1000 (gray bars). Monolayers incubated in HBSS alone serve as a negative control (white bars). A chemo-attractant gradient of fMLP (100 nM) was established to serve as a positive control for PMN movement (dashed bars). The symbol (*) represents a statistically significant difference compared to negative controls (HBSS and MC1000). **(B & C)** Transwells were pre-treated with pharmacological inhibitors CDC - 12-lipoxygenase inhibitor (50 μM), ONO-RS-082 - PLA_2 inhibitor (5 μM), RHC-80267 - DAG lipase inhibitor (50 μM), NS-398 -COX inhibitor (50 μM) or vehicle control (1:1000 DMSO) prior to infection. The number of PMNs that migrate across lung epithelial barriers H292 **(B)** and BEAS-2B **(C)** was assessed in response to infection with PAO1, MC1000, or uninfected controls (HBSS/fMLP) with or without pre-treatment of each pharmacological inhibitor. The symbol (*) represents a statistically significant difference within a treatment group comparing inhibitor to vehicle control. **(D)** The amount of PGE_2 released (ng/ml) by H292 cells in the presence or absence of PAO1 infection with or without pharmacological inhibitor pre-treatment was quantified by E.I.A.. The symbol (*) represents a statistically significant difference between uninfected (HBSS) and PAO1 infected within a treatment group. Statistical significance was determined using a two-tailed unpaired Student's T-test ($p < 0.05$). Each data point represents the average of at least three separate wells. Each experiment was performed on at least three separate occasions yielding similar results.

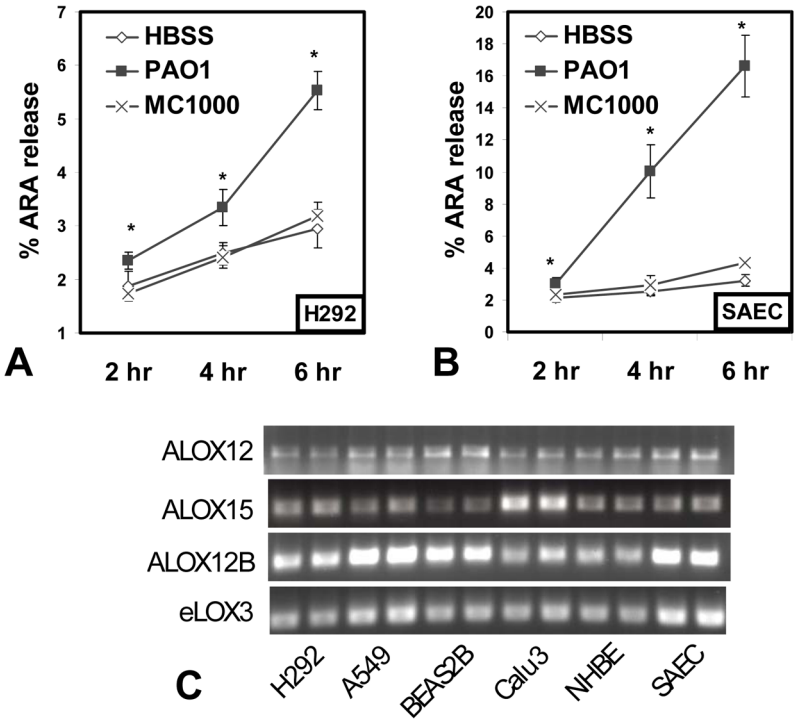

Figure 2. Arachidonic acid release following *P. aeruginosa* infection and lipoxygenase gene expression is lung epithelial cell models

The lung epithelial cell line H292 **(A)** or primary lung epithelial cells SAEC **(B)** (Table I) were incubated overnight with ^{3}H-ARA, followed by washing and treatment with HBSS alone (white diamonds), infection with *P. aeruginosa* strain PAO1 (black squares) or infection with K12 *E. coli* strain MC1000 (x symbol). Supernatants were sampled at 2 hr, 4 hr, and 6 hr post treatment/infection. The % ARA release represents the amount released into the supernatant as a percentage of the total (ARA released + cell associated ARA). The symbol (*) represents a statistically significant increase compared to HBSS control of the same time point using a two-tailed unpaired Student's T-test ($p < 0.05$) and each data point represents an average of at least three separate wells. Each experiment was performed on at least three separate occasions yielding similar results. **(C)** RNA was extracted from a panel of human lung epithelial cells (Table I) to assess gene expression by RT-PCR, shown here in duplicate sample wells. The known human 12-lipoxygenases (*alox12*, *alox15*, and *alox12B*) and the hepoxilin synthase (*elox3*) were analyzed individually using gene specific primers (Table II).

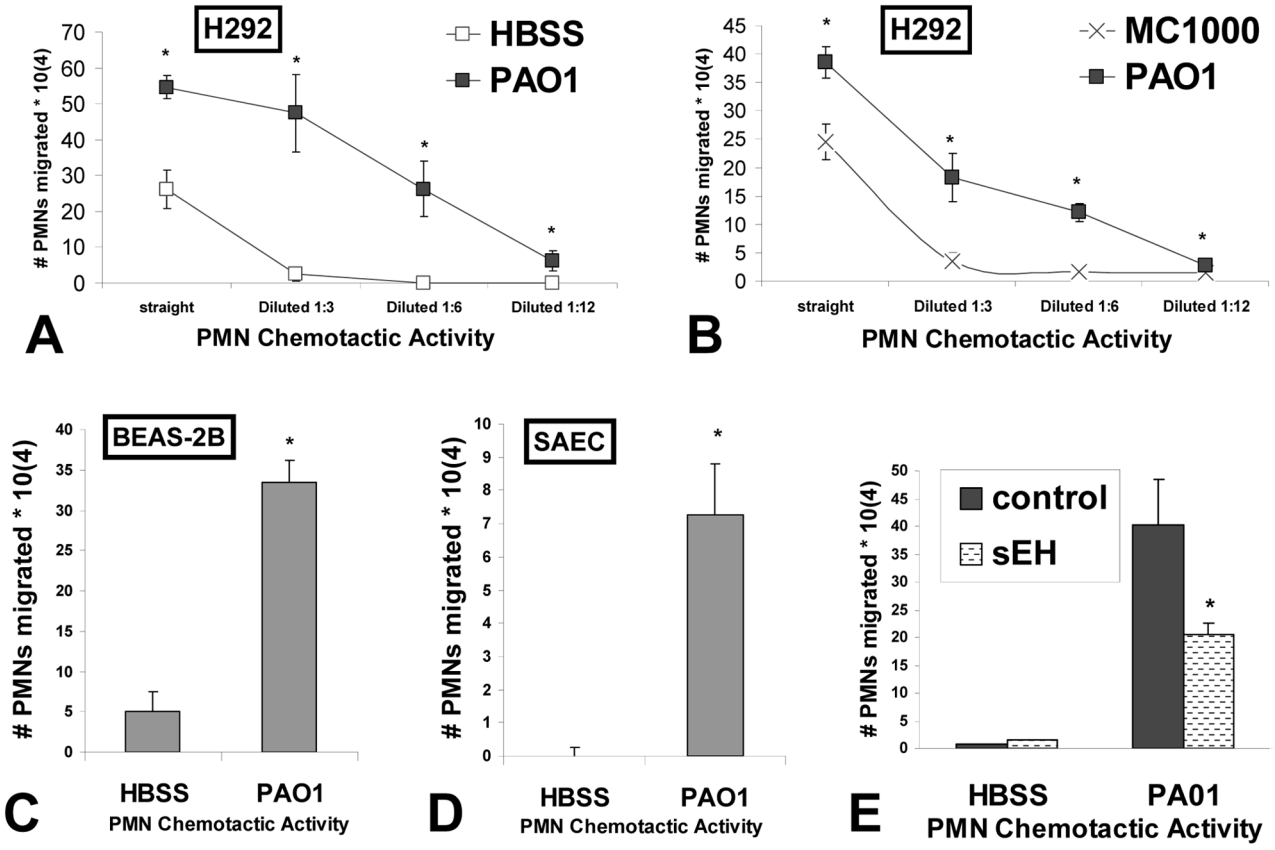

Figure 3. Lipid based chemotactic activity is increased in supernatants of various lung monolayers treated with *P. aeruginosa* and is sensitive to soluble epoxide hydrolase treatment

The amount of PMN chemotactic activity was quantified in serial dilutions of lipids extracted from the supernatants of H292 cells infected with *P. aeruginosa* strain PAO1 (black squares) and compared with serial dilutions of lipid extracted supernatants from H292 cells in the absence of infection (HBSS, white squares) **(A)** or infected with K12 *E. coli* strain MC1000 (x symbol) **(B)**. The amount of PMN chemotactic activity was quantified in lipids extracted from the supernatants of BEAS-2B **(C)** and primary lung epithelial cells SAEC **(D)** in the presence and absence of PAO1 infection. The symbol (*) represents a statistically significant difference compared to negative controls (HBSS and MC1000). **(E)** Lipids from the supernatants of uninfected (HBSS) and PAO1 infected H292 cells were extracted and split in two groups; a control group and a group subjected to treatment soluble epoxide hydrolase (sEH). Both groups were then evaluated for PMN chemotactic activity. The symbol (*) represent a statistically significant difference in PAO1 induced lipid extracted chemotactic activity when comparing sEH treated versus untreated. A two-tailed unpaired Student's T-test ($p < 0.05$) was employed to determine statistical significance. Each data point represents the average of at least three separate wells. Experiments were performed on at least two separate occasions yielding similar results.

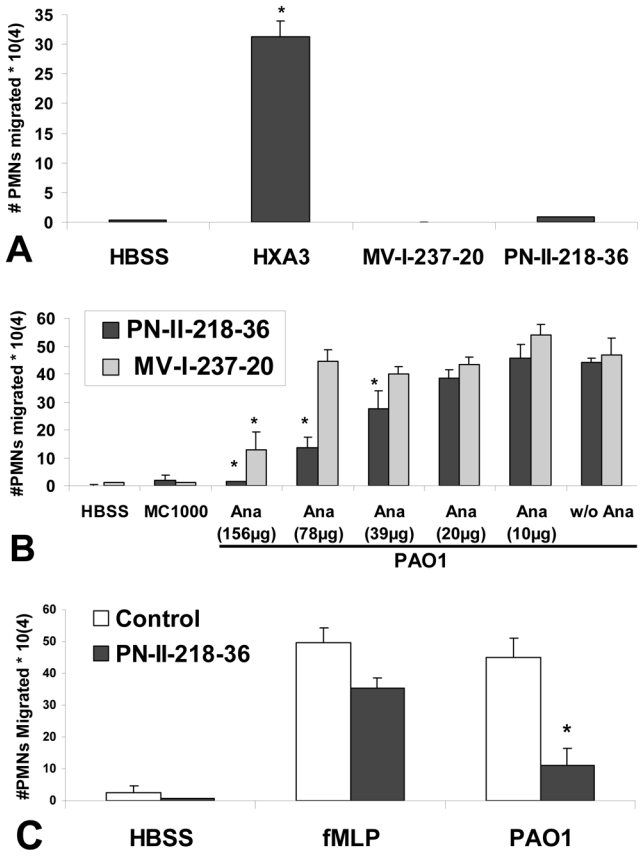

Figure 4. Stable analogues of HXA_3 lacking epoxide exhibit an inhibitory effect on P. *aeruginosa* induced PMN trans-epithelial migration

The ability of an established gradient of HXA_3 (2.5 μg/ml) and HXA_3 structural analogues (MV-I-237-20 & PN-II-218-36 (500 μg/ml)) across H292 epithelial barriers to promote PMN trans-epithelial migration is evaluated **(A)**. For **(A)**, the symbol (*) represent a statistically significant difference when compared to no gradient (HBSS) using A two-tailed unpaired Student's T-test ($p < 0.05$). Structural analogues MV-I-237-20 and PN-II-218-36, at a range of doses, were added to the apical side at the same time PMNs were added to the basolateral side to determine if either analogue impacts *P. aeruginosa* strain PAO1 induced PMN trans-epithelial migration **(B)**. The ability of PN-II-218-36 (110 μg) to influence trans-epithelial PMN migration in response to either an established gradient of fMLP (100 nM) or infection with PAO1 is evaluated **(C).** The symbol (*) represent a statistically significant difference when compared to PAO1 induced trans-epithelial migration in the absence of analogue using a two-tailed unpaired Student's T-test ($p < 0.05$). Each data point represents the average of at least three separate wells. Experiments were performed on at least two separate occasions yielding similar results.

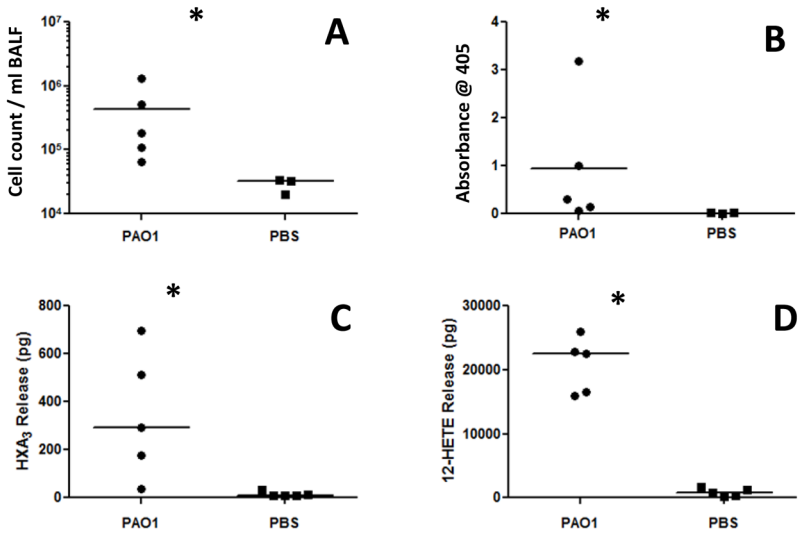

Figure 5. The number of PMNs and the concentration of 12-lipoxygenase products are increased in the BAL fluid in response to *Pseudomonas aeruginosa* intranasal infection

Mice were challenged via intranasal inoculation with *P. aeruginosa* strain PAO1 (5 mice) or PBS (5 mice) and sacrificed after 18 hours. BAL fluid was collected and analyzed for cellular infiltrate, myeloperoxidase activity, and 12-lipoxygenase eicosanoids. The quantity of cellular infiltration **(A)** MPO activity **(B)** HXA_3 **(C)** and 12-HETE **(D)** present in the airspace after infection with PA01 was compared to PBS mock infection control and differences from control reaching statistical significance using a two-tailed unpaired Student's T-test ($p < 0.05$) are represent by (*). *In vivo* experiments were performed on two separate occasions yielding similar results.

Table I

List of Cell Lines Used in Study

Cell Line	Description	Source	Media
H292	Lung epithelial pulmonary mucoepidermoid carcinoma	ATCC * CRL-1848™	RPMI-1640 * 10% HI-FBS
BEAS-2B	Normal bronchial epithelium that were transformed by an adenovirus	ATCC * CRL-9609™	DMEM *** 10% HI-FBS
A549	Type II alveolar lung epithelial carcinoma	ATCC * CCL-185™	F-12K *** 10% HI-FBS
Calu-3	Lung adenocarcinoma with epithelial morphology and phenotype	ATCC * HTB-55™	MEMα *** 10% HI-FBS
NHBE	Normal Human Bronchial Epithelium derived from healthy patient biopsy	Lonza ** CC-2640	BEGM™ ** BulletKit®
SAEC	Small Airway Epithelial Cells derived from healthy patient biopsy	Lonza ** CC-2647	SAGM™ ** BulletKit®

* American Type Tissue Culture Manassas, VA 20108 USA

** Lonza Walkersville, Inc. Walkersvile, MD 21793-0127 USA

*** Invitrogen Corporation Carlsbad, CA 92008 USA

Table II

List of Primers Used in Study

Gene	Forward (5′ - 3′)	Reverse (5′ - 3′)	Size (bp)
GAPDH	CCCATCACCATCTTCCAGGA	GTTGTCATGGATGACCTTGG	285
alox12	AAGCCCAAAGCTGTGCTAAA	TGCAGCAGGAGAGCTGAGTA	381
alox15	GAACTCAAGGTGGAAGTACC	CTTCAGGCAGGCTCAGGACG	190
alox12B	ACCGTGCAGTGCCCTCAGGA	CCGGAGTGCCAGGGTCTCGT	183
eLox3	GGCCCAGATCCCGGACACCT	GGGCTTCCTCTCTCCGCCCA	267