Distinct Cellular Sources of Hepoxilin A$_3$ and Leukotriene B$_4$ Are Used To Coordinate Bacterial-Induced Neutrophil Transepithelial Migration

Michael A. Pazos, Waheed Pirzai, Lael M. Yonker, Christophe Morisseau, Karsten Gronert and Bryan P. Hurley

*J Immunol* 2015; 194:1304-1315; Prepublished online 29 December 2014;
doi: 10.4049/jimmunol.1402489
http://www.jimmunol.org/content/194/3/1304
Distinct Cellular Sources of Hepoxilin A3 and Leukotriene B4 Are Used To Coordinate Bacterial-Induced Neutrophil Transepithelial Migration

Michael A. Pazos,*† Waheed Pirzai,*† Lael M. Yonker,*† Christophe Morisseau,‡ Karsten Gronert,§ and Bryan P. Hurley*†

Neutrophilic infiltration is a leading contributor to pathology in a number of pulmonary disease states, including cystic fibrosis. Hepoxilin A3 (HXA3) is a chemotactic eicosanoid shown to mediate the transepithelial passage of neutrophils in response to infection in several model systems and at multiple mucosal surfaces. Another well-known eicosanoid mediating general neutrophil chemotaxis is leukotriene B4 (LTB4). We sought to distinguish the roles of each eicosanoid in the context of infection of lung epithelial monolayers by Pseudomonas aeruginosa. Using human and mouse in vitro transwell model systems, we used a combination of biosynthetic inhibitors, receptor antagonists, as well as mutant sources of neutrophils to assess the contribution of each chemoattractant in driving neutrophil transepithelial migration. We found that following chemotaxis to epithelial-derived HXA3 signals, neutrophil-derived LTB4 is required to amplify the magnitude of neutrophil migration. LTB4 signaling is not required for migration to HXA3 signals, but LTB4 generation by neutrophils plays a significant role in augmenting the initial HXA3-mediated migration. We conclude that HXA3 and LTB4 serve independent roles to collectively coordinate an effective neutrophilic transepithelial migratory response. The Journal of Immunology, 2015, 194: 1304–1315.

Neutrophils are a critical component of the innate immune system and are indispensable for the clearance of many bacterial infections (1). However, uncontrolled neutrophil responses can lead to excessive inflammation and pathology, as is seen in cystic fibrosis (CF) (2, 3). CF is a congenital disorder defined by mutation of the CFTR gene. Abnormal ion regulation by dysfunctional CFTR results in chronic infection of the airway, most commonly with Pseudomonas aeruginosa (4), which can infect 70–80% of patients (5). Airway inflammation in patients with CF is dominated by persistent neutrophil infiltration and is associated with severe loss of function, respiratory failure, and ultimately mortality.

A key therapeutic strategy for treatment of CF is restraining neutrophil migration to the airspace (6). Neutrophil chemotaxis is a complex, orchestrated process involving the coordinated actions of selectins, integrins, and chemotactic signals as diverse as chemokines (IL-8), lipid mediators (leukotriene B4 [LTB4]), complement factors (C5a), matrix breakdown products proline-glycine-proline, and bacterial products (fMLP) (7, 8). Unique among known neutrophil chemoattractants is hepoxilin A3 (HXA3). HXA3 is a lipid mediator produced by mucosal epithelium and is secreted apically into luminal spaces (9) where a chemotactic gradient is formed through the tight junction complexes and recruits neutrophils across mucosal epithelial surfaces (10). Its production is triggered by pathogenic bacteria (11) and plays a necessary role in chemotaxis across both pulmonary and intestinal epithelial surfaces in vitro (9, 12). Inhibition of the HXA3 signaling pathway also has profound affects in vivo, resulting in reduced neutrophilic pathology in models of inflammatory bowel disease (13) and reduced systemic disease in a model of pneumonia (14).

HXA3-mediated chemotaxis is only a part of a coordinated recruitment cascade that must first mobilize neutrophils from the bloodstream, across the endothelium, and through the basement membrane before reaching the basolateral surface of the epithelial border. Many chemoattractants have been implicated as necessary for efficient neutrophil migration in models of pulmonary inflammation. The epithelial-derived CXC chemokine IL-8 effectively drives neutrophils from the blood into the tissue, but it is directed basolaterally and is not sufficient to drive neutrophils across the epithelium (15, 16). C5a is a complement component and anaphylatoxin that plays a role in neutrophil recruitment in a number of pulmonary inflammatory conditions (17, 18). LTB4 is a very well-studied eicosanoid that has long been known for driving the chemotaxis of neutrophils as well as other leukocytes, and it has been implicated in a number of biological mechanisms (19, 20). Despite the diversity of neutrophil chemoattractants that have been identified, an integrated concept involving the specific roles and sources of each chemoattractant has yet to emerge. Considering the large number of chemotactic signals that neutrophils may encounter in an inflammatory scenario, multistep navigation is...
necessary for successful homing (21). Chemotactic signals are pri-
oritized by downregulating the expression of alternative chemotactic recep-
tors (22, 23), suggesting that the management of multiple simultaneous signals is an important part of neutrophil biology. Furthermore, neutrophils can encourage their own migration by promoting microvascular leakage through the production of cyto-
kleine mediators (24). Once in the tissue, neutrophils cluster at sites of infection or damage. This swelling behavior organizes neutrophil localization within tissues (25, 26) and relies heavily on the pro-
duction of neutrophil-derived LTB4 (27). In a model of rheumatoid arthritis, neutrophils and synovial tissue coordinate multiple che-
motactic signals to manage waves of neutrophil recruitment (28–
30). Expanding our understanding of neutrophil recruitment cas-
cades across epithelial surfaces may allow for the development of therapeutic strategies for the treatment of patients with CF.

Given the complex nature of neutrophil recruitment mecha-
nisms, as well as the tendency for LTB4 to serve as an amplifying mediator, we sought to determine whether LTB4 played a role in bacterial-induced, HXA3-mediated neutrophil transepithelial mi-
gration. HXA3 and LTB4 are both eicosanoid neutrophil chemo-
attracants generated by the lipoxygenase family of enzymes (19, 31, 32). HXA3 plays a discrete role in mediating transepithelial migration, whereas LTB4 serves in a broad variety of functions as a leukocyte chemoattractant. We used and developed inverted transwell models of transepithelial migration to investigate the role of LTB4 in HXA3-mediated chemotaxis. We describe an axis of amplified migration that relies on neutrophil-derived LTB4 to magnify neutrophil migration following an initial response to HXA3-associated signals. This study consolidates HXA3-mediated transepithelial migration with the larger body of neutrophil mi-
gration. The relationship between HXA3 and LTB4 provides possible therapeutic targets for the modulation of neutrophil-
associated immunopathology of the airway.

Materials and Methods

Bacterial strains

*P. aeruginosa* strain PAO1 was grown aerobically in Luria-Bertani broth at 37˚C overnight. Prior to infection, cultures were washed and resuspended in HBSS and resuspended to a concentration 6 × 10^7 CFU/ml HBSS.

Human neutrophil isolation

Neutrophils were isolated from whole blood collected from healthy human volunteers (Institutional Review Board protocol no. 1999P007782) as previously detailed (33). Briefly, blood was anti-coagulated with acid citrate/dextrose. Buffy coats were obtained by centrifugation at 400 × g at room temperature. Plasma and mononuclear cells were removed by aspira-
tion, and most RBCs were removed by 2% gelatin sedimentation. Re-
sidual RBCs were removed by lysis using a cold ammonium chloride buffer. After lysis, neutrophils were washed and resuspended in HBSS without calcium or magnesium (HBSS−).

Bone marrow collection

C57BL/6j wild-type, Ltb4r1−/−, and Alox5−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in specific pathogen-free conditions. Ltb4r1−/− animals were homozygous for a tar-
geted disruption of the LTB4 receptor BLT1 (34), whereas Alox5−/− animals were homozygous for a targeted disruption of the LTB4 biosyn-
thetic enzyme 5-lipoxygenase (35). Experiments were performed on female mice aged 6–8 wk according to the guidelines of the Institutional Animal Care and Use Committee of Massachusetts General Hospital. Femurs and tibia were collected and flushed with HBSS− to collect bone marrow. RBCs were lysed using a cold ammonium chloride solution. After lysis, remaining bone marrow was washed and counted immediately prior to use.

Cell culture

Transwell inserts with 3-μm pores were purchased from Corning Life Sciences (Corning, NY). Inverted monolayers were prepared as previously described (33). Briefly, transwell inserts were flipped to an inverted position, collagen coated, and then seeded with either mouse lung epithelial cells (MLE12) or human lung epithelial cells (H292) on the underside of the transwell. Epithelial cells were allowed to attach overnight and then flipped back into transwells containing media and grown inverted until functional monolayers were established. Epithelial cells were maintained in DMEM/F12 (1:1) culture medium with 10% heat-inactivated serum and 1× antibiotics for 3–5 d for MLE12 cultures or >7 d for H292 cultures. Functional monolayers were confirmed by the maintenance of fluid resistance between apical and basolateral compartments. Additionally, all drug and infection conditions were assessed to verify their impact on ep-
ithelial viability and barrier integrity in independent experiments. Cell viability was assayed by an MTT assay (Life Technologies, Carlsbad, CA). Barrier integrity was assessed by tracking the translocation of HRP from the basolateral to apical compartments as previously described (12). None of the infection or drug treatment conditions significantly impacted epi-
thelial cell viability or barrier integrity.

Transmigration assays

Lung epithelial monolayers were grown on 24- or 96-well inverted transwell inserts (Corning Life Sciences). Transwells were first washed and equili-
brated in HBSS for 30 min prior to infection. After equilibration, transwells were again inverted and the apical surface of the epithelial cells was infected with *P. aeruginosa* 1 × 10^7 bacteria/ml for 1 h at 37˚C or mock-infected with HBSS. After infection, transwells were washed and prepared for migration. PAO1-infected cells were placed in wells containing HBSS−, as were HBSS control wells. Chemotactic gradients of IL-8 (100 ng/ml; eBioscience, San Diego, CA), LTB4 (5 ng/ml; Enzo Life Sciences, Farmingdale, NY), and mouse recombinant C5a (100 ng/ml; R&D Systems, Minneapolis, MN) were prepared in HBSS and provided at the apical chamber. Lipid fractions from epithelial supernatants were prepared as described below and serially diluted for optimal dosing. In antagonist and agonist soluble epoxide hydrolase (sEH) experiments, LY2239832 (Cayman Chemical, Ann Arbor, MI) or recombinant sEH (prepared by C. Morriseau as previously de-
scribed) (36, 37) was also added to the apical chamber at the indicated concentrations. Neutrophils (2 × 10^6/ml) were supplied to the basalateral chamber and incubated for 2 h at 37˚C. After a 2-h migration, the transwells were removed and the apical well was assayed for neutrophil content by myeloperoxidase assay (33). In cases where different neutrophil or bone marrow sources were used in the same assay, standard curves were used to control myeloperoxidase varia-
tion between sources. Migration values were normalized to HBSS controls.

Inhibitors

Epithelial drug treatments were performed immediately prior to infection. Epithelial cells were washed three times and incubated with zileuton (Sigma-Aldrich, St. Louis, MO), CDC (Enzo Life Sciences), MK886 (Enzo Life Sciences), or NS398 (Enzo Life Sciences) at the indicated concen-
trations for 1 h at 37˚C in HBSS−. After drug treatment, epithelial cells were washed three times and then infected, as above. Untreated neutrophils were then added for migration. In experiments calling for drug treatment of neutrophils, neutrophils (5 × 10^6/ml) were suspended in zileuton. CDC, MK886, or vehicle controls at the indicated concentrations for 1 h at 37˚C in HBSS−. Immediately prior to migration, neutrophils were resuspended in HBSS containing inhibitors. Neutrophil viability was confirmed by trypan blue exclusion.

Lipid extractions

Human H292 and murine MLE12 lung epithelial cells were seeded in a 162-cm² flask and grown to confluence. Confluent monolayers were washed and infected with PAO1 at 6 × 10^10 bacteria/ml in HBSS or mock-infected for 1 h at 37˚C. The monolayers were then washed three times and incubated for an additional 2 h in HBSS at 37˚C. Supernatants were collected and acidified to pH 5. In experiments where apical compartments were extracted for lipids, the plates were spun down to pellet neutrophils, and the apical supernatant was collected and acidified to pH 5. Acidified supernatants were poured through a Supelco Discovery DSC-18 SPE column (Sigma-Aldrich) and eluted with methanol. This lipid fraction in methanol was dried under a stream of nitrogen to 100 μl and stored at −80˚C for further processing. Immediately prior to the experiment, lipid fractions extracted from either epithelial cultures or from the apical supernatant of cocultured epithelium and neutrophils were then extracted in 1 ml methanol, dried under a stream of nitrogen, then finally resus-
pended in HBSS for use in assays. Lipid preparations used in epoxide hydrolase studies were incubated with 100 μg/ml sEH or vehicle control
for 2 h at 30°C prior to bioactivity assessment. Lipid fractions were serially diluted for optimal dosing.

**Calcium mobilization assays**

Neutrophils or whole bone marrow were stained with the calcium indicator fluo-4–acetoxyethyl ester (fluoro-4-AM; Life Technologies) at a final concentration of 1 μM for 30 min at 37°C and then washed in HBSS−. Whole bone marrow was further stained with anti-Ly6G-allophycocyanin (eBioscience) for 20 min on ice to identify neutrophils specifically. Stained cells were resuspended to a concentration of 2.5 × 10^6/ml. LY223982-treated neutrophils were incubated for 30 min at 2.5 μg/ml prior to analysis by flow cytometry. Immediately prior to collection, stained cells were stimulated with LTB4 (5 ng/ml), IL-8 (100 ng/ml), HBSS−, or extracted lipid preparations from infected or mock-infected epithelial cells. Lipid preparations were suspended to 400 μ HBSS− and then diluted 10-fold in neutrophil suspensions. Lipid-stimulated cells were stimulated with at least three independent lipid preparations in each condition. Median fluorescence intensity was calculated for gated neutrophils.

**Flow cytometry**

Migrated mouse neutrophils were collected from the apical compartment following 2 h of migration. The cell suspension was blocked with FBS and anti-CD16/CDC32 (1:200; BD Biosciences, San Jose, CA) for 20 min on ice. Suspensions were then stained with anti-CD45-PE (eBioscience) and anti-Ly6G-allophycocyanin (eBioscience) and then washed. Data were collected on an Accuri C6 flow cytometer (BD Biosciences) and analyzed by FlowJo software (Tree Star, Ashland, OR).

**Mixed bone marrow migrations**

Bone marrow from C57BL/6J wild-type, Alox5−/−, and Ltb4r1−/− mice was collected and processed as above. Whole bone marrow was differentially stained with CFSE (BioLegend, San Diego, CA) at a concentration of 0.25 or 2.5 μM and mixed in equal proportions. Mixed cell suspensions were then used as a neutrophil source in the transmigration assay. The apical compartment was collected following a 2-h migration, and the cell suspension was stained as above. CFSE− Ly6G+ cells were gated and the relative proportion of each population was calculated. Relative enrichment was then calculated for each migrated sample as follows: ([(% WT × N) − (% KO)]/[(% WT × N)] × 100.

**Leukotriene measurement**

LTB4 detection assays were purchased from Cayman Chemical and performed according to manufacturer’s instructions. Liquid chromatography–tandem mass spectrometry (LC-MS/MS) was performed by a triple quadrupole linear ion trap system (3200 QTRAP; AB Sciex, Framingham, MA) as previously described (39, 40).

**Statistical analysis**

Results are expressed as means ± SD. Data are representative of at least three independent data points per condition with multiple experiments yielding similar results. Single comparisons were evaluated using an unpaired two-tailed Student t test. Where noted, data were analyzed by a two-way ANOVA with a Bonferroni posttest. A p value ≤0.05 was considered significant.

**Results**

**BLT1 antagonism disrupts PAO1-mediated calcium signals**

To discriminate migration by either HXA3 or LTB4 signaling, we relied on a well-described transepithelial transwell model system (9, 33). Briefly, human lung epithelium is grown on the underside of a transwell filter with 3-μm pores. Epithelial cells spontaneously form a polarized monolayer with distinct apical and basolateral compartments (Fig. 1A). The epithelium is infected at the apical surface and bacteria are subsequently washed away. Neutrophils are supplied to the basolateral compartment and, in the presence of chemotactic signals, migrate through the filters and into the apical space. After migration, the transwell is discarded and neutrophil migration is measured by myeloperoxidase activity. In this model, P. aeruginosa infection has been demonstrated to induce endogenous epithelial production of HXA3, resulting in the migration of neutrophils in a basolateral-to-apical direction (12, 41). Imposed apical-to-basolateral gradients of LTB4 or IL-8 are also capable of driving neutrophil transepithelial migration. All chemotactic signals produced a robust migratory response (Fig. 1B).

LY223982 is a highly specific antagonist to BLT1, the primary chemotactic receptor for LTB4 on neutrophils (42). LY223982 was added to the apical space during migration to specifically antagonize LTB4-mediated signaling (Fig. 1C). As anticipated, we observed significant inhibition of LTB4-mediated migration, whereas IL-8–mediated migration was not significantly affected by the LTB4 receptor antagonist. Notably, PAO1-induced migration, mediated by HXA3, was also impacted by the LTB4 receptor antagonist, albeit to a lesser extent than LTB4 gradients at multiple doses. These data suggest that BLT1 antagonism specifically and partially inhibited PAO1-induced migration.

Lung epithelial cells respond to infection with the production of HXA3 as well as other eicosanoids such as PGE2 (39); however, we have been unable to detect the presence of LTB4 in the culture supernatant of either infected or uninfected epithelial cells. Furthermore, PAO1-mediated migration was very strongly reduced when epithelial cells were pretreated with CDC, an inhibitor of 12-lipoxygenase (the HXA3 biosynthetic enzyme), but not zileuton, an inhibitor of 5-lipoxygenase (the LTB4 biosynthetic enzyme) (Fig. 1D). Additionally, 5-lipoxygenase–activating protein inhibition with MK886 had no significant effect on migration when targeting epithelial cells (Fig. 1D).

Whereas BLT1 seems to play a role in PAO1-induced neutrophil transepithelial migration, LTB4 was not detectable in epithelial supernatants, and treatment of the epithelium with 5-lipoxygenase inhibitors did not significantly impact the ability of neutrophils to migrate. Because previous evidence suggests that LTB4 is not a relevant epithelial-derived chemotactic signal in our model, we considered two possible hypotheses to explain these results: 1) HXA3 interacts with the LTB4 receptor to signal neutrophils, or 2) LTB4 is produced by migrating neutrophils and contributes to the magnitude of the transepithelial response.

**BLT1 antagonism does not inhibit PAO1-associated calcium signals**

Given the importance of BLT1 for a robust migratory response to HXA3, we sought to determine whether HXA3 may rely on BLT1 for signaling of neutrophils. The receptor for HXA3 is unknown, but has been described as a G-protein–coupled receptor (GPCR) that induces calcium influx upon stimulation (9, 10). Because GPCRs can heterodimerize to modulate signaling (43) and BLT1 has multiple agonists (44), we considered whether BLT1 may be a required component of HXA3-mediated migration.

To determine whether BLT1 was required for HXA3-associated signaling on neutrophils, the calcium indicator fluo-4-AM was used to measure calcium mobilization in response to GPCR stimulation by various chemotactants. Calcium mobilization was strongly induced by LTB4 as well as IL-8 (Fig. 2A). To assess the calcium mobilization to HXA3-associated signals, we used lipid extracts from PAO1-infected epithelial supernatants. These supernatants are enriched for HXA3 and retain chemotactic activity dependent on HXA3 (41). PAO1 lipids induced a strong calcium mobilization in neutrophils, whereas mock-infected lipids had no detectable effect (Fig. 2A).

Calcium mobilization associated with LTB4 signaling was strongly inhibited in the presence of the specific BLT1 receptor antagonist LY223982, whereas responses to IL-8 were not diminished (Fig. 2A). Mobilization in response to lipids collected
from PAO1-infected epithelium was not impacted by the BLT1 receptor antagonist, suggesting that BLT1 is not required for HXA3-associated signaling in neutrophils.

In a similar experiment, bone marrow neutrophils were isolated from mice deficient for BLT1 (Ltb4r1^−/−). Wild-type mice responded to all chemotactic signals with calcium mobilization (Fig. 2B). As expected, mice lacking the BLT1 gene were incapable of responding to LTB4 signaling, but were fully competent in responding to PAO1-induced epithelial-derived lipid extracts. Taken together, these data suggest that BLT1 is not a necessary component of HXA3 signaling, and therefore BLT1 may play a role in complementing HXA3 signaling to drive neutrophil transepithelial migration.

HXA3 signaling is a conserved mechanism in a mouse in vitro model of transepithelial migration

Previous work has demonstrated an important role for HXA3 in neutrophil transepithelial migration in mouse models. HXA3 has been detected in response to Pseudomonas infection (41), and disruption of HXA3 signaling significantly disrupts neutrophil migration in models of gastrointestinal (13) and pulmonary inflammation (14) in mice. We adapted our transwell migration model to a fully mouse in vitro model to leverage available genetic tools and explore the functional implications of leukotriene signaling in an HXA3-mediated migration model. Briefly, the mouse lung epithelial cell line MLE12 was grown as inverted monolayers on transwell supports. As a source of neutrophils, whole primary mouse bone marrow was supplied to the basolateral compartment. Bone marrow neutrophils have previously been shown to be a functionally competent model of neutrophil migration (45). Neutrophil transmigration was measured by myeloperoxidase activity as well as flow cytometry (Fig. 3). Flow cytometry analysis of the apical compartment demonstrated that bone marrow neutrophils were selectively enriched in response to Pseudomonas infection as well as imposed gradients of LTB4 and C5a (Fig. 3A, 3B), supporting the functional specificity of the transmigration model. Myeloperoxidase was readily detectable in apical compartments following migration to infection and chemotactic gradients (Fig. 3C).

We wanted to determine whether disruption of HXA3 signaling pathways was conserved in our in vitro mouse model. First, we targeted biosynthesis of HXA3 in epithelial cells with the

**FIGURE 1.** PAO1-induced transepithelial neutrophil migration is disrupted by antagonism of the LTB4 receptor BLT1. (A) A model schematic of transepithelial neutrophil migration using lung epithelia grown on inverted transwells is shown. (B) Relative migration to PAO1-induced signals as well as control chemoattractants was quantified by a myeloperoxidase assay. (C) Migration to PAO1-induced signals or control gradients was assayed in the presence of an apical gradient of LY223982. Data are represented as migration as a percentage of vehicle control. Significance values were calculated by ANOVA. **p < 0.01**, **S** **p < 0.001. (D) H292 monolayers grown on inverted transwells were treated with pharmacological inhibitors to 12-lipoxygenase (CDC), 5-lipoxygenase (zileuton), or 5-lipoxygenase–activating protein (MK886) prior to infection or the addition of neutrophils. Data are shown as a percentage of migrating cells with respect to vehicle controls. All data are represented as means ± SD and are representative of multiple independent experiments.
12-lipoxygenase inhibitor CDC. CDC treatment of the epithelium significantly reduced migration in response to *Pseudomonas* infection, but not a LTB4 gradient (Fig. 4A).

In addition to targeting HXA3 biosynthesis with the use of pharmacological inhibitors, HXA3 signaling can be modified by directly degrading HXA3 with sEH (41). An epoxide ring is a unique structural feature of HXA3, and its hydrolysis converts HXA3 to the nonchemotactic degradative product trioxilin A3 (46, 47). Incubation of the apical compartment with sEH during migration was sufficient to significantly impair neutrophil migration (Fig. 4B). To further validate sEH targeting of HXA3 lipids, we extracted and concentrated the lipid fraction from infected and uninfected MLE12 monolayers. These extracted lipids were then assayed for their chemotactic bioactivity following incubation with soluble epoxide hydrolase (Fig. 4C). Incubation of lipid extracts with sEH significantly reduced their chemotactic bioactivity (Fig. 4D).

This PAO1-induced chemotaxis was not affected when the epithelium was incubated with selective inhibitors for either 5-lipoxygenase (Fig. 4E) or cyclooxygenase-2 (Fig. 4F). Taken together, these data suggest that bacterial-induced, HXA3-dependent neutrophil transepithelial migration is conserved in this murine in vitro model.

**Neutrophil-derived LTB4 signaling plays a significant role in HXA3-mediated migration**

Having established that epithelial-derived signaling in our mouse in vitro model is consistent with prior work, we investigated the role of LTB4 signaling by neutrophils in response to HXA3-mediated migration. Ltb4r1−/− neutrophils were severely deficient in migrating to gradients of LTB4 and were not significantly distinguishable from buffer controls (Fig. 5A). PAO1-induced migration was also severely impaired using Ltb4r1−/− bone marrow, but responses were significantly higher than either LTB4 responses or buffer controls (Fig. 5A, inset). This suggests that BLT1 is not absolutely necessary for PAO1-associated migration, but that it plays a key role in amplifying the neutrophil response to such stimuli. Interestingly, we saw a similar, although less pronounced, BLT1 dependency in C5a-mediated migration, suggesting that this is a mechanism that may be broadly used by neutrophils to amplify selected chemotactic signals.

Neutrophils are capable of releasing a variety of inflammatory factors following stimulation, including LTB4 (48). We sought to determine whether neutrophil-derived LTB4 was responsible for the BLT1-dependent migration we observed. We tested bone marrow isolated from 5-lipoxygenase–deficient mice (Alox5−/−) for their ability to respond to PAO1-induced signals (Fig. 5B). We again found a significant deficit in PAO1-induced and C5a-mediated migration, further implicating a role for neutrophil-generated LTB4 in transepithelial migration to some chemotactic signals. Alox5−/− bone marrow was fully responsive to gradients of LTB4.

Neutrophil-associated 5-lipoxygenase activity was also important in mediating robust chemotaxis in response to PAO1 infection in the human in vitro model. Human neutrophils were treated with either zileuton (Fig. 5C) or MK886 (Fig. 5D). Unlike drug treatment of the epithelium (Figs. 1D, 4E, 4F), targeting the neutrophil...
directly significantly impaired the ability of neutrophils to respond to PAO1 infection. Notably, neither LTB4- nor IL-8–mediated migration relied on 5-lipoxygenase–associated amplification for robust migration in this model. Finally, treatment of neutrophils with the 12-lipoxygenase inhibitor CDC had no significant effect on migration (Fig. 5E), in contrast to the significant inhibitory effect of CDC when pretreating the epithelium (Fig. 1D). Taken together, these data strongly suggest that neutrophil-associated 5-lipoxygenase activity is critical for a robust response to epithelial-derived HXA3.

Polymorphonuclear neutrophil–derived LTB4 contributes to chemotactic bioactivity in HXA3-induced migration

We suspected that migrated neutrophils likely produced LTB4 to amplify initial HXA3 chemotactic signaling. To test whether polymorphonuclear neutrophil (PMN)–derived LTB4 was detectable in apical compartments and amplified migratory responses to HXA3, we repeated our transepithelial migration assay in the presence and absence of neutrophils (Fig. 6A). First, we assayed this initial apical supernatant for the presence of LTB4, and C5a was also analyzed. Cells migrating to LTB4 (lower left) and C5a (lower right) were also analyzed. Cells were initially gated on CD45 to exclude cells of epithelial origin and other debris, then gated on the Ly6G.

(B) Percentage of neutrophils among CD45+ cells in the apical chamber following migration to various chemotactic stimuli was calculated. Data represent six pooled individual wells and are representative of at least three replicate experiments. (C) Relative migration was measured by myeloperoxidase assay, shown as means ± SD, and is representative of at least three independent experiments.

LTB4 release was much more strongly induced in response to PAO1-associated signals (Fig. 6C). The presence of LTB4 was confirmed by LC-MS/MS. LTB4 detected in neutrophil-containing samples migrating to PAO1 infection totaled 189.6 ± 30.5 pg/ml. No LTB4 was detected in response to mock infection or in the absence of neutrophils.

Apical supernatant was also collected and lipid components were extracted as before to assay their bioactivity. Previous lipid preparations (Figs. 2, 4D) were prepared in the absence of neutrophils and represented lipids exclusively produced by the epithelium. In this case, the apical supernatant extract contains lipids from both epithelium and migrated neutrophils. Strong migratory bioactivity was detected in samples collected from wells containing neutrophils that had migrated to PAO1-induced signals (Fig. 6D). Notably, whereas IL-8 recruited significantly more neutrophils in our assay, these neutrophils did not produce lipid-associated bioactivity above buffer background. This suggests that IL-8 does not induce significant levels of LTB4 in this model of transepithelial migration. Notably, we were able to detect a small amount of lipid bioactivity from PAO1-infected transwells lacking neutrophils (Fig. 6D, inset). This activity likely represents epithelial HXA3.

In addition to infection-induced neutrophil transepithelial migration experiments, we also performed similar experiments in which the epithelium was not infected, but instead lipid prepara-
tions from mock- or PAO1-infected epithelial cells were provided (Fig. 6E, 6F). These lipids were prepared in the absence of neutrophils. LTB4 was not detectable in lipids collected from epithelial cells in response to PAO1 or mock infection (Fig. 6E). When the HXA3-enriched lipids collected from infected epithelium are used as a chemotactic gradient, we observe heightened LTB4 generating capacity from migrating neutrophils compared with the response to a gradient of IL-8. Thus, the amplifying factor is preserved by lipid extraction of epithelial signals.

Lastly, we measured LTB4 levels from apical supernatants containing migrated neutrophils that had been treated with zileuton or vehicle control (Fig. 6G). We confirmed that zileuton treatment of neutrophils significantly reduced the amount of LTB4 in apical supernatants, which is consistent with the observed reduction in migration of zileuton-treated neutrophils (Fig. 5C).

**FIGURE 4.** PAO1-induced transepithelial migration in an MLE12 transwell model relies on HXA3 and 12-lipoxygenase signaling. (A) Relative migration was quantified after MLE12 monolayers were incubated with the 12-lipoxygenase inhibitor CDC at multiple concentrations prior to infection. (B) sEH was added to the apical compartment of transwells at multiple doses during migration to assess interference with neutrophil migration. (C) A schematic of lipid extraction and assessment of lipid chemotactic bioactivity is shown. (D) Lipids collected from MLE12 epithelium were incubated in the presence of sEH (100 μg/mL) or vehicle control for 2 h prior to evaluating their bioactivity. Lipids were serially diluted to evaluate bioactivity within a functional window. MLE12 transwells were incubated in the presence of zileuton (E) and NS398 (F) at multiple doses prior to infection. Relative migration was calculated as a function of total myeloperoxidase activity following migration. Data are represented as means ± SD and are representative of multiple independently run experiments. *p < 0.05, **p < 0.001.

LTB4 amplification skews recruitment in favor of BLT1-competent PMNs

Our data suggest that neutrophils migrating across PAO1-infected epithelial monolayers respond by secreting LTB4, thereby amplifying chemotaxis and increasing the magnitude of the responding population of neutrophils. We hypothesized that this network effect could be more directly observed using mixed bone marrow migration assays. Briefly, two genotypes of bone marrow were differentially stained with CFSE and mixed in equal proportions. The mixed population was used as a source of neutrophils in our migration assay. Following a 2-h migration, the apical compartment was sampled to determine the proportion of each population, the total migration, and total LTB4 levels.

The first combination was a mix of wild-type and Alox5−/− bone marrow. We did not observe preferential migration of either population in response to either infection with PAO1 or gradients of LTB4 or C5a (Fig. 7A). We also observed very strong migration to all three chemotactic signals (Fig. 7D). This is in contrast to the poor migration of Alox5−/− neutrophils alone (Fig. 7D). The presence of wild-type bone marrow rescues the ability of 5-lipoxygenase–deficient neutrophils to respond. This suggests that the leukotriene produced by wild-type neutrophils is capable of recruiting both genotypes of neutrophils. Indeed, we observe strong LTB4 levels in the apical compartment following migration of wild-type and Alox5−/− bone marrow mixtures (Fig. 7E).

We then migrated a combination of wild-type and Ltb4r1−/− bone marrow. As expected, in all cases Ltb4r1−/− neutrophils were deficient in responding to the chemoattractants we tested (Fig. 7B). Ltb4r1−/− neutrophils had virtually no detectable response to LTB4. Ltb4r1−/− neutrophils also represented a very
small proportion of the cells responding to either infection or C5a gradients, resulting in a population heavily skewed toward wild-type neutrophils. This skewing in favor of wild-type neutrophils in PAO1-induced and C5a gradients was significantly lower than in response to LTB4 gradients, suggesting that Ltb4r1<sup>−/−</sup> neutrophils functionally respond to their primary gradients, but do not respond to amplifying signals. In all cases the relative migration of this combination of genotypes is significantly higher than the migration of Ltb4r1<sup>−/−</sup> neutrophils alone, although much lower than either wild-type alone or the combination of wild-type and Alox5<sup>−/−</sup> (Fig. 7D). In the case of PAO1 infection and C5a gradients, this likely reflects the limited number of neutrophils capable of responding to amplifying signals, despite the significant level of LTB4 present in the apical space (Fig. 7E).

Lastly, we combined the two knockout genotypes, Alox5<sup>−/−</sup> and Ltb4r1<sup>−/−</sup>. We observed a skewing phenotype in favor of Alox5<sup>−/−</sup> neutrophils similar to the skewing in favor of wild-type neutrophils when either is combined with Ltb4r1<sup>−/−</sup> neutrophils alone, although much lower than either wild-type alone or the combination of wild-type and Alox5<sup>−/−</sup> (Fig. 7C). Predictably, Alox5<sup>−/−</sup> neutrophils were nearly all of the neutrophils responding to LTB4 gradients, but they also represented ∼80–90% of the migrating population to both PAO1 infection and C5a gradients. The migratory response is elevated compared with either knockout genotype alone and similar to the response of the wild-type/ Ltb4r1<sup>−/−</sup> combination (Fig. 7D). We also detected significant LTB4 levels in these wells (Fig. 7E). Taken together, these data suggest that both genotypes can respond to PAO1 infection and C5a gradients, Ltb4r1<sup>−/−</sup> neutrophils, once migrated, produce LTB4 and recruit Alox5<sup>−/−</sup> neutrophils, leading to a migrating population dominated by Alox5<sup>−/−</sup> neutrophils.

**Discussion**

A thorough understanding of the mechanisms and pathways of neutrophil recruitment across mucosal epithelium is of immense translational value. In the airspace alone, neutrophils play key clinical roles in the etiology of CF, bacterial pneumonia, acute respiratory distress syndrome, chronic obstructive pulmonary disease, and severe asthma, among others. Intervention strategies that manipulate the magnitude and severity of the neutrophil responses have long been recognized as key therapeutic tools in limiting neutrophil-mediated pathology (6).

HXA3-mediated chemotaxis is a necessary step in neutrophil transsepithelial migration (10, 12). HXA3 production is unique in that it is directed into the lumen of mucosal spaces, and thus it is relatively distal from the bloodstream. Its immunological niche necessitates collaboration with other signals in a chemotactic cascade. Sampling of the mucosal lumen during inflammatory events often reveals the presence of many chemoattractants that may not provide appropriate context for their ultimate role in chemotaxis. In an effort to distinguish the role HXA3 plays from the many other relevant chemoattractants, we relied on a well-described inverted transwell model system. Our immediate observation was that we were unable to disentangle HXA3 signaling from BLT1 receptor activity. Blockage of the BLT1 receptor significantly and persistently impacted the magnitude of PAO1-induced migration. This was not a result of epithelial LTB4 production. We were unable to detect LTB4 in epithelial supernatants in the presence or absence of infection, and manipulation of LTB4 biosynthetic pathways in epithelial cells had no impact on migration.

The HXA3 receptor is currently unknown, but HXA3 signaling relies on a GPCR and induces calcium mobilization in neutrophils (9, 10). We investigated whether we were able to block HXA3 signaling by either antagonizing BLT1 or using BLT1-deficient neutrophils. We were able to efficiently block LTB4 signaling in neutrophils without diminishing responses to control stimuli or HXA3-enriched lipids. These samples can be experimentally
paired with mock-infected lung epithelial supernatants, which do not contain HXA3, to gain insight into HXA3 signaling (41, 49). This maneuver is necessitated by limited availability of commercial sources of purified HXA3. Although we cannot dismiss the possibility of an unknown infection-induced lipid source of calcium signaling, these results suggest that BLT1 antagonism is not directly impacting HXA3 signaling pathways, but likely plays an indirect role in this system.

To further leverage genetic tools in exploring this mechanism, we developed a parallel murine in vitro model. Whole bone marrow was used in place of isolated neutrophils to functionally demonstrate the selective migration of neutrophils. In all conditions, the responding populations were overwhelmingly Ly6G+ neutrophils and were readily detectable by myeloperoxidase assay. In vivo mouse models have repeatedly demonstrated HXA3 and HXA3 signaling pathways as being a relevant and even critical pathway for neutrophil transepithelial recruitment (13, 14, 41). In this model, Pseudomonas-induced PMN transepithelial migration was also significantly reduced by targeting HXA3-specific pathways at the epithelium. Inhibition of HXA3 biosynthesis effectively reduced migration in this model, as in previous models. Meanwhile, inhibition of control eicosanoid synthesis pathways 5-lipoxygenase and cyclooxygenase-2 had no impact. Direct degradation of HXA3 using soluble epoxide hydrolase (41, 47) also effectively reduced PAO1-induced migration. Notably, SEH degradation of HXA3 was effective when targeting the apical space.

**FIGURE 6.** Neutrophils respond to PAO1 signals with significant upregulation of LTB4. (A) A schematic model of the workflow evaluating production of LTB4 and lipid-based bioactivity is shown. (B) The apical compartment was sampled after PMN or mock migration to PAO1 infection, IL-8 gradient, or HBSS controls. Samples were assayed for the presence of LTB4 by enzyme immunoassay (EIA). Relative migration of PMN-containing wells was also measured by myeloperoxidase and is shown as the inset. (C) LTB4 concentration of the apical compartment with neutrophil migrated samples was divided by relative neutrophil migration and normalized. Data are represented as LTB4 concentration of apical compartment as a function of relative neutrophil number. (D) Apical supernatant was collected from PAO1-infected, IL-8–migrated, and HBSS control wells with and without neutrophils. This supernatant was extracted for lipid components and then evaluated for chemotactic bioactivity. Neutrophil-containing conditions are represented with circles. The inset shows conditions where no neutrophils were provided and are represented with triangles. Significance was calculated by ANOVA. (E) The apical compartment was sampled following migration to PAO1-infected lipid preparation, IL-8 gradient, or mock-infected lipid preparation. Lipid samples were also directly sampled. LTB4 levels were measured by enzyme immunoassay. Relative migration of PMNs was also assayed by myeloperoxidase and is shown as the inset. (F) LTB4 concentration of lipid-migrated samples was divided by relative neutrophil migration and normalized. Data are represented as LTB4 generated by relative neutrophil migration. (G) Transepithelial migration was performed using zileuton-treated neutrophils, vehicle-treated neutrophils, or without neutrophils. The apical compartment was sampled following migration and assayed for the presence of LTB4 by enzyme immunoassay. Data are presented as means ± SD. Data are representative of multiple independent experiments. *p < 0.05, **p < 0.001. L.D., limit of detection of the assay.
directly and may provide insight into the potential of therapeutically targeting the airway to modulate neutrophilic pathology. Taken together, these observations confirm that HXA3 signaling is a conserved pathway in this mouse model.

HXA3-mediated chemotaxis has not previously been associated with LTB4 amplification. In both human and murine models, neutrophils deficient in either BLT1 signaling or 5-lipoxygenase activity were severely impaired in their ability to respond to PAO1 infection. Notably, migration to HXA3-associated signals was not entirely eliminated. PAO1-induced migration of Ltb4r1−/− and Alox5−/− neutrophils was above background. Inhibition of LTB4 biosynthetic pathways in human neutrophils produced similar results, whereas inhibition of neutrophil 12-lipoxygenase had no significant effect. These observations nicely integrate HXA3 with the existing body of literature. Neutrophil-derived LTB4 functions as a signal relay for neutrophil localization in many models (21, 27, 48, 50), and it functions to orchestrate neutrophil recruitment in models of rheumatoid arthritis (29, 51).

Release of LTB4 into the apical space was detectable when 5-lipoxygenase–competent neutrophils migrated, and it served to significantly enhance lipid-associated bioactivity in these preparations. LTB4 levels did not correlate well with total neutrophil migration in response to all chemotactic stimuli, suggesting that this is not a general response by neutrophils that have migrated. By correcting the total LTB4 levels detected for the relative number of neutrophils quantified by myeloperoxidase, it is evident that Pseudomonas-induced migration was associated with significantly higher levels of leukotriene production than IL-8 gradient–induced migration. Therefore, neutrophils do not blindly function to amplify their own recruitment, but they rely on signals that may be independently modulated. Both fMLP and C5a have been shown to stimulate LTB4 production in neutrophils (48), and in the present study we see LTB4 production in response to HXA3–associated migration. In this model system, neutrophils are selectively recruited across the epithelium to apically directed gradients of HXA3 and LTB4. We cannot exclude the possibility that small

FIGURE 7. LTB4 generation rescues 5-lipoxygenase deficiencies, but not BLT1 deficiencies. Transepithelial migrations were performed using mixed bone marrow populations in response to infection, as well as LTB4 and C5a gradients. Each bone marrow population was differentially labeled with CFSE prior to migration and mixed at a 1:1 ratio. After migration, the apical compartment was sampled and analyzed by flow cytometry to determine the relative proportion of each population. Combinations of (A) Alox5−/− with wild-type bone marrow, (B) wild-type with Ltb4r1−/− bone marrow, and (C) Alox5−/− with Ltb4r1−/− bone marrow was mixed to assess paracrine signaling effects. (D) Myeloperoxidase and (E) LTB4 levels were also measured from apical compartments following migration. Representative histograms, as well as quantified enrichment scores from at least three replicated experiments, are shown. Histograms are gated on Ly6G+ neutrophils. Relative migration and LTB4 levels are shown as means ± SD. *p < 0.05, **p < 0.001.
contaminating populations of cells such as lymphocytes, monocytes, or other granulocytes may also release some LTB4, but such LTB4 production would need to predominantly occur in the apical compartment to reinforce the directed gradient. In vivo, resident immune populations may actively play a role in neutrophil recruitment. Further study is needed to transition these observations to account for the complexity of the lung environment in vivo.

It is not clear whether HXA3 signaling directly induces LTB4 production or whether other factors associated with the epithelial response are necessary. This inducing factor may be of bacterial or epithelial origin, but it must persist through washing of bacteria and preparation of lipid extracts. Ultimately a better understanding of LTB4 triggering mechanisms will require studies employing a purified source of HXA3. What is evident is that LTB4 is a critical component extending and expanding the response to HXA3 migration.

When assayed in isolation, neutrophils lacking key LTB4 signaling components were unable to migrate effectively. In combination with other genotypes, we are able to see that a relatively small proportion of migrated neutrophils can compensate for signaling deficiencies. When paired with wild-type neutrophils, Alox5−/− neutrophils respond equally well, and the magnitude of the migratory response is comparable to wild-type alone. Alox5−/− neutrophil migration is effectively compensated for by having a source of LTB4 BLT1 deficiency, alternatively, is not readily compensated for, and the result is a migratory response heavily skewed in favor of BLT1-competent neutrophils. This pattern is true even in the context of Alox5−/−/Ltb4r1−/− bone marrow mixes. HXA3, as well as C5a, is capable of recruiting an initial wave of neutrophils. 5-Lipoxygenase–competent cells then produce LTB4 and recruit BLT1-competent cells to amplify the magnitude of the response. This compensation of genetic deficiency underscores the importance of neutrophil-to-neutrophil communication in mediating transepithelial migration.

Many current therapies rely on targeting neutrophil function in hopes of reducing morbidity associated with progressive lung damage (6). Nonsteroidal anti-inflammatories such as ibuprofen and BLT1 antagonists are targeted systemically with the goal of reducing neutrophil migration to the airspace (2, 52). Therapeutic doses are often associated with adverse events and poor adherence. Corticosteroid therapy is also effective at preventing lung damage in CF by limiting neutrophil migration, but it is of limited value due to similar side effects (53). A strategy of limiting neutrophil migration to sites of disease has clear therapeutic value, but adverse events are often associated with systemic immunological disruption that is inherently problematic. Targeting therapies to the mucosal surface may provide the benefits of reducing airway pathology without broadly suppressing immunity. Our in vitro model suggests that both degradation of HXA3 and antagonism of BLT1 are effective at limiting neutrophil migration when targeted to the apical space. Further understanding of the mechanisms leading to mucosal breach of neutrophils during inflammatory insults and disease can lead to the development of novel therapeutic options for patients with CF. Acute respiratory distress syndrome, pneumonia, chronic obstructive pulmonary disease, and other neutrophil-driven pulmonary insults. These observations are likely applicable to mucosal surfaces of the gastrointestinal tract, further expanding the impact to patients with any number of neutrophilic bowel disorders. Continued attention to the mechanisms driving mucosal breach of neutrophils may provide avenues for the treatment of disease associated with neutrophil-mediated pathology and opportunities to alleviate associated morbidities.

Acknowledgments
We thank Matthew Greenwood for technical assistance with LC-MS/MS analysis.

Disclosures
The authors have no financial conflicts of interest.

References


