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The role of LTB4 in neutrophil-mediated inflammation in airway epithelium infected with pseudomonas

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THE ROLE OF LTB4 IN NEUTROPHIL-MEDIATED INFLAMMATION IN AIRWAY EPITHELIUM INFECTED WITH PSEUDOMONAS

by

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DEDICATION

I would like to dedicate this work to my family and friends who have supported me throughout this learning experience and have kept me going when times were tough. Especially during this time where we are kept physically apart, they have kept me together.

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I would like to acknowledge Dr. Bryan Hurley and his lab, including Dr. Tian Lin, Brittany Wilson-Mifson, Sujatha Thundivalappil, and Dr. Eliana Drenkard. Without them, this thesis would not have been possible. I further acknowledge my thesis readers who have put the time and effort into reviewing this paper. I would also like to acknowledge the Boston University Graduate Medical Sciences faculty who taught and mentored me throughout my time here.

THE ROLE OF LTB4 IN NEUTROPHIL-MEDIATED INFLAMMATION IN AIRWAY EPITHELIUM INFECTED WITH PSEUDOMONAS

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ABSTRACT

Inflammation plays a protective function in the immune system but is also a contributor of many diseases. Airway inflammation can be the result of excessive infiltration of leukocytes, specifically neutrophils, leading to tissue damage or death. A proposed mechanism of the lipoxygenase pathway by Tamang et al. in 2012 showed Hepoxilin A_3, a neutrophil chemoattractant, can trigger the transmigration of neutrophils across airway epithelium. Activated neutrophils further produce another chemoattractant called leukotriene B4 (LTB4) to amplify the neutrophil migration to the airspace in the lungs. We investigated the role of LTB4 in neutrophil-mediated inflammation in airway epithelial infected with *Pseudomonas* by using a mouse airway transitional epithelial model cocultured with mouse neutrophils. We first established and assessed the *in vitro* coculture model using immunostaining and confocal microscope imaging to characterize the differentiation of the epithelium and RT-qPCR to investigate the gene expression of critical enzymes in the lipoxygenase pathway. Then we performed neutrophil transmigration assays *in vitro* using wild type C57BL/6 or alox5$^{-/-}$ as bone marrow polymorphonuclear cells (PMNs) cocultured with transitional airway epithelial cells. Myeloperoxidase (MPO) ELISA was performed to measure the amount of transmigrated neutrophils. Our results suggested that LTB4 had the potential of being a critical amplifier with higher migration percentages seen in C57 PMNs compared to alox5$^{-/-}$

PMNs. This was further tested in a mouse pneumonia model with wild type C57 and alox5$^{-/-}$ mice which showed a statistically non-significant difference in neutrophils transmigration to the air space in the lung based on FACS analysis, MPO and elastase 2 (ELA2) ELISA's, and lactate dehydrogenase (LDH) and LTB4 assays. *In vitro* results suggest that LTB4 has the potential in being targeted as a critical mediator and amplifier in neutrophil-mediated inflammation in airway epithelial infected with *Pseudomonas,* but further research is needed to investigate its role *in vivo*.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ALOX	Arachidonate Lipoxygenase
BAL	Bronchoalveolar Lavage
BU	Boston University
CO_2	Carbon Dioxide
DMEM/F-12	Dulbecco's Modified Eagle Medium/Modified Nutrient Mixture F-12
ELISA	Enzyme-linked Immunoassay
HBSS	Hank's Balanced Salt Solution
HXA3	Hepoxilin A_3
KO	Knock-Out
LDH	Lactate Dehydrogenase
LTB4	Leukotriene B4
MPO	Myeloperoxidase
PA	Pseudomonas Aeruginosa
PBS	Phosphate Buffered Solution
PMN	Polymorphonuclear (cells)
ROCK	Rho-associated Protein Kinase
RPM	Rotations Per Minute
RT-qPCR	Quantitative Reverse Transcription PCR
TGFB	Transforming Growth Factor Beta
WNT	Wingless/Integrated

INTRODUCTION

Immunity and the Innate System

Immunity is the capability of the host to defend against invading pathogens or remove toxins. The immune response can be divided into two different systems: the innate system and the adaptive system. While the adaptive system can take days to be effective, the innate system acts immediately to attempt to control the stimulus causing the response (Medzhitov & Janeway, 2000). Inflammation is an essential mechanism of the innate immune system as it is used to signal for healing and repair while defending against invading pathogens (Medzhitov & Janeway, 2000).

Inflammation

Inflammation is an important component in the host's immune response to stimuli such as infections, trauma, toxins, and loss of blood. It is a protective response that kills invading pathogens and clears necrotic tissue, but is also one of the main contributors in many diseases such as atherosclerosis, cancer, autoimmunity, and infections (Netea et al., 2017). The five main classifying characteristics of inflammation are redness (*rubor),* warmth *(candor)*, pain *(dolar)*, edema *(tumor),* and loss of function *(functolaesa)* (Netea et al., 2017). Overall, this process can cause physiological harm when left prolonged and uncontrolled.

Inflammation is initially induced when the host cells are stimulated by either endogenous stress signals known as damage-associated molecular patterns (DAMPs), stimulated by or pathogen-associated molecular patterns (PAMPs) by pattern recognition

receptors (PPR's) (Netea et al., 2017). The process of inflammation is complicated with a network of cascades of cytokine and chemokine release and interactions that can be amplified or inhibited. These cascades can contain mediators that aid with the amplification or inhibition process, but can quickly be destroyed or removed, ultimately changing the effect of the pathway.

Inflammation can be classified based on how long its effects are stimulated and can be categorized into two groups: acute and chronic. Acute inflammation is rapidly induced by tissue damage due to trauma, pathogens, or harmful compounds (Pahwa et al., 2022). Symptoms of acute inflammation can last for a few days and can progress into subacute inflammation lasting two to six weeks (Pahwa et al., 2022). Eventually it can progress into chronic inflammation lasting several months to a year and can vary based on the cause and ability for the body to overcome the damage done (Pahwa et al., 2022).

Further research into the cellular and molecular mechanisms of acute inflammation induced by infections can help find therapeutic targets to treat diseases caused by harmful uncontrolled or overactive inflammation. Specifically, this paper will focus on airway inflammation.

Airway Epithelial Cells and Inflammation

Inflammation can have a variety of effects on the airway and lungs. In general, acute inflammation involves vasodilation and increased blood flow. The microvascular changes allow for infiltration of leukocytes and accumulation of proinflammatory proteins and cells. However, unresolved inflammation can cause tissue damage due to

excess neutrophil and other leukocyte influx which can release free radicals (Robb et al., 2016). Examples of this inflammation in lung tissue include asthma and cystic fibrosis.

Many factors have been associated to affect airway inflammation, including disease, energy, neighborhood, stress, microenvironment, and seasonal changes (Aghasafari et al., 2019). It is also critical to study how airway inflammation can be mediated by interactions between airway epithelial cells and its environment. Although airway epithelium is specialized with a diverse set of pseudostratified cells, they have similar defense responses (Manicone, 2009). Airway epithelial lining is considered the first line of defense, being the first physical barrier pathogens encounter. Airway epithelium can also regulate leukocyte influx by adjusting chemokine gradients and producing proinflammatory cytokines or chemokines (Manicone, 2009). The main leukocyte of interest are neutrophils as they are the main innate immune cell that show excessive infiltration in many inflammatory lung diseases (Tamang et al., 2012).

Neutrophils can be described as a versatile cell unit which can change from a pro-inflammatory cell to an anti-inflammatory cell by switching what mediators are being produced (Serhan, 2007). For example, following a successful inflammatory response, neutrophils stop producing leukotriene B4 (LTB4), a pro-inflammatory mediator, to pro-resolving bioactive lipids before undergoing apoptosis (Serhan, 2007).

Neutrophil migration and transmigration in airway epithelium

Neutrophils are one of the main immune cells that target the source of inflammation and are recruited within the first few hours. They transmigrate across the

endothelial cell layer and accumulate on the site of tissue injury or infection to eventually eliminate the origin of inflammation (Wagner & Roth, 2000).

Mechanisms of neutrophil migration in the airway have been heavily researched in recent years. It is a complex phenomenon dependent on site of migration, cell type, cellular mediators, and initial inflammatory stimuli (Wagner & Roth, 2000). It is generally accepted that neutrophil transmigration is a multistep process involving sequential activation of adhesive proteins on both the neutrophil and endothelial cells (Wagner & Roth, 2000). Migration begins with the vessel wall "capturing" neutrophils from blood flowing through, then "rolling" along the vessel wall (Wagner & Roth, 2000). However, a more recent study claims that because the diameter of pulmonary capillaries are smaller than neutrophils, they are unable to roll and are forcefully shaped before finding a suitable migration site (Maas et al., 2018). With the correct stimuli, neutrophils then become firmly adhered to the endothelium to which they migrate into tissue.

Although much research has been focused on the migration of neutrophils in blood vessels, mechanisms into neutrophil transmigration has been limited. Along with adhesion and migration to the vascular walls, studies show PMN-derived proteases are released to facilitate extravascular transit (Wagner & Roth, 2000). However, it has been observed that mice deficient in PMN-derived collagenase show normal emigration into skin, peritoneum, and lungs (Betsuyaku et al., 1999). Another study investigated the different mechanisms of neutrophil transmigration and found that higher migration occurred in the physiologic basolateral-to-apical direction, and that neutrophil migration was strongest when induced by chemoattractants (Liu et al., 1996).

Overall, neutrophil transmigration is controlled by different processes and more research into the mechanisms that drive this process can help target areas of therapeutic interest. One therapeutic target has been inhibitors of the production of eicosanoids, which have been used in inflammation treatment (Calder, 2020).

Eicosanoids and Hepoxilin A_3

Eicosanoids are a group of lipid mediators that perform various biological functions. They have been shown to have roles in regulation of vascular, renal, gastrointestinal, immunity, and inflammation (Calder, 2020). Eicosanoids are mainly produced from liberated arachidonic acid from membrane phospholipids (Tamang et al., 2012). This includes leukotrienes, prostaglandins, thromboxanes, and lipoxins. During the inflammatory process, eicosanoids that are a chemoattractant can be produced to trigger chemotaxis. A potent chemoattractant of interest is Hepoxilin A_3 (HXA3), which studies have shown to drive neutrophil migration in lung epithelial barriers (Tamang et al., 2012).

A study done by Tamang et al. in 2012 showed that an increase in HXA3 production from epithelium occurred in response to *P. aeruginosa* infection and neutrophil chemotactic activity. The study also showed that pharmacological inhibitors that targeted the enzymatic pathway to produce HXA3 interfered with neutrophil transepithelial migration induced by infection. Ultimately, HXA3 is necessary to trigger chemotaxis of neutrophils to further enhance neutrophil transmigration via the lipoxygenase pathway. This pathway produces another strong chemoattractant to

neutrophils called leukotriene B4 (LTB4) and is mainly produced by neutrophils (Tamang et al., 2012).

Lipoxygenase Pathway and LTB4

LTB4 is one of the several cysteine leukotrienes generated from the lipoxygenase pathway. In 2012, Tamang et al. proposed a mechanism for this pathway for neutrophil transmigration across the airway mucosa. To initiate this pathway, infection of epithelial cells activates the phospholipase A2-beta gene (iPLA2β) to generate arachidonic acid from membrane phospholipids, which are used as a substrate to produce HXA3 with the activity of 12-lipoxygenase (12-LOX) that are encoded by either alox12 or alox15 genes. HXA3 triggers the initial migration of neutrophils across the airway epithelium. While the exact mechanism remains unclear, activated neutrophils use a different phospholipase A2 called cytosolic phospholipase A2 alpha (cPLA2α) enzyme which is activated via phosphorylation by mitogen-activated protein kinases (MAPKs) to generate arachidonic acid, the substrate for leukotriene A4 (LTA4) production with the activity of 5-lipoxygenase (5-LOX) encoded by alox5 gene. LTA4 is then converted to LTB4 by LTA4 hydrolase (LTA4H). Yonker (2017) confirmed that cPLA2α was necessary for a strong migration response and drives the release of LTB4. Inhibition of cPLA2α resulted in significant reduction in LTB4 release and total neutrophil migration (Yonker et al., 2017). LTB4 is a strong neutrophil attractant and can amplify its effects via autocrine pathway.

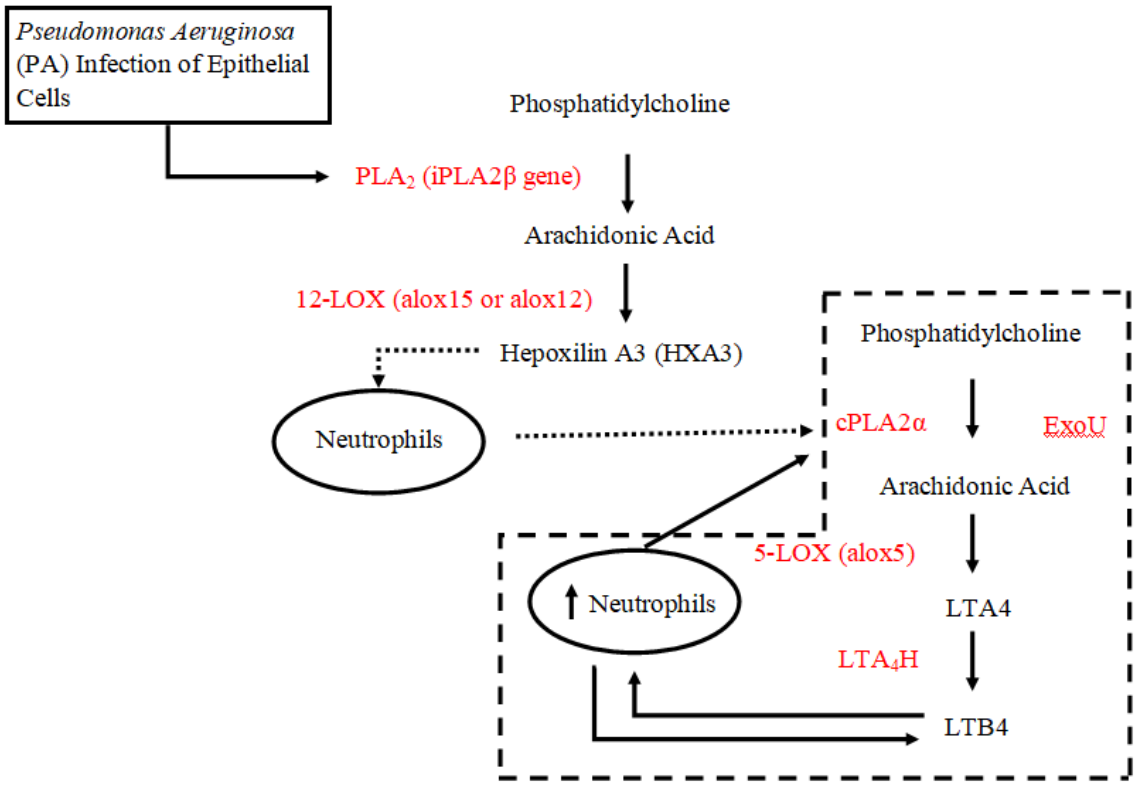


Figure 1. Lipoxygenase pathway of LTB4 production from neutrophils in airway mucosa with *Pseudomonas infection.* Proposed by Tamang et al. (2012) and supported by Yonker et al. (2017).

A study done by Lämmermann et al. in 2013 showed that LTB4 is critical in mediating neutrophil recruitment and note that LTB4 comes from several cellular sources in inflamed tissue. They suggest that the initial stages of neutrophil recruitment are more likely associated with neutrophil derived LTB4. The study also showed that neutrophils that lack the receptor for LTB4 have an impaired migration response, and there was a reduction in migration distance in neutrophils from 5-lipoxygenase-deficient mice (alox5$^{-/-}$) when compared to the wild type mice. This implicates that LTB4 plays roles in recruitment and transmigration of neutrophils.

In our studies, we investigated the role of LTB4 in neutrophil transmigration across airway epithelium by using *in vitro* culture of airway epithelial cells and *in vivo* acute pneumonia model of mice infected with *Pseudomonas.*

Clinical Significance

Research into airway inflammation has been a growing topic of interest, especially with the emergence of SARS-CoV-2. Studying the mechanisms behind airway inflammation has been critical in research of new treatments. Pharmacologic manipulation of airway inflammation has been focused on interactions between PMNs and airway epithelium. This could be further expanded by looking into manipulation of neutrophil transmigration and the lipoxygenase pathway.

Clinical research into how the lipoxygenase pathway can be used to treat certain airway inflammatory disease has been done. One study done by Gelfand in 2017 showed that drugs targeting cysteinyl leukotriene receptor 1 yielded some effectiveness in certain situations. Drugs that also targeted the 5-lipoxygenase gene has also shown some success but was limited due to adverse drug effects. Another study focusing on treating cystic fibrosis by targeting the LTB4 receptor showed a decrease in inflammation, but an increase in the risk of pulmonary related serious adverse events (Konstan et al., 2014). This study reiterates that inflammation is not entirely a harmful process, and that the lipoxygenase pathway is critical in the inflammatory process.

Better understanding the mechanisms behind airway inflammation is crucial is effectively treating airway diseases. While research has continued to shed light on what

we know about airway inflammation, they have also presented new challenges in controlling this complicated process.

SPECIFIC AIMS

Inflammation plays a significant role in the immune system as a protective function, but also as a contributor of many diseases. Current research continues looking into better methods of controlling this process. Mainly looking into key steps in the inflammation pathways, specifically, the LTB4 pathway. Manipulation of the LTB4 pathway though alox5 has been shown to effect neutrophil migration and overall, the inflammation process. While past research supports this idea, studies into the pathogen mechanism is lacking. This includes how critical LTB4 is in this process and the characterization of its importance with *in vitro* models and further understanding its role with *in vivo* models.

This paper investigates the role of LTB4 in acute inflammation of airway epithelium due to *Pseudomonas Aeruginosa* infection. Specifically, using a transitional airway epithelium model characterized by gene expression to confirm the ability for transmigration. Using the model, perform neutrophil transmigration assays to analyze differences between wild-type and alox5 knock-out. This process is further investigated in an *in vivo* model using mice of both genotypes (wild-type and alox5 KO) and analyzing the inflammatory process with various assays from fluid obtained using bronchoalveolar lavage (BAL).

MATERIAL AND METHODS

Animals

The protocol for the use of the mouse models was in accordance with review and approval by the Animal Care and Use Committee of Massachusetts General Hospital. Male and female mice 6-20 weeks old were used to isolate bone marrow cells and trachea. For *in vivo* studies, female mice 8-12 weeks old were used and assigned to the same group for the same treatment based on the same sex, genotype, and an age difference of two weeks. Both wild type C57BL/6 and alox5$^{-/-}$ mice were purchased from Jackson laboratory (Bay Harbor, ME). Mice were maintained, bred, and weaned at around 28 days old in the animal facility at Massachusetts General Hospital (MGH).

Mouse airway basal cell isolation and expansion

Preparation of basal cell culture medium

The medium was prepared under a tissue culture hood and stored at 4°C: *Basal Cell Medium:* 500 mL of Basal Cell Media Kit C-21170 (Promocell), 1.0 µM TGFB antagonist A-83-01 (Torcris Bioscience), 5 µM Rho-associated protein kinase (ROCK) inhibitor Y-27632 (Fisher Scientific), 0.5 µM WNT agonist a8301 (Fisher), 2nM Rapamycin (Fisher Scientific-APExBIO), and 5 mL of Penicillin Streptomycin solution (Gibco^{TM}).

Isolation of mouse airway basal cells

Mice were euthanized by CO_2 right before the isolation procedure. The mice were sterilized using 70% ethanol and fixed onto a wax dissection tray. The trachea was bluntly isolated and cut at the bifurcation of the mainstem bronchi. After removal of the connective tissue and esophagus, cuts were made distal to the larynx and just above the tracheal bifurcation. The isolated trachea was put in a 60 mm x 15 mm petri dish in sterile PBS (Gibco™) and cut open longitudinally. It was then placed in a 2 mL tube containing digestion buffer (1 mL/trachea) and left on an orbital shaker at 150 RPM at 37°C for about 2-4 hours. Digestion buffer was made by adding the following gradients into 20 mL of DMEM/F12 (Gibco™): 0.5 mg/mL of Pronase (Sigma-10165921001), 0.5 mg/mL DNase I (Sigma-DN25), 1% Penicillin Streptomycin solution (Gibco™), and 5 µM ROCK inhibitor Y-27632 (Fisher Scientific). A small drop of supernatant was collected and placed on a glass slide to be checked under a light microscope. The isolated basal cells existed as special clusters mixed with other single cells like leukocytes. After removal of undigested tissue, the cell suspension was centrifuged at 500xg for 5 minutes. The supernatant was removed, and the sample was resuspended and centrifuged down twice in RPMI/10%FBS (Gibco™) to wash the sample. Finally, the sample was resuspended in 2-3 mL of basal cell medium and placed in a single well on a Corning 6-well tissue culture plate. The plate was then left in an incubator with 5% CO_2 at 37°C for 3-4 days, checking daily under a light microscope for cell adhesion to the bottom of the well. After 3-4 days, cells that had not adhered to were removed by aspiration. Fresh basal medium was added to the culture and replaced with new basal medium every 2-3

days until the cells grew to 80-90% confluency. Then the cells were trypsinized and passed onto a new Corning 6-well tissue culture plate.

Passing cells

The term "passing cells" refers to the process of transferring cells from one culture plate to another to prevent the cells becoming overconfluent/overgrown. The media was aspirated from a single well and washed by adding 2 mL of warm PBS and slightly swirling the plate before aspirating again. A solution of trypsin 0.25% (Gibco™) was added to cover the entire bottom surface (1 mL/well) and placed in the incubator for 15-20 minutes until the cells were detached (round and freely floating in medium). The trypsin was then neutralized by adding 1 mL of RPMI/10%FBS (Gibco™) and carefully transferred to a 50 mL tube. The tube was then centrifuged at 500xg for 5 minutes. The supernatant was carefully aspirated, and the pellet was resuspended in 12 mL of basal cell media. The tube was mixed well by inverting three times gently, and the cell suspension was added to each well onto a Corning 6-well tissue culture plate with 2 mL/well which was pre-coated. A 6-well tissue culture plate (Corning) was prepared by coating each well in 1 mL of a 1:30 dilution of rat tail Invitrogen collagen (Gibco™) in 0.1N filtered HCl (Sigma Aldrich) and washed with PBS. The culture medium was replaced with 2-3 mL fresh medium every 2-3 days. When the cells covered 80-90% of the bottom of the well, another passing was needed to maintain optimal culture. Passing could be done heavily with a 1:3 dilution or lightly with a 1:6 dilution.

Preparation of ALI 1:2 cell culture medium

The medium was prepared under a tissue culture hood and stored at 4°C: *ALI 1:2 Medium*: ALI Basal Medium Kit (Fisher Scientific-NC0307196) includes Pneumacult ALI medium 450 mL (Stemcell Technologies Inc), 50 mL Pneumacult 10x supplement (Stemcell Technologies Inc), and 5 vials of 5 mL Maintenance Supplement (Stemcell Technologies Inc). The solution was diluted 1:2 with RPMI/F12 medium with HEPES (Gibco™).

(5 mL of Penicillin Streptomycin solution was added if ALI 1:2 medium with antibiotics was desired in solution).

Seeding cells

Basal cells were trypsinized as described above and counted. 10 µL of the cell suspension was first mixed with 10 µL of 4% trypan blue (Gibco™) and then 10 µL of the mixture was added onto a hemocytometer. Lives cells that were shown as bright in trypan blue were counted on the four corner and middle squares. The total amount of cells or concentration was then calculated appropriately. Based on the cell count, a calculated volume of basal cell medium was given to resuspend the pellet to ensure a density of $3x10^6$ cells/mL. ThinCerts™ transparent transwells with 3.0 µM pore size (Greiner Bio-One) were prepared invertedly on a sterile 150mm x 25mm petri dish. To seed the cells, 70 µL of the cell suspension was added to the apical side of the inverted transwell. The cells, now on the apical side of the transwells, were incubated with 5% CO_2 at 37°C for 5 hours.

During the incubation period, a 24-well untreated plate was prepared by adding 1 mL of basal cell medium to each well. Typically, 12 transwells were being seeded per genotype so only 12 wells were used. After 5 hours of incubation, the transwells were removed from the petri dish and placed into 1 mL of basal cell medium in the 24-well plate prepared beforehand with the apical side now facing the bottom of the well. Using the 4 mL of basal cell medium set aside from the two wells, 200 µL was added to the basolateral side of each transwell. The plate was left overnight in the incubator set at 37°C and 5% CO_2.

The following day, the transwells were checked for successful cell attachment by gently pipetting 200 µL out of the basolateral side of the transwells and assessing them under a light microscope. If unsuccessful seeding was noted with significant cell clustering, they are left in basal cell medium for another day. Two hundred microliters of fresh basal cell medium were added back to the basolateral side of the transwell before being set back into the incubator. If the seeding was successful, the medium was changed to ALI 1:2 medium with antibiotics by adding 1 mL of ALI 1:2 medium to each well on the other half of the plate and pipetting 200 µL on the basolateral side of the transwell. This process of replacing the medium was repeated every other day to maintain the cells by giving them fresh ALI 1:2 medium with antibiotics. Transwells were given 300 µL instead of 200 µL on the apical side if left for 2 days. The day before a planned transmigration, the medium was replaced with ALI 1:2 without antibiotics/penicillin streptomycin.

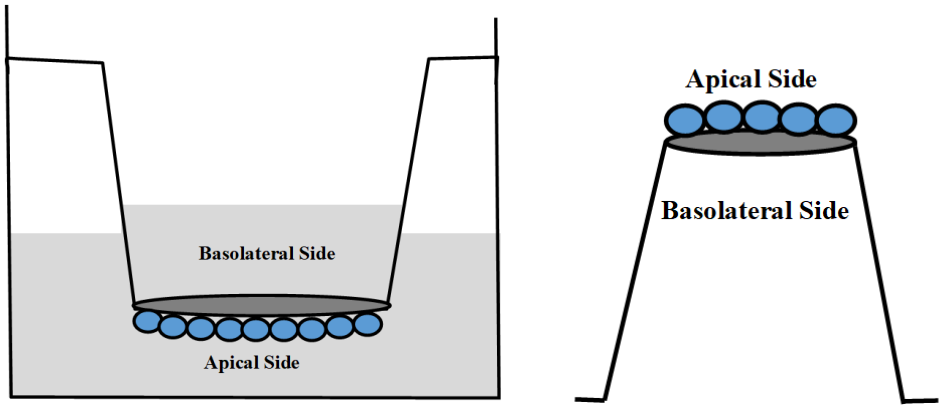


Figure 2. Transwell Orientation. The schematic shows the transwells in inverted orientation (left) in a well compared to the upright orientation (right). The epithelial layer is noted as small blue circles representing the cells and the gray representing the cell medium.

Freezing cells

Basal cells were trypsinized as described above. Instead of resuspending the cells in basal cell medium, cells from each well in a 6-well plate were resuspended in 500 µL of CryoStor media (Stem Cell) in 2 mL TTP polar cryovial tubes (MIDSCI). The sample was placed in a "Mr. Frosty" freezing container at -80°C and stored in liquid nitrogen for long term storage if needed.

Thawing cells

Frozen aliquots of cells from the -80°C freezer was rapidly thawed and immediately added to 5 mL of basal cell medium in 15 mL conical tube. The tube was then centrifuged at 500xg for 10 minutes and the supernatant was carefully aspirated. The cells were then resuspended in 12 mL of basal cell media and 2 mL of the cell solution was added to each well in a pre-coated and washed corning 6-well tissue culture plate. Then the cells were cultured as described above.

Staining for confocal analysis of mouse epithelial transitional model

Mouse epithelial cells were seeded and maintained as mentioned above. The transwells were rinsed with PBS and fixed by adding 4% PFA (ChemCruz) to cover both sides of the transwell in a 24-well tissue culture plate. They were stored at room temperature for 5-10 minutes. The transwells were again washed three times with PBS and then incubated in 0.2% Triton-X in PBS for 10 minutes. The 0.2% Triton-X was removed, and the membrane was blocked with 1% BSA/PBS (Sigma/Gibco$^{TM)}$ for over an hour at room temperature. During incubation, the primary antibody of each antibody mix (labeled A and B) was prepared in a 2 mL tube (150 uL/sample). The antibody was added based on the manufacture's protocol:

Table 1. Antibody list for confocal microscope. CK5 and CK8 antibodies were made in-house from another lab.

Antibody Mix	Primary Antibody		Secondary Antibody	Source	Fluorophore	Company
A	SSEA1	Ms_IgMkappa	Anti-Ms IgM	Goat	Alexa Flour 488	Iowa
B	CK5	Rb_IgG	Anti-rabbit IgG	Donkey	Alexa Fluor 488	(In-lab)
	CK8	Rt_IgG	Anti-Rt IGG	Rat	Alexa Fluor 594	(In-lab)

The membranes of the transwells were then cut using a scalpel and then in half with a small cut made on the upper right to mark the apical side of the membrane. Half of the membrane was placed into tube "A" and the other half from the same membrane was placed into tube "B" where the primary antibody was prepared. The membrane was submerged in the primary antibody to be incubated overnight at 4°C or two hours at room temperature. Afterwards, the membrane was washed in 1 mL 0.2% Triton-X in PBS for 5 minutes three times. From this point, light was kept away from the membranes as much

as possible. The secondary antibody was added (150 µL/sample) to another 2 mL tube, labeled appropriately, and the washed membranes were submerged and incubated at room temperature for an hour. The membranes were washed again in 1 mL 0.2% Triton-X in PBS three times. The membranes were further stained with DAPI (Invitrogen) by incubating them in 150 µL of 1:500 DAPI in water for 5-10 minutes. The membranes were then washed with 1 mL of 0.2% Triton-X in PBS again before being briefly submerged in water. The membranes were then mounted onto a glass coverslip and slide using fluoromount G (Southern Biotech). Slides were observed under a Nikon confocal microscope and analyzed by ImageJ. Sides were stored at 4°C in the dark.

Bacteria preparation

To make 1 L of Luria-Bertani (LB) broth, the following gradients were added to 1 L of de-ionized water: 10 g Tryptone (Gibco™), 5 g yeast extract (Gibco™), 5 g NaCl (Fisher Chemical), and 0.1 mL NaOH 10N (Fisher Chemical). To make LB agar plates, 15 g of Bacto™ Dehydrated Agar (BD) was added. The mixture was autoclaved on a 20-minute liquid cycle. A colony of *Pseudomonas aeruginosa* (PA14 or PA01) from a LB agar plate was obtained and mixed into a 17x100mm sterile round bottom culture tube containing approximately 3 mL of Luria-Bertani (LB) broth. PA14 was cultured for the *in vivo* model while PA01 was cultured for the *in vitro* model. A negative control sample only containing 3 mL of LB broth was also made. All culture tubes prepared were then placed in a shaker at 37°C and shaken at 200 RPM overnight.

Phosphate-buffered solution (PBS) or Hanks' Balanced Salt Solution with magnesium and calcium (HBSS+) was used to wash the bacteria. For a 1 L solution of HBSS+, 2.38 g of Hepes (Sigma), 9.75g of HBSS+ powder (Sigma) was added to 0.99 L of de-ionized water in a 1 L graduated cylinder. While the solution was being continuously mixed with a magnetic stir bar, approximately 4.95 mL of NaOH was slowly added while measuring the pH to reach 7.4. The HBSS+ was then filtered using 0.2 µm steriflip filters (Millipore). For each bacteria sample, 1 mL overnight culture was transferred to a 2 mL microcentrifuge round bottom tube and centrifuged at max speed 16,000xg for 3 minutes. The supernatant was aspirated, and the pellet was resuspended in 1 mL of HBSS+ or PBS. The bacteria were centrifuged down again at max speed 16,000xg for 3 minutes and resuspended in 1 mL HBSS+ or PBS again.

In vivo model (PA14)

A 1:10 dilution was performed by adding 50 µL of resuspended PA14 to 450 µL HBSS+ or PBS. The optical density was measured at 600 nm (OD600) using a spectrophotometer. The undiluted PA14 sample was then adjusted to an OD reading of 0.7 to get an approximate 1.3 x 10^7 colony-forming units (CFU)/mL. This was done with the equation $0.7 * 500 = x * 10 * OD\ reading$ where x is the amount needed to add to HBSS+ or PBS to total 500 uL. Then the bacteria were further diluted 1:70 in HBSS+ to get an approximate 1.9 x 10^5 colony-forming units (CFU)/mL.

In vitro model (PA01)

PA01 was resuspended in 0.6 mL HBSS+ before being further diluted 1:50 by adding 20 µL of the washed PA01 overnight to 980 µL of HBSS+.

RNA isolation and RT-qPCR

RNA isolation and RT-qPCR were performed on basal cells and transitional cells. RNeasy® Mini Kit (Qiagen-74104) was used for RNA isolation. Reverse transcription (RT) and qPCR were done following the manufacturer's protocol. Primers were designed in lab described below and bought from Eton bioscience.

Table 2. RT-qPCR primer list

Gene (Mouse)	Forward Sequence	Reverse Sequence
GAPDH	CTTTGTCAAGCTCATTTCCTGG	TCTTGCTCAGTGTCCTTGC
ALOX15	CACCGTGGTTGAAGACTCTC	TCTACAGGGAGGTCAGAGATAC
ALOX12	CCGCATAGAGAACAGTATCACC	GTGGAGTGTTTAGTCAGGAGAG
iPLA2beta	ACTGCCTTCCATTACGCTG	ATCTCCTGCTTTCCCATCTTG

Basal cells or transitional cells from one well were washed by PBS and buffer RLT was added to each well. The mixture of cells and RLT was pipetted up and down 3-5 times and added to a shredder (Qiagen). The mixture was then centrifuged at max speed 16000xg for two minutes. The same volume of 70% ethanol was then added to the lysate and was mixed well by pipetting.

Afterwards, 700 µL of each sample was added to a RNeasy Mini spin column in a 2 mL collection tube. The samples were spun down at >8,000 g for 15 seconds. Flow-through was discarded. Then 700 µL of Buffer RW1 was added to each RNeasy spin column and centrifuged again at >8,000xg for 15 seconds. Flow-through was again discarded. Once emptied, 500 µL of Buffer RPE was added to the RNeasy spin column and centrifuged again. The flow through was emptied once more before adding 500 µL of Buffer RPS to the same columns and centrifuged at >8,000xg for 2 minutes. The RNeasy spin columns were then placed in a new 2 mL collection tube and centrifuged at max speed 16000xg for one minute to dry. The dried RNeasy spin columns were placed in a new 1.5 mL collection tube and 50 µL of RNase-free water was added. Samples were spun at >8000 g for 1 minute to elute isolated RNA and left on ice.

RNA concentrations of the samples from each cell type were measured using the Nanodrop One (Thermo Scientific^{TM}) and were adjusted to the same concentrations for all the samples. An aliquot of 14 µL of each sample were then transferred into PCR tubes and labeled accordingly, along with a no-RT sample for negative control. A master mix for each sample was made for each protocol (volume per reaction) containing iScript DNase (Bio-Rad) and iScript DNase buffer (Bio-Rad). Once completed, 2 µL of the master mix was added to each sample and placed in a thermal cycler which was set to Bio-Rad's DNase reaction protocol. After the process was complete, the samples were given 4 µL of iScript Reverse Transcription Super mix (Bio-Rad) or iScript No-RT Control Super mix (Bio-Rad) based on their respective samples. They were then placed

again in the thermal cycler and set to Bio-Rad's cDNA synthesis protocol to perform reverse transcription. Samples were then stored at -80C to be further used.

On a PCR plate (Thermo Scientific™), the samples were planned out appropriately (samples, no-RT, positive controls). Positive controls came from mouse splenocytes (see splenocyte isolation below for protocol). The cDNA was diluted 1:3 with DNase/RNase free water in separate tubes before adding 3 µL to the desired location on the PCR plate. A master mix was created for each gene of interested which included 5 µL of iTaq Univerasl SYBR Green Supermix (Bio-Rad), 1 µL of the gene's forward primer, and 1 µL of the gene's reverse primer to give a total of 7 µL for each well. All primers were ordered from Eton Bioscience. Once the master mix was added to their respective wells, the PCR plate was sealed with Microseal 'B' PCR Plate Sealing Film (Bio-Rad) and then placed into the qPCR machine (7500 Fast Real-Time PCR System-Applied Biosystem). The qPCR cycle was set according to Bio-Rad's protocol. Results were exported on Microsoft Excel and GraphPad Prism.

Isolation of mouse bone marrow neutrophils

A selected number of mice from each genotype (C57 and alox5 KO) were obtained from the animal facility and sacrificed using CO_2 and cervical spine dislocation. The femoral and tibial bones were isolated from each mouse and added to a sterile 100mm x 15mm petri dish with their respective genotype. The bone marrow was then extracted into another 100mm x 15mm petri dish (keeping the genotypes separate) by flushing the bones with HBSS- without calcium and magnesium (Gibco™). This was

done by clipping both ends of the bone and inserting a 25-gauge needle inserted into a 10 mL syringe into the openings made on the clipped ends. Bone marrow cells were flushed from both sides of the bone and transferred onto a 40 µM cell strainer (Fisher Brand) on top of a 50 mL tube. The plunger from the syringe was used to gently ground pieces of bone marrow on the 40 µM cell strainer. Extra HBSS- was used to rinse the plunger, filter, and petri dish to collect all the bone marrow cells.

The 50 mL tubes with the samples were then centrifuged at 740xg for 5 minutes. The supernatant was discarded and were resuspended RBC lysis buffer using 1 mL/mouse and incubated at room temperature 1.5 minutes. To make 1 L of RBC lysis buffer, the following gradients were added to 1 L of de-ionized water: 8.29 g of ammonium chloride, 1 g of sodium bicarbonate, and 0.038 g of ethylenediaminetetraacetic acid (EDTA). Afterwards, the samples were filled up to 50 mL with HBSS- and were centrifuged at 740xg for 10 minutes. The supernatant was carefully discarded, and the cells were resuspended in HBSS- at 0.5 mL/mouse. A small amount of the cells was diluted at 1:100 and used for cell counting. The volume of cell suspension was calculated for $2x10^6$ cells/transwell. The rest of the samples were left on ice and used further for transmigration.

Neutrophil transmigration across transitional epithelium

This model was adapted from the neutrophil transepithelial migration model (Kusek, 2014). Airway basal cells were seeded on transwells and differentiated to transitional cells in ALI medium for 7±1 days. Trans epithelial electrical resistance

(TEER) values were measured using an epithelial voltohmmeter (EVOHM) (World Precision Instruments). If the TEER values were agreeable above 200 OHMs, the medium replaced with ALI 1:2 solution without antibiotics one day prior to the transmigration assay.

Overnight culture of PA01 was prepared as described above. Transitional cells on the transwells were washed by gently discarded and dipping them in pre-warmed clean HBSS+ twice. Then the cells were equilibrated in 24-well tissue culture plate with 1 mL of HBSS+ on the apical side and 0.2 mL of HBSS+ on the basolateral side. The cells were left in the incubator at 37°C with 5% CO_2 for 30-60 minutes.

After equilibrium, the transwells were removed from the wells and the HBSS+ was gently discarded. They were then placed onto a 150mm x 25mm sterile suspension culture dish with the apical side facing up. Each transwells had either 25 µL of HBSS + or PA01 added onto the apical side of each transwell. The culture dish was then placed back into the incubator set at 37°C with 5% CO_2 for an hour. During this time, 3 wash plates were prepared appropriately (based on that each transwell will be washed 3 times) in 24-well untreated plates containing 1 mL of warm HBSS+. These plates are kept at 37°C until ready to use.

Once the infection was complete, the transwells were removed from the culture plates, inverted, and added to the 24-well untreated wash plates. Each transwell was rinsed by placing them each into 1 mL of warm HBSS+ prepared on the 24-well untreated wash plates and adding 200 µL of HBSS+ to the basolateral side. They are then removed and the HBSS+ on the basolateral side was discarded out before adding it to a

another well containing 1 mL of warm HBSS+. 200 µL of HBSS+ was added to the basolateral side again, thus repeating this process. This wash process was repeated once more for a total of three washes for each transwell.

Transwells were then placed in a clean and dry well. 100 µL of HBSS+ was added back onto the basolateral side of the transwell before the addition of a calculated volume of isolated mouse PMN respectively to their genotype. The volume of mouse PMN was calculated prior as mentioned before based on the cell count to add $2x10^6$ cells for each transwell. Another 24-well untreated plate was used as the transmigration plate and filled with either 500 µL of HBSS+ or LTB4 (Cayman Chemical Company). The LTB4 was prepared freshly just before migration at a concentration of 0.2 ng/mL in HBSS+. The transwells (now with the isolated mouse PMNs) were then placed into their respective wells in the transmigration plate and left in an incubator set at 37°C and 5% CO_2 for 2 hours.

After 2 hours, the transmigration plate was removed from the incubator and looked under a light microscope for successful migration. The transwells were then tapped gently on the side of the wells and discarded. The supernatant was carefully obtained by pipetting 200 µL from each well into a 96 well plate and stored at -80°C for later cytokine assays. The migrated cells in 300 µL/well were mixed with 25 µL of Triton-X (Sigma) and 6 µL of protease inhibitor (Millipore) and then shaken on an orbital shaker at 300 RPM at 4°C for 20 minutes. Positive controls were prepared the same way by adding the same volume of neutrophils per their respective genotype into 300 µL of

HBSS+. The migrated cells were lysed to allow myeloperoxidase (MPO) release into the supernatant. The samples were then stored at -80°C for MPO ELISA later.

Mouse pneumonia model with *Pseudomonas* infection

Avertin preparation

To prepare the avertin, solid avertin 2,2,2-tribromoethanol (TBE) 99% (Invitrogen) to tertiary-amyl alcohol in a 1g/1mL ratio and stirred with a magnetic stir bar overnight until TBE was dissolved. Aliquots of 0.5 mL volumes were stored in glass vials covered in foil and sealed with parafilm at -20°C. To create a working solution of 25mg/mL, they were diluted 1:40 by slowly dripping 0.5 mL of the frozen aliquot into 20 mL of warm PBS (Gibco^{TM}) protected from light. The solution was then filter sterilized through 0.2 µm steriflip filters (Millipore) immediately before use. The dosing of Avertin was decided based on body weight of the mice by performing preliminary studies.

Intranasal infection

Pseudomonas Aeruginosa (PA14) was prepared as described above. Between six to seven mice were used for each genotype. Two mice were used for mock infection while the remaining mice were infected with PA14 for each individual experiment. After the mice were appropriately anesthetized with avertin via intraperitoneal injection, tweezers/forceps were used to pull out the tongue on one side of the mouth to prevent from airway blockage. If needed, the tongue was gently held down by the thumb from the hand holding the mice. Two 15 µL doses of either HBSS+ or PBS (mock infection) or PA14 (diluted 1:70) were administered into one of nostrils drop-wise. Mice were then

placed on their side on a mouse heating gel pad 4x6 with gauze on top. The mice were monitored for 6 hours. Generally, mice woke up in 1-2 hours post infection.

Bacteria plating and colony counts

Pseudomonas isolation agar (PIA) was made by adding the following gradients into 1 L of de-ionized water: 20 mL of Glycerol (Millipore) and 45 g of PIA powder (BD). The mixture was autoclaved on a 20-minute liquid cycle, plated, and dried appropriately. Once the plates were dry, they were stored at 4°C until needed.

After the 1:70 diluted *Pseudomonas* sample was used to infect the mice was used, it was plated on PIA plates at 1:10, $1:10^2$, $1:10^3$,$1:10^4$, $1:10^5$, and $1:10^6$ dilutions in sterile PBS under a flame using the drop plate method. The HBSS+ or PBS used for the mock infection was also plated under a flame on an LB plate using the spread plate method. The plates were left overnight in an incubator set at 37°C and 5% CO_2. The next day, the bacterial colonies were counted, and an estimated CFU/mL was calculated.

Lung homogenization and plating

For checking the amount of the bacteria in the lungs after the infection, lung homogenization and plating was performed. Mice were sacrificed according to approved protocol. The lungs were exposed by dissecting and removing the front portion of the rib cage while avoiding cutting the lung tissue itself. They were then carefully removed and placed into a 2 mL screw cap tube with a 5 mm sterile metal bead. The samples were then placed in a homogenizer, the TissueLyser LT (Qiagen), at 50 Hz for 6.5 minutes at 4°C in

a cold room. Each sample was then given 1 mL of HBSS+ and homogenization was repeated with the same settings.

Once homogenization was completed, they were diluted in 1:10 series in HBSS+. The dilutions plated were at 1:10, 1:100, and 1:1000. The plates were left overnight in an incubator set at 37°C and 5% CO_2. The next day, the bacterial colonies were counted, and an estimated CFU/lung was calculated.

Bronchoalveolar collection (BAL)

For experiments that analyzed for neutrophil infiltration, BAL was performed. After the recovery period, the mice are sacrificed according to approved protocol. The lungs and trachea are exposed without directly cutting into the lung tissue, removing any soft tissue and the esophagus. A small horizonal cut was made on the trachea and a blunt 21G catheter was placed into the trachea 0.5cm deep. A size 0 suture thread was used to tie the catheter onto the trachea to keep it in place during the procedure. A 1 mL syringe was used to flush the lungs 4 times of 0.6mL EasySep buffer (Stemcell Technologies Inc). The first wash was set aside for supernatant analysis. The other three washes were combined. One spleen was taken out for splenocytes to be positive control (see protocol below).

All the samples are then centrifuged down at 500xg for 5 minutes, and the supernatant was separated from the cell pellet. The supernatant from the first wash was saved at -80°C for cytokine ELISA, protein assay, and LDH colorimetric assay. The cells were combined and the supernatant from the remaining washes was discarded and the

cells from all four washes were combined and resuspended in 500uL in HBSS-. They were then split into two tubes of different volumes (150 µL and 350 µL).

The tube containing 150 uL was given 3 µL of protease inhibitor (Millipore) and 6 µL of 10% Triton-X (Sigma) and shaken on ice at 300 RPM for 20 minutes. The samples were stored at -80C. These samples can be used for ELA2 ELISA and MPO ELISA assay.

Flow cytometry analysis

The tube containing 350 µL will be used for flow cytometry. To make 1 mL total, 650 µL of HBSS- was added to each tube. 1 µL from the LIVE/DEAD™ fixable near-IR stain kit (Invitrogen™-L34975) was added to each sample and control (besides the unstained sample) and left on ice or room temperature in the dark for 10 minutes. Samples were then centrifuged for 5 minutes at 500xg. Supernatant was discarded and samples resuspended in 100 µL of eBioscience™ Flow Cytometery Staining Buffer (Invitrogen™). CD16/CD32 antibody (Invitrogen) was added as Fc blocker (1 µL/sample) and kept at room temperature for 15 minutes. The antibodies were then added based on manufacturer to each sample and respective controls:

Table 3. Antibody list for flow cytometry

Antibody	*Fluorophore*	*Source*	*Company-Catalog #*
CD16/CD32-Monoclonal	Unconjugated	Rat	Invitrogen-14-0161-82
CD45- Monoclonal	PE	Rat	Biolegend- 103106
CD11b- Monoclonal	PerCP-Cy5.5	Rat	Invitrogen- 45-0112-82
Ly-6G- Monoclonal	APC	Rat	Invitrogen- 17-9668-82
Siglec-5-Monoclonal	Alexa Fluor®488	Rat	Biolegend- 155523

Samples were then left on ice for 15-30 minutes. 1 mL of FACS buffer (Invitrogen) was then added and centrifuged at 500xg for 5 minutes. The supernatant was then discarded and 500 µL of IC fixation buffer diluted with FACS buffer 1:1. These samples can be stored at 4C for up to 3 days before performing FACS.

To prepare for FACS, 1 mL of FACS buffer was added to each tube and centrifuged at 500xg for 5 minutes. The supernatant was discarded and resuspended in 1 mL of FACS buffer. Samples are left on ice and now ready for flow cytometry. FACS was done on the Attune NxT Acoustic Focusing Cytometer (Thermo Fisher Scientific).

Splenocytes were isolated for flow cytometry controls because of limited cells obtained from BAL. Briefly, a spleen from any of the mice used was cut small pieces on top of a 40uM cell strainer (Fisher) set on a 50 mL tube and then grinded up using 10 mL syringe plunger to separate the cells from the tissue and washed with and washed HBSS- (Gibco^{TM}). The cell suspension was then centrifuged at 740xg for 5 minutes. The supernatant was discarded, and the cells were resuspended in RBC lysis buffer added at 2 mL/mice and left for 1.5 minutes before adding HBSS- up to 50 mL. The cells were spun down again and resuspended in 1 mL HBSS- for FACS staining. They are then split into 10 fractions in 100 µL: unstained, dead cells (heated at 65°C for 5 min), 4 for single staining with live dead staining (CD45, CD11b, Ly6G, Siglec-5), and 4 for florescence minus one for 4 antibodies (-CD45, -CD11b, -Ly6G, and -Siglec-5).

MPO and ELA2 ELISA

A 96-well EIA/RIA Plate (High Binding) from Corning Incorporated and R&D Mouse ELISA kits were used to perform either MPO or ELA2 assays. Depending on which assay was performed, the Mouse Myeloperoxidase ELISA kit (R&D- DY3667) or Mouse Neutrophil Elastase/ELA2 (R&D- DY4517-05) was used. All the antibodies, solutions, and buffers are prepared based on the manufacture's protocol according to their respective ELISA kit. The plate was covered with a new 96-well plate cover after every incubation period, except for the addition of substrate and stop solutions. Each well of the plate was first coated with 100 µL of capture antibody and was incubated overnight at room temperature or at 37°C for 3 hours. The plate was then with 400 µL of wash buffer per rinse three times. For 1 L of wash buffer, 500 µL of Tween®20 (Fisher Bioreagents) and 100 mL of PBS 10X (Lonza) was added to 900 mL of de-ionized water. It was then blocked with 300 µL of 1% BSA/PBS (Sigma/Gibco™) and left at room temperature overnight or at 4°C for a maximum of one week. The plate was then washed with the same procedure mentioned before. After the wash, 100 µL of the samples and standards were added to their respective wells and left at room temperature for 2 hours. The plate was washed again three times with wash buffer. Then, 100 µL of detection antibody was added to each well and left at room temperature for 2 hours. After the time limit, the plate was washed again three times with wash buffer before 100 µL of streptavidin-HRP was added to each well. The plate was left for 20 minutes and was stored in the dark to avoid light. The plate was rinsed again three times with wash buffer, and 100 µL of substrate solution A and B (R&D- DY999) was added. The plate was kept in a dark place to

develop for 20 minutes before 50 µL of stop solution (R&D- DY994) was added. The plate was then read in a plate reader at two wavelengths: 450 nm and 570 nm. Results were exported and analyzed in Microsoft Excel and GraphPad Prism.

LTB4 ELISA

A LTB4 ELISA kit (Caymen Chemical Company-520111) was used. All the antibodies, solutions, and buffers were prepared based on the manufacturer's protocol and a 96-well plate was provided by them. The following figure is provided for a sample plate format.

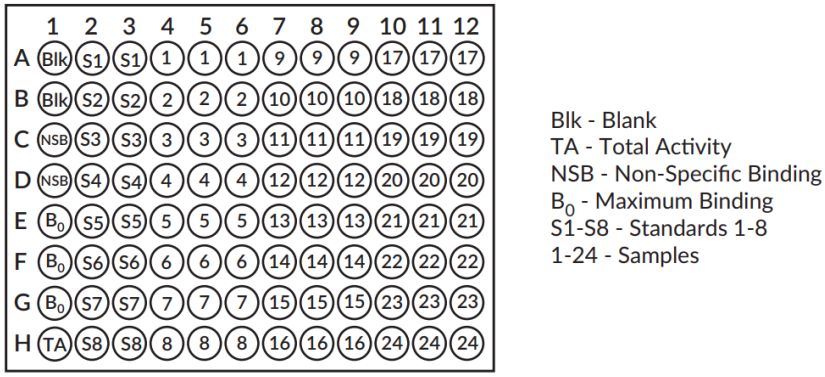


Figure 3. LTB4 ELISA sample plate format provided by Caymen Chemical Company

In the non-specific binding (NSB) wells, 100 µL of ELISA buffer (1x) was added. While in maximum binding (B_0) wells, 50 µL of ELISA buffer (1x) was added. The LTB4 standards (S1-S8) and samples (1-24) were then aliquoted appropriately by adding 50 µL to their respective wells. 50 µL of the LTB4-AChE tracer was then added to each well, except for the total activity (TA) and blank (Blk) wells. Finally, 50 µL of LTB4 ELISA antiserum was added to each well, except for the TA, NSB, and Blk wells. The plate was covered with a 96-well plate cover and left to incubate overnight at 4°C.

After incubation, the plate was emptied, and each well was washed with 400 µL of wash buffer five times. Each well then had 200 µL of Ellman's Reagent, immediately prepared beforehand, added. 5 µL of the reconstituted tracer was then added to TA wells before being covered and shaken on an orbital shaker at 400 RPM in the dark at room temperature for 90-120 minutes. The plate was then read between 405-420 nm on a plate reader and the results were exported and analyzed in Microsoft Excel and GraphPad Prism.

LDH Assay

A LDH assay kit (Fisher- 88954) was used and provided a substrate mix and assay buffer. While being protected from light, 11.4 mL of Milli-Q water was added to the substrate mix vial. Once fully dissolved, 600 µL of the assay buffer was added to form an LDH reaction mixture and stored at -20°C.

In a 96-well plate, 50 µL of sample was added to their respective wells, including a negative control which only had 50 µL of Milli-Q water. 50 µL of the LDH reaction mix was then added to each well containing a sample or blank and the plate was developed in the dark for 7 minutes at room temperature. The 96 well plate was then read at 490 nm on a plate reader and the results were exported and analyzed in Microsoft Excel and GraphPad Prism.

Statistics

Analysis was performed with Microsoft Excel and GraphPad Prism software (version 9.3.1) on Windows. Initial analysis was done with Microsoft Excel to graph for standard curves and real MPO calculations for ELISA assays in both in vivo and in vitro

models. GraphPad Prism was further used to graph the data and applied unpaired t-test to test for the significance differences between groups.

RESULTS

Cultured mouse airway epithelial transitional cells

Airway epithelium is a pseudostratified epithelium in which the composition is dependent on which part of the airway is being focused on. In large airways, the major cell types are ciliated, undifferentiated columnar, secretory, and basal cells (Crystal et al., 2008). When shifting to smaller airways, the secretory cells become Clara cells, also known as club cells (Crystal et al., 2008). Past research on this topic have used either a fully differentiated model with mouse airway epithelial cells or human pulmonary cell lines in their studies. We aimed for a simpler model that wasn't as time consuming as a fully differentiated model and wanted to make sure that the model expressed key enzymes needed to produce HXA3. To check the differentiation status of our mouse airway epithelial cell model, we stained the cells with different markers. Basal cells were stained with CK5 and differentiated cells were stained with CK8 (Schlage et al., 1998). In addition, we stained celled with club cell marker SSEA-1.

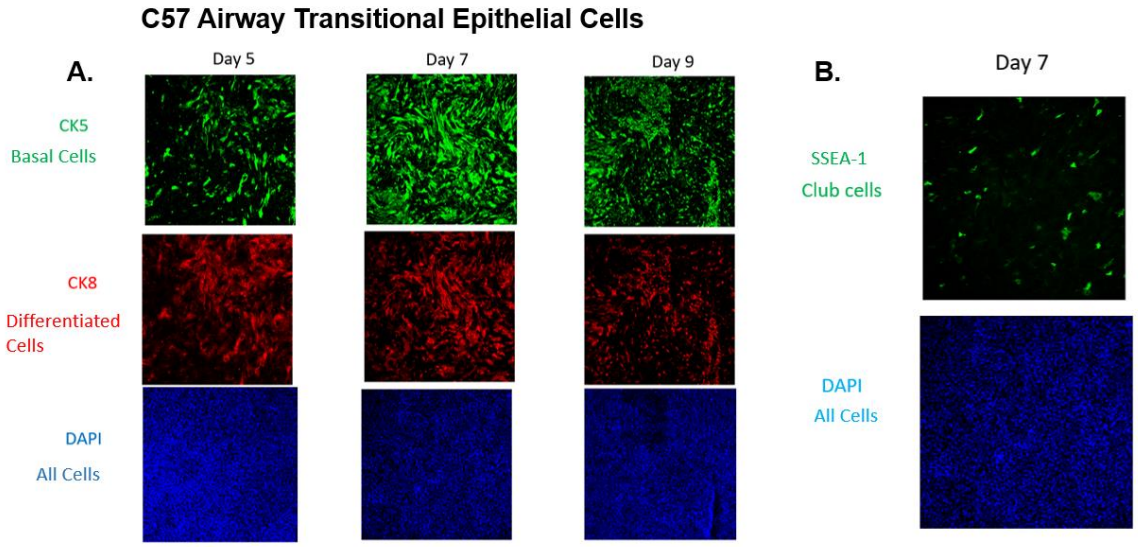


Figure 4A and 4B. Culture of mouse airway epithelial transitional cells are partially differentiated.

Staining of the mouse airway epithelial transitional cells show that they were both CK5 and CK8 positive, suggesting that they are partially differentiated **(Figure 4A)**. With club cell staining using SSEA-1, we do observe the presence of club cells on day 7 in our mouse airway epithelial transitional cells **(Figure 4B).** Both these results point to the culture of the mouse airway epithelial transitional cell model being partially differentiated.

Epithelial transitional cells express genes for HXA3 synthesis

Gene expression was evaluated to assess for the ability of these cells to produce the enzymes necessary to make HXA3. According to the proposed mechanism by Tamang et al. (2012), without the production of HXA3, we would be unable to analyze neutrophil transmigration if it were due to the alox5$^{-/-}$ neutrophils or due to our model's inability to produce HXA3. Epithelial basal cells were first assessed as to evaluate if they

possibly had a role in neutrophil transmigration. As mentioned earlier, airway basal cells are present in airway epithelium (Crystal et al., 2008). To trigger the production of HXA3, the basal cells were infected with *pseudomonas aeruginosa (PA01)*. HBSS+ was used as a control and compared basal epithelial cell expression that were infected. ΔCt is calculated relative to GAPDH, a housekeeping gene, and graphed as 1/ΔCt to present data in an intuitive format.

Table 4. Cycle threshold (Ct) of basal cell genes for HXA3 synthesis.

BASAL CELLS	GAPDH	iPLA2β	Alox5	Alox12
C57 (HBSS+)	21.63 23.26	Undetermined	Undetermined	Undetermined
C57 (PA01)	23.11 22.65	Undetermined	Undetermined	Undetermined
Alox5 (HBSS+)	21.95 21.70	Undetermined	Undetermined	Undetermined
Alox5 (PA01)	22.43 21.27	Undetermined	Undetermined	Undetermined

The results show the genes in the lipoxygenase pathway to produce HXA3 (iPLA2β, alox15, and alox12) were not expressed in epithelial basal cells. RT-qPCR data showed cycles over 35 which was concluded as undetermined or not detected. Overall, it was expected that basal epithelial cells show little to no expression of these genes. Infection with PA01 did not change expression levels.

To further define the *in vitro* model, the gene expression of epithelial transitional cells was also done to validate and define cell maturation. Using the same methods as before, qPCR was performed on cells in the transitional model cultured in ALI medium

for different days to evaluate when the cells have the optimal gene expression in the lipoxygenase pathway.

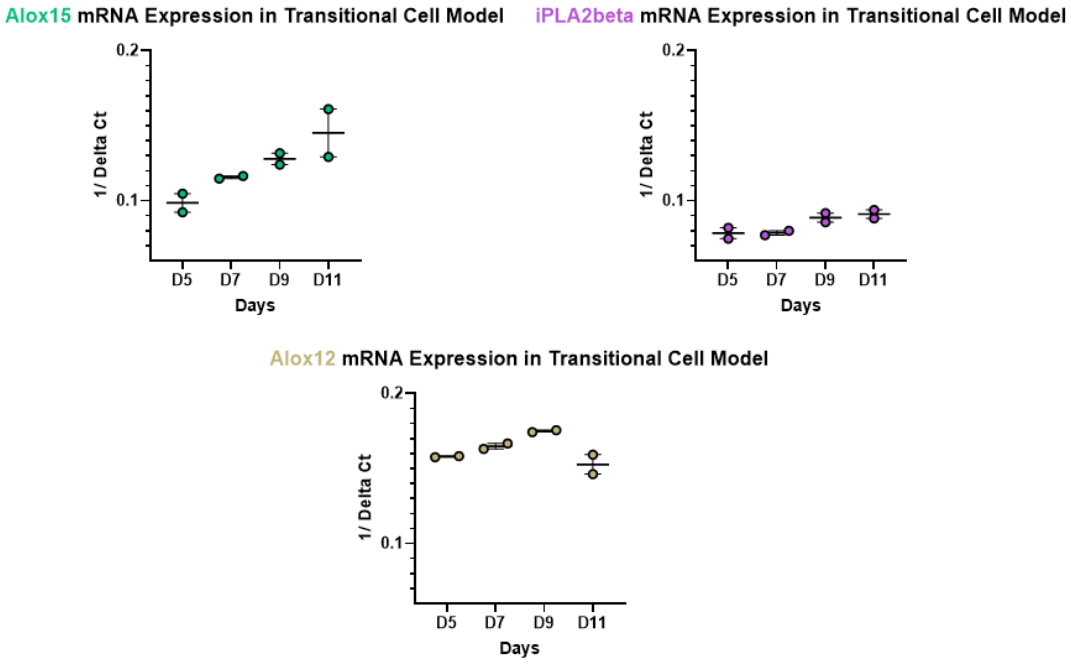


Figure 5. Epithelial transitional cells express genes for HXA3 synthesis

As expected, the data shows an increase in mRNA expressions in alox12, iPLA2beta, and alox15 from days 5 to day 9 (**Figure 5**). Both alox15 and iPLA2beta show a continuous increase in expression while alox12 shows a decrease in expression on day 11 (**Figure 5**). We decided to use the epithelial culture at 7-9 days for out *in vitro* studies.

Mouse bone marrow neutrophil transmigration across epithelial transitional cells

Different mixtures of C57 and alox5$^{-/-}$ epithelial and PMN cells were used to evaluate the effects of different genotypes on neutrophil migration. Four groups were made: C57 epithelial and PMNs, C57 epithelial and alox5$^{-/-}$ PMNs, alox5$^{-/-}$ epithelial and PMNs, and alox5$^{-/-}$ epithelial and C57 PMNs. HBSS+ was used as a negative control and LTB4 was used as a positive control.

To determine neutrophil migration in these models, one of the assays used measured neutrophil myeloperoxidase (MPO) activity, an enzyme released by activated neutrophils. The MPO activity assay is shown to directly correlate with neutrophil migration in a linear trend, thus the use of a standard curve is needed to help establish an accurate comparison along with a positive control. Wavelength were measured between 540 nm and 570 nm as recommended by the manufacture of the MPO ELISA kit and exported as reduced data to be further analyzed in Excel and GraphPad.

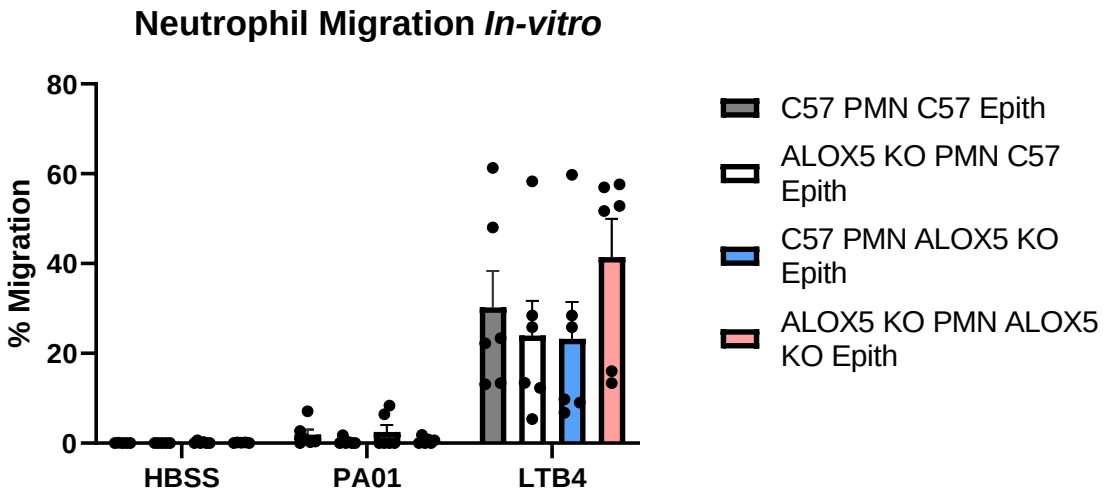


Figure 6. Mouse bone marrow neutrophils transmigration across epithelial transitional cells

Overall, the MPO activity assay shows there was mouse bone marrow neutrophil migration across epithelial transitional cells. Neutrophil migration was mildly induced when epithelial transitional cells were infected with PA14 while migration was strongly induced with LTB4 (**Figure 6**). However, there was no difference observed in migration percentage between the wild type (C57BL/6) and alox5$^{-/-}$ when induced with LTB4 (**Figure 6**).

Alox5$^{-/-}$ PMNs have decreased transmigration across epithelial transitional cells

Closer analysis was done on the migration of mouse PMNs when epithelial transitional cells were infected with PA01. Only the data points in models infected with PA01 were plotted for better visualization from **Figure 6**.

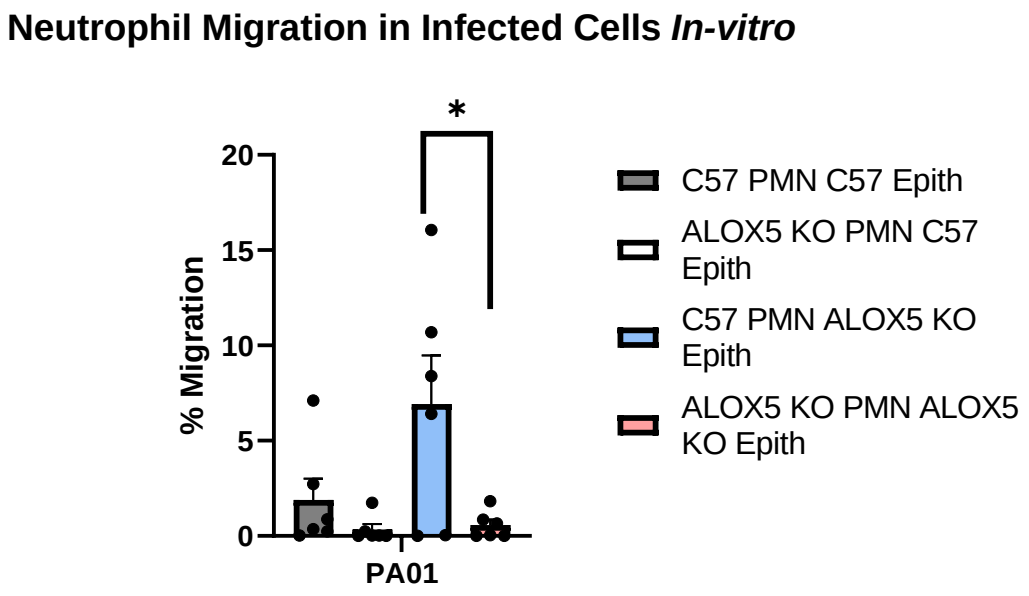


Figure 7. Alox5$^{-/-}$ PMNs have significantly decreased transmigration across epithelial transitional cells

The results show that most migration was observed in C57 PMNs, specifically with cells in alox5$^{-/-}$ epithelial cells (**Figure 7**). Alox5$^{-/-}$ PMNs with infected transitional epithelial cells show very low migration percentages; however, when compared to transitional epithelial cells that were only treated with HBSS+ they show a higher migration percentage (**Figure 7**). Overall, it seems that there is an increase in C57 PMNs migration when compared with alox5$^{-/-}$ PMNs (**Figure 7**) which is consistent with previous studies done by Yonker et al. (2017). The effects of epithelial cells seem to be minimal to which there is an increase in migration with alox5$^{-/-}$ transitional epithelial cells.

LTB4 Production in the transmigration of mouse PMNs *in vitro*

A LTB4 ELISA was done to analyze for LTB4 production in our *in vitro* model.

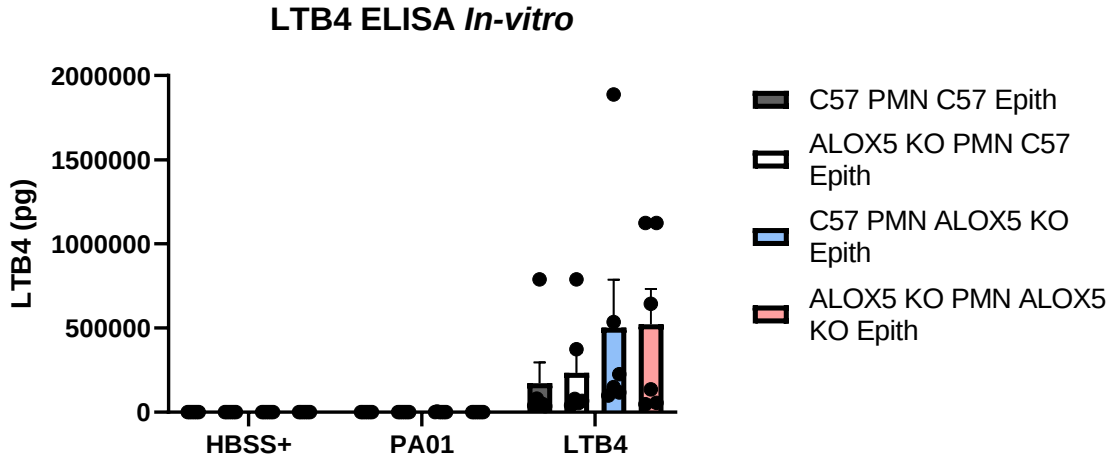


Figure 8. Mouse PMNs produce LTB4 in transmigration *in vitro*

LTB4 ELISA results showed similar trends with MPO activity assay results (**Figure 8**). Due to the large scale on the graph, the low levels detected in other transwells

are not visible. The high LTB4 concentration in the positive control is likely due to the LTB4 added to induce neutrophil migration.

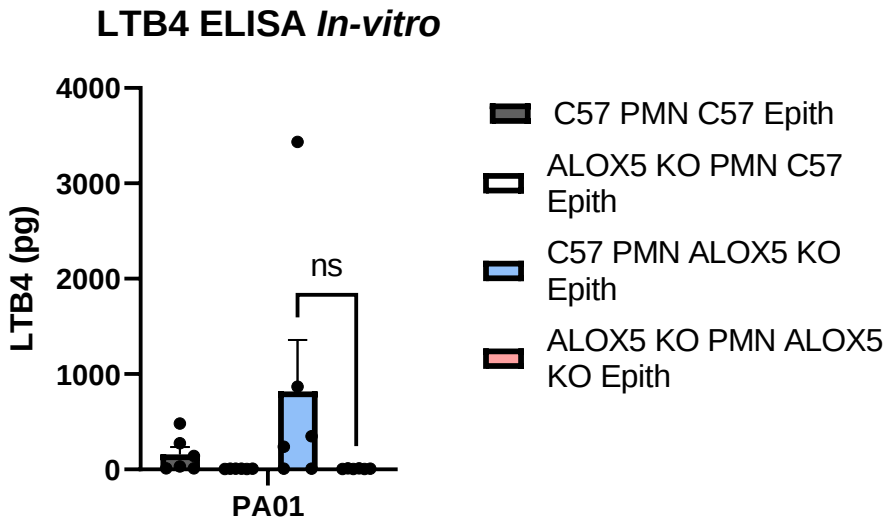


Figure 9. No significant difference in LTB4 production between C57 and alox5$^{-/-}$ PMNs

Upon closer analysis of transwells infected with PA01 from **Figure 8**, a similar trend was again seen with the MPO activity assay where the highest concentration of LTB4 was seen in C57 PMNs with alox5$^{-/-}$ epithelial cells (**Figure 9**). However, there was no statistically significant difference in LTB4 when comparing transwells with C57 PMNs versus alox5$^{-/-}$ PMNs. One key result is that transwells that had alox5$^{-/-}$ PMNs had essentially no LTB4 detection (**Figure 9**) which is again consistent with previous studies done on this pathway by Tamang et al. (2012) and Yonker et al. (2017).

Mouse pneumonia model with *pseudomonas* infection

As mentioned before, neutrophils are a hallmark to acute inflammation and have been known to have an important role in the innate immune response. Neutrophils can be assessed by evaluation neutrophil numbers and inflammatory mediator levels in

bronchoalveolar lavage (BAL). This method has been used in previous studies to investigate the inflammatory response in patients with cystic fibrosis and asthma, as well as studies using *in vivo* models.

Before experimenting with the *in vivo* model, two pilot studies were done to optimize the pneumonia mouse model. The first pilot study was done to establish a baseline of neutrophils in lungs of these mice and ensure that a mock infection did not stress the lungs to induce neutrophil migration. A second pilot study was done to test varying doses of PA14 to evaluate which dose of PA14 was effective enough to elicit a neutrophil response.

Mice were successfully infected with *Pseudomonas Aeruginosa* intranasally with no differences between C57 and alox5$^{-/-}$

To ensure mice were being successfully infected, lungs of mice that were infected with PA14 intranasally were isolated, homogenized, and plated on PIA.

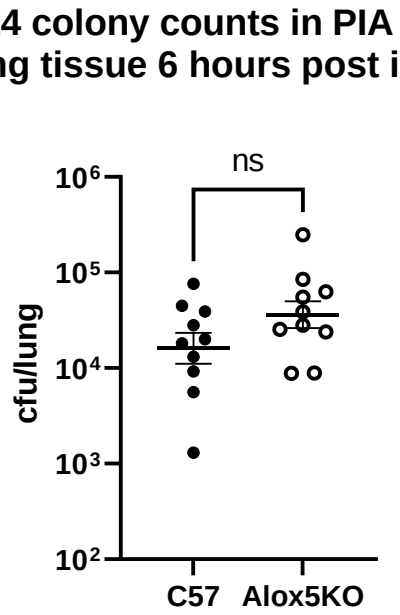


Figure 10. PA14 colony counts in PIA of mouse lung tissue 6 hours post infection.

CFU/lung counts show that mice were successfully infected with PA14 with growth shown on PIA plates and that were was no statistically significant difference between the two genotypes (**Figure 10**), suggesting that there was no different in controlling the bacterial growth between the wild type C57 and alox5$^{-/-}$ mice.

No PMNs migrated with mock infection

Mice from both genotypes either had no procedure performed on them (baseline), or had a mock infection performed where 30 µL of PBS was given intranasally in simulate infection procedure.

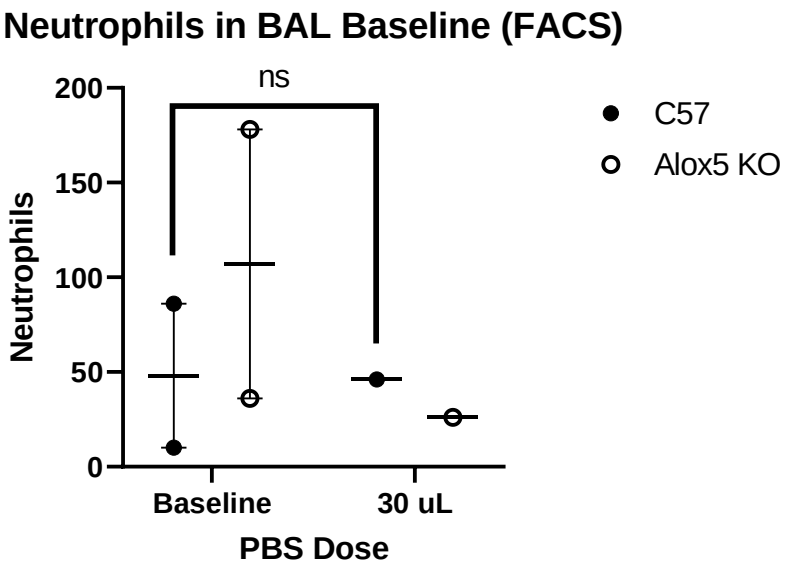


Figure 11. No PMNs migrated with mock infection

Neutrophil numbers evaluated by FACS showed that there were low numbers of neutrophil (under 200) at baseline and with mock infection with PBS in both wild type (C57) and alox5$^{-/-}$ mice (**Figure 11**). FACS data showed <2% of BAL collected were identified as neutrophils in all categories. When comparing the two genotypes, there seem to be no significant difference in neutrophil numbers.

A study done by Barletta in 2012 looked to quantify multiple leukocyte populations in the mouse lung. By using intravenous infection of fluorescently labeled anti-CD45 antibody and BAL, they estimated a 17% population of neutrophils in naïve mouse lung with an increase after lung injury (Barletta et al., 2012).

Different doses of PA14 induce PMN migration at similar levels

Mice were infected with the same methods as mentioned, but the *Pseudomonas aeruginosa* (PA14) were prepared at different doses. A high dose was only diluted to an

estimated optical density of 0.7 (~1.3 x 10^7 CFU/mL). A medium dose was done by diluting the high dose sample by 1:7 (~1.9 x 10^6 CFU/mL). A low dose was done by diluting the medium dose by 1:10 for an overall dilution of 1:70 (~1.9 x 10^5 CFU/mL). Bacteria colony counts plating on PIA plates were done to ensure PA14 was diluted properly.

Table 5. ***Pseudomonas aeruginosa (PA14)*** **colony counts on PIA and CFU actual counts at different dilutions.**

Bacterial Dilution	Plate Dilution	Average Colony Count	CFU Actual Count
OD 0.7	$1:10^6$	10	30000000
1:7	$1:10^5$	11.5	3450000
1:70	$1:10^4$	12	360000

CFU actual counts were calculated by counting colonies on PIA plate, multiplied by the plate dilution factor, and then multiplied by 3 to account for the total 30 µL given to each mouse as 10 µL was only used for drop plating. The actual counts were in the expected range of bacterial input.

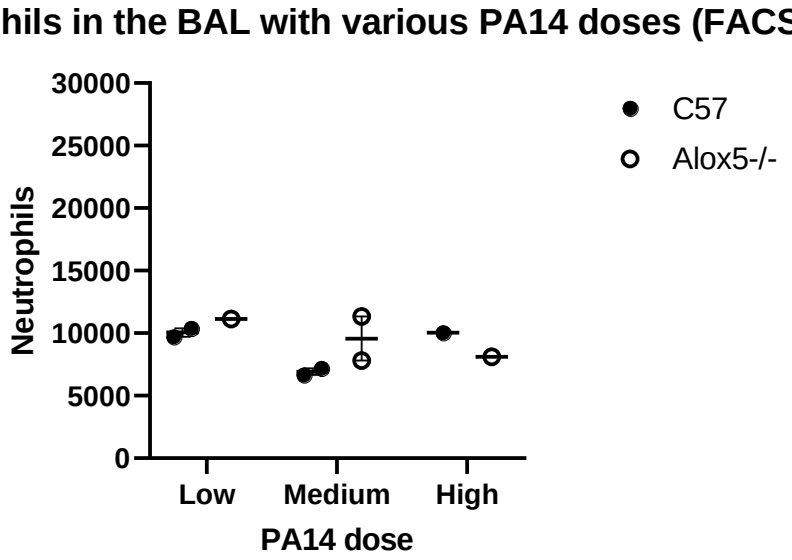


Figure 12. Different doses of PA14 induce PMN migration at similar levels

C57 and alox5$^{-/-}$ mice that were infected with different concentrations of bacteria were found to have no significant differences in neutrophils numbers when evaluated with FACS (**Figure 12**). There was also no difference in neutrophil numbers when comparing the two genotypes. We decided to use the lowest dose of bacteria in our *in vivo* model.

In vivo model demonstrates no difference in PMN transmigration across airway epithelium between C57 and alox5$^{-/-}$ mice infected with PA14

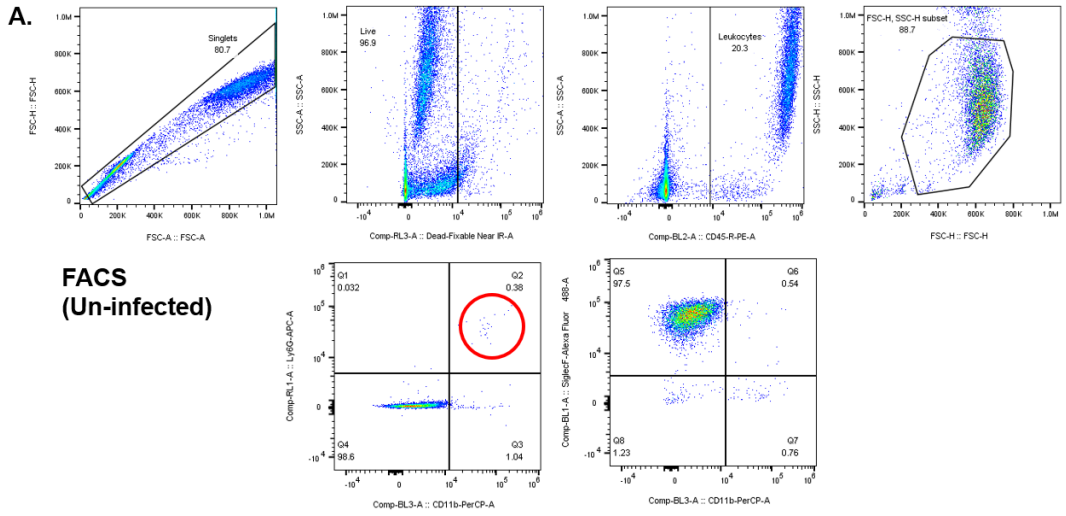
A total of 12 mice were used for each genotype with four used for mock infection and eight used for PA14 infection. Two individual experiments were performed. The results from all the mice used were pooled and graphed appropriately. Neutrophil numbers were calculated using multiple methods. Along with FACS and an MPO assay, neutrophil elastase (ELA2), was also measured to evaluate for neutrophil migration into

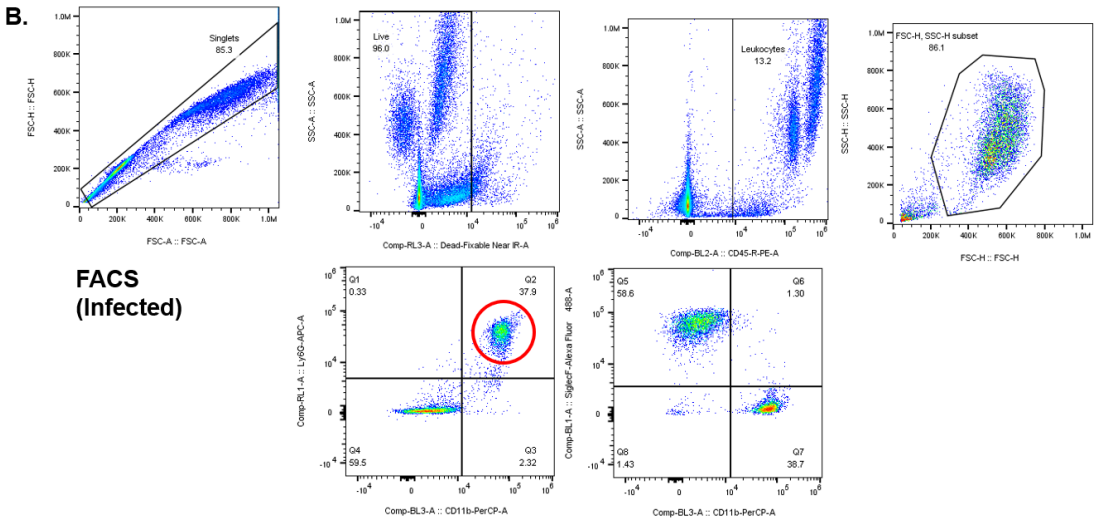
the lungs. An LTB4 assay was done to measure for LTB4 production between the two genotypes. A lactate dehydrogenase (LDH) assay, measuring level for plasma membrane damage, was also done to measure for cell death.

Table 6. *Pseudomonas aeruginosa (PA14)* colony counts on PIA and CFU actual counts for *in vivo* models.

Experiment	Bacterial Dilution	Plate Dilution	Average Colony Count	CFU Actual Count
1	1:70	$1:10^4$	22	660000
2	1:70	$1:10^4$	44.3	1330000

CFU counts were done to ensure an input of an appropriate amount of *Pseudomonas aeruginosa (PA14)* was used to infect the mice. CFU actual counts from both experiments are within reasonable range at around 6 x 10^5 CFU and within range of the bacteria dose dependent study (**Table 4**). Although some variation in the CFU actual counts exists between the two individual experiments, the variation can be seen as negligible.





Figures 13A and 11B. Gating to assess the number of neutrophils by FACS. Alveolar macrophage population marked with Siglec-F circled in black and the neutrophil population circled in red. **(A)** FACS show un-infected mice without neutrophils while **(B)** shows infected mice with neutrophil population.

FACS also accounted for alveolar macrophages using Siglec-F to confirm that they were the main leukocyte population in mock infection. Based on the literature, the alveolar compartment is made up of 93% macrophages in naïve mouse lung (Barletta et al., 2012).

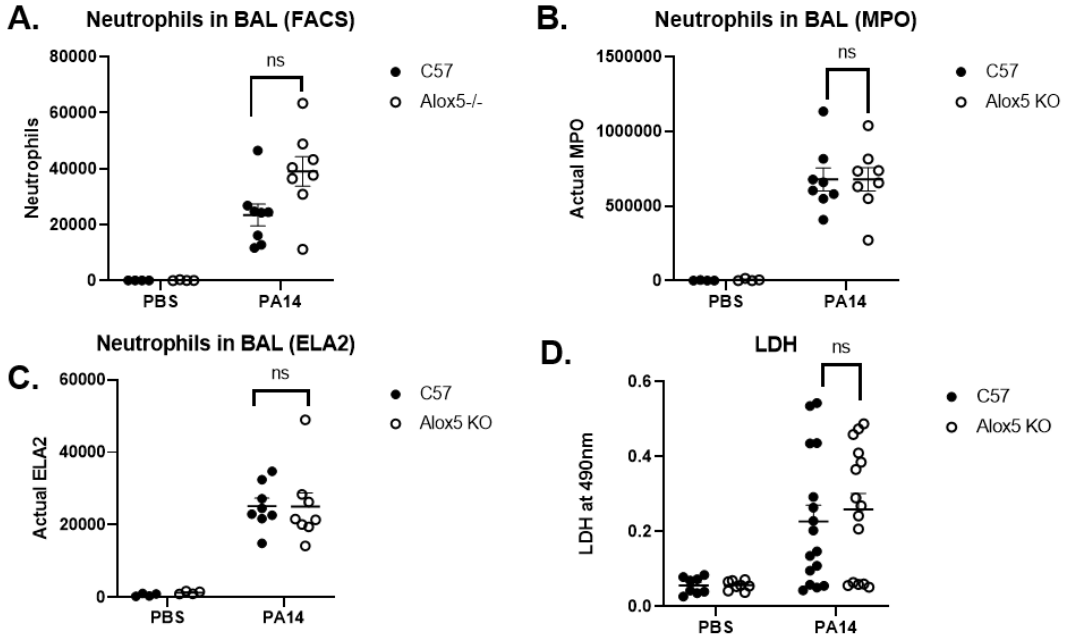


Figure 14(A-D). ***In vivo*** **model demonstrates no difference in PMN migration between C57 and alox5$^{-/-}$ mice infected with PA14**. Wild type (C57BL/6) and alox5$^{-/-}$ mice were intra-nasally infected with PA14 and assessed 6 hours after infection. (A) **Number of neutrophils were assessed by FACS,** (B) **MPO ELISA assay, and** (C) **ELA2 ELISA assay.** (D) **Cell death was measured using an LDH assay.**

All methods show successful infection and induction of neutrophils in the pneumonia mouse model with an increase in neutrophil numbers, MPO, ELA2, and LDH in mice infected with PA14 (**Figure 14A-D**). Although FACS analysis shows a slight increase in neutrophil numbers in alox5$^{-/-}$ mice, there was no statistically significant difference when compared to C57 mice (**Figure 14A**). This statistic is similar in MPO, ELA2, and LDH assays (**Figures 14-D**). The LTB4 assay showed no LTB4 production in both genotypes.

DISCUSSION

Conclusion

Continuing prior studies on the lipoxygenase pathway, we can confirm using gene expression that the transitional epithelial model provides the enzymes necessary to contribute to neutrophil transmigration when infected with *Pseudomonas.* With further differentiation of the epithelial model, an increase in gene expression of these enzymes was expected and observed. When airway epithelial basal cells differentiate into club cells, we should observe an overall increase in gene expression as they contribute more to lung functionality when compared to their basal state. Although previous studies using a similar model have used a fully differentiated epithelial model, this transitional epithelial model provides the enzymes necessary to elicit neutrophil transmigration without needing to maintain the cell culture on the transwell for 3 weeks for full differentiation. This model was then used to confirm the proposed mechanisms by Tamang et al. (2012) and previous *in vitro* data from Yonker et al. (2017).

The *in vitro* model used fits with the study done by Tamang et al. (2012) and Yonker et al. (2017) on the mechanism of the lipoxygenase pathway in which it's induction by infection of airway epithelial cells elicits neutrophil transmigration (**Figure 1**). The results are also consistent showing that LTB4 is a strong chemoattractant that can induce neutrophil transmigration (Lämmermann et al., 2013, p. 4) (**Figure 6**). Based on the proposed mechanism of the lipoxygenase pathway by Tamang et al. (2012) and data from Yonker et al. (2017), the alox5$^{-/-}$ PMNs would be unable to produce LTB4 and would be unable to amplify signaling for neutrophil recruitment, therefore, alox5$^{-/-}$ PMNs

would migrate poorly. Our results from the *in vitro* model support this mechanism as we see more migration in the wild type C57 PMNs when compared to their alox5$^{-/-}$ counterparts (**Figure 7**). The mechanism is further supported with transwells that had alox5$^{-/-}$ PMNs detected essentially no LTB4, showing that knock-out of the alox5 gene hindered the cell's ability to produce LTB4 and that more migration was seen in conditions that had LTB4 detected (**Figure 9**). However, LTB4 ELISA does show that there was no statistically significant difference when comparing C57 PMNs to alox5$^{-/-}$ PMNs (**Figure 9**). This is most likely due to the significant variation in values. When evaluating if changing the genotype of the airway epithelial cells made a difference, the results show that there was more migration in alox5$^{-/-}$ airway epithelial cells when using C57 PMNs but showed so difference with alox5$^{-/-}$ PMNs (**Figure 7**). This pattern is not reproduced when looking at the neutrophil migration trends when induced with LTB4 (**Figure 6**). While it is possible that airway epithelial could play a role in neutrophil migration, it is not possible to conclude on that and more investigation needs to be done.

The *in vivo* model developed for this study was extensively optimized within the time limit given to accurately evaluate for neutrophil migration. With a baseline neutrophil count established and an optimal bacterial dose, an accurate infection of the airway could be performed and analyzed. The overall results, including colony counts, showed that there was successful infection of the lungs, but there was no difference between C57 and alox5$^{-/-}$ mice (**Figure 14A-D**). Going back to the proposed model by Tamang et al. (2012), it is expected that alox5$^{-/-}$ mice would be unable to produce LTB4, and therefore would be unable to recruit as many neutrophils to the lungs when compared

to their C57 counterparts. Therefore, the knockout mice may have deficiency in killing the bacteria. However, we do not see this trend in any of the results. FACS analysis of neutrophil counts actually show a minimal increase in neutrophils in alox5$^{-/-}$ mice but was deemed statistically non-significant (**Figure 14A**). MPO and ELA2 assays are expected to show higher numbers in C57 mice but showed a non-significant difference (**Figure 14B and 14C**). The same trend is expected in the LDH assay where more is expected from C57 mice but again showed no significant difference between the two genotypes (**Figure 11D**). What was surprising was that the LTB4 assays showed no detection in either genotype, which could be caused by the very low production of LTB4 diluted to undetectable levels. With eicosanoids having a diverse set of biological functions, it is possible that a knockout of alox5 could have more unintentional effects than expected. While the data shows that alox5$^{-/-}$ and C57 mice show no difference in neutrophil numbers when infected with *Pseudomonas aeruginosa*, we cannot conclusively state that LTB4 does not play a critical role in neutrophil-mediated airway inflammation in our mouse model. The *in vitro* model does shows that C57 PMNs do migrate at a higher percentage than their alox5$^{-/-}$ counterparts (**Figure 7**). However, when both airway epithelial cells and PMNs are the same genotype (similar conditions to *in vivo*) that difference in migration is somehow mitigated and not significantly different (**Figure 7**).

LTB4 has the potential to be a critical target for inflammation inhibition as it is shown to be a powerful neutrophil chemoattractant. Its role in neutrophil-mediation inflammation is airway epithelium infected with *Pseudomonas aeruginosa* remains ambiguous as the *in vitro* model shows potential in being a critical mediator and amplifier

in neutrophil recruitment while the *in vivo* model shows no support to this hypothesis. It is likely that the mechanisms for the neutrophil transmigration across airway epithelium are very complex and may involve multiple factors including LTB4. The role of LTB4 could be compensated by other factors in alox5$^{-/-}$ mice with infection or could be important in stages that our mouse model at 6 hours post infection does not cover. Further research is needed to define the role LTB4 in pneumonia with *Pseudomonas aeruginosa* infection.

Limitations

Like most studies, there were limitations to what could be investigated, and that the data is part of a larger story of the role lipoxygenase pathway in airway inflammation. The main limitation is that this study was done on a mouse model. Although mice models are a powerful tool in research, there are physiological differences that can affect the overall results. Furthermore, more replicates of these *in vitro* and *in vivo* experiments would further strengthen the statistical significance of our findings. Due to time limits and COVID-19 restrictions on the animal facility, a set number of experiments had to be appropriately planned to allow for the preparation, execution, and analysis of data.

Future Directions

Further analysis of the data and consideration the limitation of our study has opened more areas for possible investigation. The *in vitro* mouse airway epithelial transitional model could be more optimized, such as researching methods that help keep TEER values more consistent across all transwells when culturing them. More

modifications to the mouse model could also be experimented with to further optimize it, including trying longer times post-infection. It is possible that analysis 6 hours post-infection is premature for complete neutrophil migration and processes to be prevalent as bacteria and neutrophils are not directly placed in contact with airway epithelial cells unlike the *in vitro* models. Other cells of the immune system, such as alveolar macrophages, also play a role in the lung's immune system and are not taken into account in the *in vitro* models. Repeat experiments with BAL extraction taking place longer than 6 hours post-infection can give more time for neutrophil migration and processes to occur and allow for a more accurate comparison between wild type and alox5$^{-/-}$ mice.

In terms of other avenues to research in this field, looking into genes upstream in the lipoxygenase pathway could be a potential study. Although most genes upstream of the lipoxygenase pathway show no expression for genes that synthesize enzymes needed to produce HXA3 in basal cells (**Table 3**), looking into possible transcellular synthesis of LTB4 where HXA3 is produced by PMNs, and the epithelial cells could assist in the production of LTB4. This idea of transcellular synthesis of LTB4 can also be applied to the transitional epithelial model by questioning if airway epithelial cells contain enzymes, such as LTA4H, to produce LTB4. By using the *in vitro* transitional epithelial model, various combination of LTA4H$^{-/-}$ cells in either the epithelial cells or PMNs with their counterpart wild type cells, it is possible to investigate if epithelial cells play a significant role in the production of LTB4. Furthermore, applying the *in vitro* transitional epithelial model to human airway epithelial cells would be advantageous to be able to apply our studies to be more relevant in a clinical setting.

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CURRICULUM VITAE