LL-37 and Citrullinated LL-37: Differential modulation of the oxylipin - chemokine axis

By

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Preface

Thesis Summary

Inflammation plays a crucial role in the host immune system, and it involves a complex network of cellular and molecular events. In the lungs, human bronchial epithelial cells release cytokines/chemokines, and other mediators such as bioactive lipids and cationic host defence peptides (CHDPs) to facilitate and regulate airway inflammation. Oxylipins are bioactive lipids that are key mediators in the process of inflammation. The human host defence peptide LL-37 enhances specific oxylipins such as prostaglandin E2 (PGE2) in fibroblasts and endothelial cells. LL-37 plays a pivotal role in inflammation by orchestrating both pro-and anti-inflammatory responses; LL-37 enhances pro-inflammatory chemokines, but also selectively suppresses inflammatory cytokines and help maintain immune homeostasis. However, the mechanisms that govern these opposing roles of LL-37 remain unclear. Under inflammatory conditions, LL-37 can get citrullinated, a post-translational modification that impairs its antimicrobial and antiviral functions. In this thesis, I investigate the interplay of LL-37 and oxylipins, and how that is altered by citrullination, in the context of airway inflammation.

In this study, a lipidomics analysis was performed to profile oxylipins enhanced in the presence of LL-37 and citrullinated LL-37 (citLL-37) in in human bronchial epithelial cells (HBEC). Subsequently, these peptide-mediated chemokine productions were examined by ELISA, transcriptional analysis by qRT-PCR, and impact of peptide on neutrophil migration was examined in a transwell assay using neutrophils isolated from human blood.

LL-37 significantly enhanced oxylipins such as PGE2, 11- and 15-HETE, 15-HETrE, and cytochrome P450-derived 9,10- and 12,13-EpOME. Whereas citLL-37 only enhanced 15-HETrE, 9,10 EpOME and 12,13-EpOME, oxylipins that negatively regulates inflammation. I showed that LL-37, but not citLL-37, enhances COX-2, a central upstream mediator of PGE2 in HBEC. Inhibition of COX-2 suppressed LL-37-induced neutrophil chemoattractants IL-8, MIP-3α, and GROα, thus establishing the link between peptide mediated oxylipin and chemokine production. I functionally confirmed that inhibition of COX-2 impairs LL-37-mediated neutrophil migration. Overall, my findings suggest that citrullination of LL-37 mitigates the peptide's ability to induce pro-inflammatory oxylipins and chemokines, thus this post-translational modification may be a molecular switch to control LL-37-mediated pro- and anti-inflammatory responses in the lungs.

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Dedications

I dedicate this thesis to my parents, whose sacrifices have made it possible for me to follow my dreams. To my mom, who was never given a fair opportunity for education, this achievement is for you.

I will continue to make you both proud!

List of Abbreviations

11,12 EET	11,12-Epoxyeicosatrienoic acid
12-HETE	12-Hydroxyeicosatetraenoic
12-HpETE	12-Hydroperoxyeicosatetraenoic
12-LOX	12-Lipoxygenase
12, 13 EpOME	12,13-Epoxyoctadecenoic acid
12,13 DiHOME	12,13-Dihydroxyoctadecenoic acid
14,15 EET	14,15-Epoxyeicosatrienoic acid
15-HETE	15-Hydroxyeicosatetraenoic
15-HpETE	15-Hydroperoxyeicosatetraenoic
15-LOX	15-Lipoxygenase
20-HETE	20-Hydroxyeicosatetraenoic
5-LOX	5-Lipoxygenase
5,6 EET	5,6-Epoxyeicosatrienoic acid
8-LOX	8-Lipoxygenase
8,9 EET	8,9-Epoxyeicosatrienoic acid
9,10 DiHOME	9,10-Dihydroxyoctadecenoic acid
9,10 EpOME	9,10-Epoxyoctadecenoic acid
AA	Arachidonic acid
ALA	α-Linoleic acid
ANOVA	Analysis of variance
APC	Antigen-presenting cell

BALF	Bronchoalveolar fluid
cAMP	Cyclic adenosine monophosphate
CHDP	Cationic host defence peptide
CIA	Collagen-induced arthritis
citLL-37	Citrullinated LL-37
CLR	C-type-lectin receptor
CNS	Central nervous system
COPD	Chronic obstructive pulmonary disease
COX	Cyclooxygenase
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
CRAMP	Cathelicidin-related antimicrobial peptide
CTL	Cytolytic
CYP P450	Cytochrome P450
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
DGLA	Dihomo-gamma (γ)-linoleic
DHA	Docosahexaenoic acid
DiHOME	Dihydroxyoctadecenoic acid
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's Phosphate-Buffered Saline

EET	Epoxyeicosatrienoic acid
ELISA	Enzyme-linked immunosorbent assay
EP1	Prostaglandin E receptor 1
EP2	Prostaglandin E receptor 2
EP3	Prostaglandin E receptor 3
EP4	Prostaglandin E receptor 4
EPA	Eicosapentaenoic acid
EpOME	Epoxyoctadecenoic acid
ERK	Extracellular-signal-regulated kinase
FPRL1	Formyl peptide receptor 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GM-CSF	Granulocyte-macrophage colony stimulating factor
GPCR	G-protein coupled receptor
GROα	Growth-related oncogene α
hβD-3	Human β-defensin 3
HBEC	Human bronchial epithelial cells
hCAP18	Human cathelicidin antimicrobial peptide 18
HETE	Hydroxyeicosatetraenoic
HpETE	Hydroperoxyeicosatetraenoic
HPLC	High-performance liquid chromatography
HRV	Human rhinovirus
IAP-2	Inhibitor of apoptosis-2

ICAM-1	Intracellular adhesion molecule-1
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgD	Immunoglobulin D
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL-1β	Interleukin-1beta
IL-10	Interleukin 10
IL-12	Interleukin 12
IL-12R	Interleukin 12 receptor
IL-15	Interleukin 15
IL-1RA	Interleukin-1 receptor antagonist
IL-23	Interleukin 23
IL-32	Interleukin-32
IL-6	Interleukin 6
IL-8	Interleukin-8
ILC	Innate lymphoid cell
JNK	c-Jun N-terminal kinase
LA	Linoleic acid
LDH	Lactate dehydrogenase
LOX	Lipoxygenase

LPS	Lipopolysaccharide
LT	Leukotrienes
LTA4	Leukotriene A4
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
MAPK	Mitogen-activated protein kinase
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIP-3α	Macrophage inflammatory protein 3α
MMP	Matrix metalloproteinase
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa B
NK	Natural killer
NLR	Nucleotide-binding oligomerization like receptor
NO	Nitric oxide
NtHi	*Non-typeable Haemophilus influenzae*
P2X7	Purinergic receptor P2X7
PAD	Peptidyl arginine deaminase
PAMP	Pattern-associated molecular patterns
PBEC	Primary bronchial epithelial cells
PBMC	Peripheral blood mononuclear cells

PGD2	Prostaglandin D2
PGE2	Prostaglandin E2
PGF2α	Prostaglandin F2 alpha
PGG2	Prostaglandin G_2
PGH2	Prostaglandin H2
PKA	Protein kinase A
PLA2	Phospholipase A2
PRR	Pattern recognition receptor
PTM	Post-translational modification
PTx	Pertussis toxin
PUFA	Polyunsaturated fatty acids
qRT-PCR	Quantitative real-time polymerase chain reaction
RA	Rheumatoid arthritis
RLR	Retinoic acid-inducible gene 1 like receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
sEH	Soluble epoxide hydrolase
sLL-37	Scrambled LL-37
SPM	Specialized pro-resolving mediator
T reg	T regulatory cell
TBX2	Thromboxane B2
TCR	T-cell receptor

TGF-β	Transforming growth factor-β
Th1	T-helper cell 1
Th17	T-helper cell 17
Th2	T-helper cell 2
TLR	Toll-like receptor
TNFα	Tumor necrosis factor α
TXA2	Thromboxane A2
VCAM-1	Vascular adhesion molecule-1
VD3	Vitamin D3

List of Figures

Figure 1. *Oxylipin metabolism* 11
Figure 2. *Schematic representation of the indirect neutrphil migration assay* 35
Figure 3. *LL-37 and citLL-37 differentially enhances cytokines and chemokines* 42
Figure 4. *Citrullination of LL-37 reduces the ability of LL-37 to induce IL-8, MIP-3α, and GROα transcriptionally* 44
Figure 5. *Citrullination of LL-37 reduces the ability of LL-37 to induce IL-8, MIP-3α, and GROα* 45
Figure 6. *Citrullination of LL-37 reduces the ability of LL-37 to induce IL-8, MIP-3α, and GROα in PBEC* 46
Figure 7. *Inhibition of GPCRs suppresses LL-37-mediated enhancement of chemokines* 48
Figure 8. *Differential modulation of COX mRNA by LL-37 and citLL-37* 50
Figure 9. *Correlation analysis between LL-37-mediated enhancement of chemokines and COX-2 mRNA* 51
Figure 10. *Inhibition of COX-2 suppresses LL-37-mediated enhancement of chemokines* 53
Figure 11. *LL-37-mediated migration of neutrophils is dependent on the COX-2 pathway* 55
Figure 12. *Inhibition of cytochrome P450 pathway suppresses LL-37 and citLL-37-mediated enhancement of chemokines* 57
Figure 13. *The interplay of LL-37 and oxylipins in immune responses of human bronchial epithelial cells* 68
Figure 14. *LL-37 drives the induction of IL-8, MIP-3α, and GROα in HBEC* 72

Supplementary Figure 1. *Time kinetics of LL-37-mediated chemokine responses at the mRNA level* 76
Supplementary Figure 2. *Time course analysis of LL-37-mediated chemokine protein abundance in HBEC* 77
Supplementary Figure 3. *Dose kinetics of LL-37-mediated chemokine response in HBEC* 78
Supplementary Figure 4. *Time course analysis of COX enzymes at the mRNA level* 79
Supplementary Figure 5. *Dose kinetics of COX-2 inhibitor, Rofecoxib on HBEC cells* 80

Supplementary Figure 6. *Percent cytotoxicity of COX-2 inhibitor on HBEC cells* 81
Supplementary Figure 7. *Dose kinetics of cytochrome P450 inhibitor, Orteronel on HBEC cells* 82
Supplementary Figure 8. *Percent cytotoxicity of cytochrome P450 inhibitor on HBEC cells* 83
Supplementary Figure 9. *Dose kinetics of GPCR inhibitor, pertussis toxin (PTx) on HBEC cells* 84

List of Tables

Table 1. *Sequences for LL-37, citLL-37, and sLL-37 peptides*. 28

Table 2. *Primers used for quantitative real-time PCR.*... 33

Table 3. *Differential enhancement of oxylipins by LL-37 and citLL-37 in HBEC.*....................... 39

Table of Contents

Preface .... *i*

Thesis Summary .... *ii*

Acknowledgements .... *iii*

Dedications .... *v*

List of Abbreviations .... *vi*

List of Figures .... *xiii*

List of Tables .... *xv*

Chapter 1: Introduction *1*

1.1 Introduction to the Immune System **2**
1.1.1 ***Innate Immunity*** 2
1.1.2 ***Adaptive Immunity*** 3
1.1.3 ***Cytokines and chemokines*** 5

1.2 Inflammation **6**

1.3 Introduction to Oxylipins **8**
1.3.1 ***Enzymatic oxidation of oxylipins*** 9
1.3.1.1 COX Pathway 9
1.3.1.2 LOX Pathway 9
1.3.1.3 CYP Pathway 10
1.3.2 ***COX/PGE$_2$ axis in inflammation*** 12
1.3.2.1 PGE$_2$ signalling 12
1.3.2.2 Influence of PGE$_2$ on immune cells 13
1.3.3 ***COX/PGE$_2$ in diseases*** 13
1.3.3.1 Airway diseases 14
1.3.4 ***Role of Cytochrome P450-derived oxylipins in the regulation of inflammation*** 14
1.3.5 ***EpOMEs and DiHOMEs in airway diseases*** 15

1.4 Cationic Host Defence Peptides (CHDPs) **16**
1.4.1 ***Overview*** 16
1.4.2 ***Human host defence peptide LL-37*** 17
1.4.3 ***LL-37-mediated immunomodulation*** 18
1.4.4 ***LL-37 in diseases*** 19
1.4.4.2 Airway diseases 20

1.5 Citrullination **21**
1.5.1 ***Overview*** 21
1.5.1.1 Physiological role of citrullination 21
1.5.1.2 Role of citrullination in immune functions 21
1.5.1.3 Effects of citrullination on LL-37 22

1.6 Interplay of oxylipins and CHDPs in inflammation **23**

1.7 Thesis Overview **25**

1.7.1 *Rationale* 25
1.7.2 *Hypothesis* 26
1.7.3 *Specific aims* 26

Chapter 2: Materials and Methods *27*

2.1 Reagents **28**
2.1.1 *Peptides* 28
2.1.2 *Cytokines and Pharmacological Inhibitors* 28

2.2 Cell Culture **29**
2.2.1 *Human Bronchial Epithelial Cells (HBEC-3KT)* 29
2.2.2 *Human Primary Bronchial Epithelial Cells (PBEC)* 30

2.3 LC-MS/MS Quantification of Oxylipins **31**
2.3.1 *Extraction and Quantification of Oxylipins* 31

2.4 Enzyme-Linked Immunosorbent Assay (ELISA) **32**

2.5 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR) **32**

2.6 Cytotoxicity Assay **34**

2.7 Neutrophil Migration Assay **34**

Chapter 3: Results *37*

3.1 Lipidomics Profile of Oxylipins **38**

3.2 Peptides LL-37 and citLL-37 selectively regulate cytokine and chemokine expression **40**
3.2.1 *Time and dose kinetics of peptide-mediated response in HBEC* 40
3.2.2 *Differential expression of cytokines and chemokines by LL-37 and citLL-37* 41
3.2.3 **Independent validation of the selected chemokine abundance in HBEC** 43
3.2.4 *Involvement of GPCRs in LL-37 and citLL-37-mediated chemokine production* 47

3.3 The relationship between the COX pathway and LL-37-mediated immunomodulatory function **49**
3.3.1 *Citrullination of LL-37 reduces the ability of LL-37 to enhance COX-2 mRNA* 49
3.3.2 *Inhibition of COX-2 suppresses LL-37-mediated enhancement of chemokines* 52
3.3.3 *Inhibition of COX-2 suppresses LL-37-mediated migration of neutrophils* 54

3.4 Relationship between cytochrome P450 pathway and LL-37 and citLL-37-mediated chemokine production **56**
3.4.1 *Inhibition of cytochrome P450 pathway suppresses LL-37 and citLL-37-mediated enhancement of chemokines* 56

3.5 Summary of results **58**

Chapter 4: Discussion **59**

4.1 Limitations **69**

4.2 Significance **70**

4.3 Future Directions **71**
4.3.1 *Mechanisms of LL-37- and citLL-37-mediated differential chemokine production* 71

4.3.2 ***Relationship of the cytochrome P450 pathway and LL-37-mediated migration of neutrophils*** 73
4.3.3 ***Quantification of citLL-37 levels in vivo*** 73
4.3.4 ***Mechanisms related to COX-2 pathway in LL-37-mediated enhancement of chemokines*** 73
4.3.5 ***Evaluation of LL-37 and COX pathways in vivo*** 74

***Chapter 5: Appendix*.. 75**

Supplementary Figures 76

References Cited..85

Chapter 1: Introduction

1.1 Introduction to the Immune System

As humans, we are constantly faced by both external and internal factors that can pose a risk to our bodies every day. Our immune system protects us from threats that could harm us. It is a complex network of cells, chemicals and processes designed to detect and combat a diverse range of pathogens such as viruses, bacteria, parasites, and foreign antigens[1]. The immune system can be broadly divided into two parts: (1) innate immunity and (2) adaptive immunity.

1.1.1 *Innate Immunity*

Innate immunity is described as the 'first line of defence' against foreign "invaders" or antigens, i.e. molecules that the body deems as "foreign". These can be infectious microbes, environmental factors, or internal molecules that is not recognized by the body as "self". The innate immune system is composed of mechanical and physical barriers that prevent the entry and establishment of antigens. For example, the lung is separated from the external environment by a barrier of epithelial cells held together by tight junctions[2]. These structural cells of the lungs produce mucus, and the movement of cilia prevent microorganisms from entering the respiratory tract. If pathogens breach the physical barriers, innate immune cells and inflammatory mediators work in concert to prevent infection. Innate immune cells can be classified as granulocytes and non-granulocytes based on whether they contain granules in their cytoplasm[3]. Granulocytes include neutrophils, mast cells, basophils, and eosinophils, while non-granulocytes consist of macrophages, natural killer (NK) cells, dendritic cells (DCs), and innate lymphoid cells (ILCs).

When an inflammatory response is initiated, neutrophils are the first responders to the site of injury. These short-lived cells possess phagocytic properties and contain granules and enzymes that aid in clearance of pathogens[1,4]. Mast cells and basophils play key roles in initiating acute inflammation, with mast cells producing cytokines and basophils releasing inflammatory mediators in response to environmental allergens[1,4]. Additionally, eosinophils possess phagocytic properties and are involved in allergy and asthma[1,4].

In contrast, macrophages are long-lived cells that serve as antigen-presenting cells (APCs), playing a crucial role in initiating and regulating immune responses. Similarly, DCs are APCs that act as messengers between the innate and adaptive immune systems, facilitating the appropriate adaptive immune response based on the antigens that develop from the innate immune response[1,4].

NK cells are another important component of the immune system with cytotoxic properties; they release perforins and granzymes to induce apoptosis, and play a major role in viral immunity[1,4]. ILCs have regulatory roles depending on their specific subsets and contribute to homeostasis and tissue repair[1,4].

It was initially thought that the innate immune system is non-specific. However, it is now known that germ-line encoded receptors known as pattern recognition receptors (PRRs) expressed on immune cells and structural cells of various tissues recognize structural / molecular similarities, pattern-associated molecular patterns (PAMPs), from pathogens as well danger signals (DAMPs) from the host[1,5–7]. This allows the innate immune system to mount a rapid response within minutes or hours of a pathogen or danger molecule being encountered[6,7]. The major PRRs include Toll-like receptors (TLRs), nucleotide-binding oligomerization (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs), and retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs)[5–7].TLRs are transmembrane proteins that recognize PAMPs like lipids, proteins, and nucleic acids from various pathogens[6]. CLRs recognize carbohydrate structure on pathogens and self-molecules[6]. NLRs and RLRs are cytosolic receptors that detect intracellular PAMPs like bacterial peptidoglycans and viral nucleic acids[6]. When initiated, PRRs trigger specific signaling cascades that lead to the production of various cytokines and chemokines. The consequent biological process known as inflammation is characterized with the production of immune messenger molecules such as cytokines and chemokines, that work in concert to coordinate the innate immune response and subsequently communicate with adaptive immune cells to mount an appropriate downstream response. The process of inflammation is essential for the resolution of infection but must be meticulously regulated and suppressed once the antigen is resolved.

1.1.2 *Adaptive Immunity*

Described as the second ‘line of defence’, the adaptive immune system is initiated when the innate immune system fails to control the infection or the antigenic stimuli. The key characteristics of the adaptive immune response are its high specificity to antigens and its capacity for memory, enabling the host to respond more quickly and effectively if re-exposed to the same antigen[1]. Lymphocytes are the key players in the adaptive immune response, specifically T cells and B cells[1,4,8]. T cells are activated by APCs to proliferate and carry out effector functions

depending on the antigen, while B cells differentiate into plasma cells to generate antibodies specific to the antigen[1].

As with the innate immune system, adaptive immune cells also use receptors to recognize antigens and mount a response. Major histocompatibility complexes (MHCs) are the receptors involved in the adaptive immune response[1,9,10]. They are responsible for presenting peptide fragments derived from self or foreign antigens to T cells[9,10]. MHC class I molecules display peptides from intracellular proteins to $CD8^+$ cytotoxic T cells[11]. In contrast, MHC class II molecules display peptides from extracellular proteins to $CD4^+$ helper T cells, which then activate other immune cells like B cells and macrophages[11]. When an immature DC digests an antigenic protein, it becomes 'mature' and travels to regional lymph nodes where it presents the antigen to a naïve T cell[1]. T cells express unique antigen-binding receptors on their membranes, known as T cell receptor (TCR)[1]. The MHC-antigen complex activates the TCR, allowing T cells to secrete cytokines and differentiate into different T cell subsets[1].

T cells can proliferate into cytotoxic T cells ($CD8^+$ T cells) or T helper (Th) cells ($CD4^+$ T cells)[1,12]. Briefly, $CD8^+$ cytotoxic T cells primarily function in eliminating cells infected by foreign antigens, such as viruses, and targeting tumor cells expressing specific antigens[1,12]. On the other hand, $CD4^+$ T cells lack cytotoxic or phagocytic properties and cannot directly eliminate infected cells or clear pathogens. Instead, they orchestrate immune responses by instructing other cells to carry out these tasks and regulating the type of immune response that develops[1,12]. $CD4^+$ T cells can further differentiate into T helper 1 (Th1), Th2, Th17, and T regulatory (T reg) cells, depending on the composition of cytokines in the inflammatory process triggered by the antigen[1,12].

In addition to producing antibodies, B cells play a role in antigen presentation and cytokine production which helps to shape the adaptive immune response[1,13,14]. B cells generate five major types of antibodies: Immunoglobulin (Ig) A, IgD, IgE, IgG and IgM[1,13,14]. These antibodies bind to antigens present on pathogen surfaces, marking them for destruction through activation of the Complement system. Antibodies label antigens for phagocytosis and subsequent pathogen elimination by immune effector cells[1,13,14]. When B cells are activated by specific foreign antigens that attach to their antigen receptors, they proliferate and differentiate into antibody-secreting plasma cells and/or memory B cells[1,13,14].

If the immune system is successful in clearing the pathogen, some T and B cells become memory cells, which can act quickly upon re-exposure with the same antigen[1]. Memory B cells stay in the body, ready to produce antibodies to eliminate the antigen.

1.1.3 *Cytokines and chemokines*

As mentioned previously, cytokines play an important role in mediating the immune response and inflammation, either by promoting or suppressing inflammation as required[15]. These are low molecular weight (5-70 kDa) proteins that are secreted by various immune cells and structural cells of different tissues. Cytokines can regulate several functions such as cell growth, proliferation, differentiation, activation, migration, and survival of immune cells[16–18]. Cytokines are grouped based on their structural similarities and the receptors that they bind to[16–18]. A hallmark of cytokine biology is that they can bind to many different cell types and cells may express more than one receptor for a given cytokine[16]. Binding to their respective receptor(s) generates a signaling cascade that influences downstream functions, either of the cell that secretes the cytokine (autocrine), on nearby cells (paracrine), or on distant cells (endocrine)[16].

Some of the major cytokines are interferons (IFN), interleukins (IL), tumor necrosis factor (TNF) and chemokines[16,19]. Chemokines are a group of small (8 to 12 kDa) molecules responsible for mediating migration of immune cells[17]. They play a crucial role in guiding immune cells to where they are needed. Chemokines mediate the immune response through their interactions with members of the 7-transmembrane, G protein-coupled receptor (GPCR) superfamily[16]. Chemokines are classified into four main families (C, CC, CXC, CXC3C) based on the arrangement of cysteine residues in their amino acid sequence[20]. The C-X-C subfamily is characterized by a variable amino acid separating the first two cysteine residues[15, 19]. In the C-C subfamily, the cysteine residues are positioned next to each other. These subfamilies can be differentiated based on their primary target cells: the C-X-C subfamily mainly attracts neutrophils, while the C-C subfamily primarily targets monocytes and T cells[16,17].

The chemokines used as readout in this thesis project are CXCL8 (IL-8), CXCL1 (GROα) and CCL20 (MIP-3α). IL-8 and growth-related oncogene α (GROα) are C-X-C family chemokines. Whereas macrophage inflammatory protein 3α (MIP-3α)[19,21–23] belongs to the CC

family of chemokines. IL-8 and GROα are produced by various cell types including macrophages and epithelial cells in response to an inflammatory stimulus such as pro-inflammatory cytokines IL-1β and TNFα[21]. IL-8 and GROα are known for their chemotactic properties, particularly their ability to induce neutrophil migration to sites of infection or injury[21]. Both these chemokines mediate their effects by binding to the GPCRs CXCR1 and CXCR2 on target cells. However, IL-8 exhibits greater specificity for the CXCR1 receptor compared to GROα, which makes it a more potent neutrophil chemoattractant[21]. On the other hand, MIP-3α binds to chemokine receptor CCR6 and is a potent chemoattractant for lymphocytes, dendritic cells, and to a lesser extent neutrophils[19,22]. MIP-3α expression can be induced by microbial factors like LPS, inflammatory cytokines like TNFα and IFNγ[19,22].

Another group of cytokines that play an important role in immune response are anti-inflammatory cytokines. These cytokines are crucial mediators of the immune system that help to maintain immune homeostasis, prevent excessive inflammation, and promote the resolution of inflammatory responses. They can suppress the production and activity of pro-inflammatory cytokines to regulate inflammation. For example, IL-10 is a cytokine with potent anti-inflammatory properties that can suppress the production of inflammatory cytokines such as TNFα, IL-6 and IL-1β[16,19]. Additionally, interleukin-1 receptor antagonist (IL-1RA) and transforming growth factor-β (TGF-β) are examples of anti-inflammatory cytokines. IL-1RA inhibits the release of IL-1-induced cytokines such as IL-6, and also blocks the stimulation of prostaglandin E2 (PGE2), an important lipid mediator in inflammation[24,25]. TGF-β also contributes to this balance by suppressing cytokine production by inhibiting macrophage and Th1 activity[16].

The cytokine network mediates immune responses by either enhancing inflammation to resolve infections or suppressing inflammation to restore immune system balance. This balance between pro-inflammatory and anti-inflammatory responses is crucial for maintaining an effective immune response.

1.2 Inflammation

Inflammation is a normal part of the host response to infection and is required for clearance of the pathogen, and regulation of this process is critical maintaining immune homeostasis[1]. The

process of inflammation aims to eliminate pathogens, clear away debris, and initiate tissue repair[15]. Inflammation involves vascular changes and a cellular phase. The vascular phase initiates the process of inflammation by also allowing the influx of immune cells into the affected area. During the this phase, upon pathogen recognition, the release of mediators such as histamine and bradykinin causes vasodilation[26,27]. This increases the blood flow to the affected area[26,27]. There is also increased permeability, allowing plasma proteins and fluids to leak into the tissue[26,27]. Together, these processes give rise to the cardinal sign associated with inflammation, heat, redness, swelling, and pain[1,27,28].

The recognition of PAMPs or DAMPs triggers an acute inflammatory phase. The acute inflammatory response is a short-term process that occurs immediately after injury or infection, lasting for a few days[1,29]. Briefly, in this phase, there is activation of immune cells and the release of inflammatory mediators that coordinate the process of inflammation. Tissue-resident macrophages and mast cells secrete various inflammatory mediators such as cytokines, chemokines, and other molecules. These mediators play a crucial role in attracting plasma proteins and other immune cells to the site of inflammation[30]. For instance, neutrophils migrate in response to chemokines and other mediators released by the initial immune cells[26]. Mediators produced by the innate immune cells further amplify the inflammatory response and recruit highly specific activated cells of the adaptive immune system to clear the pathogen or injury[31]. If the injury is minor, the acute inflammatory response will be successful in clearing the injury, and the inflammatory response will be resolved.

During the regulatory phase of inflammation, immune cells can adopt a regulatory phenotype, enabling them to suppress pro-inflammatory responses and promote anti-inflammatory actions. For example, macrophages can transition from a pro-inflammatory (M1) state to a more anti-inflammatory (M2) phenotype, releasing factors that facilitate tissue repair[32]. Additionally, molecules such as oxylipins and host defence peptides (HDPs) play critical roles in modulating inflammation to maintain immune homeostasis. Oxylipins exhibit dual roles, exerting pro-inflammatory or anti-inflammatory effects depending on the environmental milieu[28,33,34]. Host defence peptides can promote inflammation in various ways such as promoting the production of pro-inflammatory chemokines[35]. However, they can also regulate inflammation by

suppressing pro-inflammatory chemokines and enhancing anti-inflammatory cytokines[35]. These molecules therefore act as immunomodulators that fine tune the immune response.

In the cases of persistent stimuli or the body mistakenly recognizing self-components as threats, continuous lymphocyte infiltration and sustained inflammatory responses may occur, leading to chronic inflammation. Chronic inflammation can persist for months or years, leading to tissue damage and fibrosis[1,29]. Asthma is an example of a chronic respiratory disease characterized by a complex network of cellular interactions[36]. If left unregulated, this inflammation can lead to airway hyperresponsiveness and tissue remodeling, which are key features of asthma[36].

Therefore, proper regulation of the inflammatory response is essential to ensure effective pathogen clearance while minimizing tissue damage and preventing chronic inflammation. This highlights the importance of molecules that can act as immunomodulators.

In the following sections, I will focus on two immunological mediators, oxylipins and cationic host defence peptides (CHDPs) and their interplay in modulating inflammation.

1.3 Introduction to Oxylipins

Oxylipins are bioactive lipids that are generated from the enzymatic and non-enzymatic oxidation of polyunsaturated fatty acids (PUFA)[37]. PUFAs are fatty acids that contain more than one double bond on their backbone. Linoleic acid (LA) and α-linoleic acid (ALA) are two essential fatty acids that are obtained from diet[38]. LA can be further metabolized to arachidonic acid (AA) and Dihomo-gamma(γ)-linoleic acid (DGLA), whereas ALA can be further metabolized to docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA)[38,39]. The main biosynthetic pathways for oxylipins involve enzymes like cyclooxygenases (COX), lipoxygenase (LOX), and cytochrome P450 enzymes (CYPs), and non-enzymatic reactive oxygen species (ROS)[37]. A subset of oxylipins, eicosanoids, are derived from 20-carbon PUFAs such as AA and are responsible for the cardinal signs of inflammation[37]. They can act on the cells that secrete or on nearby cells. *It is important to note that there are other fatty acids that can act as precursors for the synthesis of oxylipins, however, this thesis will focus on the enzymatic oxidation of AA and LA.*

1.3.1 *Enzymatic oxidation of oxylipins*

The generation of oxylipins starts with the activation of phospholipase A_2 (PLA_2) which releases AA and LA from the lipid membrane[40]. AA and LA can act as substrates for three different metabolic pathways: (1) cyclooxygenase (COX), (2) lipoxygenase (LOX), and (3) cytochrome P450 pathway (CYP). Once formed, oxylipins can modulate biological processes by binding to receptors or by being re-esterified into lipids[28]. The three major oxylipin metabolic pathways mentioned above are briefly described below and detailed in Figure 1.

1.3.1.1 *COX Pathway*

The primary enzymes in this pathway are COX-1 and COX-2[28,41–43]. COX-1 is constitutively expressed in most tissues, whereas COX-2 can be induced in response to inflammatory stimuli, cytokines, and growth factors[40]. COX-1 and COX-2 have similar molecular weights (70-72 kDa) and share about 65% amino acid sequence homology[44]. COX-2 is primarily expressed in epithelial cells, fibroblasts, and various immune cells[45]. These enzymes catalyze the oxidation of AA into prostaglandin G2 (PGG2), an unstable intermediate that gets converted to prostaglandin H2 (PGH2)[46]. PGH2 is a precursor for various prostaglandins (PGD2, PGE2, PGF2a) and thromboxanes (TXA2, TBX2), the major products of the COX pathway[46]. COX-1 plays a role in the continuous production of prostaglandins, which are essential for maintaining homeostasis. The synthesis of prostaglandins by COX-2 is inducible by inflammation[40]. Thus, COX-2 is associated with enhancement of inflammation, and plays a key role in the axis of immune response and metabolism. Aside from their role in inflammation, prostaglandins and thromboxanes play a role in vascular homeostasis, pain perception, and other physiological processes[28,40,45].

1.3.1.2 *LOX Pathway*

Various lipoxygenase enzymes catalyze AA to form hydroxyperoxyeicosatetraenoic acid (HpETE), the main product of this pathway by the addition of an oxygen molecule[47,48]. Through the action of 5-lipoxygenase (5-LOX), 8-LOX, 12-LOX, and 15-LOX enzymes, HpETE can be converted to hydroxyeicosatetraenoic acid (HETE), leukotrienes, lipoxins, and hepoxilins. 5-LOX generates 5-HpETE and leukotriene A4 (LTA4), the precursor of pro-inflammatory leukotrienes like LTB4, LTC4, LTD4, and LTE4[47]. 12-lipooxygenase (12-LOX) and 15-lipooxygenase (15-

LOX) produce 12-HPETE/12-HETE and 15-HPETE/15-HETE, respectively[47]. It is important to note that 11- and 15-HETE can also be produced from the COX pathway, as by-products from prostaglandin synthesis[49–51]. Leukotrienes are well established for their pro-inflammatory role in inflammation, they lead to the recruitment and activation of immune cells in allergic inflammation[33]. On the other hand, HETEs are shown to have both pro- and anti-inflammatory effects depending on the environment[52,53].

1.3.1.3 *CYP Pathway*

In this pathway, AA and LA are catalyzed to epoxyeicosatrienoic acid (EET) and hydroxyeicosatetraenoic acid (HETE) by epoxygenases and ω-hydroxylases[34,54–56]. The metabolism of LA by cytochrome P450 monooxygenases produces two regioisomers: 9,10- and 12, 13-epoxyoctadecenoic acids (EpOMEs). Additionally, metabolism of AA, a downstream metabolite of LA, leads to the formation of epoxyeicosatrienoic acids (EETs)[34,54–56]. This includes four regioisomers: 5,6-, 8,9-, 11,12- and 14,15-EET, as well as hydroxyeicosatetraenoic acids (HETEs) like 20-HETE[34,54–56]. These epoxy fatty acids are then metabolized by soluble epoxide hydrolase (sEH) to generate the corresponding fatty acid diols[34,54–56]. For example, 9,10- and 12,13- EpOME are further metabolized to 9,10- and 12,13- DiHOME[57]. The exact roles of EpOMEs and DiHOMEs in inflammation are not well understood, however, they can serves as chemotactic factors for neutrophils[34,57]. Additionally, DiHOMEs have been shown to have a suppressive effect in inflammation by inhibiting neutrophil respiratory burst, highlighting their immunomodulatory functions[34,57].

Figure 1

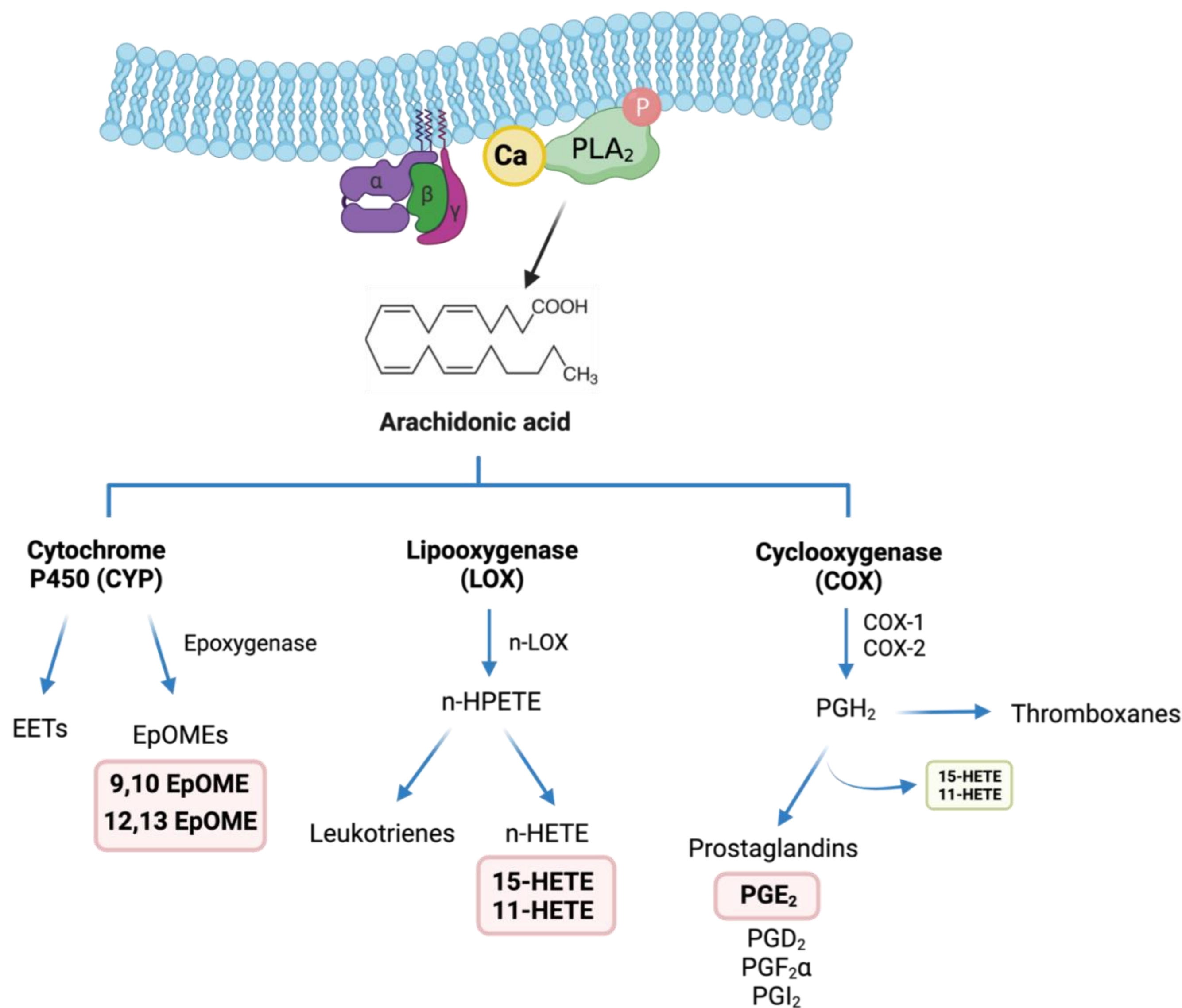


Figure 1. ***Oxylipin metabolism***. In this modified figure, oxylipins are synthesized through the oxidation of polyunsaturated fatty acids (PUFAs) via three main pathways: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP). Activation of phospholipase A_2 (PLA_2) releases the PUFA from the lipid membrane. PUFAs are further enzymatically metabolized by COX, LOX and CYP. The COX pathway generates prostaglandins and thromboxanes as its major products and hydroxyeicosatetraenoic acids (HETEs) as minor products. The LOX pathway synthesizes leukotrienes and HETEs, whereas the CYP pathway produces epoxyeicosatrienoic acids (EETs) and epoxyoctadecenoic acids (EpOMEs). *This figure was created in BioRender*.

The focus of this thesis will be on the COX and cytochrome P450 pathways. As mentioned above, oxylipins are important modulators of inflammation, therefore the following sections will focus on the opposing roles of PGE2 and EpOMEs in inflammation.

1.3.2 ***COX/PGE$_2$ axis in inflammation***

Prostaglandins were the first oxylipins to be discovered, and their role in disease has been studied extensively since it was found that aspirin, an anti-inflammatory drug, inhibits COX enzymes and the production of downstream metabolites[40]. The COX-2/PGE2 axis is a central inflammatory pathway that modulates various aspects of the immune response and inflammation through its effects on different immune cell types[28,45]. It plays a crucial role in regulating the initiation, resolution, and homeostasis of inflammation, highlighting its complex and context-dependent functions[58]. In the early stages of the inflammatory response, PGE2 and related prostanoids like PGI2 function as vasodilators, facilitating the movement of innate immune cells like neutrophils from the bloodstream into the tissue, which results in swelling and edema at the site of injury[28,28,58]. In contrast, PGE2 can inhibit the production of certain pro-inflammatory cytokines and promote the production of anti-inflammatory cytokines such as IL-10, which plays a role in the resolution of inflammation[28]. *I will further focus on the role of PGE2 in inflammation since it's the most studied prostaglandin.*

1.3.2.1 *PGE$_2$ signalling*

PGE2 is produced by various cells of the body and exerts its effects by binding to one (or a combination) of its four G protein-coupled receptors (EP1, EP2, EP3, an EP4)[28,45]. Despite their structural similarity, each receptor couples to different types of G proteins, leading to diverse downstream effects[28,45]. EP1 receptor couples to Gαq proteins, leading to activation of phospholipase C and causing the release of intracellular calcium as a second messenger[28,45]. On the other hand, EP2 and EP4 receptors primarily couple to Gαs proteins, which activate adenylyl cyclase and increase production of cAMP as a second messenger[28,45]. In contrast, the EP3 receptor predominantly couples to Gαi proteins, inhibiting adenylyl cyclase activity and thereby reducing cAMP production[28,45].

1.3.2.2 *Influence of PGE_2 on immune cells*

It is important to note that PGE2 can have both pro-inflammatory an anti-inflammatory effects depending on the cell type, concentration of PGE2, and the inflammatory environment[28,45]. PGE2 indirectly promotes tissue influx of neutrophils, macrophages, and mast cells by enhancing chemokines such as IL-8 and monocyte chemoattractant protein-1 (MCP-1)[28,45]. PGE2 acts on NK cells to suppress their cytolytic effector functions by suppressing IL-12 and IL-15[28,45]. It also suppresses the production of IFNγ by NK cells, thereby disrupting their "helper" role in DC-mediated induction of Th1 CTL responses[28,45]. Through the action of its EP2 receptor, PGE2 can limit the pathogen-killing function and phagocytosis by alveolar macrophages[28,45]. In addition, PGE2 promotes the induction of mast cells in a mechanism involving its EP1 and EP3 receptor[28,45].

PGE2 is shown to play a role in the lifespan of T cells by influencing positive and negative selection in the thymus, as well as proliferation, apoptosis, and cytokine production in mature T cells[28,45]. It can induce the secretion of cytokine such as IL-6, IL-1β, IL-8, and IL-23 in both murine and human T cells[28,45]. In contrast, PGE2 can dampen Th1 immune response by favoring Th2 subset of T cells and suppressing IL-2 and IFNγ production[28,45]. It can also have a suppressive effect on the proliferation of activated lymphocytes, reducing the expansion of immune effector cells[28,45]. In B cells, PGE2 promotes isotype-class switching, leading to increased production of IgG_1 and IgE[28,45]. Moreover, PGE2 influences antigen-presenting cells such as macrophages and dendritic cells by promoting the expression of IL-10 while inhibiting the expression of IL-12, IL-12R, TNFα, and IL-1β[28,45]. PGE2 also regulates chemokine production, suppressing the recruitment of pro-inflammatory cells while promoting the accumulation of regulatory T cells[43]. It is important to note that PGE2 levels in the initial phases of inflammation are transient and moderate[59]. However, PGE2 levels rise again during the resolution phase and eventually increase to much higher levels in the post-resolution phase[59].

1.3.3 *COX/PGE_2 in diseases*

The COX pathway and its product PGE2 play important roles in various diseases due to their involvement in inflammation and regulation of various physiological processes[28,40,45]. The role of the COX pathway and prostaglandin synthesis has been extensively studied in various diseases. There has been a lot of research examining the role of this pathway in autoimmune

diseases in particular rheumatoid arthritis. However, as the focus of my thesis work is on bronchial epithelial cells, I describe the role of COX/PGE2 in the lung and respiratory disease.

1.3.3.1 *Airway diseases*

Several studies have investigated the role of COX in airway diseases to better understand its role, including in asthma. Asthma is a chronic inflammatory respiratory disease that is characterized by airway inflammation, narrowing and hyperresponsiveness[36]. In allergic asthmatics, there is an increase of prostanoids in the bronchoalveolar fluid (BALF) compared to non-asthmatic individuals[40]. One study found that bronchial epithelial COX-2 levels were four times higher in asthmatics patients compared to healthy controls[40,60]. Corticosteroids are a common anti-inflammatory treatment option for asthmatics[36]. Studies indicate that corticosteroids can reduce COX-2 expression. For example, an *in vitro* study demonstrated that corticosteroid treatment decreased COX-2 immunoreactivity in cultured airway epithelial cells[61].

In a COX-1 knockout mouse model sensitized with OVA, there was increased airway inflammation evident by increased serum IgE levels, elevated Th2 cytokines, greater number of T cells and increased airway hyperresponsiveness. Conversely, in C57BL/6 COX-2 knockout mice, there was reduced serum IgE levels, vascular cell adhesion molecule-1 (VCAM-1) and intracellular adhesion molecule-1 (ICAM-1) compared to WT mice[40,62]. These studies highlight the complex role that the COX pathway plays in allergic inflammation, indicating the role of COX-2 in promoting responses that drive the pathophysiology of airway inflammation. This is corroborated by studies demonstrating the role of COX-2 pathway in promoting the production of mucin and goblet cell hyperplasia in chronic inflammatory respiratory diseases such as asthma and COPD[63]. These studies support the rationale of intervening in the COX-2 pathway for the suppression of airway inflammation in respiratory disease.

1.3.4 *Role of Cytochrome P450-derived oxylipins in the regulation of inflammation*

EETs and EpOMEs are the main products of the cytochrome P450 pathway. This pathway is primarily associated with resolving inflammation. EETs are well-studied for their role in

inflammation, acting as potent vasodilators that relax blood vessels by targeting smooth muscle cells[64]. EETs also enhance endothelial cell function by increasing nitric oxide (NO) production and preventing endothelial cell apoptosis[41]. They inhibit leukocyte adhesion at sites of inflammation and suppress the production of pro-inflammatory cytokines such as IL-1β,TNF-α, and IL-6 [41,65]. EETs achieve this by blocking NF-κB, a critical transcription factor involved in the expression of inflammatory genes, leading to the downregulation of inflammatory pathways[41]. Furthermore, EETs contribute to resolving inflammation by promoting the production of specialized pro-resolving mediators (SPMs) like lipoxins and resolvins, which aid in dampening inflammation and facilitating tissue repair[66].

In contrast, the roles of EpOMEs and DiHOMEs, also products of the CYP pathway, in inflammation are less well-understood. In humans, these oxylipins are produced by neutrophils and macrophages in response to bacterial challenges[57]. While both EpOMEs and DiHOMEs serve as chemotactic mediators for neutrophils, only DiHOMEs have been shown to suppress the excessive production of reactive oxygen species (ROS) in neutrophils[57]. DiHOMEs achieve this by inhibiting the neutrophil respiratory burst through a mechanism distinct from other respiratory burst inhibitors[57]. Thus, limited studies suggest that DiHOMEs can function in the negative regulation of oxidative stress and neutrophil-mediated inflammation[34].

1.3.5 *EpOMEs and DiHOMEs in airway diseases*

The exact roles of EpOMEs and DiHOMEs in respiratory diseases are not fully understood. EpOMEs and DiHOMEs are linked to chronic lung conditions caused by environmental factors. Elevated levels of EpOMEs have been observed in lung tissue and BALF following exposure of rats to inhaled oxidants, while increased plasma concentrations of DiHOMEs were observed in animal models exposed to tobacco smoke[34]. In contrast, DiHOMEs are decreased in BALF after exposure to subway air in asthmatic individuals, whereas healthy individuals show increased levels[34,67]. In another study, healthy volunteers exposed to biodiesel exhaust exhibited higher levels of plasma 9,10-DiHOME compared to those exposed to filtered air[34,68]. Furthermore, EpOMEs and DiHOMEs were found to be elevated in the BALF of female smokers with COPD compared to female smokers with normal lung function, although this increase was not observed in male smokers[34,69].

These findings suggest that EpOMEs and DiHOMEs levels are altered by various environmental factors that are linked to respiratory disease, and therefore these metabolites may play a role in the inflammatory responses to environmental exposures. Further research is needed to elucidate the specific mechanisms through which these oxylipins contribute to disease pathogenesis and progression, or perhaps play a role in the regulation of inflammation. The role of these metabolites in the process of inflammation and airway pathophysiology remains unknown.

1.4 Cationic Host Defence Peptides (CHDPs)

1.4.1 *Overview*

Cationic host defence peptides (CHDPs) are small amphipathic peptides (> 50 amino acids) with a positive charge ranging from +2 to +9 at physiological pH and are found in all living organisms, including microorganisms, plants, animals, and humans . To date, more than 3000 natural antimicrobial peptides or CHDPs have been identified, with 162 to date defined from humans (APD3; Antimicrobial Peptide Database, https://aps.unmc.edu/). These peptides have been well studied for their antimicrobial properties, however, in the last two decades these peptides have been recognized for their role beyond infection control, from immunity-related functions, to wound healing and control of inflammation[35,73,74]. It is shown that under physiological conditions, such as high salt concentration and host factors, these peptides lose their antimicrobial properties, but can still modulate the host immune response to resolve infections[35,75]. The ability of CHDPs to influence the host immune response depends on various factors, including environmental stimuli, cell and tissue type, interactions with different cellular receptors, and the concentration of the peptides[35,70]. These factors also influence the function of CHDPs in the process of inflammation, as these peptides can both facilitate and suppress inflammation. The mechanisms that control the ability of these peptides to switch between pro- and anti-inflammatory effects remains unknown.

In humans, CHDPs are synthesized by epithelial cells and secreted by immune cells such as neutrophils and mast cells[35,70,75]. CHDPs have diverse sequences, unique structure, target specificity, and can be grouped based on their three-dimensional secondary structures. They can

be categorized as α-helical linear peptides, β-sheet with disulfide bridges, cyclic peptides, and peptides with extended loop structures[35,70,75].

The two most studied human CHDPs are defensins and cathelicidins. Defensins are small cysteine-rich cationic proteins ranging from 18-45 amino acids, with three to four highly conserved disulfide bonds[35,70,75]. They are broadly categorized as α-defensins (6 major subtypes) and β-defensins (28 subtypes) based on the position of cysteine residues, peptide chain folding, and length[35,70,75]. α-defensins are produced by Paneth cells in the small intestine, whereas β-defensins are expressed by epithelial cells[35,70,75].

Cathelicidins derive their name from the presence of a conserved cathelin-like domain within the prepropeptide of this family of CHDP[35,70,75]. The prepropeptide contains an amino-terminal (N-terminal) signal peptide, a cathelin-like domain, and the carboxy-terminal domain[35,70,75]. The pro-cathelin-like domain is typically cleaved off by serine proteases after secretion, resulting in the formation of mature active peptides[76].

In humans, there is only one cathelicidin peptide, LL-37, which is an α-helical amphipathic peptide[35,70,75]. LL-37 is the mature peptide derived from hCAP18, the product of the cathelicidin gene CAMP, and this can be also cleaved into other smaller peptides resulting in several cleavage products from hCAP18[35,70,75]. hCAP18 is cleaved by proteases such as proteinase 3 to release the mature, biological functional peptide, LL-37. Cathelicidin peptide similar to LL-37, all with similar cathelin domain, is found is many other species. Similar to humans, mice also have only one cathelicidin peptide, cathelicidin-related antimicrobial peptide (CRAMP)[77]. Whereas other species such as cattle, sheep, pigs have multiple cathelicidin peptides[78]. *This thesis focuses on the human host defence peptide LL-37.*

1.4.2 *Human host defence peptide LL-37*

LL-37 is mainly produced by epithelial cells and neutrophils[35,70,75]. Neutrophils release LL-37 through degranulation, a process where they release granules, and during NETosis, a form of cell death when neutrophils release extracellular traps (NETs) composed of DNA[72]. The expression of LL-37 is regulated by infection, inflammation, and other external factors such as vitamin D3 and retinoic acid (vitamin A)[72,79,80].

LL-37 exhibits broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria[35]. It binds to the negatively charged membranes of bacteria, disrupting membrane integrity and leading to bacterial death[35]. Its α-helical shape allows it to penetrate membranes, form transmembrane pores, and cause bacterial cell death[76]. LL-37 also exhibits antiviral properties. Briefly, LL-37 can destabilize viral envelopes upon contact, damage viral proteins, and reduce infectivity[35]. For non-enveloped viruses, LL-37 binds to viral capsids to inhibit viral uncoating and nuclear entry of the viral genome, thereby suppressing viral replication[35]. In addition, recent studies have shown that LL-37 can limit the growth of clinically relevant fungi[81]. However, the direct antimicrobial functions of LL-37 occur at high localized concentrations at the site of infections and is antagonized by host factors at physiological concentrations[35]. Therefore, research in the last two decades have focused on immune functions of LL-37 in resolution of infections[82,83]. Beyond its direct antimicrobial properties, LL-37 plays a role in immune-related processes such as chemotaxis, epithelial cells activation, angiogenesis, regulation of inflammation and epithelial wound repair[35,72].

1.4.3 *LL-37-mediated immunomodulation*

LL-37 plays a crucial role in the immune response due to its multifaceted functions. It can directly interact with 16 different protein partners and indirectly interact with more than 1000 secondary effector proteins, making its role in modulating inflammation highly complex[75]. LL-37 can induce chemotaxis, modulate immune cell functions, act as a bridge between innate and adaptive immunity, and possess pro- and anti-inflammatory properties to either facilitate or negatively regulate inflammation[35,72,83].

LL-37's ability to mediate chemotaxis is one of its primary roles in facilitating inflammatory response in innate immunity. During an infection, LL-37 can directly and indirectly recruit immune cells to the site of infection. It can directly induce the recruitment of immune cells by activating the seven-transmembrane G-protein coupled receptor (GPCR), formyl peptide receptor 1 (FPRL1)[84]. LL-37 also promotes the production of chemokines to indirectly recruit immune cells to mucosal sites. Specifically, LL-37 stimulates the production of IL-8 and GROα by engaging GPCRs and the Cdc42/Rac1 GTPase pathway[85]. Additionally, LL-37 induces the

transcription of CCL4, CCL20, and CXCL1 through GAPDH in human macrophages and peripheral blood mononuclear cells (PBMCs)[86].

LL-37 can also influence other functions of immune cells at the site of infection or inflammation. LL-37 can facilitate the formation of NETs and promote bacterial clearance *in vivo* by enhancing early neutrophil responses, such as reactive oxygen species (ROS) formation[35]. However, the effect on neutrophils appears to be dependent on the time or context. [87] LL-37 has been observed to promote the internalization of chemokine receptors CXCR2 on neutrophils and monocytes, thereby dampening chemotaxis of these cells[87]. LL-37 can act as a bridge between innate and adaptive immunity by influencing functions of antigen-presenting cells. LL-37 shapes the adaptive response by modulating differentiation and function of DCs *in vitro* and *in vivo*[48,88]. LL-37 can also shape the adaptive immune response by altering the differentiation of Th subsets directly[72]. LL-37 also shapes the immune response by altering cytokine response from various cell types. However, its actions are context- dependent and influenced by the environmental milieu. For instance, LL-37 can synergize with IL-1β and granulocyte-macrophage colony stimulating factor (GM-CSF) to enhance chemokine production in PBMCs, which is classically a pro-inflammatory response[89]. In contrast, LL-37 can suppress cytokine IL-32-induced pro-inflammatory cytokines such as TNFα and IL-1β, and in contrast enhances anti-inflammatory cytokine IL-1RA in human macrophages and PBMCs[90]. In bronchial epithelial cells LL-37 can induce chemokine production and in contrast suppress specific TNFα-mediated pro-inflammatory responses[91]. Thus, LL-37's immunomodulatory properties allow it to promote or suppress inflammation as needed, as thus LL-37 is thought to play a critical role in maintaining immune homeostasis [92].

1.4.4 *LL-37 in diseases*

LL-37 plays a diverse role in infection and inflammation, contributing to various diseases. Dysregulation of LL-37 has been attributed to increased susceptibility of infectious disease, and the role of LL-37 in protecting against infections is well established with mechanisms being either directly antimicrobial or by boosting host response to resolve infections [35,93–95]. In chronic inflammatory disease, the role of LL-37 is not completely established, as the abundance of LL-37 changes based on the disease phase and severity, especially in airway disease. For example,

elevated levels of LL-37 have been found in the synovial fluid and serum of RA patients, which suggests that this peptide may contribute to the inflammatory response in RA[96]. However, this remains unclear, as a LL-37-derived immunomodulatory peptide has been shown to be protective in an animal model of inflammatory arthritis[97]. Similarly in airway disease such as COPD and asthma the role of LL-37 has not completely been defined as follows.

1.4.4.2 *Airway diseases*

LL-37 is increased in individuals with chronic respiratory diseases, including COPD and asthma[98,99]. LL-37 is present in the airways of patients with bacterial lung infections and plays a role in defence against bacterial species[100]. Airway inflammation involves the action of immune cells such as eosinophils, neutrophils, and lymphocytes, which leads to the release of pro-inflammatory mediators. LL-37 can act directly as a chemotactic factor, leading to the recruitment of immune effector cells such as neutrophils, eosinophils, and mast cells in diseases such as asthma. In an epithelial cell line, LL-37 was shown to trigger the activation of eosinophils in a P2X purinoceptor 7/epidermal growth factor receptor-dependent manner, which in turn increased the production of IL-6, IL-8, and CCL4[101]. However, animal model studies show contradictory results with cathelicidin peptides. Administration of CRAMP, the mouse ortholog of LL-37, showed increased airway hyperresponsiveness and airway inflammation in an OVA-challenged mouse model[101]. In contrast, the level of CRAMP in the lungs is decreased in the allergen house dust mite (HDM)-challenge mouse model, with exogenous administration of a cathelicidin-derived peptide alleviating HDM-mediated airway inflammation and hyperresponsiveness[102–104]. Similarly, LL-37 and its derivative peptides have also been shown to be protective by decreasing neutrophil recruitment in a model of acute lung injury[105]. These studies highlight that the role of cathelicidins remain unclear in respiratory disease characterized by airway inflammation.

Recent studies have shown that in addition to native LL-37, post-translationally modified versions of the peptide such as citrullinated LL-37 (citLL-37) are also detected in BALF in healthy individuals[106]. Moreover, enzymes result in citrullination of LL-37 are found elevated in the lungs of individuals with COPD[99]. These recent studies have led to an interest in understanding how the functions of LL-37 changes once it is citrullinated under inflammatory conditions in the lungs, which is the focus of this thesis project.

1.5 Citrullination

1.5.1 *Overview*

Under inflammatory conditions, LL-37 is susceptible to post-translational modifications (PTM) such as citrullination[107–110]. This PTM converts positively charged arginine residues into neutral citrulline, thereby reducing the overall positive charge of the peptide[107–110]. This irreversible modification is catalyzed by calcium dependent peptidyl-arginine deaminase (PAD) enzymes. There are five isoforms of the PAD enzymes (PAD1, PAD2, PAD3, PAD4, and PAD6), each with distinct substrate specificities and tissue-specific expression patterns[107,108]. For example, PAD2 and PAD4 are present in neutrophils, with PAD2 located in the cytosol and PAD4 primarily in the nucleus[107].

1.5.1.1 Physiological role of citrullination

Citrullination is involved in many biological processes such as skin keratinization and immune functions. Additionally, citrullination plays roles in the central nervous system (CNS), gene regulation, and cellular homeostasis and growth[107,110]. Specifically, PAD4-mediated citrullination of histones is important in chromatin decondensation and controlling gene regulation[107]. Citrullination has gained attention for its relevance in autoimmune diseases such as rheumatoid arthritis (RA)[107]. There are limited studies on the process of citrullination and how that may alter inflammatory responses in the lungs.

1.5.1.2 Role of citrullination in immune functions

Immune cells such as monocytes and granulocytes express PAD4 enzyme, indicating that citrullination plays a role in these cells[110]. One of the effector functions of neutrophils is their ability to form NETs and undergo NETosis. Histone hypercitrullination by PAD4 is crucial for the formation of NETs[107]. It was observed that mice deficient in PAD4 were more susceptible to bacterial infections due to reduced NET formation[111]. Studies on a macrophage cell line suggest that PAD2 interacts with inhibitor κB kinases and suppresses NF-kB activity following LPS stimulation, indicating that PAD2 may play a role in innate defence mechanisms[110]. Cytokines can also influence PAD4. It has been shown that TNFα induces the translocation of PAD4 from the

cytosol to the nucleus[110,112]. In transgenic mice overexpressing TNFα, there is increased levels of citrullinated histones and elevated nuclear PAD4[110,112]. Taken together, these studies suggest that the process of citrullination may exist as a regulatory mechanism in inflammation.

As discussed above, chemokines play a pivotal role in inflammation. Studies show that there is a change in the chemotactic activity of citrullinated chemokines. For example, citrullinated IL-8 has a reduced affinity for glycosaminoglycans, is resistant to cleavage by thrombin/plasmin, and is unable attract neutrophils to the peritoneum[113]. However, citrullinated IL-8 retains the ability to attract neutrophils into the blood stream, suggesting that citrullination of IL-8 selectively alters its inflammatory functions [113]. In addition, citrullinated chemokines have reduced affinity for their receptors. Citrullinated CXCL12 shows reduced affinity for the CXCR4 receptor, while and citrullinated CXCL10 and CXCL11 show decreased chemoattractant and signaling capabilities through CXCR3 receptor[114]. These studies suggest that the process of citrullination may have an anti-inflammatory effect, which remains to be completely understood.

1.5.1.3 Effects of citrullination on LL-37

The effects of citrullination on LL-37 is an area that is still being explored. Limited studies have evaluated the effects of citrullination on LL-37's antimicrobial and antiviral properties. The degree of citrullination, specifically how many arginine residues are citrullinated, plays a role in how PTM affects LL-37's properties. Partially citrullinated LL-37 (3cit) only slighted affected ani-bacterial activity, compared to native LL-37[99]. However, LL-37 (5cit) displayed weak antibacterial activity against both *Staphylococcus aureus* and *non-typeable Haemophilus influenzae* (NtHi)[99].

LL-37 has LPS-neutralizing properties, which protects the host from severe inflammatory responses such as those seen in septic shock. Citrullination of LL-37 significantly reduces the peptide's ability to neutralize LPS due to a reduced affinity for this endotoxin[108]. Administration of citLL-37 and LPS exacerbated sepsis, and it was suggested that citLL-37 fails to neutralize LPS by direct binding[108]. This results in heightened systemic inflammation due to increased serum levels of IL-6[108]. Citrullination of LL-37 exhibits impaired antibacterial activity against *E. coli*, diminished LPS-neutralizing ability, and reduced binding to nucleic acids[115]. In addition,

citrullination significantly alters the interaction of LL-37 with DNA, resulting in reduced activation of DCs and macrophages in response to bacterial DNA[116]. A recent study also found that citrullination of LL-37 abrogates its direct antiviral activity against human rhinovirus (HRV) [109]. HRV infection increases PAD2 protein expression and protein citrullination levels in human bronchial epithelial cells[109]. This leads to citrullination of LL-37, which, in turn impairs its antiviral properties against HRV[109]. Although studies have explored how citrullination alters the antimicrobial functions of LL-37, the effect of this PTM on the peptide's immunity-related functions and modulation of inflammation is not yet understood.

1.6 Interplay of oxylipins and CHDPs in inflammation

Limited studies have examined the interplay of CHDPs and oxylipins in inflammation, which is a complex interaction: CHDPs can trigger the production of oxylipins, and oxylipins can influence the actions of CHDPs. Both CHDPs and oxylipins have immunomodulatory properties, allowing these family of biomolecules to modulate the inflammatory response as needed, and play in role in resolution of infections and maintaining immune homeostasis as follows.

In human gingival fibroblasts, CHDP human β-defensin 3 (hβD-3) upregulates COX-2 expression in both a time and dose-dependent manner[117]. Additionally, CHDPs can induce the production of prostaglandins from immune cells. For example, LL-37 enhances COX-2 expression and PGE2 synthesis by interacting with the P2X7 receptor, ERK, and p46 JNK in human gingival fibroblasts[118]. LL-37 also triggers PGE2 synthesis in endothelial cells in a dose-dependent manner with maximal induction after 4 hours[119]. Specifically, LL-37 acts on endothelial cells to trigger calcium release and induces phosphorylation and activation of cytosolic PLA_2[119]. In keratinocytes, LL-37 upregulates COX-2 and PGE2 to aid in protection from apoptosis by inducing expression of inhibitor of apoptosis-2 (IAP-2)[120]. Additionally, LL-37 is shown to induce the release of leukotrienes from eosinophils, which are key players in asthma, although leukotrienes are not discussed extensively in this thesis[121].

LL-37 can also indirectly influence the production of oxylipins. For example, mast cells release mediators such as histamine, prostaglandins, and leukotrienes[122]. LL-37 can increase vascular permeability in the skin by activating mast cells and phosphorylating the MAP kinases

p38 and ERK[123]. LL-37 is known to induce mast cell degranulation, thereby leading to the release of lipid mediators[124].

Conversely, oxylipins can modulate the expression and functions of CHDPs. Vitamin D3 (VD3) promotes the transcription of hCAP18, precursor of LL-37[35]. PGE2 is shown to suppress vitamin D3-induced expression of hCAP18/LL-37 via EP2 and EP4 receptors in human macrophages[125]. Specifically, PGE2 suppresses VD3 expression by activating the cAMP/PKA signaling pathway which enhances a repressor protein that prevents vitamin D receptor expression[125]. Both CHDPs and oxylipins can interact with key transcription factor NF-κB, further highlighting their interconnection[32,126].

While the direct interaction between CHDPs, specifically LL-37 and oxylipins in inflammation has not been extensively studied, it is reasonable to hypothesize their interplay play a role in the process of regulating inflammation, given their role as immunomodulators. *This thesis focuses on the interplay of LL-37 and oxylipins, specifically LL-37's involvement in the COX and cytochrome P450 pathways in context of airway inflammation.*

1.7 Thesis Overview

1.7.1 *Rationale*

Inflammation is a critical component of the innate immune system, and it involves an intricate network of cellular and molecular events. Inflammation is triggered when the host becomes susceptible to invasion by foreign antigens or tissue injury[66]. In the lungs, human bronchial epithelial cells release cytokines /chemokines, and other mediators which include cationic host defense peptides (CHDP) and bioactive lipids, to facilitate and regulate airway inflammation[66,127].

Oxylipins are a group of bioactive lipids which are important soluble mediators that promote inflammation[66]. Oxylipins are formed from polyunsaturated fatty acids (PUFAs) and can be classified based on their biochemical functions[66]. A subset of oxylipins, eicosanoids, are pro-inflammatory, and are formed from the release of arachidonic acid from the lipid membrane by phospholipase A_2[128]. Eicosanoids activate specific G protein-coupled receptors (GPCRs) to trigger downstream promotion of other inflammatory mediators[66]. They act as substrates for three different pathways: cyclooxygenase (COX) pathway which generates prostaglandins, lipoxygenase (LOX) pathway which produces leukotrienes (LTs), and cytochrome P450 monooxygenases pathway which produces epoxides[46]. Prostaglandin E2 (PGE2) and the COX2 pathway are well characterized mediators of inflammation, including airway inflammation[40].

Previous studies have shown that a human CHDP, LL-37, can enhance oxylipins such as PGE2 in endothelial cells and fibroblasts[118,119]. LL-37 is critical in resolution of infections and can also modulate inflammatory responses[118]. As such, LL-37 exhibits both pro- and anti-inflammatory functions, depending on the cellular environment and kinetics of response. The pro-inflammatory functions of LL-37 such as induction of chemokines is dependent on GPCRs, but its anti-inflammatory functions seem to be independent of this pathway[85]. The molecular switch that controls the pro- and anti-inflammatory functions of LL-37 remains elusive.

Recent studies have shown that under inflammatory conditions, LL-37 can get citrullinated, a post-translational modification that can impair the antimicrobial functions of LL-37[115]. Citrullination results in conversion of positively charged arginine residues to neutral citrulline and is mediated by peptidyl-arginine deiminases (PAD) in various tissues including the lungs[110,129,129]. PAD enzymes, specifically PAD4, is essential to the formation of NETs and NETs results in

increasing the concentration of LL-37 in the lungs[107]. While citrullination of LL-37 can impair its antimicrobial and antiviral properties, the effect of citrullination on the immunomodulatory functions of LL-37, as well as the peptide's ability to enhance bioactive lipids, remains unknown. It is possible that citrullination of LL-37 mitigates its pro-inflammatory functions, including its ability to enhance pro-inflammatory lipids.

1.7.2 *Hypothesis*

Citrullination of LL-37 will alter the peptide's ability to enhance oxylipins and selectively disrupt the pro-inflammatory functions (e.g. enhancement of chemokines) of LL-37 in human bronchial epithelial cells (HBEC).

1.7.3 *Specific aims*

(Aim 1) To identify differences in LL-37- and citrullinated LL-37 (citLL-37)-mediated production of chemokines in HBEC.

(Aim 2) To examine oxylipins enhanced in the presence of LL-37 and citLL-37 in HBEC.

(Aim 3) To identify oxylipin pathway(s) involved in LL-37 and citLL-37-mediated chemokine production.

Chapter 2: Materials and Methods

2.1 Reagents

2.1.1 *Peptides*

The peptides LL-37, citrullinated LL-37 (citLL-37) and scrambled LL-37 (sLL-37) were synthesized and purchased from Innovagen AB (Lund, Sweden). The sequences of the peptides can be found in Table 1. All peptides were synthesized with >95% purity, quality control reports were obtained from the manufacturer which specified the purity by analytical HPLC and amino acid analysis, as well as provided a mass spectral analysis to confirm the molecular weight of the peptide. All peptides (1 mg per vial) were reconstituted in Hyclone™ endotoxin-free water (Fisher Scientific, Burlington, ON, CA; Cat# SH3052901) to make a working stock of 1 mg/mL, which was aliquoted in glass vials (100 µL per vial) and stored at -20 °C, for a maximum of 3 months, before use. The peptide aliquots were thawed at room temperature (RT), sonicated for 30 seconds, and vortexed for 15 seconds before use. Peptide working stocks were diluted to desired concentrations in airway epithelial cell culture medium before stimulation for cell culture experiments (detailed below).

Table 1. Sequences for LL-37, citLL-37, and sLL-37 peptides.

Peptides	Sequence
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES
citLL-37	LLGDFFCitKSKEKIGKEFKCitIVQCitKDFLCitNLVPCitTES
sLL-37	RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL

2.1.2 *Cytokines and Pharmacological Inhibitors*

Recombinant human (rh) cytokines IL-8 (carrier free, Cat# 208-IL) and GROα (CXCL1; carrier free, Cat# 275-GR) were purchased from R&D Systems (Oakville, ON, CA). These were aliquoted and stored at -80 °C before use.

Rofecoxib, a specific COX-2 inhibitor (Cat# S3043) and Orteronel, a cytochrome P450 inhibitor (Cat# S1195) were obtained from Selleckchem (Burlington, ON, CA). Rofecoxib and Orteronel were reconstituted in Dimethyl sulfoxide (DMSO; Fisher Scientific) to a working

concentration of 100 mM, according to the manufacturer's instructions. Pertussis toxin, a G-protein coupled receptor (GPCR) inhibitor (Cat# 3097/50U) was obtained from Tocris-R&D (Toronto, ON, CA), and reconstituted in endotoxin free water to a concentration of 100 ng/µL, as per manufacturer's instructions. Before use, each inhibitor was further diluted in airway epithelial cell culture medium (detailed below). Cells were pre-treated with inhibitors at concentrations indicated, 1 hour (h) prior to stimulation with peptides. Details regarding peptide and inhibitor dilutions are described in the figure legend of each experiment.

2.2 Cell Culture

2.2.1 *Human Bronchial Epithelial Cells (HBEC-3KT)*

The HBEC-3KT cell line, purchased from American Type Culture Collection (ATCC® CRL-4051™), was cultured in airway epithelial cell basal medium (ATCC® PCS-300-030™) and supplemented with bronchial epithelial cell growth kit (ATCC® PCS-300-040™), according to the manufacturer's instructions and previously described by us[130,131]. The bronchial epithelial growth kit contained 4% w/v Extract-P, 6 mM L-Glutamine, HLL supplement (containing 500 µg/mL HSA, 0.6 µg/mL Lecithin, 0.6 µM Linoleic Acid), and Airway Epithelial Cell Supplement (containing 5 µg/mL Transferrin, 1 µM Epinephrine, 5 µg/mL Hydrocortisone, 10 nM T3, 5 µg/mL rh-Insulin, 5 ng/mL rh-EGF). For passaging and plating, HBEC-3KT cells were maintained at approximately 80% confluency. Cells were trypsinized using a 1:3 dilution of 0.5% trypsin-Ethylenediaminetetraacetic acid (EDTA; Invitrogen™, Life Technologies Inc, Burlington, ON, CA) in DPBS (Gibco™, Thermo Fisher Scientific) and neutralized with 2% Fetal bovine serum (FBS; Gibco) in Dulbecco's Phosphate-Buffered Saline (DPBS), before culturing cells in T75 flasks at a density of 1.25×10^5 cells/mL. Cells were seeded at a density of 9.5×10^3 cells/mL in 12-well, 6-well and 60 mm tissue culture (TC) plates (Costar® Corning®, NY, USA), as needed for different experiments. The type of culture plate used for each experiment is detailed in the respective figure legend. The cell culture medium was replaced with airway epithelial cell basal medium (with 6 mM L-glutamine and without the growth supplements), 24 h prior to stimulation with peptides either LL-37, citLL-37, and sLL-37 (at concentrations indicated for different experiments)in the presence or absence of various inhibitors as indicated[131].

2.2.2 *Human Primary Bronchial Epithelial Cells (PBEC)*

Human PBEC were isolated from resected lung tissue from 2 anonymous donors (n=2), undergoing lung resection surgery at the Leiden University Medical Center (LUMC, The Netherlands), as previously described[132]. These cells were obtained in collaboration with Drs. Pieter Heimstra and Anne van der Does at LUMC. The donors were enrolled at LUMC with written informed consent in accordance, with approval by the LUMC institutional medical ethical committee (B20.042/Ab/ab and B20.042/Kb/kb).

PBEC were expanded in T75 TC flasks precoated with 10 µg/mL fibronectin (Sigma), 30 µg/mL PureCol (Advanced Biomatrix, California, USA) and 10 µg/mL bovine serum albumin (BSA; Sigma) in PBS (Gibco). Keratinocyte serum-free medium (KSFM; Gibco) supplemented with 25 µg/mL of bovine pituitary extract (BPE; Gibco), 0.2 ng/mL of epidermal growth factor (EGF; Life Technologies), 1:100 dilution of antibiotics Penicillin and Streptomycin (Lonza, Kingston, ON, CA) and 1 µM isoproterenol (Sigma-Aldrich, Oakville, ON, CA) was used to maintain cells until approximately 80% confluency was reached.

For plating, PBEC were trypsinized with 0.3 mg/mL trypsin (containing 1 mg/mL glucose (Gibco), 0.1 mg/mL EDTA (Gibco) and 1:100 dilution of Penicillin and Streptomycin) diluted in PBS. Subsequently, PBEC were seeded at a density of $1x10^4$ cells/mL in 12-well TC plates (Costar®) pre-coated with coating media, as described above. PBEC were cultured in a 1:1 mixture of basal bronchial epithelial cell medium (ScienCell, CA, USA), which contained bronchial epithelial cell growth supplement (ScienCell) and 1:100 dilution of Penicillin and Streptomycin, along with Dulbecco's modified Eagle's medium (Gibco), containing a 1:40 dilution of HEPES buffer (Invitrogen) and 1:100 dilution of Penicillin and Streptomycin. Additionally, the cell culture medium was supplemented with 1 nM of EC-23 (Tocris, UK). PBEC cell culture medium was replaced every 48 h until they reached approximately 80% confluency[131]. Culture medium was replaced with starvation media (without EGF, BPE, BSA), 24 h prior to stimulation with either LL-37, citLL-37, and sLL-37, at concentrations as indicated for each experiment.

2.3 LC-MS/MS Quantification of Oxylipins

2.3.1 *Extraction and Quantification of Oxylipins*

LC-MS/MS quantification of oxylipins was done in collaboration with Dr. Christopher Pascoe (Dept. Pathophysiology, University of Manitoba), in Dr. Harold Aukema's lab (Dept. Food and Human Nutritional Sciences, University of Manitoba). Briefly, HBEC-3KT cells were stimulated with either LL-37, citLL-37, and sLL-37 (0.25 μM each) and TC supernatants were collected after 24 h. 10 μL of antioxidant 0.2 mg/mL BHT, 0.2 mg/mL of EDTA, 100 μM of indomethacin, 100 μM of trans-AUCB in methanol/water (50/50, v/v) and 10 μL of deuterated mix of internal standard were added to epithelial cell media (1.5 mL). Methanol was added (20 %) and samples were adjusted to pH 3. Solid phase extraction with Strata-X SPE columns (Phenomenex, CA, USA) preconditioned with methanol and pH 3 water was used for oxylipin extraction. The samples were loaded onto columns, washed with 10 % methanol in pH 3 water, and then eluted with methanol. The extracted oxylipins were dried down under a gentle stream of nitrogen and resuspended in solvent A (Water – Acetonitrile – Acetic Acid [70:30:0.02; v/v/v]) & Solvent B (Acetonitrile – Isopropyl Alcohol [50:50; v/v]) for analysis by HPLC-MS/MS (API 4000, AB Sciex, Canada), as previously described[133]. Briefly, sample oxylipins were separated on a Nexera-XR LC-20AD XR (Shimadzu) HPLC through gradient elution: Solvent A (Water – Acetonitrile – Acetic Acid [70:30:0.02; v/v/v]) & Solvent B (Acetonitrile – Isopropyl Alcohol [50:50; v/v]) by reverse phase with a Luna column (Luna 5u C18(2) 100A; 250 x 2.00 mm, Phenomenex). The HPLC was coupled to a Qtrap 6500 (SCIEX) mass spectrometer equipped with a IonDrive Turbo V Electrospray Ion source. Polarity was set to negative mode and responses were measured using selective reaction monitoring (SRM)[134].

Oxylipin abundance was measured in cell free TC supernatant using LC-MS/MS. Oxylipins enhanced by sLL-37 were removed from the data analysis. Pairwise differential analysis was conducted on normalized $\log_2$ oxylipins values and Welch's t-test with a cut-off of $p < 0.05$ was used to select oxylipins that were significantly enhanced by LL-37 and citLL-37. Fold changes were computed for both LL-37 and citLL-37 in comparison to the unstimulated condition, and standard error was calculated to account the variability observed in each replicate.

2.4 Enzyme-Linked Immunosorbent Assay (ELISA)

HBEC-3KT cells were seeded at a density of $9.5x10^3$ cells/mL in 12-well TC plates (Costar®). Cells were stimulated with either LL-37, citLL-37, and sLL-37, at indicated concentrations, and TC supernatants were collected after 24 h. TC supernatants were centrifuged (250 x g for 5 min) to obtain cell-free samples. The abundance of IL-8 (Cat# DY208), GROα (Cat# DY275) and MIP-3α (Cat# DY360) were examined in TC supernatants using ELISA kits obtained from R&D Systems, as per the manufacturer's instructions. Briefly, capture antibodies were diluted in PBS (10 mL). Clear, high-binding 96-well Costar plates (Cat# 9018) were coated with capture antibodies (100 µL/well) and incubated overnight at RT. Plates blocked with 3 % (w/v) Bovine Serum Albumin (BSA) in PBS (200 µL/well) for 1 h at RT. TC samples and cytokine standards provided in the respective kits were added (100 µL/well) and incubated for 2 h at RT. Detection antibodies were diluted, according to instructions in the respective ELISA kits, in 3 % (w/v) BSA in PBS, 100 µL adder per well, and incubated for 2 h at RT. 0.02% Avidin-HRP (eBioscience; Cat# 18-41-0051) diluted in 3 % (w/v) BSA in PBS was added to the plate (100 µL/well) and incubated in the dark for 20 min at RT. Prior to all incubation steps, plates were washed 3 times with washing buffer containing 0.05 % Tween-20 (Sigma; Cat# P1379) in PBS. The substrate, 3,3' 5,5' Tetramethylbenzidine (TMB; Sigma; Cat # T0440), was added to the plate (100 µL/ well) for 10 – 30 min at RT, followed by the addition of 2N Sulfuric Acid (H_2SO_4) to stop the colorimetric reaction. ELISA plates were read at 450 nm (colourimetric detection) and 540 nm (background)[131].

2.5 Quantitative Real-Time-Polymerase Chain Reaction (qRT-PCR)

HBEC-3KT cells were stimulated with either LL-37, citLL-37, or sLL-37 (at concentrations as indicated), for 2, 4, 6 or 24 h (detailed in the results and figure legend of each experiment). The cells were washed with cold PBS, and cell lysate was collected by adding lysis binding solution (Fisher Scientific; Cat# AC149320050) containing MagMAX™ Lysis/Binding Solution Concentrate (Cat# AM8500). Total RNA was isolated from the cell lysates using the MagMAX™ 96 RNA isolation kit (Cat# AM1830) and the King Fisher Flex711 Automated Extraction & Purification System-GL (ThermoFisher Scientific), according to the manufacturer's

instructions. Total RNA was quantified using a NanoDrop 2000 Spectrophotometer (Fisher Scientific).

Abundance of each target mRNA was analyzed using Superscript III First-Strand Synthesis SuperMix for qRT-PCR (Cat# 11752050), as per manufacturer's instructions. Briefly, 200 ng of total RNA was reverse transcribed in a 20 μL reaction volume for 10 minutes at 25 °C, followed by 50 minutes at 42 °C, to obtain cDNA. Subsequently, the reaction was stopped by incubating the reaction mix at 85 °C for 5 minutes, followed by addition of 1 μL 2U RNaseH and incubated at 37 °C for 20 minutes. mRNA abundance was examined using the QuantStudio Real-Time PCR Systems (Applied Biosystems, CA, USA). For qRT-PCR amplification, each reaction mix contained 2.5 μL of 1 in 10 diluted cDNA template, 6.25 μL of Platinum SyBR Green qPCR-Super-Mix UDG with Rox reference, 1 μL of primer mix and 2.75 μL Nuclease-Free water (ThermoFisher Scientific; Cat# AM9937)[131]. All primers were obtained from Qiagen Inc. (Toronto, ON, CA). The Gene globe IDs of the primers used are detailed in Table 2. A melting curve analysis was performed to confirm PCR product specificity. Comparative ΔΔCt method was used to calculate relative fold changes, after normalization with the 18S RNA[135].

Table 2. Primers used for quantitative real-time PCR.

Gene	**Gene Globe ID**
Cxcl8 (IL-8)	QT00000322
Cxcl1 (GROα)	QT00199752
Ccl20 (MIP-3α)	QT00012971
Ptgs1 (COX-1)	QT00210280
Ptgs2 (COX-2)	QT00040586
18s RNA	QT00199367

2.6 Cytotoxicity Assay

A lactate dehydrogenase (LDH) assay was performed to determine cellular cytotoxicity. TC supernatants were examined for the release of the LDH enzyme using a colorimetric LDH detection assay kit from Roche Diagnostic (Laval, QC, CA), according to the manufacturer's instructions. TC supernatants obtained from cells treated with 2 % triton X100 (Sigma) at 37 °C for 30 minutes was used as a control for 100 % cytotoxicity. TC supernatants were centrifuged (250 x g for 5 minutes) to obtain cell-free samples. TC supernatants (50 μL) were incubated with the LDH substrate mix (50 μL) for ~30 minutes in the dark at RT, and the release of LDH enzyme was measured at 490 nm. Cytotoxicity was calculated relative to the TC supernatants obtained from triton-treated cells as 100 % cytotoxicity[131].

2.7 Neutrophil Migration Assay

Neutrophils were isolated from human blood obtained from healthy volunteers with informed written consent, which was approved by the University of Manitoba's Human Research Ethics Board (Protocol #: HS11105, H2010:259). Venous blood was collected in EDTA vacutainer tubes, and neutrophils were isolated using the EasySep™ Direct Human Neutrophil Isolation Kit (STEMCELL technologies, Vancouver, BC, Canada; Cat# 19666), according to the manufacturer's instructions. Briefly, 25 mL of blood was gently mixed with the isolation cocktail and 50 μL of RapidSpheres™ provided in the kit, followed by incubation at RT for 5 minutes. After incubation, DPBS (containing 1 mM EDTA, without Ca^{2+} and Mg^{2+}) was added for a final volume of 50 mL, and magnetic negative selection was performed using a magnet block for 10 minutes. The beads were washed and processed once more to ensure optimal isolation of neutrophils.

HBEC-3KT cells were stimulated with either LL-37, citLL-37, or sLL-37 (0.50 μM each peptide), in the presence and absence of pharmacological inhibitors as indicated. The TC supernatants were collected after 24 h. TC supernatants (600 μL) were added to the bottom chamber of a 12-well, 12 mm Transwell® plate with 12 (5.0 μM Pore Polyester membrane) inserts (Costar®), as shown in Figure 1. The plates were incubated at 37 °C in a 5 % of CO_2 incubator for 30 minutes. Subsequently, human blood-derived neutrophils (6 x 10^5 cells/ insert, 200 μL) were added to the upper chamber of the Transwell insert, followed by incubation for 2 h at 37 °C. As a

positive and negative control, human recombinant chemokines IL-8 (30 ng/mL) and GROα (30 ng/mL), respectively were added to the bottom chamber of the Transwell plates containing airway epithelial cells basal medium (containing 6 mM L-glutamine), based on our previous study[85]. The number of migrated neutrophils in the bottom chamber were counted using a Scepter 3.0 Handheld Automated Cell Counter (Millipore Ltd, ON, Canada) after 2 h.

Figure 2

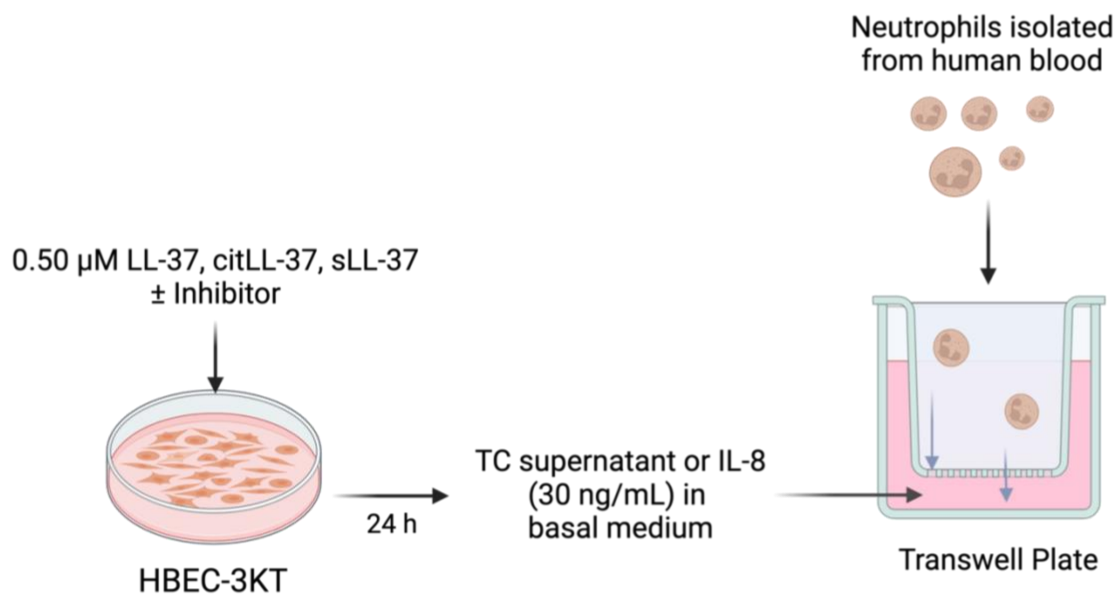


Figure 2: ***Schematic representation of the indirect neutrophil migration assay***. Human neutrophils were isolated using EasySep™ Direct Human Neutrophil Isolation Kit. HBEC-3KT cells were stimulated with 0.50 µM LL-37, citLL-37, or sLL-37 in the presence and absence of respective inhibitor. TC supernatants along with media containing IL-8 (30 ng/mL) were added to the bottom chamber of a Transwell plate. These plates were incubated at 37° C in a 5 % of CO_2 incubator for 30 minutes. Human blood-derived neutrophils (6 x 10^5 cells/ insert, 200 µL) were added to the upper chamber of the Transwell insert, followed by incubation for 2 h at 37 °C. The number of neutrophils that migrated across the transwell membrane was counted using a Scepter™ 3.0 Handheld Automated Cell Counter. *This figure was created in BioRender*

2.8 Statistical Analysis

A detailed statistical analysis is provided in each figure legend. Briefly, paired baseline correction was conducted on all experiments by subtracting outcome readouts from untreated cells from that in cells with peptides with and without inhibitors. One-Way ANOVA was used to compare IL-8, GROα, and MIP-3α protein abundance between different peptide treated conditions. Two-Way ANOVA was used to compare protein abundance of IL-8, GROα, and MIP-3α between groups in experiments with pharmacological inhibitors, in the presence and absence of the peptide treatments (LL-37, citLL-37, and sLL-37). For qRT-PCR analysis, fold changes were calculated using the comparative ΔΔCt method[135], after normalization with the 18s RNA, followed by $\log_2$ transformation. For lipidomics, pairwise differential analysis was conducted on normalized Log_2 oxylipin values. Additionally, Welch's t-test was used to determine oxylipin abundance changes between cells treated with LL-37 and citLL-37. For the Eve Technologies' Human Cytokine 96-Plex Discovery Assay® (Calgary, AB, CA) to profile cytokines and chemokines enhanced by LL-37 and citLL-37, a pairwise differential analysis was conducted on normalized $\log_2$ protein abundance values and Welch's t-test was used to determine significance. All statistical analyses were performed using GraphPad Prism (version 10.1.1; GraphPad Software). A *p*-value of $p<0.05$ was considered statistically significant.

Chapter 3: Results

3.1 Lipidomics Profile of Oxylipins

LL-37 is known to enhance oxylipins such as PGE2 in fibroblasts and endothelial cells[118,119]. Therefore, we investigated the interplay of LL-37 and oxylipins in mediating downstream inflammatory responses, and how that may change with citrullination of LL-37. To define how both LL-37 and citLL-37 alters oxylipin profile, we performed a lipidomic analysis in collaboration with Dr. Christopher Pascoe. HBEC-3KT cells were stimulated with either LL-37, citLL-37, or sLL-37 (0.25 μM each), and tissue culture (TC) supernatant was collected after 24 h (n=3). Concentration of the peptides used was based on our previous study demonstrating modulation of cytokine response by LL-37 in HBECs[91]. The lipidomics analysis revealed differential enhancement of oxylipins by LL-37 and citLL-37 as follows.

LL-37 significantly enhanced prostaglandins (>2-fold compared to unstimulated cells), specifically PGE2, 11b PGE2, Dihomo PGE2, Dihomo PGF2a, PGA2 and PGE1 (Table 1), all of which are products of the COX pathway[28,40,45]. In addition, LL-37 significantly enhanced 11-HETE (>2-fold), 15-HETE (>26-fold) and 15-HETrE (>2-fold), oxylipins produced by both the COX and LOX pathways. LL-37 also enhanced 9,10 EpOME (>5-fold) and 12, 13 EpOME (>23-fold), products of the cytochrome P450 pathway (Table 1)[34,54]. Whereas citLL-37 significantly enhanced 15-HETrE (>2-fold), 9, 10 EpOME (>2-fold), and 12, 13 EpOME (>9-fold), but not COX pathway products (Table 1). Although LL-37 and citLL-37 both enhanced 9, 10 EpOME, 12, 13 EpOME and 15-HETrE, the magnitude of induction differed significantly between the two peptides except for 15-HETrE, as indicated by the fold changes (Table 1).

PGE2 is a key mediator of inflammation, serving as a signaling molecule that initiates the inflammatory process[45]. It mediates various pathophysiological function such as fever, pain, vasodilation, and regulation of immune cell activity[45]. In contrast, EpOMEs exert anti-inflammatory functions by limiting neutrophil respiratory burst and inhibiting neutrophil chemotaxis[57]. Thus, our results demonstrating that LL-37 specifically enhanced prostaglandins, whereas citLL-37 enhanced EpOMEs, suggests that citrullination of LL-37 mitigates the peptide's ability to enhance pro-inflammatory oxylipins. Therefore, we focused on the COX pathway and the cytochrome P450 pathway to investigate how citrullination of LL-37 alters the peptide's pro-inflammatory functions.

Table 3. Differential enhancement of oxylipins by LL-37 and citLL-37 in HBEC.

Pathways	Oxylipins	LL-37		citLL-37	
		p-value	Avg Log_2 Fold Change	*p*-value	Avg Log_2 Fold Change
COX Pathway	PGE2	0.025	2.63 ± 1.19	0.261	1.28 ± 0.144
	11b PGE2	0.029	2.44 ± 1.04	0.259	1.27 ± 0.162
	Dihomo PGE2	0.021	2.34 ± 0.813	0.304	1.21 ± 0.291
	Dihomo PGF2a	0.025	1.90 ± 0.415	0.351	1.15 ± 0.178
	PGE1	0.042	2.19 ± 0.9	0.320	1.16 ± 0.102
COX/LOX Pathway	11-HETE	0.020	3.47 ± 1.04	0.107	5.50 ± 3.00
	15-HETE	0.017	26.47 ± 24.69	0.121	2.90 ± 0.92
	15-HETrE	0.021	2.49 ± 0.73	0.030	2.39 ± 0.490
Cytochrome P450 Pathway	9,10 EpOMe	0.005	5.83 ± 2.27	0.022	2.49 ± 0.522
	12,13 EpOMe	0.011	23.60 ± 13.74	0.004	9.25 ± 3.15

3.2 Peptides LL-37 and citLL-37 selectively regulate cytokine and chemokine expression

3.2.1 *Time and dose kinetics of peptide-mediated response in HBEC*

Previous studies in our lab have established the dose of LL-37 that can induce the production of chemokines at 24 h without being cytotoxic[91]. LL-37 at both 0.25 μM and 0.50 μM significantly enhanced IL-8, MIP-3α, and GROα levels in HBEC-3KT cells and was not cytotoxic[91].These concentrations are physiologically relevant as the concentration of LL-37 can range from 0.25 μM to 1 μM in individuals with neutrophilic airway inflammation[136]. Therefore, to determine the optimal time-point and concentration for assessing LL-37-mediated chemokine production for this project, I performed a time and dose kinetics study in HBEC-3KT cells.

I monitored the mRNA levels of chemokines such as IL-8, GROα, and MIP-3α at 2, 4, 6, and 24 h, in HBECs (Supplementary Figure 1). LL-37 significantly increased the mRNA abundance of MIP-3α at 2 h, and abundance of IL-8 and GROα at 4 h. Subsequently, we examined protein abundance of these chemokines in TC supernatants at 3, 6, and 24 h (Supplementary Figure 2). LL-37 enhanced the protein production of IL-8, GROα, and MIP-3α at 24 h. Based on these results, we chose 2 and 4 h as time points for evaluating mRNA abundance, and 24 h as the time point for examining protein production in TC supernatants, for IL-8, MIP-3α, and GROα.

Furthermore, to determine an optimum concentration of LL-37 for my project, I compared 0.25 μM and 0.50 μM concentration of LL-37. Both 0.25 μM and 0.50 μM LL-37 increased IL-8, MIP-3α, and GROα protein at 24 h, however, 0.50 μM LL-37 had a greater increase in chemokine production at 24 h (Supplementary Figure 3). Based on these results, I selected 0.50 μM as the optimum concentration of LL-37, citLL-37, and sLL-37 to further evaluate peptide-mediated responses, in this project.

3.2.2 *Differential expression of cytokines and chemokines by LL-37 and citLL-37*

To identify cytokines and chemokines that are differentially enhanced by LL-37 and citLL-37, a multiplex platform was used (Eve Technologies' Human Cytokine/chemokine 96-plex Discovery Assay® Array). HBEC-3KT were stimulated with 0.50 μM of either LL-37, citLL-37, or sLL-37, TC supernatants were collected after 24 h and examined for the abundance of 96 cytokines/chemokines. Among these, 22 analytes were significantly enhanced in response to LL-37 or citLL-37. LL-37 significantly enhanced MIP-3α, IL-8, fractalkine, GM-CSF, IL-6, G-CSF, IP-10, I-TAC, sCD40L, MCP-3, Eotaxin and TNFα (Figure 3). On the other hand, citLL-37 specifically enhanced SCF and IL-20 (Figure 3). Interestingly, both LL-37 and citLL-37 enhanced VEGF-A, GROα, IL-13, IL-4, MCP-1, IFNγ, LIF and sFas (Figure 3). However, the abundance of these cytokines was higher in response to LL-37 compared to citLL-37, suggesting that LL-37 was a stronger inducer of these analytes. These results were consistent with our previous work, demonstrating that LL-37 (0.25 and 0.5 μM) significantly enhanced IL-8, MIP-3α, and GROα after 24 h[91]. Overall, these results demonstrated that both LL-37 and citLL-37 can differentially enhance cytokines and chemokines in HBECs, with specific quantitative differences. Therefore, I further evaluated the levels of IL-8, MIP-3α and GROα to examine the impact of citrullination of LL-37 on these chemokine responses. Interestingly, IL-8, GROα and MIP-3α are all pro-inflammatory chemokines that can act as neutrophil chemoattractants[137,138].

Figure 3

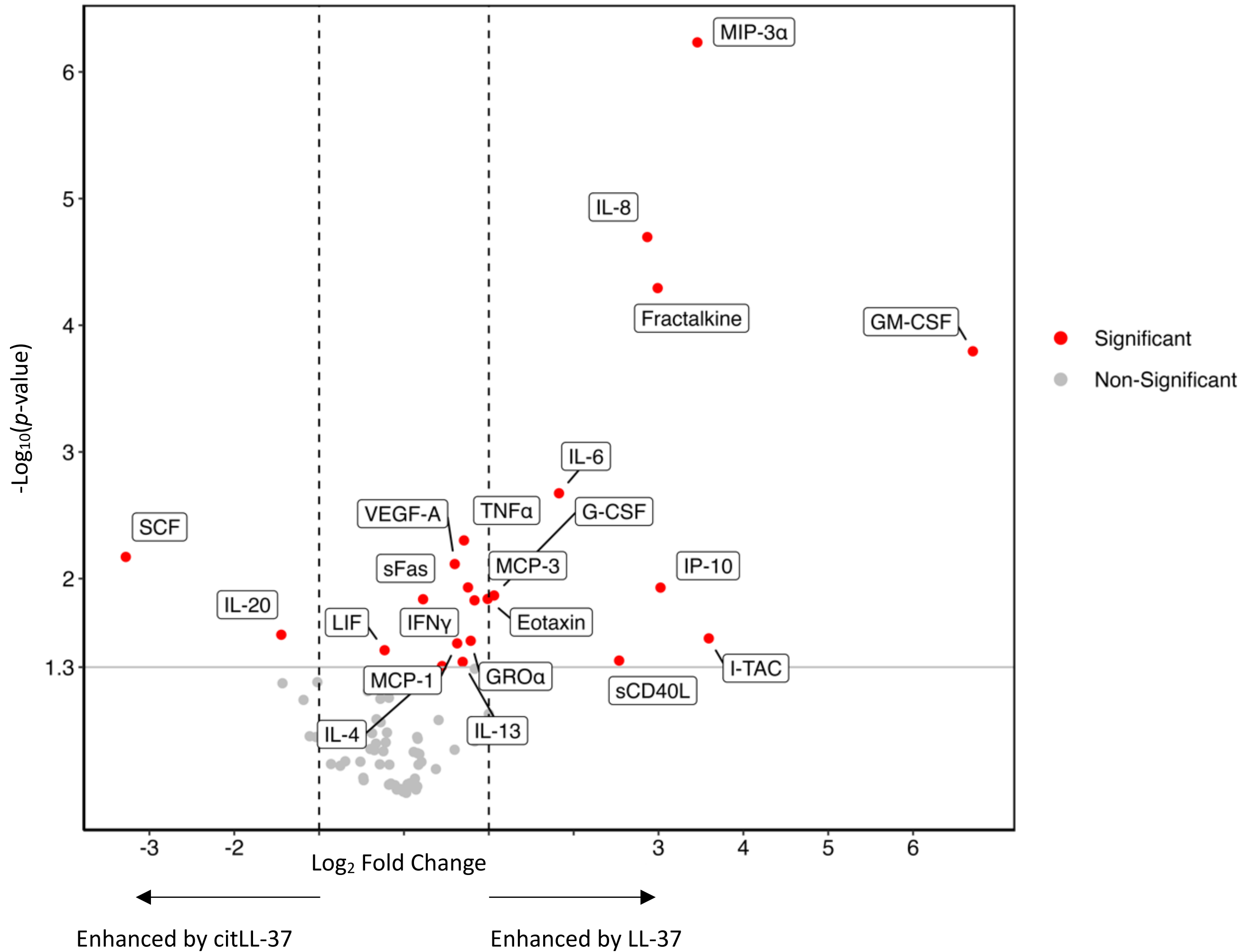


Figure 3*: LL-37 and citLL-37 differentially enhances cytokines and chemokines.* HBEC-3KT cells were stimulated with 0.50 μM LL-37, citLL-37, or sLL-37. TC supernatant collected at 24 h after stimulations were used for Eve Technologies' Human Cytokine/chemokine 96-plex Discovery Assay® Array. Paired baseline correction was conducted by subtracting outcome readouts from untreated cells from that in cells with peptides. Pairwise differential analysis was conducted on normalized log_2 protein abundance values and Welch's t-test was used to determine significance. A *p*-value of $p < 0.05$ was considered statistically significant. Each dot represents an independent experiment (n=4). Volcano plot was created using RStudio.

3.2.3 Independent validation of the selected chemokine abundance in HBEC

Next, we independently validated the abundance of IL-8, MIP-3α, and GROα at mRNA and protein levels. HBEC-3KT cells were stimulated with 0.50 μM of either LL-37, citLL-37, or sLL-37. The mRNA abundance of IL-8, MIP-3α, and GROα were evaluated using RT-qPCR after 2 h and 4 h, while protein abundance was measured in TC supernatants using ELISA after 24 h. LL-37 significantly enhanced MIP-3α mRNA at 2 h, and IL-8 and GROα mRNA abundance at 4 h (Figure 4). Citrullination of LL-37 significantly reduced the peptide's ability to enhance IL-8, MIP-3α, and GROα mRNA abundance at these time points (Figure 4). Similarly, LL-37 significantly enhanced the protein levels of IL-8, MIP-3α, and GROα after 24 h (Figure 5). Interestingly, citLL-37 also enhanced GROα abundance, however to a much lesser extent (~ 50 % reduction) compared to LL-37 (Figure 5). I further confirmed these results in human primary bronchial epithelial cells (PBEC). Consistent with these results in HBEC-3KT cell line, LL-37 increased the production of IL-8, MIP-3α, and GROα, whereas citrullination significantly suppressed LL-37' ability to enhance IL-8, MIP-3α, and GROα after 24 h in PBEC (Figure 6). In summary, these results showed that LL-37 enhances chemokines IL-8, MIP-3α and GROα at both mRNA and protein levels, whereas citrullination reduces LL-37's ability to enhance these chemokines. These results also demonstrated that citrullination does not mitigate LL-37-mediated chemokine responses, but significantly impairs the peptide's ability to induce chemokines, specifically those that are known to attract neutrophils and pro-inflammatory responses.

Figure 4

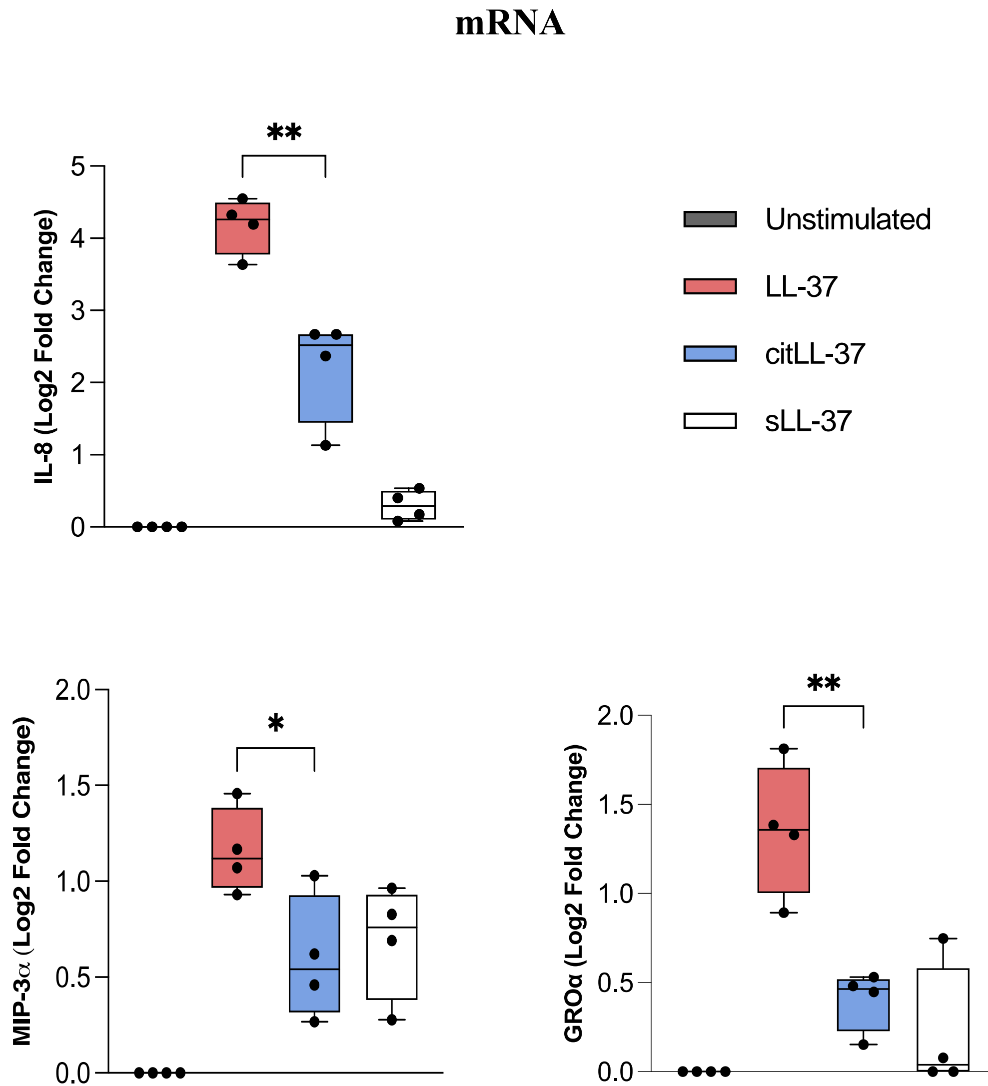


Figure 4: Citrullination of LL-37 reduces the ability of LL-37 to induce IL-8, MIP-3α, and GROα transcriptionally. HBEC-3KT cells were stimulated with 0.50 µM of LL-37, citLL-37, or sLL-37. HBEC mRNA abundance of IL-8, MIP-3α, and GROα was examined using qRT-PCR at 2 and 4 h. Relative fold changes were calculated compared to unstimulated cells normalized to 1, using the ΔΔ Ct method after normalization with 18sRNA expression, followed by log_2 transformation. Each dot represents an independent experiment (n=4), and statistical significance was determined by unpaired t-test (*$p < 0.02$, **$p < 0.003$).

Figure 5

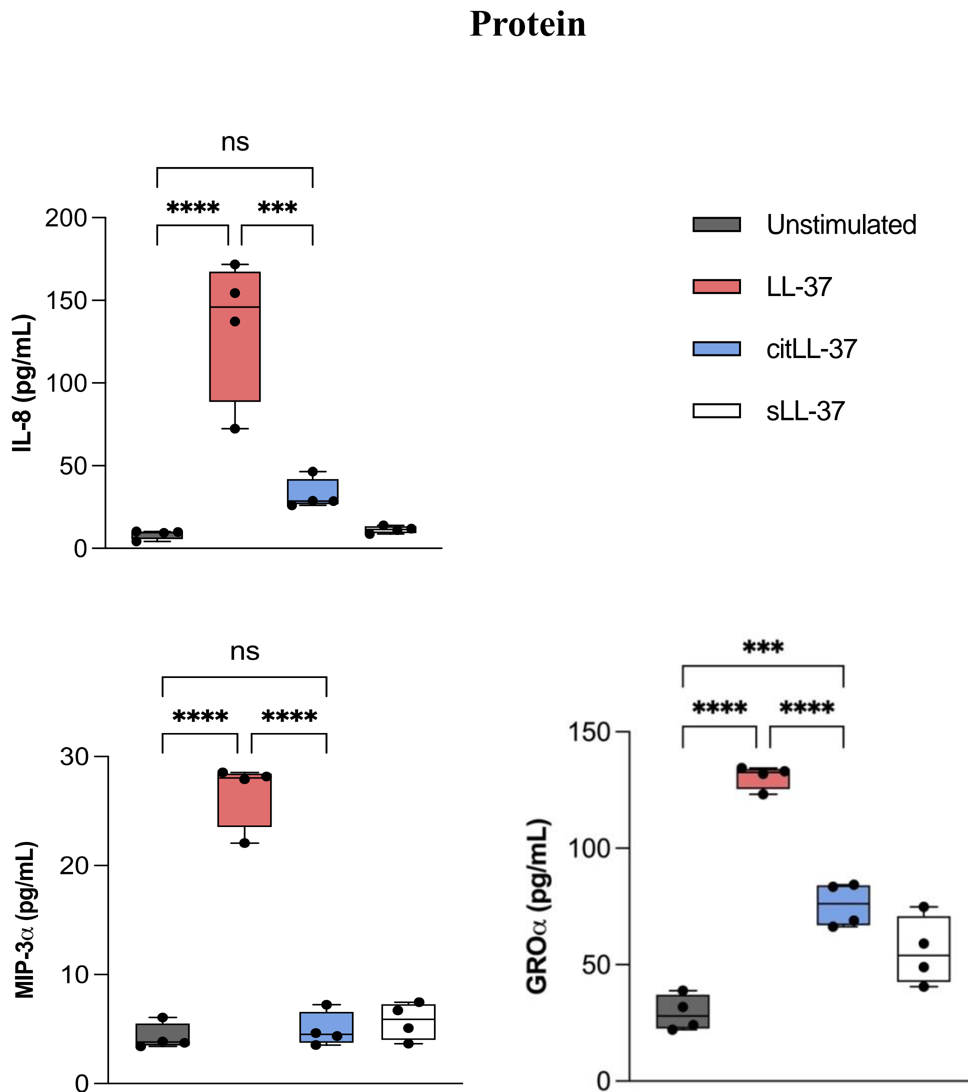


Figure 5: Citrullination of LL-37 reduces the ability of LL-37 to induce IL-8, MIP-3α, and GROα. HBEC-3KT cells were stimulated with 0.50 μM of LL-37, citLL-37, or sLL-37. IL-8, MIP-3α and GROα protein abundance was examined in tissue culture supernatants by ELISA after 24 h. Each dot represents an independent experiment (n=4), and statistical analysis was done using One-Way Anova (***p< 0.0002, ****p< 0.0001).

Figure 6

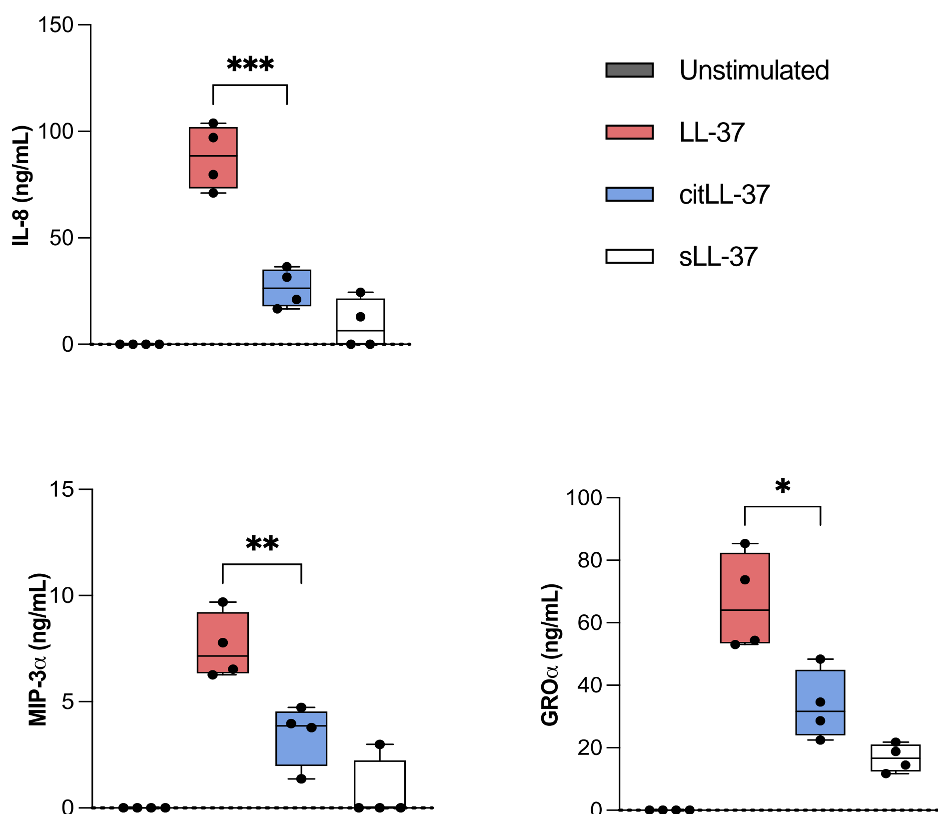


Figure 6: ***Citrullination of LL-37 reduces the ability of LL-37 to enhance IL-8, MIP-3α, and GROα in PBEC.*** Human primary bronchial epithelial cells (PBEC) were stimulated with 0.50 µM of LL-37, citLL-37, or sLL-37 for 24 h. Tissue culture supernatant was used to examine IL-8, MIP-3α, and GROα protein abundance by ELISA. Baseline correction was done by subtracting unstimulated cells from each condition. Each dot represents an independent experiment (n=4), and statistical analysis was done using unpaired t-test (*$p < 0.01$, **$p < 0.008$, ***$p < 0.0004$).

3.2.4 *Involvement of GPCRs in LL-37 and citLL-37-mediated chemokine production*

As shown above, there are differences in LL-37 and citLL-37-mediated chemokine enhancement of IL-8, MIP-3α, and GROα. LL-37 is shown to directly interact with 16 protein partners, including GPCRs[75]. We hypothesized that LL-37 and citLL-37 may interact with different protein partners, resulting in varying levels of chemokine induction. Previous work in our lab showed that LL-37's enhancement of pro-inflammatory chemokines IL-8 and GROα is dependent on GPCRs in human monocytes[85]. However, LL-37-mediated enhancement of IL-1RA, an anti-inflammatory cytokine was independent of GPCRs[85]. Therefore, to determine if LL-37 and citLL-37 use GPCRs to enhance chemokine production in human bronchial epithelial cells, we used pertussis toxin (PTx), a broad inhibitor of GPCRs. First, I performed a dose response analysis of PTx in HBEC, based on the IC50 of the inhibitor provided by the manufacturer. I examined a range of concentrations of PTx (100, 50, 25, and 12.5 ng/mL) for cellular cytotoxicity and percent inhibition on LL-37-mediated enhancement of chemokines (Supplementary Figure 9). Based on these results, 25 ng/mL was used for further analysis. HBEC-3KT cells were pre-treated with 25 ng/mL PTx for 1 h, followed by stimulation with either 0.50 μM LL-37, citLL-37, or sLL-37. The abundance of chemokines IL-8, MIP-3α and GROα was examined in TC supernatants by ELISA.

Consistent with previous results (Figures 4, 5 and 6), LL-37 significantly enhanced the abundance of IL-8, MIP-3α, and GROα, while citLL-37 modestly enhanced GROα. Presence of PTx significantly suppressed the levels of LL-37-mediated IL-8, MIP-3α and GROα (Figure 7). However, the presence of PTx did not significantly impact citLL-37's ability to enhance GROα (although the trend showed a decrease) (Figure 7). These results suggest that LL-37 interacts strongly with GPCRs to induce chemokine production, whereas citLL-37 may bind weakly to GPCRs or interact with different protein partners to induce chemokine production. Further studies are needed to identify the specific protein partners for both LL-37 and its citrullinated form in bronchial epithelial cells.

Figure 7

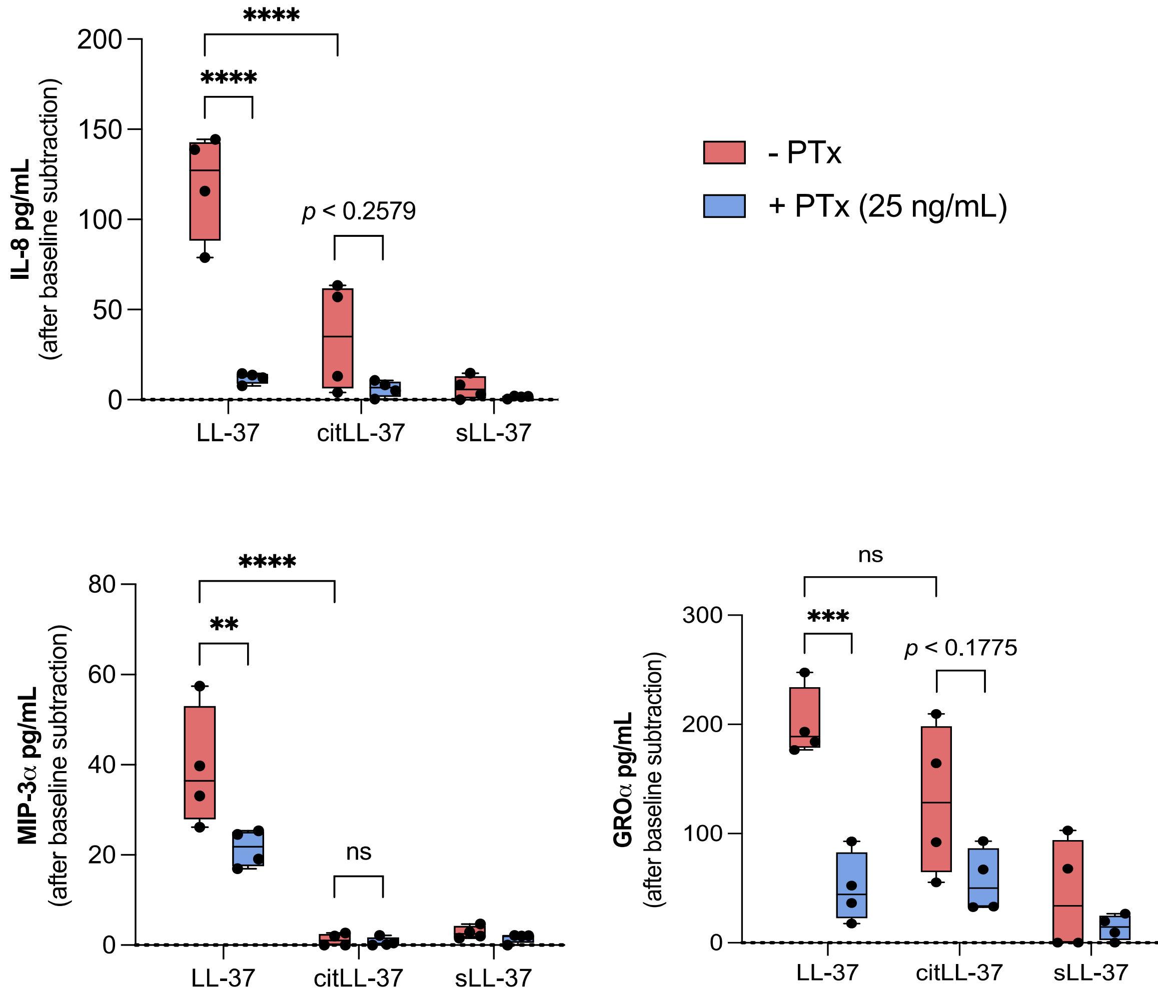


Figure 7. *Inhibition of GPCRs suppresses LL-37-mediated enhancement of chemokines.* HBEC-3KT were pre-treated with GPCR inhibitor PTx (25 ng/mL) for 1 h. Subsequently, cells were stimulated with 0.50 µM of peptides LL-37, citLL-37, or sLL-37, for 24 h. TC supernatant were examined for the production of IL-8, MIP-3α, and GROα, by ELISA. Baseline correction was performed by subtracting unstimulated cells from each condition. Each dot represents an independent experimental replicate (n=4). Statistical significance was measured using Two-Way ANOVA (***p*< 0.005, (****p*< 0.0005, *****p*< 0.0001).

3.3 The relationship between the COX pathway and LL-37-mediated immunomodulatory function

3.3.1 *Citrullination of LL-37 reduces the ability of LL-37 to enhance COX-2 mRNA*

As shown in section 3.1, LL-37 enhanced prostaglandins such as PGE2, a product of the COX pathway, and this response was mitigated with citrullination (citLL-37). Therefore, to further study the relationship between LL-37 and PGE2, I focused upstream of PGE2 and examined the COX pathway in HBEC-3KT cells. The mRNA abundance of COX-1 and COX-2 were assessed using RT-qPCR after 2, 4, 6 and 24 h (Supplementary Figure 4). LL-37 (0.50 μM) significantly enhanced COX-2 expression after 4 h, while citrullination of LL-37 reduced the peptide's ability to enhance COX-2 (Figure 8). In contrast, LL-37 or citLL-37 did not enhance COX-1 mRNA abundance (Figure 8). Furthermore, correlation plots revealed a positive relationship between LL-37-mediated enhancement of COX-2 mRNA, and mRNA levels of IL-8, MIP-3α and GROα (Figure 9). Based on these results, I further examined whether LL-37's ability to enhance these chemokines is dependent on the COX-2 pathway using specific pharmacological inhibitors.

Figure 8

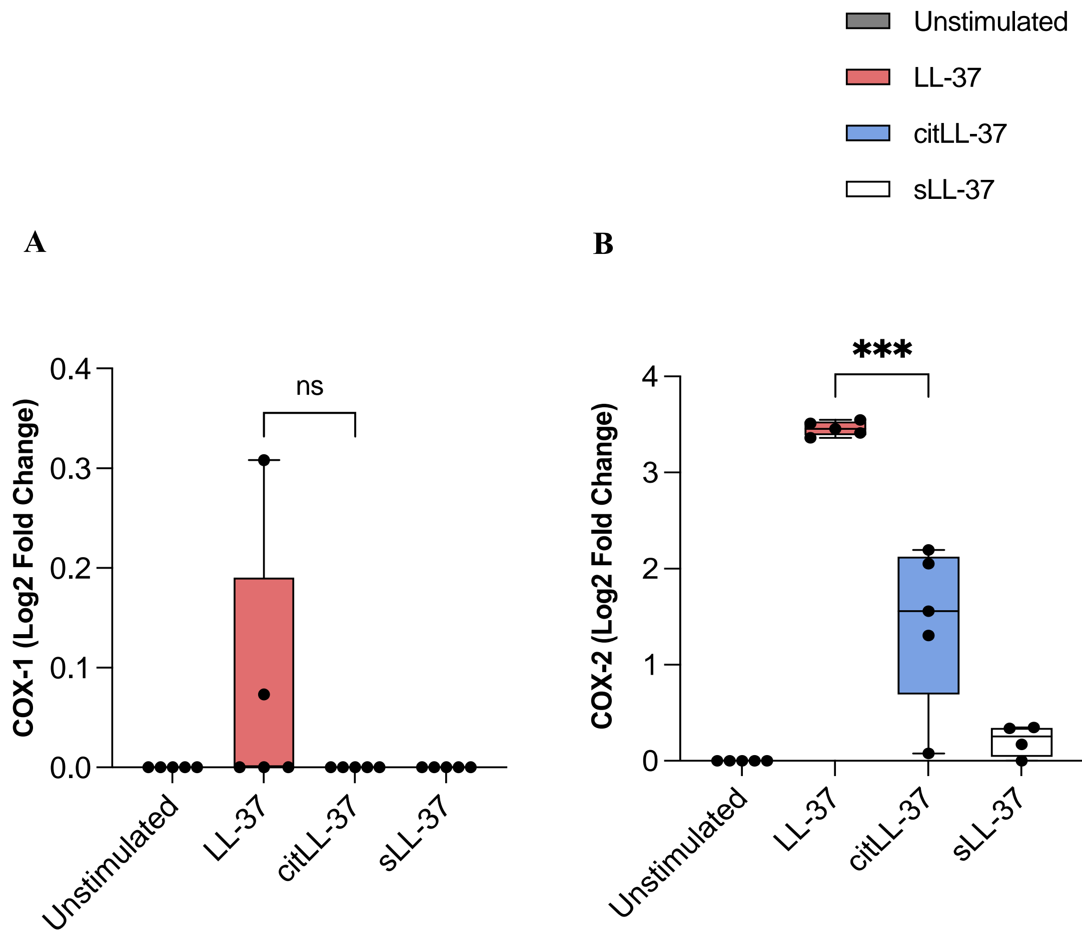


Figure 8: ***Differential modulation of COX mRNA by LL-37 and citLL-37***. HBEC-3KT cells were stimulated with 0.50 μM of LL-37, citLL-37, or sLL-37. **(A)** COX-1 and **(B)** COX-2 mRNA abundance was examined using qRT-PCR after 4 h. Relative fold changes were calculated compared to unstimulated cells normalized to 1, using the ΔΔ Ct method after normalization with 18s RNA expression, followed by $\log_2$ transformation. Each dot represents an independent experiment, and statistical significance was determined by unpaired t-test (***$p < 0.0007$).

Figure 9

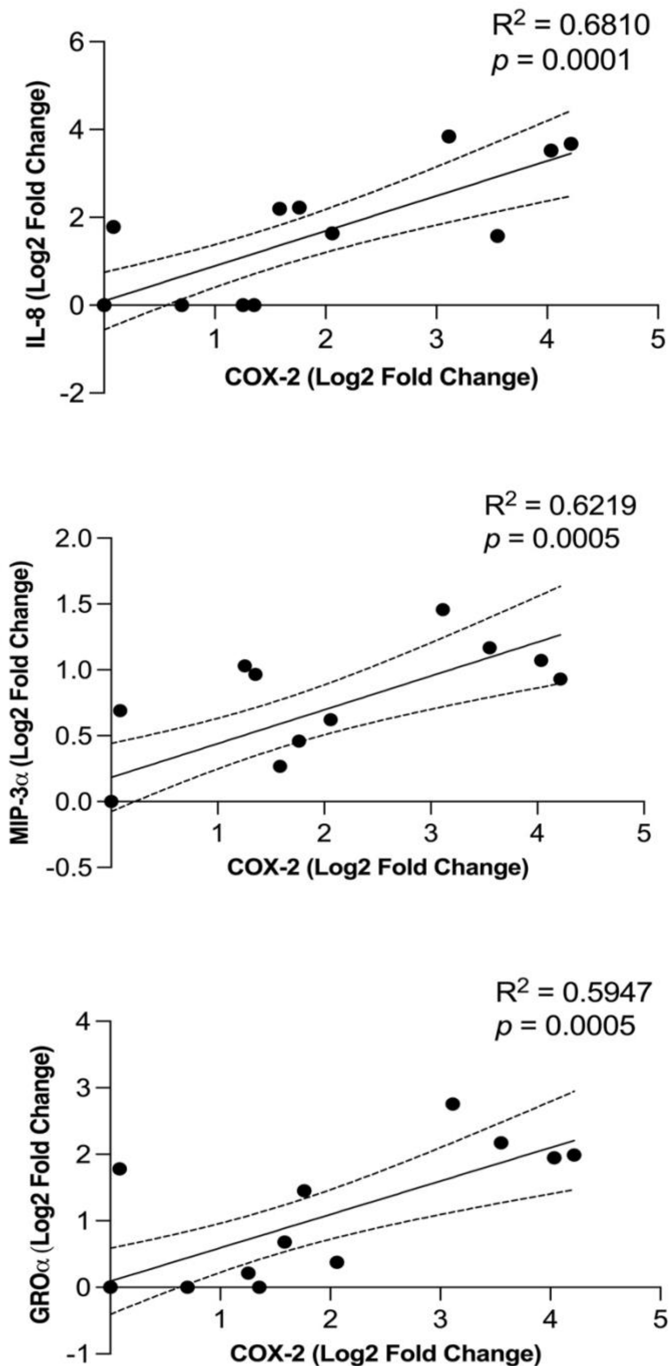


Figure 9: ***Correlation analysis between LL-37-mediated enhancement of chemokines and COX-2 mRNA.*** HBEC-3KT cells were stimulated with 0.50 μM of either LL-37, citLL-37, or sLL-37 for 2 and 4h. Pearson's correlation analysis was performed to determine the correlation between COX-2 and chemokines mRNA abundance, and a $p < 0.05$ was considered statistically significant.

3.3.2 *Inhibition of COX-2 suppresses LL-37-mediated enhancement of chemokines*

To investigate the relationship between LL-37-mediated chemokine response and the COX-2 pathway, I used a COX-2 specific inhibitor, rofecoxib[139,140]. First, I performed a dose response analysis of rofecoxib in HBEC, based on the IC50 of the inhibitor provided by the manufacturer. I examined a range of concentrations of rofecoxib (10, 20, 40 nM) for cellular cytotoxicity and percent inhibition on LL-37-mediated enhancement of chemokines (Supplementary Figures 5 and 6). Based on these results, 20 nM rofecoxib was used for further analysis. HBEC-3KT cells were pre-treated with 20 nM rofecoxib for 1 h and then stimulated with 0.50 μM of either LL-37, citLL-37 or sLL-37, TC supernatants were collected after 24 h and used to examine the abundance of chemokines by ELISA.

Consistent with previous results (Figures 4, 5 and 6), LL-37 significantly enhanced the abundance of IL-8, MIP-3α, and GROα, while citLL-37 modestly enhanced GROα. Presence of the COX-2 inhibitor rofecoxib significantly suppressed the levels of LL-37-mediated IL-8, MIP-3α and GROα (Figure 10). In contrast, rofecoxib showed no impact on the ability of citLL-37 to enhance GROα (Figure 10). These results indicate that LL-37-mediated chemokine response is dependent on the COX-2 pathway, whereas citLL-37-mediated GROα response is independent of this pathway. These results align with the lipidomics data demonstrating enhancement of PGE2, an oxylipin downstream of COX-2, in response to LL-37 but not citLL-37 in HBEC (Table 3).

Figure 10

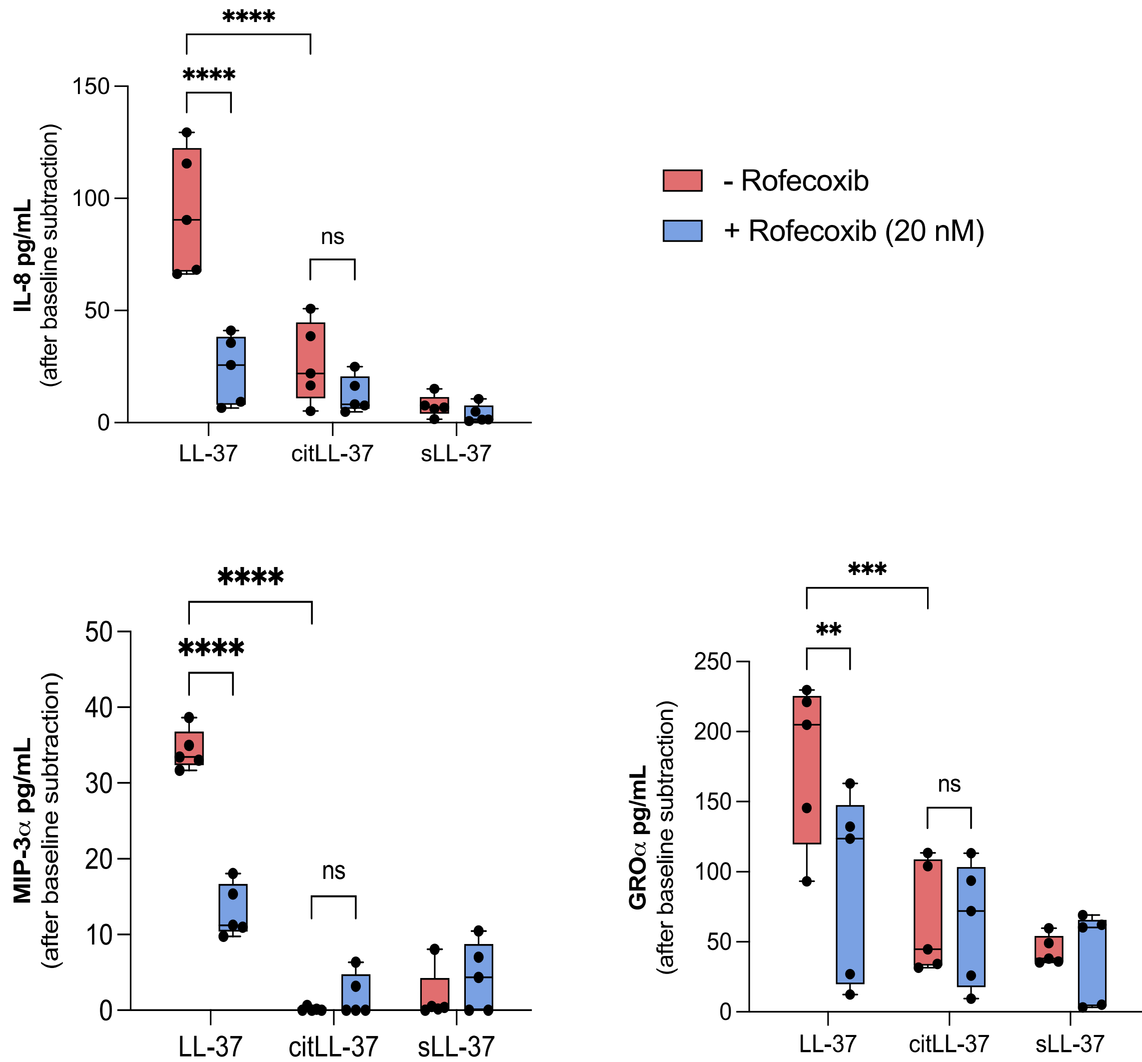


Figure 10: ***Inhibition of COX-2 suppresses LL-37-mediated enhancement of chemokines.*** HBEC-3KT were pre-treated with COX-2 inhibitor Rofecoxib (20 nM) for 1 h. Subsequently, cells were stimulated with 0.50 μM of peptides LL-37, citLL-37, or sLL-37, for 24 h. TC supernatant were examined for the production of IL-8, MIP-3α and GROα, by ELISA. Baseline correction was performed by subtracting unstimulated cells from each condition. Each dot represents an independent experimental replicate (n=5). Statistical significance was measured using Two-Way ANOVA (**$p < 0.006$, ***$p < 0.0005$, ****$p < 0.0001$).

3.3.3 *Inhibition of COX-2 suppresses LL-37-mediated migration of neutrophils*

Chemokines IL-8, MIP-3α and GROα are neutrophil chemoattractants[17,137]. Therefore, I used a neutrophil migration assay to confirm LL-37's ability to induce neutrophil migration, and to functionally assess the impact of COX-2 inhibition in LL-37-mediated neutrophil migration. HBEC-3KT cells were pre-treated with rofecoxib (20 nM), subsequently the cells were stimulated with 0.50 μM of either LL-37, citLL-37, or sLL-37, in the presence and absence of rofecoxib (20 nM). TC supernatants were collected after 24 h and used in the basal chamber of a Transwell plate for neutrophil migration assay (Refer to Chapter 2, Figure 2). Human recombinant IL-8 (30 ng/mL) was used as a positive control as IL-8 induces robust neutrophil migration. TC supernatants obtained from cells treated with LL-37 significantly induced neutrophil migration compared to unstimulated cells, which was significantly suppressed in the presence of rofecoxib (Figure 11). Conversely, TC supernatants obtained from cells treated with citLL-37 did not induce neutrophil migration, and this was unaffected by presence of rofecoxib (Figure 11). These results confirm that cells treated with LL-37 produce extracellular factors, including chemokines, that can robustly induce neutrophil migration, and that this function is dependent on the COX-2 pathway. Moreover, citrullination of LL-37 mitigates the peptide's ability to induce neutrophil migration. Taken together, this data suggests that both citrullination of LL-37 and inhibition of COX-2 pathway may lead to the impairment of LL-37's ability to facilitate neutrophil recruitment to the site of inflammation.

Figure 11

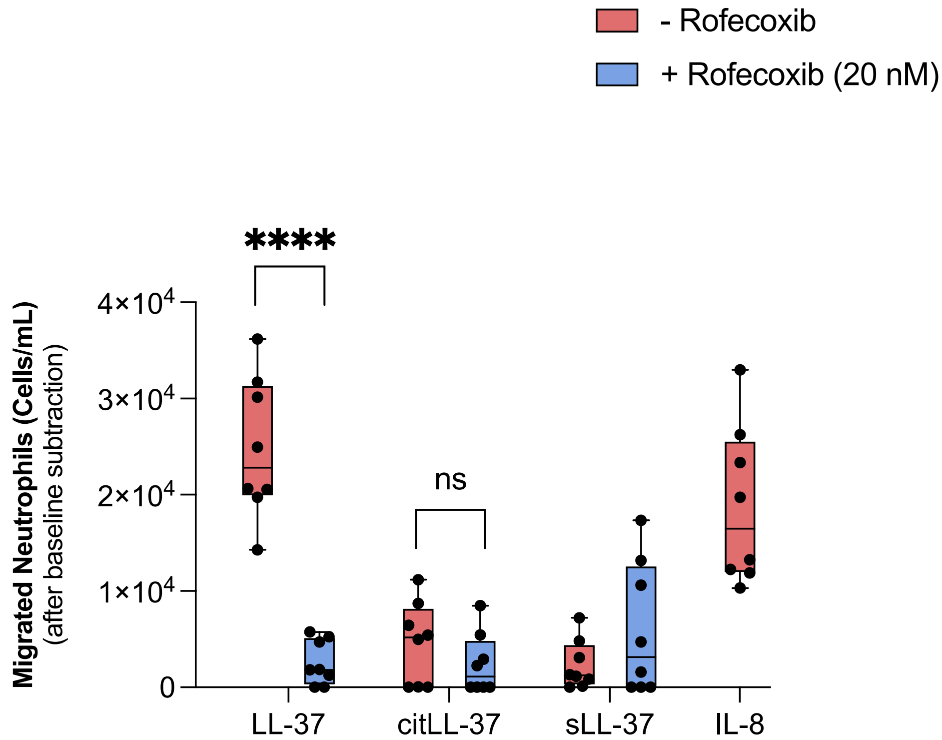


Figure 11: ***LL-37-mediated migration of neutrophils is dependent on the COX-2 pathway.*** Neutrophils were isolated from human blood from 2 independent donors using a method of negative selection. HBEC-3KT were pre-treated with COX-2 inhibitor Rofecoxib (20 nM) for 1 h. Subsequently, cells were stimulated with 0.50 µM of peptides LL-37, citLL-37 or sLL-37 and tissue culture (TC) supernatants were collected after 24 h. TC supernatants were used in the bottom chamber of the Transwell plates. IL-8 (30 ng/mL) was used as a positive control. Neutrophils isolated from human blood, from two independent donors with two technical replicates each, were placed in the upper chamber. Neutrophil migration was determined after 3 h by counting the neutrophils in the bottom chamber using a cell scepter. Baseline correction was performed by subtracting unstimulated cells from each condition. Each dot represents an independent replicate (n=4; two technical replicates from each donor neutrophils). Statistical significance was measured using Two-Way ANOVA (****$p < 0.0001$).

3.4 Relationship between cytochrome P450 pathway and LL-37 and citLL-37-mediated chemokine production

3.4.1 *Inhibition of cytochrome P450 pathway suppresses LL-37 and citLL-37-mediated enhancement of chemokines*

Next, I wanted to investigate the involvement of cytochrome P450 pathway in peptide function, as both LL-37 and citLL-37 enhanced 9, 10 EpOMe and 12, 13 EpOMe, products of this pathway in the lipidomics data (Table 3). To investigate how inhibition of the cytochrome P450 pathway would affect LL-37 and citLL-37-mediated enhancement of chemokines, I used orteronel, a cytochrome P450 inhibitor. I performed a dose response analysis of orteronel in HBEC, based on IC50 of the inhibitor. I examined a range of concentrations of orteronel (12.5, 25, 50 nM) for cellular cytotoxicity and percent inhibition on LL-37 and citLL-37-mediated enhancement of chemokines (Supplementary Figures 7 and 8 respectively). Based on these results, HBEC-3KT cells were pre-treated with 12.5 nM of orteronel for 1 h, followed by stimulation with 0.50 µM of either LL-37, citLL-37, or sLL-37. TC supernatants were collected after 24 h and used to examine the abundance of chemokines by ELISA. The presence of orteronel significantly reduced LL-37's ability to enhance these chemokines (Figure 12). Moreover, presence of orteronel reduced citLL-37's ability to enhance GROα (Figure 12). These results showed that LL-37 and citLL-37-mediated enhancement of chemokines is dependent on the cytochrome P450 pathway, which was consistent with the lipidomics data indicating the enhancement of oxylipins of this pathway by both the forms of LL-37.

Figure 12

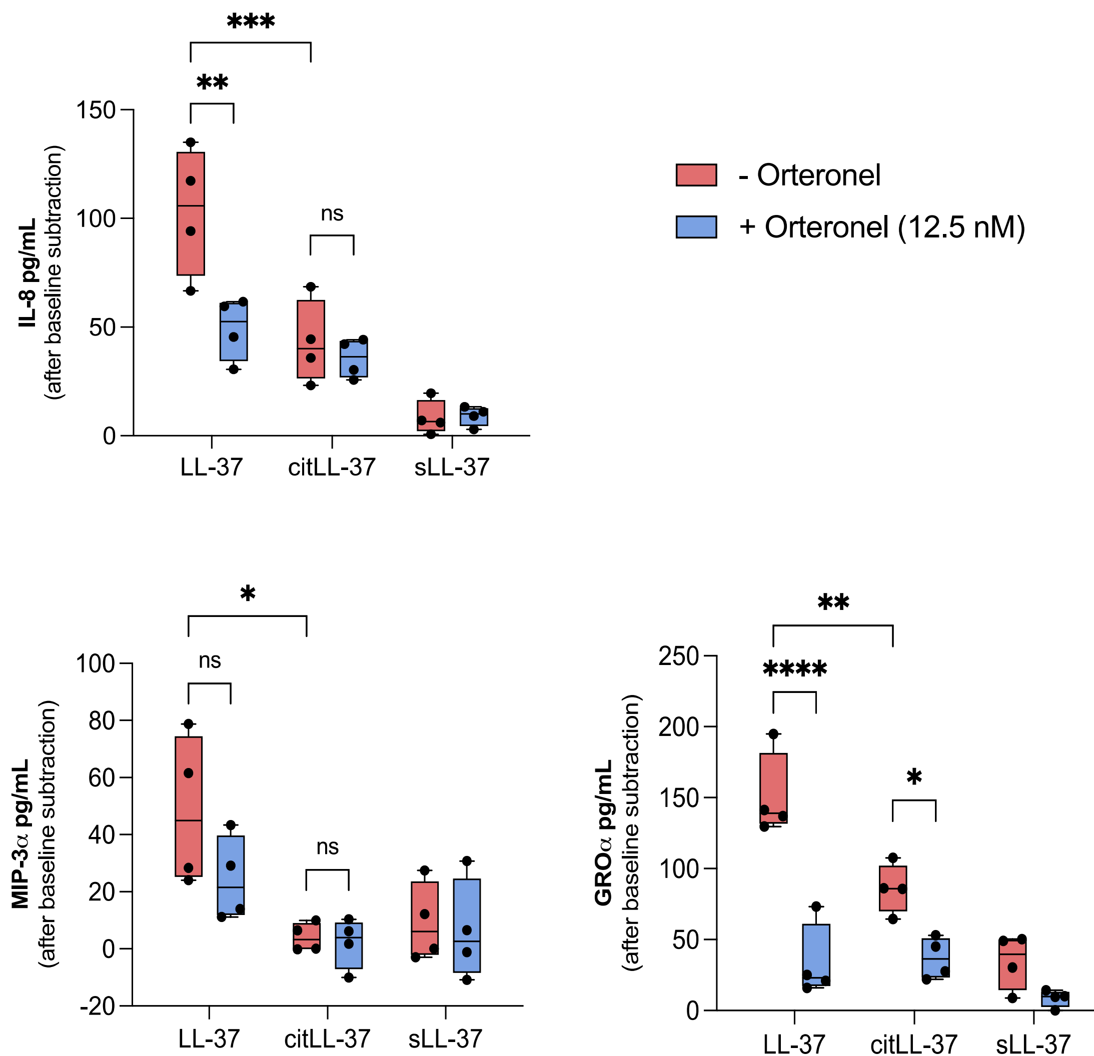


Figure 12: ***Inhibition of cytochrome P450 pathway suppresses LL-37 and citLL-37-mediated enhancement of chemokines***. HBEC-3KT were pre-treated with Cytochrome P450 inhibitor Orteronel (12.5 nM) for 1 h. Subsequently, cells were stimulated with 0.50 µM of peptides LL-37, citLL-37, or sLL-37, for 24 h. Tissue culture supernatant were examined for the production of IL-8, MIP-3α, and GROα by ELISA. Baseline correction was performed by subtracting unstimulated cells from each condition. Each dot represents an independent replicate (n=4). Statistical significance was measured using Two-Way ANOVA (**p*<0.02, ***p*<0.005, ****p*< 0.0005, **** *p*< 0.0001).

3.5 Summary of results

My data highlights the interplay of LL-37 and oxylipins in bronchial epithelial cells and establishes the role of LL-37 in immunometabolism. It demonstrates the involvement of the metabolic pathways like COX-2 and cytochrome P450 pathway in LL-37 and citLL-37-mediated enhancement of chemokines. Specifically, inhibition of both COX-2 and cytochrome P450 pathways suppressed LL-37's ability to enhance IL-8, MIP-3α, and GROα. Moreover, inhibition of COX-2 pathway suppressed LL-37-mediated migration of neutrophils, suggesting that LL-37's ability to induce neutrophil migration may be dependent on the COX-2 pathway. Furthermore, my data highlights the impact of citrullination on LL-37's immunomodulatory functions in bronchial epithelial cells. Firstly, citrullination of LL-37 reduced the peptide's ability to enhance pro-inflammatory oxylipins such as PGE2. Secondly, citrullination of LL-37 diminished the peptide's ability to enhance pro-inflammatory chemokines such as IL-8, MIP-3α, and GROα. Additionally, my data suggests that the differential enhancement of chemokines by LL-37 and citLL-37 may be due to their interactions with different receptors.

Chapter 4: Discussion

In this thesis, I highlight the interplay with LL-37 and oxylipins in human bronchial epithelial cells, emphasizing LL-37's role in immunometabolism. I also demonstrate how citrullination, a post-translational modification, alters LL-37's immunomodulatory functions, specifically the peptide's ability to enhance oxylipins and chemokines. I identified the involvement of metabolic pathways, specifically COX-2 and cytochrome P450 pathways in LL-37-mediated enhancement of chemokines. Secondly, my data showed that citrullination of LL-37 reduced the peptide's ability to enhance pro-inflammatory oxylipins such as prostaglandins and chemokines that mediate neutrophil recruitment. To my knowledge, this is the first study to define a role of LL-37 in immunometabolism in bronchial epithelial cells, linking lipid profile with the peptide's ability to induce chemokines, specifically in the context of airway inflammation.

Current literature demonstrates the critical roles of both LL-37 and oxylipins in inflammation, with both acting as immunomodulators to promote or suppress inflammation, depending on the cellular milieu[37,72]. Limited studies have highlighted the association of LL-37 and oxylipins in different cell types. For example, in human gingival fibroblasts, LL-37 enhances COX-2 and PGE2 by interacting with P2X7 receptor, ERK, and p47 JNK[118]. In endothelial cells, LL-37 triggers PGE2 release in a dose-dependent manner[119]. In keratinocytes, LL-37 upregulates COX-2 and PGE2 to aid in protection from apoptosis[120]. My results corroborate these studies in context of airway inflammation, where I show that LL-37 enhances COX-2 and PGE2 abundance in human bronchial epithelial cells. In the lungs, airway epithelial cells are crucial in host defence, preventing allergens and other environmental threats from entering. These cells secrete various molecules, such as host defence peptides and cytokines/chemokines to aid in the immune responses that aim to resolve various environmental factors such as allergens and/or pathogens. These environmental factors play a role in the exacerbation of various respiratory disease. In airway diseases such as asthma and COPD, both LL-37 and PGE2 play a role. Therefore, to our knowledge, this study is the first to provide insight into the interplay between LL-37 and COX-2/PGE2 in bronchial epithelial cells.

PGE2 is one of the most abundant COX products produced by the airway epithelium and smooth muscle[40]. During the initial phase of inflammation, PGE2 acts as a vasodilator to facilitate the influx of immune cells to the site of injury[28,58]. The effects of PGE2 are dependent on which PGE2 receptor is activated, the environment and concentration of PGE2 present[28]. In acute

exacerbations of asthma, PGE2 can lead to bronchodilation, thereby relieving symptoms[141]. However, chronic levels of PGE2 in the airways can lead to persistent inflammation and airway remodeling, characteristics of severe asthma[141]. In addition, PGE2 can stimulate mucus production by epithelial cells leading to airway obstruction, thereby worsening asthma symptoms[63,142]. Similar to PGE2, the function of LL-37 seems to be different in acute v/s chronic inflammation.

Although the exact role of LL-37 in airway diseases is still being elucidated, previous studies provide insight into the abundance and some functions of the peptide in different respiratory disease. LL-37 is increased in individuals with COPD and asthma, and present in the airways of patients with bacterial lung infections[98–100]. However, there are contradictory reports on the role of LL-37 in lung inflammation. For example, LL-37 was shown to promote lung remodeling in a model of smoking-mediated COPD[143]. In contrast, another study showed that LL-37 protects epithelial barrier dysfunction and contributes to maintenance of tight junctions in airway epithelial cells[144]. Nevertheless, in the airways, LL-37 acts as chemotactic factor, recruiting inflammatory cells like eosinophils, neutrophils, and mast cells[35]. It also stimulates epithelial cells to produce pro-inflammatory cytokines/chemokines, further amplifying the inflammatory response[72]. Results from my studies suggest that LL-37-mediated activation of the COX-2 pathway is required for LL-37-induced chemokine production and neutrophil migration, thus establishing a role of LL-37-COX-2 axis in airway inflammation. In patients with severe asthma, there is a need for new treatment options as some patients do not respond to commonly used asthma treatments such as corticosteroids. It has been shown that corticosteroids can decrease COX-2 mRNA[61]. I have identified LL-37 as being a key regulator of COX-2 function. Therefore, further investigation into the mechanism of LL-37 mediated enhancement of COX-2 could lead to identification of novel therapeutic targets. Targeting LL-37 and its receptors in patients with elevated LL-37 and COX-2 who exhibit glucocorticoid resistant asthma as a treatment option is not plausible, as LL-37 plays a critical role in anti-inflammatory mechanisms, maintaining of tight junctions and immune homeostasis in the lungs. How LL-37 can play a role in opposing functions in the process of inflammation remains unknown, which may be regulated by citrullination of the peptide (discussed below in this section).

I also showed the association of LL-37 with other oxylipins associated with airway inflammation, such as those in the LOX and CYP pathways. The LOX pathway is known for the

production of leukotrienes, pro-inflammatory mediators associated with the pathogenesis of asthma[33]. Inhibitors of 5-LOX, the enzyme responsible for leukotriene production, is approved for asthma treatment[145]. It has been shown that LL-37 can induce release of leukotrienes in eosinophils[101]. Here I show, to my knowledge for the first time, that LL-37 can enhance other products of the LOX pathway, such as 11-HETE, 15-HETE, and 15-HETrE. Additionally, it is shown that 11-HETE- 15-HETE, 15-HETrE can be produced as by-products in the COX pathway during prostaglandin synthesis. LL-37's ability to enhance oxylipins that can be generated from multiple pathways highlights its intricate role in oxylipin synthesis.

11- and 15-HETE are both pro-inflammatory oxylipins, however 15-HETE can also exhibit anti-inflammatory properties depending on the inflammatory milieu[146,147]. Both 11- and 15-HETE can lead to the recruitment and activation of immune cell such as neutrophils, eosinophils, and macrophages[52,148,149]. Previous studies indicated that 15-HETE may contribute to airway hyperresponsiveness and bronchoconstriction, which are characteristic features of asthma and COPD[150,151]. On the other hand, 15-HETrE is also shown to exhibit anti-inflammatory properties, such as suppressing COX-2 and PGE2 synthesis in prostatic adenocarcinoma cells[152]. It also inhibits 5-LOX, the enzyme responsible for leukotriene synthesis[145]. Therefore, similar to LL-37, 15-HETE may function as a modulator of inflammation, with both pro- and anti-inflammatory functions dependent on the cell and tissue environment. Thus, it is possible that mechanisms related to LL-37's ability to modulate inflammation by enhancing both pro- and anti-inflammatory responses may be related to its interplay with oxylipins. Therefore, pro-inflammatory oxylipins identified in this study to be enhanced in response to LL-37 may serve as potential drug targets for lung inflammatory disease such as asthma.

In this study, I also show that LL-37 enhances 9,10- and 12, 13 EpOME, products of the CYP pathway. Oxylipins are very short-lived and rapidly metabolized to secondary metabolites. Through the action of the enzyme sEH, EpOMEs can be metabolized to DiHOMEs[34]. Although these have been linked to chronic lung conditions caused by environmental factors, their role in airway inflammation remain unknown. Elevated levels of EpOMEs have been observed in lung tissue and BALF following inhaled oxidant exposure in rats, while increased plasma concentrations of DiHOMEs were observed in animal models exposed to tobacco smoke[34]. Conversely, DiHOMEs are decreased in BALF after exposure to subway air in asthmatic

individuals, whereas health individuals show increased levels[34,67]. In another study, healthy volunteers exposed to biodiesel exhaust exhibited higher levels of plasma 9,10-DiHOME compared to those exposed to filtered air[34,68]. EpOMEs and DiHOMEs serve as chemotactic mediators for neutrophils[34]. However, DiHOMEs have been shown to inhibit neutrophil respiratory burst, a process in which neutrophils rapidly release ROS to degrade pathogens they have phagocytosed[153]. Thus, the pro-inflammatory functions of LL-37 may be mediated by the enhancement of EpOMEs by driving immune cell chemotaxis. In contrast, anti-inflammatory functions of LL-37 maybe by producing EpOMEs that are rapidly metabolized into immune-suppressive DiHOMEs, which require further investigation as this study did not investigate the levels of DiHOMEs. It would also be interesting to investigate the effect of LL-37 on the enzyme sEH, which converts EpOMEs to DiHOMEs. It is likely that the function of LL-37 in maintaining immune homeostasis by promoting pro- or anti-inflammatory response as needed, involves the EpOMEs and the CYP pathway.

Taken together, I demonstrate that LL-37 can enhance oxylipins derived from the COX, LOX, and CYP pathways. However, the precise mechanism through which LL-37 enhances these oxylipins remains unknown. One possibility is that LL-37 enhances oxylipin production through its involvement in lipid peroxidation. Oxylipins are products of PUFA oxidation, a complex process where PUFAs undergo reactions with oxygen to form new compounds[37]. ROS are pivotal in initiating lipid peroxidation[154]. LL-37 has been observed to stimulate NAPDH oxidase in neutrophils and macrophages, leading to increased ROS production[155]. Therefore, LL-37 may enhance ROS levels, facilitating their interaction with PUFAs to generate oxylipins. LL-37's ability to enhance oxylipins demonstrates its role in modulating the immune response, by enhancing pro-inflammatory oxylipins to initiate inflammation or enhancing oxylipins that have suppressive effects to regulate inflammation. This also highlights a novel role of LL-37 in immunometabolism. Immunometabolism refers to the interplay between the immune system and metabolic processes. It involves how metabolic pathways and metabolites influence immune cell function and how immune responses, in turn, affect metabolic processes. Here, I show that LL-37-mediated chemokine production (IL-8, MIP-3α, and GROα) depends on metabolic pathways such COX-2 and CYP P450 in bronchial epithelial cells.

During airway inflammation, epithelial cells can produce chemokines including IL-8, MIP-3α, and GROα. These chemokines serve to attract immune cells to the site of infection or injury[19,21,22]. Chemokines are pivotal in mediating inflammation by influencing the cells that produce them or by facilitating the recruitment of other immune cells[19,21,22]. LL37's ability to enhance these chemokines represents a mechanism through which it can promote inflammation, which is a key mechanism in the peptide's antimicrobial functions[35]. The link between LL-37-inducing chemokines and oxylipins is unknown. A previous study demonstrated that bradykinin-induced IL-8 was dependent on the COX pathway[156]. My findings are the first study to reveal that LL-37-mediated chemokine enhancement is dependent on the COX-2 and CYP pathways. This dependency suggest that LL-37 may stimulate the production of these chemokines by generating specific prostaglandins and/or other oxylipins. Regarding the COX-2 pathway, a plausible mechanism for the interplay with LL-37-mediated chemokine production may be that LL-37-mediated enhancement of COX-2 leads to the synthesis of PGE2, which acts in an autocrine manner by binding to its EP receptors, and triggering the secretion of IL-8, MIP-3α, and GROα. Further investigation is required to fully understand how these pathways contribute to LL-37-mediated chemokine enhancement.

Inflammation is a tightly regulated process, where the amount and duration of inflammation needs to be controlled. Although pro-inflammatory responses are required for efficient immune function, if this is not resolved it can lead to chronic inflammatory diseases. While LL-37 can negatively regulate inflammation by suppressing pro-inflammatory chemokines and enhancing the production of anti-inflammatory cytokines in various cell types, there may be other mechanisms that modulate LL-37's pro-inflammatory properties[35]. Citrullination of the peptide may be one such mechanism involved in controlling LL-37's pro-inflammatory responses. During inflammation, neutrophils enter the airway lumen, where these cells undergo cell death resulting in an increase of PAD enzymes[107]. Neutrophils perform various functions, including the release of NETs and LL-37[72]. Within such inflammatory environment, PAD enzymes can mediate the citrullination of LL-37[108,115]. My results provide an insight into the effects of citrullination on the immunomodulatory functions of LL-37 in the lungs, which was previously unknown.

Limited studies have explored the impact of citrullination on the antimicrobial and antiviral properties of LL-37. Citrullination causes a loss of positive charge of LL-37, hindering its ability

to bind to charged molecules. LL-37 protects the host from severe inflammatory responses, such as those seen in septic shock, by binding to endotoxin like LPS[108]. However, citrullination reduces the peptide's ability to neutralize LPS due to decreased binding capacity. Additionally, citrullination impairs LL-37's antimicrobial properties against *E. coli*[115]. It also significantly alters the interaction of LL-37 with DNA, leading to reduced activation of DCs and macrophages in response to bacterial DNA[116]. Furthermore, recent literature indicates that citrullination diminishes the antiviral activity of LL-37 against HRV[109]. HRV infection increases PAD2 protein expression and protein citrullination levels in human bronchial epithelial cells, thereby abrogating LL-37's direct activity against HRV infections in these cells[109]. These studies indicate that under inflammatory conditions where LL-37 can get citrullinated, this modification of the peptide impairs the antimicrobial and antiviral functions of the peptide. The effect of citrullination on LL-37's immunomodulatory properties, specifically its ability to enhance oxylipins and pro-inflammatory chemokines has not been addressed before.

In this thesis, I show that citrullinated LL-37 enhances 15-HETrE, 9,10- and 12,13 EpOME, products of the LOX and CYP pathways, respectively. I also showed that citLL-37 loses its ability to enhance pro-inflammatory oxylipins such as prostaglandins and HETEs. In addition, I show that citrullination of LL-37 significantly reduces the peptide's ability to enhance pro-inflammatory chemokines such as IL-8, MIP-3α, and GROα. It is possible that citrullination acts as a regulatory mechanism to control the pro-inflammatory functions of LL-37. This is supported by my findings that citLL-37 retains its ability to enhance certain oxylipins, especially those that exhibit anti-inflammatory responses. For instance, citLL-37 can enhance 15-HETrE, which acts to suppress pro-inflammatory oxylipins such as prostaglandins and leukotrienes, thus regulating oxylipins that are implicated in airways diseases like asthma[145,152]. Additionally, citLL-37 can enhance EpOMEs, which can act as chemotactic molecules and weakly activate neutrophil respiratory burst, while DiHOMEs limit inflammation by inhibiting neutrophil respiratory burst[57]. Uncontrolled production of ROS during neutrophil respiratory burst can be harmful, as ROS can damage cellular components such as lipids, proteins, and DNA, contributing to tissue damage through oxidative stress[157]. The enhancement EpOMEs and subsequently DiHOMEs may serve to regulate this process.

Given that LL-37 enhances chemokines that act as neutrophilic chemoattractants, and LL-37 enhances neutrophil migration, all of which is significantly decreased in response to citLL-37, citrullination may serve a regulatory mechanism to control the pro-inflammatory properties of LL-37 in airway inflammation. Neutrophil-mediated inflammatory responses play a critical role in chronic inflammatory respiratory diseases. For example, steroid-unresponsive neutrophilic asthma is a common immunophenotype, where neutrophils are the key drivers in the pathogenesis of this disease[158]. The process of NETosis, a mechanism involving the release of NETs, can be detrimental to the airways[159]. NETs contain proteases like neutrophils elastase and myeloperoxidase, which can degrade extracellular matrix components and damage airway tissues [160–162]. The persistent presence of NETs and associated inflammatory responses can cause structural changes in the airways, include thickening of the airway wall and narrowing of the airways[160–162]. Thus, the findings in this thesis suggest that citrullination may be a process to enhance the anti-inflammatory and protective functions of LL-37 during airway inflammation in the lungs.

It is important to keep in mind that the degree of citrullination may play a role in how this PTM affects LL-37's immune properties. Recent study show that partially citrullinated LL-37 (3 arginine residues citrullinated; 3cit) only slighted affected ani-bacterial activity, compared to native LL-37[99]. However, fully citrullinated LL-37 (with all 5 arginine amino acids modified; 5cit) displayed weak antibacterial activity against both *Staphylococcus aureus* and *non-typeable Haemophilus influenzae* (NtHi)[99]. It has been observed that LL-37 with varying degrees of citrullination (3cit and 5cit) can be detected in the airways of healthy individuals. Investigating how different levels of citrullination influence the immunomodulatory functions of LL-37 would be interesting and could provide insights into the role of differential citrullination in disease.

In this thesis, I demonstrate distinct differences in the immunomodulatory functions between LL-37 and citLL-37. Previous research has shown that LL-37 interacts with Cdc42 Rho GTPase through GPCRs to enhance the production of chemokines like IL-8 and GROα in human monocytes[85]. However, LL-37's enhancement of the anti-inflammatory cytokine IL-1RA occurs independently of this mechanism[85]. My findings demonstrate that LL-37-mediated enhancement of IL-8, MIP-3α, and GROα in human bronchial epithelial cells is dependent on GPCRs, whereas citLL-37's ability to enhance GROα is not affected by GPCR inhibition. This suggests that citrullination of LL-37 may alter its interaction with specific protein partners, however further

investigation is needed to understand why these differences arise with citrullination, and to determine if LL-37 and citLL-37 interact with different protein partners. It is also possible that citrullination reduces LL-37's affinity for its receptors.

Previous studies have indicated that citrullination can diminish the affinity of chemokines for their receptors. For example, citrullinated CXCL12 shows reduced affinity for the CXCR4 receptor, while citrullinated CXCL10 and CXCL11 show decreased chemoattractant and signaling capabilities through CXCR3 receptor[114]. Interestingly, I observed that citrullination significantly reduced LL-37's ability to enhance chemokines. For example, citLL-37 could enhance the abundance of GROα protein, however, to a much lesser extent than LL-37. Therefore, it is possible that citLL-37 may bind weakly to its receptor, affecting its immunomodulatory functions such as production of specific chemokines. This is supported by previous study from the Mookherjee lab demonstrating that receptors for the pro- and anti-inflammatory functions of LL-37 are different[85]. Another possibility is that the peptide is degraded, as it has been observed that citrullination makes LL-37 more prone to degradation by bacterial proteases[99,106]. It's possible that a shortened lifespan could reduce the peptide's ability to bind to its receptor, resulting in decreased levels of chemokines. However, as citLL-37 can retain some of the functions of LL-37, such as enhancing oxylipins that play a role in anti-inflammatory process, it is likely that citLL-37 may engage with different receptors and/or pathways compared to the native peptide leading to differential downstream functions. It would thus be interesting to investigate if citLL-37 retains its ability to enhance anti-inflammatory cytokines and related signaling pathways compared to the native peptide in future studies.

In conclusion, in this thesis I have defined the interplay of LL-37 and oxylipins in human bronchial epithelial cells, highlighting a role of LL-37 in immunometabolism in the lungs (Figure 13). By demonstrating LL-37's involvement in fatty acid oxidation, this study opens new avenues to explore the potential role(s) of LL-37 in other aspects of immunometabolism, such as oxidative phosphorylation. Furthermore, this thesis provides evidence to support the possible mechanism that citrullination serves as a regulatory switch to suppress LL-37's pro-inflammatory functions, thereby contributing to immune homeostasis. Overall, my findings enhance our understanding of LL-37's multifaceted roles in immune responses and metabolic processes.

Figure 13

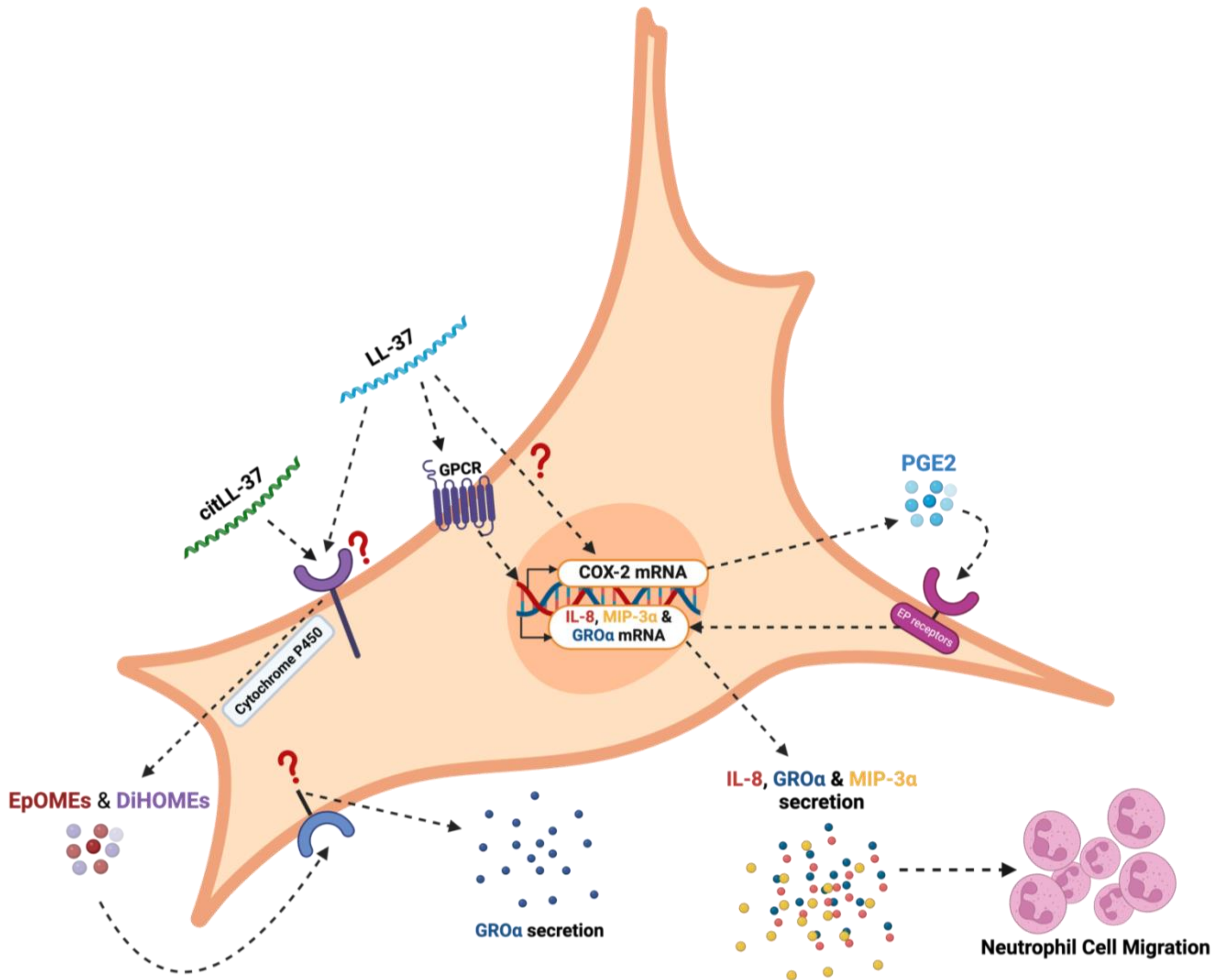


Figure 13. ***The interplay of LL-37 and oxylipins in the immune responses of human bronchial epithelial cells***. In this proposed mechanism we show that LL-37 enhances chemokine (IL-8, Groα, and MIP-3α) production and subsequent neutrophil migration through COX-2-PGE2 pathway. LL-37 mediated chemokine (IL-8, Groα, and MIP-3α) production is dependent on the COX-2 and cytochrome P450 pathways. Whereas citrullinated LL-37 enhances Groα through cytochrome P450 pathway, to a lesser extent compared to LL-37.

4.1 Limitations

A major limitation of this study is that all experiments performed were *in vitro* using human bronchial epithelial cells. While the results of this study provide some mechanistic insights into the interplay between LL-37 and oxylipins, and how this process is affected by citrullination, it does not necessarily reflect what would happen *in vivo* in an animal model of airway inflammation. *In vitro* studies are controlled; however, in a biological system, the environmental milieu plays a critical role in the response observed. Therefore, further studies to examine cathelicidins and oxylipins in an animal model of airway inflammation is warranted.

Another limitation is that this study does not elaborate on the kinetics of oxylipin response; This study only examined oxylipins production at 24 h. It is possible that some oxylipins were missed because their production was too low to detect at this time point. Analyzing earlier and later time points could be interesting, as oxylipin levels can be transient and may peak at different times. Also, citrullination of LL-37 may alter the kinetics of peptide-mediated oxylipin response, which was not examined in this study.

Another limitation is that this study did not investigate the role that COX-1 pathway in LL-37-mediated chemokine production. While this study had a strong rationale for investigating the COX-2 pathway, as LL-37 did not enhance COX-1 expression and only COX-2 in my findings, a pharmacological inhibitor that targets both isoforms of the COX enzymes may be beneficial. In line with this, end products such as PGE2 and 9,10 EpOME were not measured when the COX-2 and CYP pathways were inhibited. Because oxylipins are difficult to measure and can be rapidly metabolized into secondary products, measuring them by immunoassays such as ELISA are challenging, and can be done reliably by mass spec (which was used for oxylipin profiling in this study). Additionally, this study did not measure the levels of DiHOMEs or conduct functional tests involving the CYP pathway, which warrants further investigation.

Furthermore, this study did not investigate the effect of citrullination on LL-37's ability to enhance anti-inflammatory cytokines. It would be interesting to explore whether citrullinated LL-37 can retain its ability to enhance anti-inflammatory cytokines.

4.2 Significance

This thesis demonstrates the interplay between LL-37 and oxylipins in bronchial epithelial cells, highlighting LL-37's role in immunometabolism in the lungs. To our knowledge, this is the first study to elucidate LL-37's involvement in immunometabolism, linking its oxylipin profile to the induction of chemokine, in the context of airway inflammation. Our findings reveal a novel mechanism by which LL-37-mediated enhancement of COX-2 pathway is critical in the production of neutrophil chemoattractants and neutrophil migration by the peptide, suggesting potential new avenues for asthma therapeutics. Additionally, I demonstrate that citrullination serves as a regulatory mechanism controlling LL-37's pro-inflammatory properties. Given their broad roles in infection and inflammation, therapies mimicking host defence peptides are under development. Our study highlights how citrullination affects LL-37's immunomodulatory functions, contributing new insights to peptide biology and paving the way for novel therapeutics strategies.

4.3 Future Directions

4.3.1 *Mechanisms of LL-37- and citLL-37-mediated differential chemokine production*

As mentioned, I observed differences in LL-37 and citLL-37-mediated chemokine enhancement. Another hypothesis to explain this difference is that LL-37 may outcompete citLL-37 in binding to its receptors. To test this hypothesis, I conducted a competitive study; HBEC-3KT cells were pretreated with 0.50 μM LL-37 or citLL-37 for 30 mins, followed by stimulation with the reciprocal peptide. In independent experiments, I also added 0.50 μM LL-37 and citLL-37 simultaneously. As previously shown, I observed that citrullination reduced the peptide's ability to enhance IL-8, MIP-3α, and GROα. However, there was no significant difference in IL-8, MIP-3α, and GROα production in the combinatorial conditions (Figure 14). Interestingly, in the combinatorial conditions, the levels of IL-8, MIP-3α, and GROα were very similar to stimulation with LL-37 alone. These preliminary experiments suggest that regardless of which peptide is added first, LL-37 is driving the induction of IL-8, MIP-3α, and GROα. It is possible that LL-37 and citLL-37 may be interacting with different receptors or citLL-37 loses its ability to bind strongly to LL-37 receptor(s), therefore resulting in decreased chemokine production. This provides a strong rationale to perform studies to identify the direct interacting protein partners of LL-37 and citLL-37 in human bronchial epithelial cells.

Figure 14

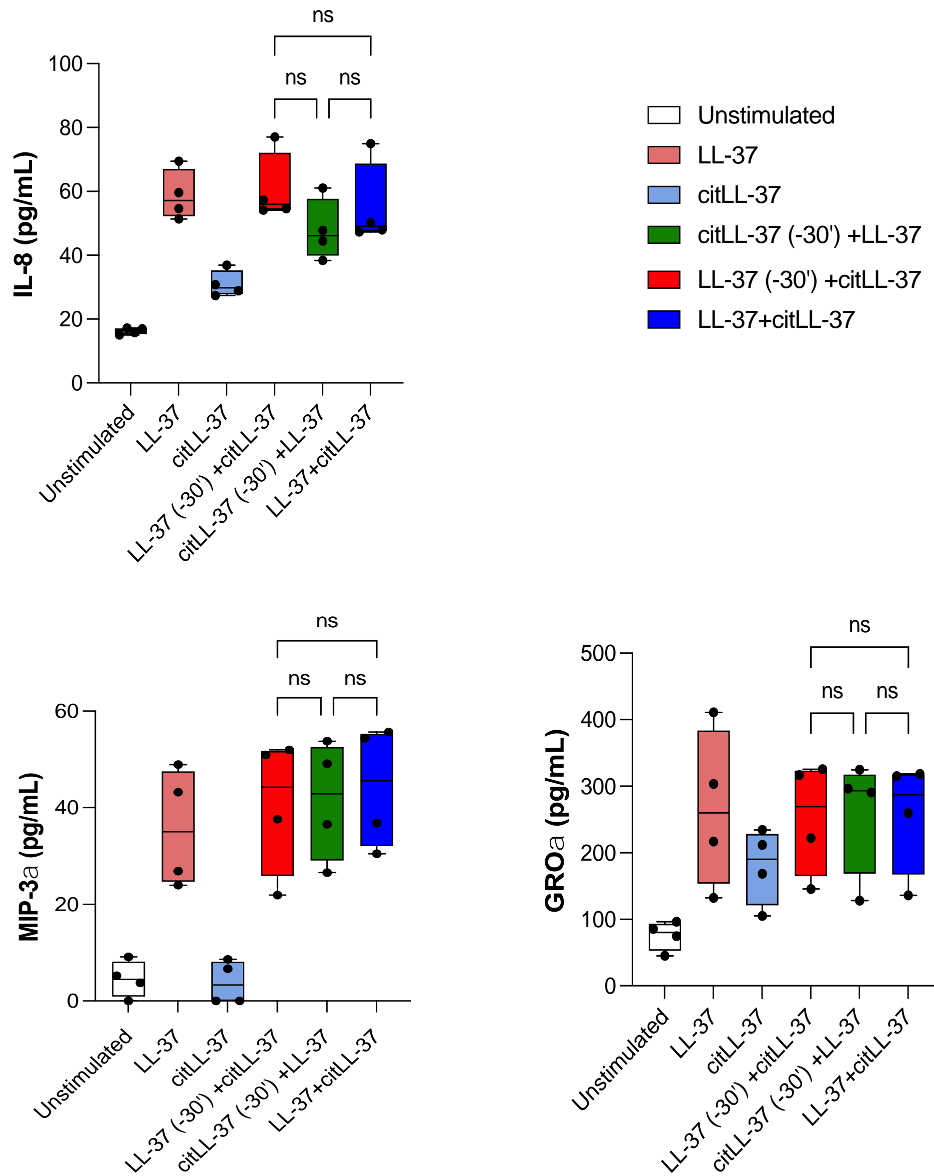


Figure 14: ***LL-37 drives the induction of IL-8, MIP-3α, and GROα in HBEC.*** HBEC-3KT cells were stimulated with 0.50 µM of peptides LL-37 and citLL-37, for 24 h. Abundance of IL-8, MIP-3α and GROα was examined in tissue culture supernatants by ELISA. Each dot represents an independent experimental replicate (n=4). Statistical significance was measured using One-Way ANOVA.

4.3.2 *Relationship of the cytochrome P450 pathway and LL-37-mediated migration of neutrophils*

As shown above, inhibition of cytochrome P450 pathway reduced LL-37-mediated enhancement of neutrophil chemoattracts IL-8, MIP-3α, and GROα. Therefore, it would be interesting to investigate whether inhibition of cytochrome P450 pathway affects LL-37-mediated migration of neutrophils, being that these chemokines can serve as neutrophil chemoattractants. This would provide insight into an additional mechanism by which LL-37 mediates neutrophil migration, further highlighting its role in immunometabolism.

4.3.3 *Quantification of citLL-37 levels in vivo*

Its previously shown that citLL-37 is present in the airways of healthy individuals, but the exact levels have not been quantified. Given LL-37's involvement in both airway inflammation, it would be valuable to quantify citrullinated cathelicidin CRAMP (the mouse ortholog of LL-37) and compare it to the abundance of the native peptide in the lungs, using a house dust mite (HDM)-challenge model of airway inflammation. Different HDM-challenged models can be used to further determine the relative abundance of these peptides in acute v/s chronic inflammation, and eosinophilic v/s neutrophilic inflammation. This would provide insight into the extent of citrullination occurring in different inflammatory conditions in the lungs, and the number of arginine residues affected. Similarly, it could help determine if citrullinated LL-37, similar to citrullinated antibodies in rheumatoid arthritis (RA)[96], contributes to pathogenesis of RA (the scope of the findings of this thesis can be expanded to other diseases such as RA).

4.3.4 *Mechanisms related to COX-2 pathway in LL-37-mediated enhancement of chemokines*

Based on my findings, LL-37 enhances various products from the COX pathway, notably PGE2, and upregulates COX-2 transcriptionally. To determine if LL-37-mediated chemokine production relies on COX-2 activity, we used an upstream approach to inhibit COX-2, the primary producer of PGE2. Our findings showed that COX-2 inhibition significantly suppressed LL-37-mediated chemokine production. However, the mechanism by which COX-2 inhibition affects LL-37-mediated chemokine production remains unclear. One hypothesis is that LL-37 induces PGE2

production, and that PGE2 acts in an autocrine manner to stimulate chemokine production. PGE2 exerts its effects through four receptors (EP1, EP2, EP3, and EP4), each influencing downstream signaling pathways. To further investigate this, inhibiting PGE2 receptors could provide insights into how PGE2 signaling contributes to LL-37's modulation of chemokine production.

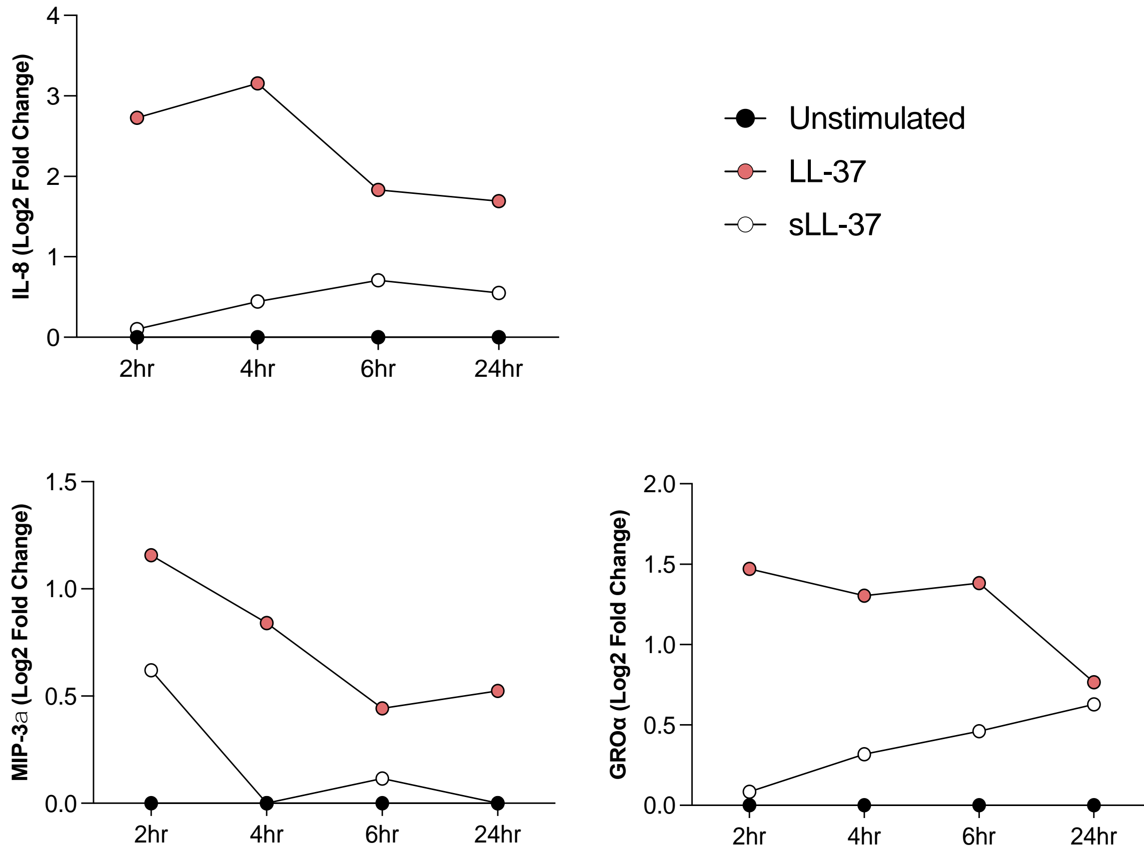
4.3.5 *Evaluation of LL-37 and COX pathways in vivo*

Based on the results in this thesis, it would be beneficial to confirm the findings of this study in an *in vivo* model of airway inflammation using HDM-challenge. Using immunohistochemistry, it would also be interesting to identify the major producers of oxylipins in the lung epithelial cells in this disease model. This technique would allow for spatial localization and identification of prostaglandin-producing cells within the tissue. Additionally, it would be interesting to investigate how knocking down COX would affect disease pathogenesis, given that some prostaglandins have both pro- and anti-inflammatory properties. However, it is likely that knockdown of COX-2 may impair the induction of airway inflammation *in vivo*. It is thus challenging to select an appropriate animal model to confirm the findings in this study with a knockout or knock down approach.

Chapter 5: Appendix

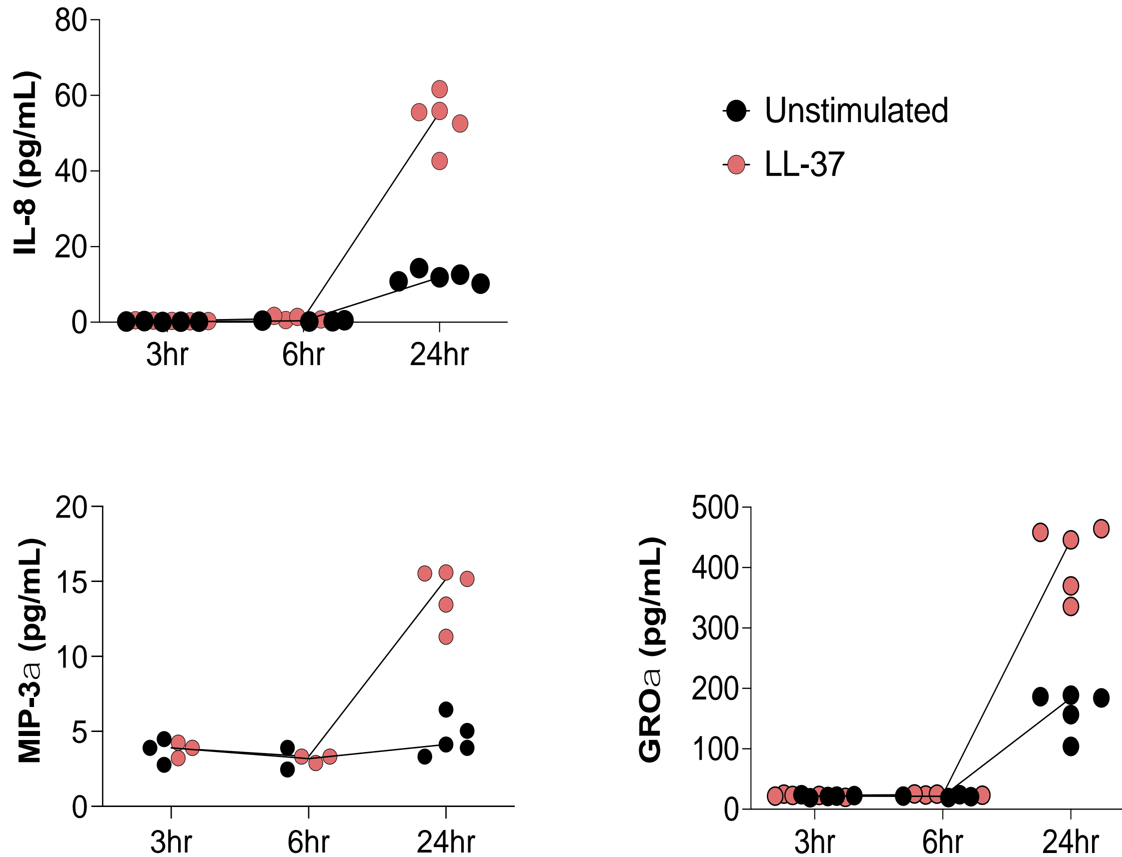
Supplementary Figures

Supplementary Figure 1



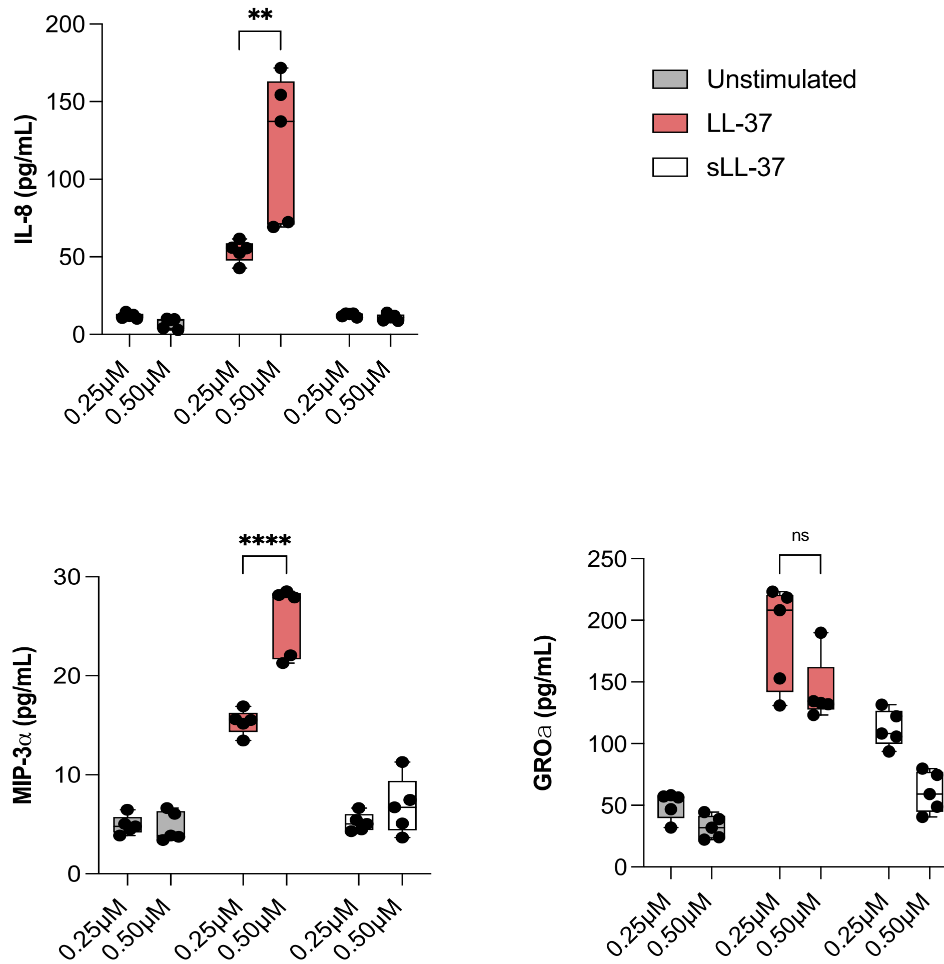
Supplementary Figure 1: Time kinetics of LL-37-mediated chemokine responses at the mRNA level. HBEC-3KT were stimulated with 0.25 μM LL-37 or sLL-37, and total RNA was collected after 2, 4, 6, and 24 h. mRNA abundance of IL-8, GROα, and MIP-3α was measured using RT-qPCR. Relative fold changes were calculated compared to the expression in unstimulated cells normalized to 1, using the ΔΔ Ct method after normalization with 18s RNA expression, followed by log_2 transformation. Each dot represents the mean of four independent experiments (n=4).

Supplementary Figure 2



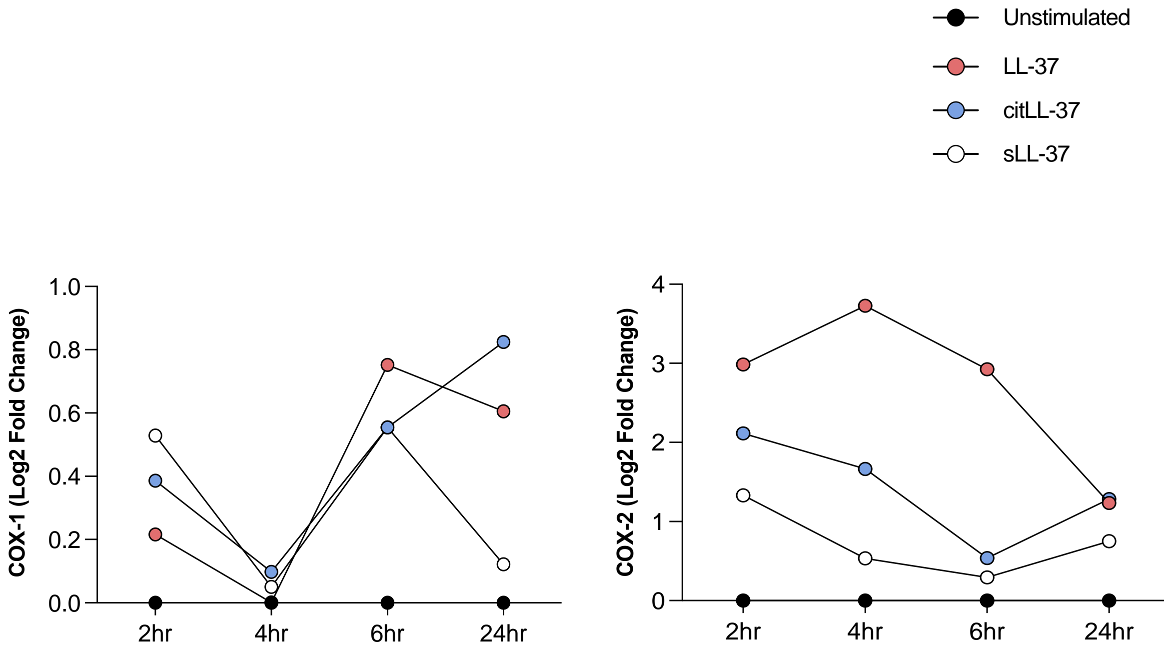
Supplementary Figure 2: Time course analysis of LL-37-mediated chemokine protein abundance in HBEC. HBEC-3KT were stimulated with 0.25 µM LL-37 and tissue culture supernatant was collected after 3, 6, and 24 h. The abundance of IL-8, GROα, and MIP-3α was measured using ELISA. Each dot represents an independent experiment (n=5).

Supplementary Figure 3



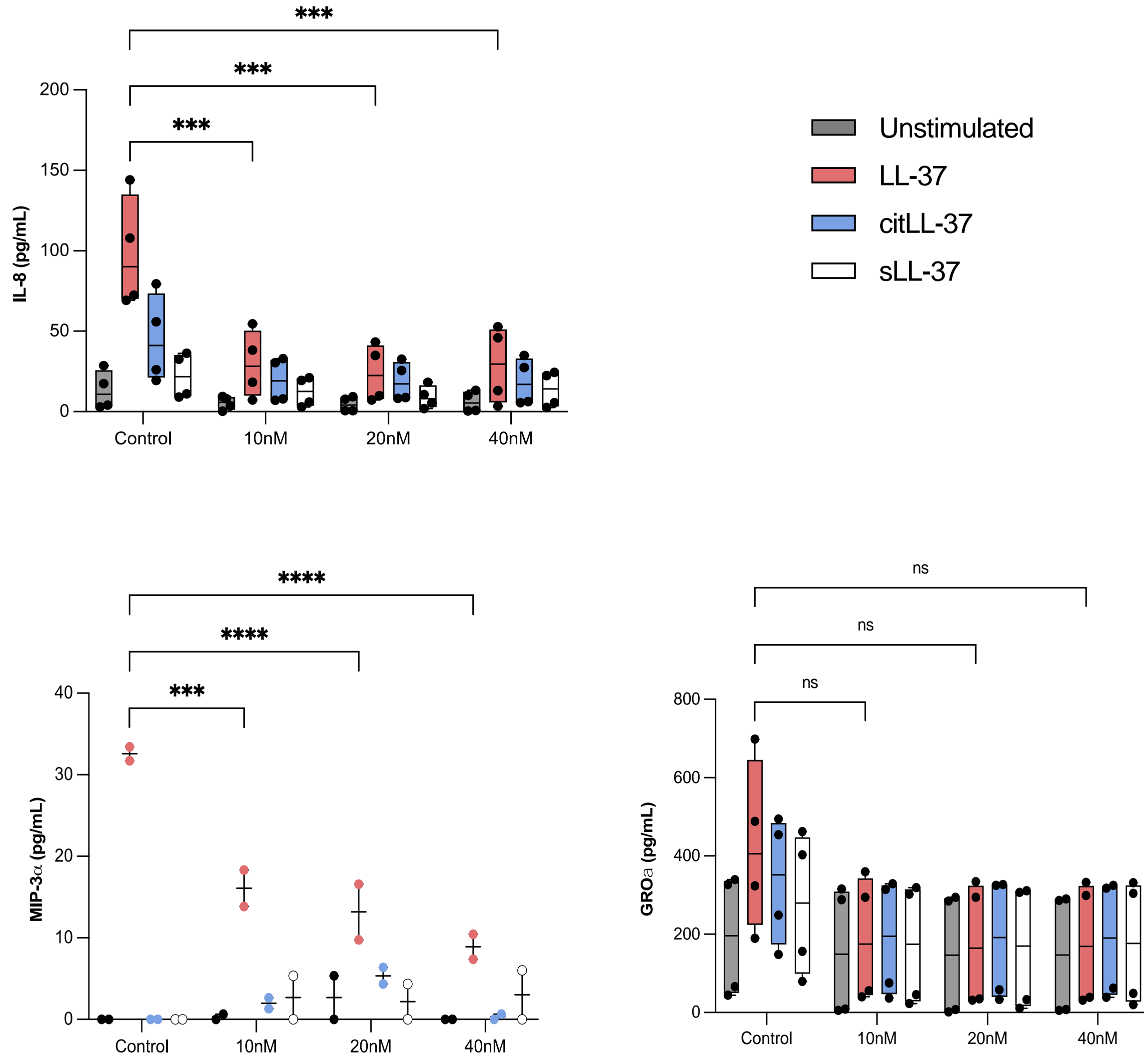
Supplementary Figure 3: Dose kinetics of LL-37-mediated chemokine response in HBEC. HBEC-3KT were stimulated with 0.25 µM and 0.50 µM LL-37 and sLL-37 after 24 h. TC supernatant was measured for the abundance of IL-8, MIP-3α, and GROα using ELISA. Each dot represents an independent experiment (n=5), and statistical analysis was determined by Two-Way Anova (**p< 0.004 and ****p< 0.0001).

Supplementary Figure 4



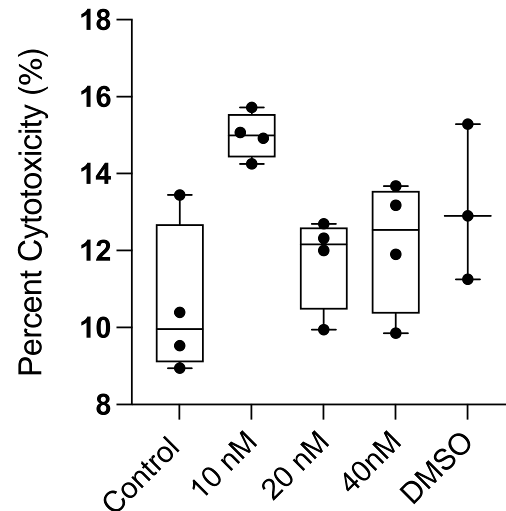
Supplementary Figure 4: Time course analysis of COX enzymes at the mRNA level. HBEC-3KT were stimulated with 0.25 µM LL-37, citLL-37, and sLL-37 and total RNA was collected after 2, 4, 6, and 24 h. mRNA abundance of COX-1 and COX-2 was measured using RT-qPCR. Relative fold changes were calculated compared to the expression in unstimulated cells normalized to 1, using the ΔΔ Ct method after normalization with 18s RNA expression, followed by $\log_2$ transformation. Each dot represents the mean (n=4).

Supplementary Figure 5



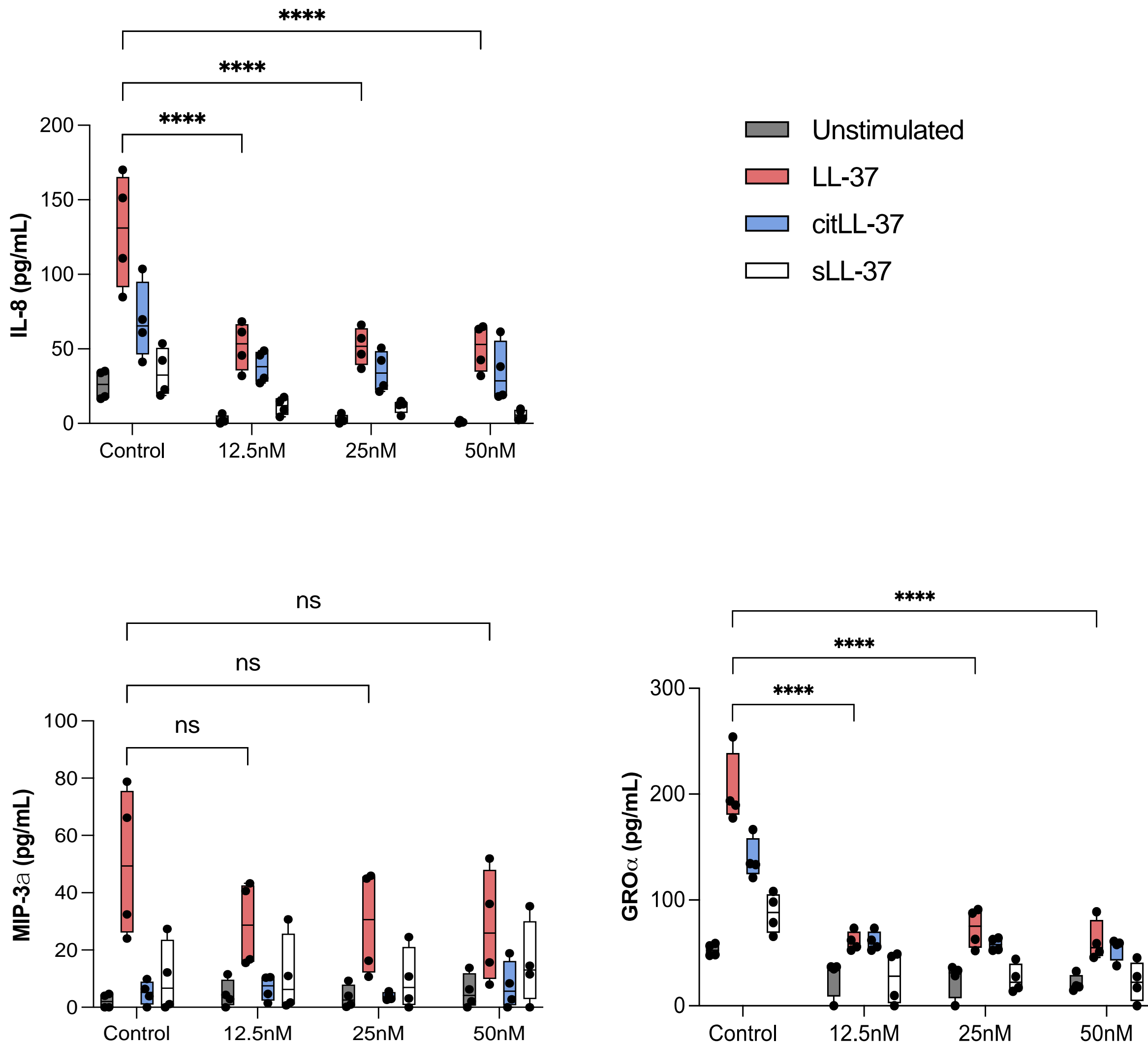
Supplementary Figure 5: Dose kinetics of COX-2 inhibitor, Rofecoxib on HBEC cells. HBEC-3KT cells were pre-treated with 10 nM, 20 nM, and 40 nM rofecoxib 1 h prior to stimulation with peptides. Cells were stimulated with 0.50 µM LL-37, citLL-37, and sLL-37 for 24 h. Protein abundance of IL-8, GROα, and MIP-3α in the presence of rofecoxib were measured using ELISA. Concentration of 20 nM rofecoxib was selected for future experiments based on percent inhibition of LL-37-mediated chemokine response. Each dot represents an independent experiment (n=4). Statistical significance was measured using One-Way ANOVA (***p<0.0004 and **** p<0.0001).

Supplementary Figure 6



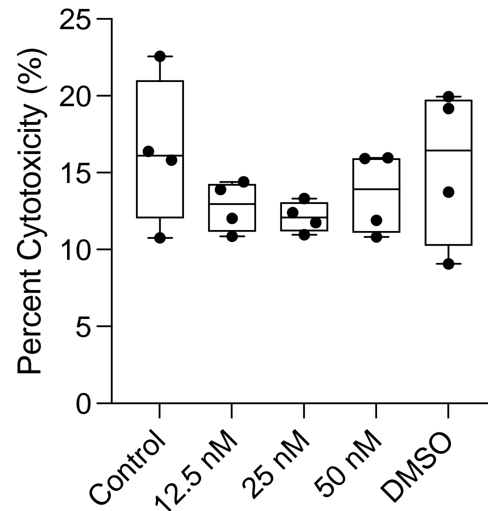
Supplementary Figure 6: Percent cytotoxicity of COX-2 inhibitor on HBEC cells. HBEC-3KT cells were pre-treated with 10 nM, 20 nM, and 40 nM rofecoxib for 1 h prior to stimulation with peptides. HBEC cells were stimulated with 0.50 μM LL-37, citLL-37, and sLL-37 for 24 h. 20 nM DMSO (vehicle control) was also tested to determine the cytotoxicity of DMSO on HBEC cells. LDH release was monitored in the TC supernatant as a marker for cellular cytotoxicity. Each dot represents an independent experiment (n=4).

Supplementary Figure 7



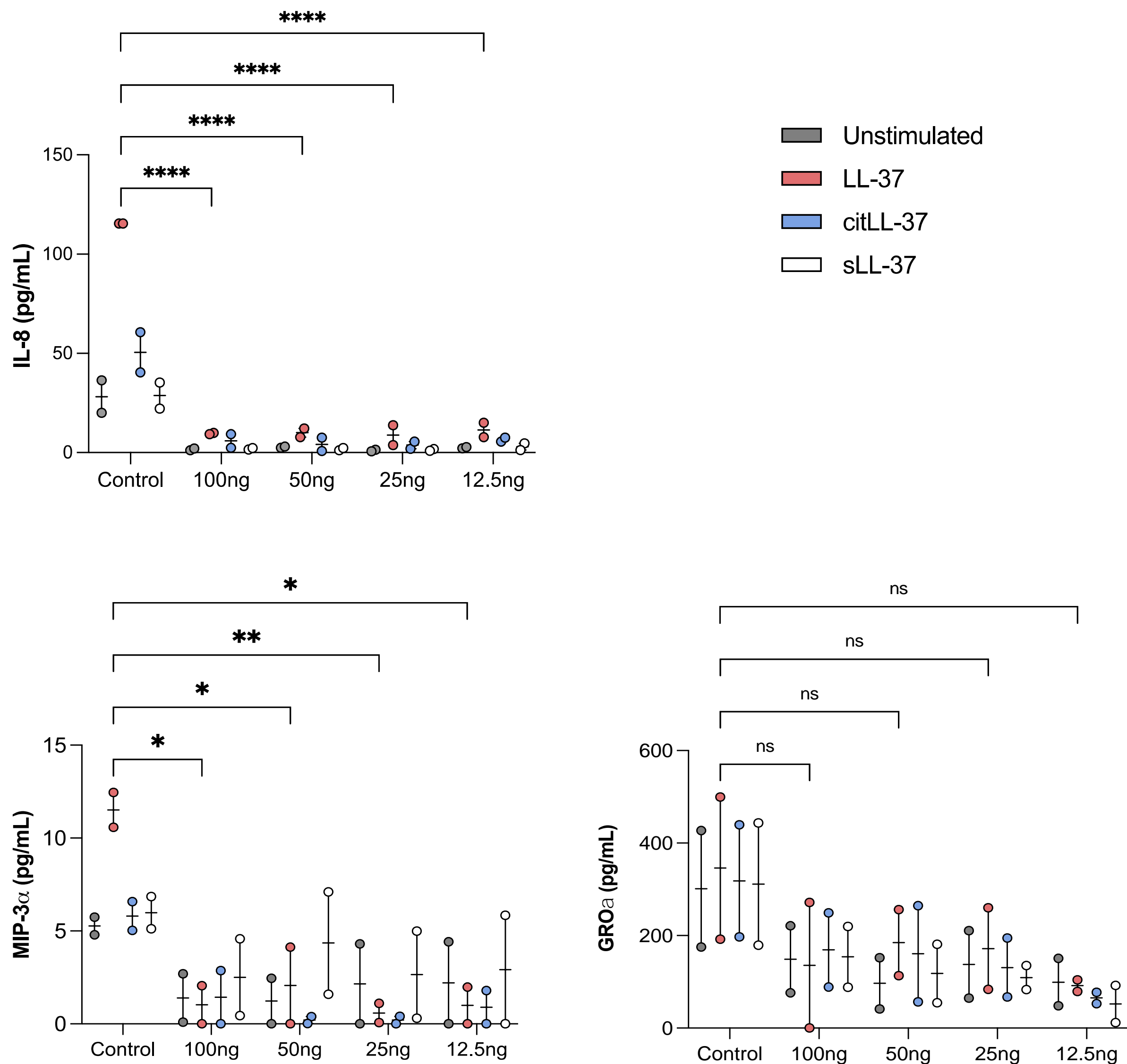
Supplementary Figure 7: Dose kinetics of cytochrome P450 inhibitor, Orteronel on HBEC cells. HBEC-3KT cells were pre-treated with 12.5 nM, 25 nM, and 50 nM orteronel 1 h prior to stimulation with peptides. Cells were stimulated with 0.50 µM LL-37, citLL-37, or sLL-37 for 24 h. Protein abundance of IL-8, GROα, and MIP-3α in the presence and absence of orteronel were measured using ELISA. Concentration of 12.5 nM orteronel was selected for future experiments based on percent inhibition of LL-37-mediated chemokine response. Each dot represents an independent experiment (n=4). Statistical significance was measured using Two-Way ANOVA (**** p<0.0001).

Supplementary Figure 8



Supplementary Figure 8: Percent cytotoxicity of cytochrome P450 inhibitor on HBEC cells. HBEC-3KT cells were pre-treated with 12.5 nM, 25 nM, and 50 nM orteronel for 1 h prior to stimulation with peptides. HBEC cells were stimulated with 0.50 µM LL-37, citLL-37, and sLL-37 for 24 h. 12.5 nM DMSO (vehicle control) was also tested to determine the cytotoxic effects of DMSO on HBEC cells. LDH release was monitored in the TC supernatant as a marker for cellular cytotoxicity. Each dot represents an independent experiment (n=4).

Supplementary Figure 9



Supplementary Figure 9: Dose kinetics of GPCR inhibitor, pertussis toxin (PTx) on HBEC cells. HBEC-3KT cells were pre-treated with 12.5, 25 and 50 ng/mL PTx 1 h prior to stimulation with peptides. Cells were stimulated with 0.50 µM LL-37, citLL-37, or sLL-37 for 24 h. Protein abundance of IL-8, GROα, and MIP-3α in the presence of PTx were measured using ELISA. Concentration of 25 ng/mL orteronel was selected for future experiments based on percent inhibition of LL-37-mediated chemokine response. Each dot represents an independent experiment (n=4). Statistical significance was measured using Two-Way ANOVA (**** p<0.0001).

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