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**ANTIMICROBIAL PEPTIDES AND  
EICOSANOIDS: ROLES AND INTERACTIONS  
IN INFLAMMATION AND HOST DEFENSE**

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Antimicrobial peptides and eicosanoids: roles and interactions in inflammation and host defense  
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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*To my family*

致我的家人



## ABSTRACT

Antimicrobial peptides (AMPs) have a broad spectrum of activities and exert their functions by both direct killing of microbes through the interaction with the microbial membrane, and modulation of innate and adaptive immunity of the host. In humans, cathelicidins and defensins represent the major families of AMPs. The only human cathelicidin is LL-37, a cationic,  $\alpha$ -helical peptide with 37 amino acids in its sequence.

Eicosanoids are a class of oxygenated hydrophobic compounds derived mainly from arachidonic acid (AA). The main pathways of eicosanoid biosynthesis are cyclooxygenase (COX) pathway and lipoxygenase (LOX) pathway, which metabolize AA into bioactive lipid mediators, namely prostaglandins, thromboxanes, leukotrienes and lipoxins. Eicosanoids exert important functions in normal homeostasis as well as in various pathological conditions. In the present thesis, we investigated the roles and interactions of AMPs and eicosanoids in inflammation and host defense towards bacteria, more specifically, in regulation of macrophage functions in innate immunity.

Efficient phagocytosis is an important step in the clearance of invading bacteria and host defense. We demonstrate that LL-37 up-regulates the expression of Fc $\gamma$  receptors (Fc $\gamma$ Rs) CD64 and CD32, leading to an enhanced phagocytosis capacity of IgG-opsonized bacteria in human macrophages. Using the subcutaneous air pouch model in cathelicidin deficient mice, we further demonstrate the effect of LL-37 on the expression of Fc $\gamma$ Rs and bacterial phagocytosis *in vivo*.

LL-37 interacts with host cells in many ways. In our study, LL-37 internalization by human macrophages is characterized and we could also demonstrate that macrophages take up LL-37 derived from neutrophils. Further studies show that LL-37 internalization contributes to intracellular bacteria killing by macrophages. Together with the finding that LL-37 enhances bacterial phagocytosis, we conclude that LL-37 enhances the ability of human macrophages to kill bacteria via promoting bacterial phagocytosis, as well as via lysosome accumulation, and ROS production triggered by internalized LL-37.

Macrophages can be triggered to produce large amounts of eicosanoids, participating in discrete stages of inflammation. We observe that LL-37 induces a biphasic release of eicosanoids from human macrophages. At early time points (1 h) LTB<sub>4</sub> is produced, while induction of COX-2 expression and TXB<sub>2</sub> and PGE<sub>2</sub> production is observed at a late phase (8 h). The purinergic receptor P2X<sub>7</sub>R is involved in LL-37 triggered early phase eicosanoid production in human macrophages. Furthermore, LL-37 internalization seems to be required for eicosanoid production. More importantly, we confirm the involvement of cathelicidin in eicosanoid production *in vivo*.

Prostaglandin (PG)E<sub>2</sub> is a multifunctional lipid mediator in host defense. Our studies show that PGE<sub>2</sub> suppresses the basal level of AMPs in human macrophages, and also VD3-induced expression of cathelicidin. The effect of PGE<sub>2</sub> on AMP expression is transduced via EP2/EP4-cAMP-PKA regulated downstream transcription factors CREM/ICER and VDR. Of clinical relevance, we report that PGE<sub>2</sub> impairs VD3-induced expression of cathelicidin and concomitant activation of autophagy during Mtb infection, and facilitates intracellular Mtb growth in human macrophages. Collectively, our findings indicate that PGE<sub>2</sub> plays deleterious roles in human Mtb infection.

Together, the results of the present thesis reveal the modulatory effects of LL-37 and PGE<sub>2</sub> in macrophage functions towards bacteria, from multiple perspectives. Moreover, the interactions of AMPs and eicosanoids in macrophages expand our understanding of the inflammatory mediator network and could provide opportunities for future pharmacological intervention in infectious or inflammatory diseases.

## LIST OF SCIENTIFIC PAPERS

- I. Wan M, van der Does AM, **Tang X**, Lindbom L, Agerberth B, Haeggstrom JZ. *Antimicrobial peptide LL-37 promotes bacterial phagocytosis by human macrophages*. J Leukoc Biol, 2014. 95(6): p. 971-81.
- II. **Tang X**, Basavarajappa D, Haeggstrom JZ, Wan M. *P2X7 Receptor Regulates internalization of antimicrobial peptide LL-37 by human macrophages that promotes intracellular pathogen clearance*. J Immunol, 2015. 195(3): p. 1191-201.
- III. Wan M, Soehnlein O, **Tang X**, van der Does AM, Smedler E, Uhlen P, Lindbom L, Agerberth B, Haeggstrom JZ. *Cathelicidin LL-37 induces time-resolved release of LTB4 and TXA2 by human macrophages and triggers eicosanoid generation in vivo*. FASEB J, 2014. 28(8): p. 3456-67.
- IV. Wan M\*, **Tang X\***, Rekha RS, Muvva SSV J, Brighenti S, Agerberth B, Haeggström JZ. *PGE<sub>2</sub> suppresses antimicrobial peptide hCAP18/LL-37 expression in human macrophages via EP2/EP4: potential implications in the treatment of M. tuberculosis infection*. Manuscript

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- I. Wan M, **Tang X**, Haeggström JZ. *Host Defense Peptides and the Eicosanoid Cascade*. In: Epanand RM, editor. *Host Defense Peptides and Their Potential as Therapeutic Agents*. Cham: Springer International Publishing; 2016. p. 139-58.
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## LIST OF ABBREVIATIONS

AMP	Antimicrobial peptide
LOX	Lipoxygenase
COX	Cyclooxygenase
FcγR	Fcγ receptor
PG	Prostaglandin
MPS	Mononuclear phagocyte system
ROS	Reactive oxygen species
RNS	Reactive nitrogen species
NK cell	Natural killer cell
PRR	Pattern recognition receptor
PMN	Polymorphonuclear leukocyte
PAMP	Pathogen associated molecular pattern
DAMP	Damage associated molecular pattern
HMGB1	Chromatin-associated protein high-mobility group box 1
TLR	Toll-like receptor
TNF	Tumor necrosis factor
IL	Interleukin
NO	Nitric oxide
NOS	nitric oxide synthase
nNOS	neuronal NOS
eNOS	endothelial NOS
iNOS	Inducible NOS
EMP	Erythromyeloid progenitor
HSC	Hematopoietic stem cell
CCR2	CC-chemokine receptor 2
CX <sub>3</sub> CR1	CX <sub>3</sub> C-chemokine receptor
CCL2	CC-chemokine ligand 2
LPS	Lipopolysaccharide
DC	Dendritic cell
MDP	Monocyte-macrophage dendritic cell progenitor
M-CSF	Macrophage colony-stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
FLT3L	FMS-like tyrosine kinase 3 ligand
HSC	Hematopoietic stem cell
MCP-1	Monocyte chemotactic protein 1

CAMP	Cathelicidin antimicrobial peptide
LTA4H	LTA4 hydrolase
LTC4S	LTC4 synthase
CysLT	Cysteinyl leukotriene
LX	Lipoxin
12-LOX	12-lipoxygenase
15-LOX	15-lipoxygenase
15S-HETE	15S-hydroxy-eicosatetraenoic acid
15R-HETE	15R-hydroxy-eicosatetraenoic acid
ATL	Aspirin-triggered lipoxins
cPLA2	Cytosolic phospholipase A2
sPLA2	Secreted PLA2
iPLA2	Calcium-independent PLA2
NSAID	Nonsteroidal anti-inflammatory drug
MAPKAP	Mitogen-activated protein kinase-activated protein
PMA	Phorbol myristate acetate
MAPEG	Membrane associated protein in eicosanoid and glutathione metabolism
PGDS	Prostaglandin D synthase
PGFS	Prostaglandin F synthase
PGIS	Prostacyclin synthase
TXAS	Thromboxane A synthase
cAMP	Cyclic adenosine monophosphate
PGT	Prostaglandin transporter
12-HHT	12(S)-hydroxy-5-cis-8, 10-trans-heptadecatrienoic acid
RPM	Resident mouse peritoneal macrophage
mPGES-1	Microsomal prostaglandin E synthase-1
15-PGDH	15-hydroxy-prostaglandin dehydrogenase
GSK3	Glycogen synthase kinase 3
HBP	Heparin-binding protein
HNP	Human neutrophil peptide
dTHP-1 cell	Differentiated THP-1 cell
HMDM	Human monocytes derived macrophages
MM6	Mono Mac 6
Antibody	Ig
<i>Cnlp</i> <sup>-/-</sup>	Cathelicidin-deficient
s.c.	Subcutaneous

sLL-37

Scrambled LL-37

CME

Clathrin-mediated endocytosis



# 1 INTRODUCTION

The immune system defends the host against infection by two distinct mechanisms, *viz.* innate immunity and adaptive immunity. In most living organisms, innate immunity is fundamental and crucial for host defense, mainly due to its sentinel effect and fast reaction to kill invading pathogens. A normal functioning innate immune system is highly dependent on the activities of macrophages, one of the most abundant and multifunctional immune cell populations in the human body. Macrophages are evolutionary conserved phagocytes that have a history of more than 500 million years [1] and were discovered by Nobel laureate Elie Metchnikoff in late 19th century as part of the mononuclear phagocyte system (MPS). Derived from embryonic progenitors [2-6] or adult blood monocytes [1, 7], tissue resident macrophages work as sentinel cells under steady state and become activated once encountering invading pathogens. The activated macrophages kill pathogens by phagocytosis and release of antimicrobial agents, as well as secretion of inflammatory mediators to recruit neutrophils and monocytes from blood to extravascular sites of infection. Meanwhile, the permeability of blood vessels increase, followed by extravasation of plasma into the infected tissues and a rapid delivery of host defense effector cells and molecules to the sites of injury. This process is named inflammation, described in AD40 by its four cardinal signs: rubor (redness), calor (heat), dolor (pain), and tumor (swelling). Well-controlled inflammation contributes to pathogen elimination and tissue restoration. However, inappropriate or excessive inflammation may lead to acute and chronic inflammatory diseases.

AMPs and eicosanoids are two families of potent immune effector molecules. In this thesis, we focus on the roles and interactions of those two families *in vitro* and *in vivo*, to get a deeper understanding of inflammatory mediator network.

## 1.1 INNATE IMMUNITY

Innate immunity is an old host defense strategy, which exists in plants, fungi, insects and primitive multicellular organisms. It serves as the first line of defense, rapidly but not specifically eliminating the invading pathogens. Innate immunity comprises epithelial barriers and mucosa, innate immune cells in tissues and circulation, complement system, cytokines and other inflammatory molecules. Once a pathogen succeeds in breaching the anatomical barriers of the host, the effector molecules of innate immunity, such as AMPs start to act immediately by either directly killing the pathogen or decreasing its activity. In the infected tissue, the invading pathogens are recognized and eliminated by tissue phagocytes- mainly macrophages- and elicit a series of cellular responses, such as generation of reactive

oxygen species (ROS) and nitrogen species (RNS), induction of cytokines, chemokines and release of other inflammatory mediators, to recruit neutrophils and monocytes from the circulation, trigger and maintain a local inflammatory response. Meanwhile, the complement system is activated and mediates pathogen opsonization allowing pathogens to be more readily taken up and killed by phagocytes. Moreover, natural killer (NK) cells recognize and bind to virus infected cells and tumor cells to induce their apoptosis [8, 9].

### **1.1.1 Phagocytes and Pattern Recognition Receptors (PRRs)**

Phagocyte recruitment to sites of infection or injury is a key cellular process in inflammation. The most abundant phagocytes are polymorphonuclear leukocytes (PMNs), which accounts for 40-70% of leukocytes in human peripheral blood [10]. They are short-lived and are recruited early in the inflammatory responses. PMNs, together with monocytes and macrophages constitute the professional phagocyte population, coordinating with each other to kill invading microbes and regulate inflammatory process (initiation, progression and resolution) [11].

PRRs are a series of receptors expressed in phagocytes and recognize mainly two classes of molecules: (i) the microbe-derived pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) on Gram-negative bacteria. (ii) the tissue damage derived damage-associated molecular patterns (DAMPs), such as chromatin-associated protein high-mobility group box 1 (HMGB1) and heat shock proteins. Among the many PRRs, Toll-like receptors (TLRs) represent one of the best-characterized families responsible for sensing invading pathogens [12]. The name TLRs comes from its homologue found in *Drosophila* called Toll [13]. Those receptors are expressed on human phagocytes and recognize structurally conserved PAMPs. There are at least ten human TLRs and they recognize a series of different microbial ligands. For instance, LPS on Gram-negative microbes activates TLR4 via LPS binding protein and CD14, whereas Gram-positive PAMP lipoteichoic acid activates TLR2 [14, 15]. TLR5 has been reported to recognize flagellin in flagellated bacteria, while TLR9 and TLR3 have been shown to recognize CpG motifs of bacterial DNA and viral double-stranded RNA, respectively [9, 16-18].

In most of the cases, the activation of TLR leads to the translocation of transcription factor NF- $\kappa$ B through the adaptor molecule, MyD88 [19]. The translocation of NF- $\kappa$ B promotes production of a series of inflammatory cytokines, including but not limited to tumor necrosis factor (TNF) and interleukin 6 (IL-6) [9]. The activation of TLRs and enhanced production of



cytokines are potent steps in bacteria elimination, however, could potentially induce deleterious effect on the host such as contribution to sepsis [15].

### **1.1.2 Effector molecules in innate immunity**

#### **1.1.2.1 ROS and RNS**

ROS and RNS are produced as important parts of innate immune response. The examples of intracellular ROS include superoxide anion radical ( $O_2^-$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $\cdot OH$ ), ozone ( $O_3$ ) and singlet oxygen ( $O_2$ ). There are several major sources of ROS within cells, such as mitochondria, endoplasmic reticulum (ER) and NADPH oxidases (NOXs). RNS refer to nitric oxide (NO) and the reactive molecules derived from NO. Intracellular NO can be synthesized by nitric oxide synthases (NOSs). There are three NOS isoforms identified in humans, viz. neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). ROS and RNS play diverse roles in homeostasis, as well as in host defense and inflammation. For example, ROS and RNS can be generated during the early response to infection and kill pathogens, by interacting with microbial components [20, 21]. Furthermore, ROS and RNS also play roles in immune-modulation, such as regulate apoptosis of macrophages and neutrophils [22, 23].

Apart from ROS and RNS, there are other families of effector molecules such as lysozyme, cytokines and chemokines, which will not be further described here. The focus of the present thesis-AMPs and eicosanoids will be described in detail in the following part of the introduction.

## **1.2 MACROPHAGES**

Macrophages are residential immune effector cells. Upon inflammation, blood monocytes are recruited to the inflamed tissue where they are differentiated into tissue specific macrophages [24]. Macrophages are distributed throughout the body tissues and form a heterogeneous cell population with high plasticity, although two distinct activation states of macrophages have been well defined: the pro-inflammatory (M1) state and the anti-inflammatory (M2) state [25]. In innate immunity, macrophages kill invading pathogens and modulate inflammatory processes, by several strategies, such as phagocytosis, secretion of antimicrobial molecules and production of inflammatory mediators [26-28]. Surface markers have been used to characterize human macrophages, for instance, CD11b, CD68, and CD163 (**Table 1**).

Markers	Comments
CD11b	Also expressed on neutrophils
CD68	Expressed on all macrophages
MAC2	
CD11c	Expressed on many monocytes-derived cells, such as specific populations of DCs
IL-4Ra	Expressed on many macrophages, but also lymphocytes and other cell types
CD163	Expressed on most tissue macrophages

**Table 1** Receptors commonly used to distinguish human macrophages

### 1.2.1 Macrophage ontogeny and tissue distribution

Tissue macrophages do not necessarily share the same origins, arising either from embryonic progenitors, such as macrophages in yolk sac and fetal monocytes, or from adult blood monocytes [29]. In the 1960s, Van Furth proposed that tissue macrophages are differentiated from blood monocytes derived from hematopoietic stem cells (HSCs), which has remained as the prevailing view in the macrophage field. In the last decade, however, this view has been challenged by several publications, claiming that many macrophages are established during embryonic development and persist and self-renewed in tissues [30-32]. The embryonic precursors for yolk sac and fetal monocytes were recently discovered as yolk sac erythromyeloid progenitors (EMPs) [4, 33], but controversies on the actual origin of fetal monocytes still remains [34]. Nevertheless, fetal monocytes and yolk sac macrophages are important for fetal development and homeostasis since absence of fetal macrophages leads to abnormal development and functions of brain and lung [35-37]. The classical HSC derived macrophages differ from yolk sac derived macrophages by the expression of transcription factor MYB [31] and there is different composition of those two macrophage subtypes in different tissues. Till now, it is still unclear whether there are functional differences between tissue macrophages of different origins.

Macrophages are distributed throughout the body tissues. Specialized tissue macrophages include but are not limited to microglia (brain), alveolar macrophages (lung), kupffer cells (liver), osteoclasts (bone), langerhans cells (skin), splenic macrophages (spleen) and histiocytes (interstitial connective tissue). Based on their anatomical locations and phenotypes, specialized tissue macrophages exhibit differences in their morphology and functions.

#### 1.2.1.1 Monocyte recruitment into tissues of inflammation

Monocytes are a heterogeneous population of peripheral blood leukocytes, representing 5-10% of the nucleated cells in the blood [29]. In mice, high expression of LY6C and CD11b

identifies a monocyte subset, which is usually recognized as LY6C<sup>hi</sup> or inflammatory monocyte subset. These monocytes represent 2%-5% of circulating leukocytes in healthy mice, express high level of CC-chemokine receptor 2 (CCR2) and CX<sub>3</sub>C-chemokine receptor 1 (CX<sub>3</sub>CR1), and are recruited to the inflammatory sites upon infection and inflammation [38]. In contrast to this subset, the other major monocyte population expresses low levels of LY6C and CCR2, but high level of CX<sub>3</sub>CR1. Those LY6C<sup>lo</sup> monocytes mainly crawl along the luminal surface of small vessel endothelium and participate in a process referred to as patrolling [39, 40].

In humans, the subtypes of monocytes are defined based on the expression pattern of CD14 (a co-receptor for the detection of bacterial lipopolysaccharide (LPS) and CD16 (FcγRIII): (i) “Classical” CD14<sup>hi</sup>CD16<sup>-</sup> monocytes, representing up to 90-95% of peripheral blood monocytes in healthy individuals, resembling the LY6C<sup>hi</sup> monocytes in mice based on their gene expression [41, 42], (ii) “non-classical” CD14<sup>+</sup>CD16<sup>+</sup> monocytes, which resemble the LY6C<sup>lo</sup> monocytes, regarding their *in vivo* patrolling [41, 43-45].

In adults, monocytes are derived from HSCs, more specifically, from monocyte-macrophage dendritic cell (DC) progenitors (MDPs) [46]. During inflammation, those MDPs derived monocytes are recruited to the inflammatory sites. The recruitment process of monocytes follows the general paradigm of leukocyte trafficking, which involves rolling, adhesion and transmigration. This process is highly dependent on the adhesion molecules such as integrins [47] and also chemoattractants such as CC-chemokine ligand 2 (CCL2) and leukotriene B<sub>4</sub> [38, 48]. The recruited monocytes are stimulated by various growth factors and differentiate into macrophages and DCs. By utilizing transgenic mouse models and high-throughput screening, the role of macrophage colony-stimulating factor (M-CSF) has been established for macrophage differentiation, whereas granulocyte-macrophage colony-stimulating factor (GM-CSF) and FMS-like tyrosine kinase 3 ligand (FLT3L) have been reported as growth factors for DC formation and maintenance [49, 50]. However, controversies still remain regarding the role of GM-CSF in macrophage and DC maturation.

### **1.2.2 M1 and M2 macrophages**

Based on the tissue microenvironment, macrophages can acquire distinct polarized phenotypes, namely classically activated macrophages (M1) and alternatively activated macrophages (M2). Though there are literatures showing different opinions on macrophage classification [26, 45, 51], this dichotomous concept of macrophage activation still remains the most generally used classification method especially for *in vitro* macrophage research

[52-54]. The macrophage M1 phenotype is usually obtained by pro-inflammatory stimuli such as LPS or interferon- $\gamma$  (IFN- $\gamma$ ), which induce an increased production of inflammatory cytokines and antimicrobial agents, and promote T helper 1 (T<sub>H</sub>1) response [55]. In contrast, the M2 phenotype is usually associated with tissue repair, thus the name “healing macrophage”. M2 macrophages are stimulated by anti-inflammatory cytokines such as IL-4 or IL-13, leading to a decreased production of pro-inflammatory cytokines in macrophages and an increased T helper 2 (T<sub>H</sub>2) response [56, 57]. **Table 2** summarizes several markers for distinguishing M1 and M2 macrophages.

<b>M1-macrophages</b>	<b>M2-macrophages</b>
MARCO	SOC2
SOCS3	IRF4
Ptgs2 (COX-2)	CXCL13
NOS2	CCL12
IL12b	KLF4
IDO1	CCL18
IL23a (Il23p19)	
CCL15	

**Table 2** Markers commonly used to distinguish M1 and M2 macrophages

### 1.2.3 Macrophage Phagocytosis

Phagocytosis of pathogens is one of the key steps for the initiation of an innate immune response [58]. It starts with the recognition of PAMPs by PRRs. The PRRs that are expressed in macrophages include mannose receptors and an endocytic receptor CD205 that recognize mannans, CD14 that binds LPS and lipoteichoic acid in Gram-negative and Gram-positive bacteria, respectively. They also include the scavenger receptor A family that recognizes bacteria lipid and polyanionic ligands, as well as TLRs that signals the presence of various PAMPs and lead to the translocation and activation of NF- $\kappa$ B and the interferon regulatory transcription factor 3 (IRF-3), resulting in production of a series of pro-inflammatory cytokines and other mediators [59, 60].

Opsonization is a preparatory step for bacteria phagocytosis, which facilitates the removal of microbes by neutrophils and macrophages. Opsonins, such as antibodies and complement proteins specifically recognize ligands on infectious agents and through their generic domains, bind to the Fc receptors and complement receptors expressed on phagocytes. There are three Fc receptors on macrophages recognizing IgG: Fc $\gamma$ RI (CD64), Fc $\gamma$ RII (CD32) and

Fc $\gamma$ RIII (CD16). CD64 is a high-affinity receptor for monomeric IgG, while CD16 and CD32 have low affinity to monomeric IgG but bind to multiplexes more effectively. The complement receptors involved in uptake of opsonized particles by macrophages are mainly CR1 (CD35) that binds to C3b and C4b, CR3 (CD11b/CD18) that binds to inactivated C3b (iC3b) and C4b, and CR4 (CD11c/CD18).

#### **1.2.4 Macrophage secretion**

Macrophages secrete various molecular components to regulate inflammation and host defense. The secretome of macrophages highly depends on their activation status and the microenvironment. For instance, under pro-inflammatory conditions, macrophages are activated into a M1 phenotype and secrete more pro-inflammatory cytokines and mediators such as TNF- $\alpha$ , IL-1, IL-6, IL-8, and IL-12 [61]. Alternatively, macrophages are triggered by anti-inflammatory stimuli and secrete cytokines such as IL-10 and TGF- $\beta$ . Macrophages are also resident cells that initiate chemotaxis of other immune cells by secreting chemoattractants, such as CXCL1, CXCL2, CCL5 and leukotriene B<sub>4</sub> (LTB<sub>4</sub>).

Macrophages produce large amount of eicosanoids. Like cytokines, the profile of eicosanoids differs between macrophages of different tissue origins, with different activation status. Using a lipidomic approach, eicosanoid profiles have been determined and compared in murine macrophages from different tissue origins and murine macrophage cell line (RAW264.7) in response to TLR4 stimulation [62]. An earlier study has also reported eicosanoid profiling in RAW264.7 cells activated by different TLR agonists [63]. The lipidomic studies suggest that murine macrophages produce mostly COX products such as PGD<sub>2</sub>, PGE<sub>2</sub> and PGF<sub>2 $\alpha$</sub> .

As tissue sentinel cells, macrophages also produce antimicrobial agents to kill pathogens, such as antimicrobial peptides, lysozyme, reactive oxygen species (ROS), nitric oxide (NO), and other effector molecules. Collectively, these molecules released into infected sites contribute to the maintenance of a unique microenvironment for tissue homeostasis and inflammatory processes.

### **1.3 AMPs**

AMPs are peptides with a broad spectrum of antimicrobial activities [64]. So far, more than 2600 antimicrobial peptides have been identified from animals, plants, bacteria and other organisms (APD: <http://aps.unmc.edu/AP/main.php>). These peptides are evolutionary conserved molecules and are usually cationic small peptides (12-50 amino acids) with an amphipathic structure [65]. Although there are great diversity among peptides from different

organisms, significant conservation of amino-acid sequences can be recognized in the preproregion of the precursor molecules [66]. AMPs exert their functions by both direct killing of microbes through the interaction with the microbial membrane [66], and modulation of innate and adaptive immunity of the host [27]. Two major families of naturally occurring AMPs in mammals have been distinguished; *viz.* defensins and cathelicidins.

### **1.3.1 The direct antimicrobial activity of AMPs**

The direct antimicrobial activity is a unique feature of the AMPs, based on the interaction of AMPs with the membranes of microbes, best understood for bacteria. Bacterial membranes are organized in a way such that the outermost leaflet of the bilayer is heavily populated by lipids with negatively charged phospholipid head groups, which provide binding sites for positively charged AMPs [67, 68]. Moreover, the amphipathic nature of the peptides allows them to be incorporated into the lipid bilayer of the bacterial membrane [69]. A model that explains the activity of most AMPs is the Shai-Matsuzaki-Huang (SMH) model [67, 70, 71]. The model proposes the interaction of the peptide with the membrane, followed by displacement of lipids, alteration of membrane structure, and in certain cases entry of the peptide into the interior of the target cell [66].

### **1.3.2 The immunomodulatory effect of AMPs**

At the site of infection or injury, resident cells and recruited immune cells are stimulated by microbes to release AMPs [72]. In addition to direct interaction with the invading organisms, AMPs play important roles in recruiting leukocytes including neutrophils, monocytes and T cells, by acting as a chemokine or inducing the expression of other chemokines and cytokines, such as IL-8, monocyte chemoattractant protein 1 (MCP-1) and IFN- $\alpha$  [65]. Furthermore, AMPs also participate in the endogenous inflammatory process by interacting with the endogenous or recruited immune cells and inflammatory mediators. For instance, cathelicidin has been shown to inhibit TLR4 and CD44 mediated induction of cytokine release in DCs and macrophages [73, 74]. In addition, it has been reported that human cathelicidin LL-37 promotes LTB<sub>4</sub> release from human neutrophils [75] and macrophages [76]. AMPs have also been reported to promote wound healing, by stimulating keratinocyte migration required for re-epithelialization [77], promoting angiogenesis [78, 79], and inhibiting TGF-induced collagen expression and fibrosis [79].

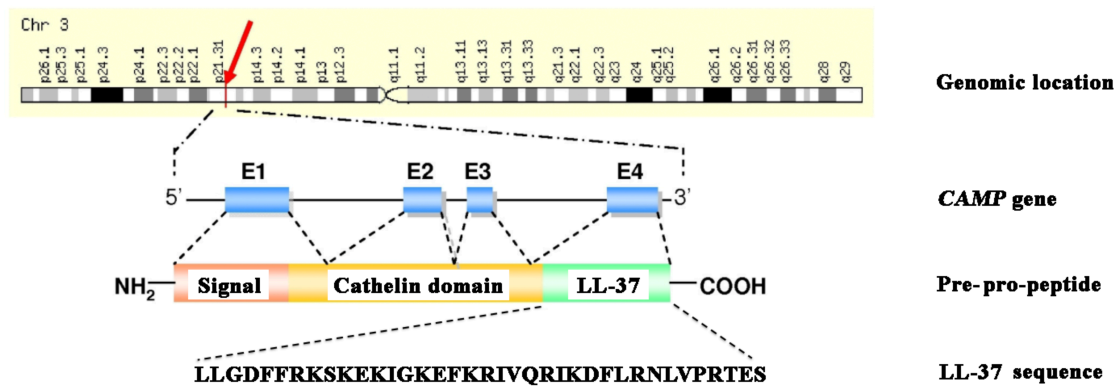
### 1.3.3 Cathelicidin

Cathelicidins are a major family of AMPs that exist in mammals [80], but are also found in other species, such as fish [81], birds [82], and snakes [83]. This family of AMPs carries a highly conserved pre-proregion at the N-terminal, consisting of a N-terminal signal peptide (29-30 residues) and a conserved cathelin domain (99-114 residues), followed by diverse antimicrobial residues at the C-terminus [84]. The C-terminal domain, which becomes active when cleaved off from the proregion, displays a broad spectrum and potent antimicrobial activity [84]. As most AMPs, cathelicidins are diverse in both length and sequence. They can form  $\alpha$ -helix (LL-37),  $\beta$ -hairpins (pig protegrin 1-5) or sometimes extended helices (porcine PR-39) [80].

#### 1.3.3.1 LL-37

##### Structure

LL-37 is the only cathelicidin identified in humans. It is predominantly produced in neutrophils and cells of epithelial surfaces [85, 86], but also in monocytes and macrophages, lymphocytes, mast cells and NK cells [87, 88]. It is synthesized as pre-pro-peptide, which contains a signal sequence at the N-terminus, followed by a conserved cathelin domain and the sequence of LL-37 at the C-terminal domain. The pre-pro form of LL-37 is encoded by the gene Cathelicidin antimicrobial peptide (*CAMP*), which is located in the third chromatin and consists of four exons. Exon 1-3 encode the signal protein and the cathelin domain, while exon 4 encodes the active form of LL-37 [86] (**Fig.1**). The signal protein is mainly responsible for directing the peptide for exocytosis and is removed afterwards. The remaining peptide is entitled hCAP18. To generate the active form LL-37, hCAP18 is cleaved off by proteinase, such as proteinase 3 in neutrophils [89], and adopts an  $\alpha$ -helical conformation. The mature LL-37 is a cationic peptide that contains an amphipathic  $\alpha$ -helix at the central part of LL-37 (residues 11-32) [90]. It contains 37 amino acids starting with two leucine residues at the N-terminal region, hence the name LL-37. The orthologues of LL-37 is mCRAMP (mouse), rCRAMP (rat) and CAP-18 (rabbit), which are also  $\alpha$ -helical peptides and are good models to mimic the effect of LL-37 *in vivo* [91-94].



**Figure 1** Schematic structure and sequence of LL-37 and *CAMP* gene, modified from [95]

### Induction of hCAP18/LL-37

Expression of hCAP18/LL-37 can be induced by several different compounds. Of the most important are 1,25-dihydroxyvitamin D3 (VD3) and histone deacetylase (HDAC) inhibitors.

The up-regulation of *CAMP* gene and peptide expression by VD3 has been observed in many cells, such as keratinocytes [96], lung/colonic epithelial cells [97], macrophages [96, 98], and neutrophils [99]. The induction of LL-37 expression by VD3 relies on the transcription factor vitamin D receptor (VDR), which could bind to a consensus vitamin D response element (VDRE) in the promoter region of *CAMP* gene. Since VDRE is absent in mouse cathelicidin gene *Cnlp*, mCRAMP expression is not influenced by VD3 [96]. The prominent function of VD3 triggered LL-37 is exemplified by the study from Liu et al. In this study, they demonstrate that TLR activation of human macrophages up-regulate the expression of the VDR and the vitamin D-1-hydroxylase genes, leading to induction of human cathelicidin and killing of intracellular *Mycobacterium tuberculosis* (Mtb) [100].

Another major family that has been anticipated for the induction of cathelicidin expression is HDAC inhibitor family. Several members in this family have been reported as AMP inducers, such as butyrate [101], phenylbutyrate [102] and entinostat [103]. The induction of gene expression by HDAC inhibitors relies on the histone acetylation of the chromatin structure, which facilitates binding of transcription factors at the promoter regions. The signaling pathways involved in this process include mitogen-activated protein kinase (MAPK) and NF- $\kappa$ B pathways [104]. For examples, the induction of LL-37 by butyrate is associated with inhibition of NF- $\kappa$ B pathway, and the recruitment of transcription factors such as activator protein 1 (AP-1), PU.1 [105], vitamin D receptor (VDR) [106] to the promoter of *CAMP* gene. Recently, Kalkani et al. has reported that phenylbutyrate induces cathelicidin expression via VDR [107].

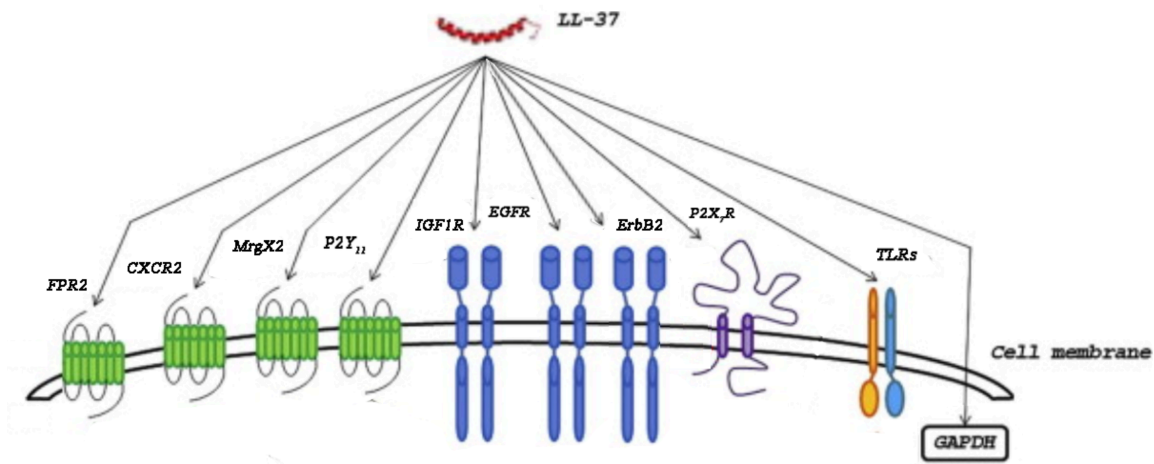


## Receptors

To date, several receptors have been reported to mediate the functional response elicited by LL-37. The best characterized one is formyl peptide receptor 2 (FPR2), which belongs to a class of G protein coupled receptors (GPCRs) and can be triggered by LL-37 to mediate the chemotaxis of neutrophils, monocytes, eosinophils and T-cells by [78, 108, 109]. Apart from this, FPR2 also triggers the production of cysteinyl leukotriene production from eosinophils and mediate a pro-angiogenic effect of LL-37 in endothelial cells [78, 110]. To be noted, LL-37 is not the sole ligand for FPR2, which can also be activated by anti-inflammatory and pro-resolving mediators such as lipoxin A<sub>4</sub> (LXA<sub>4</sub>) and Annexin A1 (AnxA1) to mediate varied biological effects [111]. In addition to FPR2, several other GPCRs have been indicated for LL-37 mediated responses, namely purinergic receptor P2Y<sub>11</sub> (P2Y<sub>11</sub>), CXC chemokine receptor type 2 (CXCR2) and Mas-related gene X2 (MrgX2) [112-114]. Recently, Wu et al have reported a new mechanism for LL-37 mediated effect, by increasing the incorporation of a CXCR<sub>4</sub> into lipid rafts and promoting the CXCR<sub>4</sub> linked downstream effect [115].

Another potent receptor of LL-37 is the purinergic receptor P2X<sub>7</sub>R, originally identified as a receptor for extracellular ATP, mediates inflammatory responses elicited by LL-37, such as the maturation and release of IL-1 $\beta$  and IL-8 [116, 117]. Furthermore, P2X<sub>7</sub>R is involved in the maintenance and re-establishment of the intestinal barrier integrity contributed by LL-37 [118]. Recently, it has also been reported to induce autophagy upon *Mycobacterium tuberculosis* (Mtb) infection [119]. The mechanism behind the activation of P2X<sub>7</sub>R by LL-37 is not fully understood. One hypothesis is that LL-37 and P2X<sub>7</sub>R interact via direct ligand/receptor binding. This theory is supported by the fact that many LL-37 mediated function can be suppressed by P2X<sub>7</sub>R inhibitors [76, 120, 121]. Another report has proposed that LL-37 and P2X<sub>7</sub>R interactions involve the transmembrane segment binding [122], which is supported by the capacity of LL-37 to insert into the host cell membrane [123].

LL-37 also transactivates epidermal growth factor receptor (EGFR), a member of receptor tyrosine kinases (RTKs), to promote IL-8 release from airway epithelial cells, as well as induce keratinocytes migration [77, 124]. Two other members of this family has been linked to LL-37, for its role in breast cancer, *viz.* the Insulin-Like Growth Factor 1 Receptor (IGF1R) and ErbB2 [125, 126]. In addition, LL-37 has been reported to interact with TLRs and GAPDH in phagocytes to modulate immune responses [73, 127-130]. **(Fig.2)**



**Figure 2** LL-37 receptors, modified from [131]

### **Biological activities of LL-37 and its interaction with other immune mediators**

LL-37 exerts both direct antimicrobial activity and immunomodulatory activity. The cationic property and hydrophobic residues of LL-37 allow its binding to the bacteria membrane and form transmembrane pores, further resulting in bacterial lysis [132-134]. LL-37 is effective against gram positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermidis*, Group A *Streptococcus* (GAS) and *Listeria monocytogenes* [72, 134], as well as in gram negative bacteria such as *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Proteus mirabilis* [134] and *Escherichia coli* [135]. To be noted, the bacteria killing effect of LL-37 on *Mycobacterium tuberculosis* (Mtb) has been reported as a major mechanism behind the anti tuberculosis agent VD3 which induces the expression of LL-37 [100, 136]. Furthermore, LL-37 exhibits anti-fungal and anti-viral activities [134, 137]. In vitro studies indicate that the direct antimicrobial activities of LL-37 may be affected by environmental factors such as pH and ionic strength [94].

The immunomodulatory effects of LL-37 can be generally categorized into pro-inflammatory and anti-inflammatory, based on the microenvironment and pathophysiological background [138]. For the pro-inflammatory function, LL-37 works as a chemokine to induce the migration of neutrophils and eosinophils [109], directs M1 macrophage polarization [139], stimulates inflammatory responses such as inflammasome activation [140], and activates endosomal TLR7 and TLR9 as well as type I IFN production [128, 141]. LL-37 is also effective in promoting the release of pro-inflammatory mediators. For instance, LL-37 enhances COX-2 expression and PGE<sub>2</sub> production in human gingival fibroblasts through P2X<sub>7</sub>R [142], as well as 5-LOX expression and LTB<sub>4</sub> production from neutrophils through FPR2 [75, 76]. On the other hand, LL-37 also possesses anti-inflammatory effects. LL-37

has been reported to downregulate TLR4- mediated responses by binding to its ligand LPS [143], as well as interrupting TLR4 complex in macrophages and dendritic cells [73, 143, 144]. However, the effect of LL-37 on TLR4 responses depends on the way of LPS exposure. In LPS primed macrophages, LL-37 increases the release of TNF- $\alpha$ , activates inflammasome, IL-1 $\beta$  and IL-18 production [116, 140, 145]. Furthermore, LL-37 also plays a role in protecting intestinal epithelia against pathogen-mediated intestinal inflammation [118, 146].

#### **1.3.4 Defensins**

Defensins are another family of evolutionary related vertebrate AMPs, with a characteristic  $\beta$ -sheet-rich fold and a framework of disulphide-linked cysteines [147]. They are widely distributed in vertebrates, including humans, and produced by various cell types, such as leukocytes [148-151] and epithelial cells [150, 151]. There are three families of defensins:  $\alpha$ -defensins,  $\beta$ -defensins and  $\theta$ -defensins. [152]. Currently, there are 6  $\alpha$ -defensins and 4  $\beta$ -defensins identified in man.  $\beta$ -defensins (hBD-1–hBD-4) are widely expressed in epithelium and leukocytes, and their expression is constitutive or inducible based on the site of expression. Defensins have been reported as effector molecules in host defense against bacteria, fungi, protozoa and enveloped viruses [147].

### **1.4 EICOSANOIDS**

Metabolism of arachidonic acid (AA) leads to several families of lipid mediators, including prostaglandins, thromboxanes, leukotrienes and lipoxins, along two major pathways, the lipoxygenase (LOX) and the COX pathways. Those mediators, collectively known as eicosanoids, exert potent biological activities in the maintenance of hemostasis, regulation of blood pressure, renal function, reproduction as well as host defense [153]. Meanwhile, they are also the key effector molecules in many acute and chronic inflammatory responses.

#### **1.4.1 Eicosanoid biosynthesis**

##### **1.4.1.1 Prostaglandins (PGs) and thromboxanes (TXs)**

At the endoplasmic reticulum (ER) and nuclear membrane, AA is presented to COX isozymes and converted into the intermediate PGH<sub>2</sub> [154]. COX exists as two isoforms, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues, while COX-2 expression is kept at a low level under normal conditions, but can be greatly enhanced by inflammatory stimuli, such as endotoxins, cytokines, growth factors, and carcinogens [155]. The intermediate PGH<sub>2</sub> is further metabolized into PGE<sub>2</sub>, PGD<sub>2</sub>, PGF<sub>2 $\alpha$</sub> , PGI<sub>2</sub> (prostacyclin),

and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) by various downstream enzymes, whose expressions are regulated in a cell-specific fashion. Those downstream metabolites are collectively known as prostanoids [156]. (Fig. 3)

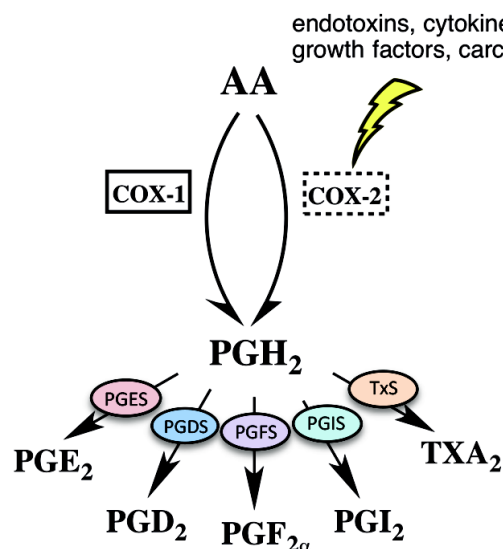
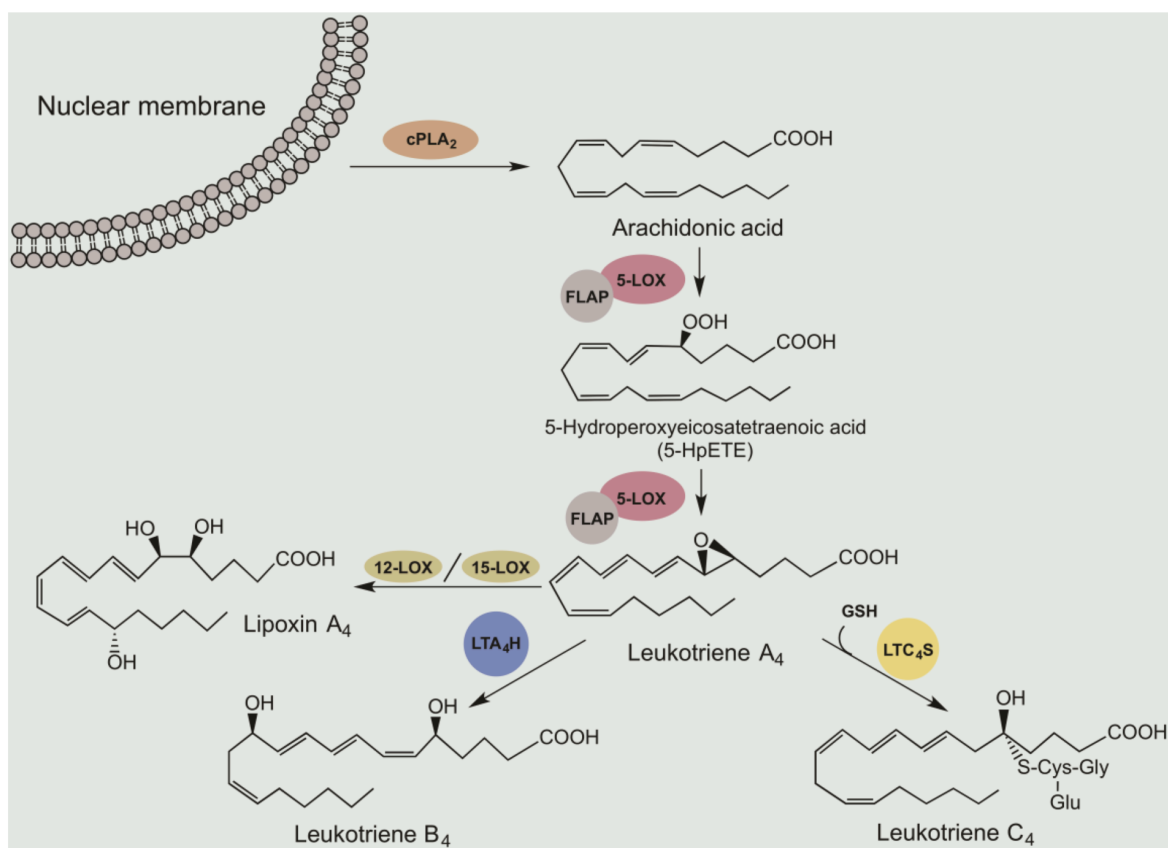


Figure 3 Biosynthesis of prostaglandins and thromboxane A<sub>2</sub>, modified from [157]

#### 1.4.1.2 Leukotrienes

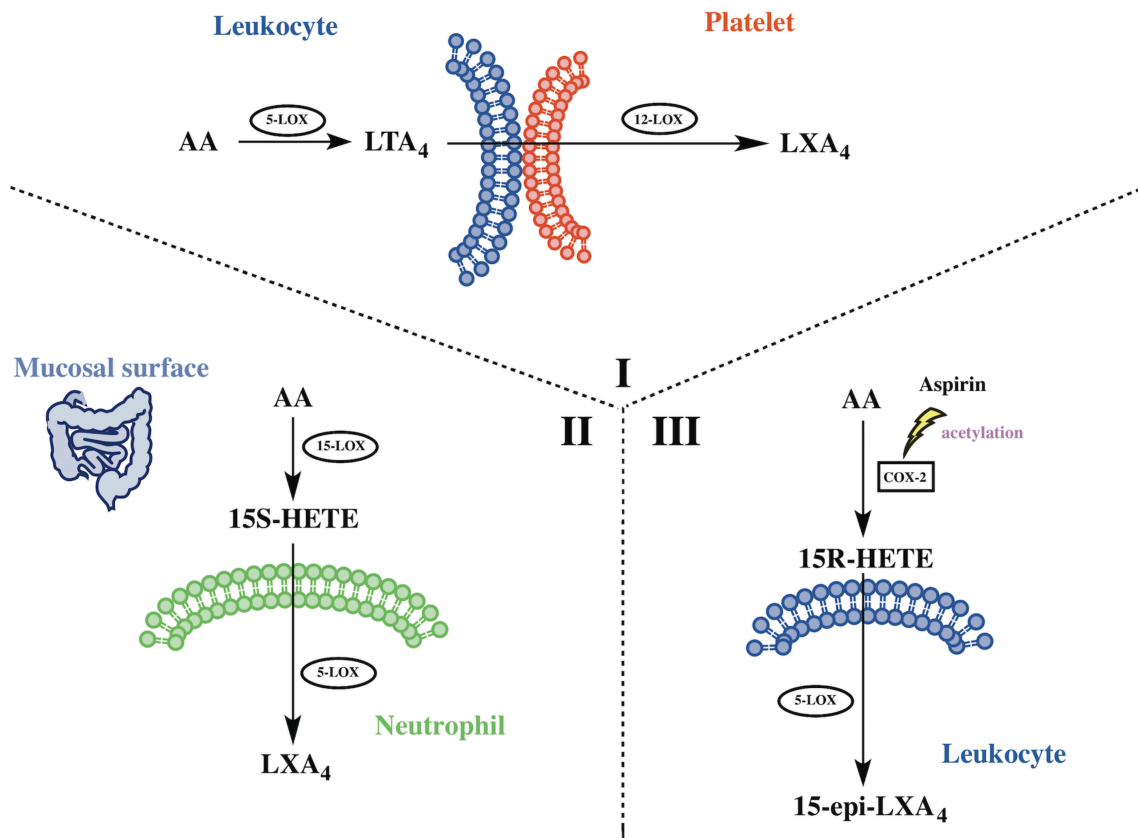
The leukotriene biosynthesis also starts from AA. Unlike prostanoids, leukotrienes are predominantly produced in leukocytes. In response to a variety of inflammatory stimuli leukocytes begin to synthesize leukotrienes. Increased levels of  $i[Ca^{2+}]$  triggers translocation of 5-LOX to the nuclear envelop, where 5-LOX associates with 5-lipoxygenase-activating protein (FLAP), leading to the conversion of AA to the highly unstable intermediate leukotriene (LT) A<sub>4</sub> (LTA<sub>4</sub>). LTA<sub>4</sub> rapidly undergoes further transformations with the assistance of two downstream enzymes. Thus, LTA<sub>4</sub> is metabolized LTB<sub>4</sub>, a potent neutrophil chemoattractant, through the action of LTA<sub>4</sub> hydrolase (LTA<sub>4</sub>H). Alternatively, LTA<sub>4</sub> may also be conjugated with GSH by LTC<sub>4</sub> synthase (LTC<sub>4</sub>S) to generate LTC<sub>4</sub>, which is further metabolized to LTD<sub>4</sub> and LTE<sub>4</sub>. LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> are collectively referred to as cysteinyl leukotrienes (cys-LTs), and they comprise the well-known bioactive principle termed “slow-reacting substance of anaphylaxis” because of its slow and sustained smooth muscle contracting activities [154]. (Fig. 4)



**Figure 4** Biosynthesis of LTB<sub>4</sub> and LTC<sub>4</sub>

### 1.4.1.3 Lipoxins

Lipoxins are a family of pro-resolving lipid mediators discovered by Dr. Serhan and colleagues in 1984 [158]. They are all trihydroxylated derivatives of AA generated via a common epoxide intermediate [159]. The two major metabolites, lipoxin (LX) A<sub>4</sub> (LXA<sub>4</sub>) and LXB<sub>4</sub> are positional and geometrical isomers that possess potent bioactions, although the functions of LXA<sub>4</sub> is by far the most studied and well characterized [160]. In humans, lipoxin biosynthesis, with few exceptions, requires cell-cell interactions. There are mainly three routes for lipoxin biosynthesis. The first route involves peripheral blood leukocyte-platelet interactions. The enzyme 5-LOX in leukocytes metabolizes AA to LTA<sub>4</sub>, which is then released from leukocytes and further transformed by adherent platelets to LXA<sub>4</sub> via 12-lipoxygenase (12-LOX) [161, 162]. The second route is initiated at mucosal surfaces by 15-lipoxygenase (15-LOX) that transforms AA to 15*S*-hydroxy-eicosatetraenoic acid (15*S*-HETE), which is then rapidly taken up by neutrophils and subsequently metabolized to LXA<sub>4</sub> via 5-LOX [163, 164]. In addition, it has also been discovered that aspirin-acetylated COX-2 can transform AA to 15*R*-hydroxy-eicosatetraenoic acid (15*R*-HETE), which can be taken up by leukocytes and converted via 5-LOX to 15-epi-LXA<sub>4</sub>, also called aspirin-triggered lipoxins (ATL) [164]. **(Fig. 5)**



**Figure 5** Transcellular biosynthesis of lipoxins, adopted from [157]

## 1.4.2 Cellular control of eicosanoid biosynthesis-key enzymes

### 1.4.2.1 Cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>)

AA is the precursor of the eicosanoid cascade. The majority of intracellular AA is esterified at the sn-2 position of phospholipids. Phospholipase, typically PLA<sub>2</sub>, hydrolyzes phospholipids and release free fatty acid [153]. There are mainly three types of PLA<sub>2</sub>: secreted PLA<sub>2</sub> (sPLA<sub>2</sub>), calcium-independent PLA<sub>2</sub> (iPLA<sub>2</sub>) and cytosolic PLA<sub>2</sub> (cPLA<sub>2</sub>) [165]. The regulation of AA release from cellular membranes is not fully understood, however, cPLA<sub>2</sub> still remains the key player in eicosanoid production, since cells lacking cPLA<sub>2</sub> are generally devoid of eicosanoid synthesis [154, 166].

cPLA<sub>2</sub> is widely distributed at a relatively constant level in most of human tissues [167]. During eicosanoid biosynthesis, the 85 kDa protein cPLA<sub>2</sub> is activated and translocates to membranes, in response to intracellular Ca<sup>2+</sup> mobilization, and selectively hydrolyze AA esterified in the sn-2 position of phospholipids [153, 168]. The crystal structure of cPLA<sub>2</sub> shows that the enzyme contains two distinct, independent domains: the N-terminal C2 domain and the C-terminal catalytic domain [169]. The active site is in the catalytic domain, which undergoes conformational changes upon membrane binding [169]. On the other hand,

maximal cPLA<sub>2</sub> activation requires not only intracellular calcium mobilization, but also sustained phosphorylation of the protein [170]. The currently known phosphorylation sites in cPLA<sub>2</sub> are located in the catalytic domain including Ser505, Ser515 and Ser727, phosphorylated by MAPK [170, 171], calcium-calmodulin kinase II [172, 173], and MAPK-regulated kinase MNK-1 [173], respectively.

#### **1.4.2.2 COX-1 and COX-2**

COX-1 and COX-2, also called prostaglandin endoperoxide H synthases-1 and -2, oxygenates AA to PGH<sub>2</sub>, which is the key step in prostaglandin biosynthesis [155]. Crystal structures of both COX-1 and COX-2 have been determined and show a high degree of similarity [174]. However, COX-2 harbors a side pocket in the active site that allows efficient binding of COX-2 selective inhibitors [175]. Previously COXs were considered to be homodimers both structurally and functionally. However, a recent study shows that COX-1/COX-2 heterodimers may also exist, whereas their roles in biology need to be elucidated [176]. In addition, there is significant cross-talk between monomers of homodimeric COX-1 and COX-2 such that one subunit becomes allosteric and the other catalytic upon binding of substrate and/or inhibitors [177, 178]. Structurally, each monomer consists of three domains: an epidermal growth factor (EGF) domain of 50 amino acids at the N terminal, a neighboring membrane binding domain (MBD) of about 50 amino acids, and a large C-terminal globular catalytic domain with about 460 amino acids [174].

COX-1 is expressed constitutively in most tissues and cells. Under physiological conditions, COX-1 is the dominant enzyme responsible for prostanoid production, whereas COX-2, induced by inflammatory stimuli, hormones and growth factors, is a more important source for prostanoid biosynthesis in inflammation and in proliferative diseases, such as cancer [179, 180]. COX-1 and COX-2 are targets of nonsteroidal anti-inflammatory drugs (NSAIDs). These drugs are competitive inhibitors for the active sites in both of the COX isozymes, leading to the inactivation of one monomer of the COX dimer and shutdown of prostanoid production [181].

#### **1.4.2.3 5-LOX**

The oxygenation of AA to generate LTA<sub>4</sub> is comprised of two chemical steps catalyzed by 5-LOX [182]. The first is a classical lipoxygenation at C-5 of AA generating 5(S)-hydroperoxy-6-*trans*-8,11,14-*cis* eicosatetraenoic acid (5-HpETE), followed by the second step, formation of LTA<sub>4</sub> by dehydration of 5-HpETE [153, 183]. 5-LOX is one of six

lipoxygenases in mammals and is expressed in various leukocytes, such as neutrophils, eosinophils, monocytes/macrophages, mast cells, B-lymphocytes and dendritic cells [184].

5-LOX is a soluble monomeric enzyme composed of 673 amino acids with a molecular mass of about 78 kDa [185]. The polypeptide chain is divided into two domains, an N-terminal regulatory domain and a catalytic C-terminal domain that harbors one atom of nonheme iron, involved in catalysis [186]. The crystal structure of human 5-LOX was presented in 2011 by Gilbert et al. and revealed, similar to other LOX enzymes an N-terminal  $\beta$ -sandwich (residues 1–120) and an iron-containing C-terminal catalytic domain (residues 121–673). The N-terminal domain of 5-LOX is composed of two 4-stranded antiparallel  $\beta$ -sheets and has been shown to bind several regulatory factors such as  $\text{Ca}^{2+}$ , phosphatidylcholine, coactosin-like protein and dicer, which suggests that this domain facilitates the association of 5-LOX with membranes during translocation and catalysis (153, 154).

In resting cells, 5-LOX resides either in the cytosol or in a nuclear soluble compartment [184]. Both nuclear import- and export- sequences of 5-LOX have been identified [187, 188]. Once triggered by cell stimulation, such as priming by glycogen and cytokines, 5-LOX translocates to the nuclear membrane. Upon translocation, 5-LOX works in concert with FLAP, leading to the enzymatic metabolism of AA to  $\text{LTA}_4$ . Intracellular mobilization and phosphorylation of 5-LOX are two major processes for the regulation of 5-LOX activities [189].

5-LOX can be phosphorylated *in vitro* on three residues: Ser-271, by mitogen-activated protein kinase-activated protein (MAPKAP) kinase 2, Ser-663 by ERK2, and Ser-523 by PKA catalytic subunit [184, 190-192]. The phosphorylation of 5-LOX by MAPKAP kinase 2 and ERK2 trigger 5-LOX activity and translocation of the enzyme to the nuclear membrane, leading to induction of leukotriene biosynthesis. This can be confirmed by *in vitro* studies of stress-stimulated leukocytes and phorbol myristate acetate (PMA)-primed Mono Mac 6 (MM6) cells [191, 193]. To be noted, recent findings suggest that 5-LOX trafficking may be different in neutrophils between genders, which leads to different leukotriene production [194].

#### **1.4.2.4 Other enzymes involved in eicosanoid biosynthesis**

Besides the enzymes mentioned above, there are several other enzymes that also play important roles in eicosanoid biosynthesis.  $\text{LTA}_4\text{H}$ /aminopeptidase is a cytosolic, 70 kDa, bifunctional zinc enzyme. On the one hand,  $\text{LTA}_4\text{H}$  converts  $\text{LTA}_4$  into the proinflammatory



LTB<sub>4</sub>, on the other it cleaves and inactivates the chemotactic tripeptide Pro-Gly-Pro, thus playing roles in both the initiation and resolution phases of inflammation [195]. LTC<sub>4</sub>S, which conjugates LTA<sub>4</sub> with GSH to form LTC<sub>4</sub>, belongs to a family of integral membrane proteins with the acronym MAPEG (membrane associated protein in eicosanoid and glutathione metabolism) [196, 197]. FLAP is another member of the same family and assists 5-LOX during the conversion of AA to LTA<sub>4</sub>.

In prostanoid biosynthesis, various bioactive prostaglandins and thromboxane are generated through the actions of upstream COXs as well as downstream terminal enzymes such as prostaglandin D synthases (PGDSs), prostaglandin E synthase, which is a third member of the MAPEG family, prostaglandin F synthases (PGFSs), prostacyclin synthase (PGIS) and thromboxane A synthase (TXAS). Each terminal enzyme is responsible for the production of a specific prostanoid, in a cell-specific manner.

### **1.4.3 Eicosanoid receptors**

#### **1.4.3.1 Prostanoid receptors**

Once synthesized, cellular prostanoids are released through a specific prostaglandin transporter (PGT), and potentially by other uncharacterized transporters [198]. The released prostanoids binds to a series of GPCRs, such as EP1-EP4 that bind PGE<sub>2</sub>, DP1 and DP2 that bind PGD<sub>2</sub>, and receptors that bind PGF<sub>2α</sub>, PGI<sub>2</sub>, and TXA<sub>2</sub> (FP, IP, and TP, respectively) [154, 180]. Prostanoid receptors couple with various intracellular signaling pathways and second messengers that mediate the prostanoid-triggered bioactivities. For instance, the transduction of PGE<sub>2</sub> signaling between cells activates different signaling pathways through EP1 to EP4. EP1 activates phosphatidylinositol metabolism via G<sub>q</sub>, leading to the formation of inositol triphosphate with mobilization of intracellular free calcium [180]. EP2 and EP4 activate adenylyl cyclase via G<sub>s</sub>, increasing intracellular cyclic adenosine monophosphate (cAMP) [180]. EP3 relates to elevation of intracellular Ca<sup>2+</sup>, inhibition of cAMP generation, and activation of the small G protein Rho, via G<sub>i</sub> or G<sub>13</sub> [199].

#### **1.4.3.2 Leukotriene receptors**

LTB<sub>4</sub> mediates cellular signaling mainly via two GPCRs (BLT1 and BLT2). BLT1 is a 43 kDa GPCR with high specificity and affinity for LTB<sub>4</sub> (K<sub>d</sub>: 0.15-1 nM) [200]. This receptor is expressed on neutrophils but also on a variety of other inflammatory cells, including lymphocytes and mast cells [201-205]. In resting endothelial cells, BLT1 is expressed at a low level, but can be induced by LPS and LTB<sub>4</sub> [206]. Another GPCR for LTB<sub>4</sub> is BLT2, the

homologue of BLT1 with a higher  $K_d$  value (23 nM) for  $LTB_4$  and a broader range of ligands, including 12-HETE and 15-HETE [153, 207]. Unlike BLT1, BLT2 is ubiquitously expressed in various tissues. It has been reported that 12(S)-hydroxy-5-*cis*-8, 10-*trans*-heptadecatrienoic acid (12-HHT), a side-product during thromboxane synthesis from  $PGH_2$ , is an endogenous high-affinity ligand for BLT2 [208]. Although its physiological role has not been fully elucidated yet, recent evidence indicate that it may exert anti-inflammatory and wound healing functions [209, 210].

Cys-LTs exert their biological functions mainly through two GPCRs, CysLT1 and CysLT2. Though a third receptor was found in 2008, as a high affinity receptor for  $LTE_4$ , this receptor is predominantly expressed in brain and sense purynergic ligands [211]. CysLT1 contains 336 amino acid residues and is expressed in various tissues and cells, such as smooth muscle cells and macrophages, peripheral blood, spleen and lung [212-215]. CysLT1 receptor binds  $LTD_4$  with a high affinity, followed by  $LTC_4$  then  $LTE_4$  in decreasing order of potency [153]. The classical bioactivity elicited by cys-LTs, such as smooth muscle contraction, increased vascular permeability, and plasma leakage, is mediated through CysLT1 signaling, which is the target for antiasthma drugs such as montelukast. The CysLT2 receptor contains 345 amino acids. It binds to  $LTC_4$  and  $LTD_4$ , but has a lower affinity with  $LTE_4$ . This receptor has a wide distribution such as heart, brain, endothelial cells, lymphocytes, and has been shown to play a role in regulation of vascular permeability and neuronal signaling in the gut [216, 217].

#### **1.4.4 Eicosanoids in host defense**

Studies in mammals have demonstrated that eicosanoids affect the immune response by modulating cellular differentiation, migration, phagocytosis, and cytokine/chemokine production. It has been reported that zymosan and *Candida albicans* induce cPLA<sub>2</sub> activation and eicosanoid production in macrophages via different signaling mechanisms [218-220]. Moreover, a recent report investigated the functional consequences of cPLA<sub>2 $\alpha$</sub>  activation and the effect of endogenously produced eicosanoids on gene expression in response to *C. albicans* by comparing cPLA<sub>2 $\alpha$</sub> <sup>+/+</sup> and cPLA<sub>2 $\alpha$</sub> <sup>-/-</sup> resident mouse peritoneal macrophages (RPMs). The results suggest that killing of *C. albicans* was impaired in cPLA<sub>2 $\alpha$</sub>  deficient RPMs. Meanwhile, *C. albicans*-stimulated cPLA<sub>2 $\alpha$</sub>  activation and the early production of prostanoids promote an autocrine pathway in RPMs that affects the expression of genes involved in host defense to dampen inflammation [221]. In addition, it has also been shown that AA stimulates human neutrophils to release AMPs to strongly impair bacterial growth

[222]. To be noted, eicosanoids could be involved in lactose and phenylbutyrate (PBA)-induced human cathelicidin expression in human epithelial cell line HT-29 since a PLA<sub>2</sub> inhibitor significantly suppressed lactose/PBA-induced peptide expression [223].

#### **1.4.5 PGE<sub>2</sub>, a multifunctional lipid mediator**

Within the eicosanoid family, PGE<sub>2</sub> represents the best characterized and one of the most potent prostanoids in the inflammatory milieu. It is produced by most tissues and cells in the human body and acts as an autocrine and paracrine lipid mediator through its four GPCRs, EP1-EP4. [154].

PGE<sub>2</sub> has long been recognized as a multi-functional inflammatory mediator, participating in the regulation of blood flow and renal filtration, hematopoiesis, neuronal signaling, vascular permeability and smooth muscle function [224-227]. PGE<sub>2</sub> has a paradoxical role in regulating inflammatory responses. It can promote the inflammatory processes by enhancing local vasodilatation and stimulating immune cell chemotaxis and activation [228-231]. However, PGE<sub>2</sub> also dampens innate immunity and antigen-specific immunity by suppressing Type I immunity, promoting the recruitment of immune-suppressive cells and modulating cytokine release [232-234].

##### **1.4.5.1 PGE<sub>2</sub> biosynthesis and degradation**

PGE<sub>2</sub> is produced de novo from membrane-released arachidonic acid and its production can be triggered by growth factors, cytokines, mechanical trauma and other stimuli [154]. The metabolism of AA by COX isozymes leads to the generation of unstable endoperoxide PGH<sub>2</sub>, which can be further metabolized by the inducible microsomal prostaglandin E synthase-1 (mPGES-1) [235]. Two other PGES have been reported but found to contribute very little, if at all, to the biosynthesis of PGE<sub>2</sub> [236]. On the other hand, PGE<sub>2</sub> is chemically relatively stable but has a rapid turnover rate *in vivo* [237, 238]. The degradation of PGE<sub>2</sub> is controlled by the degrading enzyme 15-hydroxy-prostaglandin dehydrogenase (15-PGDH). 15-PGDH catalyzes the first step in the degradation of PGE<sub>2</sub>, oxidizing the prostanoid 15-hydroxyl group to a ketone, and thereby abrogating its binding to PGE<sub>2</sub> receptors [239, 240]

##### **1.4.5.2 PGE<sub>2</sub> receptors and downstream signaling pathways**

The diverse effects of PGE<sub>2</sub> may be partially accounted for by the varied expression of their receptors (EP1-EP4), and heterogeneity in the coupling of these receptors to the intracellular signal transduction pathways [241]. Of the four receptors, EP3 and EP4 represent high-

affinity PGE<sub>2</sub> receptors ( $K_d < 1$  nM), whereas EP1 and EP2 binds to PGE<sub>2</sub> with lower affinity ( $K_d < 10$  nM) [242].

Signaling through EP2 and EP4 has been implicated in the anti-inflammatory and immune suppressive activity of PGE<sub>2</sub>, through cAMP/PKA/CREB pathway [234, 243-245]. The signaling of EP4 and EP2 is triggered by different concentrations of PGE<sub>2</sub> and differs in duration of activation. Apparently due to a longer C-terminal tail of the EP4 receptor compared with the EP2 receptor, EP4 signaling is rapidly desensitized when induced by PGE<sub>2</sub>, whereas EP2 is resistant to agonist-induced desensitization, indicating a longer period of PGE<sub>2</sub> triggered EP2 signaling [234]. In addition to their nominal activation of the cAMP pathway, EP2 and EP4 have also been suggested to activate other intracellular signaling pathways. Stimulation of EP4 (but not EP2) by PGE<sub>2</sub> leads to activation of a PI3K-dependent ERK1/2 pathway [246], and the PI3K signaling is also involved in EP4 mediated Glycogen synthase kinase 3 (GSK3)/ $\beta$ -catenin signaling [247]. EP2 has also been reported to activate the GSK3/ $\beta$ -catenin signaling pathway, however, in a PKA-dependent, PI3K-independent manner [247]. Furthermore, EP2 has also been implicated in transactivation of EGF receptor, leading to increased migration and invasion of colon cancer cells [248, 249].

The low-affinity receptor EP1 has been reported to couple to mobilization of intracellular calcium [250, 251], and the high-affinity receptor EP3, which has at least eight splice variants in humans, has been reported to couple with G<sub>i</sub>, leading to the inhibition of intracellular cAMP [252, 253].

### **1.4.5.3 The effect of PGE<sub>2</sub> on macrophage biology**

#### **Regulation of phagocytosis and pathogen clearance by PGE<sub>2</sub>**

Macrophages are potent immune effector cells playing key roles in phagocytosis and pathogen clearance. Acting in a EP2 dependent manner, PGE<sub>2</sub> has been reported to inhibit alveolar macrophage phagocytosis through the induction of the cAMP signaling pathway [254]. Further study on this issue reveals that PGE<sub>2</sub> suppresses phagocytosis via Epac but not PKA, both of which are downstream signaling molecules in the cAMP pathway [255], and PI3K signaling plays an important role in Fc $\gamma$ R phagocytosis which is mediated by PTEN [256]. Moreover, Hubbard et al. have reported that PGE<sub>2</sub> could suppress macrophage phagocytic ability through IL-1R-associated kinase (IRAK)-M, a known inhibitor of MyD88-dependent IL-1R/TLR signaling [257]. Further work shows that phagocytosis can be restored by the inhibition of COX[258]. Together, these evidences demonstrate that PGE<sub>2</sub> is a suppressor of macrophage phagocytosis.

Besides the influence on bacterial phagocytosis, PGE<sub>2</sub> has also been shown to suppress macrophage bactericidal ability and the inflammatory process by regulating NADPH oxidase and release of ROS [259, 260]. Moreover, PGE<sub>2</sub> suppresses the activation of macrophages by inhibiting the production of nitric oxide radicals [261, 262].

### **PGE<sub>2</sub> and macrophage polarization, cytokines and inflammatory mediator release**

PGE<sub>2</sub> alters macrophage cytokine responses and promotes an immunosuppressive phenotype. Though at early stages of inflammation, PGE<sub>2</sub> is believed to enhance monocyte recruitment [228], while the activation of macrophages are inhibited by PGE<sub>2</sub> via EP2/PKA signaling [263]. Furthermore, PGE<sub>2</sub> has been suggested to inhibit TNF- $\alpha$  expression both in murine and human macrophages [257, 264]. Also, it has been shown that PGE<sub>2</sub> regulates leukotriene production in alveolar macrophages [257, 265]. In the tumor microenvironment, PGE<sub>2</sub> has been implicated in regulating IL-10 and IL-12 production [266], leading to an immunosuppressive phenotype of macrophages [267]. This was further demonstrated by the rescue of IL-10 and IL-12 balance and restoration of antitumor activity after specific inhibition of COX-2 [266].

### **PGE<sub>2</sub> and monocytic myeloid-derived suppressor cells**

Myeloid-derived suppressor cells (MDSC) represents a heterogeneous population of immature myeloid cells including immature precursors of macrophages, neutrophils and dendritic cells, capable of suppressing immune responses [268, 269]. MDSCs express CD34, common myeloid marker CD33, macrophage/DCs marker CD11b, and IL4R $\alpha$  (CD124), but lack expression of the lineage markers of DCs and other mature myeloid cells [270, 271]. Human MDSCs are defined as CD33<sup>+</sup>Lin<sup>-</sup>HLA<sup>-</sup>DR<sup>/low</sup> cells, and can be subdivided into CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>high</sup> monocytic and CD11b<sup>+</sup>Ly6G<sup>+</sup>Ly6C<sup>low</sup> granulocytic MDSCs. It has been reported that in murine colon adenocarcinoma-38 and GL261 murine glioma, > 90% tumor-infiltrating CD11b<sup>+</sup> cells were of the CD11b<sup>+</sup>F4/80<sup>+</sup> monocytic MDSCs, which bear both M1 and M2 phenotypes [272].

PGE<sub>2</sub> has been reported to play a central role in the development and accumulation of MDSCs through a CXCL12-CXCR4 pathway in ovarian cancer [269]. *In vitro*, PGE<sub>2</sub> has also been suggested as an inducer of monocytic MDSC generation from peripheral blood monocytes. Further *in vitro* studies on MDSCs indicate that the PGE<sub>2</sub> triggered EP2/EP4 - cAMP/PKA/CREB signaling pathway is involved in the generation of MDSCs [273]. On the other hand, COX-2, one of the inducible enzymes in PGE<sub>2</sub> biosynthesis, has also been associated with development of MDSCs in several tumor models. *In vitro* coculture of human

melanoma cells with monocytes triggers CD14<sup>+</sup> monocytes to acquire a MDSC-like phenotype and immunosuppressive properties, via COX-2/PGE<sub>2</sub> and STAT-3 signaling [274]. The association of COX-2 and MDSCs has also been investigated in nasopharyngeal carcinoma, where COX-2 promotes MDSCs expansion and further leads to cancer metastasis [275].

To be noticed, MDSC expresses high level of COX-2 and is one of the major sources for PGE<sub>2</sub> production in the tumor microenvironment, which forms a positive feedback loop to further sustain a suppressive phenotype of MDSCs. In this loop, COX-2 plays an essential role for the functional stability of MDSCs [276].

## **1.5 INTERACTIONS BETWEEN AMPs AND EICOSANOIDS IN INNATE IMMUNITY**

As two large families of immune effector molecules, AMPs and eicosanoids have been demonstrated to interact with each other to regulate the immune response. A previous study in our group shows that LL-37 promotes LTB<sub>4</sub> production by human neutrophils [277]. It has also been reported that hBDs including hBD-2,-3 and -4 induce PGD<sub>2</sub> production in mast cells [278-280]. In turn, eicosanoids could work as an external regulator of AMPs and further influence host defense against microbes. For instance, PGD<sub>2</sub> induces hBD-2 and hBD-3 production in human keratinocytes [281, 282]. Studies on human neutrophils also show that LTB<sub>4</sub> induces the release of human AMPs, including  $\alpha$ -defensins, cathepsin G, elastase, lysozyme C, and LL-37 via the BLT1 receptor [277, 283]. Based on the finding of reciprocal regulation between some specific AMPs and eicosanoids, a regulatory feedback loop between LTB<sub>4</sub> and LL-37 has been proposed to operate at an inflammatory locus, which may further expand the existing inflammatory responses [75, 277]. Given the fact that macrophages are tissue resident immune cells which act at the frontline against infection, and represent major target cells in various acute and chronic inflammatory states, studies of the mechanisms and roles of AMPs and eicosanoid interactions might provide us with new strategies for pharmacological interventions in host defense and inflammation.

## 2 AIMS

My PhD work started from the observation that LL-37 promotes the capacity of differentiated monocytic cell line THP-1 (dTHP-1) to phagocytize bacteria from where we began to explore the role of LL-37 on macrophage functional responses relevant to innate immunity. The specific objectives of my PhD studies were:

- To elucidate how LL-37 affects macrophage bactericidal activities by influencing phagocytosis (Paper I) .
- To characterize LL-37 internalization by human macrophages and investigate its functions (Paper II).
- To demonstrate the effects and regulatory mechanisms of LL-37 induced eicosanoid production in human macrophages (Paper III).
- To determine the effect and mechanisms of action of PGE<sub>2</sub> on AMP expression in human macrophages and its potential role in Mtb infection (Paper IV).





### 3 METHODOLOGY

The methods employed in this thesis are listed below. For detailed protocols of the methods, please see papers as indicated.

Isolation of neutrophils and monocytes	Paper I, II, III, IV
Culturing of MM6 and THP-1 cells	Paper I, II, III, IV
Phagocytosis assay	Paper I
Intracellular killing assay	Paper I, II
Immunocytochemistry	Paper I, II, III, IV
Flow cytometric analysis	Paper I, II
SDS-PAGE and Western blot analysis	Paper I, II, III, IV
Real-time PCR	Paper III, IV
ELISA	Paper III, IV
Intracellular calcium mobilization	Paper III
Lentivirus based shRNA transfection	Paper II, IV
ROS detection assay	Paper II
<i>In vivo</i> subcutaneous air pouch model	Paper I
<i>In vivo</i> peritonitis model	Paper III



## 4 RESULTS AND DISCUSSION

Based on the objectives of the current study, the major observations can be divided into three parts: (i) Paper I and Paper II demonstrate beneficial effects of LL-37 on macrophages' ability to kill bacteria, by promoting phagocytosis of bacteria and internalization of LL-37 into intracellular compartments, leading to induction of ROS production and lysosome formation. (ii) In Paper III, the role of LL-37 in regulation of macrophage eicosanoid production is described. (iii) In Paper IV, we investigate the induction of LL-37 expression by a potent inflammatory mediator, *viz.* PGE<sub>2</sub>, under in vitro conditions with or without Mtb-infection. Paper III and paper IV collectively provide evidence for the mutual regulation and interactions of the eicosanoid and AMP families, in host defense and inflammation.

### 4.1 LL-37 enhances bacteria killing activity of human macrophages (Paper I&II)

In an attempt to determine the effect of LL-37 on the ability of human macrophages to phagocytize bacteria, the synthetic LL-37 was added and incubated together with human monocyte derived macrophages (HMDMs) or dTHP-1 cells. The results from bacteria killing assay showed that macrophages treated with LL-37 exhibited increased ability to kill both Gram-positive and Gram-negative bacteria.

Increasing evidence indicate that LL-37 plays an important role in modulating immune responses in addition to directly binding and killing bacteria. The importance of LL-37 in immunomodulation is supported by the observation that synthetic LL-37 variant with an ablated bacteria killing activity plays beneficial roles in host defense against *Staphylococcus aureus* and *Salmonella typhimurium* infection [284]. Expanding evidence has also shown that LL-37 exhibits a weak direct antimicrobial activity under physiologically relevant conditions [285, 286]. Given the role of LL-37 in host defense [87, 287, 288], understanding the mechanisms by which LL-37 can contribute to innate immune responses is of great importance.

As an endogenous antimicrobial agent, LL-37 has been found in various epithelial and mucosal surfaces, as well as in most of the body fluids in man. It has been reported that under physiological conditions, a concentration of 2-5 µg/ml has been observed for LL-37 in mucosal surfaces and body fluids [289]. Another report suggests that LL-37 concentrations vary among different body fluids in healthy donors, *i.e.* 0.7-27 µg/ml in saliva and 0.9-2.3 µg/ml in plasma [290]. In our studies, we have shown that LL-37 promotes bacterial killing by human macrophages under conditions relevant to normal physiological states and to certain infections.

#### 4.1.1 LL-37 promotes bacterial phagocytosis by human macrophages (Paper I)

Efficient phagocytosis of bacteria is an important step in the clearance of invading bacteria and host defense. It has previously been reported by our group that LL-37 elevates bacterial

phagocytosis by human neutrophils [277]. Moreover, neutrophil-derived heparin-binding protein (HBP) and human neutrophil peptide 1–3 (HNP-1–3) have been suggested to boost bacterial phagocytosis by macrophages [291]. In this project, we aimed to investigate the effect of LL-37 on bacterial phagocytosis by macrophages and to reveal the mechanisms that mediate this action.

### **LL-37 increases the phagocytic capacity of Gram-negative bacteria in human macrophages, associated with an up-regulation of TLR4 and CD14 expressions**

To study the effects of LL-37 on phagocytosis of bacteria in human macrophages, LL-37 was incubated together with dTHP-1 cells, followed by washing steps and exposure of macrophages to bacteria. Our results showed that LL-37 selectively enhanced phagocytosis of nonopsonized *E. coli* but not nonopsonized *S. aureus*.

TLR signaling pathways are involved in recognition and phagocytosis of bacteria [292-294]. Among many TLRs, TLR4, together with CD14, are associated with cellular recognition of LPS, whereas TLR2 is associated with recognition of Gram positive bacteria [14, 15]. Our results showed that expression of TLR4 and CD14 was up-regulated in LL-37 treated dTHP-1 cells, which was in line with the finding of the increased phagocytosis induced by LL-37 in *E.coli* but not *S.aureus*.

### **LL-37 up-regulates the FcγRs CD64 and CD32, leading to an enhanced phagocytic capacity of IgG-opsonized bacteria in human macrophages**

In innate immunity, efficient uptake and phagocytosis of bacteria by macrophages are achieved by opsonization of pathogens with antibody (Ig) or complement proteins. Particles opsonized with IgG are recognized by FcγRs [295, 296]. In our study, we analyzed the expression of three major FcγRs (CD16, CD32, and CD64) on the surface of macrophages, before and after LL-37 treatments. Our results showed that dTHP-1 cells expressed high levels of CD32 and CD64, whereas CD16 expression was at a very low level. Our data showed that LL-37 elevated CD32 and CD64 expression, in a non-synchronized manner. This result was in line with the finding that LL-37 enhanced phagocytosis of IgG-opsonized *E. coli* and *S. aureus* in dTHP-1 cells. In concordance, CD64 antibody blocked LL-37 induced bacterial phagocytosis, which further confirmed the involvement of FcγR in LL-37 promoted bacteria phagocytosis in human macrophages.

### **FPR2/ALX is involved in LL-37 enhanced bacteria phagocytosis in human macrophages**

LL-37 exerts its immunomodulatory effect via several receptors, such as FPR2/ALX [108], P2X<sub>7</sub>R [116], and EGFR [77]. We found that pretreatment of dTHP-1 cells with the FPR2/ALX antagonist peptide WRW4 totally abolishes LL-37-enhanced bacterial phagocytosis of dTHP-1 cells, while the GPCR inhibitor pertussis toxin showed a similar effect. Furthermore, WRW4 also blocked LL-37-induced CD32 expression on dTHP-1 cells. In contrast, inhibitors of P2X<sub>7</sub>R or EGFR had no effect on LL-37-promoted bacterial

phagocytosis. Enhanced bacterial phagocytosis was also detected after dTHP-1 cells were incubated with the specific FPR2/ALX agonist WKYMVm peptide. Together, these results demonstrate the involvement of FPR2/ALX in LL-37 enhanced bacterial phagocytosis in human macrophages.

#### **4.1.1.1 Macrophages from *Cnlp*<sup>-/-</sup> mice exhibit suppressed bacterial phagocytosis**

To obtain evidence for the involvement of LL-37 in regulating bacterial phagocytosis *in vivo*, we conducted experiments on *Cnlp*<sup>-/-</sup> mice, transgenic mice deficient in murine cathelicidin (mCRAMP). We used the dorsal subcutaneous (s.c.) air pouch model with injection of TNF- $\alpha$  on both WT and *Cnlp*<sup>-/-</sup> mice. After 24 h, leukocytes in pouch lavage were collected and the macrophage population was obtained by cell adherence. In our hands, the relative amounts of different subtypes of leukocytes were similar in TNF- $\alpha$ -induced pouch lavage from WT and *Cnlp*<sup>-/-</sup> mice ( $\approx$ 15% neutrophils,  $\approx$  10% monocytes, and  $\approx$  60% macrophages). Our results revealed that phagocytosis of IgG-opsonized *S. aureus* was significantly suppressed in macrophages from *Cnlp*<sup>-/-</sup> mice compared with WT mice (Fig. 6A). Moreover, the expression of CD14 and Fc $\gamma$ Rs on adhered leukocytes from *Cnlp*<sup>-/-</sup> mice was significantly lower than that from WT mice. These results agreed with the *in vitro* findings regarding the effect of LL-37 on bacterial phagocytosis in macrophages.

#### **4.1.2 Internalization of LL-37 by human macrophages promotes intracellular bacterial clearance (Paper II)**

To be noted, we also identified in Paper I that LL-37-treated human macrophages exhibited an enhanced intracellular killing of *S. aureus*, although LL-37 had no effect on phagocytosis of this bacterial species. Therefore, we continued to investigate the mechanisms mediating LL-37 promoted intracellular bacterial killing activity in human macrophages.

In this project, we observed that human macrophages take up extracellular LL-37. Moreover, the internalized LL-37 co-localized with the intracellular bacteria in endosomes and lysosomes of human macrophage. These phenomena led to our hypothesis that LL-37 internalization could contribute to the ability of human macrophages to kill bacteria.

#### **LL-37 internalization by human macrophages**

Macrophages are residential sentinel cells for invading pathogens. During infection, neutrophils are recruited to the infectious site where they encounter the invading pathogens, and immediately release their preformed granules containing antimicrobial agents, including hCAP18/LL-37. Thus, the residential macrophages may be surrounded by LL-37 in a high level at infectious or inflammatory sites. By culturing human macrophages with neutrophil conditioned medium that contained released antimicrobial agents from neutrophils, we were able to show that LL-37 released by human neutrophils was internalized by human macrophages. When human macrophages were incubated with FAM-labeled LL-37, the fluorescence intensity of the cells increased in a manner dependent on LL-37 dose and

incubation time. However, the fluorescence intensity of the macrophages did not change significantly if they were treated with the endocytosis inhibitor cytochalasin B before incubation with FAM-labeled LL-37, which suggested that internalization of LL-37 by human macrophages is an endocytic process. In addition, we treated the macrophages with sequence scrambled LL-37 (sLL-37). Unlike LL-37, sLL-37 was not internalized by human macrophages, which indicates that this process might be mediated by specific receptor(s).

### **Clathrin- and caveolae/lipid raft–dependent endocytosis pathways are involved in LL-37 internalization by human macrophages**

Endocytosis is characterized by internalization of molecules from extracellular space into intracellular compartments. Based on the participation of different surface molecules and intracellular compartments, two major types of endocytosis have been well defined: the classical, clathrin-mediated endocytosis (CME) pathway and the nonclassical, clathrin-independent, but lipid raft-dependent pathway.

Both the inhibitors of CME and caveolae/lipid raft endocytosis pathway suppressed LL-37 internalization in HMDMs and dTHP-1 cells, which suggested that both clathrin-dependent and caveolae/lipid raft–dependent pathways are involved in LL-37 endocytosis by human macrophages.

CME represents the classical strategy of particle internalization mediated through clathrin-coated vesicles. This pathway encompasses the internalization of nutrients, antigens, growth factors, and receptors [297]. The particles internalized via clathrin-coated vesicles are engaged into the endosome-lysosome system, which could either end up with degradation in lysosome or sorted for recycling back to the plasma membrane (or the Golgi) via recycling endosomes. However, emerging evidence shows that clathrin independent pathways also exist. One form of clathrin-independent endocytosis relies on cholesterol-rich membrane domains, such as lipid rafts and caveolae. This type of endocytosis exists in the multiple endocytic processes, such as virus and bacteria entry into host cells and internalization of sphingolipids, endothelin and growth hormones [298]. The scaffolding protein caveolin-1 has been reported as a key component in the formation of caveolae, since the lack of caveolin-1 in null mice leads to the absence of caveolae [299, 300].

Our results showed that internalized LL-37 localized in the CME associated intracellular compartments, namely endosomes, lysosomes and the Golgi apparatus. Moreover, internalized LL-37 partially co-localized with markers of lipid rafts, caveolae and clathrin. Those evidence further demonstrate the involvement of both CME and lipid raft/caveolae dependent endocytosis pathways.

### **P2X<sub>7</sub>R is associated with clathrin-dependent endocytosis of LL-37 by human macrophages**

The exclusive internalization of LL-37 but not sLL-37 by human macrophages suggested that this process is mediated by specific receptor(s). To this end, pharmacological tools and gene

depletion were utilized to investigate the receptor involvement. Our results showed that inhibitors of P2X<sub>7</sub>R significantly suppressed LL-37 internalization by human macrophages. Meanwhile, P2X<sub>7</sub>R knock-down (KD) dTHP-1 cells exhibited a lower level of LL-37 internalization, compared with control cells. Furthermore, partial co-localization of LL-37 and P2X<sub>7</sub>R has been observed in dTHP-1 cells. Together, these lines of evidence showed that P2X<sub>7</sub>R participated in the process of LL-37 internalization by human macrophages.

P2X<sub>7</sub>R is highly expressed in macrophages, microglia, and certain lymphocytes. This receptor has been reported to mediate the influx of Ca<sup>2+</sup> and Na<sup>+</sup> ions, as well as the release of proinflammatory cytokines. However, it has been reported that LL-37 enters human PBMCs independent of P2X<sub>7</sub>R [130]. Our results have showed that human PBMCs express much less P2X<sub>7</sub>R than human macrophages. Therefore, the discrepancy of the P2X<sub>7</sub>R involvement in LL-37 internalization by PBMCs and macrophages is possibly due to the lower expression of P2X<sub>7</sub>R in PBMCs than HMDMs. It also indicates that P2X<sub>7</sub>R may play different and context-dependent roles in LL-37-related responses in monocytes and macrophages.

The activation of P2X<sub>7</sub>R has been reported to trigger several downstream signaling pathways, including PLD, MAPK, and PI3K signaling pathways [301]. We found that PI3K and Panx-1 might be involved in P2X<sub>7</sub>R mediated LL-37 internalization. Panx-1 is a P2X<sub>7</sub>R-associated protein and appears to be the large pore or is responsible for activation of the large pore of P2X<sub>7</sub>R [302]. Interestingly, Panx-1 has also been reported for the recognition and intracellular delivery of bacterial molecules and caspase-1 activation [303, 304].

A previous report has shown that ATP stimulated P2X<sub>7</sub>R internalization occurs through the clathrin domain [305]. Accordingly, we also observed that the LL-37/P2X<sub>7</sub>R complex primarily co-localized with clathrin. In addition, the inhibitor of CME (dynasore) exerts no inhibitory effect on LL-37 internalization by P2X<sub>7</sub>R-KD dTHP-1 cells, whereas the inhibitor of caveolae/lipid rafts (nystatin) suppressed the internalization of LL-37 in both control and P2X<sub>7</sub>R-KD cells. Taken together, our results suggest that P2X<sub>7</sub>R-mediated LL-37 internalization is primarily associated with CME, which is consistent with the fact that CME tends to be a receptor-mediated endocytosis pathway [297].

### **LL-37 internalization enhances the bacteria killing ability of human macrophages**

In Paper I, we have demonstrated that LL-37 enhanced the ability of human macrophages to kill bacteria. Here we hypothesized that LL-37 internalization worked as a mechanism for LL-37 enhanced bacteria killing ability. In our experiments, we observed a significantly lower level of LL-37 internalization, at lower temperature and shorter LL-37 exposure time, compared with cells treated with LL-37 under normal experimental conditions. In agreement with our hypothesis, cells, which were loaded with less intracellular LL-37, exhibited a significantly suppressed bacteria killing activity. Moreover, the promoting effect of LL-37 on bacterial killing was diminished in P2X<sub>7</sub>R KD dTHP-1 cells, compared with control vector transfected dTHP-1 cells. These results suggest that internalization is a strategy for LL-37 to

modulate innate immune responses of human macrophages. In addition, we observed co-localization of internalized LL-37 and the phagocytized bacteria in endosomal and lysosomal compartments, which suggests that LL-37 might encounter and eliminate pathogens directly in these organelles.

### **LL-37 internalization enhances intracellular ROS activity and lysosome accumulation in human macrophages**

To further investigate the mechanisms by which internalized LL-37 may enhance clearance of bacteria in macrophages, we measured the quantity of several antimicrobial effector molecules in dTHP-1 cells with or without LL-37 treatment. According to our measurements, LL-37 significantly enhanced intracellular ROS levels and lysosome accumulation. Moreover, pretreatment with endocytosis inhibitor nystatin and dynasore, as well as P2X<sub>7</sub>R inhibitor, significantly suppressed the effect of LL-37 on ROS and lysosome accumulation. Together, these results suggest that LL-37 internalization might contribute to intracellular ROS and lysosome accumulation in human macrophages. In addition, the enhanced bacteria killing ability in LL-37 treated dTHP-1 cells was abolished when those cells were pretreated with ROS inhibitor. This result demonstrated that ROS production induced by internalized LL-37 contributed to the intracellular bacterial killing by human macrophages.

### **4.2 LL-37 regulates eicosanoid production by human macrophages (Paper III)**

In this study, we investigated the role of LL-37 in the regulation of eicosanoid production in human macrophages. We measured several eicosanoids, including LTB<sub>4</sub>, cysteinyl LTs (cys-LTs), PGI<sub>2</sub> (analyzed as 6-keto PGF<sub>1α</sub>), PGE<sub>2</sub>, and TXA<sub>2</sub> (analyzed as TXB<sub>2</sub>), from LL-37 treated and non-treated macrophages at different time points. We found that the production of LTB<sub>4</sub> and TXB<sub>2</sub> were enhanced most significantly in LL-37 treated macrophages at early (1 h) and late (6 h) time points, respectively. Furthermore, LL-37 promoted LTB<sub>4</sub> and TXB<sub>2</sub> production of HMDMs in a dose-dependent manner; while sLL-37 did not evoke any significant responses.

#### **LL-37 induces a quick response (1 h) of LTB<sub>4</sub> production in human macrophages**

Phospholipase A<sub>2</sub>, in particular group IV cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>), mediates agonist-induced AA release, which is the rate-limiting step in the biosynthesis of eicosanoids [306]. Activation of cPLA<sub>2</sub> requires phosphorylation by p38 MAPK and an increase in intracellular calcium [171, 307]. 5-LOX catalyzes oxygenation of AA, resulting in LT biosynthesis. 5-LOX activity is regulated by several factors, including intracellular Ca<sup>2+</sup>, translocation of 5-LOX from the cytosol to the nuclear membrane, phosphatidyl choline, CLP, and phosphorylation of 5-LOX [308].

According to our observations, the incubation of human macrophages with LL-37 rapidly evoked intracellular calcium mobilization, activated ERK1/2 and p38 MAPKs, which contributed to phosphorylation of cPLA<sub>2</sub> and 5-LOX as well as translocation of 5-LOX from



cytosol to the nucleus in human macrophages. These changes in the intracellular signaling pathways lead to an enhanced production of LTB<sub>4</sub> from human macrophages.

#### **P2X<sub>7</sub>R is a candidate receptor involved in LL-37 induced early production of LTB<sub>4</sub>**

Our finding that sLL-37 does not promote eicosanoid production from HMDMs indicates that LL-37-induced responses are mediated by specific receptor(s). By further using pharmacological tools, we demonstrated the involvement of P2X<sub>7</sub>R in LL-37 induced early production of LTB<sub>4</sub>.

#### **LL-37 elicits COX-2 expression and TXB<sub>2</sub> (and PGE<sub>2</sub>) production in human macrophages**

LL-37 also induced TXB<sub>2</sub> production at early time points (1 h), however TXB<sub>2</sub> production steadily increased and peaked at a late time point around 6-8 h. The early production of TXB<sub>2</sub> was mediated via the LL-37-P2X<sub>7</sub>R-cPLA<sub>2</sub>-COX-1 axis, since COX-1 specific inhibitor SC-560, but not COX-2 inhibitor celecoxib inhibited TXB<sub>2</sub> production at the early time phase. On the other hand, TXB<sub>2</sub> production at late time points was contributed by both COX-1 and COX-2 enzymes, which was evidenced by inhibition of TXB<sub>2</sub> production by both COX-1 and COX-2 inhibitors. In addition to TXB<sub>2</sub>, we have also observed an increased PGE<sub>2</sub> production after 8 h exposure of macrophages to LL-37.

Several lines of evidence indicate that the COX isozymes regulate different phases of prostanoid biosynthesis in activated cells [309-311]. In line with this notion, we observed more abundant COX-1 than COX-2 in human macrophages at resting states. When incubated with LL-37, COX-2 expression began to increase at both mRNA and protein levels, leading to an increased level of COX-2-derived TXA<sub>2</sub> and PGE<sub>2</sub>. Further studies also showed that internalization of LL-37 by HMDMs contributed to LL-37-induced COX-2 activation and TXA<sub>2</sub> production.

#### **Cathelicidin elicits LTB<sub>4</sub> and TXA<sub>2</sub> production *in vivo***

To determine the effect of LL-37 on eicosanoid production *in vivo*, we designed two experiments. First, C57BL/6 WT mice were injected intraperitoneally with mCRAMP (mouse LL-37 orthologue), using PBS and mTNF $\alpha$  as negative and positive controls. In this model, injection of mCRAMP led to significantly increased levels of both LTB<sub>4</sub> and TXB<sub>2</sub> in the peritoneal lavage fluid. In the second experiment, acute peritonitis was induced in WT and cathelicidin-deficient (*Cnlp*<sup>-/-</sup>) C57BL/6 mice by *i.p.* injection of mTNF $\alpha$ . After 4 h, significantly lower levels of LTB<sub>4</sub> and TXB<sub>2</sub> were detected in lavage fluids from *Cnlp*<sup>-/-</sup> mice, compared to WT mice. However, since LL-37 has also been reported to trigger LTB<sub>4</sub> production in PMNs [75], it is hard to interpret our *in vivo* findings in a cell-type specific fashion.

### **4.3 PGE<sub>2</sub> suppresses hCAP18/LL-37 expression (Paper IV)**

PGE<sub>2</sub> is produced by most tissues and cells in the human body and acts in an autocrine or paracrine fashion to exert its functions [154]. The paradoxical role of PGE<sub>2</sub> in inflammation and host defense has been discussed above in section 1.4.5. For instance, PGE<sub>2</sub> can promote the inflammatory processes by enhancing local vasodilatation, and stimulating the immune cell chemotaxis and activation [228-231]. However, in certain conditions, PGE<sub>2</sub> also dampens innate immunity and antigen-specific immunity [232-234]. Herein, we report a deleterious role of PGE<sub>2</sub> in human Mtb infection via inhibition of AMP expression, especially VD3-induced hCAP18/LL-37 in human macrophages.

#### **PGE<sub>2</sub> suppresses the expression of AMPs in human macrophages**

HMDMs and human monocytic cell line Mono Mac 6 (MM6) were used in this study. To obtain differentiated MM6 cells, TGF- $\beta$  and VD3 were incubated together with MM6 cells for 96 h. We found that hCAP18/LL-37 expression was significantly decreased in PGE<sub>2</sub> treated macrophages. In addition, PGE<sub>2</sub> also inhibited mRNA expression of human hBD-2 in human macrophages. PGE<sub>2</sub> effectively suppressed VD3-induced LL-37 expression, at both mRNA and protein levels. Furthermore, mPGES1 deficient MM6 cells, which produced less PGE<sub>2</sub> than control cells, expressed significantly higher levels of hCAP18/LL-37, indicating an inhibitory role of autocrine PGE<sub>2</sub> on LL-37 expression.

#### **PGE<sub>2</sub> inhibits LL-37 expression via EP2 and EP4**

We measured the mRNA expression of EP receptors on HMDMs. Cultured HMDMs express high levels of EP2 and EP4, but low levels of EP1 and EP3. Using a series of pharmacological tools and MM6 cells with EP2 or EP4 gene depletion, we demonstrated that both EP2 and EP4 are involved in PGE<sub>2</sub> mediated suppression of hCAP18/LL-37 in human macrophages. It has been reported that EP2/EP4 mediate various immunosuppressive effects of PGE<sub>2</sub> [234, 243-245]. Our results are in line with this notion.

#### **PGE<sub>2</sub> regulates the expression of transcription factors of *CAMP* gene**

cAMP responsive element modulator (CREM), also called inducible cAMP early repressor (ICER), is the direct phosphorylation target of PKA [312]. Quantitative mRNA analysis showed that PGE<sub>2</sub> induced CREM/ICER expression in human macrophages. Further studies demonstrated the involvement of EP2/EP4, cAMP and PKA in PGE<sub>2</sub>-induced CREM/ICER expression. In addition, the inhibitory effect of PGE<sub>2</sub> on LL-37 expression was diminished in CREM/ICER KD MM6 cells. Taken together, these data depicted a PGE<sub>2</sub>- EP2/EP4-cAMP- PKA-CREM/ICER-hCAP18/LL-37 signaling cascade, involved in the regulation of hCAP18/LL-37 expression by PGE<sub>2</sub>.

*CAMP* gene is a target of the transcription factor VDR [96], whose expression is up-regulated by VD3 treatment [313]. We observed that VD3 induced VDR protein expression in human macrophages and this increase was significantly downregulated in the presence of

PGE<sub>2</sub>. Further experiments showed that PGE<sub>2</sub> suppressed VDR expression via a EP2/EP4-cAMP- PKA-VDR axis. To be noted, the inhibitory effect of PGE<sub>2</sub> on VDR protein expression was diminished in CREM/ICER KD MM6 cells, which indicate the involvement of CREM/ICER in the regulation of VDR expression.

### **PGE<sub>2</sub> impairs hCAP18/LL-37 expression in both M1- and M2-polarized macrophages**

Macrophages exhibit varied molecular expression profiles and activities, under different activation states. To further investigate our findings of PGE<sub>2</sub> regulated hCAP18/LL-37 expression in macrophages, we polarized HMDMs with different cytokines to obtain M1 and M2 macrophage phenotypes. In these cells, PGE<sub>2</sub> suppressed hCAP18/LL-37 expression in both M1 and M2 macrophages, with a more profound effect on M2 macrophages. This result was in line with our finding that PGE<sub>2</sub> triggers a more profound increase in CREM/ICER expression and decreased VDR expression in M2-polarized macrophages.

### **PGE<sub>2</sub> elevates Mtb growth in human macrophages**

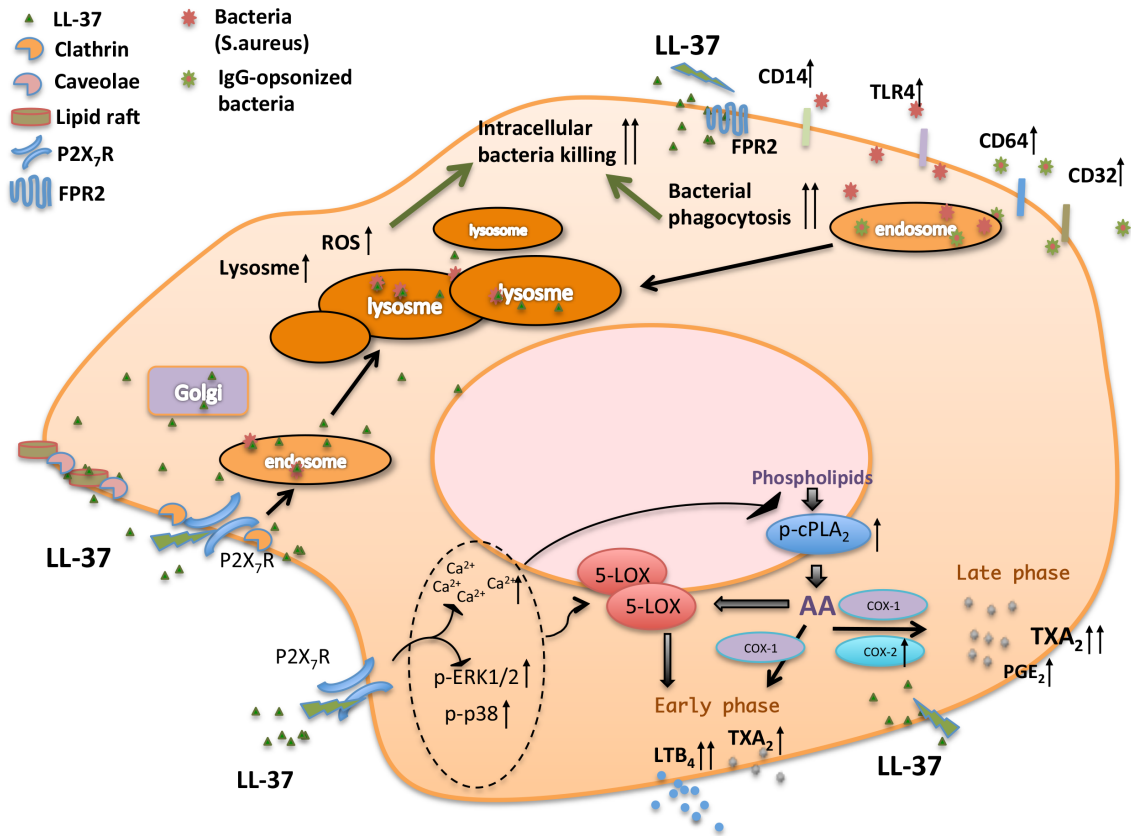
In spite of several previous publications claiming a protective effect of PGE<sub>2</sub> for the host in mouse models of Mtb infection, our data showed that PGE<sub>2</sub> treatment elevated Mtb growth in human macrophages. A recent report has shown that cathelicidin deficient mice exhibited increased susceptibility to Mtb infection compared to wild type mice [314]. In addition, it is well known that AMPs, especially human cathelicidin, is induced in human macrophages by VD3 [96, 99, 100, 315]. On the other hand, autophagy activation is required for VD3-mediated anti-Mtb activity in human macrophages and cathelicidin mediates VD3-induced autophagy (17,18). In line with this, PGE<sub>2</sub> significantly suppressed VD3 induced expression of hCAP/LL-37 and autophagy markers LC3, Atg5 and Beclin 1 in Mtb-infected macrophages.

## 5 CONCLUSIONS

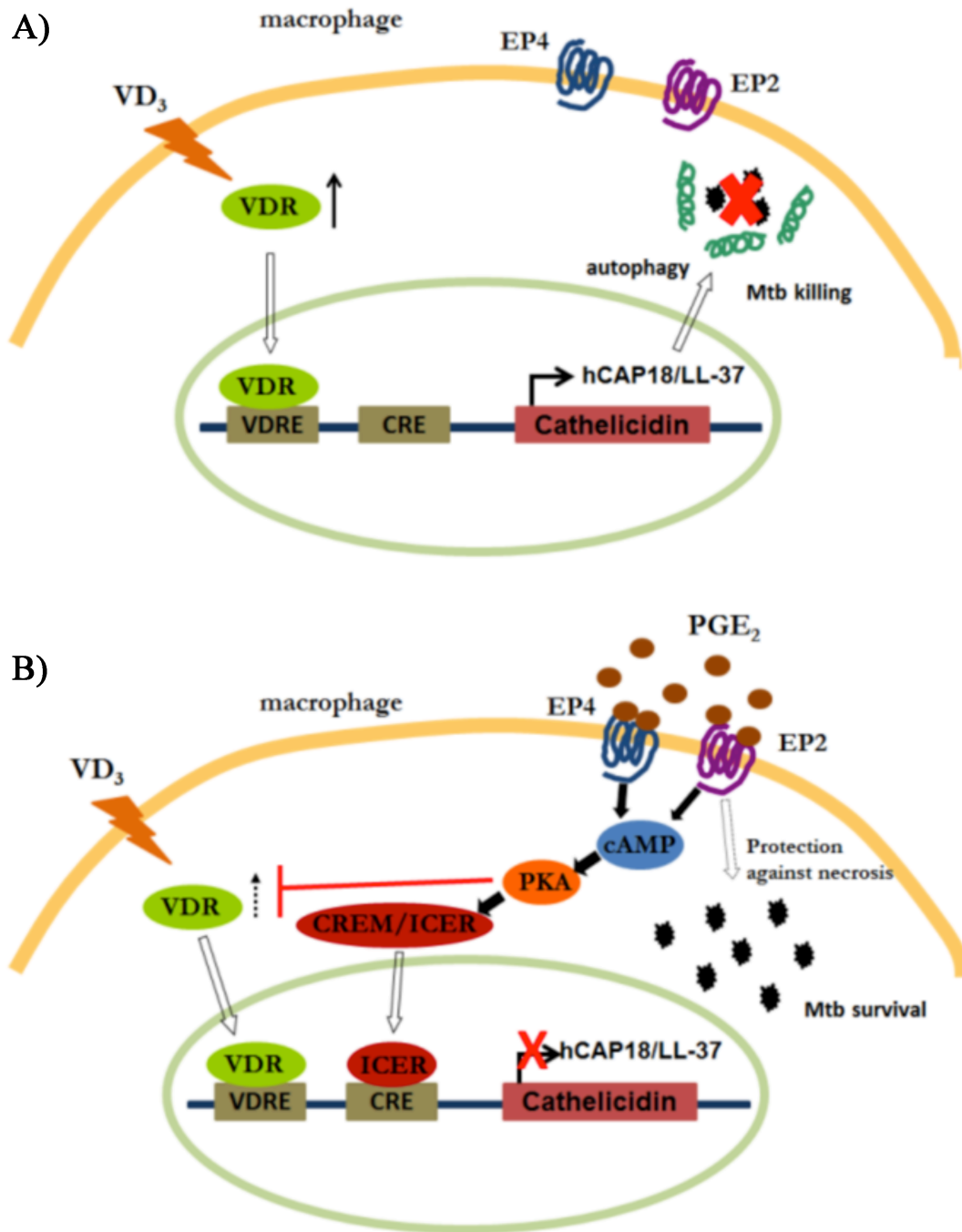
This thesis has been focusing on two major families of inflammatory mediators -AMPs and eicosanoids, regarding their roles in inflammation and host defense. Based on the major observations of the four studies, we conclude the following:

- LL-37 enhances bacterial clearance by human macrophages via promoting bacterial phagocytosis by human macrophages, as well as via entry of LL-37 into the macrophage intracellular compartments, which triggers ROS and lysosome accumulation.
- LL-37 triggers LTB<sub>4</sub>, TXA<sub>2</sub> and PGE<sub>2</sub> production in human macrophages, in a dose and time dependent manner. Short-term (1 h) exposure to LL-37 induces LTB<sub>4</sub> production, via activation of cPLA<sub>2</sub> and 5-LOX enzyme activity. The activated cPLA<sub>2</sub> also leads to an increased early phase TXA<sub>2</sub> production, via COX-1. Meanwhile, LL-37 increases COX-2 expression in human macrophages, resulting in a late phase induction of TXA<sub>2</sub> and PGE<sub>2</sub> production, which might be partially dependent on LL-37 internalization.
- LL-37 internalization contributes to intracellular bacterial killing of macrophages, as well as LL-37-triggered COX-2 expression in human macrophages.
- The purinergic receptor P2X<sub>7</sub>R is involved in LL-37 internalization as well as LL-37 triggered early phase eicosanoid production in human macrophages. P2X<sub>7</sub>R mediated LL-37 internalization is associated with a clathrin-dependent endocytosis pathway.
- PGE<sub>2</sub> exhibits deleterious effects on Mtb infected macrophages, by suppressing expression of the antimicrobial peptide LL-37 via an EP2/EP4-cAMP-PKA signaling pathway. Activation of cAMP leads to increased expression of CREM/ICER and decreased expression of VDR, two transcription factors that regulate hCAP18/LL-37 expression.

The schematic models of this thesis are depicted in Fig. 6 and Fig. 7. **Fig. 6** summarizes the effects of LL-37 on bacterial phagocytosis, intracellular bacteria killing, and eicosanoids production in human macrophages, as well as the characterization of LL-37 internalization. **Fig. 7** illustrates the regulation of cathelicidin expression by PGE<sub>2</sub> via EP2/EP4 in human macrophages.



**Figure 6** A schematic model of LL-37 regulating bacterial phagocytosis, intracellular bacteria killing, and eicosanoids production in human macrophages, as well as an illustration of LL-37 internalization.



**Figure 7** A schematic model of PGE<sub>2</sub> suppressing cathelicidin expression in human macrophages via EP2/EP4. (A) VD<sub>3</sub> treatment induces VDR expression, leading to more VDR binding to VDRE in the promoter region of *CAMP* gene in human macrophages. As a result, human cathelicidin expression and autophagy is enhanced, contributing to intracellular Mtb killing. (B) Surrounding PGE<sub>2</sub> binds to EP2 and EP4, which activates cAMP/PKA-signaling pathway to promote the repressor CREM/ICER expression and reduce VDR expression. Increased binding of CREM/ICER to CRE and reduced binding of VDR to VDRE in the promoter region of *CAMP* gene lead to reduced VD<sub>3</sub>-triggered human cathelicidin expression and autophagy, resulting in increased Mtb survival.

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