From Inflammation to Apoptosis with Focus on Non-Classical Immune Cells

Bankell, Elisabeth

2024

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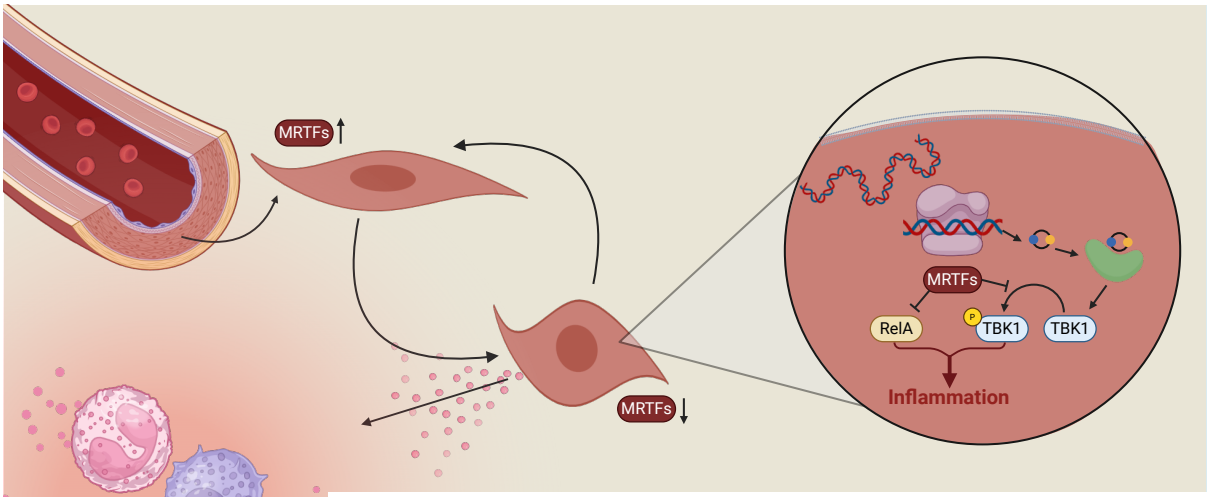

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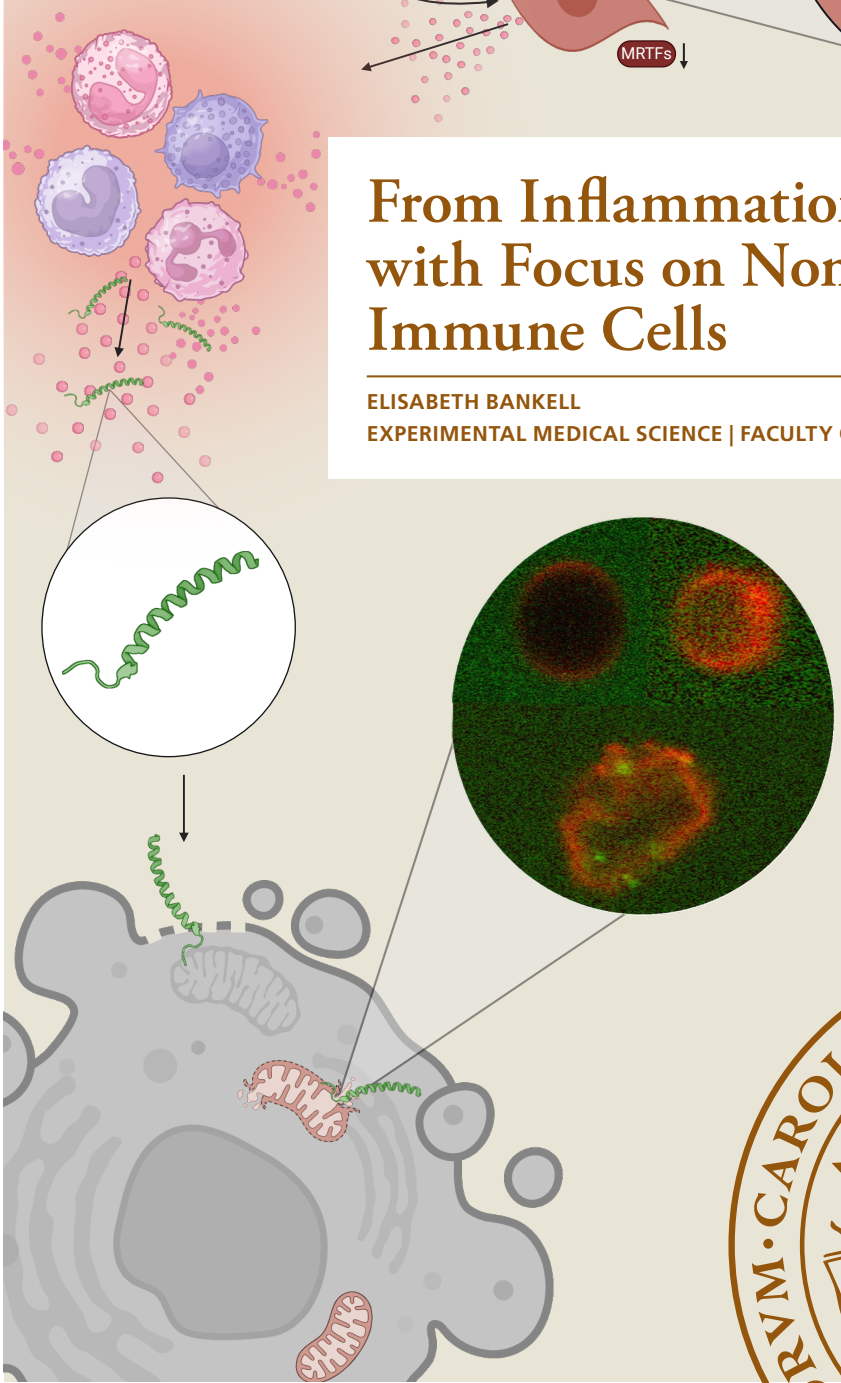
EXPERIMENTAL MEDICAL SCIENCE | FACULTY OF MEDICINE | LUND UNIVERSITY



Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

ELISABETH BANKELL studied Molecular Biology at Lund University, where she also earned her master's degree in Molecular Genetics and Biotechnology. As part of her bachelor's studies, she spent a year on exchange at Nanyang Technological University, Singapore, where she studied Molecular Biology and Life Science. In 2021, she began her PhD at Lund University under the supervision of Prof. Bengt-Olof Nilsson, working within the Vascular Physiology group.

Her research focuses on innate immunity and the signaling pathways involved in inflammation. Her thesis provides new insights into the regulation of inflammatory responses, particularly how non-immune cells, such as vascular smooth muscle cells, contribute to immune regulation. These findings could contribute to the development of novel therapies for inflammatory diseases by targeting dysregulated immune responses.

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Experimental Medical Science

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2024:139
ISBN 978-91-8021-637-1
ISSN 1652-8220

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Elisabeth Bankell

LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 5th of December at 09.00 in Belfragesalen, BMC D15, Department of Experimental Medical Science, Klinikgatan 32, Lund, Sweden

Faculty opponent
Professor Johan Bylund
Department of Oral Microbiology and Immunology,
University of Gothenburg

Organization: LUND UNIVERSITY, Faculty of Medicine, Department of Experimental Medical Science

Document name: Doctoral Dissertation **Date of issue:** December 5, 2024

Author(s): Elisabeth Bankell **Sponsoring organization:**

Title and subtitle: From Inflammation to Apoptosis with Focus on Non-Classical Immune Cells

Abstract:

The innate immune system is our first line of defense against harmful threats, relying on leukocytes to recognize general danger signals and respond rapidly. The inflammatory response activates pathways to eliminate threats and restore homeostasis, which often results in apoptosis to eliminate injured cells and prevent prolonged inflammation. Leukocytes secrete pro-inflammatory cytokines and chemokines to recruit more immune cells, and produce host defense peptides (HDP), including LL-37, which permeabilizes pathogen membranes, leading to cell death. However, high concentrations of LL-37 can also cause host cell cytotoxicity. Non-immune cells, such as smooth muscle cells (SMCs), also contribute to inflammation by producing pro-inflammatory cytokines. SMCs can exist in a contractile (differentiated) or synthetic (dedifferentiated) state, with the latter contributing to pro-inflammatory cytokines and associated with disease. Myocardin-related transcription factors (MRTFs) regulate the SMC contractile phenotype but their impact on inflammation has been inconclusive.

This thesis explores how SMCs contribute to the inflammatory microenvironment, focusing on the anti-inflammatory role of MRTFs. We found that MRTFs interact with key proteins of pro-inflammatory pathways, such as RelA/p65 in NF-κB signaling (paper I) and TBK1 in the cGAS-STING pathway (paper II), reducing inflammation in SMCs from both vasculature and airways. Additionally, we studied LL-37's role in inducing host cell cytotoxicity, revealing that it permeabilizes mitochondrial membranes, leading to caspase-independent apoptosis (paper III and IV).

In conclusion, this thesis provides new insights into the regulation of inflammatory responses, highlighting the role of non-immune cells like SMCs in immune regulation. We have demonstrated that MRTFs reduce inflammation by modulating key signaling pathways, and that LL-37 can induce host cell cytotoxicity. These findings enhance our understanding of immune regulation and could contribute to developing novel therapies for inflammatory diseases by targeting dysregulated immune responses.

Key words: Innate immunity, host defense peptides, LL-37, smooth muscle cells, myocardin-related transcription factors, inflammation, cGAS-STING, NF-κB, apoptosis

Classification system and/or index terms (if any) Supplementary bibliographical information

Language English **Number of pages**: 99

ISSN and key title: 1652-8220

ISBN: 978-91-8021-637-1

Recipient's notes Price Security classification

Signature Date 2024-10-28

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ISBN 978-91-8021-637-1
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

To my family ♥

“Then, I was inspired
Now I’m, sad and tired
Listen, surely I’ve exceeded expectations
Tried for three years, seems like thirty
Could you ask as much from any other man?”

- Gethsemane (I Only Want To Say) by Andrew Lloyd Webber

“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”

- Marie Curie

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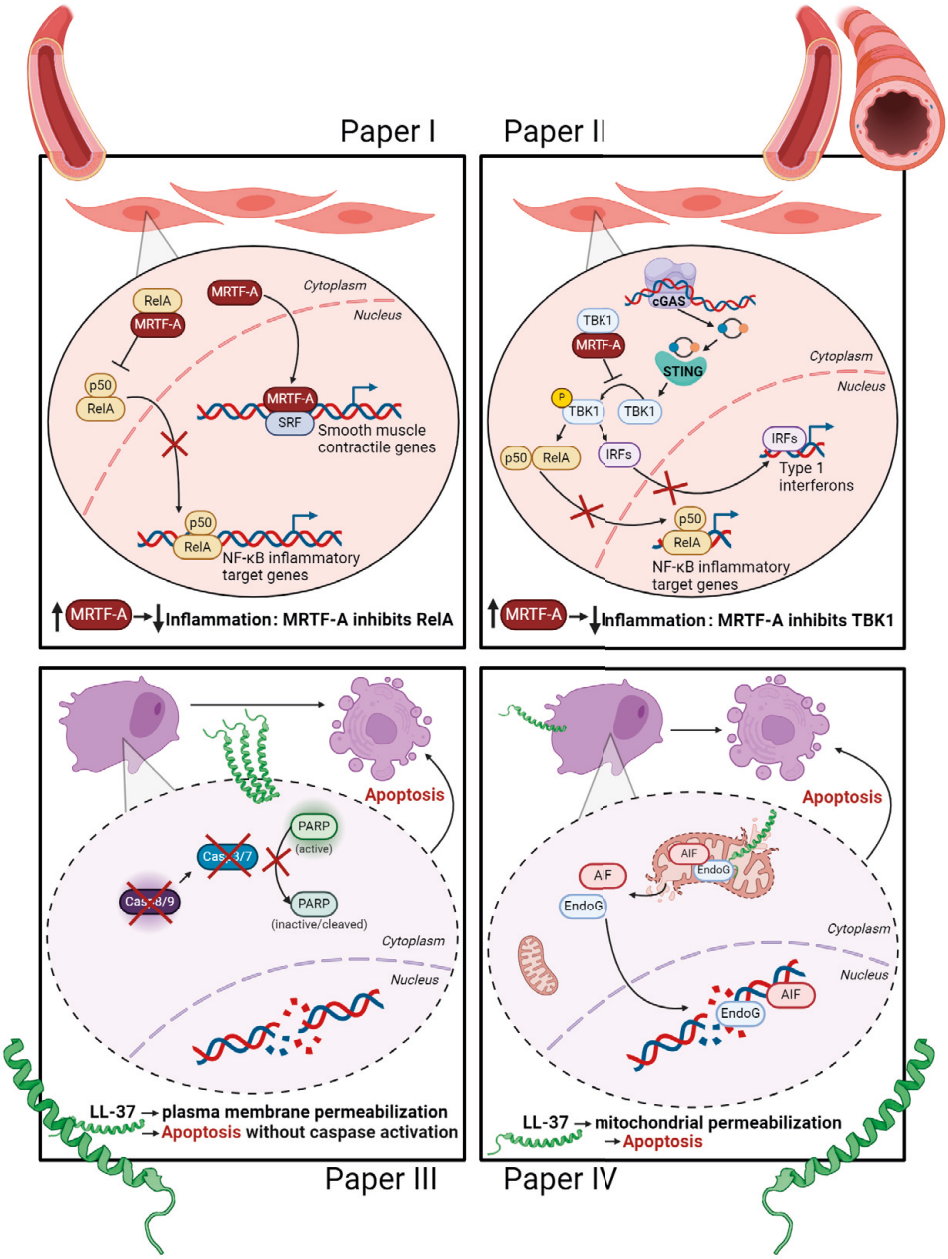
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Abstract

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Populärvetenskaplig sammanfattning

Inflammation är kroppens svar på när något inte är som det ska, exempelvis när vi får en infektion eller skadar oss. Det börjar när våra immunceller känner igen tecken som signalerar fara. Dessa tecken kan komma utifrån och vara exempelvis delar av bakterier eller virus. Men de kan också komma inifrån, exempelvis när våra egna celler är skadade och går sönder och det läcker ut proteiner eller DNA som annars enbart hade funnits inuti cellen. Inflammation som sker i frånvaro av bakterier eller virus kallas för en steril inflammation. Under inflammationen utsöndrar immunceller olika proteiner, så kallade cytokiner, för att signalera fara och för att rekrytera fler immunceller till det inflammerade området. Syftet med inflammationen är att eliminera hotet och att återställa kroppens balans. I vissa sjukdomar kan inflammationen dock bli kronisk och fortsätta trots att den ursprungliga skadan är reparerad. Detta kan ske om kroppen felaktigt identifierar sina egna celler som skadliga, eller om inflammationen inte regleras på rätt sätt.

I den här avhandlingen har jag studerat det medfödda immunförsvaret. Jag har dels fokuserat på hur icke-immunceller, såsom glatta muskelceller, kan signalera fara och bidra till en inflammatorisk miljö. Glatta muskelceller är ansvariga för ofrivilliga rörelser i organ som tarmarna och i blodkärlen. I normala fall är de glatta muskelcellerna i kärlväggen kontraktila, vilket innebär att de spänner sig för att minska kärlets storlek vilket ökar blodtrycket, eller slappnar av så att kärlets väggar utvidgas och blodtrycket minskar. Vid sjukdom har man sett att de glatta muskelcellerna tappar den kontraktila förmågan och istället utsöndrar cytokiner. Myokardin-relaterade transkriptionsfaktorer (MRTFs) är proteiner som ser till att de glatta muskelcellerna är i sitt kontraktila tillstånd. I åderförkalkning har man sett att nivåerna av MRTFs i glatta muskelceller sjunker. Exakt hur MRTFs bidrar till den inflammatoriska miljön har varit omdiskuterat, vissa studier har visat att MRTFs ökar det inflammatoriska svaret medan andra har visat att MRTFs minskar inflammation. I denna avhandling visar vi att glatta muskelceller som har höga nivåer av MRTFs utsöndrar en mindre mängd inflammatoriska cytokiner. Detta beror på att MRTFs binder till och blockerar viktiga komponenter i inflammatoriska signalvägar, vilket leder till att dessa stängs av.

I avhandlingen har jag även studerat hur den antimikrobiella peptiden LL-37 kan skada kroppens egna celler. Våra immunceller har olika sätt att hantera skadliga ämnen, varav ett sätt är att utsöndra antimikrobiella peptider. Dessa peptider har som funktion att tränga igenom bakteriers cellväggar och se till att dessa går sönder och att bakterien dör. Höga halter har dock visat sig kunna orsaka skada på våra egna celler. Höga koncentrationer av den viktiga antimikrobiella peptiden LL-37 har kopplats till flera kroniska inflammatoriska sjukdomar, bland annat psoriasis, där höga halter hittats i hudsåren. I avhandlingen visar vi att höga koncentrationer av LL-37 kan orsaka skada på kroppsegna celler genom att tränga sig igenom cellmembranet vilket leder till att viktiga proteiner inuti cellen läcker ut. Vi ser även

att LL-37 kan orsaka programmerad celldöd (apoptos) utan att involvera caspaser, som normalt är associerade med denna process. Vi fann också att LL-37 orsakar skada på mitokondrier, cellens energifabrik, vilket leder till att proteiner som bryter ner cellens DNA frigörs, vilket i sin tur leder till apoptos. Apoptos är ett sätt för cellen att dö på kontrollerat sätt att utan att orsaka mer inflammation. Apoptos är också en vanlig sista utväg för skadade celler under en inflammation, som ett sätt att återställa balansen igen.

Sammanfattningsvis har detta arbete ökat förståelsen för hur vårt medfödda immunförsvar reglerar inflammation och eliminerar skadade värdceller. Vår forskning kan förhoppningsvis bidra till utvecklingen av nya läkemedel för att hjälpa patienter med inflammatoriska sjukdomar.

List of Papers

This thesis is based on the following papers, referred to by Roman numerals:

I. Liu L*, **Bankell E***, Rippe C, Morén B, Stenkula KG, Nilsson B-O, Swärd K. Cell Type Dependent Suppression of Inflammatory Mediators by Myocardin Related Transcription Factors. Front Physiol. 2021 Oct; 12:732564. doi: 10.3389/fphys.2021.732564. PMID: 34671275.

II. **Bankell E**, Liu L, van der Horst J, Rippe C, Jepps TA, Nilsson B-O, Swärd K. Suppression of smooth muscle cell inflammation by myocardin-related transcription factors involves inactivation of TANK-binding kinase 1. Sci Rep. 2024 Jun;14(1):13321. doi: 10.1038/s41598-024-63901-3. PMID: 38858497.

III. **Bankell E**, Dahl S, Gidlöf O, Svensson D, Nilsson B-O. LL-37-induced caspase-independent apoptosis is associated with plasma membrane permeabilization in human osteoblast-like cells. Peptides. 2021 Jan;135:170432. doi: 10.1016/j.peptides.2020.170432. PMID: 33129893.

IV. **Bankell E**, Liu X, Lundqvist M, Svensson D, Swärd K, Sparr E, Nilsson B-O. The antimicrobial peptide LL-37 triggers release of apoptosis-inducing factor and shows direct effects on mitochondria. Biochem Biophys Rep. 2021 Dec;29:101192. doi: 10.1016/j.bbrep.2021.101192. PMID: 34988298.

* Authors contributed equally.

Published Papers Not Included in the Thesis

I. Aidoukovitch A, **Bankell E**, Davies JR, Nilsson B-O. Exogenous LL-37 but not homogenates of desquamated oral epithelial cells shows activity against *Streptococcus mutans*. Acta Odontol Scand. 2021 Aug;79(6):466-472. doi: 10.1080/00016357.2021.1892180. PMID: 33687301.

II. Aidoukovitch A, **Bankell E**, Svensson D, Nilsson B-O. Vitamin D triggers hCAP18/LL-37 production: Implications for LL-37-induced human osteoblast cytotoxicity. BBRC. 2024 Jun;712-713:149962 doi: 10.1016/j.bbrc.2024.149962. PMID: 38642493.

Selected Abbreviations

AIF	Apoptosis inducing factor
AMPs	Antimicrobial peptides
cGAS	Cyclic GMP-AMP synthase
DAMPs	Damage-associated molecular patterns
dsDNA	Double-stranded DNA
dsRNA	Double-stranded RNA
ECM	Extracellular matrix
hCAP18	Human cathelicidin antimicrobial protein 18
HDPs	Host defense peptides
IFNs	Interferons
IL	Interleukin
LPS	Lipopolysaccharide
MRTFs	Myocardin-related transcription factors
MRTF-A	Myocardin-related transcription factor A
MRTF-B	Myocardin-related transcription factor B
MYOCD	Myocardin
NF-κB	Nuclear factor kappa B
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern-recognition receptors
SMCs	Smooth muscle cells
SRF	Serum response factor
STING	Stimulator of interferon genes
TBK1	TANK-binding kinase 1
TLRs	Toll-like receptors

Prologue

Over the past two decades, our understanding of the immune system has advanced considerably. The earlier belief that the immune system's sole function was to distinguish between 'self' and 'non-self' to defend against invading pathogens has evolved. We now understand that inflammation can arise even in the absence of pathogens, a phenomenon known as sterile inflammation. Sterile inflammation can be triggered by tissue and cell damage and can lead to chronic inflammation if prolonged. Inflammation is an important process aimed to restore homeostasis, but excess inflammation, without the resolution phase, can result in cell death and tissue degeneration including loss of function.

While immune cells with their molecules and proteins are central to the immune system, it is now more widely recognized that cells not traditionally considered part of the immune system also may have important immunological roles. These non-classical immune cells can modulate immune responses, regulate inflammation, and contribute to tissue repair.

This thesis will focus specifically on the components of innate immunity and the roles of non-classical immune cells, exploring their contributions to immune defense, tissue homeostasis, and inflammation.

Introduction

The Immune System

The immune system is a complex network of disease-fighting cells, distributed throughout the blood, lymphatic system, tissues, and organs of the body. These cells protect the host by distinguishing between 'self' and 'non-self', identifying and neutralizing harmful agents such as invading pathogens, foreign substances, and damaged cells (1).

The immune system can be classified into two main categories: innate immunity and adaptive immunity. Innate immunity, a highly conserved system, present across a wide range of species, provides the first line of defense against foreign substances, pathogens or damaged cells without needing to recognize their specific identities. This broad-spectrum defense mechanism is why innate immune responses are also referred to as a generalized and fast defense system. In contrast, adaptive immunity provides a more specialized response. It relies on the specific recognition of foreign substances or cells by lymphocytes, leading to targeted attacks. While adaptive immunity is slower to respond than innate immunity, it offers a tailored defense that improves with repeated exposure to the same pathogen (1–3).

Despite their differences, innate and adaptive immune responses are interconnected and synergistic. Components of the innate immune system provides signals that activate the cells responsible for adaptive responses, ensuring a coordinated and effective defense against potential threats (1,3).

Cells Involved in the Immune Response and Cellular Communication

The Immune Cells

The immune system is composed of various white blood cells collectively known as leukocytes. Unlike red blood cells (erythrocytes), leukocytes can exit the circulatory system and enter tissues to perform their functions (1). Leukocytes are classified into two main groups: myeloid cells and lymphoid cells. Myeloid cells

include neutrophils, basophils, eosinophils, and monocytes, which are primarily involved in innate immune responses. These cells are essential for the rapid and broad-spectrum defense mechanisms, such as phagocytosis and the release of inflammatory mediators (1,2).

Lymphoid cells consist of several types of lymphocytes, including B lymphocytes (B cells), T lymphocytes (T cells), and natural killer (NK) cells. The lymphocytes are connected to the adaptive immune response, characterized by its ability to recognize and target specific pathogens. Despite belonging to the lymphoid lineage, NK cells operate within the innate immune system (Figure 1).

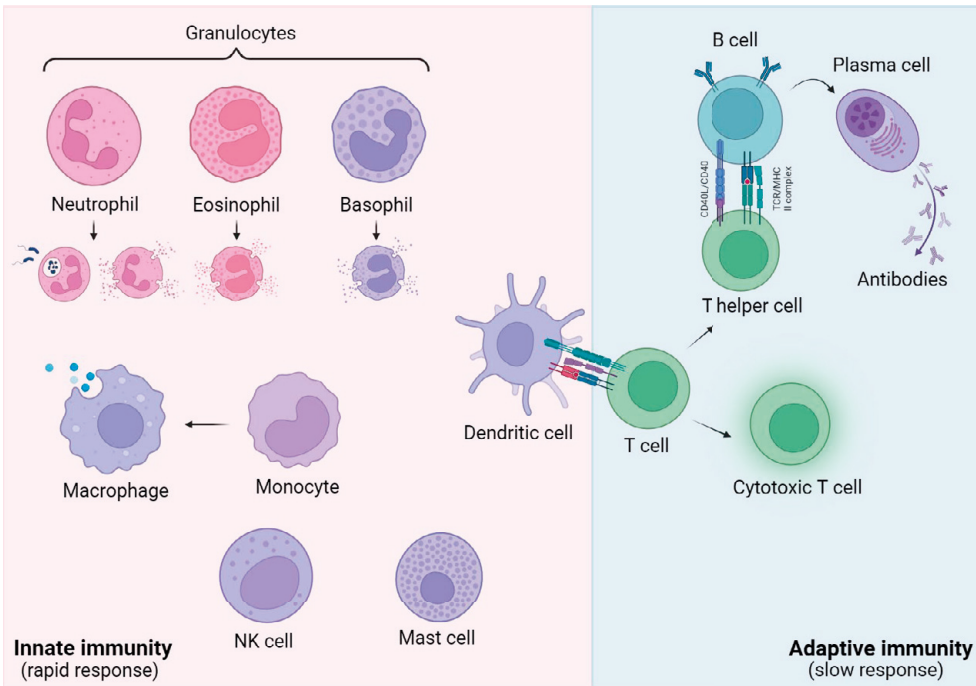

Figure 1. Overview of immune cells in the innate and adaptive immune systems. The innate immune system (left) includes granulocytes (neutrophils, eosinophils, basophils), which provide rapid responses, and phagocytes (macrophages, neutrophils), which engulf pathogens. Dendritic cells present antigens to T cells, linking innate and adaptive immunity. Natural killer (NK) cells target infected and tumor cells, while mast cells release histamine during allergic reactions. The adaptive immune system (right) includes B cells, which produce antibodies via plasma cells, and T cells, which include T helper cells that coordinate immune responses and cytotoxic T cells that directly kill infected and abnormal cells. Adaptive immunity provides a slower but more targeted response to pathogens. Illustration created in BioRender.com.

Neutrophils

Neutrophils are the most abundant type of leukocyte, primarily found circulating in blood. During early stages of infection or injury, the production and release of neutrophils from the bone marrow are increased, and they leave the capillaries and

enter the affected tissues (1). Once inside the tissues, neutrophils engulf pathogens through a process called phagocytosis, where parts of the cytoplasmic membrane forms vesicles around pathogens, cellular debris, or foreign particles. The engulfed pathogens are then destroyed within these endocytotic vesicles by proteases, oxidizing compounds, and cationic proteins (2,4).

Basophils and Mast Cells

Basophils and mast cells are relatively few compared to other leukocytes, yet they are important in severe immune responses (2). Basophils releases heparin, an anticoagulant, at infection sites, which aids in flushing out the infected area through increased circulation. Both basophils and mast cells secrete histamine, which promotes the attraction of other immune cells and proteins to the site of infection, amplifying the immune response and moreover causing vasodilation (1).

Eosinophils

Eosinophils are present in the blood and mucosal surfaces lining the gastrointestinal, respiratory, and urinary tracts. Their primary function is to protect the host from parasitic infections. Eosinophils combat parasites by releasing toxic substances and, to a lesser extent, through phagocytosis (1).

Monocytes and Macrophages

Monocytes are circulating phagocytes that remain in the bloodstream for a short period before migrating into tissues, where they differentiate into macrophages. Macrophages are widely distributed throughout nearly all organs and tissues. Their primary role is to carry out phagocytosis. These cells are strategically located in areas where they are most likely to encounter pathogens, such as the skin and the internal surfaces of the respiratory and digestive tracts (1).

Lymphocytes

Lymphocytes are key players in the adaptive immune response, consisting of various cell types with specialized roles. B cells produce antibodies and, upon encountering an antigen, differentiate into plasma cells that secrete these antibodies. The antibodies then bind to pathogens, marking them for destruction by other immune cells. Cytotoxic T cells target and destroy infected or abnormal cells, such as those infected with viruses or tumor cells. They recognize antigens presented on major histocompatibility complex (MHC) class I molecules and, upon binding, release perforins, which creates pores in the target cell, allowing granzymes to enter and induce apoptosis. Helper T cells coordinate immune responses by releasing signaling molecules, cytokines, that activate B cells and cytotoxic T cells. T helper cells recognize antigens presented on MHC class II molecules (2).

Natural Killer Cells

NK cells, although morphologically similar to lymphocytes, do not have specific antigen receptors. They identify abnormal cells through two mechanisms. First, NK cells have immunoglobulin receptors that bind to antibody-coated targets, initiating antibody-dependent cellular cytotoxicity. Second, NK cells recognize the absence of MHC class I molecules on target cells. If MHC class I is missing, NK cells are activated to lyse the target by releasing perforins and granzymes to trigger apoptosis. In healthy cells, MHC class I molecules bind to NK cell receptors and inhibit cell death. However, tumors and virus-infected cells often downregulate MHC class I, making them less detectable by cytotoxic T cells, while becoming more susceptible to destruction by NK cells (2).

Dendritic Cells

Dendritic cells are known for their ability to capture, process, and present antigens to initiate adaptive immune responses. These highly motile cells are distributed throughout nearly all tissues, with a high concentration at barrier sites such as the digestive tract, where internal and external environments meet. Upon encountering and phagocytosing pathogens, dendritic cells become activated, migrate via lymphatic vessels to secondary lymphoid organs, and present processed antigens and active T cells (1).

Cytokines and Chemokines

The immune cells secrete a diverse array of signaling proteins that regulate host cell proliferation and function in innate and adaptive immune responses. These proteins are collectively known as cytokines, each with its own unique name. Unlike hormones produced by specialized glands, cytokines are generated by various individual cells. Their actions primarily occur at the sites of secretion but can in some cases enter the blood stream and exert effects on distant organs and tissues (1).

Cytokines are crucial in linking the components of the immune system. They form a chemical communication network that facilitates cross-talk between different immune cells, ensuring the precise timing and coordination of immune functions. Most cytokines are secreted by multiple types of cells, both immune cells and non-immune cells, often resulting in cascades of cytokine secretion, where one cytokine stimulates the release of another. Each cytokine can act on a wide range of target cells, amplifying and fine-tuning the immune response. Depending on their function, cytokines can have pro-inflammatory or anti-inflammatory effects, or act as chemoattractants, stimulating the migration of specific immune cells known as chemotaxis. Cytokines with chemoattractant properties are known as chemokines (1,5).

Chemokines are classified into four subfamilies: CXC, CC, CX_3C, and XC, with the "C" representing cysteine residues in their amino acid sequences. CXC chemokines, also known as α-chemokines, are distinguished by the presence of a single amino acid between the first two cysteine residues (C-X-C), while CC chemokines, or β-chemokines, have two adjacent cysteines (C-C). The CX_3C chemokine subfamily is defined by the presence of three amino acids between the cysteine residues (C-X-X-X-C), and XC chemokines lack the first and the third cysteines of the motif (6). The α-chemokines primarily act as chemoattractants for neutrophils, while β-chemokines mainly attract monocytes and macrophages to the site of inflammation (5,6). Common pro-inflammatory cytokines studied in my papers and their classifications and functions can be seen in table 1.

Table 1. Pro-inflammatory cytokines.
Overview of key pro-inflammatory cytokines studied in this thesis. The table highlights cytokines that play crucial roles in mediating inflammatory responses.

Cytokine	*Family*	*Subfamily*	*Function*	*Inflammatory role*
IL-1β	Interleukin	-	Promotes inflammation; induces fever, and activates endothelial cells and leukocytes	Pro-inflammatory
IL-1α	Interleukin	-	Promotes inflammation; induces fever, and activates endothelial cells and leukocytes	Pro-inflammatory
IL-6	Interleukin	-	Induces acute phase proteins	Pro-inflammatory
CCL2 (MCP-1)	Chemokine	CC	Recruits monocytes to sites of inflammation	Pro-inflammatory
CXCL8 (IL-8)	Chemokine	CXC	Chemoattracant for neutrophils, basophils, and T cells	Pro-inflammatory
CXCL1 (GRO-α)	Chemokine	CXC	Chemoattractant for neutrophils; involved in inflammation and wound healing	Pro-inflammatory
CXCL3	Chemokine	CXC	Chemoattractant for neutrophils	Pro-inflammatory
CXCL5	Chemokine	CXC	Attracts neutrophils and promotes angiogenesis during inflammation	Pro-inflammatory
LIF (Leukemia Inhibitory Factor)	Interleukin-6 Family	-	Involved in immune response regulation and may have roles in inflammation and autoimmunity	Pro-inflammatory

Non-immune cells can produce a variety of cytokines and other signaling molecules, which contribute to the overall regulation of the surrounding microenvironment (7–9). An example of this is cellular senescence. Cellular senescence occurs when cells permanently stop dividing in response to various stressors, such as DNA damage, oxidative stress, or oncogene activation (9,10).

Contrary to being inactive, senescent cells develop altered secretory activities where they secrete a broad array of cytokines, chemokines, growth factors, and proteases, known as the senescence-associated secretory phenotype (SASP) (9). Among the key elements of the SASP are the upregulation and secretion of pro-inflammatory cytokines, particularly interleukin-6 (IL-6) and interleukin-1 (IL-1α and -β) in senescent cells. These cytokines can influence neighboring cells through cell-surface receptors, activating inflammatory pathways. Additionally, senescent cells secrete chemokines like IL-8 (*CXCL8*), GRO-α (*CXCL1*) and MCP-1 (*CCL2*), to attract immune cells (11). The factors secreted as part of the SASP not only promote inflammation but also alter the extracellular matrix and influence the behavior of neighboring cells. This process can lead to chronic inflammation, and thereby contribute to age-related diseases (9).

Non-Classical Immune Cells

In addition to immune cells, various non-immune cells contribute to immune regulation during inflammation and tissue damage. These cells, primarily responsible for maintaining structural and physiological functions, can become active participants in immune processes through the secretion of cytokines, chemokine, and other signaling molecules (12).

Fibroblasts are essential for maintaining the structural integrity of connective tissues. However, they also contribute to immune responses by expressing cell surface receptors, so called Toll-like receptors (TLRs), enabling them to detect pathogens or damage signals. When activated, fibroblasts produce pro-inflammatory cytokines and chemokines that recruit immune cells to sites of injury or infection. This capacity to modulate immune activity links fibroblasts directly to processes such as wound healing and fibrosis (8,13).

Epithelial cells, forming the outer surfaces of organs including the skin, lungs, and gastrointestinal tract, also contribute to immune defense. They act as both a physical barrier and an active participant in immune surveillance. They detect pathogens through TLRs and other receptors, producing antimicrobial peptides and cytokines that initiate immune responses (8,14).

Endothelial cells, lining the interior of the blood vessels, are key players in regulating immune cell trafficking. They express adhesion molecules that allow immune cells to adhere to the vascular wall and migrate into tissues in response to infection or injury (15). Additionally, endothelial cells release cytokines that regulate inflammation and vascular permeability (16).

Smooth muscle cells (SMCs) are another example of non-immune cells that can influence the immune response. These cells are responsible for the contractility of blood vessels, airways, and other organs, and can shift from a contractile phenotype to a synthetic, pro-inflammatory, phenotype in response to injury or disease. This

shift involves the production of cytokines and chemokines that attract immune cells, contributing to inflammation in diseases such as atherosclerosis and asthma (16–18).

Smooth Muscle Cells

Smooth muscle is one of the three main types of muscles in the body, alongside skeletal and cardiac muscle. While skeletal muscle primarily controls voluntary movements and cardiac muscle drives the rhythmic contraction of the heart, smooth muscle plays a role in regulating involuntary internal processes in for example the cardiovascular system and the gastrointestinal tract (19).

SMCs are found surrounding various hollow organs and structures that undergo changes in volume, such as blood vessels, the stomach, intestines, bladder, airways, and uterus. These cells are essential for the proper functioning of these organs by contracting or relaxing to accommodate changes in volume (Figure 2). For instance, in blood vessels, SMCs adjust the diameter of the vessel wall, thereby regulating blood flow, vascular tone and blood pressure. SMCs are spindle-shaped and have a diameter ranging from 2-10 μm and lengths that can vary between 50-400 μm, depending on their location in the body (19).

Unlike skeletal and cardiac muscle, smooth muscle lacks the distinctive cross-striated banding pattern created by sarcomeres, which are formed by the regular alignment of actin and myosin filaments in skeletal muscles. This absence gives smooth muscle its “smooth” appearance. Another unique feature of SMCs is their single nucleus and their capacity to divide throughout life (19).

Smooth Muscle Structure and Function

Smooth muscle is regulated by a range of factors, including hormones, autocrine and paracrine agents, and other local chemical signals. Contraction is primarily triggered by receptor activation or mechanical stretching, which in turn activates the contractile proteins actin and myosin. A change in membrane potential, whether through action potentials or the activation of stretch-dependent ions channels, can also initiate contraction (20).

The contraction process begins when calcium ions enter the cell, activating myosin light chain kinase (MLCK). MLCK phosphorylates the myosin light chain, enabling the formation of cross-bridges between myosin and actin, which leads to muscle contraction. Relaxation is dependent on myosin phosphatase, causing dephosphorylation of myosin light chains. One of the key features of smooth muscle is its ability to sustain contraction for long periods, often with minimal energy

demand, making it highly efficient in maintaining functions such as vascular tone (20).

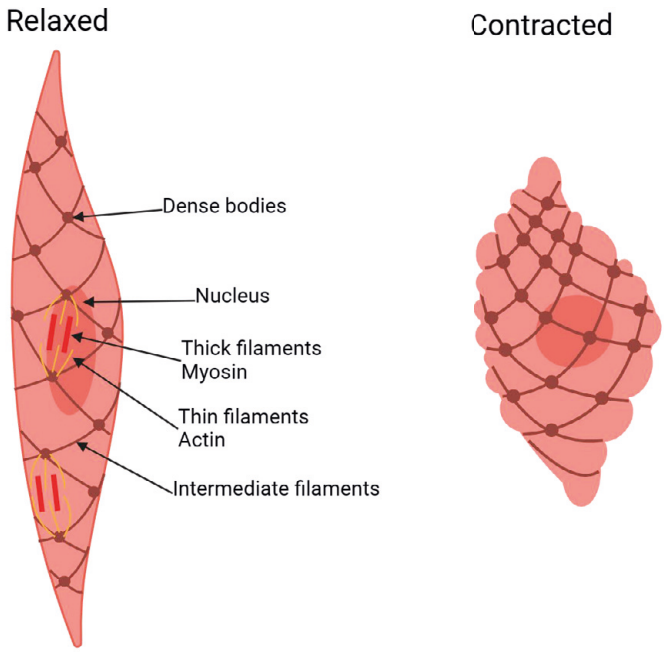

Figure 2. Structural organization of smooth muscle cells. Smooth muscle cells possess an arrangement of thick (myosin) and thin (actin) filaments, which are organized diagonally across the cell. These filaments are anchored to either the plasma membrane or dense bodies within the cytoplasm. Upon activation, the actin and myosin filaments slide past one another, leading to the shortening and thickening of the cell. This structural arrangement facilitates smooth muscle contraction, enabling critical physiological functions such as vasoconstrictions and vasodilation. Illustration created in BioRender.com.

Structurally, SMCs are often interconnected, forming sheetlike layers that can contract in a coordinated manner. Actin filaments in smooth muscle are anchored to regions called dense bodies within the cytoplasm and dense bands located on the cell membrane, which function similarly to the Z-lines in skeletal muscle by providing anchorage points for actin. The myosin filaments alternate between the actin filaments in a side-polar arrangement, allowing smooth muscle to contract more extensively and with greater flexibility than skeletal or cardiac muscle (Figure 2) (19).

SMCs are typically classified into two types: single-unit or multiunit. Single-unit SMCs, found in structures like the gastrointestinal tract, small blood vessels, and

the uterus, contract in a coordinated fashion due to the presence of gap junctions that electrically couple the cells, enabling them to function as a single unit. In contrast, multiunit SMCs, found in structures like large arteries and airways, contract more independently, with each cell receiving separate nerve supply. It is important to note, however, that most smooth muscles display a combination of characteristics from both single-unit and multiunit types, representing a spectrum of properties rather than only belonging to one category (19).

Phenotypic Switching

SMCs are highly specialized and differentiated in adult tissues, with their primary role being to maintain organ function through contraction and relaxation. In their mature state, SMCs show a low rate of proliferation, migration, and express a unique repertoire of contractile proteins, ions channels, and signaling molecules that are essential for smooth muscle contraction (21). Several genes specific to SMCs have been identified and are widely used as markers for their differentiated state. These include smooth muscle-specific isoforms of contractile proteins, such as smooth muscle α-actin (*ACTA2*) and smooth muscle myosin heavy chain (*MYH11*), which are critical components of the contractile apparatus (21–23).

One distinguishing feature of mature SMCs is their plasticity. Unlike skeletal and cardiac muscle cells, which are terminally differentiated and lose their capacity to alter their phenotype (24), SMCs retain the ability to adapt to environmental cues and extracellular signals. This plasticity allows SMCs to respond to physiological changes and external stimuli by shifting between different functional states (25).

Under normal conditions, SMCs maintain a contractile phenotype, focused on their primary function of regulating organ contraction. However, in response to injury or inflammation, they can undergo phenotypic switching to a synthetic phenotype (Figure 3) (26). In this state, SMCs show increased proliferation and migration, along with enhanced synthetic activity, producing extracellular matrix (ECM) proteins and cytokines (27). Phenotypic modulation is crucial in pathological processes such as vascular remodeling in atherosclerosis (23,25) and airway remodeling in chronic respiratory diseases (18,28). The ability of SMCs to shift between contractile and synthetic states highlights their dynamic nature and their central role in both tissue homeostasis and disease progression (29).

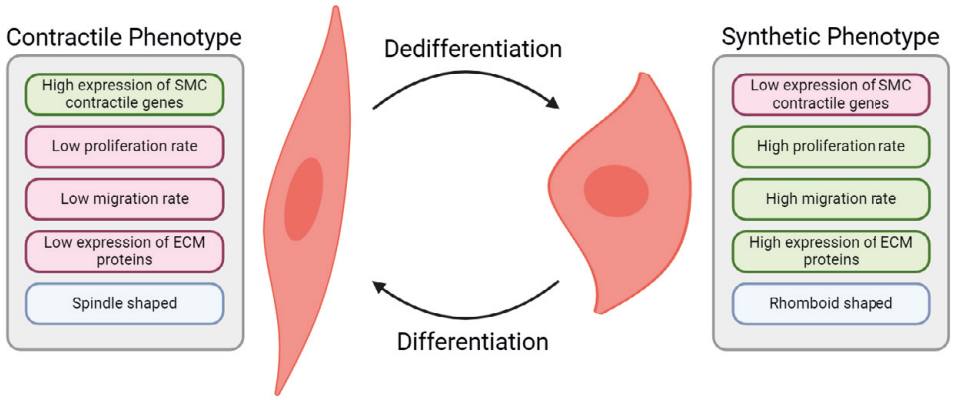

Figure 3. Phenotypic modulation of smooth muscle cells. Smooth muscle cells (SMCs) show phenotypic plasticity, switching between a differentiated (contractile) state and a dedifferentiated (synthetic) state in response to various stimuli. In the contractile phenotype, cells show high expression of contractile genes, have low rates of proliferation and migration, and express minimal extracellular matrix (ECM) proteins, maintaining a spindle shape. In contrast, SMCs in the synthetic phenotype show low expression of contractile genes, increased proliferation and migration, and higher expression of ECM proteins, adopting a rhomboid shape. This modulation plays a role in tissue repair and pathological processes such as vascular remodeling and atherosclerosis. Illustration created in BioRender.com.

Atherosclerosis

Cardiovascular diseases are among the leading causes of death worldwide (30,31). Atherosclerosis, a chronic inflammatory disease affecting the arterial wall, accounts for more than 80% of cardiovascular disease-related mortality (32).

During the progression of atherosclerosis, SMCs undergo phenotypic switching, transitioning from their contractile state to a synthetic phenotype. This shift allows SMCs to migrate from the medial layer of the artery into the intima, where they proliferate and secrete ECM components such as collagen and elastin, forming the fibrous cap that stabilizes the atherosclerotic plaque (Figure 4) (25,33). The fibrous cap is vital for plaque stability, preventing rupture and the associated risk of thrombosis. In addition to their role in fibrous cap formation, SMCs can also take on macrophage-like characteristics, including the ability to engulf oxidized low-density lipoproteins (LDL) (34). This leads to the formation of SMC-derived foam cells, which contribute to lipid accumulation, promote the expansion of the necrotic core, and worsen the inflammatory environment within the plaque, ultimately increasing the risk of plaque instability and rupture (33,34).

One of the factors influencing SMC behavior is the mechanical environment. Research has demonstrated that changes in mechanical stretch or ECM stiffness can induce phenotypic modulations in SMCs. For example, when SMCs are removed from their native environment and cultured *in vitro* on rigid plastic or collagen I substrates, they often lose their contractile characteristics and adopt a more

proliferative, synthetic phenotype (35–37). This change is partly due to the absence of mechanical cues and ECM components that regulate SMC function *in vivo* and can mimic the phenotypic modulation observed in atherosclerosis (25).

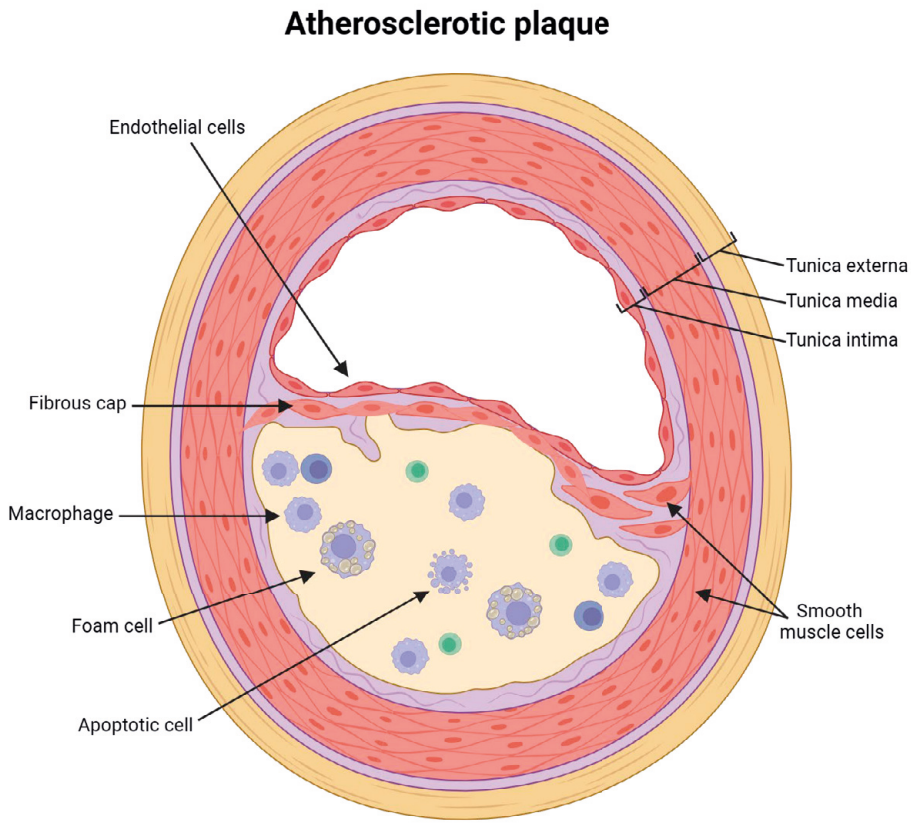

Figure 4. Structure of an atherosclerotic plaque. Atherosclerotic plaques feature a fibrous cap that separates the plaque from the arterial lumen. Beneath the cap is the necrotic core, composed of dead cells, lipid deposits, and extracellular debris. Immune cells, including macrophages, infiltrate the lesion, driving plaque progression and instability. Smooth muscle cells are also involved in the remodeling of the plaque, with some migrating from the media to the intima, contributing to plaque stability. Foam cells, derived from macrophages, accumulate within the necrotic core as they engulf oxidized LDL cholesterol, and these cells play a significant role in the development of the plaque. Illustration created in BioRender.com.

Smooth Muscle Cell Transcriptional Regulation

The transcriptional program that controls SMC identity is tightly regulated by a complex network of transcriptional regulators (25). This network consists of transcription factors and their associated co-regulators, including co-activators and

co-repressors, which either promote or suppress target gene expression. The co-regulators lack the DNA-binding domains and cannot directly bind to DNA. Instead, they modulate gene expression by interacting with transcription factors.

A key transcription factor in smooth muscle differentiation and gene expression is serum response factor (SRF), a member of the MADS-box family of transcription factors. SRF binds with high affinity to CArG boxes, which are A/T-rich regulatory elements located in the promoter regions of smooth muscle-specific genes. Many genes that encode contractile proteins contain at least two CArG boxes in their promoter regions, underscoring the importance of SRF in driving the expression of these genes (38–42).

To promote the contractile phenotype of SMCs, SRF interacts with co-activators from the myocardin-related transcription factor (MRTF) family, which includes myocardin (*MYOCD*), myocardin-related transcription factor A (MRTF-A/*MRTFA*), and myocardin-related transcription factor B (MRTF-B/*MRTFB*) (25,42).

Myocardin-Related Transcription Factors

Myocardin, the founding member of the MRTF family, is recognized as a master regulator of SMC differentiation (25). Myocardin expression is primarily restricted to cardiac and smooth muscle cells, whereas MRTF-A and MRTF-B, the other members of the MRTF family, are more broadly expressed across various cell types. MRTF-A is most abundant in fibroblasts and SMCs, while MRTF-B is primarily expressed in endothelial cells (43). Upon activation, MRTF-A and MRTF-B translocate from the cytoplasm to the nucleus, where they influence gene expression.

The activity of MRTF-A and MRTF-B is closely linked to actin dynamics (44–46), and their regulation is sensitive to mechanical cues, such as stretch and matrix stiffness (47,48). In vascular SMCs, the cytoskeleton undergoes continuous remodeling, with actin filaments assembling and disassembling through polymerization and depolymerization processes. Actin occurs in two forms: monomeric globular actin (G-actin) and polymeric filamentous actin (F-actin) (44). Actin polymerization can be triggered by various signaling pathways, and pharmacological agents can modulate this process. For example, Jasplakinolide stabilizes F-actin (49), while Latrunculin B promotes actin depolymerization by preventing the assembly of G-actin into F-actin (50).

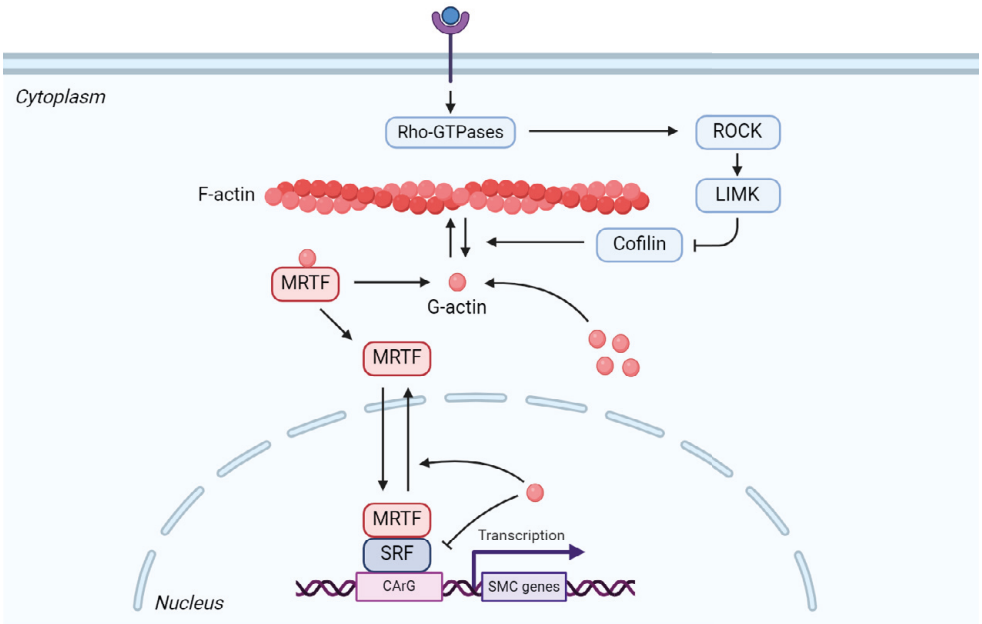

Figure 5. Regulation of MRTF-A by actin polymerization. MRTF-A activity is controlled by the dynamics of the actin cytoskeleton. When actin exists in its globular (G-actin) form, MRTF-A is retained in the cytoplasm. Upon activation of Rho-GTPases, actin polymerizes into filamentous actin (F-actin), which reduces the binding of MRTF-A to G-actin. This release allow MRTF-A to translocate into the nucleus, where it interacts with SRF to drive the transcription of SMC contractile genes. Illustration created in BioRender.com.

MRTF-A and MRTF-B have RPEL motifs at the N terminus, which bind to G-actin and inhibit their nuclear translocation and activation (44). Upon actin polymerization, G-actin dissociates from the RPEL motif, allowing MRTF-A and MRTF-B to translocate to the nucleus and activate the expression of target genes as described in Figure 5 (51,52).

In contrast to the other MRTFs, myocardin is constitutively expressed in the nucleus (25). Disruption of the myocardin gene in SMCs of adult mice has been shown to result in arterial aneurysms, indicating its critical role in maintaining vascular integrity (53). Interestingly, myocardin expression deceases when SMCs switch from the contractile to the synthetic phenotype, a transition that is implicated in atherosclerosis progression (54,55).

In addition to their role in regulating SMC contractility, recent studies suggest that MRTFs also have anti-inflammatory effects, inhibiting the expression of pro-inflammatory cytokines and chemokines (55,56). However, conflicting evidence suggests MRTFs may also promote inflammation under certain conditions (57–59).

The opposing roles of MRTFs in inflammation may depend on the specific MRTF family member, the cell type, or the inflammatory stimulus. Since atherosclerosis is common and may cause stroke and myocardial infarction, and smooth muscle phenotypic switching occurs in its progression, further research is needed to determine whether MRTFs act as pro- or anti-inflammatory regulators in the vascular wall.

Innate Immunity

The innate immune system provides the body's first line of defense against potential threats, responding rapidly and non-specifically to a wide range of danger signals and molecular patterns associated with harm. These patterns can include specific classes of carbohydrates and lipids found in microbial cell walls, as well as endogenous danger signals, such as nucleic acids or proteins, released by the body's own cells when they are damaged or under stress. Plasma membrane receptors on immune cells, along with various circulating proteins, recognize and bind to these molecular patterns, triggering a cascade of innate immune responses. This system includes both physical barriers at the body's surfaces and the inflammatory response to injury or infection (1).

First Line of Defense

Although they are not classified as immune responses, the body's first line of defense against various external threats consists of the barriers provided by surfaces exposed to the external environment. Intact skin is particularly effective, as it prevents the entry of most pathogens and harmful substances, such as chemicals, toxins, and physical damage. Additional surface defenses include the hairs at the entrance of the nose and the reflex actions of coughing and sneezing, which help remove irritants, dust, and potentially harmful substances from the respiratory system. Various glands, such as those in the skin, salivary glands, and lacrimal glands, contribute more actively to immunity by secreting antimicrobial substances. These secretions may contain antibodies, enzymes like lysozymes, which break down bacterial cell walls, and lactoferrin, an iron-binding protein that starves bacteria of the iron necessary for their survival (1).

Antimicrobial peptides, also called host defense peptides, play a crucial role in this first line of defense. Produced by epithelial cells in the skin and mucosal areas, these peptides help to directly kill pathogens at the site of entry. The mucus produced by the epithelial linings of the respiratory and upper gastrointestinal tracts also contain antimicrobial agents, including these peptides. The mucus is sticky and will trap particles and prevent them from entering the bloodstream. In the upper respiratory

tract, these trapped particles are either moved up to the pharynx by ciliary action and swallowed, or they are phagocytosed by macrophages within the various linings (1).

Lastly, the acidic environment of the stomach can kill many pathogens, though some bacteria are able to survive and colonize the large intestine, where they contribute to beneficial gastrointestinal functions (1).

PAMPs

Cells involved in innate immunity can sense 'self' from 'non-self' by recognizing molecular features commonly present on pathogens. These features are called pathogen-associated molecular patterns (PAMPs).

Key PAMPs include various microbial nucleic acids, such as DNA with unmethylated CpG motifs, double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and 5'-triphosphate RNA. Additionally, PAMPs include lipoproteins, surface glycoproteins, and specific membrane components like peptidoglycans, lipoteichoic acid, lipopolysaccharide (LPS), and glycosylphosphatidylinositol (60,61).

DAMPs

In addition to distinguishing between 'self' and 'non-self', the cells involved in innate immunity can recognize danger or damage signals that indicate tissue stress or injury. These signals are known as damage-associated molecular patterns (DAMPs). DAMPs are endogenous molecules released by damaged or stressed cells, which can initiate and sustain immune responses even in the absence of an infectious agent. They are crucial in responding to situations such as trauma, ischemia, cancer, and other forms of tissue damage.

DAMPs consist of various proteins, molecules and nucleic acids that are typically contained within cells under normal conditions. However, when these components are released due to cellular damage, they serve as danger signals, activating inflammatory and immune responses that work to restore tissue homeostasis (60–62).

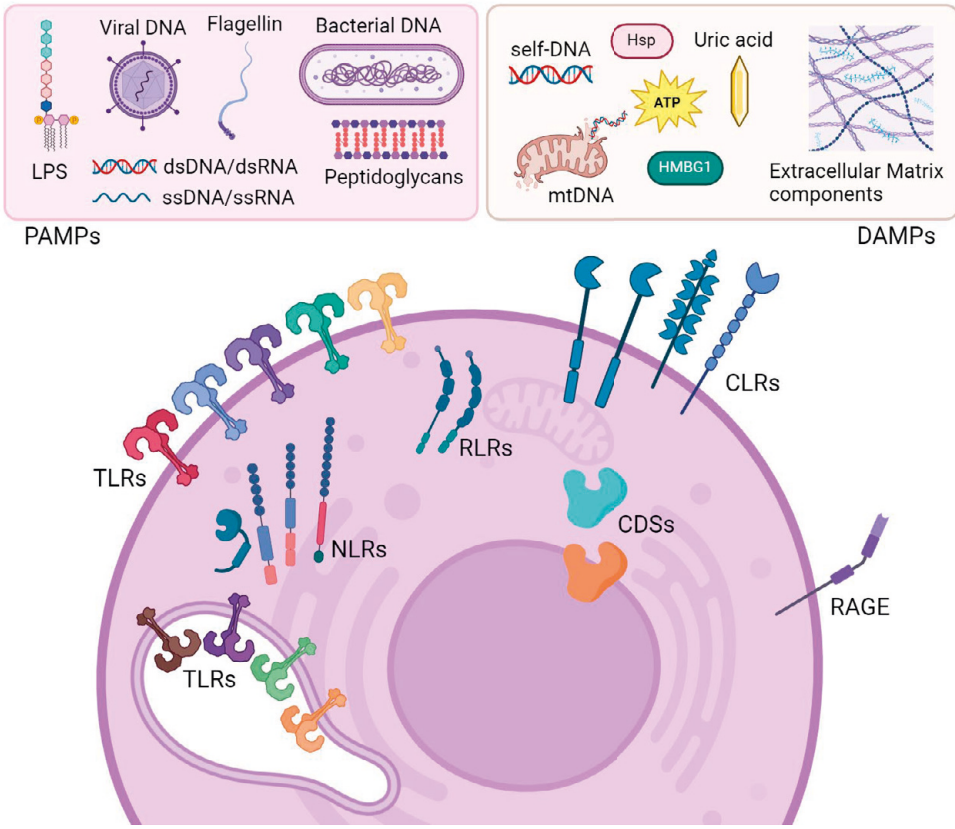

Figure 6. Pattern-recognition receptors (PRRs) and their ligands. Major PRR families, including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), Nod-like receptors (NLRs), RIG-I-like receptors (RLRs), and cytosolic DNA sensors (CDSs), recognize pathogen-associated molecular patterns (PAMPs) such as LPS, viral RNA/DNA, and bacterial peptidoglycans, initiating innate immune responses. Damange-associated molecular patterns (DAMPs), including self-DNA, mitochondrial DNA (mrDNA), ATP, and extracellular matrix components, are released during cellular stress or injury, contributing to sterile inflammation. Illustration created in BioRender.com.

Pattern-Recognition Receptors

Most PAMPs and DAMPs bind to specific receptors known as pattern-recognition receptors (PRRs) (Figure 6). PRRs are expressed on various cells, including macrophages, dendritic cells, neutrophils, and epithelial cells. They are crucial components of the innate immune system, providing a rapid and effective means of identifying potential threats before the adaptive immune response is activated.

When PRRs bind to these molecular patterns, they trigger signaling cascades that initiate an immune response. This response includes the production of pro-inflammatory cytokines, chemokines, and other mediators that recruit and activate

additional immune cells at the site of infection or injury. The activation of PRRs is not only essential for initiating the innate immune response but also for shaping and directing the adaptive immune response that follows (60–62). An overview of different PRRs involved in innate immunity and their characteristics can be seen in table 2.

Table 2. Overview of key pattern-recognition receptors (PRRs)
A summary of key PRRs, their recognition of PAMPs or DAMPs, as well as their cellular localization and type of pathogen or cellular feature they respond to.

PRR	*PAMP/DAMP*	*Type of PAMP/DAMP Recognized*	*Recognized Pathogen/Cellular Feature*	*Cellular Localization*
Toll-Like receptors (TLRs)				
TLR1	PAMP	Bacterial triacylated lipoproteins	Bacteria	Plasma membrane
TLR2	PAMP	Bacterial lipooeotides, peptidoglycans, lipoteichoic acid	Bacteria, mycobacteria, fungi	Plasma membrane
TLR3	PAMP	Double-stranded RNA (dsRNA)	Viruses	Endosomal membrane
TLR4	PAMP/DAMP	Lipopolysaccharide (LPS), Heat shock proteins (HSPs), HMGB1	Gram-negative bacteria, endogenous stress signals	Plasma membrane
TLR5	PAMP	Bacterial flagellin	Bacteria	Plasma membrane
TLR6	PAMP	Bacterial dyiacylated lipoproteins	Bacteria, mycobacteria	Plasma membrane
TLR7	PAMP	Single-stranded RNA (ssRNA)	Viruses	Endosomal membrane
TLR8	PAMP	Single-stranded RNA (ssRNA)	Viruses	Endosomal membrane
TLR9	PAMP	Unmethylated CpG DNA	Bacteria, viruses	Endosomal membrane
TLR10	DAMP			
C-type Lectin Receptors (CLRs)				
Dectin-1	PAMP	β-glucans	Fungi	Plasma membrane
Dectin-2	PAMP	High mannose structures	Fungi	Plasma membrane
Mannose receptor	PAMP	Mannose, fructose, N-acetylglucosamine	Fungi, bacteria, viruses	Plasma membrane
DC-SIGN	PAMP	Mannose-rich glycans	Viruses, bacteria	Plasma membrane
Nod-Like Receptors (NLRs)				
NOD1	PAMP	Peptidoglycan	Gram-negative bacteria	Cytosol
NOD2	PAMP	Muramyl dipeptide	Bacteria	Cytosol
NLRP3	PAMP/DAMP	Bacterial toxins, ATP, uric acid, ROS	Bacteria, cellular stress	Cytosol
NLRC4	PAMP	Bacterial flagellin	Bacteria	Cytosol
AIM2	DAMP	Cytosolic double-stranded DNA (dsDNA)	Viral or bacterial DNA, self-DNA	Cytosol

RIG-I-like Receptors (RLRs)				
RIG-I	PAMP	Viral dsRNA, ssRNA with 5'-triphosphate	RNA viruses	Cytosol
MDA5	PAMP	Long viral dsRNA	RNA viruses	Cytosol
LGP2	PAMP	Viral RNA	RNA viruses	Cytosol
Cytosolic DNA sensors (CDSs)				
cGAS	DAMP	Cytosolic DNA	Viral or bacterial DNA, self-DNA	Cytosol
IFI16	DAMP	Viral and host DNA	Viral DNA, self-DNA	Cytosol/Nucleus
Receptor for Advanced Glycation End-products (RAGE)				
RAGE	DAMP	Advanced glycation products, HMGB1, S100 proteins	Cellular stress, inflammation	Plasma membrane

Host Defense Peptides

Host defense peptides (HDPs) are small, cationic, and amphipathic molecules, typically consisting of 12-50 amino acids. These peptides, produced by all complex organisms as part of their innate immune response, have a broad range of functions, including both antimicrobial and immunomodulatory activities (63,64). Historically referred to as antimicrobial peptides (AMPs) due to their ability to directly target and kill microbes, they are now more commonly known as HDPs to reflect their broader role in host defense beyond just antimicrobial action. Many HDPs are synthesized as inactive precursors (propeptides or prepropeptides) and are stored in the granules of granulocytes. Upon secretion, these precursors are proteolytically cleaved into their active forms (63).

HDPs are classified into two major families: defensins and cathelicidins. Defensins are characterized by their beta-sheet-rich structure and the presence of disulfide bonds, which stabilize their conformation and maintain their function in challenging environments. In humans, 12 defensins have been well characterized, and can be further divided into two subtypes: α-defensins, which consists of β-strands, are predominantly found in neutrophils and Paneth cells within the gut, whereas β-defensins, which comprises both helices and β-strands in their secondary structure, are mainly expressed by epithelial cells, though also found in various leukocytes (65).

Cathelicidins are defined by their α-helical structure under physiological conditions. Unlike defensins, where multiple subtypes exist, only one single cathelicidin has been identified in humans: the human cathelicidin antimicrobial protein 18 (hCAP18). This precursor protein is cleaved extracellularly to generate the active peptide LL-37 (66,67).

LL-37

The precursor protein hCAP18 and its active form, LL-37, were independently identified by three research groups in 1995 (67–69). The name hCAP18 is derived from its structural similarity to CAP18, an 18 kDa cationic antimicrobial peptide identified in rabbits. The gene encoding hCAP18, known as *CAMP*, is located on chromosome 3 and consists of four exons. The first three exons encode the signal sequence and the cathelin domain, while the fourth exon encodes the active LL-37 peptide (70).

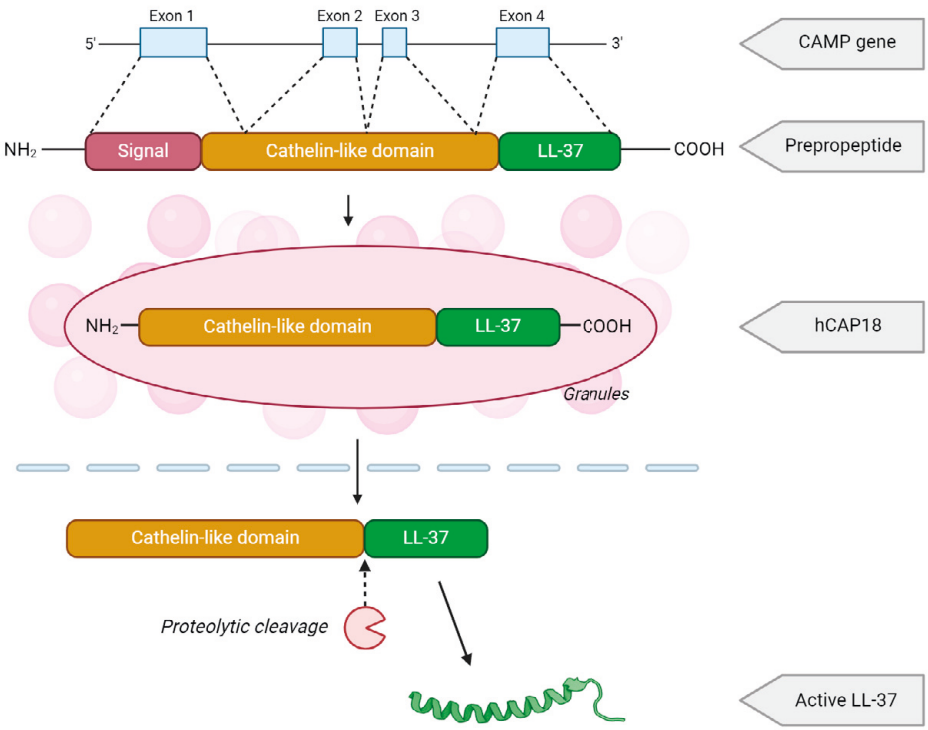

Figure 7. Synthesis and processing of the antimicrobial peptide LL-37. The *CAMP* gene encodes the precursor prepropeptide, which consists of a signal peptide, a cathelin-like domain, and the LL-37 domain. This precursor is stored as hCAP18 within granules of immune cells. Upon cellular activation, hCAP18 is secreted via exocytosis and subsequently undergoes proteolytic cleavage, releasing the active form of LL-37. Illustration created in BioRender.com.

hCAP18 is primarily expressed by neutrophils (69,71), but it is also produced by various cell types, including epithelial cells (71,72), keratinocytes (73), and leukocytes such as monocytes (71,74), macrophages (71), mast cells (75), NK-cells

(74), T-cells (74) and B-cells (74). The conversion of hCAP18 to its active form, LL-37, occurs through extracellular cleavage by proteinase 3 (76), kallikrein 5, and kallikrein 7 (77). The expression of LL-37 can be upregulated by bacterial products, human β-defensin-2, and the vitamin D metabolite 1,25-dihydroxyvitamin D3 (66,78). LL-37 is found at varying concentrations across different cells, tissues, and body fluids, reflecting its broad functional spectrum (66,79).

Structure of LL-37

LL-37 obtains its name from its length of 37 amino acids, with the first two residues being leucine. It is a cationic peptide with a net charge of +6 at physiological pH, containing a high proportion of basic and hydrophobic amino acids. Structurally, LL-37 transitions from an unstructured monomer in pure water to an α-helical conformation under physiological conditions, such as in the presence of lipids, salt, or at a high peptide concentration (79–81). Its monomeric form features a helical region with a disordered C-terminus. LL-37 is amphipathic, with hydrophobic residues on one side and hydrophilic, mainly positively charged residues on the opposite side, this facilitates its interaction lipid membranes, particularly those of pathogens. The positively charged surface of the peptide allows it to interact with negatively charged molecules, such as bacterial cell wall components and nucleic acids. LL-37 can form dimers, trimers, and higher-order oligomers in a concentration-dependent manner, allowing it to minimize the exposure of its hydrophobic surfaces (Figure 8) (66,82).

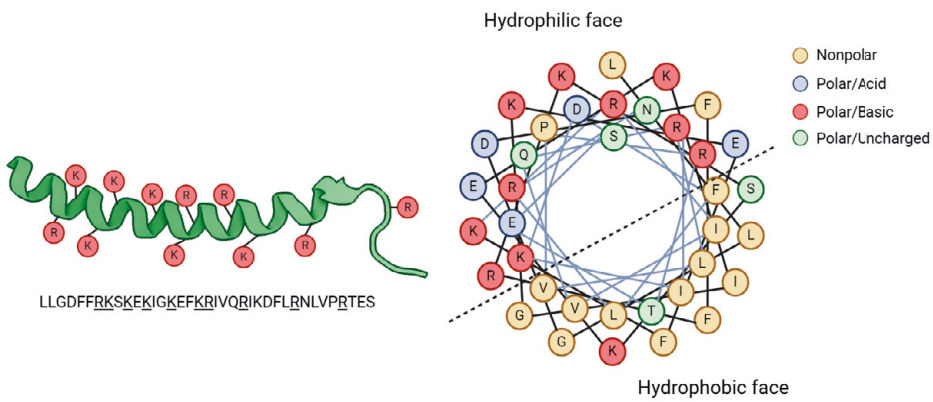

Figure 8. Stucture of LL-37. LL-37 is an amphipathic α-helical peptide composed of 37 amino acids, within a helical structure shown on the left (PDB: 2K6O). The helical wheel diagram on the right highlights the amphipathic nature of LL-37, where positively charged residues (Lys (K), Arg (R)) are grouped on one side of the helix (red), while hydrophobic residues (yellow) are located on the opposite side. This organization is essential for its interaction with negatively charged molecules and membranes. Illustration created in BioRender.com.

Antimicrobial and Antiviral Activities

LL-37 is known for its broad-spectrum antimicrobial activity, targeting Gram-positive and Gram-negative bacteria, biofilm (83,84), viruses (85), fungi (86), and parasites (87). Its positive charge allows it to interact with negatively charged phospholipids in the membranes of pathogens, disrupting the membrane integrity through permeabilization, which leads to microbial cell lysis (66).

Several models have been proposed to explain the mechanism by which LL-37 permeabilizes membranes. In the "carpet" model, LL-37 acts in a detergent-like manner, forming micelles that disrupts the membrane. In another model, LL-37 inserts itself transiently into the lipid bilayer, creating "toroidal pores". In the "barrel-stave" model, LL-37 aggregates into channel-like structures that insert themselves into the bacterial membrane, forming transmembrane pores (82). Beyond its membrane-disrupting activity, LL-37 can bind to and neutralize bacterial endotoxins, such as LPS, a component of Gram-negative bacterial cell walls (67). The minimum inhibitory concentration (MIC) of LL-37 has been reported to range from 1-10 μM against various Gram-positive and Gram-negative bacteria (88–90).

Immunomodulatory Activity

In addition to its antimicrobial activity, LL-37 has a role in modulating immune responses (91). The peptide exhibits both pro-inflammatory and anti-inflammatory activities depending on the surrounding microenvironment. LL-37 modulates immune responses by regulating the production of anti- and pro-inflammatory cytokines, increasing the expression of chemokines, controlling the production of reactive oxygen species (ROS) and nitrogen species (NOS), and by enhancing antigen presentation and leukocyte differentiation (79,91).

As an alarmin, LL-37 recruits and activates immune cells to sites of infection or damage (92). By interacting with the formyl peptide receptor-like 1 (FPRL1) receptor, it promotes the migration of neutrophils, eosinophils, monocytes, and T-cells, creating a positive feedback loop in which neutrophils release more LL-37 (93,94). LL-37 can also function as a pro-inflammatory factor by indirectly influencing the increased expression of cytokines such as IL-8, IL-1β, and, type I interferons (IFNs), while downregulating IL-10, an anti-inflammatory cytokine (95,96).

LL-37 binds to nucleic acids, such as DNA and RNA, influencing TLR signaling by transporting these nucleic acids into endosomes, where they are recognized by TLR3, TLR7, TLR8, and TLR9. At the same time, LL-37 regulates TLR4 signaling by binding to LPS, preventing TNF expression and protecting the host from excessive inflammatory responses (71,96).

LL-37 can exhibit anti-inflammatory effects by inhibiting the formation of the AIM2 inflammasome and suppressing the expression of IFN- γ, TNF-α, IL-4 and IL-12 (79,97). In wound healing, LL-37 promotes angiogenesis, stimulates IL-18 production by keratinocytes, and induces IFN-γ secretion, facilitating tissue repair and epithelial cell migration. LL-37 also activates stromal cell-derived factor 1-α and vascular endothelial growth factor-α, aiding in epithelial regeneration (98,99).

LL-37 in Disease

LL-37 is expressed in various concentrations across tissues and body fluids. Upregulation of LL-37 is often observed in inflamed or infected tissues, and abnormal levels have been detected in several autoimmune and inflammatory diseases as shown in Figure 9 (66).

Although LL-37 preferentially binds to membranes of pathogenic microorganisms, it can also exhibit cytotoxic effects on host cells, but usually at higher concentrations due to the presence of cholesterol in the membrane (100). However, LL-37 has been reported to permeabilize cellular membranes of host cells at concentrations similar to its MIC value for bacteria. LL-37 concentrations between 13-15 μM can induce cell death in immune cells (81), while lower concentrations (around 4 μM) have been reported to affect other cell types, such as osteoblasts (101,102).

Skin Diseases

The skin, a critical physical barrier, produces LL-37 through keratinocytes and epithelial cells. During inflammation, leukocytes at the site can also contribute to LL-37 levels in the skin (96).

Psoriasis, an autoimmune skin disorder characterized by red, inflamed lesions, shows abnormal, very high LL-37 concentrations (>100 μM) in the lesions (103). However, it is not clear whether the elevated levels of LL-37 promote disease progression or represent a compensatory response to reduce inflammation and tissue damage. While LL-37 can inhibit inflammasome activation and reduce IL-1β expression (104,105), it may also promote leukocyte migration, keratinocyte proliferation, and type I IFN production, contributing to disease progression (106).

In rosacea, another chronic inflammatory skin disease, high concentrations of LL-37 and the LL-37 generating enzyme kallikrein 5 are found in lesions. In this disease the elevated LL-37 level has been implicated in contributing to the progression of rosacea (97,107,108).

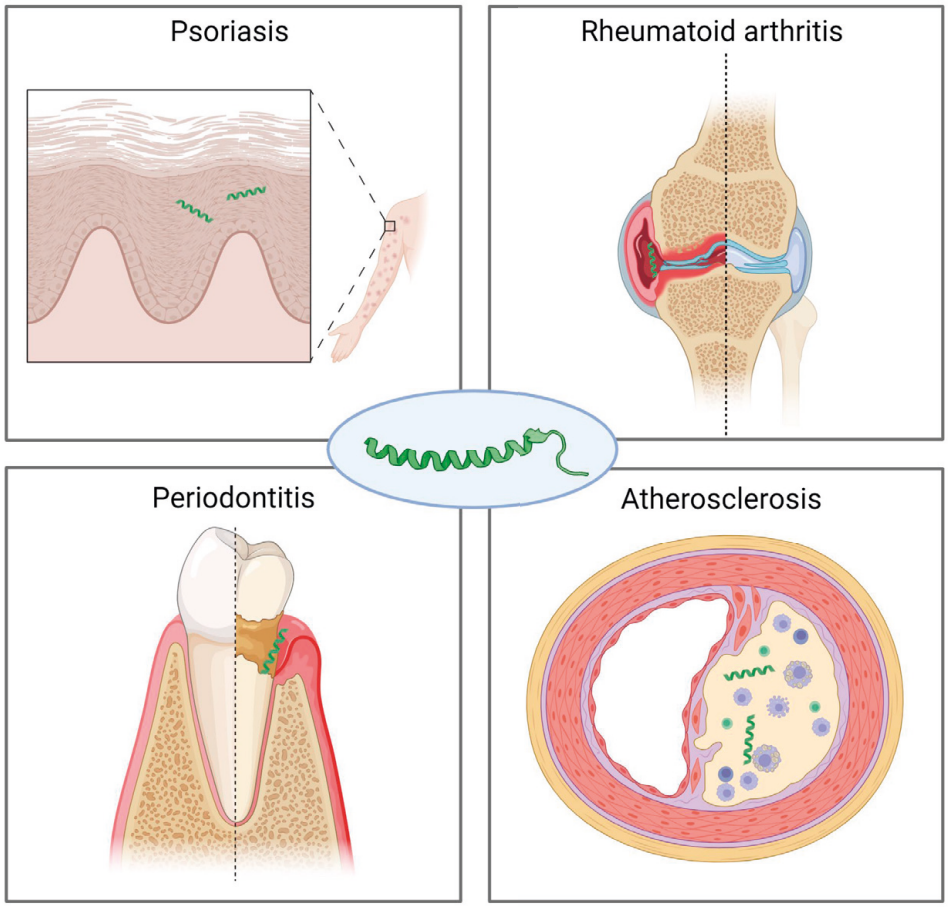

Figure 9. Elevated LL-37 levels associated with various inflammatory diseases. High concentrations of the antimicrobial peptide LL-37 have been implicated in several inflammatory and autoimmune diseases. In psoriasis (top left), LL-37 may trigger autoimmune responses in skin. In rheumatoid arthritis (top right), elevated LL-37 contributes to joint inflammation and tissue destruction. In periodontitis (bottom left), LL-37 is associated with inflammation and tissue degeneration around the teeth. In atherosclerosis (bottom right), LL-37 contributes to plaque formation and progression of the disease. Illustration created in BioRender.com.

Bone Degeneration

Elevated levels of LL-37 have been associated rheumatoid arthritis (RA), an autoimmune connective tissue disease characterized by joint destruction and loss of function (109,110). One of the early indicators of RA is low bone mass or reduced bone mineral density (111). Studies have shown that LL-37 can induce apoptosis in osteoblast, which potentially could contribute to the bone degeneration observed in the progression of RA (101,102). In a rat model of arthritis, rCRAMP, the rodent orthologue of LL-37, was found to be upregulated in granulocytes. When the

neutrophils from this model were transferred into control rats, the recipients developed arthritis, suggesting that LL-37 may have a role in the disease progression (112).

Periodontitis, a chronic inflammatory disease affecting the tissues supporting the teeth, can lead to the destruction of the periodontal attachment and alveolar bone, potentially resulting in tooth loss (113). The disease is initiated by microbial infection and plaque formation on the tooth surface, causing inflammation of the gingiva. In severe cases, characterized by deepened gingival pockets and loss of periodontal attachment, elevated levels of LL-37 and proteinase 3 have been detected in the gingival crevicular fluid of patients with periodontitis (114). Furthermore, a correlation has been observed between deeper gingival pockets and higher concentration LL-37, indicating that the expression of LL-37 is linked to the severity of periodontal disease (115).

Vascular Inflammation

Atherosclerosis is an inflammatory disease in which the activation of innate immune pathways plays an important role in the disease development (33). It has been proposed that LL-37, bound to immune complexes, promotes the production of type I IFN, thereby contributing to the progression of atherosclerosis (116). Elevated levels of LL-37 have been observed in human atherosclerotic aortas, where it is closely associated with neo-intima macrophages (117,118). In a mouse model of atherosclerosis using $ApoE^{-/-}$ mice, mCRAMP, the murine orthologue of LL-37, was found to be upregulated in neutrophils within the carotid arteries of mice fed with a high-fat diet. Interestingly, when these mice were crossed with $mCRAMP^{-/-}$ mice, the absence of mCRAMP led to reduced plaque size and macrophage recruitment, suggesting LL-37 plays a role in recruiting immune cells to atherosclerotic plaques and modulating the local cytokine environment to promote lesion development (119).

LL-37 also impacts endothelial cells, including their activation and upregulation of intracellular adhesion molecule-1 (ICAM-1). This facilitates the recruitment of immune cells to the plaque (120). While this mechanism is important for processes such as the generation of new blood vessels and wound vascularization, it may contribute to pro-thrombotic events in atherosclerotic lesions. Interestingly, LL-37 has shown protective effects in-stent rethrombosis by promoting re-epithelialization, which limits the proliferation and migration of vascular SMCs in the tunica intima layer (121). These findings suggest that LL-37 has a dual role in atherosclerosis. On one hand, it promotes inflammation and plaque development, while on the other, it may facilitate and promote vascular healing.

Diseases Associated with Reduced LL-37 Production

While upregulation of LL-37 is more frequently observed in various diseases, there are some cases where decreased levels of LL-37 are found. One example is

Kostmann syndrome, a rare and severe congenital neutropenia disorder characterized by the absence of mature neutrophils and recurring severe bacterial infections from early life (122). Patients with Kostmann syndrome often suffer from periodontal disease, and due to the lack of neutrophils, LL-37 is undetectable in their saliva (123). Despite treatment with granulocyte-colony stimulating factor to stimulate neutrophil production, these neutrophils do not produce LL-37, and the patients continue to experience periodontal disease (123).

Another rare genetic disorder, Papillon-Lefèvre syndrome, is also associated with periodontal disease. This condition is characterized by a deficiency of proteinase 3, the enzyme that processes the hCAP18 precursor protein into its active form, LL-37 (124). The lack of LL-37 in these syndromes suggests that LL-37 might have different roles in periodontal disease.

Inflammation

Inflammation is a fundamental biological response to harmful stimuli. It serves as a crucial defense mechanism in which the immune system recognizes threats and initiates a cascade of reactions aimed at removing the source of harm and restoring homeostasis.

The inflammatory response is triggered when PRRs on immune and non-immune cells detect danger signals, such as PAMPs or DAMPs. Upon activation, these receptors induce the release of cytokines and chemokines, which serve as signaling molecules to recruit immune cells to the site of injury or infection. Inflammation can be initiated by infectious agents, such as pathogens, which are recognized as ‘non-self’ based on their PAMPs. However, inflammation can also arise from non-infectious stimuli, leading to what is termed sterile inflammation. This occurs when the immune system responds to DAMPs, endogenous signals from damaged or stressed cells, initiating a response similar to that caused by infection (125).

The primary goal of inflammation is to remove harmful stimuli, initiate tissue repair, and restore normal function (126). Without this critical response, infections would persist, wounds would remain unhealed, and overall survival would be compromised. While inflammation is essential for maintaining health, improper regulation or prolonged inflammation can have detrimental effects. If the inflammatory response is dysregulated or persists for an extended period of time, it can result in tissue damage and contribute to the development of chronic diseases.

Inflammation can be divided into two main types: acute and chronic.

Acute Inflammation

Acute inflammation is the body's immediate response to the harmful stimuli, designed to remove and eliminate the cause of injury and promote repair. This type of inflammation lasts for a short period of time, ranging from a few hours to a few days, and is characterized by the classical signs: redness, heat, swelling, pain, and loss of function. These symptoms arise due to increased blood flow, vascular permeability, and the recruitment of immune cells to the affected area (125).

The first immune cells to arrive at the site of injury or infection are typically neutrophils. These cells, together with other immune cells, work to neutralize the harmful agents and initiate the healing process. Once the threat is gone, the resolution phase begins, ending the inflammatory response, and homeostasis is restored (125).

Chronic Inflammation

Chronic inflammation occurs when the underlying cause of inflammation persists or when the immune response becomes dysregulated. Unlike acute inflammation, chronic inflammation is long-lasting, often persisting for months or even years. It is characterized by the consistent presence of leukocytes and lymphocytes, as well as ongoing tissue damage and fibrosis (125).

Over time, this persistent inflammatory state can contribute to the progression of various chronic diseases, including atherosclerosis, RA, and certain cancers. In these conditions, inflammation, which is initially protective, becomes harmful and plays a key role in driving disease progression (125).

Inflammatory Pathways

The activation of innate immune responses is orchestrated through complex signaling pathways that translate extracellular danger signals into intracellular reactions. When PRRs recognize pathogens or cellular damage through PAMPs or DAMPs, a cascade of downstream signaling events is triggered. These cascades lead to the transcriptional activation of genes encoding inflammatory cytokines, chemokines, and other mediators essential for escalating an immune response. Among the most critical signaling pathways involved in this process are the NF-κB and cGAS-STING pathways.

The NF-κB Pathway

The NF-κB (nuclear factor kappa B) pathway plays a central role in immune regulation, inflammation, and cell survival. This pathway is a master regulator of immune responses, ensuring the rapid and coordinated expression of genes involved in inflammation and defense. NF-κB is a family of transcription factors that translocate into the nucleus upon activation, where they initiate the transcription of various target genes involved in immune and inflammatory processes.

The NF-κB family consists of five members: RelA (p65), RelB, c-Rel, p50 and p52 (127). These proteins typically form dimers, with RelA/p50 dimer being the most studied and widely involved in inflammatory responses. In their inactive state, NF-κB dimers remain in the cytoplasm by inhibitory proteins known as IκBs (inhibitors of κB), which bind to their nuclear localization signals. Activation of the NF-κB pathway is regulated primarily through the IκB kinase (IKK) complex, which consists of three subunits: IKKα, IKKβ, and IKKγ. The IKK complex phosphorylates IκB proteins, leading to their ubiquitination and subsequent degradation by the proteasome. This releases the NF-κB dimers, allowing them to translocate to the nucleus and bind to specific DNA sequences in the promoter region of target genes (127,128).

The NF-κB pathway can be activated by various stimuli, including PAMPs recognized by different TLRs, pro-inflammatory cytokines, and cellular stress signals. The canonical pathway is the most well-characterized form of NF-κB activation, primarily involving the RelA/p50 dimer. In this pathway, an external stimulus activates the IKK complex, leading to the phosphorylation and degradation of IκBα, which releases the RelA/p50 dimer, allowing it to enter the nucleus and initiate transcription of pro-inflammatory genes (Figure 10).

In addition to the canonical pathway, there is also a non-canonical NF-κB pathway, which is activated by a smaller range of stimuli. Unlike the canonical pathway, the non-canonical pathway predominantly relies on IKKα and NF-κB-inducing kinase (NIK). Instead of degrading IκB, this pathway processes the p100 precursor protein into p52, which pairs with RelB to form dimers (Figure 10). This pathway primarily regulates genes involved in adaptive immunity (127).

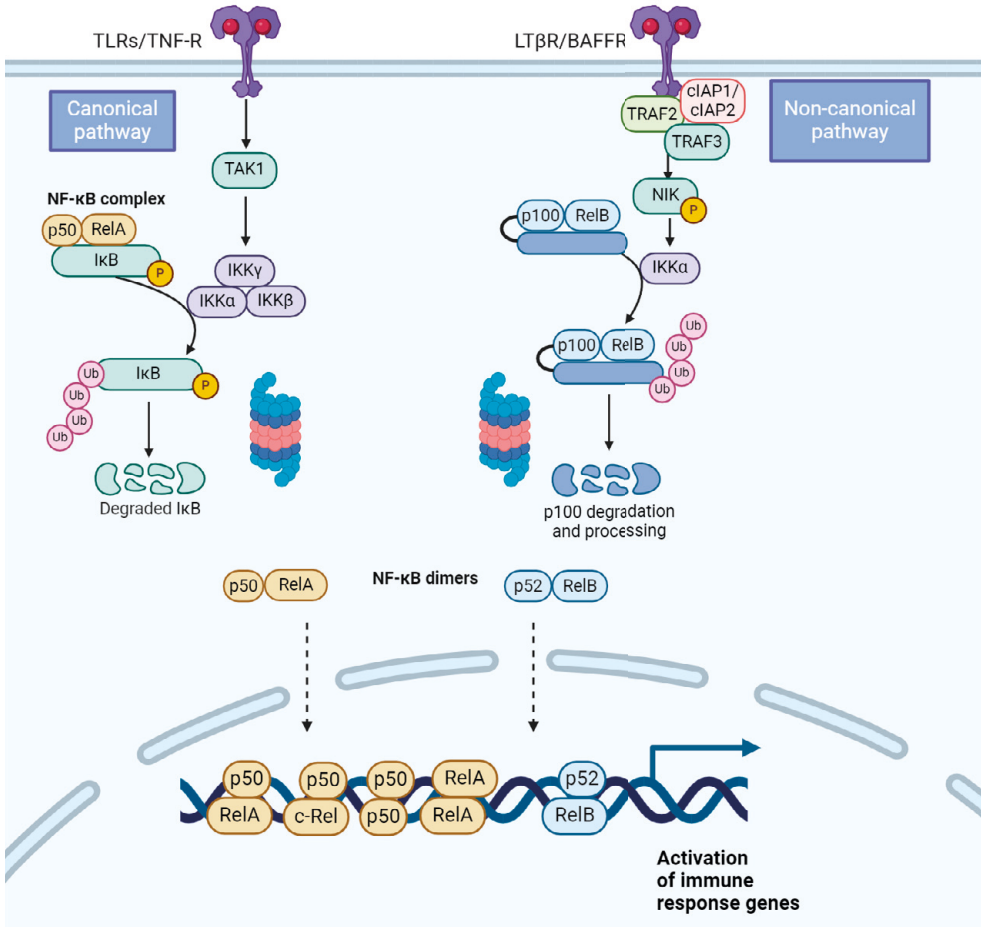

Figure 10. The canonical and non-canonical NF-κB pathways. There are two distinct signaling pathways of NF-κB activation. In the canonical pathway (left), activation through receptors such as TRLs leads to the phosphorylation and subsequent degradation of IκB by the IKK complex, allowing NF-κB dimers, primarly composed of p50/RelA, to translocate into the nucleus and initiate the transcription of target genes. In the non-canonical pathway (right), other receptors will activate NIK, which phosphorylates IKKα, leading to the processing of the p100/RelB complex into the active p52/RelB dimer. The p52/RelB dimer then translocates to the nucleus to activate the target genes. Illustration created in BioRender.com.

The NF-κB pathway is tightly regulated to prevent excessive or chronic inflammation, which can result in tissue damage and contribute to various diseases. One key mechanism of negative feedback is the resynthesis of IκBα, which is one of the NF-κB target genes. This newly synthesized IκBα enters the nucleus, binds

to NF-κB dimers, and shuttles it back to the cytoplasm, thereby ending the inflammatory response (127).

Despite these regulatory mechanisms, dysregulated or prolonged activation of the NF-κB pathway is implicated in a number of chronic inflammatory conditions, including RA, inflammatory bowel disease, and atherosclerosis. Thus, the NF-κB pathway represents a critical target for therapeutic intervention in diseases characterized by excessive inflammation.

The cGAS-STING Pathway

The cGAS-STING pathway plays a vital role in the innate immune system by detecting cytosolic DNA, a danger signal often associated with infections, particularly from viruses. Beyond recognizing pathogen-derived DNA, this pathway is also capable of sensing endogenous self-DNA released into the cytoplasm due to mitochondrial stress, DNA damage, or other cellular injuries, making it a crucial mediator of sterile inflammation. Its activation is important for initiating the production of type I IFNs and other pro-inflammatory cytokines, important for immune defense and the inflammatory response (129).

At the heart of this pathway is the cyclic GMP-AMP synthase (cGAS), a cytosolic sensor that is activated when it detects double-stranded DNA (dsDNA) in the cytoplasm. DNA in the cytoplasm is a signal of abnormality, as DNA is typically located and confined to the nucleus or mitochondria. Upon binding to dsDNA, cGAS undergoes a conformational change and catalyzes the synthesis of a cyclic dinucleotide, cyclic GMP-AMP (cGAMP), which acts as a secondary messenger (Figure 11) (129,130).

Stimulator or interferon genes (STING) is an adaptor protein located on the membrane of the endoplasmic reticulum (ER) and transmits the signal from cGAS to downstream immune responses. Once cGAMP is produced, it binds to and activates STING. This activation causes STING to translocate from the ER to the Golgi apparatus, where it recruits signal molecules, including various kinases (129,130).

The activated STING complex subsequently triggers activation of TANK-binding kinase 1 (TBK1), which phosphorylates interferon regulatory factor 3 (IRF3). Phosphorylated IRF3 forms dimers and translocate to the nucleus, where it initiates the expression of type I IFN genes, driving the antiviral response. Additionally, the cGAS-STING pathway can activate the NF-κB pathway via IKK complex (131). During its journey from the ER to the Golgi, STING recruits various signaling proteins, among them the IKK complex, leading to the production of pro-inflammatory cytokines (Figure 11) (129,130).

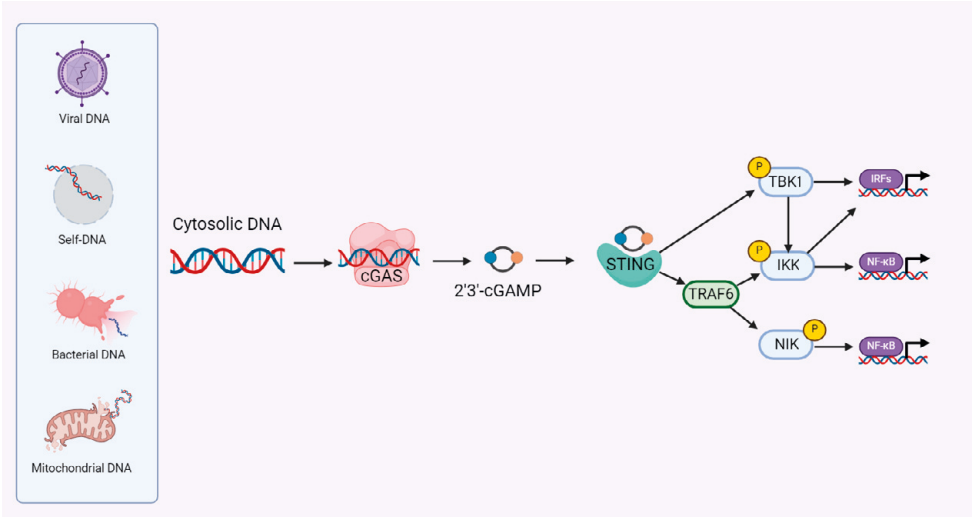

Figure 11. The cGAS-STING pathway. The cyclic GMP-AMP synthase (cGAS) detects cytosolic DNA from various sources, including viral DNA, self-DNA, bacterial DNA and mitochondrial DNA. Upon DNA binding, cGAS catalyzes the production of 2'3'-cyclic GMP-AMP (cGAMP), which acts as a secondary messenger that activates STING (stimulator of interferon genes). STING, in turn, activates downstream signaling targets such as TBK1, leading to the phosphorylation and activation of IRF3 and type I IFNs expression and NF-κB target genes. Illustration created in BioRender.com.

Similar to the NF-κB pathway, the cGAS-STING pathway is tightly regulated to avoid overactivation. Dysregulation of this pathway can lead to chronic inflammation or autoimmune diseases. For instance, in systemic lupus erythematosus (SLE), the cGAS-STING pathway is abnormally activated as it recognizes self-DNA from damaged cells, triggering chronic inflammation. Recent studies have also linked chronic activation of the cGAS-STING pathway to atherosclerosis, where DNA from dying cells within plaques activates the pathway, contributing to ongoing inflammatory responses and disease progression (130).

Apoptosis

Apoptosis is a form of programmed cell death that occurs in a controlled and regulated manner, allowing cells to die without causing harm to surrounded tissues. The process is important in maintaining tissue homeostasis, development, and immune responses by removing damaged, infected, or unnecessary cells. Apoptosis is distinct from necrosis, another form of cell death, where cells die due to injury or disease and release harmful substances that can cause inflammation (132).

Apoptosis can be characterized by key features. Apoptotic cells undergo shrinkage and form membrane-bound vesicles called apoptotic bodies. The apoptotic cell also

undergoes DNA fragmentation, where the DNA of the cell is cleaved into small fragments. Unlike necrosis, the cell membrane remains intact, preventing release of cellular contents into the extracellular environment. Apoptotic bodies are recognized and engulfed by macrophages, ensuring that no inflammatory response is triggered (132).

There are two primary pathways through which apoptosis is triggered, the intrinsic (mitochondrial) pathway and the extrinsic (death receptor) pathway. Both pathways merge in activating caspases, a family of proteases that orchestrate the degradation of the cell.

The Intrinsic and Extrinsic Pathways

The intrinsic pathway is triggered by internal signals such as DNA damage, oxidative stress, or cellular stress. This pathway is regulated by the Bcl-2 family of proteins, which control the permeability of the mitochondrial membrane. The intrinsic pathway is initiated by internal stress that activates pro-apoptotic proteins like Bax and Bak. Bax and Bak form pores in the outer mitochondrial membrane, leading to the release of cytochrome c from the mitochondrial cytoplasm. Cytochrome c binds to apoptotic protease activating factor 1 (Apaf-1), forming a complex called the apoptosome. The apoptosome then recruits and activates caspase-9, which in turn activates caspase-3 and other executioner caspases, leading to the cleavage of important proteins for cell survival, such as PARP and subsequently apoptosis (Figure 12) (133).

The extrinsic pathway is initiated by extracellular signals that activate cell surface death receptors. The binding of ligands to their death receptors leads to receptor activation. The activated death receptor recruit adapter proteins like Fas-associated death domain (FADD), forming death-inducing signaling complex (DISC). DISC activates caspase-8, which in turn activates downstream executioner caspases, such as caspase-3, leading to apoptosis (Figure 12).

Prolonged or unresolved inflammation can lead to apoptosis of immune cells or other tissue cells, serving as a regulatory mechanism to limit excessive immune responses and prevent tissue damage. This process helps to terminate inflammation and promote tissue repair.

Dysregulation of apoptosis can lead to various pathological conditions. Excessive apoptosis can contribute to tissue degeneration in diseases like neurodegeneration and autoimmune diseases, and reduced apoptosis can lead to uncontrolled cell proliferation and survival, contributing to cancer and chronic inflammation (133).

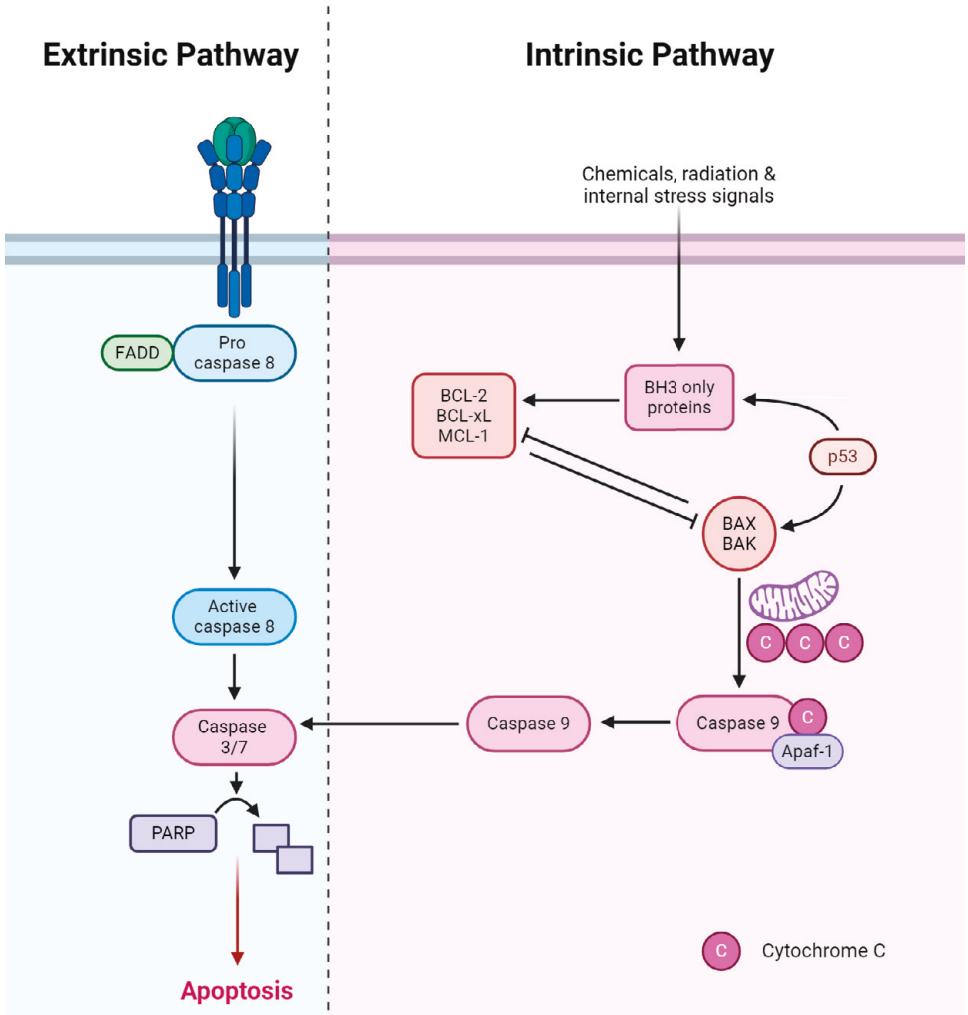

Figure 12. The extrinsic and intrinsic apoptotic pathways. The extrinsic and intrinsic apoptotic pathways are the two primary pathways leading to apoptosis. The extrinsic apoptotic pathway is initiated by death receptors, which recruits FADD and pro-caspase 8, leading to the activation of caspase-8. This, in turn, activates downstream executioner caspases (caspase-3/7), leading to cell death. The intrinsic pathway, triggered by internal signals such as cellular stress, involves mitochondrial outer membrane permeabilization. This releases cytochrome C, which binds to Apaf-1 and forms the apoptosome, leading to caspase-9 activation. Caspase-9 subsequently activates executioner caspases. Illustration created in BioRender.com.

Caspase-Independent Apoptosis

Caspase-independent apoptosis represents an alternative form of programmed cell death that operates without the activation of caspases, the proteases commonly associated with the classical apoptotic pathways. This pathway provides an

important backup mechanism for cell death, particularly in situations where the caspase-dependent pathways are inhibited or insufficient (134).

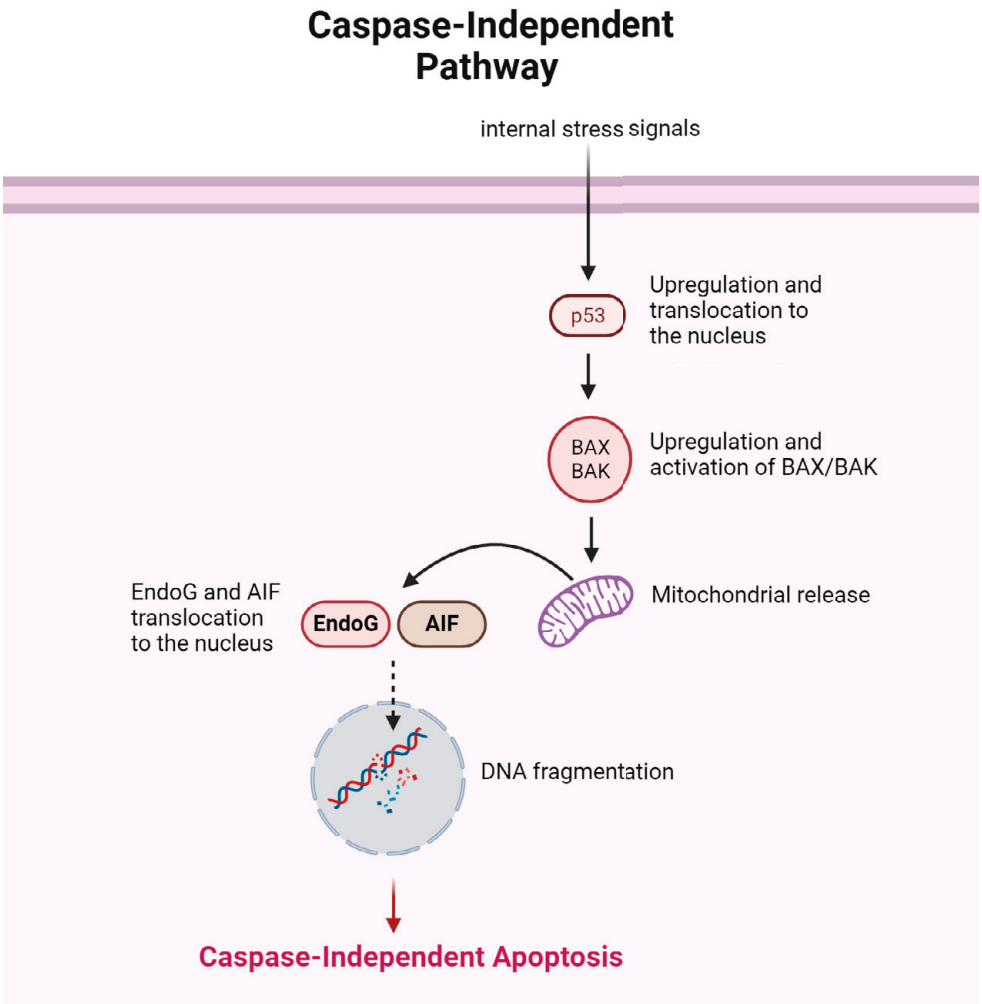

Figure 13. Caspase-independent pathway. This pathway is initiated by internal stress signals such as DNA damage or oxidative stress, leading to the activation of p53 which translocates to the nucleus and activates pro-apoptotic proteins BAX and BAK. These proteins facilitate mitochondiral membrane permeabilization, resulting in the release of mitochondrial proteins such as Endonuclease G (EndoG) and Apoptosis-Inducing Factor (AIF). Once release into the cytosol, AIF and EndoG translocate to the nucleus, where they induce chromatin condensation and DNA fragmentation, promoting caspase-independent apoptosis. Illustration created in BioRender.com.

Like the intrinsic apoptotic pathway, mitochondria play a central role in caspase-independent apoptosis. Upon receiving a death signal, specific proteins are released

from the mitochondria, initiating the cell death process. One of the primary proteins involved in this pathway is apoptosis-inducing factor (AIF). Under normal conditions, AIF is located within the mitochondrial intermembrane space, but upon stress or damage, it is released into the cytoplasm and subsequently translocates to the nucleus. Once in the nucleus, AIF induces chromatin condensation and DNA fragmentation, key hallmarks of apoptosis. Another important mitochondrial protein involved in this pathway is the Endonuclease G (EndoG), which is also released from the mitochondria in response to apoptotic stimuli. Like AIF, EndoG translocates to the nucleus, where it contributes to the degradation of nuclear DNA, promoting cell death in a capsase-independent manner as depicted in Figure 13 (134,135).

Necroptosis and Pyroptosis

Necroptosis is a form of caspase-independent programmed cell death that combines features of both apoptosis and necrosis (136,137). While apoptosis is a non-inflammatory, tightly regulated process, necroptosis results in cell lysis and the release of intracellular contents, triggering a strong inflammatory response, much like necrosis. However, unlike necrosis, necroptosis is a controlled process and is often considered a backup mechanism when apoptosis is inhibited.

Necroptosis is initiated by the activation of receptor-interacting protein kinases (RIPK1 and RIPK3). Typically, death receptor activation leads to apoptosis through the caspase-8-dependent extrinsic pathway. However, when caspase-8 is absent or inhibited, necroptosis is activated. In this pathway, RIPK1 interacts with RIPK3 to form a complex known as the necrosome. RIPK3 then phosphorylates mixed linage kinase domain-like protein (MLKL), which translocates to the plasma membrane, leading to membrane rupture and eventually cell death (136–138).

Unlike apoptosis, which preserves membrane integrity, necroptosis results in membrane rupture and the release of DAMPs, which further promote inflammation by activating the immune system. Necroptosis is particularly important when pathogen-infected cells or cancerous cells evade apoptosis, and in various inflammatory diseases.

Pyroptosis, on the other hand, is another form of programmed cell death closely associated with inflammatory responses. It is typically triggered by intracellular pathogens, such as bacteria and viruses, and is mediated by caspase-1 through the activation of inflammasomes that detect danger signals, including PAMPs and DAMPs (139).

Upon activation, inflammasomes cleave inactive pro-caspase-1 into its active form, caspase-1. Active caspase-1 then processes the pro-inflammatory cytokines IL-1β and IL-18 into their active forms, which are released into the ECM to promote inflammation. Simultaneously, caspase-1 cleaves gasdermin D (GSDMD), creating

a pore-forming fragment that inserts into the plasma membrane. This results in membrane rupture, cell swelling, and the release of intracellular contents (140).

Unlike apoptosis, which is immunologically silent, pyroptosis is a highly inflammatory form of cell death. The release of cytokines and DAMPs during pyroptosis stimulates a strong immune response, aiding in the clearance of pathogens and damaged cells. Pyroptosis is therefore necessary for immune defense but can also contribute to excessive inflammation if not regulated (140).

The interplay between inflammation and programmed cell death is important for resolving infections and initiating tissue repair. While inflammation is essential for defense and repair, it frequently leads to cell death as a resolution mechanism to remove damaged cells and prevent excessive immune activation. Apoptosis, in particular, ensures that cell death occurs in a controlled manner without triggering further inflammation. Thus, the balance between inflammation and cell death is vital to maintaining immune homeostasis and preventing chronic inflammation or tissue damage.

Aims

The overall aim of this thesis was to explore key aspects of innate immunity in non-classical immune cells, focusing on the role of the host defense peptide LL-37 in inducing host cell cytotoxicity, as well as the regulatory role of myocardin-related transcription factors (MRTFs) in pro-inflammatory innate immunity pathways in smooth muscle cells (SMCs).

The specific aims of the individual studies included in this thesis were as follows:

I. To investigate the effect of MRTFs on inflammation signaling pathways in human arterial disease.

II. To determine whether MRTFs can downregulate the cGAS-STING pathway in SMCs.

III. To examine the pathways involved in LL-37-induced cell death in human osteoblast-like cells.

IV. To explore whether LL-37 induces cell death by permeabilizing the mitochondrial outer membrane.

Material and Methods

In this section, I provide an overview of the key methods and techniques used throughout this thesis. For comprehensive details, please refer to the methodology section in each respective paper.

Cell Culture (Study I-IV)

In this thesis, human cell cultures were used in all studies as a primary method for investigating cellular processes *in vitro*. Cell culture offers a controlled environment that allows precise regulation of experimental conditions, enabling reproducibility and targeted manipulation of specific variables. While cultured cells lack the complex interactions seen in tissues and *in vivo* models, this simplified system provides a cost-effective and efficient alternative for studying isolated cellular mechanisms.

Throughout this thesis, both commercially available cell lines and primary human cells were used (see table 3). Cell lines are ideal for general mechanistic studies and high-throughput experiments due to their stability and cost-effectiveness. Primary cells offer greater physiological relevance, closely mimicking *in vivo* biology, but are more technically challenging and have a limited lifespan, restricting their use to a finite number of passages. Although *in vitro* studies have their limitations, human cells are preferred over animal models for studying innate immunity, as they more accurately reflect human-specific immune responses, enhancing the relevance and translatability of finding clinical applications.

All cells were cultured in a water-jacked cell incubator at 37 °C with 5% CO_2, and seeded into culture plates the day before experiments were conducted.

Table 3. Overview of cell types used throughout the thesis.

Name	*Origin*	*Type*	*Culture media*	*Paper*
hCASMCs	Coronary artery SMC	Primary	M231 + 5% SMGS	I-II
THP-1	Monocyte	Cell line	RPMI Glutamax + 10% FBS	I
hBrSMCs	Bronchial SMC	Primary	Vascular Cell Basal Medium + SMC Growth kit	II
MG63	Osteoblast-like cell	Cell line	DMEM/Ham's F12 + 10% FBS	III-VI

Overexpression and Silencing (Study I-II)

Overexpression and gene silencing are powerful techniques for understanding gene functions. Overexpression involves introducing and elevating the expression of a gene of interest to study how increased protein levels influence cellular behavior and signaling pathways. This method provides insights into the role of the gene in physiological processes. Conversely, gene silencing, often achieved through RNA interference techniques such as short hairpin RNA (shRNA), reduces gene expression, enabling the exploration of the consequences of reduced or absent gene function. This approach is valuable for identifying the contribution of the gene to various cellular mechanisms.

In papers I-II, I have used adenoviral vectors for both overexpression and silencing. Adenoviral vectors offer several advantages over plasmid vectors, which typically require toxic reagents to increase cell membrane permeability and facilitate transfection. Adenoviral vectors enable a more efficient delivery of genetic material across a wide range of cell types, including primary cells that are generally resistant to plasmid-based transfection. Additionally, adenoviral vectors no not integrate into the host genome, and gene expression is typically achieved within 48 h, making them well-suited for transient gene expression and short-term experiments. However, despite being replication-deficient, adenoviral vectors carry a higher biosafety risk compared to plasmids. To mitigate the potential risk of host cell immune activation by the viral vector itself, we investigated the impact of null-virus transduction on pro-inflammatory target genes and found no increase in inflammatory responses in our experimental setting.

In paper I, a plasmid vector was used for overexpression in THP-1 cells. While plasmid vectors are more cost-effective and safer due to their non-viral nature, they are generally less effective compared to viral vectors and are typically most effective in cell lines compared to primary cells.

Gene Expression Analysis

Transcriptomics Analysis (Study I-II)

In papers I and II, we utilized an RNA-sequencing (RNA-seq) dataset that was generated prior to my studies (43). RNA-seq is a powerful technique for analyzing the transcriptome, which represents the complete set of RNA transcripts produced by the genome under specific conditions. In this particular dataset, RNA was extracted from hCASMCs overexpressing myocardin. By comparing gene expression between myocardin-overexpressing cells and hCASMCs transduced

with control null-virus, we identified numerous potential target genes of myocardin, which require further validation using other experimental methods.

In general, RNA-seq involves several key steps. First, RNA is isolated from the cells or tissues of interest and is then converted into complementary DNA (cDNA) via reverse transcription. The cDNA is fragmented into smaller pieces, and specific adapters are ligated to both ends of these fragments, which are essential for sequencing and enable the cDNA to bind to the flow cell during the sequencing process. The sequencing itself is performed using fluorescently labeled nucleotides that are incorporated into the growing DNA strand, and the fluorescent signal from each nucleotide is captured by a camera. This process occurs in parallel with millions of fragments, generating a substantial volume of sequence data.

The sequencing output consists of short reads, typically ranging from 50-150 bp in length, which are aligned to a reference genome or transcriptome. RNA-seq enables the comparison of gene expression between different conditions, such as treated versus untreated samples, allowing for the identification of genes that are upregulated or downregulated.

RT-qPCR (Study I-II)

To investigate the expression of target genes, I extracted RNA from cell lysates and performed reverse transcription quantitative polymerase chain reaction (RT-qPCR), a widely used technique that combines reverse transcription (RT) and quantitative PCR (qPCR). Initially, the purified RNA is converted into cDNA by the enzyme reverse transcriptase. The cDNA is then amplified during qPCR. For the RT-qPCR reactions in this thesis, I have used a one-step RT-qPCR method, where both RT and qPCR reactions occur within the same tube, simplifying the procedure.

During qPCR, primers specifically designed for the target gene bind to its corresponding DNA sequence, which is then amplified in cycles. SYBR Green, a fluorescent dye, binds to the amplified DNA, and fluorescence increases proportionally with the amount of DNA produced. As SYBR Green binds to any double-stranded DNA (dsDNA), it is crucial to perform melting curve analysis to verify the specificity of the qPCR product and avoid detecting non-specific products.

The number of cycles required for the fluorescence signal to reach a detectable level is referred to as the cycle threshold (Ct) value, which is used to quantify the initial amount of target gene expression. To account for sample-to-sample variations, I normalize the data to 18S ribosomal RNA (18S), a highly stable and consistently expressed housekeeping gene.

Protein Detection

Western Blotting (Study I-IV)

Western blotting is a widely used technique for detecting and analyzing specific proteins in a sample through antibody-based detection. The process begins with protein extraction using a lysis buffer containing sodium dodecyl sulphate (SDS), which disrupts cell membrane and releases proteins. The total protein concentration is then quantified using a Lowry-based colorimetric assay, to ensure equal loading of protein in each lane of the gel.

Proteins are separated by size using SDS-PAGE electrophoresis, where SDS denatures the proteins and gives a uniform negative charge, allowing them to migrate through the gel in response to an electric current. Smaller proteins move faster, while larger ones travel more slowly. After electrophoresis, the separated proteins are transferred to a nitrocellulose membrane through an electric field, enabling them to move out of the gel and bind to the membrane.

To minimize non-specific antibody binding, the membrane is blocked with a solution containing casein. The membrane is then incubated with a primary antibody specific to the target protein, followed by a secondary antibody conjugated to horseradish peroxidase (HRP), that recognizes and binds to the primary antibody. HRP is an enzyme that generates a chemiluminescent signal upon substrate addition. This signal allows visualization and imaging of the protein bands.

The intensity of the protein bands correlates with the protein levels and can be quantified. Endogenous loading controls, such as housekeeping proteins, are crucial to ensure accurate comparisons. Western blotting provides information about the relative protein levels and can detect post-translational modifications. However, the success of the technique relies on the quality and specificity of the antibodies used.

Dot blotting (Study I, II and VI)

In papers I, II, and VI, I have performed dot blotting, a simplified immunoblotting technique used to detect proteins in a sample without the need for gel electrophoresis. In this method, small droplets of the sample are applied directly onto a nitrocellulose membrane, forming discrete dots. Once the membrane dries, the proteins are immobilized. Similar to Western blotting, the membrane is then blocked to prevent non-specific binding, followed by incubation of primary antibody specific to the target protein and a secondary antibody for detection. Dot blotting is advantageous for its speed and simplicity, as it bypasses protein separation and requires minimal sample volume. However, unlike Western blotting, dot blotting does not provide information about protein size or molecular weight,

and it can sometimes have lower specificity. Quantifying protein levels is also more challenging, making this technique better suited for qualitative or presence/absence detection.

ELISA (Study I)

In paper I, enzyme-linked immunosorbent assay (ELISA) was used to quantify the levels of the pro-inflammatory cytokines MCP-1 and IL-8 in hCASMCs transduced with null or myocardin-related transcription factor A (MRTF-A) viruses, under control conditions or in response to lipopolysaccharide (LPS) treatment.

A sandwich ELISA was used for this purpose. This method relies on the "sandwich" formation between a capture antibody, the target antigen, and a detection antibody. First, the ELISA plate is coated with a capture antibody specific to the target antigen, which is immobilized to the well surface. A blocking buffer is applied to prevent non-specific binding. The sample is then added, allowing the target antigen to bind to the capture antibody. After washing off unbound proteins, a biotinylated detection antibody is added, which binds to a different epitope on the antigen. Streptavidin conjugated to HRP is subsequently added, binding to the biotinylated detection antibody. Following another wash step, a substrate is added, and the HRP enzyme catalyzes a color change reaction proportional to the antigen concentration. Finally, a stop solution is added to stop the reaction, turning the color to yellow, and the absorbance is measured at 450 nm.

To quantify the target proteins (MCP-1 and IL-8), a standard curve is generated using known concentrations of the proteins, allowing the absolute amount of protein in a sample to be calculated in pg/ml. ELISA is a highly sensitive technique that offers superior quantitative measurement of protein concentrations compared to the other immunoblotting methods. However, it requires high-quality, contaminant-free samples, as impurities can affect the accuracy of the results. The method also relies on the antibody quality, with potential cross-reactivity and a risk for false positives.

Immunocytochemistry (Study I, and IV)

Immunocytochemistry (ICC) is a widely used technique for detecting and visualizing specific proteins within individual cells using antibodies that specifically bind to the target protein. In my studies, cells were cultured on coverslips and treated with adenoviral vectors for overexpression (paper I) or with LL-37 and MitoTracker (paper IV) to study the effects on protein localization. After the experimental treatments, cells were fixed with 4% paraformaldehyde, which preserves cellular structures and proteins in their native state.

Following fixation, the cells were permeabilized using Triton X-100 to allow the antibodies to access intracellular proteins. To minimize non-specific antibody binding, a blocking buffer containing bovine serum albumin (BSA) was applied. The cells were then incubated with a primary antibody designed to specifically bind to the protein of interest. After excessing primary antibody was washed off, the cells were incubated with a fluorescently labeled secondary antibody, which binds to the primary antibody. The fluorescent signal was then detected using a fluorescence microscope, enabling the detection of the localization and distribution of the target protein within the cell. ICC is a good method for highly specific visualization of protein distribution within cells. By using different fluorophores, the technique can be used to detect multiple proteins simultaneously, making it ideal for co-localization studies. Much like immunoblotting, ICC relies on the quality and specificity of the antibodies used, and poor-quality antibodies can lead to non-specific results. Fixing cells provides a static snapshot and cannot be used to study dynamic processes in live cells.

Co-Immunoprecipitation (Study I-II)

Co-immunoprecipitation (Co-IP) is a powerful technique used for studying protein-protein interactions. By using antibodies to isolate a target protein from a sample, Co-IP enables the identification of proteins that interact with the target, providing insight into protein complexes and potentially revealing novel binding partners. In papers I and II, Co-IP was performed to investigate interactions between MRTF-A and key signaling proteins, including RelA in the NF-κB pathway and TANK-binding kinase 1 (TBK1), which is involved in both NF-κB signaling and the cGAS-STING pathway.

The process begins with an antibody, specific to the target protein, immobilized on resin in a column. In these studies, antibodies against MRTF-A were used. After overexpression of MRTF-A, cells were lysed in a buffer designed to preserve protein structure and interactions. The lysate is then applied to the column, where the antibody binds to the target protein and its interacting partners. Following several wash steps to remove non-specifically bound proteins, the target protein and associated proteins are eluted and analyzed by immunoblotting to confirm and identify co-precipitated proteins.

Co-IP is valuable for capturing protein interactions in their native cellular environment, making it highly relevant for studying physiological complexes. However, as with all antibody-based techniques, the success of Co-IP depends on the quality of the antibodies, and non-specific binding can lead to noise and false-positive results.

Cell Cytotoxicity Assays

MTT Assay (Study III)

In paper III, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was used to evaluate cell viability after treatment with different concentrations LL-37. This colorimetric assay assesses cell viability and proliferation by measuring the metabolic activity of cells, which reduces yellow MTT into purple formazan crystals. The reduction occurs in the mitochondria of viable cells, making the assay dependent on mitochondrial function.

For this experiment, cells were seeded in a 96-well plate and treated with varying concentrations of LL-37 dissolved in dimethyl sulfoxide (DMSO), while control cells received DMSO only. After 3 h of treatment, MTT reagent was added and incubated for 1 h to allow uptake and conversion. Metabolically active cells converted the MTT dye into purple formazan crystals. Following incubation, the culture medium was removed, and the formazan crystals were solubilized using DMSO. The resulting purple color, proportional to viable cells, was measured at 540 nm. Higher absorbance values indicated greater metabolic activity.

The MTT assay provides a straightforward and quantitative measure of cell viability and is adaptable for high-throughput screening. However, since it reflects only metabolic activity, it cannot differentiate between increased cell numbers and enhanced metabolic rates. Therefore, it is important to complement these findings by counting the number of cells per well in each experiment.

LDH Assay (Study III)

In paper III, the LDH (lactate dehydrogenase) assay was performed to evaluate cell membrane integrity and assess cytotoxicity following LL-37 treatment. The LDH assay is a colorimetric method that detects LDH, an enzyme normally found in the cytoplasm. When the cell membrane is damaged, LDH is released into the surrounding medium, making it a reliable indicator for membrane disruption.

In this experiment, cells were treated with varying concentrations of LL-37 for 30 minutes. After incubation, the cell culture medium was collected, and NADH and pyruvate solutions were added. If LDH was present in the medium, it catalyzed the conversion of pyruvate to lactate, oxidizing NADH to NAD+. The absorbance of NADH at 340 nm was measured, with a decrease in absorbance indicating higher LDH levels and thus greater membrane permeabilization. Controls included a positive control, where sonication induced maximum LDH release, and a negative control (DMSO-treated cells) to measure background LDH levels.

The LDH assay is a rapid and easy method for quantitatively assessing membrane integrity. However, it detects general membrane damage and cannot differentiate between types of cell death.

Subcellular Fractionation (Study IV)

In paper IV, subcellular fractionation was performed and optimized (141) to investigate the effects of LL-37 on mitochondrial membrane integrity. This technique separates cellular components based on their size and density, allowing for the isolation of specific organelles, including nuclei, cytoplasm and mitochondria.

After treating the cells with LL-37 (using DMSO as a negative control), cells were lysed using mechanical disruption via a Dounce homogenizer to break open the cells while preserving the integrity of the organelles. The homogenization was performed on ice to prevent protein degradation. The lysate was then subjected to differential centrifugation, which separates cellular components according to their size and density. This allowed the collection of distinct fractions containing the nuclei, cytoplasm, and mitochondria. The purity of each fraction was verified via Western blot analysis using specific marker proteins for each cellular compartment. However, it is important to note that Western blot analysis only detects the target proteins being investigated, meaning there may be impurities present in the sample that go undetected.

Results and Discussion

In this section, the key findings from the included papers are summarized, and the results are briefly discussed. For more detailed information, please refer to the original publications.

Paper I

Cell Type Dependent Suppression of Inflammatory Mediators by Myocardin Related Transcription Factors

The role of myocardin-related transcription factors (MRTFs) in smooth muscle cells (SMCs) has traditionally been linked to regulating smooth muscle differentiation, primarily through activation of contractile genes. However, recent studies suggest both anti-inflammatory and pro-inflammatory roles of MRTFs, particularly in vascular inflammation, though the results have been inconsistent (56,57,59,142–145). Given the reduction of MRTF levels observed in atherosclerosis (142), we hypothesized that this decrease may contribute to vascular inflammation. The objective of this study was to investigate the influence of MRTFs on inflammatory signaling pathways, findings which may have an impact on human arterial disease.

Using a previously generated RNA-seq dataset (43), we found that myocardin overexpression in human coronary artery SMCs (hCASMCs) significantly reduced several pro-inflammatory transcripts, including *CCL2*, *CXCL8*, *IL6*, and *IL1B*. This anti-inflammatory effect was validated by adenoviral overexpression of MRTF-A, MRTF-B, and myocardin in hCASMCs, all of which consistently suppressed these inflammatory mediators (Figure 14A).

To extend these findings *in situ*, we analyzed transcriptomic data from the GTExPortal (146) to assess correlations between MRTFs and inflammatory mediators in human arteries. While myocardin and MRTF-A exhibited a negative correlation with inflammatory transcripts, MRTF-B did not, suggesting potential functional divergence among MRTF family members despite their shared anti-inflammatory effects in vitro. Based on these observations, and due to limitations in myocardin antibody performance, we focused our work on MRTF-A.

We further examined the anti-inflammatory effect of MRTF-A under pro-inflammatory conditions by stimulating hCASMCs with lipopolysaccharides (LPS). Remarkably, MRTF-A continued to suppress inflammatory mediators even in the presence of LPS (Figure 14B), indicating that its anti-inflammatory function is preserved regardless of inflammatory activation. Additionally, silencing MRTF-A using a short hairpin construct led to increased expression of the pro-inflammatory transcripts (Figure 14C), confirming the specificity of MRTF-A's regulatory role in inflammation.

Interestingly, the anti-inflammatory effect of MRTF-A varied depending on the cell type. While MRTF-A suppressed inflammatory mediators in hCASMCs, it had the opposite effect in human bladder SMCs (Figure 14D) and had minimal to no effect in endothelial cells or monocytes. This suggests that the function of MRTF-A may be context-dependent, influenced by cell type and perhaps by specific phenotypic state of SMCs. Previous studies have reported that SMCs in culture often transition to a synthetic phenotype, which has lower MRTF expression compared to SMCs *in situ* (35). The phenotypic state of SMCs can be influenced by the stiffness of the culture well, with plastic surfaces promoting the synthetic phenotype. This makes plastic a more relevant model for studying atherosclerosis. In our experiments, overexpression of MRTFs in hCASMCs cultured on plastic consistently led to a reduction in inflammation, though this effect may vary depending on the specific culture conditions and the phenotypic state of the cells.

Mechanistically, we explored the interaction between MRTF-A with the NF-κB signaling pathway, previously reported in pulmonary artery SMCs (56). Co-immunoprecipitation (co-IP) experiments revealed that MRTF-A interacts with both RelA (canonical NF-κB pathway, Figure 10) and RelB (non-canonical pathway, Figure 10), suggesting that MRTF-A may inhibit NF-κB signaling by sequestering these transcription factors and preventing their pro-inflammatory activity (Figure 14E). We hypothesized that MRTF-A could interact with either SRF, its canonical binding partner, or one of the Rel proteins (Figure 14F). While silencing RelA attenuated the anti-inflammatory effects of MRTF-A, it did not fully reverse them, indicating that additional mechanisms are likely involved (Figure 14G). When SRF was silenced, only *IL1B* and *CXCL8* transcripts were increased (Figure 14H). Interestingly, neutralization of *IL1B* has been found to reduce cardiovascular mortality in clinical trials (147).

Overall, our findings indicate that MRTFs, particularly MRTF-A, have broad anti-inflammatory effects in vascular SMCs mediated, at least in part, by interactions with the Rel proteins of the NF-κB pathway, though other pathways are likely involved. In vascular inflammatory diseases, SMCs likely contribute by producing pro-inflammatory cytokines and chemokines that recruit immune cells to resolve inflammation, rather than functioning directly as immune cells themselves. These results highlight the potential of MRTFs as therapeutic targets in inflammatory diseases such as atherosclerosis.

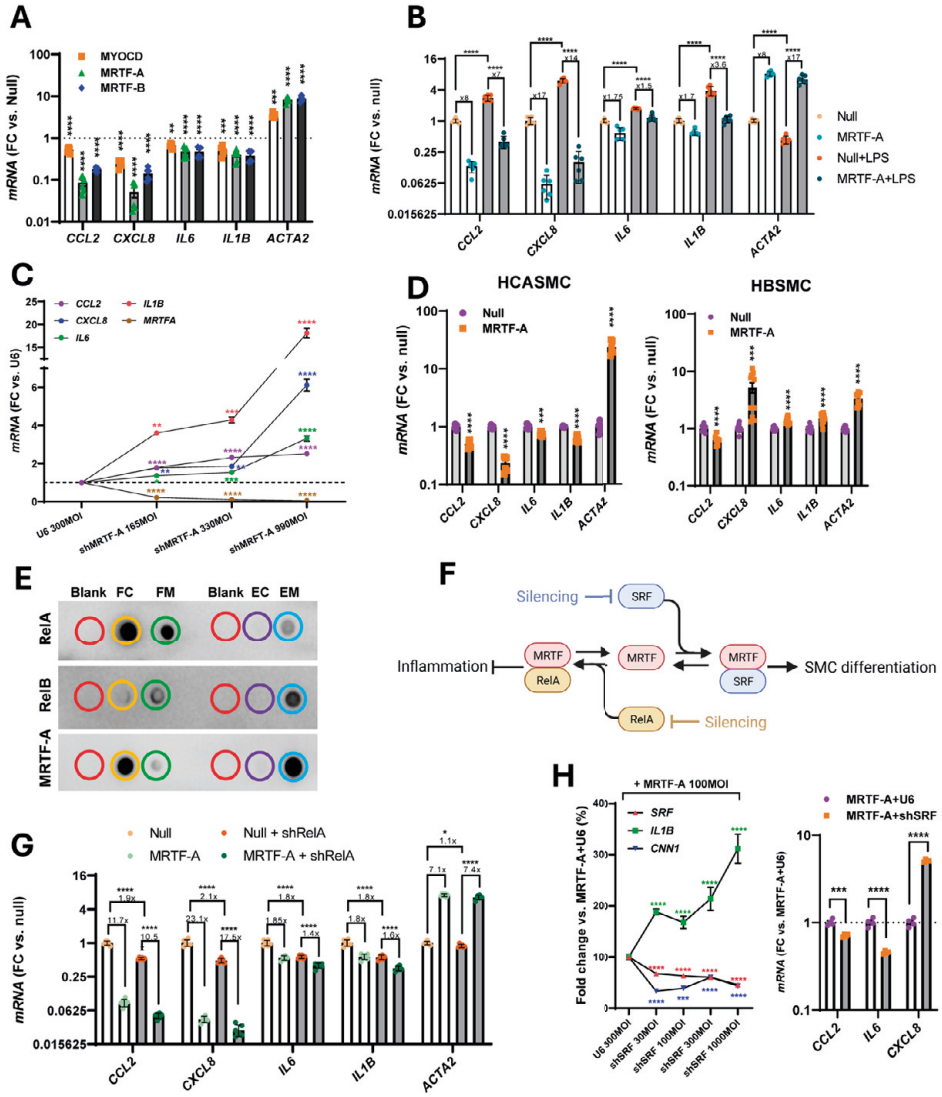

Figure 14. Anti-inflammatory effects of MRTFs in hCASMCs. (A) Overexpression of myocardin, MRTF-A and MRTF-B in human coronary artery smooth muscle cells (hCASMCs) significantly recuded pro-inflammatory mediators, such as *CCL2*, *CXCL8*, *IL6* and *IL1B*. **(B)** MRTF-A's anti-inflammatory effect persisted even in the presence of LPS, indicating its sustained role under pro-inflammatory conditions. **(C)** MRTF-A silencing increased the expression of these pro-inflammatory transcripts, highlighting its regulatory role in suppressing inflammation. **(D)** MRTF-A's effects were cell type-specific, as it suppressed inflammatory mediators in hCASMCs but increased them in human bladder SMCs (hBSMCs). **(E)** Co-immunoprecipitation experiments showed interactions between MRTF-A and RelA, suggesting that MRTF-A might inhibit NF-κB signaling by sequestering RelA. **(F)** It was hypothesized that MRTF-A might interact with either SRF or RelA. **(G)** Silencing of RelA attenuated, but did not fully reverse the anti-inflammatory effect of MRTF-A, implying the involvement of additional mechanisms. **(H)** Silencing of SRF selectively increased *IL1B* and *CXCL8* transcripts, suggesting that SRF is involved in the regulation, while other transcripts like *CCL2* and *IL6* is regulated independently of SRF.

Paper II

Suppression of Smooth Muscle Cell Inflammation by Myocardin-Related Transcription Factors Involves Interaction of TANK-Binding Kinase 1

In paper II, we built upon the findings from paper I, where MRTFs were shown to have anti-inflammatory effects, partly by inhibiting RelA in the NF-κB pathway. In paper II, we aimed to further investigate the mechanisms underlying these anti-inflammatory properties. We hypothesized that MRTFs could suppress inflammation by modulating the cGAS-STING pathway, known for its activation through the TANK-binding kinase 1 (TBK1). TBK1 activation leads to the expression of type I interferons (IFNs) via interferon regulatory factor 3 (IRF3), and it also plays a role in the activation of the NF-κB pathway.

To explore this, we conducted experiments on hCASMCs and human bronchial SMCs (hBrSMCs), given the high expression of STING in both the vascular and pulmonary systems. The connection to the STING-associated vasculopathy of infancy (SAVI), a rare autoimmune disease marked by vascular and pulmonary symptoms (148), further reinforced the relevance of this pathway in our study.

Using an RNA-seq dataset of myocardin-overexpressing hCASMCs, we identified a reduction in 56 cGAS-STING target genes, suggesting that myocardin overexpression inhibits cGAS-STING signaling (Figure 15A). Correlation analysis using arterial tissues from the GTExPortal (146) confirmed that *MYOCD*, *MRTFA*, and *SRF* negatively regulate cGAS-STING target genes (Figure 15B), while *MRTFB* showed no such effect (Figure 15B).

In hBrSMCs, overexpression of MRTF-A resulted in a significant reduction of pro-inflammatory cGAS-STING target genes (Figure 15C). The suppression of key inflammatory mediators, *CCL2* and *CXCL8*, occurred in both a time- and dose-dependent manner (Figure 15D). These findings were further validated in both hBrSMCs and hCASMCs through western blot analysis, which demonstrated that MRTF-A reduced TBK1 phosphorylation, a key enzyme in the cGAS-STING pathway (Figure 15E). Pharmacological inhibition of TBK1 using amlexanox reversed the anti-inflammatory effects of MRTF-A (Figure 15F), whereas STING inhibition (H-151) had little to no effect. This pointed to TBK1 as the specific target of MRTF-A.

Further mechanistic insight was provided by co-IP and proximity ligation assays (PLA) (Figure 15G-H), which revealed that MRTF-A binds to TBK1 in SMCs. The co-IP data showed interactions between MRTF-A and total TBK1 and phospho-TBK1 (Figure 15G), while the PLA data indicated enhanced MRTF-A and total TBK1 interactions after overexpression, both in the cytoplasm and nucleus (Figure 15H, top). Interestingly, the interactions between MRTF-A and phospho-TBK1 was not significantly altered upon overexpression (Figure 15H, bottom), which may

reflect weaker binding affinity or reduced levels of phospho-TBK1 in MRTF-A-overexpressing cells.

TBK1 is a key enzyme in many inflammatory pathways, including those mediated by type I IFNs and NF-κB. MRTF-A's specific inhibition of TBK1, independent of upstream STING activation, raises new questions about whether MRTF-A (and potentially myocardin) could modulate other TBK1-dependent signaling pathways. Given the involvement of TBK1 in inflammatory diseases, the ability of MRTF-A to suppress TBK1 activity presents intriguing therapeutic possibilities. Further studies could explore MRTF modulation as a strategy to mitigate atherosclerosis or other TBK1-associated conditions, particularly those where dysregulated inflammatory response plays a critical role.

The findings in paper II demonstrate that the anti-inflammatory effects of MRTF-A may involve binding and inhibition of TBK1, a key enzyme for many pro-inflammatory pathways such as the cGAS-STING pathway, and the NF-κB pathway. This study adds to the understanding of how MRTFs regulate inflammation in SMCs and identifies a novel mechanism by which MRTFs could protect the vascular wall from inflammation.

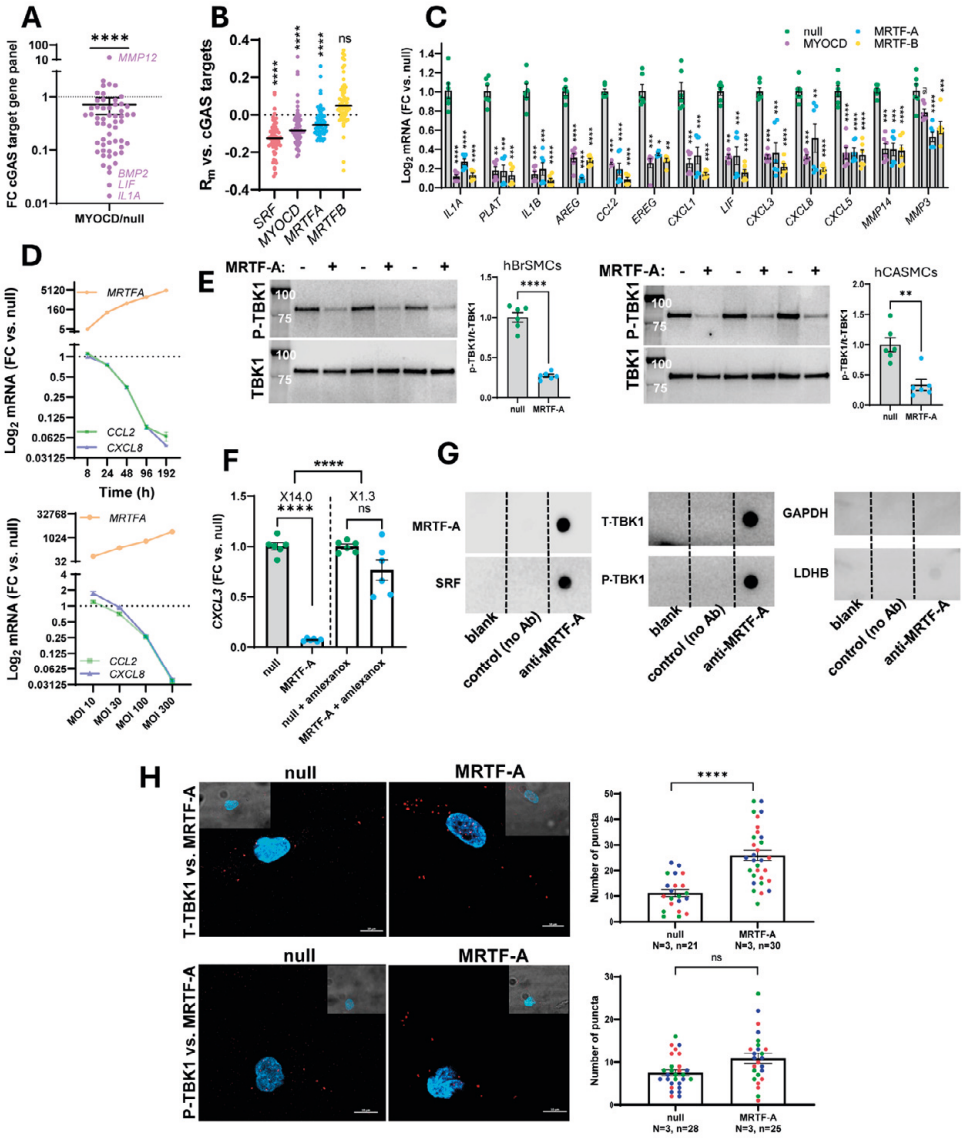

Figure 15. MRTF-A inhibits TBK1 activation in human smooth muscle cells. (A) RNA-seq data from hCASMCs overexpressing myocardin showed a significant reduction in 56 cGAS-STING target genes, suggesting a regulatory role of myocardin in cGAS-STING signaling. **(B)** Correlation analysis GTExPortal confirmed that *MYOCD*, *MRTFA* and *SRF* negatively correlated with cGAS-STING taget genes, while *MRTFB* did not. **(C)** In hBrSMCs, MRTF-A overexpressing significantly reduced pro-inflammatory cGAS-STING target genes. **(D)** This suppression of *CCL2* and *CXCL8* was time- and dose-dependent. **(E)** Western blot analysis showed that MRTF-A reduced TBK1 phosphorylation (p-TBK1), indicating reduced activation. **(F)** TBK1 inhibition by amlexanox reversed MRTF-A's anti-inflammatory effects. **(G)** Co-immunoprecipitation revealed MRTF-A binding to both total and p-TBK1. **(H)** Proximity ligations assays showed increased interactions between MRTF-A and total TBK1, but not p-TBK1, suggesting that MRTF-A may primarily target TBK1, or reduced levels of phospho-TBK1 after MRTF-A overexpression.

Paper III

LL-37-Induced Caspase-Independent Apoptosis Is Associated with Plasma Membrane Permeabilization in Human Osteoblast-Like Cells

In this study, we investigated the pro-apoptotic effects of the human host defense peptide LL-37 on human MG63 osteoblast-like cells. High concentrations of LL-37 (4-10 μM) reduced cell viability and number in a concentration-dependent manner (Figure 16A-B). Morphological changes, such as membrane blebbing and cell shrinkage, which are hallmarks of apoptosis, were evident following treatment with 10 μM LL-37 for 24 h (Figure 16A). However, lower concentrations (1 μM), did not impact cell viability or number (Figure 16B).

To assess whether LL-37-induced cytotoxicity was associated with apoptosis, we conducted TUNEL staining and Annexin V flow cytometry (Figure 16C-D). LL-37 (4 μM) increased the population of early apoptotic cells, indicated by Annexin V positive cells (Figure 16D), and caused DNA fragmentation observed by positive TUNEL staining (Figure 16C). These results indicated apoptotic cell death. However, LL-37 did not trigger cleavage of caspase-3 or PARP, two key markers of classical caspase-dependent apoptosis (Figure 16E). This indicates that LL-37-induced apoptosis in MG63 cells follows a caspase-independent mechanism.

Further investigation suggested that the effects of LL-37 might be linked to plasma membrane permeabilization. Both LL-37 and the detergent Triton X-100 significantly decreased cell viability and increased LDH release, a marker of membrane damage (Figure 16F). LL-37 is thought to permeabilize membranes by forming pores. At higher concentrations, once it reaches a critical concentration at the membrane surface, LL-37 acts like a detergent. In this study, LL-37 permeabilized the membrane at 4 μM, whereas Triton X-100 required substantially higher concentrations (130-180 μM), suggesting different mechanisms of membrane permeabilization. Like LL-37, Triton X-100 induced DNA fragmentation (Figure 16G) and an increase in Annexin V positive cells. Despite inducing membrane permeabilization and apoptosis, neither LL-37 nor Triton X-100 triggered the cleavage of PARP (Figure 16H), confirming that both agents promote caspase-independent apoptosis.

One notable consequence of plasma membrane permeabilization is the potential leakage of damage-associated molecular patterns (DAMPs) from cells. DAMPs could further amplify the inflammatory response by activating innate immune receptors such as Toll-like receptors (TLRs) and the inflammasome. Released DAMPs can also induce other programmed cell deaths, raising the possibility that LL-37 might induce necroptosis or pyroptosis. Both necroptosis and pyroptosis are characterized by plasma membrane rupture and the release of inflammatory mediators, which may contribute to a broader inflammatory environment.

It would be interesting for future studies to explore whether LL-37 triggers necroptosis or pyroptosis alongside with apoptosis and how these pathways might interact in tissues experiencing chronic inflammation. This could deepen our understanding of how LL-37 contributes to inflammatory diseases and its potential role for therapeutic intervention.

LL-37-induced cytotoxicity in host cells may have a functional role. It has been proposed that damaged or infected cells are more susceptible to LL-37-induced cytotoxic effects (149,150). This mechanism could serve as a way for the innate immune system to resolve inflammation by clearing damaged cells that would otherwise contribute to prolonged inflammation.

These findings suggest that LL-37 induces apoptosis in MG63 cells through a caspase-independent pathway, primarily associated with plasma membrane permeabilization. This mechanism could play a role in tissue damage seen in inflammatory conditions, such as periodontitis, where high local concentrations of LL-37 have been observed.

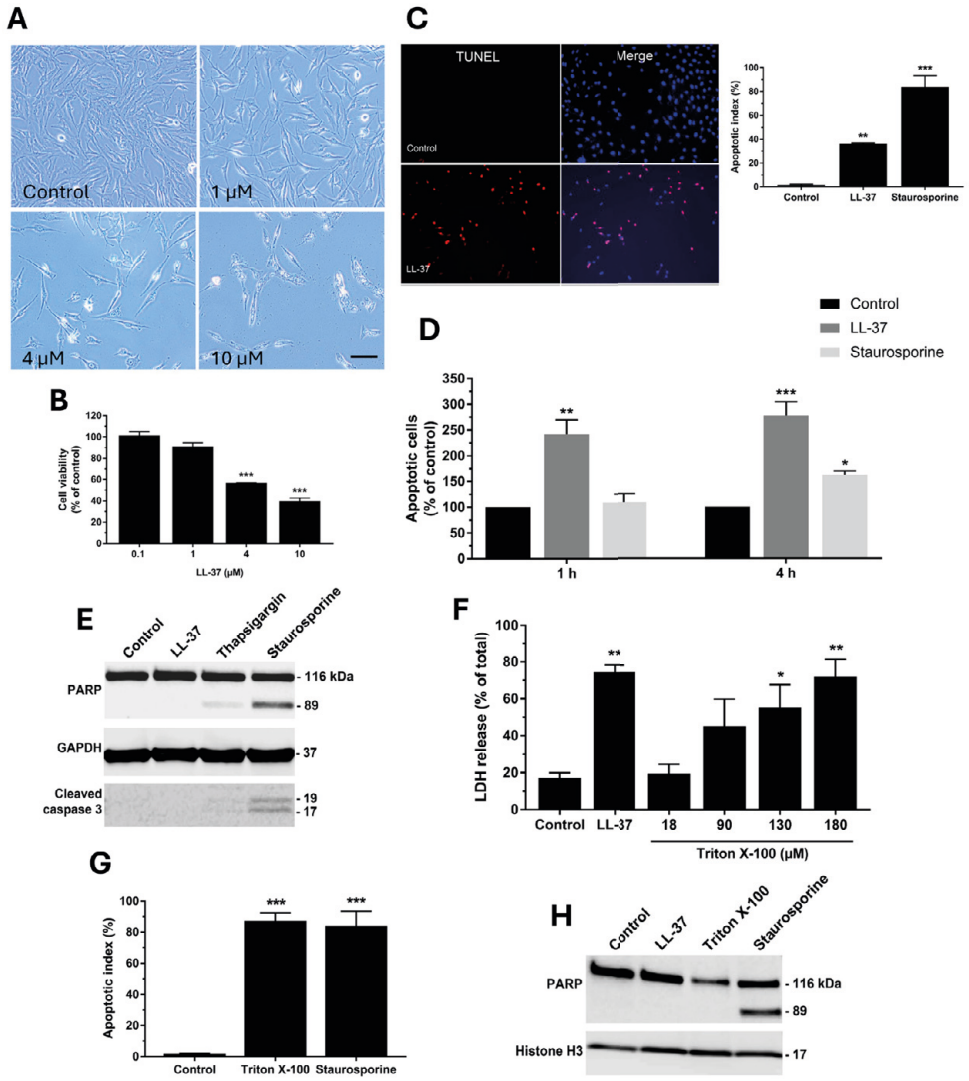

Figure 16. LL-37 induces caspase-independent apoptosis associated with membrane permeabilization. (A) LL-37 treatment reduces the number of MG63 cells in a concentration-dependent manner. Morphological changes characterized by apoptosis, such as membrane blebbing and cell shrinkage, are observed following treatment with 10 μM LL-37. **(B)** High concentration of LL-37 significantly reduces cell viability, while low concentration (1 μM) do not impact cell viability or cell number. **(C)** Positive TUNEL staining indicates that LL-37 induces DNA fragmentation, a hallmark of apoptosis. **(D)** Annexin V flow cytometry confirms that LL-37 increases the population of early apoptotic cells. **(E)** LL-37 treatment does not trigger PARP or caspase-3 cleavage, suggesting apoptosis occurs through a caspase-independent pathway. **(F)** Both LL-37 and Triton X-100 decrease cell viability and increase LDH release, with LL-37 permeabilizing the membrane at a lower concentration than Triton X-100. **(G)** Triton X-100 also induces DNA fragmentation, similar to LL-37, as shown by positive TUNEL staining. **(H)** Neither LL-37 nor Triton X-100 induce PARP cleavage, confirming that both promote caspase independent apoptosis, which may be linked to membrane permeabilization.

Paper VI

The Antimicrobial Peptide LL-37 Triggers Release of Apoptosis-Inducing Factor and Shows Direct Effects on Mitochondria

In paper VI, we further explored the mechanisms behind LL-37-induced apoptosis, building on the caspase-independent pathway studied in paper III. Specifically, we aimed to investigate the direct interactions of LL-37 with mitochondrial membranes, which had not been extensively studied.

We first examined the uptake and intracellular distribution of LL-37 in MG63 cells using immunocytochemical analysis. Following treatment with LL-37 (4 μM) for 60 minutes, cytoplasmic immunoreactivity was observed, indicating rapid internalization of the peptide. LL-37 primarily accumulated in the perinuclear region of the cytoplasm, co-localizing with mitochondria, as indicated by MitoTracker staining (Figure 17A). Notably, this mitochondrial localization was observed as early as 20 minutes after LL-37 exposure, highlighting the peptide's rapid interaction with mitochondrial structures.

To assess whether LL-37 permeabilizes mitochondrial membranes and facilitates the release of mitochondrial proteins, we performed subcellular fractionation and Western blot analysis. Treatment with LL-37 (8 μM for 2 h) induced the release of apoptosis-inducing factor (AIF) into the cytosol, while cytochrome C oxidase subunit IV (COX IV), a marker of the inner mitochondrial membrane, remained unaffected (Figure 17B). This suggests that LL-37 selectively permeabilizes the outer mitochondrial membrane, allowing AIF release while preserving inner membrane integrity.

Further experiments using isolated mitochondria demonstrated that LL-37 increases the release of both AIF and cytochrome C, as shown by dot blot analysis (Figure 17C). LL-37-treated mitochondria released 50% more AIF and three times more cytochrome C compared to controls, providing direct evidence of the permeabilizing effects of LL-37 on mitochondrial membranes.

To further examine the underlying mechanism, we investigated whether LL-37 directly interacts with lipid membranes similar to those of mitochondria. Using model lipid vesicles (both giant and small unilamellar vesicles), we demonstrated that LL-37 induces membrane permeabilization in a concentration-dependent manner, leading to the release of encapsulated dye from the vesicles. When vesicles were prepared with the dye on the outside, LL-37 facilitated its leakage into the vesicle interior, indicating a loss of membrane integrity (Figure 17D). At higher concentrations, LL-37 caused complete vesicle rupture, reinforcing its potent membrane-disrupting capacity.

An interesting point to raise is the fact that mitochondrial membranes share similarities with bacterial membranes, particularly in the presence of the lipid

cardiolipin, which is unique to these membranes. The endosymbiotic theory suggests that mitochondria originated from an ancestral bacterium that was engulfed by a eukaryotic cell, becoming an integral part of the cell structure. Given that LL-37 is an antimicrobial peptide, that targets bacterial membranes, it is tempting to speculate that this evolutionary connection could explain its affinity for mitochondrial membranes. LL-37 may recognize mitochondrial membranes as resembling bacterial membranes, thereby triggering permeabilization and apoptosis in a caspase-independent manner. Additionally, it would be interesting to explore whether mitochondrial membrane disruption by LL-37 could also release DAMPs, potentially activating other signaling pathways.

However, in our study, experiments with membrane vesicles revealed that LL-37 permeabilized all vesicles, regardless of their lipid composition, suggesting that hydrophobic properties may have a more critical role than the specific charge of lipid components. It is important to note that these experiments provide an all-or-nothing result. Further investigation into the extent to which LL-37 permeabilizes different cellular membranes would be valuable for a deeper understanding of its effects.

Overall, these findings show that LL-37 rapidly accumulates in mitochondria, permeabilizes mitochondrial membranes, and triggers the release of pro-apoptotic factors such as AIF and cytochrome C. This suggests that LL-37 induces apoptosis through direct effects on mitochondria, contributing to its cytotoxic properties, particularly in inflammatory diseases where LL-37 levels are elevated.

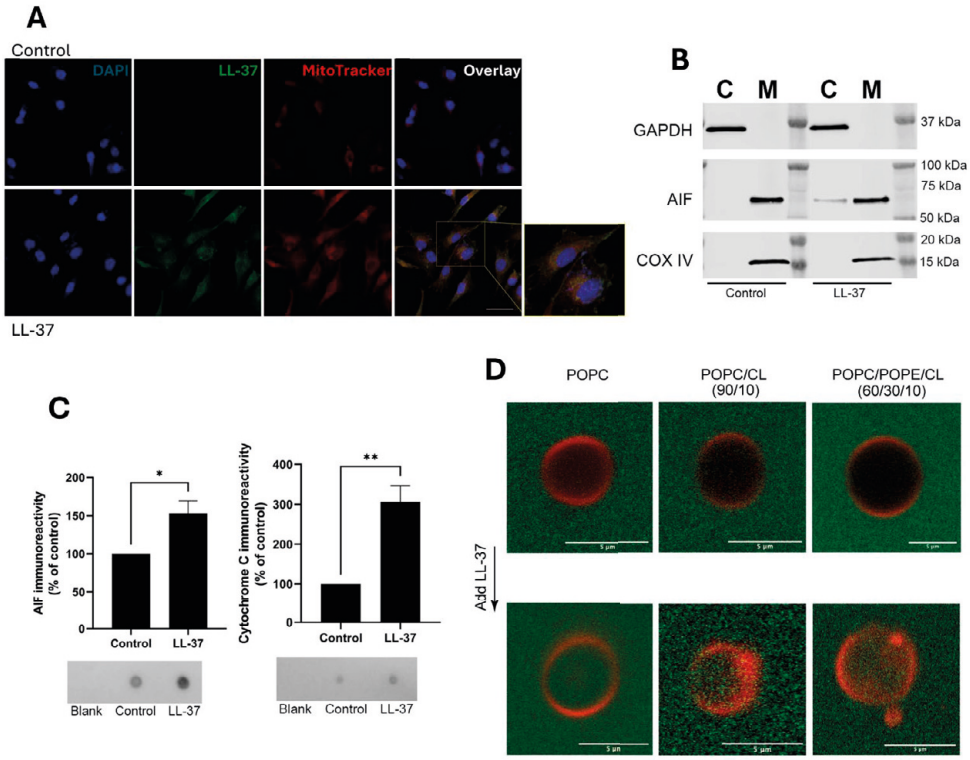

Figure 17. LL-37 induces mitochondrial membrane permeabilization and release of apoptosis inducing factor (AIF). (A) Immunocytochemical analysis showing rapid internalization of LL-37 in MG63 cells. LL-37 co-localizes with MitoTracker in the perinuclear region, indicating mitochondrial accumulation. **(B)** Western blot analysis of subcellular fractions showing that LL-37 treatment leads to the release of AIF into the cytosol, suggesting outer mitochondrial membrane permeabilization. No release of cytochrome C oxidase subunit IV (COX IV), a marker of inner mitochondrial membrane integrity, was observed, indicating preserved inner membrane integrity. **(C)** Dot blot analysis of isolated mitochondria treated with LL-37 showing increased release of AIF and cytochrome C, confirming mitochondrial membrane permeabilization by LL-37. **(D)** Model lipid vesicle experiments demonstrate that LL-37 permeabilizes membranes in a concentration-dependent manner, leading to the leakage of dye across the lipid vesicles.

Concluding Remarks

Innate immunity encompasses a wide range of cellular responses and activates various inflammatory pathways. Many inflammatory and autoimmune diseases stem from uncontrolled and persistent sterile inflammation. Gaining a deeper understanding of the triggers behind sterile inflammation and the mechanisms that regulate these inflammatory pathways is critical for identifying novel approaches to modulate the immune response and promote tissue regeneration. The findings presented in papers I-VI underscore the significant roles that myocardin-related transcription factors (MRTFs) and LL-37 play in innate immune response of non-classical immune cells. By elucidating their functions, these studies contribute to the broader understanding of inflammatory and autoimmune diseases and may pave the way for novel therapeutic targets.

Key conclusions from this thesis include:

- MRTFs demonstrate a cell type-specific anti-inflammatory effect in human coronary artery smooth muscle cells (hCASMCs) through interaction with RelA and inhibition of NF-κB signaling.
- MRTFs suppress inflammation in both vascular and airway smooth muscle cells by inhibiting TBK1, a key kinase involved in the activation of the NF-κB and cGAS-STING pathway.
- LL-37 induces cell death via a caspase-independent apoptotic pathway, which is associated with plasma membrane permeabilization in host cells.
- LL-37 permeabilizes mitochondrial membranes, triggering the release of pro-apoptotic factors such as apoptosis-inducing factor (AIF) and cytochrome C, which in turn induces apoptosis in host cells.

Future Perspectives

Sterile inflammation is commonly associated with chronic inflammation and autoimmune diseases. Understanding the mechanisms behind this type of inflammation and identifying new therapeutic targets holds great promise for the future. Based on the findings from this thesis, several potential angles for future research have emerged.

One promising direction is the development of MRTF-based therapies aimed at preventing the transition of smooth muscle cells (SMCs) to the synthetic state, thereby maintaining vascular integrity and potentially preventing diseases like atherosclerosis. MRTF levels drop with age, and age is a risk factor of many vascular diseases. By targeting MRTFs, we could modulate the balance between synthetic and contractile phenotypes in vascular diseases. As shown in the thesis, MRTFs regulate not only the phenotype of SMCs but also their inflammatory responses. This suggests that MRTF modulation could be explored as a therapeutic strategy, not only to maintain vascular health, but also to reduce inflammation.

Additionally, the binding of MRTFs to TANK-binding kinase 1 (TBK1), as demonstrated in the thesis, highlights another future research topic. It remains to be explored whether MRTFs interact with TBK1 or other key signaling molecules in different tissues and cell types, expanding the therapeutic potential of MRTF modulation beyond the vascular system. MRTF-A and MRTF-B is expressed by many different cell types in different tissues, exploring their regulatory role beyond contractility is of interest. TBK1 is involved in multiple inflammatory pathways, understanding how MRTFs regulate TBK1 activity in different context could reveal new pathways for controlling inflammation in diverse diseases.

The host defense peptide LL-37 has shown dual roles depending on the concentration and cellular environment. While high concentrations of LL-37 induce apoptosis of host cells, lower concentrations have been shown to promote wound healing and the resolution of inflammation. Future studies could focus on dose-dependent therapies using LL-37 or its derivatives, harnessing its pro-healing properties while minimizing its cytotoxic effects.

Exploring non-apoptotic cell death pathways, such as necroptosis and pyroptosis, also presents an interesting future research direction. While LL-37 induces caspase-independent apoptosis by permeabilizing the cellular membrane, it could potentially

also trigger other forms of cell death, such as necroptosis and pyroptosis, which are known to play roles in sterile inflammation.

Furthermore, chronic inflammation caused by DAMPs becomes increasingly prevalent with age as the body's autophagy systems decline. The aging process is accompanied by an increase in sterile inflammation across various organs and tissues. For example, neuroinflammation is believed to play a key role in the progress of neurodegenerative diseases such as Alzheimer's disease. Identifying inflammatory pathways involved in sterile inflammation and developing targeted therapies could hold the key to treating these age-related inflammatory diseases. Current anti-inflammatory drugs, such as the glucocorticoids, for example dexamethasone, often have broad effects and significant side effects, as they target pathways like NF-κB at an upstream level. Therefore, a more focused approach targeting specific inflammatory pathways may yield more effective treatments with fewer side effects.

Lastly, the complex interactions between various inflammatory pathways suggest that future research should aim to map out the connections between these pathways more comprehensively. Many inflammatory pathways are interconnected in highly complex systems and understanding how they influence each other could lead to the identification of more disease-specific therapeutic targets. This would allow for the development of drugs that more precisely inhibit the inflammatory pathways implicated in specific diseases, potentially modernizing the treatment of chronic inflammatory conditions.

By addressing these points, future research has the potential to pave the way for novel, more effective therapies that can better manage sterile inflammation and its consequences in various diseases.

Acknowledgements

The projects of this thesis would not have been possible and my time as a PhD student would not have been the same, without the support of colleagues, friends and family. For that I am grateful and would like to express my warmest gratitude in particular to the people mentioned below.

First and foremost, I would like to thank my main supervisor **Bengt-Olof Nilsson**. I could not have asked for a more enthusiastic and supportive supervisor. Thank you for believing in me and for always having your door open when I have questions or want to discuss ideas. When I started as a master student in your lab, little did I know about the PhD journey I was about to take on. During my four years of studies, I have become a more confident and independent researcher thanks to you, and I cannot thank you enough for your guidance during this journey. You have always been a very present supervisor, waiting with excitement for the results and the following discussion, which I am truly grateful for. You have taught me that patience and a positive attitude are key traits for success, both in academia and in life in general. I am very grateful for your mentorship and the opportunity to pursue my PhD studies in your group.

Next, I would like to thank my co-supervisor **Karl Swärd**. Even though you are not my main supervisor on the paper, you have been like a second main supervisor to me during my four years of studies. Thank you for being the force forward. You have taught me to think big, novel, and dare to aim at the more challenging research questions. You always have great ideas and are the force that pushes the boundaries of the research questions. I am so grateful for your support during these years.

I would also like to thank my co-supervisor, **Catarina Rippe**. I cannot imagine what my time in the lab would have been like without you. Thank you so much for always being available for discussing research related questions as well as giving personal advice and believing in me. Thank you for being the person who lights up the room and makes the work environment wonderful. I would also like to thank you for being one of the few people that believed in me during Vasaloppet and for dragging me out on roller skis to prepare me for the race.

Katarzyna Kawka, you have been the best officemate one could ask for during these years. Thank you for always supporting and believing in me. You are the glue that holds everything together in this larger research group. Thank you for your personal advice and general discussions about life. We have been laughing, crying,

and probably been talking way too loudly for all the people on the floor to hear, and I do not know what my PhD journey would have been without you.

Next, I would like to thank **Sara Bodahl** and **Alexandra Aidoukovitch**. You two have been my best friends in the lab during my PhD journey. Thank you for welcoming me into the group, for supervising me and showing me around in the beginning. Thank you for all the nice lunches in the sun and for all the fun times we have shared.

Li Liu, I remember when you started your PhD studies when I was still a master student in the group, and we have since then shared much of this journey together. Thank you for great collaboration on projects and for being a good friend and colleague.

Olivia Ritzwall Losand, thank you for being so positive and kind. You are a breath of fresh air in this work environment!

Thank you to the rest of my colleagues in the larger Vascular Physiology group. **Sebastian Albinsson** and **Johan Holmberg**, thank you for sharing your scientific thoughts and valuable advice. And sorry for disturbing you with my loud conversations with Katarzyna. Thank you to all present and former group members that have been part of my PhD journey, **Per Hellstrand**, **Katarzyna Krawczyk**, **Azra Alajbegovic**, **Fatima Daoud**, **Marycarmen Arévalo Martinez**, and **Francesco de Luca**. Thank you for all the interesting conversations and scientific discussions over the years, as well as the social gatherings, lunches, fika breaks, parties, and much more. You have all contributed to making this a fantastic place to work.

I would also like to express my gratitude to the collaborators contributing to the hard work behind every published study. Thank you, **Daniel Svensson,** for sharing your knowledge and being helpful during the projects. **Olof Gidlöf**, for helping with the flowcytometry experiments. **Emma Sparr**, **Martin Lundqvist**, and **Xiaoyan Liu**, for providing expertise in the biophysical experiments as well as teaching and showing me how the experiments were performed. Thank you **Björn Morén** and **Karin Stenkula** for imaging experiments. Thank you, **Thomas Jepps** for letting me visit your lab and **Jennifer van der Horst** for teaching me how to perform PLA.

I would like to thank all the colleagues at D12 for contributing to a nice working place and a good work environment.

Katarzyna Said Hilmersson and **Monika Bauden**, thank you both for always being so inclusive and for all the nice lunches and fika breaks we have shared.

Thank you to **Anja Meissner** and your group members for great discussions during our shared group meetings. Especially thanks to **Lotte Vanherle** for always coming with great advice and questions during the meetings. Thank you, **Frank Matthes**, **Lisa Teresa Porschen**, and **Sevilay Şahoğlu Göktaş**.

Thank you, **Lena Uller** and group members for interesting discussions and fun times. Thank you, **Samuel Cerps**, for all your help with ELISAs and interesting discussions over the years. **Sangeetha Ramu**, thank you for being so helpful and kind. Thank you **Sofia Malm Tillgren** and **Mandy Menzel** for making the work environment a great place.

And a huge thanks to all the other present and former colleagues contributing to making D12 the best place to work, **Jonas**, **Daisy**, **Premkumar**, **Renan**, **Julia M**, **Hodan**, **Björn**, **Kreema**, **Selvi**, **Siv**, **Mario**, **Neha**, **Fredrik**, **Joanna**, **Julia T**, **Cecilia**, **Frida**, **Sofia M**, **Ellen**, **Nicole**, **Carin**, and **Martin**. Thank you all for the Christmas parties, Friday breakfasts, and lunch times.

Especially thanks to the members of the Running Club at the floor, **Catarina**, **Katarzyna**, **Li**, **Lotte**, **Mario**, **Frida**, and **Sofia M**. For helping me stay healthy and sane during the covid pandemic.

I also would like to thank my friends outside of work, thank you to my best girls **Emelie**, **Alisha**, **Frida**, and **Ilona** (and of course your partners **Tim**, **Mattis**, **Erik**, and **Johan**). Thank you for always believing in me and for your endless support. This journey would not have been the same without your encouragement along the way! Thank you all, for the laughs, fun events, dinners, parties, kräftskivor, surströmmingsskivor, escape rooms, and crazy nights. You are the best!

Thank you to my family for their endless support and love. **Mom** and **Dad**, thank you for always believing in me, and for supporting my career choices. You two are my true role models. Even though you probably never expected me to pursue an academic career, you have taken an interest in my research by asking questions and being curious. Thank you for encouraging me to become the best version of myself. Thank you to my younger sister, **Evelyn**. I am so proud of being your sister and your support throughout life has made me the person I am today.

Lastly, I would like to thank my one true love and partner in crime, **Emil Axell**. Thank you for listening to my complaints, ideas and thoughts. Thank you for being my sparring partner and my rock in all situations. We have shared the PhD journey together, all the ups and downs, and I am so grateful for making this journey with you. Thank you for dragging me along on your crazy ideas and travels. Sometimes you give me an ulcer, by being the most spontaneous and crazy person. But you are my person, and I am so glad you want me to come along. With this I would also like to thank your mother, **Pia**, for being a strong and independent woman, a role model and a supportive person during the years I have known you. And finally, your colleagues at KC for always being friendly and inviting me to beer club and barbeque nights after work!

This thesis would not have been possible without you all, so once again thank you!

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