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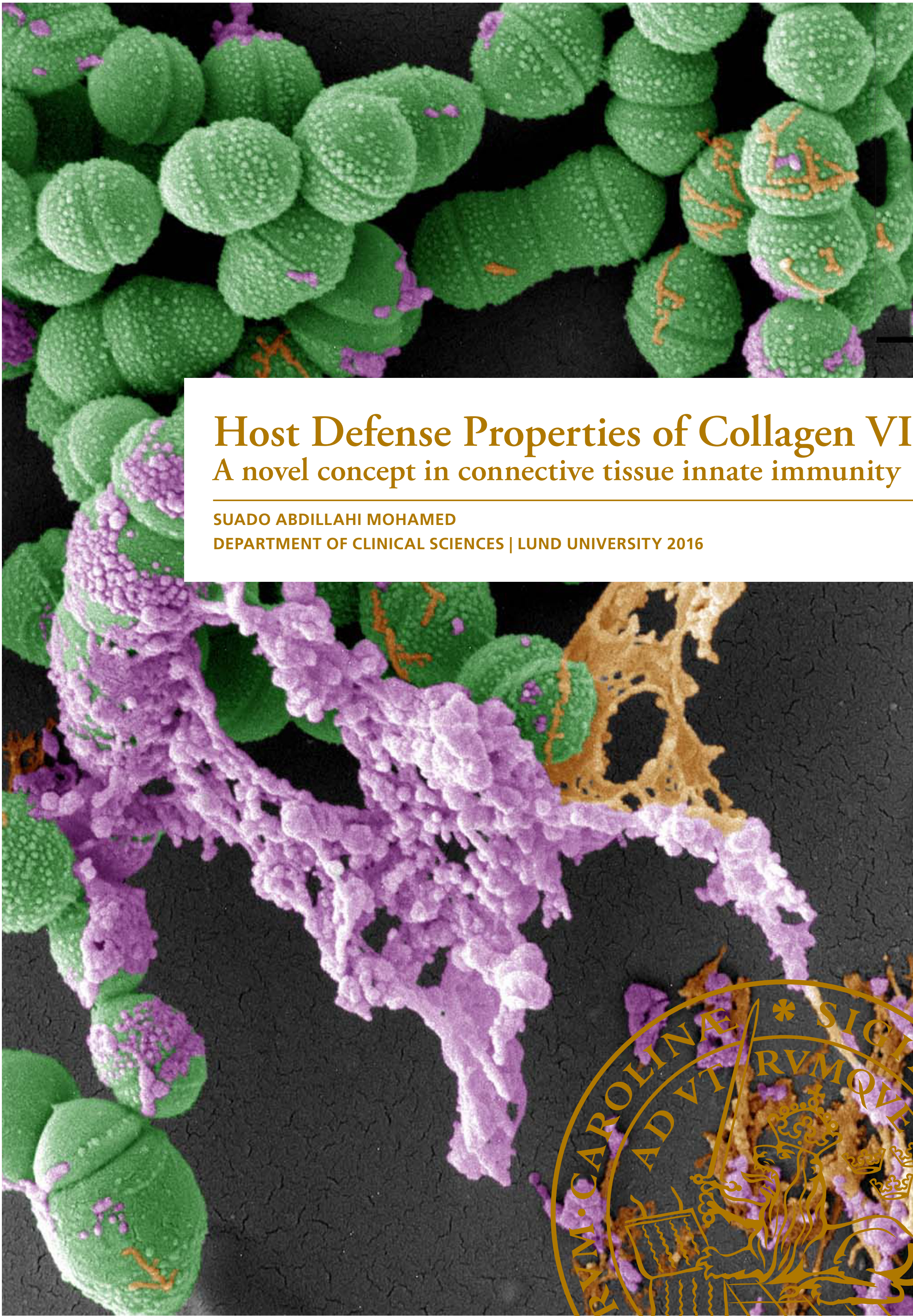

Host Defense Properties of Collagen VI

A novel concept in connective tissue innate immunity

SUADO ABDILLAHI MOHAMED

DEPARTMENT OF CLINICAL SCIENCES | LUND UNIVERSITY 2016

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Suado Abdillahi Mohamed

LUND UNIVERSITY

DOCTORAL DISSERTATION

By due permission of the Faculty of Medicine, Lund University, Sweden, this doctoral thesis will be defended on April 29th 2016 at 9:15 AM in Belfragesalen, Biomedical Center, Lund, Sweden

Faculty opponent

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Key words: **Innate immunity, antimicrobial peptides, collagen VI, chronic obstructive pulmonary disease**

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Cover image: Pseudo-colour scanning electron microscopic image of *Streptococcus pyogenes* (green) subjected to collagen VI treatment. Purple indicates the leakage of the cytoplasmic content. Image courtesy of Matthias Mörgelin, PhD.

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Bismillahi-rahmani-rahim

To my parents and my grandparents ♥

"*Aqoon la'aan waa iftiin la'aan*" means
"*To be without knowledge is to be without light*"

– Somali proverb –

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Preface

> If you know just what you are looking for, finding it can hardly count as a discovery, since it was fully anticipated. But if, on the other hand, you have no notion of what you are looking for, you cannot know when you have found it.
>
> – Steve Sharpin –

I have always been fascinated by our immune system and its ability to constantly protect and defend us against harmful threats posed by our environment. While, writing this thesis, I remember the time when I stayed with grandparents in the countryside in Somalia and I fell and scratched my knees. Since the closest health care centre was miles away, my grandparents went instead to collect leaves from nearby trees. They cleaned the wound and put the crushed leaf material on top of it and told my mom that it would help the body to heal the wound. The Somali nomads, such as my grandparents and their ancestors, have been practising and relying on traditional medicine for a long time and it has become an important part of their lives. However, my grandparents and the generations before them never knew, and never will know, what is in the crude plant material that prevented infections and promoted wound healing?

Today, I am writing a PhD thesis about that same subject. The only difference here is that I am focusing on how components in our connective tissues can protect us from bacterial infections. When I began my PhD, I did not know what to anticipate and nor did I know it would lead to the discovery of a novel host defense molecule, collagen VI. This journey has been immensely rewarding and it has not only given me the opportunity to explore but also to contribute to the world of science. I could not have accomplished all of this without my research group and our wonderful collaborators. Thank you!

I hope whoever reads this book finds it both enjoyable and thought-provoking.

Suado M. Abdillahi

Lund, 24th of March 2016

List of original papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals:

I. **Abdillahi SM**, Balvanović S, Baumgarten M, Mörgelin M. Collagen VI encodes antimicrobial activity: novel innate host defense properties of the extracellular matrix. *J Innate Immun*. 2012; 4(4):371-6.

II. **Abdillahi SM***, Bober M*, Nordin SL, Hallgren O, Baumgarten M, Erjefält J, Westergren-Thorsson G, Bjermer L, Riesbeck K, Egesten A, Mörgelin M. Collagen VI is upregulated in COPD and serves both as an adhesive target and a bactericidal barrier for *Moraxella catarrhalis*. *J Innate Immun*. 2015; 7(5):506-17.

III. **Abdillahi SM**, Tati R, Nordin SL, Baumgarten M, Hallgren O, Bjermer L, Erjefält J, Westergren-Thorsson G, Singh B, Riesback K, Mörgelin M. Collagen VI is a bactericidal barrier against *Haemophilus influenzae in vivo* in chronic obstructive pulmonary disease (COPD). Submitted to *PLoS Pathogens*.

IV. **Abdillahi SM**, Maaβ T, Kasetty G, Strömstedt AA, Baumgarten M, Tati R, Walse B, Wagener R, Schmidtchen A, Mörgelin M. Collagen VI contains multiple host defense peptides with potent *in vivo* activity. Submitted to *J Antimicrob Chemother*.

V. **Abdillahi SM**, Tati R, Strömstedt AA, Baumgarten M, Schmidtchen A, Mörgelin M. Mode of action and immunomodulatory effects of collagen VI-derived host defense peptides. Manuscript.

* These authors contributed equally to this work.

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Abbreviations

AMPs	Antimicrobial peptides
BM	Bethlem myopathy
COPD	Chronic obstructive pulmonary disease
CRAMP	Cathelin-related antimicrobial peptide
DCD-1L	Dermcidin-derived peptide 1L
ECM	Extracellular matrix
GAGs	Glycosaminoglycans
GAS	Group A *Streptococcus*
GOLD	Global Initiative for Chronic Obstructive Lung Disease
HDPs	Host defense peptides
HBD	Human β-defensin
LPS	Lipopolysaccharides
LTA	Lipoteichoic acid
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSCRAMMs	Microbial surface components recognizing adhesive matrix molecules
NTHi	Non-typeable *Haemophilus Influenzae*
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
TLRs	Toll-like receptors
UCMD	Ullrich congenital muscular dystrophy
VWA	von Willebrand factor A-like domains
VWF	von Willebrand factor

Abstract

Rapid and powerful host defense mechanisms are essential in order to overcome harmful actions of pathogenic bacteria. Antimicrobial peptides (AMPs) and proteins are vital effector molecules of the fast-acting innate immune system and exist virtually in all living organisms. They exert a broad spectrum of natural antibiotic activity, but also play important immunomodulatory functions in the host. During the past few decades, host defense molecules have gained remarkable attention as alternative treatments for bacterial infections due to the growing bacterial resistance to current antibiotics.

This thesis sheds light on an intriguing and novel aspect of innate immunity in the context of connective tissues, where collagen VI emerges as a host defense molecule. Collagen VI is an extracellular matrix protein that forms complex microfibrillar networks in most connective tissues. The best studied form of collagen VI is a heterotrimer comprised of three α-chains, α1(VI), α2(VI) and α3(VI), where the majority of these α-chains are flanked by globular domains that share homology with von Willebrand factor type A (VWA) domains. The results presented in this thesis demonstrates that tissue-purified collagen VI exhibits a broad spectrum of antibacterial activity against Gram-positive and Gram-negative bacteria by disrupting the bacterial membranes and causing leakage of intracellular components, which subsequently leads to cell death. Interestingly, the expression of collagen VI was upregulated in the airways of chronic obstructive pulmonary disease (COPD) patients compared to healthy individuals. Upon airway epithelial damage in COPD, we found that collagen VI is exposed and serves both as an adhesive substrate and an antibacterial barrier for a number of pulmonary pathogens. In order to gain deeper insight into the antimicrobial nature of the collagen VI molecule, we identified and characterized cationic sequence motifs in the VWA domains of the α3(VI)-chain. These peptides showed a significant antibacterial activity against Gram-positive and Gram-negative bacteria *in vitro* and *in vivo*. Furthermore, some of them also displayed wound healing and anti-endotoxic properties *in vitro*.

In conclusion, these data reveal for the first time in detail how extracellular matrix proteins, such as collagen VI, provide host defense mechanisms against bacterial infections in connective tissues. These findings also suggest a novel role for collagen VI-derived peptides in innate immunity and provide templates for the development of peptide-based antibacterial therapies.

Chapter 1 – A short introduction to host defense

> Human subtlety will never devise an invention more beautiful, more simple, or more direct than does Nature – because in her inventions, nothing is lacking – and nothing is superfluous.
>
> – Leonardo da Vinci –

Infectious diseases – a serious global health issue

Over the last century, the morbidity and mortality of many infectious diseases has been significantly reduced due to improved hygiene and the development of antibiotics and vaccination programs (1-3). Despite these advances, however, infectious diseases are today one of the leading causes of morbidity and mortality around the globe (4). One of the major challenges is the increase of resistant pathogenic bacteria to conventional antibiotics (5-7). Therefore, it is crucial to expand our knowledge of host-pathogen interactions in order to find novel antimicrobial therapies to fight infectious diseases. This thesis is focused on a novel role of collagen VI in innate immunity and its antimicrobial actions against several important human pathogens in the respiratory tract as well as in the skin.

The host-microbe interplay

Throughout our lifespan, we encounter numerous microorganisms which are either beneficial (commensals) or pose a threat (pathogens) to our survival (8, 9). Interestingly, our bodies contain ten times more microbes than our own cells. The majority of these microbes are important for our health and wellbeing and we are in a symbiotic relationship with them. They provide several essential functions in the host including the production of certain vitamins, energy harvesting and maintaining epithelial integrity (10). Another significant role for them is to prevent pathogens in adhering to and colonizing a particular niche, which is also referred to as "colonization resistance" (11). In the skin, the commensals are able to prevent colonization of pathogenic microbes such as group A *Streptococcus* (GAS), *Staphylococcus aureus*, *Pseudomonas* spp and *Candida albicans*. For instance, the binding of *Staphylococcus epidermidis* to keratinocyte receptors inhibits pathogenic *S. aureus* adhesion (12, 13). The release of fatty acids from sebum breakdowns by *Propionibacterium acnes* induces an acidic environment, which inhibits the growth of *Streptococcus pyogenes* (14). This delicate system is not always in balance and may lead to disease due to alterations in the microbial communities (15). Initial contact with microorganisms can take place through a variety of routes such as external or internal body surfaces. Airborne microorganisms can gain access through airway mucosa (16) while the gastrointestinal mucosa provides a route of entry for microorganisms in food and water (17). External injury to the skin by wounds or burns may allow microbes to bypass the skin barrier and thus directly cause deep tissue infections (18). Furthermore, direct contact between individuals may also pave the way for infections in the skin and reproductive mucosa (19). Despite these exposures, we rarely get sick. This is due to our immune system's amazing ability to rapidly sense harmful pathogens and prevent infections from taking place.

The immune system

The immune system consists of two complex branches, the innate and the adaptive immune system, which are the cornerstones of human defense against infectious diseases. On the other hand, more primitive organisms such as invertebrates and plants are completely dependent on protection by innate immunity, which highlights the importance of this unique system (20, 21). Even though there are major differences between the innate and adaptive immune systems, they collaborate during an immune response and enhance each other's activity.

The innate immune system is characterized by its instant and highly unspecific response to invading pathogens and the induction of an adaptive immune response. In contrast to the innate response, the adaptive immune response is much slower and can take several days to react to a new invading pathogen (see Figure 1) (22, 23). Bacteria are able to divide every 20 minutes and could thus easily outnumber the host if no measurements were taken by the host defense at the early stages of infection. Therefore, it is essential that the innate immune response is active in the first crucial hours and days after exposure to a new pathogen. The adaptive immune response is dependent on the activation of lymphocytes (T cells and B cells) and the production of highly specific antibodies against various pathogens. Even though the lymphocytes are the main players in the adaptive immune response, the antigen presenting cells (APCs) play an important role in its activation. The strength of this system rests in its ability to create immunological memory after exposure to a specific pathogen and provide a long-lasting protection against that particular pathogen (22). This process is also the fundamental principal of vaccination.

Although these two systems provide rapid and long-term protection against all the different types of microbial pathogens in our environment, some pathogens still have the ability to circumvent the immune surveillance and cause infections (24-27).

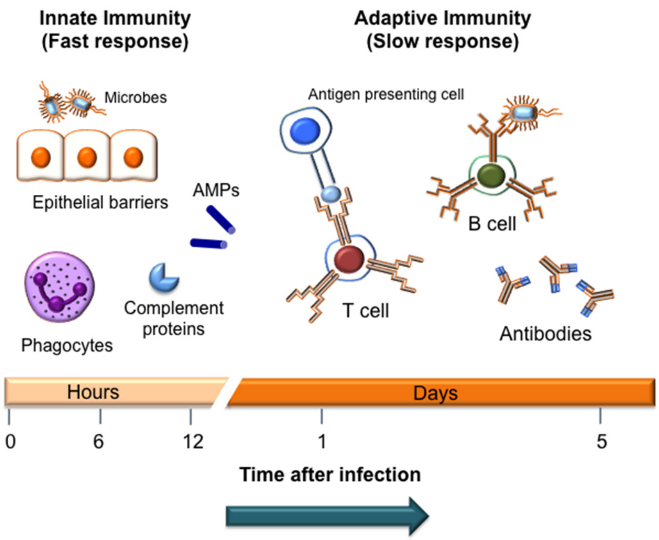

Figure 1.
A simplified schematic overview of components involved in the innate and adaptive immune response.

The innate immune system

Although we are exposed to a myriad of microbes, we seldom develop any symptoms and this is due to the instantaneous actions of our innate immune system against pathogenic microbes. The defense mechanisms of innate immunity are evolutionary old and have evolved a multitude of weapons and strategies to combat microbial pathogens and their toxins. It constitutes the first line of defense against invading pathogens and exists in all living organisms. Without the presence of innate immunity, we would be more prone to life-threatening infections.

Components of innate immunity

Structural barriers such as the skin and the mucosal surfaces provide a remarkable protection against potential pathogens. The high salt concentration, low pH and dry condition of the skin as well as tears from the eyes, clear pathogens before an infection can be established. In addition, epithelial cells produce a range of antimicrobial peptides (AMPs) including HBD-2 (28), which effectively inhibit and kill intruders. The mucus layer and the cilia in the airways are able to trap and remove inhaled pathogens, whereas the acidic secretions in the stomach create a rough environment for intruding pathogens and destroy them. If these barriers become breached by physical damage and a pathogen is able to invade the host, another component of innate immune system is quickly activated, forming an immunological barrier. This particular defence mechanism is comprised of the complement and the coagulation systems, acute-phase proteins and professional phagocytes. An important key player is the neutrophil, which is the first immune cell recruited to the site of infection, where it engulfs and destroys invading pathogens by employing antimicrobial molecules (29). It has been more than a century ago since Élie Metchnikoff first noticed the properties of these cells in innate immunity. His discovery of phagocytosis, "mobile cells" (phagocytes) that ingest and kill invading pathogens, was awarded the Nobel Prize in 1908 and had a significant impact on biomedical research ever since (30). Taken together, the components of innate immunity provide an important protection for the host and pose serious threat to the invading pathogen (31).

Recognition of pathogens

The detection and identification of foreign substances is vital to allow innate immunity to eliminate them, before they can cause substantial damage to the host. The innate immune response is triggered by recognition of components that are unique to microbes. These microbial structures are known as pathogen-associated molecular patterns (PAMPs) and are important for survival of the microbe and cannot be altered. Cells of innate immunity are able to recognize PAMPs through conserved germ line encoded recognition receptors called pattern recognition receptors (PRRs) (32). The most characterized PRRs in mammalians are Toll-like receptors (TLRs), which are found on different cell types including macrophages, neutrophils and epithelial cells, (33, 34). TLRs were first discovered in the fruit fly (*Drosophila melanogaster*) by Hoffmann (35) in late twentieth-century. They recognize a wide range of conserved microbial structures such as lipopolysaccharides (LPS) of Gram-negative bacteria (36), lipoteichoic acid (LTA) in Gram-positive bacteria (37), bacterial DNA (38) and flagellin (39). The innate immunity not only detects harmful pathogens, but also endogenous molecules, which are known as damage-associated molecular patterns (DAMPs) from tissues undergoing damage (40, 41). Upon recognition of a microbial structure by PRRs, intracellular signalling pathways are triggered which leads to the production of pro-inflammatory cytokines, chemokines and immune cell activation (42, 43).

Chapter 2 – Antimicrobial peptides

Discovery of AMPs

Our knowledge of the innate immune system in multicellular organisms has expanded over the last decades and so has the field of antimicrobial peptides. It has been almost 90 years since Alexander Fleming first discovered the antimicrobial activity of lysozyme, which was also the first natural antimicrobial protein isolated from humans (44). The discovery of penicillin by Fleming in the late 1920s (45) and streptomycin by Selman Waksman and his associates in the 1940s, initiated the "Golden age of antibiotics" and the interest in the therapeutic use of natural host antibiotics such as lysozyme was lost (46, 47). Nonetheless, it was not until the mid-1960s, the modern era of antimicrobial peptide research, when the discovery of cationic polypeptides (later called defensins) by Hussein Zeya and John Spitznagel occurred (48). In the 1980s, the interest in the field was greater the ever. Hans G. Boman discovered cecropins from hemolyph of the silk moths, (*Hyalophora cecropia*) (49), magainins from the skin of the African clawed frog (*Xenopus laevis*) were discovered by Micheal Zasloff (50) and mammalian defensins were isolated by Robert I. Lehrer and Michael E. Selsted (51, 52). Ever since, the field of AMPs has been well studied and thousands of AMPs (The Antimicrobial Peptide Database, aps.unmc.edu/AP/main.php) have been isolated and characterized from different organisms, which all have been reported to play an important role in the host immune defense. The ability of AMPs to induce killing of microbial pathogens has long been thought to be their main function but recent studies have shown that they can also modulate the immune system. Given this fact, many researchers have shifted the terminology from AMPs to host defense peptides (HDPs) (53). Furthermore, with increased antibiotic resistance worldwide, AMPs are getting a remarkable amount of attention as potential next-generation antibiotics (54).

AMPs – an ancient defense weapon

Antimicrobial peptides are powerful weapons of the innate immune system, which provide a rapid and non-specific immune response to invading pathogens (55, 56). This defense system evolved over 2.6 billion years ago (57) and is fundamental to all kind of living organisms, including vertebrates, invertebrates, prokaryotes and plants

(55, 58). The presence of AMPs in insects (59) and plants (60), provide strong evidence as to why these multicellular organisms are able to resist infections. In bacteria, however, they are used as a weapon in order to compete against other microbial species for survival (61).

In general, AMPs have been described to exert a broad-spectrum of antimicrobial activity against various types of targets such as Gram-positive bacteria, Gram-negative bacteria (56), fungi (62), protozoa (63), viruses (64) and even cancer cells (65). The biological effects among AMPs vary greatly, where some have been shown to be pathogen specific, such as cecropin P1 (66), while others act synergistically (67). However, their molecular mechanisms of action are often not completely elucidated.

In humans, they are found on external surfaces such as the skin or the mucosal linings (68), which are most susceptible to infection. They are also found in the granules of neutrophils and can be released in response to an infection (69). These peptides are constitutively expressed in the tissues, but they can also be triggered in response to PAMPs, bacteria or inflammatory-mediators e.g. tumour necrosis factor α (TNF-α) (61).

Despite their often diverse evolutionary origin, most AMPs share common physicochemical properties, which are important for their direct antimicrobial nature. They are generally characterized as small polypeptides of less than 50 amino acids residues with an overall positive charge ranging from +2 to +9. Another important characteristic feature is that they contain ≥ 30% hydrophobic residues. The combination of these properties allows them to adopt an amphipathic structure, which is an important prerequisite for bacterial interaction and killing (55, 70-72). A recent study by Andersson *et al.* (73) shows that many AMPs also contain a heparin-binding region (Cardin-Weintruab motifs (74, 75)), which can be used as an indicator to find novel peptides with antimicrobial activity. As discussed in Brogden *et al* (56), AMPs are a diverse group of molecules and can be divided into subgroups according to their amino acid composition and structure. Structurally, they are divided into four major groups I) linear cationic α-helical peptides, II) peptides with β-sheet structures that are stabilized by disulphide bridge, III) extended peptides and IV) peptides with loop structures. These structures are also essential for their broad and narrow antimicrobial activity (70, 72). AMPs with α-helical and β-sheet structures are the most predominant ones in nature but α-helical peptides such as magainins are among the best reviewed ones (76). Many AMPs adopt their active structure when they are in close contact with bacterial membranes. Cationic motifs are not the only naturally occurring AMPs. There also exist anionic AMPs such as dermcidin-derived peptide (DCD-1L), which is found in human sweat (77, 78). In addition to AMPs, proteins also exert antimicrobial properties. Lysozyme was the first human antimicrobial protein described by Fleming, and later more proteins with antimicrobial activity were discovered, such as lactoferrin (79) and secretory leucocyte protease inhibitor (SLPI) (80) in the airway secretions.

The defensins and cathelicidins are the two major groups of AMPs in humans (81). They are gene-encoded and synthesized as propeptides and then proteolytically cleaved to release the potent antimicrobial peptide. Defensins are cysteine-rich cationic polypeptides (82), which play a major role in vertebrate, invertebrate and plant host defense. In mammalians, they are found in many different tissues and cells including neutrophils, epithelial cells and keratinocytes. These peptides are grouped into three subfamilies according to their structure; α-defensins, β-defensins and θ-defensins (67), the latter one being found only in non-human primates (83). There are six different α-defensins in humans, with the first four being isolated from neutrophils and named human neutrophil peptide (HNP) 1-4. Human defensins (HD) 5 and 6 are mainly produced by Paneth cells in the intestines (67), whereas human β-defensins (HBD) 1-4 are expressed in various epithelial cells (84).

Cathelicidins are a diverse group of peptides and are represented in all mammalians species. Interestingly, only one cathelicidin has been found in human, LL-37 (85). The proform, hCAP-18, of LL-37 is stored in the granules of circulating neutrophils and is cleaved by proteinase-3 to the active form (86). LL-37 is known to adopt an α-helical conformation and has a broad spectrum of antimicrobial activity (see Figure 2) (85, 87). It has also been reported that the biological activities of many AMPs, including LL-37, can be lost in the presence of physiological relevant conditions such as salt concentrations (88, 89), glycosaminoglycans (GAGs) (90) and plasma (91).

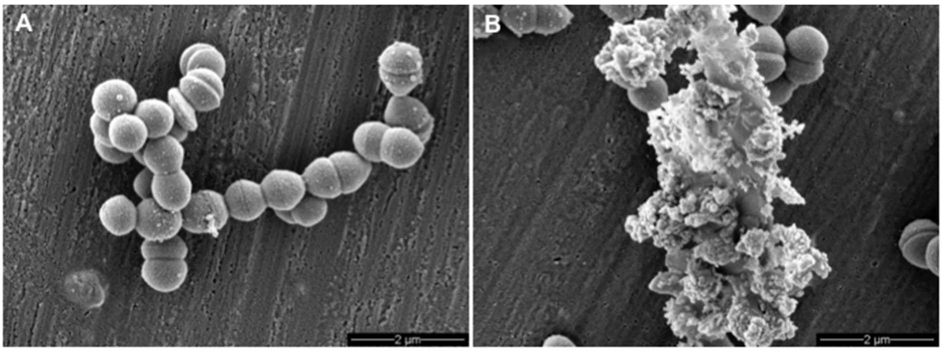

Figure 2.
Scanning electron microscopic image of *S. pyogenes* treated with LL-37. (A) In normal growth medium, the bacterial membrane is intact. (B) In the presence of LL-37, the bacterial membrane is disrupted and extensive leakage of cytoplasmic material is observed. Scale bar = 2 µm.

In addition to their antimicrobial properties, AMPs are also known to have other functions in host defense such as immunomodulatory activities (92). The immunomodulatory functions of AMPs have been well studied for the last few decades and numerous intriguing discoveries have been made. They have been shown to be chemotactic (93), promote phagocytosis (61), neutralize endotoxin (e.g. LPS) (94, 95), enhance wound healing (96), stimulate angiogenesis (97) and regulate the production of pro-inflammatory cytokines (98). These studies also demonstrate that AMPs form a link between innate immunity and adaptive immunity (99), which is pivotal in the clearance of an infection. Interestingly, these spectra of immunomodulatory properties of AMPs have made researchers question whether the antimicrobial activities of these peptides are their true primary function in the host (98).

Furthermore, dysregulation of AMP production in humans has also been shown to contribute to several inflammatory diseases. For instance, high levels of LL-37 are known to be associated with psoriasis (100) whereas low levels of LL-37, HBD-2 and HBD-3 (101) increase the susceptibility to skin infections caused by *S. pyogenes* and *S. aureus* in atopic dermatitis (AD) (102). To prove the biological importance of AMPs, several *in vivo* studies with transgenic mice have been carried out. One example is the study performed by Nizet *et al* (103), where they deleted the *Cnlp* gene encoding the cathelin-related antimicrobial peptide (CRAMP) in mice. When these mice were challenged with *S. pyogenes*, they developed more severe and persistent skin infections as compared to their wild-type littermates. This study highlights the importance of CRAMP and CRAMP-related molecules in host defense.

Mechanisms of AMP action

The mode of action varies between AMPs and is dependent on amino acid sequence, membrane lipid composition as well as peptide concentration. Different methods have been employed to study the activity of these peptides on whole microbial cells. These structure-activity studies reveal that the action of AMPs can be divided into two groups; membrane-active and non-membrane active (76). Regardless of which mechanistic class a given AMP belongs to, the initial interaction between the peptide and the bacterial cell membrane is a prerequisite for bacterial killing. This interaction is due to the electrostatic force between the positively charged peptide and the polyanionic bacterial cell surface, which is defined by LPS in Gram-negative bacteria or LTA in Gram-positive bacteria (70, 104, 105). Moreover, the presence of acidic phospholipids (phosphatidylglycerol (PG), phosphatidylserine (PS) and cardiolipin (CL)) adds additional negative charge to the surface of these microbes. Their activity is also dependent on amphipathicity, which allow them to interact and initiate perforation of microbial membranes (106). Since bacterial membranes are the main targets of AMPs, they have been termed "the Achilles heel of microbes" (55). From an evolutionary point of view, the fundamental differences in lipid composition between prokaryotes and eukaryotes enable host defense peptides to select and kill microbes. Generally, most mammalian cytoplasmic membranes are composed of zwitterionic lipids such as phosphatidyletanolamine (PE), phosphatidylcholine (PC) and sphingomyelin (SM), which neutralize the overall net charge (106). In addition, they contain cholesterol, which stabilizes the lipid bilayer and prevents AMPs binding to the membrane and thus cytotoxicity (107). In contrast to normal cells, human cancer cells are preferentially targeted and killed by AMPs (e.g. NK-2) and this is due to the high expression of anionic molecules on their cell surface (108, 109).

It is generally agreed that AMPs have the ability to destroy bacterial membranes. Several models describing their mode of action have been proposed (see Figure 3A). The carpet model (110-113) involves the assembly of peptides at the bilayer surface in a carpet-like fashion. When the threshold concentration of the peptide is reached, it disrupts the membrane like a detergent. This model was first described for dermaseptin S (114) from the frog *Phyllomedusa sauvageii*. In the barrel-stave model, a cluster of peptides is inserted perpendicular to the membrane bilayer and forms a transmembrane pore that leads to extensive membrane rupture and cytoplasmic leakage (110, 115). DCD-1L has been shown to act via this model (116). The killing mechanism of the toroidal model (111, 115, 117) is similar to the barrel-stave model, but the peptides are still associated with the phospholipid head groups even when they are inserted into the bilayer. In this context, it is also interesting to mention that both LL-37 (118) and magainin-2 (119) achieve killing according to the toroidal model. On the other hand, the aggregate model (110, 120) is more similar to the toroidal model. The only difference is that the peptides are less oriented when they are inserted into the lipid bilayer.

Several other studies have shown that highly cationic peptides are able to traverse the bacterial membrane without causing any sign of membrane damage and interference with intracellular components. These studies have demonstrated that AMPs can inhibit several key elements within the bacteria such as the synthesis of nucleic acid, protein (121), cell wall or its enzymatic activity (122) (see Figure 3B).

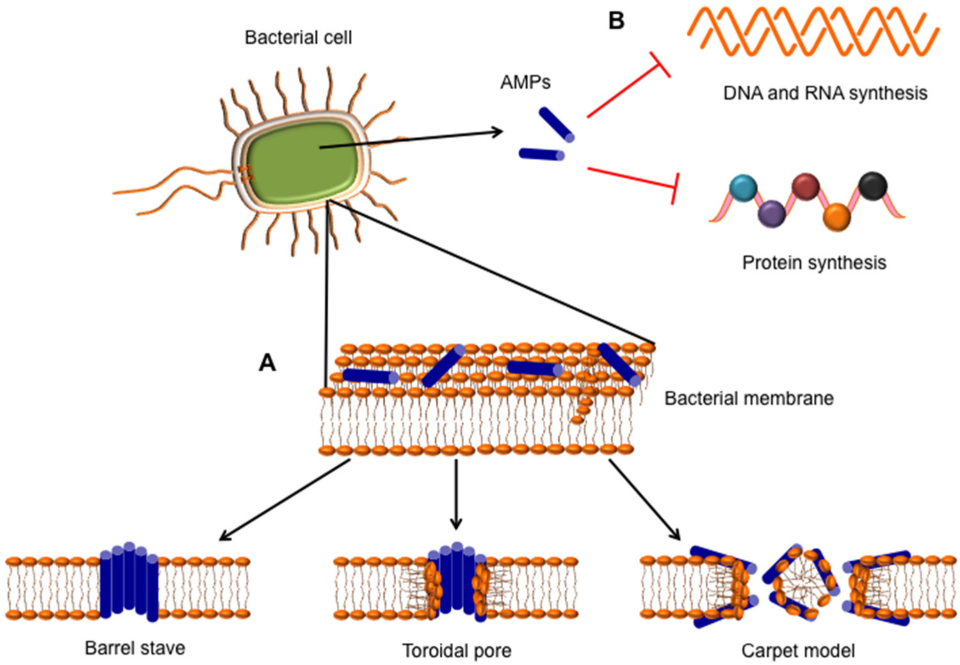

Figure 3.
Modes of action of antimicrobial peptides. (A) Membrane-active AMPs cause damage to bacterial membranes. (B) Non-membrane active AMPs target intracellular components and inhibit their activity. Modified from Jong-kook *et al.* (123).

AMPs as therapeutic agents

The increasing bacterial resistance towards many conventional antibiotics has drawn the attention of many academic researchers and pharmaceutical companies towards AMPs (124, 125). As mentioned earlier, today more than two thousand AMPs have been isolated from tissues or identified by *in silico* sequence analysis. Their diverse structure, broad spectrum of activity with prompt action, low bacterial resistance and low cytotoxicity against human cells provide a gold mine for researchers to design the perfect antibacterial agent to replace conventional antibiotics. AMP-based drugs are interesting candidates for topical as well as systemic applications. They can be used: (I) as a single antibiotic treatment, (II) in combination with conventional antibiotics, or (III) as immunostimulatory agents that boost the immune system. They are also promising therapeutic agents for autoimmune disorders, cancer and other infectious diseases. Despite the promising results from *in vitro* studies and animal disease models, there are several hurdles that need to be overcome before these peptides can be successfully brought to the market. Their sensitivity to proteases and significant decrease of antimicrobial activity under physiological conditions *in vivo* restrict their use in therapy. Another important issue is a detailed understanding of their immunogenicity, mode of action, interaction with immune/inflammatory cells and the potential cytotoxicity of highly cationic peptides, which largely remain elusive. Generally, human cells are resistant to AMPs, but at higher concentrations, some peptides have been shown to be cytotoxic (72, 126, 127). The last major challenge is the high cost associated with AMP production. The production of just one gram of AMP can cost up to 400US$, while for a conventional antibiotic it can be less than 1US$. Therefore it is necessary to find cost effective ways to synthesize AMPs in a large-scale production (128).

During the last few decades, several AMPs with therapeutic potential have been developed but these have subsequently failed in clinical trials. The first AMP that successfully completed a Phase III clinical trial was Pexiganan (MSI-78) by Magainin Pharmaceutical Inc. Pexiganan is a modified version of magainin-2 and was proven to cure 90% of infected diabetic foot ulcers. In 1999, the Food and Drug Administration (FDA) rejected it, because the effects of Pexiganan were no better than already existing antibiotic treatments. Many more AMP-based drugs, including Iseganan (IB-367, a synthetic analog of protegrin I from pig) faced the same fate as Pexiganan and did not pass Phase III studies (57). Despite these setbacks, the interest in AMPs is still high and there are more AMP-based drugs in the pipeline. However, more studies are needed to prove whether we can use these ancient molecules as templates for new antibacterial drugs to treat infections diseases.

Chapter 3 – Connective tissues, an emerging field in innate host defense

Extracellular matrix

Our body is composed of trillions of cells and each cell is a fundamental building block for various types of tissues and organs. The connective tissues are ubiquitous in our body and exert important functions such as supporting, anchoring and connecting body structures and organs. Within connective tissues, the cells are surrounded by a complex meshwork of extracellular macromolecules also known as extracellular matrix (ECM). The ECM is a highly dynamic material that undergoes continuous turnover in order to maintain the biological and structural integrity of cells and tissues. It is also responsible for regulating a variety of cellular events. Although the topological and biochemical composition of the ECM in each tissue is unique, there are many components that are common for all different connective tissues. The ECM is composed of various types of proteins and polysaccharides, which are secreted by local cells and assembled into unique structures in the extracellular space. The most abundant class of ECM components are the structural proteins, which consist of collagen and elastin. Other important components of the ECM are proteoglycans and the specialized non-collagenous proteins such as laminin, fibronectin and fibrillin (129). In this thesis, we are focusing on a member of the collagen family, more specifically collagen VI.

Collagen superfamily

Collagens are a heterogeneous family of structural proteins that are abundantly found in mammalian ECM. In humans, nearly one third of the body's protein content and approximately three-quarters of the dry weight of the skin are composed of collagens (130, 131). Interestingly, the Oxford Dictionary (1893) described collagen as "the constituent of connective tissue which yields gelatin on boiling" (132). This definition refers to ancient times, when people used to boil animal skin and sinews to extract gelatin (denatured collagen) and use it as glue. It is now known that collagen acts as intercellular glue that holds cells together and thereby provides structural integrity and mechanical strength to a wide range of tissues (133).

It was not until the 1950s that the modern era of collagen research started, when Hall and Schmitt characterized the collagen fibril using electron microscopy. The first models explaining the triple helical conformation of collagen was independently proposed by Ramachandran and Kartha (134), as well as others (135, 136). As described in Yamada *et al.* (137), the hallmark of the collagen family is defined by their distinct and common compositional and structural properties, which include; (I) the typical configuration of the molecule, where the three α-helices are intertwined to form a right-handed triple helix structure; (II) the collagenous domains consist of repeating Gly-Xaa-Yaa triplets, where proline and hydroxyproline frequently represent the Xaa-Yaa, respectively; (III) the ability to form supramolecular organizations and interact with other ECM proteins. These properties give the collagen fibers remarkable strength, which is crucial for the structural support of connective tissues and major organs in the body. The triple-helix structure of collagen also makes it highly resistant to proteolytic cleavage (138, 139).

Collagen provides rigidity, elasticity and strength for various tissues such as bone, cartilage, tendon and skin, in order to resist shear or pressure force (140). In addition to their physical function, collagens are also involved in a myriad of biological functions including cell adhesion, migration and tissue repair (139). Due to their diverse locations in tissue, they are synthesized by different cell types such as fibroblasts, myofibroblasts, osteoblasts, and chondrocytes. Other cell types that have also been reported to produce collagen are epithelial, endothelial, muscle and Schwann cells (140). The importance of collagens in different tissues have been highlighted by the vast number of diseases associated with genetic abnormalities in the collagen molecule (139).

Today, there exist at least 28 different types of collagens which have been identified and characterized in vertebrates. Most of these are further categorized into subfamilies based on their structure and supramolecular assemblies in the extracellular space. A summary of the subfamilies is shown in Table 1, which consist of fibrillar collagens, network-forming collagens, the FACITs (Fibril-associated collagens with interrupted triple helices), anchoring fibril collagens, MACITs (Membrane associated collagens with interrupted triple helices) and MULTIPLEXINs (Multiple triple helix domains and interruptions). In addition to these collagen groups, there exist several other proteins that contain collagenous domains but these do not fulfil the criteria for collagens. The distribution of a given collagen within different tissues varies greatly; some have a more restricted tissue distribution pattern, while others are present in virtually all tissues (130, 139, 141).

Table 1.
Different types of collagens found in vertebrates. Modified from Shoulders *et al.* (130) .

Type	Class	Distribution
I	Fibrillar	Abundant and widespread: dermis, bone, tendon, ligament
II	Fibrillar	Cartilage, vitreous
III	Fibrillar	Skin, blood vessels, intestine
IV	Network	Basement membranes
V	Fibrillar	Widespread: bone, dermis, cornea, placenta
VI	Network	Widespread: bone, dermis, cornea, cartilage
VII	Anchoring fibrils	Dermis, bladder
VIII	Network	Widespread: dermis, brain, heart, kidney
IX	FACIT	Cartilage, cornea, vitreous
X	Network	Cartilage
XI	Fibrillar	Cartilage, intervertebral disc
XII	FACIT	Dermis, tendon
XIII	MACIT	Endothelial cells, dermis, eye, heart
XIV	FACIT	Widespread: bone, dermis, cartilage
XV	MULTIPLEXIN	Capillaries, testis, kidney, heart
XVI	FACIT	Dermis, kidney
XVII	MACIT	Hermidesmosomes in epithelia
XVIII	MULTIPLEXIN	Basement membrane, liver
XIX	FACIT	Basement membrane
XX	FACIT	Cornea (chick)
XXI	FACIT	Stomach, kidney
XXII	FACIT	Tissue junctions
XXIII	MACIT	Heart, retina
XXIV	Fibrillar	Bone, cornea
XXV	MACIT	Brain, heart, testis
XXVI	FACIT	Testis, ovary
XXVII	Fibrillar	Cartilage
XXVIII	-	Dermis, sciatic nerve

Collagen VI

Collagen VI is a remarkable constituent of the collagen superfamily due to its unique set of characteristics. It is widely distributed in all interstitial connective tissues and is often associated with basement membranes. The major functions for collagen VI involve cell attachment and anchoring interstitial structures to the surrounding matrix (142-144). Collagen VI was originally isolated from human aortic intima after pepsin digestion by Chung *et al.* (145) and was thus referred to as intima collagen. Thereafter, it was isolated from cirrhotic liver (146), human and bovine placenta (147-149), calf skin (150) and uterus (151) by other groups. In humans, the predominant form of collagen VI is composed of three distinct α-chains namely α1(VI), α2(VI) and α3(VI) (see Figure 4A) that are encoded by the *COL6A1*, *COL6A2*, *COL6A3* genes, respectively (152-154). However, more recently, three additional collagen VI genes (*COL6A4*, *COL6A5* and *COL6A6*) were found in both mice and human. These new genes, which encode for α4(VI), α5(VI) and α6(VI), chains share sequence homology with the α3(VI) chain. Unlike the other collagen VI chains, these are highly tissue-specific (155, 156). Moreover, the α1(VI) and α2(VI) chains have a similar molecular mass of around 120 kDa, while α3(VI) is much larger with 260 kDa. Despite their variation in molecular mass, each α-chain is characterized by a 105 nm long triple-helical region flanked by two large N- and C-terminal regions. The globular regions contain several domains that share homology with von Willebrand factor A-like domains (VWA). These VWA domains are involved in the matrix-matrix interactions and cell-matrix interactions (157). Only 8 of 28 collagens are known to carry these domains in their sequence (141, 158). The α1(VI) and α2(VI) chains consist of one N-terminal (N1) and two C-terminal (C1-C2) VWA domains, whereas the α3(VI) is much larger and is comprised of 10 N-terminal (N10-N1) VWA domains and two C-terminal VWA domains. In addition, the α3(VI) chain has three C-terminal domains (C3-C5) that share homology with proline rich salivary gland proteins, fibronectin type III repeats and the Kunitz family of serine protease inhibitors. Several studies have been carried out that show that C-terminal domains of collagen VI are essential for assembly and extracellular microfibril formation (159-161). In contrast, N-terminal N1-N5 are important for collagen VI suprastructure (162). Because of its unique structure with four tightly interwined triple helices, collagen VI is resistant to degradation by bacterial collagenase and several matrix metalloproteinases (MMPs), which commonly cleave other collagens. However, serine proteinases secreted by neutrophils and mast cells are able to cleave and thus degrade intact collagen VI (163).

During recent decades, the assembly of collagen VI has been extensively studied by implementing both biochemical and electron microscopy analysis. The assembly of collagen VI is a complex process, where the three α-chains associate intracellularly to form a triple-helical monomer. Subsequently, the monomers align in an antiparallel fashion to form dimers, which are stabilized by disulfide bonds. By lateral association, the dimers then form tetramers and are secreted into the extracellular space. Here, the tetramers aggregate end-on-end to form beaded microfibrils, which become a part of the extended supramolecular matrix assemblies (164, 165) (see Figure 4B and C).

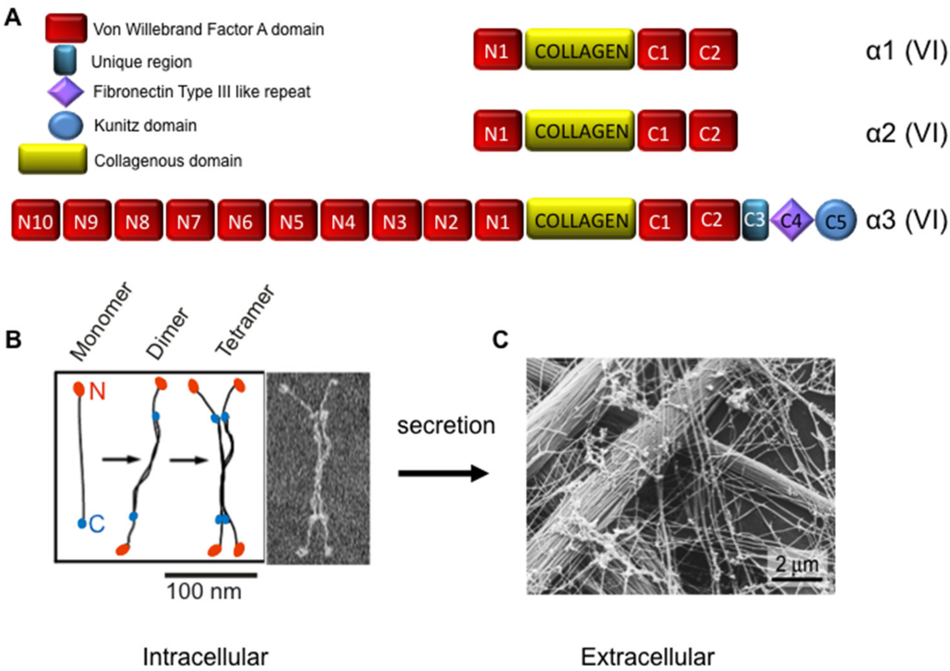

Figure 4.
(A) Schematic diagram of collagen VI structure. Collagen VI is composed of three distinct α-chains (α1, α2 and α3). Each chain contains several domains that share homology with the A-type domains found in von Willebrand factor. In addition, the α3-chain contains a proline-rich repeat domain, a type III fibronectin repeat domain and a domain that inhibits Kunitz proteases. (B) The assembly of collagen VI involves the formation of monomers to dimers and then by lateral assocations they form tetramers. After secretion, the tetramers aggregate end-on-end to form beaded microfilaments. (C) In the ECM, they form independent networks in conjunction to the large fibrillar collagens such as collagen I.

Collagen VI does not only have cell adhesion properties, but also interacts with several other extracellular matrix components, including collagen I (142), II (166) and XIV (167), perlecan (168), hyaluronan and heparin sulphate (169). For a long time collagen VI has been recognized as a basement membrane anchoring molecule by interacting with collagen IV (170). In addition, a recent study by Groulx *et al.*

(171) demonstrates that collagen VI is, in fact, also a basement membrane component in the gut epithelial basement membrane. Moreover, Wiberg *et al.* (172, 173) showed that the binding of collagen VI microfibrils to small leucine-rich proteoglycans (SLRPs) like decorin and biglycan leads to interaction with matrilins and subsequently to other interaction partners.

Apart from providing structural support for cells in the ECM, other interesting biological roles for collagen VI have been described. It has been reported to regulate signalling pathways for apoptosis (174), autophagy (175), proliferation, angiogenesis (176), fibrosis and inflammation (176, 177). Interestingly, collagen VI has been regarded as a potential biomarker for hepatic fibrosis (178) and cancer (179) diagnosis. Another study shows that macrophages are able to synthesize collagen VI and that it has an essential role in tissue repair (180). This is also supported by an earlier study performed by Oono *et al.* (181), showing that collagen VI is upregulated in normal wound healing. Other findings also suggest that collagen VI may have neuroprotective effects against Alzheimer's disease (182). Recently, we described collagen VI as a host defense molecule in connective tissues and a potential treatment for bacterial infections (183, 184).

Collagen VI plays a vital role in maintaining the structural integrity of skeletal muscles. Mutations in the collagen VI genes have been linked to several major human muscle diseases including Bethlem myopathy (BM) and Ullrich congenital muscular dystrophy (UCMD) (185). BM is a dominant inherited disorder characterized by mild muscular weakening and wasting in shoulders, pelvis, upper arms and legs (186, 187). In comparsion, UCMD is an autosomal recessive disorder that displays a severe congenital muscle weakness with proximal joint contractures and distal hyperlaxity (188, 189).

Extracellular matrix proteins - a double-edged sword in bacterial pathogenesis and innate host defense

Adherence to host tissues is a crucial step for any given pathogen in order to establish successful infection. Bacterial adhesion to the host cells occurs through piliated and nonpilated bacterial adhesins (190, 191). The epithelial surfaces of the skin, upper and lower airways and gastrointestinal tract, and urinary tract are the first structures to encounter invading pathogens. Below the epithelial cell layer is a thin supporting sheet, the basement membrane, which contains ECM proteins such as laminin and collagen. Upon epithelial injury due to a physical or chemical force, the pathogens gain access to the ECM, which may lead to colonization, deep tissue penetration and persistent infection. Furthermore, bacterial and viral infections of the mucosal surfaces can also expose the underlying ECM by permeabilizing the epithelium with their proteolytic enzymes and toxins (see Figure 5). It is evident that the ECM proteins do not only provide adhesion for host cells but also for invading pathogens. Over the past decades, a vast number of microbial pathogens have been identified to adhere to the host ECM constituents and often this adherence contributes to their virulence. These bacteria are able to exploit the host cells' adhesion system by coating themselves with ECM components in order to evade the host immune defense. Bacterial binding to the ECM proteins represents an important initial adhesion mechanism for their survival and colonization in the host. Adhesion to the host ECM is mediated by specific adhesins called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs), which is important for the establishment of different bacterial diseases (192-194). One of the best studied systems of MSCRAMM-ECM interaction is the binding of *S. aureus* surface proteins to fibrinogen (clumping factor; ClfA and ClfB), fibronectin (Fibronectin binding protein; FnBP) and collagen (collagen binding protein; Cna) (195-197). The binding of fibronectin to *S. aureus* was first reported by Kuusela in 1978 (198) and paved the way for extensive research in the field. The human skin pathogens *S. aureus* and *S. pyogenes,* as well as the gastric pathogen *Helicobacter pylori*, have also been demonstrated to utilize host laminin (199-201) and GAGs such as heparin and heparin sulphate to adhere to host tissues (193, 202, 203). The binding of vitronectin to *Eschericiha coli*, *S. aureus* and *Streptococcus pneumoniae* has been reported to promote better adhesion to epithelial cells (204). However, several pathogens have been shown to recruit collagen via prebound fibronectin (205). More recently, *S. pyogenes*, *S. pneumoniae, Moraxella catarrhalis* and *Haemophilus influenzae* have been demonstrated to directly interact with collagen VI (184, 206). For *S. pyogenes*, the binding was mediated by M1 protein (206).

In addition to the role of ECM components in bacterial pathogenesis, there is increasing evidence that they may also protect the tissues from invading pathogens. Sarikaya *et al.* (207) have showed that components of the ECM scaffold display potent antibacterial activity against Gram-positive and Gram-negative bacteria. Similar results were also obtained by both Brennan *et al.* (208) and *Smith et al.* (209), when they studied ECM extracts from other tissues. Interestingly, the antibacterial activity has been linked to degradation products of the ECM and not to the intact molecules in the ECM (210). In addition to antimicrobial properties, *in vitro* studies have revealed that peptides derived from the ECM scaffold possess chemotactic and angiogenic activities (211). Recently, Ilknur Senyürek and her colleagues demonstrated that peptides generated from laminin exert host defense and wound healing properties (212, 213). In this work, we describe for the first time that the collagen VI holoprotein, as well as cationic peptides derived from VWA domains of α3(VI), serve as a bactericidal barrier for both Gram-positive and Gram-negative bacteria *in vivo* (183, 184).

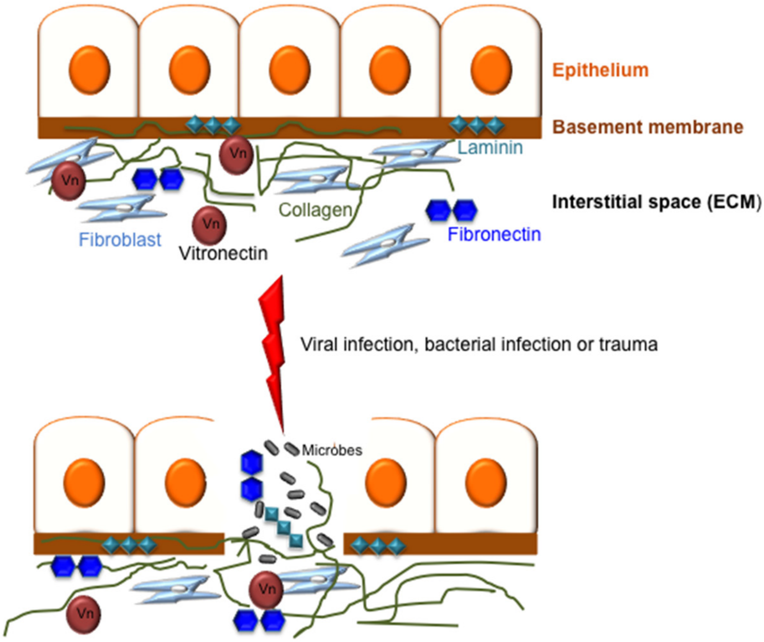

Figure 5.
Disruption of the physcial barriers (skin or mucosal surfaces) by trauma or viral or bacterial infection may expose extracellular matrix components and allow microbes to gain access to deeper tissues.

Taken together, these findings introduce mammalian ECM as a novel branch of innate immunity. Despite this fact, the ECM proteins may function as a double-edged sword, on the one side serving as powerful effector molecules of innate immunity and on the other side contributing to bacterial adhesion to tissue. Interestingly, this field of research is in its infancy and many more ECM proteins and derivatives thereof with host defense properties remain to be discovered. More importantly, in each case, their biological *in vivo* relevance needs to be further investigated in detail.

Chapter 4 – Host defense and ECM modifications in chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is a growing health issue that is currently ranked as the third leading cause of death, after ischaemic heart disease and stroke, worldwide (214). According to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) report in 2011, it is also associated with significant healthcare costs and social burden. COPD is a lung disorder defined by chronic airflow limitation that arises as a result of remodelling of the airways (obstructive bronchiolitis) and destruction of the lung parenchyma (emphysema), which is usually due to noxious particles from cigarette smoking. Although cigarette smoking is the best studied risk factor in COPD, there are also other risk factors such as genetic predisposition (e.g. α-1 antitrypsin deficiency), air pollution, occupational exposure and early childhood infections and asthma that may contribute to the development of COPD. The long-term exposure to cigarette smoke or other noxious particles has been shown to interfere with the innate and adaptive immune defense by eliciting an inflammatory cascade in the lungs, which leads to chronic inflammation and subsequently destruction of the lung tissue architecture. Interestingly, the inflammation process persists even after the patient quits smoking. Apart from pulmonary features, COPD is also frequently associated with other systemic effects including anemia, cardiovascular diseases, osteoporosis and weight loss (215). The degree of severity of COPD can be classified into four categories according to GOLD based on the value of the forced expiratory volume in one second (FEV_1). The GOLD stages (I-IV) reflect a spectrum ranging from mild (I) to severe (IV) COPD condition (216).

Under physiological conditions, tissue repair is a tightly regulated process. However, during the course of COPD, the process becomes pathological due to the persistent immunological trigger by cigarette smoke. In contrast to normal lung tissue, the lungs of COPD patients display numerous structural alterations including increased mucus production, defective mucociliary clearance and epithelial disruption (see Figure 6). The release of potent cytokines and chemokines recruits inflammatory cells (neutrophils, macrophages and CD8+ T cells) to the damaged tissues. These cells produce proteolytic enzymes and generate oxidants that cause additional injury to the tissues, which enhances the extent and severity of the disease. Furthermore, the production of transforming growth factor β (TGF-β) by epithelial cells in the small airways has been shown to contribute to other important features of COPD such as lung fibrosis (217) (see Figure 6). Smoking does not only cause permanent damage to the structural airway integrity but it also alters the cellular immune response, which increases susceptibility to infections. Another important hallmark of COPD are frequent exacerbations, acute "worsening of the patient's baseline condition", which is usually triggered by a viral or bacterial infection. Studies have shown that approximately 50% of the exacerbations are caused by bacterial infections in the lower airways. The most common bacterial species associated with exacerbations are non-typeable *H. influenzae* (NTHi), *S. pneumoniae* and *M. catarrhalis*. At later stage of the disease, *P. aeruginosa* becomes more important. Persistent bacterial colonization (chronic bronchitis) has been shown to enhance the level of inflammatory mediators, which may contribute to chronic airway inflammation and progression of the disease. Bacterial proteases and surface proteins have also been shown to induce tissue damage directly or by activating host inflammatory mechanisms. However, the underlying mechanisms of exacerbations are still poorly understood (218-222).

As the disease advances, the loss of subepithelial basement membrane integrity results in thickening of airways walls due to increased deposition of ECM molecules by fibroblasts or myofibroblasts (223, 224). Several studies have been performed to investigate the inflammatory response in COPD compared to structural remodelling and modifications of ECM (225) and even less studies have focused on collagen subtypes in COPD airways (226). Collagens I and III, which are important in maintaining the structural integrity of lung tissue, have been shown to be upregulated in the central airways (in basement membranes, lamina propria and adventitia) during a mild to moderate disease state. An increase of total collagen has also been found in bronchiolar tissue of COPD patients with a moderate to severe disease state. These data imply that structural remodelling of the ECM plays an important role in the airflow obstruction and in disease progression (225). In a recent study, our group demonstrated that collagen VI is highly upregulated in the airways of COPD patients and exposed upon tissue damage (184).

In summary, the development and progression of COPD is determined by several factors such as genetic susceptibility, exposure to harmful particles, exposure to pathogens, inflammation and other unknown factors. Furthermore, there is no single effective treatment for COPD. Influenza and pneumococcal vaccinations are generally administered to individuals with a COPD diagnosis to prevent infections. In addition, they also obtain symptom management medications such as bronchodilators and a combination of corticosteroids and antibiotics to dampen inflammation and infections in acute exacerbations. However, the long-term use of corticosteroids may interfere with host defense and increase the risk for infections. The use of antibiotics has also been controversial, since exacerbations can be caused both by viral and bacterial infections. Therefore, alternative therapies are necessary for long-term management of COPD (216, 221).

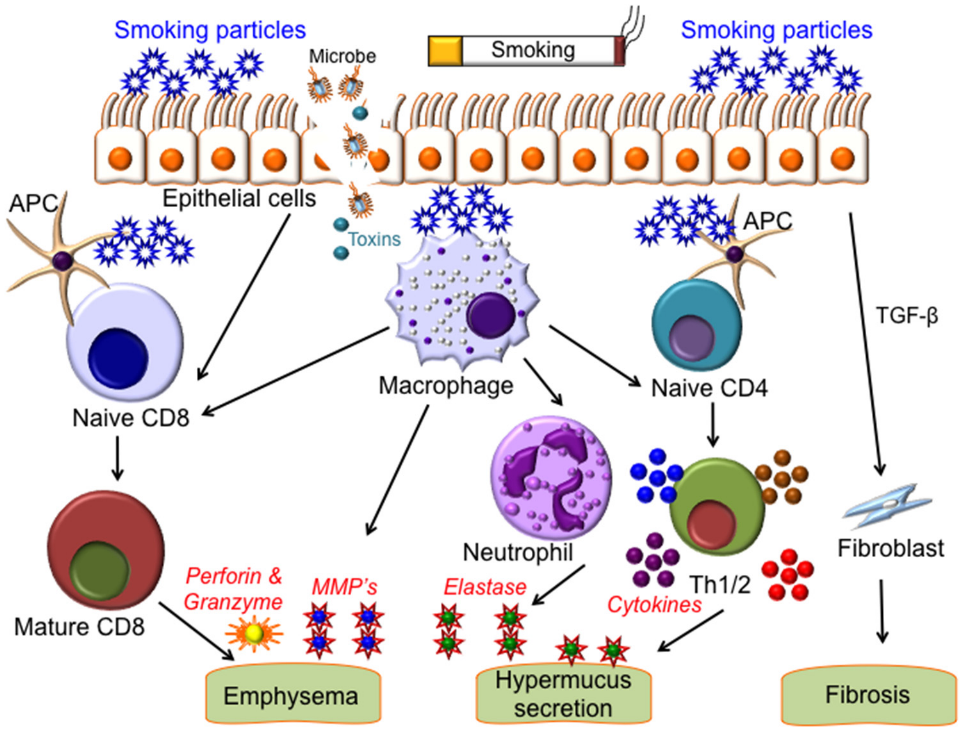

Figure 6.
Effects of cigarette smoke in the lungs. Noxious particles from cigarette smoke activate macrophages and epithelial cells to release several chemotactic factors that recruit inflammatory cells to the site of injury. These inflammatory cells and macrophages release proteases that cause emphysema and hypermucus secretion. The release of transforming growth factor-β by epithelial cells promotes fibroblast proliferation, which in turn leads to fibrosis in the small airways. Alteration of the cellular immune response also increases susceptibility to infections. Modified from Lane *et al*. (227).

Chapter 5 – Present investigation

Paper I

Collagen VI encodes antimicrobial activity: novel innate host defense properties of the extracellular matrix

Background

Collagen VI is a ubiquitous component of the mammalian extracellular matrix. It is virtually found in all connective tissues and often seen in close association with basement membranes. This intriguing molecule is composed of three distinct polypeptide chains α1(VI), α2(VI) and α3(VI), but more recently three additional tissue-specific chains (α4(VI), α5(VI) and α6(VI)) were discovered. Generally, the collagen VI α-chains are organized as dumb-bell-shaped monomers, where a short extended triple-helical region is flanked by two large N- and C-terminal globular regions. These globular regions contain several domains homologous to the A-type domain found in von Willebrand factor (155, 156, 228). In a previous study by Andersson *et al.* (73) it was shown that von Willebrand factor (VWF) harbors cationic sequence motifs associated with heparin-binding that may confer antimicrobial properties. The link between VWA domains and antimicrobial properties prompt us to investigate whether such features were also present in collagen VI.

Aim

- To investigate whether collagen VI has antimicrobial properties

Results and conclusions

In this study, we show for the first time the antimicrobial activity of tissue-purified collagen VI against human oral pathogens. The results obtained from viable count assays demonstrated that collagen VI induced dose-dependent killing of group A, C and G streptococci in physiological conditions. Bacterial killing was achieved through membrane destabilization and the leakage of cytoplasmic content. Using isogenic mutants of *S. pyogenes*, we investigated the correlation between the expression of M1 protein and protein H at the bacterial surface and collagen-VI induced killing. These mutant strains are known to either lack the expression of M1 protein (BMJ11), H protein (BMJ27.6) or both H and M1 protein (BMJ71). Interestingly, the M1 protein expressing strains AP1 and BMJ27.6, were more prone to killing by collagen VI than the M1 mutants. These findings indicate that the interaction between collagen VI and M1 protein at the bacterial surface may play a key role during the elimination of group A streptococcus. In summary, these data disclose a previously unknown role for collagen VI in innate host defense.

Paper II

Collagen VI is upregulated in COPD and serves both as an adhesive target and a bactericidal barrier for *Moraxella catarrhalis*

Background

M. catarrhalis is an important human respiratory pathogen that frequently causes middle ear infections in infants and children. In adults, it is mostly associated with a number of clinical manifestations, but is also recognized as an important pathogen in COPD (229). In 2012, COPD was ranked by the World Health Organization (WHO) as one of the top three deadliest diseases in the world (214). It is defined as a progressive chronic inflammatory disease commonly caused by cigarette smoking but also by other factors. An important hallmark of COPD are frequent exacerbations, which are often caused by bacterial infections. These exacerbations are characterized by enhanced airway inflammation, which results in tissue damage and deterioration of lung function. The loss of epithelial integrity exposes the underlying ECM, which in turn facilitates pathogens to gain access to the connective tissues and cause deeper tissue infections (221). Several ECM proteins including fibronectin, laminin, vitronectin and collagens have been classified as targets for Gram-positive and Gram-negative bacteria (194). Recently, our group identified collagen VI as an adhesive substrate for pulmonary pathogens such as *S. pyogenes* and *S. pneumoniae* (206).

Aims

- To determine the expression of collage VI in normal lung tissues and in COPD lung tissues
- To examine the interaction between collagen VI and *M. catarrhalis* in COPD lung tissues *ex vivo*
- To assess the antimicrobial activity of collagen VI against *M. catarrhalis* and other important respiratory pathogens in COPD *in vitro*

Results and conclusions

The results showed that collagen VI is upregulated in COPD distant to basement membranes and exposed upon epithelial damage. *M. catarrhalis* displayed strong adhesion to collagen VI microfibrils secreted by pulmonary fibroblasts from COPD patients, which promotes bacterial invasion and colonization during the course of infection. This interaction was specifically mediated by the collagen α2-chain, followed by membrane disruption and killing. Collagen VI was also recruited to the bacterial surfaces of *H. influenzae*, *S. pneumoniae* and *P. aeruginosa* and similar killing effects were observed. Together, these data demonstrate that collagen VI has a dual role in connective tissues, where the α2-chain is targeted by *M. catarrhalis*. On the other hand, collagen VI prevents bacteria from disseminating into the host tissue, followed by killing and elimination.

Paper III

Collagen VI is a bactericidal barrier against *Haemophilus influenzae in vivo* in chronic obstructive pulmonary disease (COPD)

Background

Non-typeable *Haemophilus Influenzae* (NTHi) is an important human commensal, which is present in the nasopharynx of most healthy pre-school children, but it is also a major mucosal pathogen causing a repertoire of respiratory infections. More importantly, it is linked to persistent bacterial colonization of the lower respiratory tract of COPD patients and known as a major cause of COPD exacerbations. Furthermore, studies have shown that the presence of NTHi in COPD patients causes disruption of ciliary activity and epithelial injury, which leads to impaired mucociliary clearance (230). NTHi possess a number of bacterial surface factors including PE and Hap that interact with host components such as ECM to promote bacterial colonization and persistent infection (231). As mentioned earlier, we have shown that collagen VI is upregulated in COPD (184) and represents an adhesive substrate for Gram-positive and Gram-negative respiratory pathogens, but also exerts antimicrobial properties (184, 206).

Aims

- To examine whether collagen VI induces killing of NTHi *in vitro*
- To identify NTHi adhesins that interacts with collagen VI
- To further investigate the interaction between NTHi and collagen VI microfibrils in a COPD mouse model *in vivo*

Results and conclusions

Here, we showed that NTHi bound more extensively to the collagen VI-rich region in the subepithelial lamina propria in the airways of COPD patents compared to healthy individuals. In another experimental setup, NTHi adhered mostly to ECM fibrils secreted by COPD fibroblasts and to a much less extent, healthy fibroblasts. Interestingly, collagen VI caused rapid killing of NTHi by targeting the membranes and inducing membrane blebbing, destabilization and leakage of cytoplasmic content under physiological pH and ionic strength. A similar effect was also observed when mice were challenged with NTHi *in vivo*. Bacterial killing was mediated by the specific binding of the NTHi surface proteins PE and Hap to the VWA domains of collagen VI. We also demonstrated that PE partially protected NTHi from being killed, while NTHi strains lacking the expression of PE were more prone to killing by ECM in murine and human airways. Taken together, these data shed light on the biological importance of collagen VI as an adhesive substrate for NTHi in COPD, but also as an antibacterial barrier *in vivo*. The data also highlight the importance of the extracellular matrix as an emerging branch of innate host defense.

Paper IV

Collagen VI contains multiple host defense peptides with potent *in vivo* activity

Background

Antimicrobial peptides (AMPs), also known as host defense peptides (HDPs), are small, cationic, gene-coded natural peptide antibiotics that exist in all living organisms. AMPs display potent antibacterial, antifungal, antiparasitic and antiviral properties and play an important role in innate host defense. The mode of action of AMPs varies, some target the membrane and disrupt it through diverse mechanisms, while others interfere with intracellular targets. The growing problem of antibiotic resistance has made AMPs interesting candidates for development of novel antibiotics due to their wide spectrum of antimicrobial activity, together with a low bacterial resistance mechanism (57). We have recently discovered the antimicrobial activity of collagen VI holoprotein against several important human pulmonary pathogens (184). Although its antimicrobial activity has been studied, it is still unknown which part of the molecule is responsible for antibacterial action and its biological relevance in host defense *in vivo*.

Aims

- To investigate the antimicrobial activity of collagen VI against several Gram-positive and Gram-negative bacteria
- To determine which part of collagen VI exerts antimicrobial activity
- To identify, synthesise and characterize putative antimicrobial peptides from collagen VI
- To elucidate the biological relevance of collagen VI and its derivatives in skin infections *in vivo*

Results and conclusions

In this work, we further unravel the antimicrobial activity of collagen VI by employing different approaches. Here, we show that collagen VI exerts a broad spectrum antimicrobial activity against a number of Gram-positive and Gram-negative bacteria. Furthermore, the recombinantly expressed VWA of this molecule binds to negatively charged surfaces such as heparin and bacterial membranes. These VWA domains displayed a dose-dependent killing against *S. pyogenes* by membrane disruption and extravasation of cytoplasmic content. *In silico* sequence and structural analysis revealed that the three α-chains of collagen VI contain cationic and amphipathic sequence motifs, which may explain the antimicrobial nature of this molecule. Templates for novel antimicrobial peptides were chosen from the N2, N3 and C1 domains of 3α-chains. Interestingly, these peptides demonstrated a significant bactericidal activity against *S. pyogenes*, *S. aureus*, *E. coli* and *P. aeruginosa* in skin infections *in vivo*. High levels of collagen VI and its peptides were also found in skin biopsies from patients with group A streptococcus infection. These findings suggest a novel role for collagen VI-derived peptides in innate host defense, which could serve as potential antibacterial agents to treat skin infections.

Paper V

Mode of action and immunomodulatory effects of collagen VI-derived host defense peptides

Background

As stated earlier, the increase of resistant pathogenic bacteria to conventional antibiotics has become a serious threat to public health worldwide (232). Therefore, it is essential to find novel therapies to treat infectious diseases. One promising therapeutic area that has gained enormous attention for the past few decades is AMPs. These peptides are ubiquitous in nature and represent an important component of innate host defense. Besides their antimicrobial properties, these peptides are also known to have immunomodulatory functions such as being chemotactic, neutralizing endotoxins and enhancing wound healing (98, 233, 234). In paper IV, we showed that peptides derived from VWA domains of the collagen VI α3-chain display antimicrobial activity *in vitro* and *in vivo*.

Aims

- To examine the binding of collagen VI-derived peptides to bacteria and bacterial surface protein e.g. LPS
- To further explore the antibacterial activity of collagen VI-derived peptides in physiological conditions such as salt and human plasma

- To investigate the cytotoxic effects of collagen VI-derived peptides
- To determine whether collagen-VI derived peptides possess anti- endotoxic and wound healing properties

Results and conclusions

In this study, which is a follow-up study of paper IV, we describe that novel antimicrobial peptides derived from collagen VI interact and bind to *S. aureus* and *P. aeurginosa*. In addition, the peptides strongly interacted with LPS, which might at least partly explain their interaction with Gram-negative bacteria. Upon binding to LPS, the peptides changed their conformation and adopted either a random coil or a mixture of alpha-helix or beta-sheet structure. The collagen VI-derived peptide SFV33 exerted a strong antibacterial activity against *P. aeruginosa* in plasma conditions as compared to LL-37 and other peptides. In salt conditions, SFV33 displayed concentration-dependent killing of both *P. aeruginosa* and *S. aureus.* However, all peptides were not active against *S. aureus* in plasma. At the ultrastructural level, bacterial killing was caused by disruption of bacterial membranes, resulting in disintegration and ejection of cytoplasmic components. The peptides did not cause cytotoxic effects against human cells at the concentrations used for bacterial killing. Interestingly, the majority of the peptides promoted wound healing, while only the GVR28 peptide showed anti-endotoxic effects *in vitro*. Collectively, these findings suggest that collagen VI-derived peptides play a role in connective tissue innate immunity against bacterial infections. Furthermore, they are powerful mediators and modulators of inflammatory processes and could thus serve as templates for designing efficient antibacterial and wound healing agents.

Chapter 6 – Future perspectives

The discovery of antibiotics in late 1920s gave us hope for a future where infections would be controlled and cured. However, today, that scenario is about to change due to the growth of bacterial resistance to conventional antibiotics (232, 235). As a result, fewer and fewer antibacterial agents are available to treat severe bacterial infections. Most hospital-acquired infections are associated with dangerous bacterial species such as methicillin-resistant *S. aureus* (MRSA) and multidrug resistant Gram-negative bacteria (235). Hence, it is essential to improve our knowledge of host-pathogen interactions in order to find alternative antibacterial therapies before we reach a post-antibiotics era. One promising area of research is the naturally occurring antibiotics (AMPs/HDPs), which represent an important part of the innate host defense in all living organisms including humans (55).

The interplay between ECM proteins and bacteria has long been recognized to be of importance for bacterial pathogenesis (194, 236). In this work, we extended this knowledge and studied the role of ECM in innate host defense. Here, we describe collagen VI as the first ever discovered extracellular matrix holoprotein which confers innate host defense properties to connective tissues. Moreover, our data suggest that this molecule encodes a dual function in host defense, like molecular flypaper, as an adhesive substrate that entraps pathogens and a bactericidal agent that kills them. These data, in fact, add to a growing body of evidence supporting ECM as a novel domain of innate immunity. More importantly, we hope that this knowledge will initiate extensive studies in the field to uncover more ECM proteins with similar properties and to address their significance to protect the host from invading pathogens.

The discovery of collagen VI in host defense also prompted us to search for AMP motifs within the collagen VI molecule. Our studies demonstrated that collagen VI-derived peptides exerted a wide spectrum of antibacterial properties *in vivo*. In addition, some of them also exhibited wound healing and anti-endotoxic properties *in vitro*. However, it is not yet clear how these potent peptides are generated from collagen VI during wound healing. It is also interesting to investigate whether these peptides are generated in the airways of COPD patients, where inflammation is prominent. Another important aspect that needs further investigation is their ability to modulate the immune system. In preliminary studies, we found that they also kill multidrug resistant strains such as MRSA, *P. aeruginosa*, *S. epidermidis, Klebsiella* *pneumoniae* and *E. coli*. To this end, these peptides represent a new class of AMPs/HDPs in the ECM that could provide promising alternative treatment for bacterial infections.

Another interesting application for collagen VI and its derivatives lies in the area of biomaterials. Post-surgical infections associated with implanted medical devices are quite common and can give rise to serious complications. These infections often involve bacterial colonization and biofilm formation on the implants surface, which have been reported to be very difficult to treat (237). Therefore, therapies that can prevent bacterial adherence and colonization and thus biofilm formation on implants are greatly needed. Indeed, preliminary data in recent studies indicate that bioactive collagen VI-coated surfaces eradicated pathogens as compared to non-coated surfaces, which might minimize bacterial infections and improve tissue integration and long-term stability of the implant.

Populärvetenskaplig sammanfattning – popularized summary in Swedish

Vi lever i en värld full av mikroorganismer, så som bakterier, virus och svampar. Vår egen kropp är inget undantag, det uppskattas att vi har 10 gånger fler bakterieceller än egna celler. De allra flesta bakterier i vår kropp lever i symbios med oss och har stor betydelse för vårt välbefinnande. Bakteriefloran i mag-tarmkanalen hjälper oss bland annat att ta upp näringsämnen och nomalfloran på huden hindrar sjukdomsframkallande bakterier (patogener) från att bosätta sig och orsaka infektioner.

Med tanke på den mängd mikroorganismer vi utsätts för varje dag så är det mirakulöst att vi ändå kan hålla oss friska. Detta beror på att vårt immunförsvar ständigt är alert och kan skilja mellan ofarliga mikroorganismer, som normalt ska finnas från skadliga, sjukdomsframkallande mikrober och oskadliggöra dessa innan vi ens hinner bli sjuka. Under evolutionens gång har vårt immunförsvar utvecklat olika typer av försvarsmekanismer för att hindra invasion och hejda spridning av olika sjukdomsframkallande bakterier. De fysiska barriärerna (såsom huden och våra slemhinnor) och immunceller utgör en fundamental del av vårt medfödda immunförsvar.

De intakta fysiska barriärerna utmanas ständigt av sjukdomsframkallande bakterier och när en skada uppstår kan dessa lätt ta sig in i kroppen. För att kunna ta sig djupare in i vävnaden och kolonisera måste bakterierna först binda till celler och proteiner som finns i det extracellulära matrixet (ECM). ECM består av ett nätverk av olika proteiner och kolhydrater som ger stöd till celler i de olika vävnaderna i kroppen. Kollagen är det mest förekommande proteinet i ECM och utgör nästan 30% av kroppens totala vävnadsproteiner. Kollagenfamiljen är stor och består av 28 medlemmar som bildar unika fiberstrukturer i ECM och ger exempelvis bindvävnaden sin fina struktur och förmågan att stå emot slitande krafter. I denna avhandling, fokuserar jag på en unik medlem hos kollagenfamiljen, kollagen VI. Den består av tre peptidkedjor som är snurrande runt varandra för att bilda en trippelhelixstruktur. I varje ände av denna struktur finns stora globulära domäner som är väsentliga för interaktioner med andra proteiner. Dessa globulära domäner har en struktur som liknar von Willebrands faktor (VWF, ett viktigt protein vid blodkoagulation) och en tidigare studie har visat att VWF har förmåga att döda sjukdomsframkallande bakterier.

En annan viktig del av vårt medfödda immunförsvar utgörs av evolutionärt bevarade små proteiner, så kallade antimikrobiella peptider (AMPs). Hittills har man identifierat och karakteriserat sammanlagt över 2000 peptider hos olika arter. Hos människan utgör AMPs den första försvarslinjen mot sjukdomsframkallande mikroorganismer och agerar direkt, innan det specifika immunförsvaret hinner aktiveras. AMPs har en bred och kraftfull naturlig antibiotisk effekt mot bakterier, virus och svampar och fungerar genom att till exempel bilda porer och förstöra mikroorganismernas ytterhölje (membran). Eftersom peptiderna är positivt laddade attraheras de till bakteriemembran som oftast är negativt laddade, vilket leder till att bakterieceller kan oskadliggöras snabbt. AMPs kan antingen genereras kontinuerligt eller frisättas vid inflammation eller vävnadsskada. Förutom att dessa peptider har bakteriedödande effekt, kan de också påverka vårt immunförsvar på olika sätt. De kan rekrytera immunceller till infektionshärden för att snabbt avlägsna infektionen och även stimulera sårläkningsprocessen och aktivera det specifika immunförsvaret.

Under de senaste årtiondena, har intresset för AMPs vuxit. Dels för att de utgör ett kroppseget antibiotikum och dels för att de inte triggar igång resistensutveckling hos bakterier i samma grad som konventionella antibiotika gör. Antibiotikaresistens är ett allt mer växande problem i världen och kan leda till att infektioner blir långvariga och svåra att behandla. Detta medför inte bara mer lidande för patienterna utan också större ekonomiska kostnader för samhället. Därför är det angeläget att hitta nya strategier och behandlingsmetoder för att bekämpa antibiotikaresistenta bakterier.

I mitt **första avhandlingsarbete** har vi kunnat visa att kollagen VI har en bakteriedödande effekt mot grupp A, C och G streptokocker genom att förstöra deras ytterhölje. Vi kunde också visa att denna effekt var dosberoende vid kroppsliknade miljö såsom vid fysiologisk salthalt och pH. Vi visade även att ytproteinet M1 på grupp A streptokocker medför ökad känslighet mot kollagen VI avdödning. I stora drag visar vår studie att kollagen VI kan ha en betydelsefull roll i vårt medfödda immunförsvar i bindvävnaden.

I mitt **andra och tredje avhandlingsarbete** undersökte vi den antibakteriella effekten hos kollagen VI mot sjukdomsframkallande luftvägsbakterier vid kroniskt obstruktiv lungsjukdom (KOL). KOL är en tilltagande inflammatorisk lungsjukdom som främst orsakas av cigarrettrökning. Den kroniska inflammationen som uppstår vid KOL leder till att luftvägarna förstörs, lungfunktionen blir allt sämre och patienterna riskerar även allvarliga bakterieinfektioner. Vi har kunnat visa att produktionen av kollagen VI är förhöjd i KOL-patienternas lungor jämfört med friska individer. Eftersom den fysiska barriären i luftvägarna är förstörd vid KOL så exponeras kollagen VI i bindvävnaden och blir därmed tillgänglig för bakterier. Vi har även kunnat visa att kollagen VI har förmåga att fånga och oskadliggöra de vanligaste bakterierna (*Moraxella catarrhalis* och *Haemophilus influenzae*) som koloniserar de nedre luftvägarna hos KOL-patienter. Vi har också visat att det är α2-kedjan hos kollagen VI som står för bindningen till *M. catarrhalis*. I dessa två studier kunde vi understryka att kollagen VI har en skyddande effekt mot bakterieinfektioner hos KOL-patienter.

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Sammanfattningsvis är min förhoppning att denna avhandling kan öka förståelsen för hur beståndsdelar i vår bindvävnad, såsom kollagen VI, kan skydda oss mot bakterieinfektioner. Jag hoppas även att man i framtiden kan utnyttja dessa kunskaper till utveckling av nya strategier för att behandla infektioner orsakade av antibiotikaresistenta bakterier.

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>
> – John F. Kennedy –

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My interest in research, particularly in the field of microbiology and immunology, has led me to my PhD work at the Faculty of Medicine at Lund University. Over the last four years, I have been studying a specific component in our connective tissues, collagen VI. Here, I describe for the first time that collagen VI kills pathogenic bacteria and thereby protects us against bacterial infections. In the future, we can harness this knowledge to develop new antibacterial treatments.

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