Universidade do Minho
Escola de Engenharia

Reinaldo Rodrigues Ramos

Recombinant expression and activity of cationic antimicrobial peptides

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Recombinant expression and activity of cationic antimicrobial peptides

Tese de Doutoramento
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Trabalho efectuado sob a orientação do
Doutor Miguel Gama
e do
Doutora Lucília Domingues

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ABSTRACT

Recombinant expression and activity of cationic antimicrobial peptides

In the past 60 years, antibiotics have been critical in the fight against infectious disease caused by microorganisms. The increasing bacterial resistance to antibiotics is a serious public health problem. Much research has been dedicated to the development of new classes of antibiotics to overcome this situation.

Antimicrobial peptides (AMPs) are generally defined as peptides of less than 50 amino acid residues, bearing positive charge due to multiple lysine and arginine residues and with 50% or more of hydrophobic residues. AMPs have aroused great interest due to their broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria, fungi, protozoa and some viruses and their ability to overcome bacterial resistance.

The major problem for the chemical production of AMPs is the elevated cost. Recombinant expression techniques for large-scale production of these peptides would represent an economically more viable approach. Therefore, strategies to produce AMPs at affordable cost are much required for the exploitation of their therapeutic potential. This was one of the main objectives of this thesis: the recombinant production and purification of two AMPs (Magainin-2 and LL37) using a family 3 carbohydrate-binding module (CBM3) from *Clostridium thermocellum* as fusion partner. The use of the CBM3 could reduce the purification costs using cellulose as affinity matrix. The other purpose was to evaluate the biological activities of recombinant LL37 and its therapeutic effects in mice models of wound healing, after topical application.

The general introduction of this thesis is presented in chapter 1 and includes a bibliographic revision of: 1) the antimicrobial peptides; 2) a brief revision of carbohydrate-binding modules and their potential as fusion partners; 3) The frog AMP magainin-2; 4) the human AMP LL37; and 5) the wound healing process.

In chapter 2, the expression of the AMP magainin-2 (MAG2) is described. MAG2 was fused to the N- and C-termini of the CBM3 containing a linker sequence (LK-CBM3), and formic acid recognition site between the two modules, for chemical cleavage. The recombinant protein MAG2-LK-CBM3 was expressed in *Escherichia coli* BL21 (DE3) and LK-CBM3-MAG2 in *E. coli* M15 (pREP4). The N-terminal MAG2 from the first construction was successfully cleaved and purified from the fusion partner LK-CBM3. However, the peptide showed no antibacterial activity against *E. coli* K12. The negatively charged C-terminal aspartic acid left from the acid cleavage may be the cause for the absence of antimicrobial activity. The expression of C-terminal MAG2 from the second construct was not successful. The peptide may have suffered proteolysis during the recombinant expression.

Chapter 3 describes the expression of the human LL37. The protein was also cloned to the N- and C-termini of LK-CBM3 and a formic acid recognition site was introduced between the two modules, allowing the isolation of LL37 after chemical cleavage. The recombinant proteins were expressed in *E. coli* BL21 (DE3) and solubilized with Triton X-100. The purification was achieved using cellulose CF11 fibers, taking advantage of the CBM3 specific affinity for cellulose; after hydrolysis with formic acid, LL37 was further purified by reverse-phase HPLC. Only the recombinant LL37 obtained from the C-terminally fused protein (LK-CBM3-LL37) showed antibacterial activity against E. coli K12, with a MIC of 180 μg/ml.

The biological activity of the recombinant P-LL37 with a N-terminus proline resulting from the chemical cleavage was confirmed in chapter 4. Also, wound healing experiments were performed in dexamethasone-treated mice to study the effect of LL37 on angiogenesis and wound regeneration. P-LL37 neutralized the activation of macrophages by lipopolysaccharide (LPS) and induced proliferation, migration and tubule-like structures formation by endothelial cells. The topical application of synthetic or recombinant LL37 on mice wounds increased vascularization and re-epithelialization, accelerating the healing process.

After the confirmation that P-LL37 preserves its biological activities and accelerated wound healing, we tested the adjuvant activity of CRAMP and P-LL37 using *Candida albicans* protease Sap2 as antigen. We also evaluated the wound healing effects of topically added LL37 on diabetic mice. These experiments are described in the chapter 5. The administration of CRAMP or P-LL37 with Sap2 did not increase the production of Sap2-specific serum IgG antibody, *i.e.* no adjuvant activity was detected for both peptides. The topical application of LL37 on diabetic mice wounds did not accelerate the healing process very significantly. However, histological analysis after 13 days showed encouraging results. In fact, they revealed that the wounds treated with LL37 were smaller and presented several new skin annexa that could originate new hair, which may translate a more effective tissue regeneration.

RESUMO

Expressão recombinante e atividade de péptidos antimicrobianos catiónicos

Nos últimos 60 anos, os antibióticos têm sido cruciais no combate contra doenças infecciosas causadas por microrganismos. A resistência crescente destes microrganismos aos antibióticos tornou-se um sério problema de saúde pública. Para combater esta situação, muita investigação tem sido desenvolvida para produzir novos tipos de antibióticos.

Os péptidos antimicrobianos (Antimicrobial Peptides - AMPs) são geralmente definidos como péptidos com menos de 50 aminoácidos, com carga positiva devido à presença de múltiplas lisinas e argininas e com mais de 50% de aminoácidos hidrofóbicos. Os AMPS têm gerado grande interesse pelo facto de apresentarem um largo espectro de atividade antimicrobiana contra bactérias Gram-positivas e Gram-negativas, fungos, protozoários e pela capacidade de ultrapassarem os mecanismos de resistência a antibióticos.

A síntese química para a produção de AMPs apresenta custos elevados; alternativamente, as técnicas de expressão recombinante podem ser mais viáveis, economicamente, para uma produção em larga escala, permitindo assim a introdução desta nova geração de antibióticos na prática clínica. Este foi então um dos principais objectivos desta tese: a produção recombinante de dois péptidos antimicrobianos (Magainina-2 e LL37) usando como proteína de fusão um domínio de ligação a carbohidratos de família 3 (Carbohydrate-binding module - CBM3), proveniente de *Clostridium thermocellum*. A utilização deste CBM3 poderá reduzir os custos do processo de purificação usando celulose com matriz de afinidade. O outro objectivo consistiu na avaliação das propriedades biológicas do péptido recombinante LL37 e o seu potencial terapêutico na cicatrização de feridas após aplicação tópica.

A introdução geral desta tese está apresentada no capítulo 1 e inclui uma revisão bibliográfica de: 1) péptidos antimicrobianos; 2) uma breve revisão dos CBMs e do seu potencial como proteínas de fusão; 3) o AMP de rã magainina-2; 4) o AMP humano LL37; e 5) o processo de cicatrização/regeneração de feridas.

O capítulo 2 descreve a clonagem do AMP magainina-2 (MAG2) nos lados N- e C-terminal do CBM3, incluindo o espaçador (LK-CBM3). Foi ainda introduzido na construção, entre os dois módulos, um local de reconhecimento para hidrólise química com ácido fórmico. A proteína recombinante MAG2-LK-CBM3 foi expressa em *Escherichia coli* BL21 (DE3) e a proteína LK-CBM3-MAG2 em *E. coli* M15 (pREP4). A MAG2 no N-terminal da primeira construção foi expressa, clivada e purificada com sucesso. No entanto, o péptido não apresentou atividade antimicrobiana contra *E. coli K12*. A presença de um resíduo carregado negativamente (aspartato) no lado C-terminal da MAG2, poderá causar a ausência de atividade. Este resíduo não está presente no péptido nativo, resultando da introdução da sequência aspartato-prolina na

construção, para permitir a hidrólise ácida. A expressão da MAG2 no lado C-terminal do LK-CBM3 não teve sucesso. O péptido poderá ter sofrido proteólise durante a expressão em *E. coli*.

No capítulo 3 descreve-se a expressão do AMP humano LL37. Este foi também clonado nos lados N- e C-terminal do LK-CBM3. De igual modo, introduziu-se o local para hidrólise química com ácido fórmico entre os dois módulos, para permitir a separação da LL37. As duas proteínas recombinantes foram expressas em *E. coli* BL21 (DE3) e solubilizadas com Triton X-100. A purificação foi efectuada usando fibras de celulose CF11, beneficiando da afinidade específica do CBM3 para este material; após hidrólise com ácido fórmico, LL37 foi purificada por RP-HPLC. A LL37 proveniente do C-terminal da proteína de fusão (LK-CBM3-LL37) foi a única a apresentar atividade antimicrobiana contra *E. coli* K12, com uma MIC de 180 µg/ml.

As atividades biológicas do péptido recombinante P-LL37, com uma prolina no lado N-terminal resultante da hidrólise química, foram confirmadas no capítulo 4. Além disso, o efeito da LL37 na angiogénese e cicatrização de feridas foi avaliada em ratinhos tratados com dexametasona. P-LL37 neutralizou a ativação de macrófagos por lipopolissacáridos e induziu a proliferação, migração e formação de vasos em células endoteliais. A aplicação tópica de LL37 sintética ou recombinante nas feridas de ratinhos aumentou a vascularização e re-epitelialização, acelerando o processo de cicatrização.

Após ter sido confirmado que o péptido recombinante P-LL37 conserva as suas atividades biológicas e acelera o processo de cicatrização, foi testado o efeito adjuvante dos péptidos CRAMP e P-LL37 para efeitos de vacinação, usando a protease Sap2 de *Candida albicans* como antigénio. Além disso, foi avaliado o efeito da aplicação tópica de LL37 nas feridas de ratinhos diabéticos. Estas experiências estão descritas no capítulo 5. A administração de CRAMP ou P-LL37 juntamente com Sap2 não aumentou a produção de anticorpos específicos anti-Sap2. Isto é, não foi detectada atividade adjuvante para nenhum dos dois péptidos. A aplicação tópica de LL37 nas feridas de ratinhos diabéticos não acelerou o processo de cicatrização de maneira muito significativa. No entanto, as análises histológicas das feridas após 13 dias revelaram dados encorajadores. De facto, estas análises mostraram que as feridas tratadas com LL37 eram menores e apresentavam vários anexos de pele que poderão originar novos pêlos, traduzindo uma mais eficiente regeneração dos tecidos.

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List of Abbreviations

Ab	Antibody
Ag	Antigen
AMP	Antimicrobial Peptide
bFGF	Basic Fibroblast growth factor
BMDM	Bone marrow-derived macrophages
BrdU	5-bromodeoxyuridine
BSA	Bovine serum albumin
CBD	Cellulose-binding domain
CBM	Carbohydrate-binding module
CNBr	Cyanogen bromide
CRAMP	Cathelin-related antimicrobial peptide
DC	Dendritic cell
DCA	Deoxycholic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ECGS	endothelial cell growth supplement
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
FBS	Fetal bovine serum
FPRL-1	Formyl Peptide Receptor Like-1
hBD	Human β-defensing
hCAP18	Human cationin antimicrobial peptide 18
HD	human α-defensins
HGF	Hepatocyte growth factor
HIF1α	Hypoxia-inducible transcription factor 1α
HIV	Human immunodeficiency virus

HMEC	Human microvascular endothelial cell
HNP	Human neutrophil peptide
HSV	Herpes Simplex Virus
HUVEC	Human umbilical vein endothelial cell
i.d	Intradermal
i.p	Intraperitoneal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMAC	Immobilized metal ion affinity chromatography
IPTG	isopropyl-β-D-thiogalactopyranoside
JV	Junin Virus
Ka	Equilibrium affinity constant
KGF	Keratinocyte growth factor
LB	Luria-bertani broth
LBP	LPS-binding protein
LPS	Lipopolysaccharide
MAG	Magainin
MALDI-TOF	Matrix-assisted laser desorption/ionization-time of flight
MAPK	Mitogen-activated protein kinase
MCP-1	monocyte chemotactic protein-1
MIC	Minimum inhibitory concentration
MIP-3	Macrophage inflammatory protein 3-α
MSC	Mesenchymal stem cell
MTS	3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium
MVD	Microvessel density
Mw	Molecular weight
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ODN	Oligodeoxynucleotide
OVA	Ovalbumin
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cell

PDGF	Platelet-derived growth factor
pI	Isoelectric point
PMN	Polymorphonuclear neutrophil
PMSF	Phenylmethanesulfonylfluoride
RNA	Ribonucleic acid
RP-HPLC	Reverse phase high-performance liquid chromatography
Sap	Secreted aspartic proteinase
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the means
s-LL37	Synthetic LL37
TFA	Trifluoracetic acid
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

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PUBLICATIONS

This thesis is based on the following original research or review articles:

Chapter 1: Reinaldo Ramos, Lucília Domingues, Miguel Gama. **LL37, a human antimicrobial peptide with immunomodulatory properties.** Accepted for publication in the book titled: *Science against microbial pathogens: communicating current research and technological advances.* MICROBIOLOGY BOOK SERIES – 2011 Edition

Chapter 3: Reinaldo Ramos, Lucília Domingues, Miguel Gama. ***Escherichia coli* expression and purification of LL37 fused to a family III carbohydrate-binding module from *Clostridium thermocellum*.** *Protein Expression and Purification* 71(1), 1-7, 2010.

Chapter 4: Reinaldo Ramos, João Pedro Silva, Ana Cristina Rodrigues, Raquel Costa, Luísa Guardão, Fernando Schmitt, Raquel Soares, Manuel Vilanova, Lucília Domingues and Miguel Gama. **Wound healing activity of the human antimicrobial peptide LL37.** *Peptides*, In Press.

Chapter 5: Reinaldo Ramos, João Pedro Silva, Raquel Costa, Luísa Guardão, Raquel Soares, Augusto Faustino, Manuel Vilanova and Miguel Gama. **Studies on the bioactivity of the human antimicrobial peptide LL37.** Paper in preparation for submission.

CHAPTER 1

GENERAL INTRODUCTION

1.1 ANTIMICROBIAL PEPTIDES

The increasing bacterial resistance against common antibiotics is a growing concern for the public health. Resistance began to emerge as early as the 1950s with multiresistant microorganisms such as *Staphylococcus aureus*, *Pseudomonas aeruginosa* or *Enterococcus sp.,* in large part as the result of antibiotic overuse [1]. The exposition of living organisms to potentially harmful microorganisms is constant through contact, ingestion and inhalation [2]. Survival involves several levels of host defense mechanisms and the contact with microorganisms originates different reactions [3]:

A. The invading microorganisms are eliminated by innate host defense mechanisms without inflammatory response;

B. The host defenses are crossed over and innate immune system molecules are upregulated. These have direct antimicrobial activity and attract inflammatory cells and cells of the adaptive immune system resulting in the elimination of the microorganisms;

C. Microbes surpass innate and adaptive immunity. The consequences are a strong inflammatory response and death of the host.

Much research has been dedicated to the development of new classes of antibiotics to overcome bacterial resistance; research focusing on the structures and functions of antimicrobial peptides (AMPs) has greatly increased in recent years. AMPs are an important part of the innate immune system of most living organisms against invading pathogens with direct antimicrobial and mediator function. They play an important role in all the situations described above by providing the initial host defense mechanism. They are generally defined as peptides of less than 50 amino acids residues with positive charge, due to multiple lysine and arginine residues and with 50% or more of hydrophobic residues [4]. In addition to positively charged AMPs, peptide antibiotics with a negative charge at neutral pH have also been identified [5, 6]. Some of these peptides seem to originate as cleavage products from larger proteins. AMPs have aroused great interest due to their broad spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria [7], fungi [8] and protozoa [9] with minimum inhibitory concentrations (MIC) as low as 0.25-0.4 μg/ml [10]. Certain cationic peptides have been shown to inhibit the replication of enveloped viruses such as influenza A virus [11], vesicular stomatitis virus (VSV) and human immunodeficiency virus (HIV-1) [12]. Moreover, these peptides

have potential to overcome bacterial resistance [2]. Therefore, AMPs or their derivatives can represent potentially new classes of antimicrobial drugs.

1.1.1 Classification and source of antimicrobial peptides

AMPs are found in all forms of life including bacteria, fungi, plants, insects, birds, crustaceans, amphibians and mammals and a single animal can contain different classes of peptides [13]. So far, more than 1700 peptides have been identified (http://aps.unmc.edu/AP/main.php). They can be divided into two classes based on the mechanism of their cellular synthesis: non-ribosomally synthesized and ribosomally synthesized peptides [14]. The first class is largely produced by bacteria and some are already used for clinical applications: polymyxin B and colistin (polymyxin E, colimycin) have been developed mainly for topical applications. Cyclosporin is widely used as immunosupressor. The non-ribosomally synthesized tripeptide ACV is the precursor of penicillin and cephalosporins [15]. Ribosomally synthesized peptides are produced by all organisms (including bacteria) as an important component of their host defense. Ribosomally synthesized AMPs are gene-encoded peptides, meaning that one gene codes for one peptide. AMP genes of vertebrates have a characteristic intron-exon structure with regulatory elements in their promoter regions (figure 1.1). The primary translational product is a prepropeptide consisting of an N-terminal signal sequence for targeting to the endoplasmic reticulum, a pro-segment, and a C-terminal cationic peptide that has antimicrobial activity after cleavage (figure 1.1). The pro-segment is generally anionic and may have several biological functions such as assisting the correct folding of the C-terminus, intracellular trafficking or inhibition of the activity of the mature peptide. AMPs are stored in cells as propeptides or mature C-terminal peptides [15].

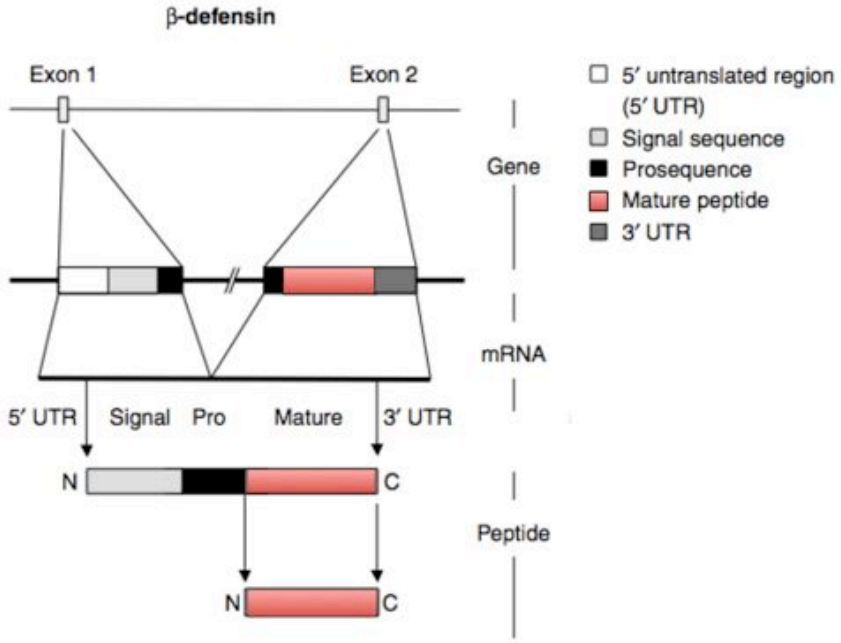

Figure 1.1. Gene of a vertebrate antimicrobial peptide and processing. The C-terminus represents the part of the molecule with antimicrobial activity: the β-defensin. The gene is represented schematically (adapted from Koczulla *et al.* [15]).

AMPs can be grouped according to their size, conformational structure or predominant amino acid sequence; nevertheless, the diversity of these molecules is so great that it is difficult to categorize them in a generally accepted classification. On the basis of their 3D structure, peptide antibiotics can be classified in four major groups, with the first two being the most common in nature [10]:

- Group I: β-sheet structures stabilized by disulfide bridges.
- Group II: linear peptides with a α-helical structure.
- Group III: extended peptides.
- Group IV: peptides with loop structures.

The representative structures from each of these classes are illustrated in figure 1.2.

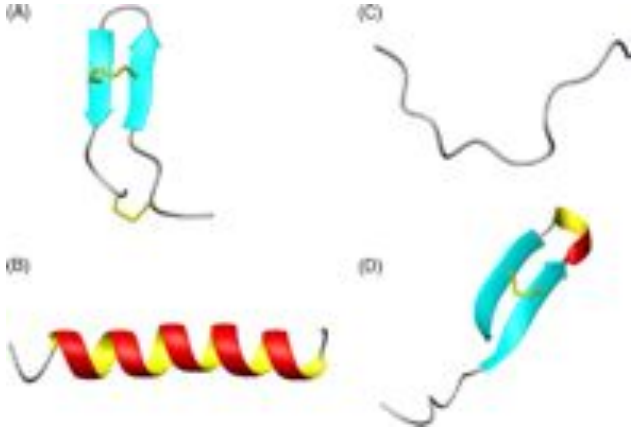

Figure 1.2. Structural classes of antimicrobial peptides: (A) β-sheet, tachyplesin I; (B) α-helical, magainin 2; (C) extended, indolicidin; (D) loop, thanatin. Disulfide bonds are indicated in yellow (picture from Powers *et al.* [4]).

AMPs from insects and other invertebrates

A better understanding of the peptide production in response to pathogenic invasions came from the fruit fly, *Drosophila melanogaster*. Infection in this insect rapidly induces the production of a lineage of peptides namely drosomycin, cecropins, diptericin, drosocin, attacin and metchnikowin by the fat body cells [16]. Cecropins are 3, 4 kDa linear amphipathic peptides with activity against protozoa, metazoan parasites, bacteria and fungi [17]. Drosomycin and metchnikowin are potently antifungal while others exhibit antibacterial properties [18]. In certain species such as the ant, *Pachycondyla goeldii*, about 15 different peptides demonstrating antibacterial and insecticidal properties have been isolated from its venom. These peptides, named ponericidins, range from 1.8 to 3.3 kDa and share sequence similarities with cecropins, mellitins and dermaseptins [19].

Insect defensins from various species have been characterized. These peptides are generally composed of 34–46 amino acid residues with exception of the 51-residue defensins characterized in bees [20]. All insect defensins share a consensus motif of six cysteines which form intramolecular disulfide bonds and all have the same cysteine pairing: Cys1-Cys4, Cys2-Cys5 and Cys3-Cys6 [16]. So far, many more AMPs from insects (188) have been identified. Table 1.1 summarizes some examples of insect AMPs that can be divided in the following groups:

- The cysteine-containing peptides namely insect defensins, drosomycin, cecropins, sapecins and thanatin;
- The proline-rich peptides like drosocin, lebocins and formaecins;
- The gloverins (holotricins, diptericins), glycine-rich molecules.

Table 1.1. Antimicrobial peptides from insects (adapted from Reddy *et al.* [2] and Bulet *et al.* [16])

Source	Peptide	Number of amino acids	Antibacterial activity
Stomoxys calcitrans	Smd 1	46	G^+, G^-
	Smd 2	40	G^+, G^-
Drosophila melanogaster	Drosomycin	44	F
	Drosocin	19	G−
	Diptericin	83	G−
	Metchnikowin	26	F
Phormia terranovae	Defensin-α	40	G^+, G^-
Sarcophaga peregrina	Sapecin-α	40	G^+, G^-
	Sapecin-β	34	G^+, G^-
	Sapecin-c	40	G^+, G^-
Aedes aegypti	Defensin-α	40	G^+, G^-
	Defensin-β	40	G^+, G^-
Apis mellifera	Mellitin	26	G^+, G^-, H
	Defensin	51	G^+, G^-
	Royalisin	51	G^+, G^-
Acalolepta luxuriosa	Acaloleptin	71	G^+, G^-
Hyalophora cecropia	Cecropin	37	G^-
Lycosa carolinensis	Lycotoxin	27	G^+, G^-
Cupiennius salei	Cupiennin	35	G^+, G^-
Podisus maculiventris	Thanatin	21	G^+, G^-
Tenebrio molitor	Tenicin 1	43	G^+, G^-
Bomby mori	Lebocin 1	32	G^+, G^-
	Lebocin 2	32	G^+, G^-
	Lebocin 3	32	G^+, G^-
Myrmecia gulosa	Formaecin 1	16	G^-
	Formaecin 2	16	G^-
Holotrichia diomphalia	Holotricin 3	84	F

G^+: gram-positive; G^-: gram-negative; F: antifungal; H: haemolytic.

Apart from insects, other invertebrates possess antimicrobial peptides. Tachyplesins, 17,18 amino acid long peptides isolated from the haemocytes of the Japanese horseshoe crab, *Tachypleus tridentatus*, are bactericidal against Gram-positive and Gram-negative bacteria [21]. Polyphemusin, an isoform of tachyplesin has been purified from the haemocytes of *Limulus polyphemus*, a related horse shoe crab [22]. Both are cysteine-rich peptides with similar sizes and intramolecular disulfide-pairing patterns. Another peptide, a defensin with 8 kDa, was purified from the haemocyte granules of *T. tridentatus* [23] showing significant homology with mammalian β-defensin.

Androctonin, a 25-residue AMP has been isolated from the scorpion *Androctonus australis* by Ehret-Sabatier *et al* [24]. This highly cationic peptide has four cysteine residues involved in the formation of two intramolecular disulfide bridges and is active against Gram-positive and Gram-negative bacteria and fungi. Penaeidins, peptides with 47 to 62 residues, were purified from shrimps by Destoumieux and colleagues [25] and Cuthbertson *et al.* [26]. These AMPs exhibit activity against Gram-positive bacteria and filamentous fungi but not against *Candida albicans* or *Saccharomyces cerevisiae* [27].

AMPs from plants

Plants are constantly exposed to a large variety of microorganisms, such as viruses, fungi, bacteria, protozoa, mycoplasma and nematodes [28]. The survival in these conditions requires quick defense mechanisms, which include the synthesis of defense peptides and proteins with antimicrobial properties. The major groups of AMPs in plants are thionins and defensins.

The thionins are basic peptides (pI>8), with low molecular weight (~5 kDa), rich in basic and sulfur-containing residues (arginine, lysine and cysteine). They share high sequential and structural similarities besides presenting toxic effects against bacteria, fungi, yeast, animal and plant cells. Purothionins are basic polypeptides with antimicrobial properties that are present in the endosperm of wheat and other cereal species. Antimicrobial activity was demonstrated in phytopathogenic bacteria such as: *Pseudomonas*, *Xanhtomonas*, *Erwinia* e *Corynebacterium* [29]; yeast [30] and phytopathogeninc fungi [31]. Besides their activity against microorganisms, Carrasco *et al.* [32] described

cytotoxic activity of thionins on mammalian cells. Several *in vivo* functions have been proposed for thionins, like the regulation of proteins [33, 34].

Plant defensins were initially known as γ-thionins because they showed a similar size (5 kD) and the same number of disulfide bridges (four) as α- and β-thionins. They were isolated in the 90s from wheat, barley, sorghum, radishes and other species of *Brassicaceae* [28]. Later, it was concluded that the γ-thionins actually show a low degree of similarity with other thionins. Therefore, Broekaert *et al.* [35] suggested to include the γ-thionins into a superfamily of antimicrobial peptides, with representatives in vertebrates, invertebrates and plants, renaming them as plant defensins. Plant defensins are 45 to 54 amino acids long, have a net positive charge, and show clear, although relatively limited, sequence conservation. Many plant defensins present a powerful fungicide action [35], but bacteria are mostly unaffected, with some exceptions such as *Bacillus subtilis* [36], *Pseudomonas solanacearum* and *Clavibacter michigansis* [37].

AMPs from animals

AMPs in animals are expressed in several tissues, polymorphonuclear leukocytes, macrophages and mucosal epithelial cells; they can also be secreted into circulating fluids (e.g. the bloodstream or the haemolymph), which deliver AMPs to infection sites.

Some examples of antimicrobial peptides from animals are listed in table 1.2, according to their structure. The two major groups of AMPs in mammals are defensins and cathelicidins.

Mammalian defensins are cationic, relatively arginine-rich, nonglycosylated peptides with a molecular weight of 3.5-4.5 kDa and contain six cysteines that form three characteristic intramolecular disulfide bridges [38]. According to their structure, defensins can be divided into three classes: α-defensins, β-defensins and θ-defensins [15].

α-Defensins are 29-35 amino acids long, contain three disulfide bridges in a 1-6, 2-4, 3-5 alignment and reveal a triple stranded β-sheet structure with a β-hairpin, which contains cationic amino acids. At the present time, six human α-defensins have been identified. Human neutrophil peptides 1-4 (HNP 1–4) are found in the azurophil granules of neutrophils. The two other human α-defensins 5 and 6 (HD 5–6) are primarily found in Paneth cells of the small intestine. They are active at 1-100 μg/ml against Gram-positive bacteria, Gram-negative bacteria, mycobacteria, fungi and enveloped viruses [39].

Table 1.2. Antimicrobial peptides from animals (adapted from Koczulla *et al.* [15])

Peptide	Species, organ	Activity
Group I: β-sheet structures stabilised by two or three disulphide bridges		
Protegrin	Pig, intestine	Antimicrobial
Defensins	Vertebrates, immune cells, epithelia	Antimicrobial, chemoattractant
θ-Defensins	Monkeys, neutrophils	Antimicrobial
Group II: Linear, α-helical peptides without cysteines		
Bombinins	Frog, skin	Antimicrobial
LL-37	Human, neutrophils, epithelial cells	Antimicrobial, chemoattractant, wound healing, angiogenesis
Magainins	Frog, skin	Antimicrobial
Group III: extended peptides		
PR-39	Pig, intestine, neutrophils	Antimicrobial, angiogenesis, wound healing
Bac5, Bac7	Cow, neutrophils	Antimicrobial
Group IV: Peptides with loop structures		
Bactenecin	Cow, neutrophils	Antimicrobial
Ranalexin	Frog, skin	Antimicrobial

β-Defensins have 36-42 residues and are arginine-rich with broad antimicrobial, antiviral and cytotoxic activity. Although there is low sequence homology with α-defensins, their tertiary structure is very similar. Therefore, this new family of AMPs was named β-defensins. β-defensins reveal a disulfide alignment of 1–5, 2–4, 3–6, and have been isolated from several species [40]. The first human β-defensin, called human β-defensin 1 (hBD-1), was originally isolated from haemofiltrate of patients undergoing dialysis treatment and is expressed constitutively in epithelial cells of the urinary and respiratory tract [41]. Human β-defensin 2 (hBD-2) was isolated from psoriatic skin and is widely expressed in epithelia, leukocytes and the bone marrow [42]. hBD-3 was isolated from

human lesional psoriatic scales and cloned from keratinocytes [43]. Schuttle *et al.* identified 28 new human and 43 new mouse β-defensin genes in five syntenic chromosomal regions by screening human and murine genome databases [44].

A novel class of defensins, were isolated from rhesus monkey neutrophils and named θ-defensins according to their circular molecular structure [45].

Besides their antimicrobial activity, defensins possess other properties like antitumoral activity, cells proliferation, chemoattraction of immune cells, stimulation of cytokines and expression of adhesion molecules [40].

Cathelicidins are a family of antimicrobial peptides derived from proteins, that contain a highly conserved signal sequence and pro-region but show substantial heterogeneity in the C-terminal domain. Because of the similarity of their proregions to cathelin, a 12 kDa protein from porcine leukocytes, this family of antimicrobial peptides was named cathelicidins [46]. The C-terminal domain that encodes the mature peptide with antimicrobial activity can range from 12 to 97 amino acid residues. To date, cathelicidins have only been found in mammals, in the neutrophils of horses, cattle, pigs, sheeps, rabbits and mice. They are particularly abundant in porcine and bovine neutrophils [47]. The only human cathelicidin, LL-37 or hCAP-18, was isolated from human bone marrow [48]. The processing of cathelicidin preforms to mature antimicrobial peptides occurs by proteolytic cleavage of the propiece upon degranulation of neutrophils. This cleavage is mediated by elastase in cattle and pigs and proteinase 3 in humans [47]. Cathelicidins have a diverse range of antimicrobial activity against Gram-negative and Gram-positive bacteria [49], fungi [50, 51], parasites [52, 53] and enveloped viruses [54]. In mammals, cathelicidins with α-helical, extended or loop structure, and β-sheet peptides with 2 or 3 disulfide bridges have been identified [47].

Cathelicidins have first been identified for their antimicrobial activities; however, additional biological functions have been documented. These activities include wound repair mechanisms, chemoattractant properties that recruit inflammatory cells, induction of angiogenesis, interaction with lipopolysaccharides (LPS), and induction of cytolysis [47].

1.1.2 Functions of AMPs

Antimicrobial activity

The antimicrobial activity of peptide antibiotics was deduced from *in vitro* tests against microorganisms. AMPs have a broad-spectrum activity against Gram-positive and Gram-negative bacteria as well as against fungi and enveloped viruses. The minimal inhibitory concentrations of the more effective peptides are in the range from 0.1–10 µg/ml. AMPs show synergistic activity with other host defense molecules, such as lysozyme and lactoferrin.

The exact mechanism by which AMPs exert their antimicrobial properties is yet unclear, but it is generally accepted that cationic AMPs interact by electrostatic forces with the negatively charged bacterial membrane phospholipids, leading to its disruption. In the case of microbes, the anionic lipids are present on the outer surface of the membrane whereas for mammalian cells, anionic lipids are present along the cytoplasmic side of the membrane. This feature might account for their preferential activity against bacteria. Another major difference in the membranes chemical composition that distinguishes prokaryotic and eukaryotic cells is that the latter are abundant in sterols. The presence of membrane-stabilizing cholesterol has been shown to protect human erythrocytes from magainin 2 [55]. Among the several mechanisms proposed, the most widely accepted are the “barrel-stave”, “carpet” and “toroidal” models [56].

The “barrel-stave” model describes the formation of transmembrane channel/pores by groups of amphipathic α-helical peptides, where the hydrophobic surfaces interact with the aliphatic chain of the membrane phospholipids and the hydrophilic parts point inside producing an aqueous pore [57]. This pore formation can be confirmed by stepwise conductivity increases in channel measurement. The transmembrane pore formation involves the binding of peptide monomers to the membrane in a helical fashion, followed by insertion of the helices into the hydrophobic core of the membrane. Progressive recruitment of additional monomers increases the pore size leading to leakage of cell contents and thereby death of the cell (Figure 1.3A). Pore formation accompanies the reorientation of the helix from the parallel state to the perpendicular membrane spanning state.

In the “carpet model”, peptides accumulate on the bilayer surface. This model explains the activity of antimicrobial peptides such as ovispirin that orientate parallel (‘in-plane’) to

the membrane surface [58]. In the “carpet model”, peptides accumulate on the bilayer surface. Peptides are electrostatically attracted to the anionic phospholipid head groups at numerous sites covering the surface of the membrane in a carpet-like manner. At high peptide concentrations, surface-oriented peptides are thought to disrupt the bilayer by acting like a detergent, eventually leading to the formation of micelles [59].

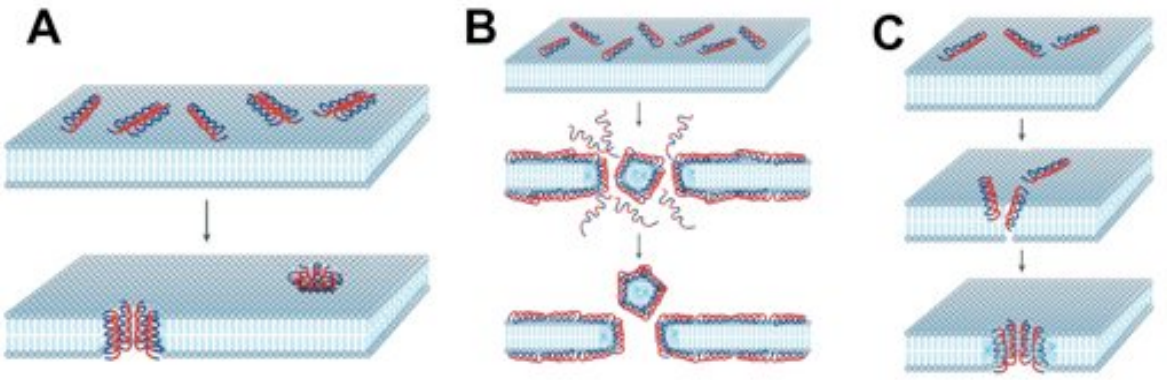

Figure 1.3. Mechanisms of antimicrobial activity. **A** – Barrel-stave model; **B** – Carpet model; **C** – Toroidal model. Hydrophilic regions of the peptide are shown coloured red, hydrophobic regions of the peptide are shown coloured blue (adapted form Brogden *et al.* [56]).

In the “toroidal-pore model”, antimicrobial peptide helices insert into the membrane and induce the lipid monolayers to bend continuously through the pore so that the water core is lined by both the inserted peptides and the lipid head groups. This type of transmembrane pore is induced by magainins, protegrins and melittin [60, 61]. The “toroidal” model differs from the “barrel-stave model” as the peptides are always associated with the lipid head groups even when they are perpendicularly inserted into the lipid bilayer.

Dathe *et al.* [62] suggested that these mechanisms do not represent three completely different modes of action, but instead, there is a continuous graduation between them. Initially, the transmembrane potential and pH gradient are destroyed, the osmotic regulation is affected and respiration is inhibited [56].

The mechanism of bacterial killing by anionic peptides is not known. Anionic peptides require zinc for maximal activity and complex with it [63]. Brogden *et al.* [64] suggest that zinc may form a cationic salt bridge that allows the peptide to overcome the net negative charge on the microbial surface. Then, the peptide penetrates the outer membrane

without inducing any morphological changes [65]. Once in the cytoplasm, they may attach to ribosomes and inhibit ribonuclease activity, like aspartic acid polymers do, leading to the precipitation of cytoplasmic proteins. Killing occurs within 30 min [66].

Although the formation of ion channels, transmembrane pores and extensive membrane rupture eventually leads to the lysis of microbial cells, there is increasing speculation that these effects are not the only mechanisms of microbial killing. There is increasing evidence that antimicrobial peptides have other intracellular targets. Figure 1.4 illustrates the intracellular action of several AMPs from different sources.

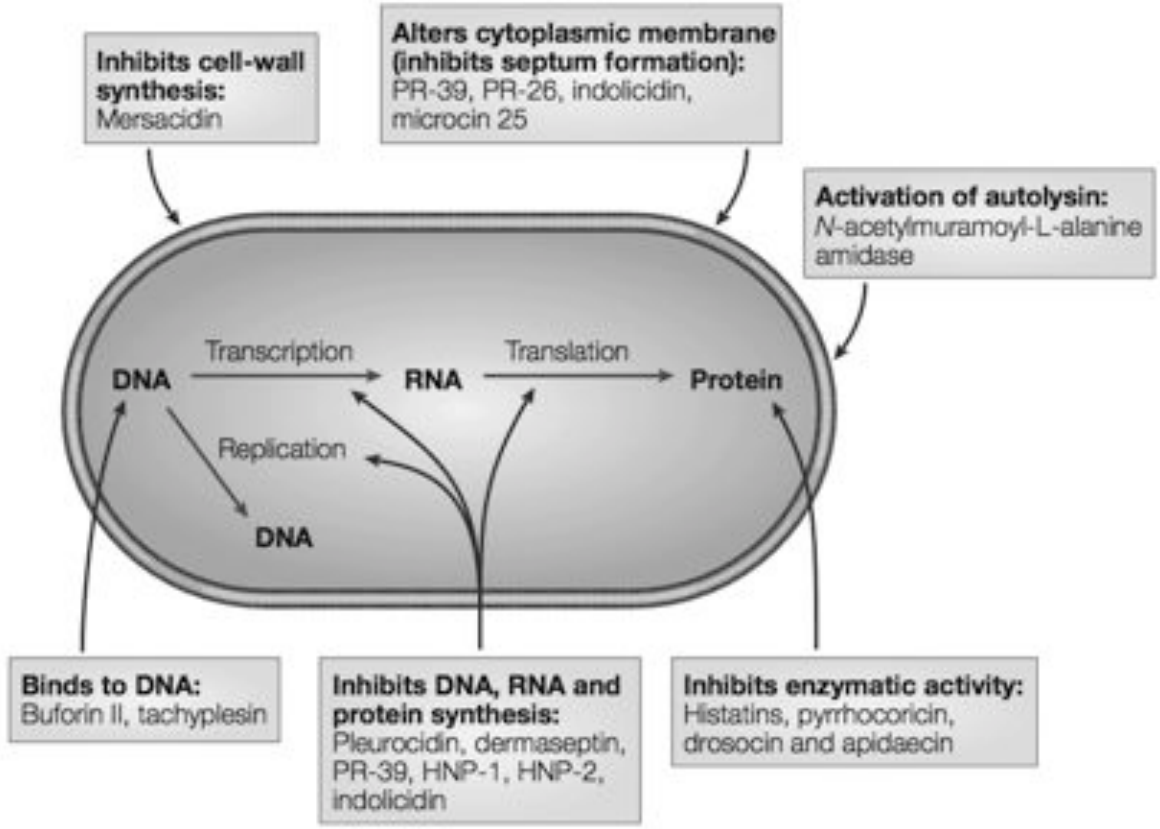

Figure 1.4. Mode of action for intracellular antimicrobial peptide activity. In this figure *Escherichia coli* is shown as the target microorganism (from Brogden *et al.* [56]).

The action of cationic antimicrobial peptides is not limited to direct killing of microorganisms. Instead, they possess an impressive variety of additional activities that impact particularly on innate immune responses and inflammation.

Immunomodulatory properties

Defensins and cathelicidins show chemoattractant properties on different cell types such as monocytes, T lymphocytes and dendritic cells (DC) [67]. Moreover, β-defensins induce the production of several chemokines and cytokines such as monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein 3-α (MIP-3), RANTES, interleukin-6 IL-6), IL-10, interferon-inducible protein 10, tumor necrosis factor α (TNF-α) and IL-1, mainly in keratinocytes. hBD-3 induces the higher expression of these cytokines and chemokines compared with hBD-1, -2, and -4 [68]. At low concentrations α-defensins induce the proliferation of fibroblasts and epithelial cells in the airways [69]. Also, defensins are able to induce phenotypic and functional changes in DC [70]. These experimental observations highlight the relevance of AMPs bridging the innate and adaptive immunity. Table 1.3 summarizes the effect of several AMPs on host cells and inflammatory/immune processes. LL37, the only human cathelicidin, also shows several immunomodulatory properties that will be discussed in more detail further ahead.

Table 1.3. Effect of cationic antimicrobial peptides on host cells and inflammatory/immune processes (adapted from Hancock and Diamond [13])

Peptide	Effect	Possible role in immunity
LL37; PR-39; TAP; LAP; α- and β-defensins	Induction during inflammation	Synthesis triggered by situations that might involve infection
CAP11; Magainin 2	Promote histamine release from mast cells	Stimulate increase in blood vessel permeability
α -defensins	Induce IL-8 in airway epithelial cells	Recruitment of neutrophils
PR-39	Chemotactic activity for neutrophils; Induce cell surface matrix proteogylcans, syndecans 1 and 4	Increase phagocytic activity; Wound healing
LL-37; HNP-1, -2 and –3; histone H2B fragments	Induction in wounds and blisters	Wound healing
Guinea-pig defensins	Increased expression of CD11b,c and ICAM-1	Increase adherence of neutrophils
Rabbit defensins NP-1,2	Promote non-opsonic phagocytosis by macrophages	Bacterial clearance
Defensin I and II;	Inhibit fibrinolysis by tissue plasminogen activator	Bacterial clearance; activation of macrophages
Defensin; SB-37	Stimulate mitogenic effect for fibroblasts and epithelial cells; stimulate fibroblast growth	Wound healing
Histatin 3; pro Bac7	Inhibition of furin proprotein convertase/cathepsin L protease	Inhibit tissue injury during inflammation
BMAP-27; BMAP-28; Lactoferricin	Apoptosis in the U937 and RAW264.7 macrophage lines and in in vitro- activated human lymphocytes	Elimination of cells with intracellular bacteria, virus-infected cells and cancer cells
Defensins	Attenuates steroidogenesis	Blunt the release of immunosuppressive cortisol during stress from infection; upregulated tissue inflammation
ProBac7; defensins	Chemotactic activity for monocytes	Recruitment of monocytes, which differentiate into inflammatory macrophages
Many peptides	Neutralize LTA responses in macrophages	Antisepsis
HNP-1, -2; LL-37	Chemotactic activity for T cells	Recruit T helpers and initiate cellular immune responses
Defensins; HNP-1, -2 and -3	Enhancement of IFN-γ, IL5, K6, K10 and proliferative responses by T-helper cells cytokine secretion, increased systemic IgG but not IgA	Promotion of acquired systemic immune defenses (not mucosal immune defenses)

Abbreviations: IFN, interferon; Ig, immunoglobulin; IL, interleukin; LAP, lingual antimicrobial peptide; LTA, lipoteichoic acid; LPS, lipopolysaccharide; TAP, transporters associated with antigen processing

1.1.3 AMPs as drugs

AMPs have a number of potential advantages as future therapeutics. As already stated, in addition to their broad spectrum of antimicrobial activity and rapid killing of microbes, they neutralize endotoxin and are unaffected by classical antibiotic resistance mechanisms. Moreover, high levels of defensins have been reported in several pulmonary disorders such as idiopathic pulmonary fibrosis, alveolar proteinosis, acute respiratory distress syndrome, lung transplantation, and panbronchiolitis, among others [71]. The exact role of defensins in these lung disorders is not fully understood, but they are probably involved in the modulation of inflammatory response. Furthermore, Crohn's colitis is associated with an impaired induction of hBD-2 and hBD-3 [72]. Also, a deficient expression of α-defensins HD-5 and HD-6 in Paneth cells from patients with Crohn's ileal disease has been described [73].

Several studies have demonstrated a cytotoxic activity of AMPs against cancerous cells. Three mechanisms have been proposed, namely: (1) lysis of the cell membrane, (2) activation of extrinsic apoptotic pathways, and (3) inhibition of angiogenesis [74]. Most research conducted in this field has centered on cecropins and magainins, but other AMPs like defensins, cathelicidin, and lactoferricin are also being studied.

AMPs have proven to play an important role in wound repair and angiogenesis. Cathelicidins and defensins hBD-2 and hBD-3, rapidly and dramatically increase at the wound edges leading to wound repair. A recent study has shown that hBD-2 stimulates migration, proliferation and tube formation of endothelial cells in wounds, *in vitro*, leading to accelerated wound closure [75]. The induction of angiogenesis through the use of AMPs could generate new treatments to improve wound healing and to fight diseases.

Having clearly a great potential, the tight regulation of AMPs should nevertheless be considered, since elevated amounts may lead to a chronic inflammatory process, as it has been demonstrated for psoriasis and rosacea [76, 77].

Due to their capacity to modulate both innate and adaptive immunity, considerable efforts have been made to exploit their therapeutic potential. Novel peptides for drug development are generally produced by modification of natural peptides. Table 1.4 shows some AMPs that have been or are currently being tested in clinical and preclinical trials.

Table 1.4. Companies involved in the development of antimicrobial peptides as drugs (adapted from Koczulla and Bals [15] and Guaní-Guerra *et al.* [78])

Company Name	Peptide	Clinical use
Migenix	MBI-226	Catheter sepsis
	MX-594AN	Acne
Intrabiotics	PG-1	Treatment of peritoneal infections caused by *P. aeruginosa* and *S. aureus*. Treatment of pneumonia.
Novozymes	Plectasin	Microbicidal activity against antibiotic-resistant bacteria
Dermegen	P-113	Oral candidiasis, mucositis; lung spray
	D2A21	Burn wounds, infected wounds
Genaera	Pexiganan (MSI-78)	Infected diabetic food ulcers
Xoma Corp.	Neuprex	Meningitis
Cubist Pharmaceuticals	Daptomycin	Sepsis
AM	Lactoferricin-B	Antifungal
Entomed	Heliomycin	Antibacterial
Trimeris	Enfuvirtide (T-20)	HIV

The major problem of chemical synthesis for the production of AMPs is the elevated cost. It is possible to chemically synthesize most AMPs, but the costs exclude most peptides from clinical development. Therefore, biological expression strategies have been developed to circumvent this problem. Biological production routes using different microbial host systems such as bacteria, yeast and insect cells have been increasingly reported in recent AMP production studies [27, 79-84].

In a different approach, the coding sequence of AMPs can also be transferred into the target cells by means of adenoviral gene transfer [85, 86]. Finally, the production of AMPs can be induced by stimulants administered by topic or systemic routes. Fehlbaum *et al.* [87] induced the expression of epithelial β-defensin with l-isoleucine and several of its analogs. This promising technique would avoid the possible toxicity and adverse systemic reactions, as well as the difficulty to deliver them in integral form to the desired sites of action [78].

1.2 CARBOHYDRATE-BINDING MODULES (CBMS) AS FUSION PARTNERS

Much research has been dedicated to AMPs as a new class of antibiotics. However, the chemical synthesis of these peptides carries high costs. Recombinant expression techniques for the large-scale production of AMPs can be much more viable economically, if the bio-production system is adequately optimized to increase the peptide expression yield while minimizing costs. Biological methods will also ease the production of peptides that require correct folding and disulfide bonds formation for antimicrobial activity (*e.g.* protegrin, θ-, α- and β-defensin, bactenesin, and hepcidin), which is difficult to achieve using chemical synthesis due to the typical low amounts of correctly-oxidized products [88]. Unfortunately, the expression of heterologous genes in *E. coli* often fails due to the toxicity of the molecule of interest towards the host. This problem is particularly common in the case of antimicrobial peptides. Also, AMPs are very sensitive to intracellular proteases. The lethal effects of antimicrobial peptides on host (mostly *E. coli*) and proteolysis can often be circumvented by expressing the molecule of interest as part of a fusion protein (*e.g.* in conjunction with glutathione S-transferase (GST) [89] or thioredoxin [90, 91]).

In recombinant expression systems, the purification of the proteins is generally the most critical step. Although very effective, chromatography is a slow process with high costs due to expensive matrixes. Therefore, fusion partners that would allow the overexpression of the AMPs and low purification costs are essential for the large-scale production of these peptides.

Regarding their characteristics, carbohydrate-binding modules (CBMs) are promising fusion partners for the recombinant expression of AMPs.

Many enzymes that hydrolyze insoluble carbohydrates share a common structure composed of a catalytic domain linked to an independent CBM. CBMs were initially classified as cellulose binding domains (CBDs), based on the initial discovery of several modules that bind cellulose [92, 93]. However, additional modules in carbohydrate-active enzymes that bind carbohydrates other than cellulose but that meet the CBM criteria are continually being found, hence the need to reclassify these polypeptides using a more

inclusive terminology. CBMs are present in a large variety of enzymes, within a wide range of species, from archea, bacteria, viruses, fungi, plants and mammals. CBMs have affinity for crystalline cellulose, non-crystalline cellulose, chitin, β-1,3-glucans and β-1,3-1,4-mixed linkage glucans, xylan, mannan, galactan and starch as well as cell-surface glycans [94]. A CBM is defined as a contiguous amino acid sequence within a carbohydrate-active enzyme with a discrete fold having carbohydrate binding activity [95]. They are classified into families based on amino acid sequence, binding specificity, and structure. The CAZy database provides an updated list of the CBM-containing proteins, arranged by CBM family. Actually, 64 families of CBMs have been identified (http://www.cazy.org/Carbohydrate-Binding-Modules.html#nb3 - June 2011).

The CBMs are composed of 30 to about 200 amino acids and exist as a single, double, or triple domain in one protein. Their location within the parental protein can be both C- or N-terminal and is occasionally centrally positioned within the polypeptide chain [95]. CBMs can be found in both hydrolytic and nonhydrolytic proteins. Proteins that possess hydrolytic activity (e.g., cellulases and xylanases) are generally composed by a catalytic module and one or more CBMs that are normally joined by relatively unstructured linker sequences. The CBMs present in proteins that do not possess hydrolytic activity comprise part of a scaffolding subunit that organizes the catalytic subunits into a cohesive multienzyme complex known as a cellulosome. Cellulosomes are extracellular multiprotein complexes, first identified in the early 1980s on the anaerobic thermophilic bacterium *Clostridium thermocellum* [96]. It is composed of numerous kinds of cellulases and related enzyme subunits, which are assembled in a large non-catalytic protein that acts as a scaffoldin. The assembly of the cellulosome is facilitated by the high-affinity recognition between the scaffoldin cohesion and the enzymes dockerin molecules. Another scaffoldin module - the cellulose-binding domain - is responsible for binding to the substrate [97].

According to the typology of the binding site CBMs, a classification in three types, A, B or C has been proposed [94]:

- **Type A**: CBMs that bind to insoluble, highly crystalline cellulose and/or chitin. They display aromatic amino acid residues forming a planar hydrophobic surface that interacts with the glucosyl-pyranose ring of the substrate;
- **Type B**: the largest type of CBMS that bind to less-ordered plant structural polysaccharides such as amorphous cellulose, mannan or xylan. The binding sites are extended, often described as grooves or clefts, and comprise several subsites able to accommodate the individual sugar units of the polymeric ligand;
- **Type C**: these CBMs have a solvent-exposed binding pocket or blind canyon, small binding sites that interact with mono or disaccharides.

CBMs have three general roles with respect to the function of their cognate catalytic modules [94]:

- **Proximity effect**: they promote the association of the enzymes with the substrate, increasing their effective concentration;
- **Targeting function**: they have selective substrate affinity, distinguishing different crystalline, amorphous, soluble and non-soluble polysaccharides;
- **Disruptive function**: they can mediate non-catalytic disruption of fibers, rendering the substrate more susceptible to enzymatic hydrolysis.

Many applications have been reported for CBMs, as reviewed by Moreira and Gama [98]. They have been used in the paper, textile and food industries; as probes for protein-carbohydrate interaction and microarrays; as bioremediation and biomedical tools; they can also be used to modify physical and chemical properties of composite materials.

Bioprocessing is the major application for CBMs, given that large-scale recovery and purification of biologically active molecules continue to be challenges for many biotechnological products. Biospecific affinity purification (affinity chromatography) has become one of the most rapidly developing divisions of immobilized affinity ligand technology. Several works describe the use of CBMs as a tag for recombinant protein purification [99-107]. Depending on the binding reversibility, different applications may be

envisaged. CBMs with reversible binding are powerful tools for low-cost affinity purification, while irreversible CBMs can be used for enzyme immobilization [108] or as a tool to adsorb bioactive peptides to carbohydrate-based materials [109, 110].

The family 3 carbohydrate-binding module (CBM3) is the cellulose-binding module of the scaffolding protein CipA from *C. thermocellum.* It is a type A CBM and the crystal structure showed that its 155 amino acid residues fold into a single, compact domain that has an overall prismatic shape with approximate dimensions of 30Å×30Å×45Å. The CBM3 belongs to the all-β family of proteins and is arranged in two antiparallel β sheets that stack face-to-face to form a β-sandwich with jellyroll topology [111]. It has high affinity for cellulose with an equilibrium affinity constant (K_a) for Avicel of 7.7×10^6 M^{-1} [112]. Guerreiro *et al.* have demonstrated that the CBM3 can be used as a successful fusion partner for recombinant expression in *E. coli*. The authors reported the high-level and soluble expression of four antimicrobial peptides fused to the CBM3 [100]. Moreover, the recombinant proteins retained the capacity to bind cellulose. Therefore, the use of CBM3 as fusion partner could permit the expression and purification of recombinant peptides by a fast and inexpensive method, using cellulose for purification.

1.3 THE ANTIMICROBIAL PEPTIDE MAGAININ-2

Frog skin has been used for medicinal purposes for centuries and is still used today in South American countries [113]. Magainins, a family of antimicrobial peptides present in the skin of the African clawed frog, *Xenopus laevis*, were first identified in 1987 by Michael Zasloff [114]. The author observed that it was extremely rare for non-sterile surgical wounds to develop infections in the microbially contaminated water-filled tanks to which the animals were returned immediately after surgery. Infections were not seen on the cut margins of the wound, at the sites of suture placement, or within the communicating subdermal space or peritoneum. He proposed that the antimicrobial peptides were responsible for the protection against infection characteristic of wound healing in this animal.

These host defense peptides are secreted by granular glands in the skin of the frog in response to tissue injury and are also known as PGS-peptides (peptide beginning with glycine and ending with serine) [115]. Later, Kreil *et al.* reported that these peptides are expressed not only within the granular glands of the skin but also in the cells of the gastric mucosa and intestinal tract [116]. The generation of the antimicrobial peptides is achieved through proteolysis of longer peptide precursors that are presumably secreted on to the skin surface before cleavage.

Magainins are cationic (+3 to +5) AMPs comprised of 21 to 27 amino acid residues that create an α-helical secondary structure characterized by separate cationic and hydrophobic faces. These peptides have been reported to have a wide spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi and also induce osmotic lysis of protozoa [114]. They are also known to facilitate wound closure and reduce inflammation [117]. The most important peptides that belong to this family are magainin-1 and magainin-2 (MAG-1 and MAG-2). Both AMPs are composed of 23 amino acid residues and only differ in two amino acids in the positions 10 and 22 with molecular weights of 2409.87 and 2466.93 Da respectively.

MAG-1: H_2N-GIGKFLHSA**G** KFGKAFVGEI M**K**S-COOH

MAG-2: H_2N-GIGKFLHSA**K** KFGKAFVGEI M**N**S-COOH

cDNA analysis showed that MAG-1 and MAG-2 are both present in a single precursor protein of approximately 160 amino acids which contains one copy of MAG-1 and two

copies of MAG-2. Both AMPs are flanked by proteolytic cleavage sites, an arginine residue adjacent to the amino-terminal glycine and a lysine-arginine dipeptide adjacent to the carboxyl-terminal serine [114].

Magainin-2 has 4 lysines and 1 glutamate resulting in a positive charge of +3 at pH 7. The peptide is water soluble, unstructured and highly flexible in aqueous solution. However, MAG-2 forms an amphipathic α-helix in membrane environment [118]. The antimicrobial effect is attributed to the disruption of the bacterial membrane. MAG-2 induces transmembrane pores that conform to the toroidal model [119]. At low solution concentrations, MAG-2 orients relatively parallel to the lipid bilayer surface. When the concentration increases, MAG-2 molecules insert into the lipid bilayer and adopt a transmembrane orientation [120]. The resulting pores lead to diffusion of intracellular ions and small molecules, which is followed by the collapse of the transmembrane electrochemical gradient and then microbial cell death. It has been shown experimentally that magainin-2 is about 5−10 times more potent than magainin-1. The MIC of MAG-2 against most microorganisms is inferior to 100 μg/ml but the peptide is not haemolytic up to at least 150 μg/ml against human erythrocytes [121].

Magainins also possess antiviral properties. MAG-1 and MAG-2 exhibit inhibitory action toward Herpes Simplex Virus 1 and 2 (HSV-1 and HSV-2) at reduced and non-cytotoxic concentrations for epithelial cells (lower than 50 μg/mL) but are inactive against Junin Virus (JV) [122]. Recent works showed the capacity of MAG-2 to enhance transdermal delivery by perturbing stratum corneum lipids in the skin [123, 124]. Transdermal delivery is an attractive method to administer drugs that avoids the pain of injection, reduces the enzymatic degradation and facilitates sustained delivery for up to many days. Potent analogues of MAG-2 have been obtained by substitutions of glycine or serine with alanine residues, and an amidation at the C-terminus, which resulted in enhanced α-helical structure and antimicrobial activity. Studies with pronase digestion suggested that the higher antimicrobial activity may be due to differential susceptibility to proteolysis, in the presence of membranes [125]. Laughlin and Ahmad showed that MAG2-amide was able to inhibit ATP synthase by 60% in *E. coli* while MAG-2 reduced this activity by only 20% [126]. One of these potent analogues, magainin-A, showed spermicidal activity *in vitro* and *in vivo*. The minimum concentration of MAG-A required to immobilize spermatozoa within 20 seconds under *in vitro* conditions was 50 μg/mL in rat and 200 μg/mL in rabbits. However, in the case of monkeys the concentration required was five-fold higher compared to that of rabbits and the effect was found to be both time- and dose-dependent [127]. Monkeys are the only animal with a reproductive tract similar to that of

women, they are a suitable model for postcoital testing [128]. Therefore, Aranha *et al.* evaluated the contraceptive efficacy of MAG-A in monkeys as an *in vivo* model. The results indicated that 1 mg of MAG-A was sufficient to block completely sperm motility: none of the monkeys became pregnant as they returned to cyclicity in the following month. The repeated intravaginal administration of contraceptive doses of MAG-A caused no adverse effects on the morphology of vaginal epithelial cells or on hematologic and serum biochemical profiles [129]. However, Sengupta *et al.* recently reported that the administration of MAG-A *in vitro* resulted in attenuation of differentiation, enhancement in apoptosis and loss of viability in early placental villi trophoblast cells in primary culture and might adversely affect the process of placentation and pregnancy outcome [130].

In spite of great advances in cancer therapy, there is considerable current interest in developing anticancer agents with a new mode of action because of the development of resistance by cancer cells towards current anticancer drugs. A growing number of studies have shown that some AMPs exhibit a broad spectrum of cytotoxic activity against cancer cells. Such studies have considerably enhanced the significance of AMPs. MAG-2 shows cytotoxic activity against several human bladder cell lines with an average IC50 of approximately 200 μM but has no effect on normal murine or human fibroblasts [131]. Takeshima *et al.* reported that a magainin-2 derivative is able to permeabilize and cross the cell membrane of HeLa human cervical carcinoma cells [132]. MAG-2 and its more potent synthetic analogues (magainins A, B, and G) cause the rapid lysis of both hematopoietic and solid tumor cell lines at concentrations that are 5–10 fold lower than magainin concentrations that are lytic for normal human peripheral blood lymphocytes or neutrophils. Magainins lyse tumor cells by forming ion-conducting α-helical channels in the cancer cell membrane. Among the synthetic magainin analogues, magainin-G exhibited superior selectivity for human cancer cells whereas magainin-B is the most potent of the synthetic magainin analogues in terms of cytotoxicity [133]. Magainins A and G inhibit the growth of human small cell lung cancer cell lines, including drug-resistant tumor cell variants, with an average IC50 of approximately 9 μM. Also, MAG-A or MAG-G had an additive effect when used with the chemotherapeutic agents DDP and VP-16. effects. Two additional synthetic magainin analogues (MSI-136, comprised of L-amino acids and MSI-238, comprised of D-amino acids) were found to have superior *in vitro* cytotoxic activity against human lung carcinoma cells in comparison to native MAG-2 and to increase survival of ovarian teratoma-bearing mice. MSI-238 was more effective *in vivo* than MSI-136 or magainin-2, most likely because of decreased susceptibility to proteolytic degradation [134]. Soballe *et al.* demonstrated that MSI-511 (another synthetic all-D-

amino acid magainin analogue) was more lytic for 17 human melanomas than for normal melanocytes. Moreover, the intratumoral injection of MSI-511 completely eradicated human melanoma cells grown as subcutaneous xenografts in immune-deficient mice [135]. MAG-2 only exhibits cytotoxicity at high concentrations probably because of the limited cell membrane-binding due to its low cationic charge. Therefore, one strategy for improving peptide binding is to increase the number of positive charges on the peptide by amino acid substitution or conjugation with a cationic peptide. Liu *et al.* [136] reported the enhancement of cytotoxicity of MAG-2 in tumor cells by conjugation with bombesin, a 14-amino acids peptide isolated from amphibian skin [137].

These activities, namely the antitumoral, make MAG-2 and analogues promising candidates for therapeutic applications.

1.4 THE HUMAN CATHELICIDIN LL37

("LL37, a human antimicrobial peptide with immunomodulatory properties" accepted for publication in the book titled: **Science against microbial pathogens: communicating current research and technological advances**)

There are two major groups of antimicrobial peptides in humans: defensins and cathelicidins. All cathelicidins share a similar structure characterized by a highly conserved N-terminal domain of about 100 amino acid residues. This cathelin-like domain is flanked by a signal peptide (approximately 30 residues long) on its N-terminus, and by an antimicrobial peptide region on its C-terminus.

1.4.1 Structure and cellular expression of LL37

The only member of the cathelicidins family found to date in humans is LL37/hCAP18, an 18 kDa peptide encoded by the gene *CAMP* (GeneBank ID 820). It was first described in 1995 in bone marrow cells [138]. The name hCAP18 was chosen because of its close relationship to the cationic antimicrobial peptide found in rabbit, which has a molecular weight of 18 kDa and is therefore called Cationic Antimicrobial Peptide CAP18. The gene for the human cathelicidin hCAP18 is a compact gene of 1963 bp located in chromosome 3, and is composed of four exons. Exons 1 to 3 code for the signal sequence and the cathelin domain, while exon 4 codes for the antimicrobial peptide [139] (figure 1.5).

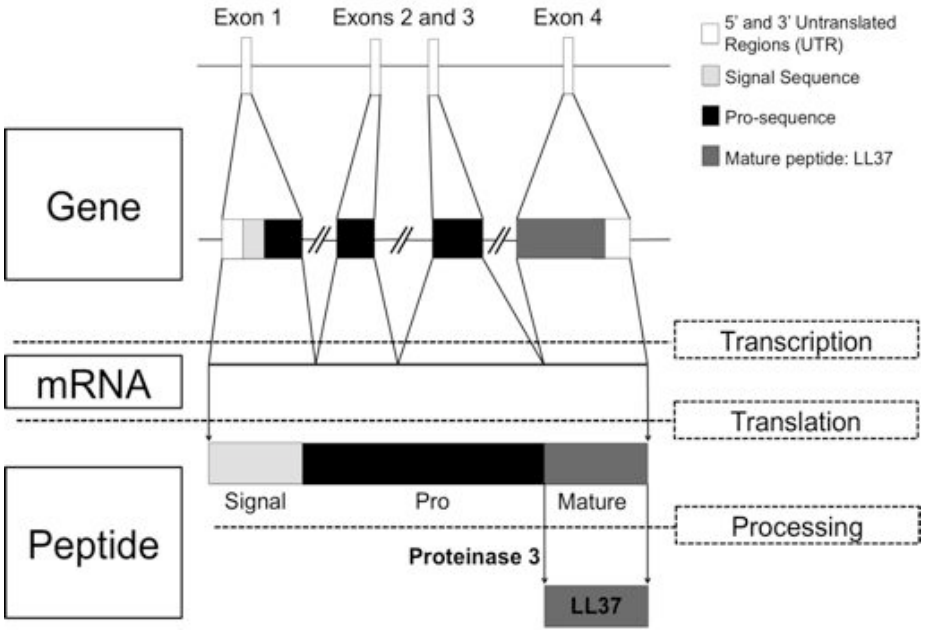

Figure 1.5. Schematic structure of the LL37/hCAP18 gene and its processing into LL37 peptide.

The mature antimicrobial peptide has 37 amino acid residues starting with two leucines (NH_2-LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH), hence being named LL37. It does not contain any cysteine or tryptophan residue and has a molecular weight of ~4.5 kDa. The peptide is derived by extracellular proteolysis of proteinase 3 from the C-terminal end of hCAP18 [140], differing from most cathelicidins that are cleaved by elastase [141, 142]. Initially, the active mature peptide was considered to be the 39-residue FALL39, based on the presence of a typical KR cleavage site [143]. However, Gudmundsson *et al.* [139] used anti-FALL39 immunoglobulin to locate the mature peptide and isolated it from granulocytes after degranulation. The structural analysis determined the mature peptide to be LL37. Nevertheless, two research groups have described other processed peptides. Sorensen *et al.* reported that hCAP-18 in seminal plasma is processed to generate the 38-amino acid antimicrobial peptide ALL38 by the prostate-derived protease gastricsin, when incubated at the vaginal pH. Indeed, ALL38 was found in the vagina following sexual intercourse and the antimicrobial activity of ALL38 against a variety of microorganisms tested was equivalent to that of LL37 [144]. Murakami *et al.* [145] and Yamasaki *et al.* [146] showed that after secretion onto the skin surface or sweat, the *CAMP* gene product is processed by a serine protease-dependent mechanism

into multiple novel antimicrobial peptides distinct from the cathelicidin LL37. These peptides (RK31, KS30, LL29, KS22 and KR20) showed enhanced antimicrobial action, acquiring the ability to kill skin pathogens such as *Staphylococcus aureus* and *Candida albicans*.

At physiological pH, LL37 has a positive net charge of +6. The peptide has 6 lysines and 5 arginines that carry 11 positive charges, while 3 glutamates and 2 aspartates residues bear 5 negative charges. It is relatively disordered in aqueous solution, but folds into an amphipathic α-helix in other environments, such as in contact with lipid membranes [147]. Guangshun Wang studied the structure of ^{13}C,^{15}N-labeled LL37 by three-dimensional triple-resonance NMR and reported that the helical region covers residues 2 to 30, while the C-terminus is mobile [148]. All antimicrobial peptides are amphipathic, meaning that they have hydrophobic and hydrophilic regions, allowing the electrostatic interaction with anionic bacterial membranes. Figure 1.6 illustrates the three-dimensional structure of LL37. The hydrophobic residues are shown in red and are aligned on one side of the peptide.

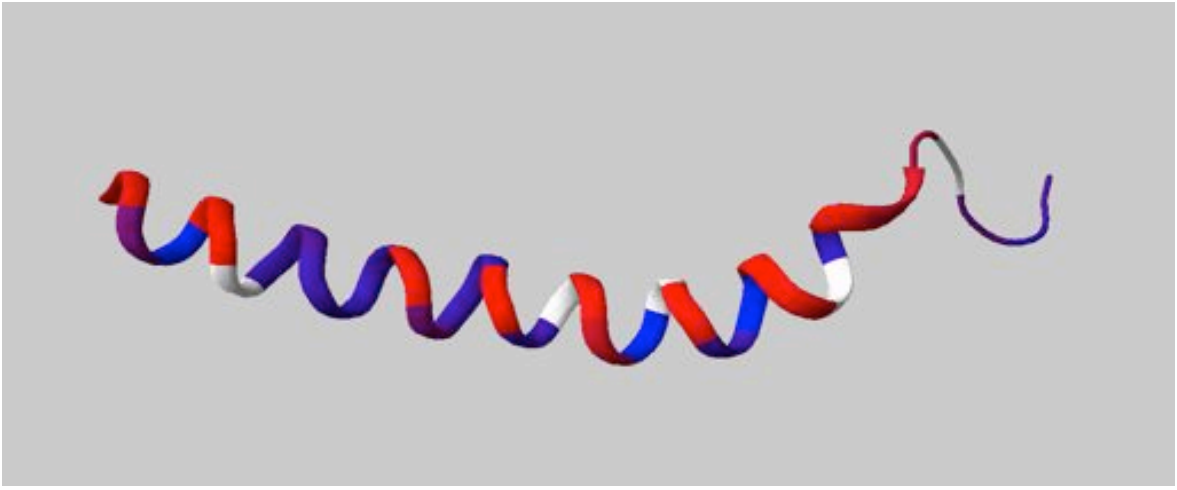

Figure 1.6. α-helical structure of LL37. Hydrophobic residues are shown in red.

LL37 and its precursor, hCAP18, are found at different concentrations in very different cells, tissues and body fluids, predominantly in leukocytes and various epithelial linings. But the expression of hCAP18/LL37 has been reported in many other tissues or body fluids like breast milk, sweat, wound fluids, saliva, gingiva, testis, spermatozoa, seminal plasma, amniotic fluids and more, as well summarized by Durr *et al.* [143]. In a recent

work, Laudien *et al.* also reported the expression of LL37 and defensins in the nasal epithelium providing a chemical defense shield [149]. AMPs are also present in ear wax preventing bacteria and fungi from causing infections in the external auditory canal [150]. The quantification of hCAP18/LL37 concentrations *in vivo* is challenging. Sorensen *et al.* developed a specific enzyme-linked immunosorbent assay (ELISA) for the detection of hCAP18 in cells, plasma and urine. They determined that the amount of hCAP18 in neutrophils is 0.627 μg/10^6 cells and is barely excreted in the urine. The plasma level is 1.18 μg/ml, several fold higher than for other neutrophil specific granule proteins [151]. In the airway fluids, the levels of LL37 are estimated to be 2 μg/ml in adults and 5 μg/ml in neonates.

The down and upregulation of LL37 have been identified in many diseases. A higher concentration of the peptide is more common in the presence of infections. In fact, in pulmonary infections the levels of LL37 are augmented two or three times [152]. A low expression of LL37 is associated with skin disorders like atopic dermatitis [153] or chronic ulcers [154]. Putsep *et al.* [155] reported that the neutrophils from patients with morbus Kostmann syndrome are deficient in LL37. However, in acute inflammation, LL37 concentrations can dramatically increase, reaching 1.5 mg/ml in the skin lesions of psoriasis patients [156]. High concentrations of LL37 have also been associated to other inflammatory disorders like lupus erythematosus, contact dermatitis or inflamed synovial membranes [143, 157]. Gilliet and Lande [158] proposed that these inflammatory disorders are caused by the inappropriate recognition of self-nucleic acids with induction of interferon responses by plasmacytoid dendritic cells (pDCs). In normal conditions self-DNA released by dying cells is unable to enter pDCs and is degraded rapidly by extracellular DNases. LL37 binds to self-DNA and forms aggregated particles that are resistant to degradation by DNases and able to enter pDCs through proteoglycan-mediated attachment of LL37 to the cell membrane and lipid-raft-mediated endocytosis. Aggregated self-DNA particles are retained in early endocytic compartments to trigger induction of interferons (IFNs) by activating endosomal toll-like receptor 9 (TLR-9) as if they were viruses. Type I IFNs trigger local maturation of myeloid dendritic cell and activation of autoreactive T cells leading to the formation of psoriatic skin lesions. In this process, activated Th17 cells produce IL-22 and IL-17 that stimulate psoriatic keratinocytes to sustain the expression of cationic AMPs including LL37, which further forms complexes with self-DNA that is abundantly released in psoriatic skin lesions by apoptotic cells. As a result, LL37/self-DNA complexes constantly promote dendritic cell maturation and activation of autoreactive T cells, providing a self-sustaining feedback

mechanism that amplifies and maintains autoimmune skin inflammation in psoriasis. Leung *et al.* measured the serum concentrations of LL37 in eczema children patients and reported that the concentration of the peptide increased with the eczema severity. They considered that LL37 might be a biomarker for the severity of childhood eczema [159].

The expression of LL37 is upregulated by several stimuli, such as pro-inflammatory cytokines, growth factors, nutrients, and bacterial products, especially at inflammation and repair sites. The mechanisms regulating the LL37 production are not fully understood. Recently, the roles of the vitamin D_3 and the hypoxia response pathways in the regulation of LL37 production in leucocytes and keratinocytes have been described. Vitamin D_3 is naturally produced in the skin during exposure to sunlight, and is activated by hydroxylases CYP27A1 and CYP27B1 to generate the biologically active 1,25-dihydroxyvitamin D_3 [158], which is a potent inducer of the antimicrobial protein cathelicidin in isolated human keratinocytes, monocytes, neutrophils and gingival epithelial cells [160-162]. As sunlight induces immunosuppression increasing the vulnerability to infection, Vitamin D_3, potentially to balance this effect, stimulates the synthesis of LL37 in skin and circulating phagocytic cells [163]. Moreover, Hata *et al.* reported that oral administration of vitamin D for 21 days induced cathelicidin production in atopic dermatitis lesional skin [164].

Hypoxia-inducible transcription factor 1α (HIF1α) also plays an important role in LL37 cellular expression. In fact, HIF1α-null mouse neutrophils show a dramatic reduction in the levels of mouse cathelin-related antimicrobial peptide (CRAMP) [165]. Also, the siRNA knock down of HIF1α in human keratinocytes decreased the expression of hCAP18 [166]. On the other hand, Stroinigg and Srivastava [167] reported that the human beta-defensin 2 (HBD-2), present in significant amounts in human milk, greatly increased LL37 expression in colon and breast epithelial cells. Moreover, the exposure of human primary bronchial epithelial cells to IL-4 and IL-13 cytokines during mucociliary differentiation increased mRNA expression of hCAP18/LL37 and hBD-2 [168].

Finally, microorganisms can also regulate the expression of LL37. Bacterial products from both Gram-positive and Gram-negative bacteria were shown to increase LL37 production in cultured sinus epithelial cells [169]. Butyrate, a product of bacterial microflora in the colon, induces LL37 production [170]. LL37 cellular expression was also found to be augmented by *Pseudomonas aeruginosa* flagellin [171], fungal allergens in nasal tissue from chronic rhinosinusitis patients [172], and *Helicobacter pylori* in gastric epithelium [173]. On the other hand, other pathogens reduce the expression of the peptide: *Shigella*

flexneri suppress LL37 production [174]; *Neisseria gonorrhoeae*, a human pathogen causing the sexually transmitted disease gonorrhea, that preferentially attach to and invade epithelial cells of the genital tract was found to down-regulate the expression of LL37, indicating that pathogenic *Neisseria* may gain a survival advantage in the female genital tract by decreasing LL37 expression [175]; Cholera toxin and labile toxin, the major virulence proteins of *Vibrio cholerae* and enterotoxigenic *Escherichia coli*, respectively, are predominantly responsible for the suppression of LL37 and hBD-1 expression in intestinal epithelial cells both *in vitro* and *in vivo* [176].

1.4.2 Functions of LL37

LL37 was initially recognized for its antimicrobial properties. Nevertheless, it has been found to have additional defensive roles such as regulating the inflammatory response and chemo-attracting cells of the adaptive immune system to wound or infection sites, binding and neutralizing LPS, promoting re-epithelialization and wound closure and more as illustrated in figure 1.7.

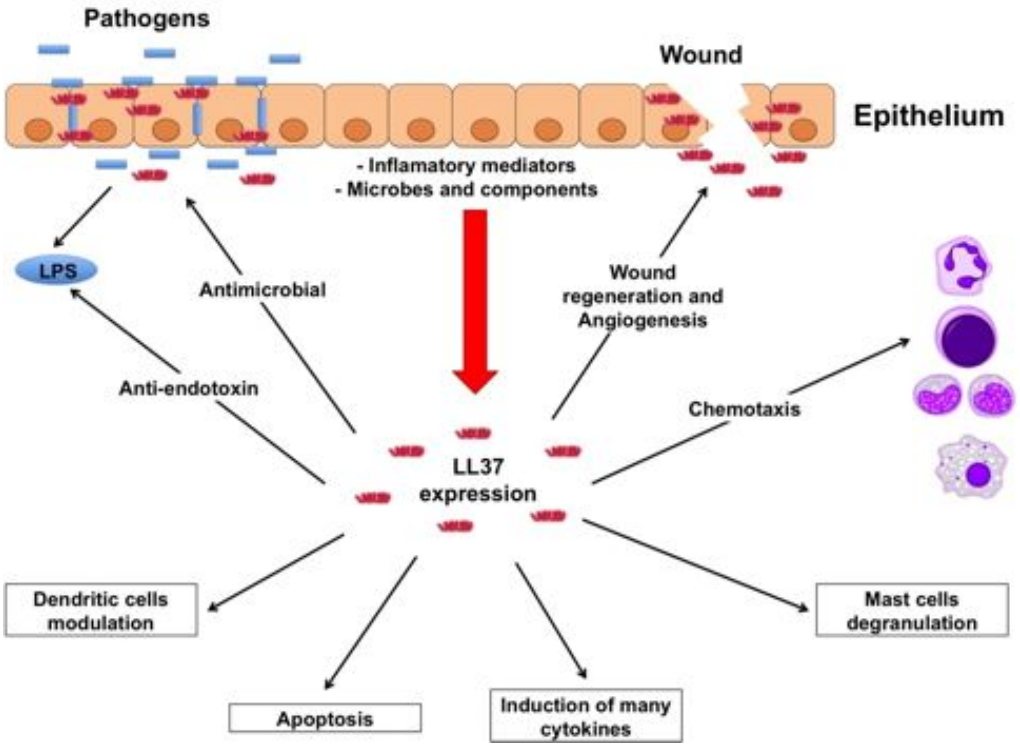

Figure 1.7. Biological activities of LL37.

Antimicrobial activity

The antibacterial activity of LL37 has mostly been studied *in vitro* using the synthetic peptide. The reported activities of LL37 vary in several studies, indicating that it is sensitive to differences in the experiment conditions, such as salt, pH and the phase of bacterial growth. Nevertheless, LL37 has a broad range of activity against both Gram-negative and Gram-positive bacteria. LL37 exhibits potent activity against *Escherichia coli*, *Salmonella typhimurium and Salmonella minnesota*, *Pseudomonas aeruginosa, Neisseria gonorrhoeae, Klebsiella pneumoniae* and group A *Streptococcus* with MIC values of 0.6-7.6 μg/ml (0.1-2 μM) [177], 0.2-3.6 μg/ml (0.1-0.8 μM) [177], 0.9-5.7 μg/ml (0.2-1 μM) [177], 0.9 μg/ml (0.2 μM) [175], 4.2 μg/ml (0.9 μM) [178], and 5-72 μg/ml (1-16 μM) [179] respectively, while low activity has been detected against the yeast *Candida albicans* (MIC >250 μg/ml or 56 μM) [180] and *spirochaete* (MIC>145 μg/ml or 32 μM) [181]. Besides its low activity against *C. albicans,* LL37 was still able to reduce the *C. albicans* infectivity by inhibiting its adhesion to plastic surfaces, oral epidermoid cells, and urinary bladders of female BALB/c mice. The inhibitory effects of LL-37 on cell adhesion and aggregation were mediated by its preferential binding to mannan, the main component of the *C. albicans* cell wall, and partially by its ability to bind chitin or glucan, which underlie the mannan layer [182]. LL37 has also been shown to possess some antiviral activity against herpes simplex virus [183] and vaccinia virus [184] and inhibits HIV-1 replication in peripheral blood mononuclear cells, including primary CD4(+) T cells [185]. Additionally, LL37 inhibits the formation of *P. aeruginosa* bacterial biofilms [186]. Like most other AMPs, LL37 kills microorganisms by membrane disruption, resulting in lysis. By using solid-state NMR data and differential scanning calorimetry, Wildman *et al.* proposed that LL37 covers the surface of the membrane, resulting in toroidal pore formation with subsequent leakage of the cytoplasmic content [187]. LL37 can also be cytotoxic to eukaryotic cells, including both erythrocytes and leukocytes. However, the cytotoxic concentrations (>58 μg/ml or 13 μM) are generally higher than the concentrations required for elimination of microorganisms [188].

The MIC values of LL37 determined *in vitro* are similar with the physiological concentrations observed during infectious and inflammatory disorders. Several studies reported the antimicrobial activity of LL37 to be noticeably reduced in high salt concentrations, under serum conditions, in the presence of a lung surfactant preparation as well as in the presence of artificial tears. However, *in vivo*, LL37 acts together with other antimicrobial components, i.e. hBD-2 [76], lysozyme and lactoferrin [189], to show optimal killing capacity. Since the reported MIC values are determined for LL37 alone,

there might be an underestimation of the antimicrobial activity under *in vivo* conditions, where synergistic effect with other antimicrobial components has to be considered. In fact, the role of LL37 in disease has been demonstrated in several reports. Nizet *et al.* [190] showed that mice with disrupted *Cnlp*, the gene coding for CRAMP, bear increased susceptibility to skin infections. Since CRAMP is similar to LL37 in structure, tissue distribution and antimicrobial activity, the CRAMP knockout model is a useful one for studying the function of the human cathelicidin. The *in vivo* role of LL37 in preventing infections is also supported by an observation made in patients with morbus Kostmann syndrome. These patients lack LL37 in the saliva neutrophils, which may contribute to increased susceptibility to oral infections. A different approach to evaluate the role of LL37 in certain diseases is to overexpress the peptide. As example, an adenovirus vector was utilized to transfer the LL37-gene systemically or into trachea of mice [191]. Furthermore, the vector was inserted into human bronchial xenograft, derived from patients with cystic fibrosis [192]. The overexpression of LL37 augmented the killing of bacteria in all these cases.

Lipopolysaccharide neutralizing properties

Lipopolysaccharides (LPS) endotoxins are heteropolymeric components of the outer layer membrane of Gram-negative bacteria with strong immunotoxic properties. LPS play an important role in pathogenesis of many exogenous respiratory diseases, including organic dust toxic syndrome and chronic illnesses such as chronic obstructive pulmonary disease, asthma or allergic alveolitis (hypersensitivity pneumonitis) [193]. LPS is released upon cell death, activating mononuclear phagocytes (monocytes and macrophages) to produce and release proinflammatory cytokines such as TNF-α, IL-6, and others [194]. This event is initiated by binding of LPS to LPS-binding protein (LBP), followed by a second binding of the LPS-LBP complex to CD14, the primary receptor of LPS, which is expressed mainly on macrophages [195]. The LPS-CD14 complex initiates intracellular signaling by interacting with the transmembrane protein TLR-4, which activates the NF-kB transcription factor, resulting in the production and secretion of pro-inflammatory cytokines [196]. The massive release of LPS can lead in extreme cases to endotoxic shock and therefore, death.

Some AMPs, including LL37, have the capacity to neutralize LPS by binding it with high affinity, inhibiting LPS interaction with LBP and/or CD14 [197, 198]. Another mechanism

has also been suggested, where LL37 binds directly to CD14, inhibiting the association of LPS with its receptor. LL37 significantly inhibited the expression of specific proinflammatory genes upregulated by NF-kB in the presence of LPS in human monocytes [199]. It reduced TNF-α and nitric oxide levels produced by LPS and IFN-γ-polarized mouse bone marrow-derived macrophages [200]. Also, LL37 suppressed the LPS-induced apoptosis of endothelial cells [201]. Kandler *et al.* showed that LL37 also blocked the effect of flagellin and lipoteichoic acid on dendritic cells [202]. The antiendotoxic effect of LL37 has been confirmed *in vivo* using rat as a model system, making it a promising candidate for treatment of endotoxin shock or sepsis that is associated with a high rate of human death, for which there is yet no effective treatment [203].

Immunomodulatory properties

In addition to its antimicrobial activity and ability to neutralize LPS, LL37 plays a central role in innate immunity. It is chemoattractant for mast cells [204], monocytes, T lymphocytes and neutrophils [205]. Formyl Peptide Receptor Like-1 (FPRL-1) is the only receptor found to activate direct migration of immunological cells to a site of infection. As leukocytes participate in both innate and adaptive immunity, the fact that LL37 can chemoattract human leukocytes may provide one additional mechanism by which LL37 contributes to host defense against microbial invasion, by participating in the recruitment of leukocytes to sites of infection. This mechanism is potentially important *in vivo*, because the chemotactic activity of LL37, unlike its antimicrobial action, is not significantly inhibited by the presence of human serum. The activation of FPRL-1 requires relatively high concentrations of LL37 (10^{-5} M) when compared to other classical chemoattractant agents, suggesting a low-affinity peptide-receptor interaction [206]. This implies that cellular FPRL-1 mediated recruitment by LL37 *in vivo* may be active only when a threshold concentration of the peptide is reached following upregulation of the LL37 gene in epithelial cells or after massive release from invading neutrophils. Therefore, LL37 can potentially reach its optimal chemotactic concentration at local inflammatory sites.

LL37 modulates cellular immune responses by stimulation of chemokine production. LL37 activates airway epithelial cells as demonstrated by activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) and increases release

of the potent chemoattractant IL-8. LL37 transactivates the epidermal growth factor receptor (EGFR) via metalloproteinase-mediated cleavage of membrane-anchored EGFR-ligands [207]. LL37 also induces IL-8 secretion through phosphorylation and activation of the MAPKs, extracellular signal-regulated kinase 1/2 (ERK1/2) in monocytes [208]. Human airway smooth muscle cells also respond to LL37 by releasing IL-8, suggesting that LL-37 is a regulator of the inflammatory process in various inflammatory lung diseases [209]. Yoshioka *et al.* [210] reported that LL37 also causes functional changes in mast cells. Mast cells in the skin are involved in the innate immune system response against microbial infections via Toll-like receptors, such as TLR-4, which that is known to recognize LPS. They observed that LL37 increased the level of TLR-4 mRNA and TLR-4 protein, and induced the release of IL-4, IL-5 and IL-1β from mast cells. Additionally, mast cells show degranulation upon stimulation with LL37. Degranulation releases pro-inflammatory mediators, such as histamine and prostaglandins, into the surrounding tissue, thus increasing vascular permeabilization and promoting the infiltration of inflammatory cells [211]. LL37 also interacts with the purinoceptor $P2X_7$, predominantly expressed on monocytes, macrophages and DCs and induces the processing and release of the potent cytokine IL-1β [212] and IL-8 in human gingival fibroblasts [213].

LL37 shows different effects on apoptotic cell death of different cell types. LL37 promotes apoptosis in various T-cells, smooth muscle cells and epithelial cells, while it is a potent inhibitor of human neutrophil apoptosis [188, 214]. The inhibition of neutrophils apoptosis may be a survival strategy, leading to an increase of viable neutrophils at the site of infection, which is beneficial for the host during bacterial invasion. It has been proposed that the underlying mechanism for the inhibition of apoptosis by LL37 is mediated via FPRL-1 and $P2X_7$ receptors, resulting in the inhibition of caspase 3 activity [215]. Lau *et al.* [216] reported that the apoptosis of airway epithelial cells is inhibited by human serum.

Finally, Davidson *et al.* [217] identified LL37 as a potent modifier of dendritic cells differentiation, bridging innate and adaptive immune responses. LL-37-derived DCs displayed significantly up-regulated endocytic capacity, modified phagocytic receptor expression and function, up-regulated costimulatory molecule expression, enhanced secretion of Th-1 inducing cytokines, and promoted Th-1 responses *in vitro*. Moreover, when internalized by immature human DCs, LL37 caused phenotypic changes, characterized by an increased expression of the antigen-presenting molecule HLA-DR, and the costimulatory molecule CD86 [218].

Angiogenesis and wound healing

The healing of wound is a complex process that involves different steps: haemostasis, inflammation, remodeling/granulation tissue formation and re-epithelialization. Angiogenesis is critical to wound repair. Newly formed blood vessels participate in provisional granulation tissue formation and provide nutrition and oxygen to growing tissues. Angiogenesis, in response to tissue injury, is a dynamic process that is highly regulated by signals from both serum and the surrounding extracellular matrix environment [219].

LL37 is importantly involved in tissue healing processes, especially revascularization and cell growth. LL37 induces angiogenesis mediated by FPRL-1 expressed on endothelial cells. Koczulla *et al.* [220] reported that the application of LL37 stimulated neovascularization in a chorioallantoic membrane assay and in a rabbit model of hind-limb ischemia. The peptide directly activates endothelial cells, resulting in increased proliferation and formation of vessel-like structures in cultivated endothelial cells. Also, decreased vascularization during wound repair was observed in mice deficient for CRAMP. LL37 induces wound healing, proliferation, and migration of airway epithelial cells. Therefore, the peptide is likely involved in the regulation of tissue homeostasis in the airways [221]. It also promotes the migration of keratinocytes via EGFR transactivation [222]. More importantly, upon injury, a large increase in the expression of cathelicidins in human and murine skin after sterile incision was observed and returned to normal levels as the wound closed [179]. The appearance of cathelicidins in skin was due to both synthesis within epidermal keratinocytes and deposition from granulocyctes that migrate to the site of injury. In human burns, higher mRNA levels of hCAP18/LL37, hBD-2 and hBD-3 were detected [223]. The importance of LL37 in re-epithelialization was clearly demonstrated by Heilborn *et al.* [224]. The authors showed that the use of antibodies against LL37 inhibited re-epithelialization in a concentration-dependent manner in a non-inflammatory *ex vivo* wound healing model and that the peptide is lacking in chronic ulcers. In a recent work, Carretero *et al.* [225] demonstrated that LL37 significantly improved re-epithelialization and granulation tissue formation by *in vivo* adenoviral transfer of the peptide to excisional wounds in ob/ob mice. According to Gronberg *et al.* the proteolytic environment of chronic wounds does not seem to prevent the therapeutic use of topical LL37. The authors showed that LL37 was not degraded by matrix metalloproteinase-9, and was fairly resistant to proteolytic cleavage *ex vivo* by incubation with wound fluid from non-healing venous leg ulcers [226].

As already stated, LL37 promotes cells proliferation and angiogenesis. Unfortunately, angiogenesis is also necessary for cancerous tumors to keep growing and spreading. LL37 has emerged as a novel modulator of tumor growth and metastasis in carcinogenesis in ovarian, lung, breast and prostate cancers [227-230]. In fact, LL37 expression is upregulated in these tumors. However, in gastric cancer, the peptide inhibits cell proliferation [231]. LL37 also exhibits antitumor effects on epidermoid carcinoma cells, suggesting that the effect of LL37 on cancer phenotypes depends on the tissue origin of the tumor [232]. Although LL37 is demonstrated to promote cancer metastasis, fragments of the peptide have tumor inhibitory effects. These findings suggest that the fragments of LL37 have the potential to be developed into anticancer agents [233]. As an alternative strategy, LL37 has been shown to enhance the antitumor effects induced by CpG oligodeoxynucleotides (ODNs) through stimulating the tumor-suppressing activity of natural killer cells [234, 235]. Unmethylated CpG sites can be detected by TLR-9 on pDCs and B lymphocytes in humans to detect intracellular viral, fungal, and bacterial pathogen DNA, resulting in the secretion of proinflammatory cytokines, which may contribute to the immune response to the pathogen [236]. The potent immunostimulatory effects of TLR-9 activation by CpG ODNs have resulted in enormous interest in the potential uses of these TLR-9 agonists as adjuvants for clinical therapy [237]. Hurtado and Peh reported that LL37 promotes the rapid sensing of CpG ODNs by B lymphocytes and pDCs but not T cells. These results are relevant to contemporary studies of TLR9 agonists as adjuvants for vaccines and cancer therapy [238].

LL37 is a multifunctional host defense peptide. Besides its antimicrobial activity, it stimulates a complex group of responses in many cells, either directly or through modulation of cellular responses to microbial compounds and other immune mediators. A better understanding of the biological activity of LL37 and its interactions with other immune mediators will permit to create strategies and opportunities for therapeutic intervention in infectious and inflammatory diseases.

1.5 WOUND HEALING

Wound healing, or wound repair, is an intricate process in which the skin (or another organ-tissue) repairs itself after injury. Adult skin consists of two tissue layers: a keratinized stratified epidermis and an underlying thick layer of collagen-rich dermal connective tissue providing support and nourishment (dermis) [239]. Because the skin serves as a protective barrier against the environment, any break/injury sets in motion the wound healing process, characterized by four distinct, but overlapping phases: hemostasis (not considered by some authors), inflammation, proliferation and remodeling [240, 241]. The different phases of wound healing are represented in figure 1.8.

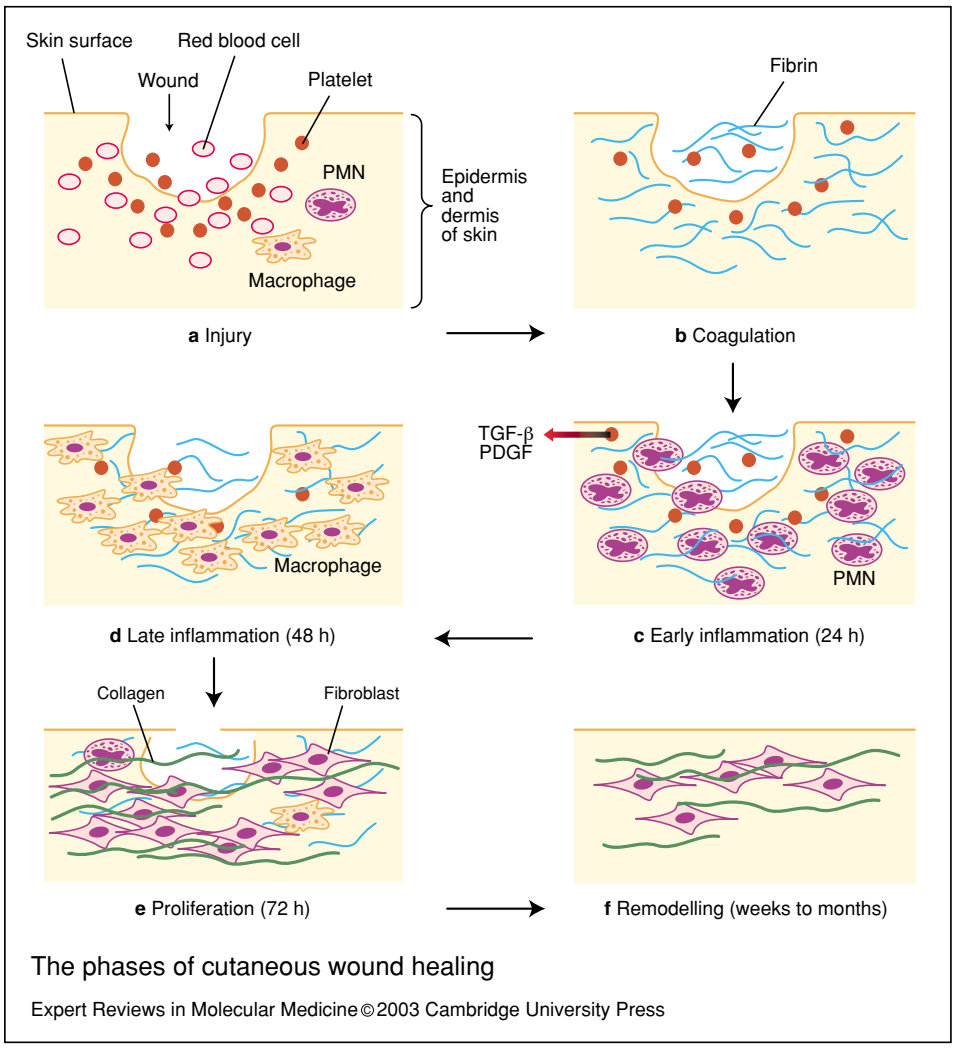

Figure 1.8. The phases of normal wound healing.

1.5.1 Hemostasis

Most wounds to the skin will cause leakage of blood from damaged blood vessels. The formation of a clot then serves as a temporary shield protecting the denuded wound tissues and provides a provisional matrix over and through which cells can migrate during the repair process. The clot consists of platelets embedded in a mesh of cross-linked fibrin fibers derived by thrombin cleavage of fibrinogen, together with smaller amounts of extracellular matrix (ECM) proteins like plasma fibronectin, galectins, osteopontin, tenascins, thrombospondins and vitronectin [242].

1.5.2 Inflammatory phase

Platelets not only release the clotting factors needed to control the bleeding and loss of fluid and electrolytes but they also provide cytokines and growth factors, that initiate the healing response. The two most important signals are platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) [243]. The PDGF is involved in the chemotaxis of neutrophils, macrophages, smooth muscle cells and fibroblasts. In addition it also stimulates the mitogenesis of the fibroblasts and smooth muscle cells. TGF-β attracts macrophages and stimulates them to secrete additional cytokines including bFGF (basic fibroblast growth factor), PDGF, TNF-α and IL-1. In addition, TGF-β further enhances fibroblast and smooth muscle cell chemotaxis and modulates collagen and collagenase expression [241].

Polymorphonuclear neutrophils (PMNs) normally begin arriving at the wound site within minutes of injury. Neutrophils are the next predominant cell marker in the wound within 24 hours after injury. The major function of the neutrophils is to remove foreign material, bacteria and non-functional (apoptotic) host cells and damaged matrix components that may be present in the wound site [244]. Neutrophils usually undergo apoptosis once they have completed their tasks and are, in turn, engulfed and degraded by macrophages [245].

The mast cell is another marker cell of interest in wound healing. Mast cells release granules filled with enzymes, histamine and other active amines and these mediators are responsible for the characteristic signs of inflammation around the wound site [246].

By 48 hours after injury, fixed tissue monocytes become activated to become wound macrophages that are essential for effective wound healing; if macrophage infiltration is prevented, then healing is severely impaired [247]. Macrophage tasks include phagocytosis of any remaining pathogenic organisms and other cell and matrix debris. Macrophages also secrete a number of factors such as growth factors and other cytokines. These factors attract cells involved in the proliferation stage of healing to the area, thus amplifying the earlier wound signals released by degranulating platelets and neutrophils. The growth factors involved in wound healing are described in table 1.5. At the end of the inflammatory phase the number of neutrophils and macrophages decreases and fewer inflammatory factors are secreted. The disappearance of macrophages is essential for the beginning of the next phase. In fact, Newton *et al.* showed that the presence of macrophages delayed wound contraction of an *in vitro* wound healing model [248].

1.5.3 Proliferative phase

The steps in the proliferative phase do not occur in a series but rather partially overlap in time. The proliferative phase is characterized by the formation of granulation tissue, epithelialization, and angiogenesis. As the proliferative phase progresses, the TGF-β released by the platelets, macrophages and T lymphocytes becomes a critical signal. TGF-β is considered to be a master control signal that regulates a host of fibroblast functions [249]. Granulation tissue consists of new blood vessels, fibroblasts, inflammatory cells, endothelial cells, myofibroblasts, and the components of a new, provisional ECM. TGF-β has a three-pronged effect on ECM deposition [250]. First, it increases transcription of the genes for collagen, proteoglycans and fibronectin thus increasing the overall production of matrix proteins. At the same time TGF-β decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates the protease inhibitor TIMP - tissue inhibitor of metalloproteinase [251]. Other cytokines considered to be important are interleukins, bFGFs and TNF-α (Table 1.5).

Table 1.5. Growth factors involved in the wound healing process (adapted from Martin [239])

Growth factor	Source	Effects
Epidermal growth factor (EGF)	Activated macrophages Salivary glands Keratinocytes	Keratinocyte and fibroblast mitogen Keratinocyte migration Granulation tissue formation
Hepatocyte growth factor (HGF)	Mesenchymal cells	Epithelial and endothelial cell proliferation Hepatocyte motility
Transforming growth factor (TGF-α)	Activated macrophages T-lymphocytes Keratinocytes	Hepatocyte and epithelial cell proliferation Expression of antimicrobial peptides Expression of chemotactic cytokines
Basic Fibroblast growth factor (bFGF-1, -2 and -4)	Macrophages Mast cells T-lymphocytes Endothelial cells Fibroblasts	Fibroblast chemotaxis Fibroblast and keratinocyte proliferation Keratinocyte migration Angiogenesis Wound contraction Matrix deposition
Keratinocyte growth factor (KGF)	Keratinocytes	Keratinocyte migration, proliferation and differentiation
Platelet derived growth factor (PDGF)	Platelets Macrophages Endothelial cells Smooth muscle cells Keratinocytes	Granulocyte, macrophage, fibroblast and smooth muscle cell chemotaxis Granulocyte, macrophage and fibroblast activation Fibroblast, endothelial cell and smooth muscle cell proliferation Matrix metalloproteinase, fibronectin and hyaluronan production Angiogenesis Wound remodeling Integrin expression regulation
Vascular endothelial growth factor (VEGF)	Keratinocytes; macrophages	Angiogenesis
Transforming growth factor-β (TGF-β1, -β2 and – β3)	Platelets T-lymphocytes Macrophages Endothelial cells Keratinocytes Smooth muscle cells Fibroblasts	Granulocyte, macrophage, lymphocyte, fibroblast and smooth muscle cell chemotaxis Fibroplasia Matrix metalloproteinase production inhibition Keratinocyte proliferation Antiscarring TIMP synthesis
Interleukin-1 (IL-1α and –β)	Neutrophils	Early activators of growth factor; expression in macrophages,keratinocytes, and fibroblasts
Tumor necrosis factor-α (TNF-α)	Neutrophils	Early activators of growth factor; expression in macrophages,keratinocytes, and fibroblasts

The process of epithelialization is stimulated by the presence of EGF and TGF-α that are produced by activated wound macrophages, platelets and keratinocytes [241]. The epidermal covering is reconstituted from the edges of the wound and from the cut remnants of hair follicles [239]. Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab (a hard coating on the skin formed during the wound healing reconstruction phase) resulting in its removal. The high metabolic activity at the wound site increases the demand for oxygen and nutrients. This is achieved through angiogenesis and stimulated by VEGF, bFGF, TGF-α, angiopoietin, and mast cell tryptase [252]. As already stated, LL37 is also an angiogenic factor that plays an important role in wound healing.

Contraction is a key phase of wound healing and starts when fibroblasts have differentiated into myofibroblasts [253] that contain the same actin as smooth muscle cells [254]. As the actin in myofibroblasts contracts, the wound edges are pulled together and fibroblasts lay down type III collagen to reinforce the wound [240].

1.5.4 Maturation and remodeling

At the end of the granulation phase, fibroblasts begin to commit apoptosis, converting granulation tissue from an environment rich in cells to one that consists mainly of collagen [240]. At least 23 individual types of collagen have been identified to date but type I is predominant in the scar tissue of skin. Type III collagen is prevalent during proliferation but it is gradually degraded and the stronger type I collagen is laid down in its place [255]. In normal tissue collagen is a strong and highly organized molecule. However, collagen fibers formed in scar tissue are much smaller and have a random appearance. Therefore, the scar tissue is always weaker and will break apart before the surrounding normal tissue. The regained tensile strength in a wound will never approach normal. In fact, the maximum tensile strength that a wound can ever achieve is approximately 80% of normal skin [241]. Then, the activity at the wound site is reduced, and the blood vessels that are no longer needed are removed by apoptosis. Thus, the scar looses its red appearance [245]. Finally, in the process of collagen remodeling, collagen degradation also occurs [256].

1.5.5 Fibrosis and chronic ulcers

Fibrosis can be defined as the replacement of the normal structural elements of the tissue by distorted, non-functional and excessive accumulation of scar tissue due to the aberrant production of cytokines [257]. Many clinical problems are associated with excessive scar formation. For example, keloids and hypertrophic scars in the skin, tendon adhesions, transmission blockage following nerve injury, scleroderma, Crohn's disease, esophageal strictures, urethral strictures, capsules around breast implants, liver cirrhosis, pulmonary fibrosis, endomyocardial fibrosis atherosclerosis and fibrotic non-union in bone [241].

Keloids are a type of scar with an abnormal production of collagen. In fact, fibroblasts isolated from keloids produce about 2 to 3 times more collagen as compared to fibroblasts isolated from normal skin in the same patients [258]. Also TGF-β is expressed at greater levels in keloid fibroblasts when compared with normal human dermal fibroblasts [259]. Hypertrophic scars are also characterized by excessive accumulation of scar collagen. Contrary to keloids, hypertrophic scars regress in time. This can be explained by the presence of myofibroblasts in hypertrophic scars [260]. Fibrosis is also characterized by an augmentation of mast cells that contain specialized enzymes capable of processing procollagen [261].

Chronic non-healing dermal ulcers such as pressure ulcers contribute significantly to the morbidity and even mortality of many patients [262]. Wounds that do not heal within three months are often considered chronic. They generally remain in the inflammatory phase for too long. In fact, a biological marker of these wounds is the excessive infiltration of neutrophils. The neutrophils release significant amounts matrix metalloproteinases and elastase that are responsible for destruction of the connective tissue matrix and PDGF and TGF-β [263, 264]. These chronic ulcers will not heal until the chronic inflammation is reduced.

1.5.6 Diabetes mellitus impaired healing

Diabetic foot ulcers, a leading cause of amputations, affect 15% of people with diabetes mellitus. The foot ulcer is a leading cause of hospital admissions for people with diabetes in the developed world and is a major morbidity associated with diabetes, often leading to pain, suffering, and a poor quality of life for patients [265]. Diabetes is the prototypical model of impaired wound healing. Several factors contribute to wound healing deficiencies in individuals with diabetes. These include decreased or impaired growth factor production, angiogenic response, macrophage function, collagen accumulation, epidermal barrier function, quantity of granulation tissue, keratinocyte and fibroblast migration and proliferation, number of epidermal nerves, bone healing, and balance between the accumulation of ECM components and their remodeling by matrix metalloproteinases [265].

Angiogenesis seems to be the most compromised phase in diabetes [266]. Several angiogenic growth factors such as bFGF and TGF-β have been described, but VEGF is unique for its effects on multiple components of the wound healing cascade, including angiogenesis and recently shown epithelialization and collagen deposition [267]. The impairment in VEGF synthesis and release at the wound site has been claimed as the crucial event for the altered production of new blood vessels. In fact, one of the mechanisms underlying the healing impairment in diabetic mice is thought to result from a defect in VEGF regulation, during gene expression [268]. In agreement with these observations, therapeutic interventions aimed at restoring the impaired VEFG production have succeeded in ameliorating the wound healing process in experimental model of diabetic mouse [269-272]. Therefore, the restoration of the angiogenesis process seems fundamental to reduce impaired healing in diabetes. As already stated, the AMP LL37 promotes angiogenesis *in vivo* and *in vitro* and is important for re-epithelialization [220, 224]. Thus, the therapeutic use of this peptide for the treatment of impaired wound healing should be considered.

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CHAPTER 2

ESCHERICHIA COLI EXPRESSION AND PURIFICATION OF MAG2 FUSED TO A FAMILY III CBM FROM *CLOSTRIDIUM THERMOCELLUM*

ABSTRACT

Magainin-2 (MAG2) is a polycationic antimicrobial peptide isolated from the skin of the African clawed frog *Xenopus laevis*. It has a wide spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi and induces osmotic lysis of protozoa. MAG2 also possesses antiviral and antitumoral properties. These activities make this peptide a promising candidate for therapeutic applications. Recombinant expression systems are necessary for the affordable production of large amounts of the biologically active peptide. In this work, MAG2 has been cloned to the N- and C-termini of a family III carbohydrate-binding module fused to the linker sequence (LK-CBM3) from *Clostridium thermocellum*; a formic acid recognition site was introduced between the two modules for chemical cleavage of the peptide.

The recombinant protein MAG2-LK-CBM3 was expressed in *Escherichia coli* BL21 (DE3) and LK-CBM3-MAG2 in *E. coli* M15 (pREP4). The N-terminal MAG2 from the first construction was successfully cleaved and purified from the fusion partner LK-CBM3. However, the peptide showed no antibacterial activity against *E. coli* K12. The negatively charged C-terminal aspartic acid left from the acid cleavage may be the cause for the absence of antimicrobial activity. The expression of C-terminal MAG2 from the second construct was not successful. We believe that the peptide has suffered proteolysis during recombinant expression.

2.1 INTRODUCTION

Bacterial resistance to antibiotics, first noticed back in the 1950s, is a growing concern for public health [1]. Due to their mechanism of action, the antimicrobial peptides (AMPs) represent an attractive alternative as human therapeutics. AMPs are part of the innate immune system and represent an important component of immune defense. They have a large spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi [2] and viruses like influenza A [3] or human immunodeficiency virus (HIV-1) [4]. Moreover, certain peptides may possess anticancer activity [5]. AMPs are generally defined as cationic, amphipathic peptides, with less than 50 amino acids, including multiple arginine and lysine residues [6] and show inhibitory concentrations (MIC) as low as 0.25-4 µg/ml [7]. They are widely distributed in nature, from insects to plants and animal species; 1755 AMPs have been identified so far (http://aps.unmc.edu/AP/main.php; May 2011).

Magainins are AMPs isolated from the skin of the African clawed frog *Xenopus laevis* [8]. Magainins are comprised of 21 to 27 amino acid residues that create an α-helical secondary structure characterized by separate cationic and hydrophobic faces [5]. Magainin-2 (GIGKFLHSAKKFGKAFVGEIMNS), a polycationic peptide with a net positive charge of 3, has been well studied for its killing action on various micro- organisms, as well as on tumor cells [9-11]. It acts by permeabilizing their membranes, either in a detergent-like manner or by making well-structured channels [12, 13]. Magainin-2 and its more potent synthetic analogues (magainins A, B, and G) cause the rapid lysis of both hematopoietic and solid tumor cell lines at concentrations 5–10 fold lower than those lytic for normal human peripheral blood lymphocytes or neutrophils [5].

Many bacterial and fungal enzymes that hydrolyze insoluble carbohydrates share a common structure composed of a catalytic domain linked to a carbohydrate-binding module (CBM). CBMs that are specific for insoluble cellulose (Cellulose Binding Domain – CBD) represent the predominant category. The CBMs can be grouped into distinctive families on the basis of amino acid sequence similarities [14]; 64 families of CBMs have been recognized so far (http://www.cazy.org/fam/acc_CBM.html; June 2011).

Family-III CBDs normally comprise ~150 amino acids residues, have been identified in many different bacterial enzymes, and also in non-hydrolytic proteins [15]. *Clostridium thermocellum* produces a multi-enzyme complex of cellulases and hemicellulases, termed the cellulosome, which is assembled by the scaffoldin protein CipA. Binding of the cellulosome to the plant cell wall is driven by the action of the CipA family 3 CBM

(CBM3), which presents high affinity for crystalline cellulose [16]. CBM3 belongs to the all-β family of proteins and is arranged in two antiparallel β sheets that form a β sandwich with jellyroll topology [15].

Since AMPs are usually short peptides, chemical synthesis could be one approach for producing them. However, such an approach has not been practical due to its high cost. The recombinant expression of AMPs requires the use of fusion constructs to reduce the toxicity towards the expression host. In this work, the fusion of MAG2 with the CBM3 was attempted as an approach for the large scale production of AMPs.

2.2 MATERIALS AND METHODS

2.2.1 Construction of expression vectors

The gene encoding CBM3 fused to the endogenous CipA N-terminal linker sequence (LK) has previously been cloned in the expression vector pET-21a (Novagen), in our laboratory [16]. Two expression vectors were constructed with MAG2, either at the N-terminal (MAG2-LK-CBM3) of the pET-21a vector or C-terminal (LK-CBM3-MAG2) of the pQE-30 vector (Figure 2.1). These two vectors were constructed to allow the cloning of MAG2 at the N- or C-termini of the LK-CBM3 while keeping a terminal His6-tag.

The nucleotide sequence of MAG2 was engineered to allow codon optimization in *E. coli*. The sequences encoding the peptide were included in the primers in fusion with the gene of a family 3 CBM. The pET-21a-LK-CBM3 was used as template. For the MAG2-LK-CBM3 construction, the following primers were used: 5'- GGA ATTC **CAT ATG** GGT ATT GGT AAA TTT CTG CAT AGC GCG AAA AAA TTT GGT AAA GCG TTT GTG GGT GAA ATT ATG AAC AGC GAT CCG ACA CCG ACC AAG GGA GCA -3' (forward primer) and 5'- CAC **CTC GAG** TTC TTT ACC CCA TAC AAG AAC -3' (reverse primer). For the LK-CBM3-MAG2 construction, the primers used were: 5'- CGC **GGA TCC** ACA CCG ACC AAG GGA GCA -3' (forward primer) and 5´-C **GAG CTC** TTA GCT GTT CAT AAT TTC ACC CAC AAA CGC TTT ACC AAA TTT TTT CGC GCT ATG CAG AAA TTT ACC AAT ACC CGG ATC TTC TTT ACC CCA TAC AAG -3´(reverse primer). *Nde*I, *Xho*I (MAG2-LK-CBM3), *BamH*I and *Sac*I (LK-CBM3-MAG2) recognition sites are shown in bold. In order to allow the chemical cleavage with formic acid, proline and aspartate residues

were introduced (underlined). PCR reactions were performed using the DNA Polymerase VENT (Stratagene). The PCR reactions were performed as follows: preheating at 95ºC for 2 min, 30 cycles at 95ºC for 30s, 30s at 52ºC and 30s at 72ºC, followed by a final elongation stage at 72ºC for 10 min. The amplified products were recovered from 1% agarose gel, digested with *Nde*I and *Xho*I or *BamH*I and *Sac*I and ligated into pET-21a or pQE-30 plasmids, respectively. The plasmids were sequenced to ensure that no mutations had occurred during the PCR. Figure 2.1 represents MAG2-LK-CBM3 and LK-CBM3-MAG2 constructions.

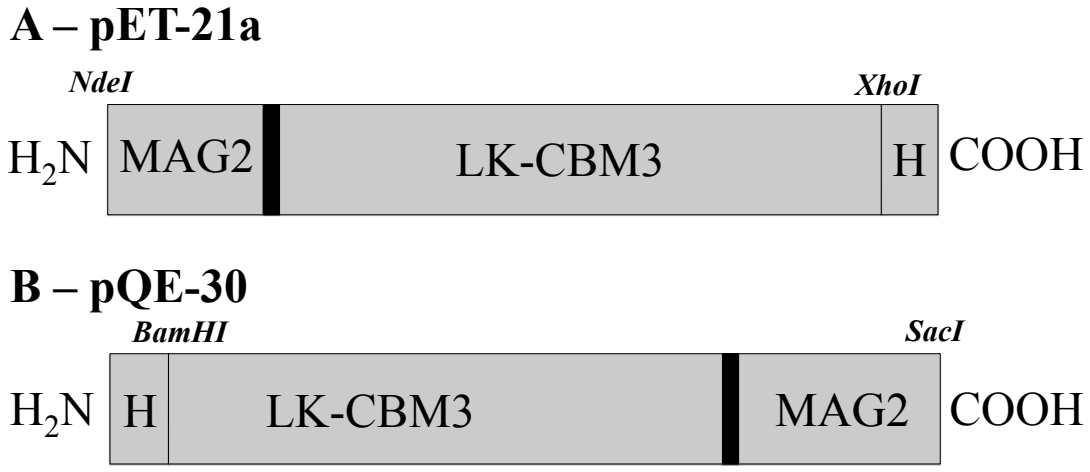

Figure 2.1. Schematic representation of MAG2-LK-CBM3 (A) and LK-CBM3-MAG2 (B) constructions. The AspPro site for chemical cleavage with formic acid is shown in black; H: His-tag.

2.2.2 Expression and purification of fusion proteins

The recombinant plasmid pET-21a-MAG2-LK-CBM3 was transformed into the *E. coli* strain BL21 (DE3) (Novagen) and pQE-30-LK-CBM3-MAG2 into *E. coli* M15 (pREP4) (Quiagen) for protein expression. The bacterial strains were grown in Luria-Bertani broth (LB) containing 100 µg/ml of ampicillin or 100 µg/ml of ampicillin plus 50 µg/ml of kanamycin, at 37ºC, to mid-exponential phase (OD_{595}=0.6). Expression of the fusion proteins was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and the culture was incubated overnight at 37ºC. The culture harboring *E. coli* M15 was incubated overnight at 25ºC after induction with IPTG 1 mM. The cells were centrifuged at 6000 rpm for 15 min at 4ºC, resuspended in Tris–HCl pH 7.0, 20 mM NaCl, 5 mM $CaCl_2(2H_2O)$ buffer (CBM buffer) and sonicated in ice for 6 min.

The cells were centrifuged at 12000 rpm, for 30 min at 4ºC and the soluble fraction was collected.

The His6-tagged recombinant proteins were purified by immobilized metal ion affinity chromatography (IMAC), using 5 mL Niquel His-Trap Columns (GE Health). The column charged with 0.1 M $NiSO_4$ was equilibrated with 20 mM of Na_3PO_4, 500 mM NaCl, 40 mM Imidazole, pH 7.4. The cell extracts were loaded into the column, which was washed with the equilibration buffer. Finally the recombinant proteins were eluted with 20 mM Na_3PO_4, 500 mM NaCl, 300 mM Imidazole, pH 7.4. After purification, proteins buffer was exchanged into CBM buffer, using PD10-columns (GE Health).

The recombinant proteins were also purified on cellulose CF11 (Sigma), exploiting the CBM3 cellulose-binding properties, as follows: 20 ml of cell-free extracts were mixed with 2 g of cellulose and incubated, with agitation, for 1h at 4ºC. The cellulose with bound proteins was then centrifuged and washed 5 times with CBM buffer.

2.2.3 Formic acid cleavage and MAG2 purification

In order to cleave MAG2 of the recombinant protein, a 50% formic acid solution (20 ml of distilled water and 26 ml 88% formic acid) was applied directly to cellulose with the adsorbed fusion proteins. In the case of proteins purified by IMAC, formic acid was mixed with the purified protein (20 ml of pure protein and 26 ml of formic acid 88%). The mixture was then incubated for 24h at 50ºC. The supernatant was separated after centrifugation at 12000 rpm, for 30 min, and lyophilized to remove formic acid. The cleavage was confirmed by SDS-PAGE using a 16.5% Tris-Tricine gel.

The purification of MAG2 was achieved by reverse-phase HPLC, using an YMC C18 preparative column (250×30 mm) equilibrated with aqueous acetonitrile (5%)/0.1% trifluoracetic acid (TFA). The peptide was eluted using a linear gradient of acetonitrile from 5% to 90% at a flow rate of 5 ml/min. The elution was monitored at 215 nm. Several peaks were collected and analyzed by SDS-PAGE using a 16% Tris-Tricine gel. The fraction corresponding to pure MAG2 was lyophilized.

The purification of MAG2 was also attempted through ultrafiltration in a Centricon with 5 kDa molecular weight cut-off (Millipore). The filtrate was collected and analyzed by 16% Tris-Tricine gel SDS-PAGE.

The concentration of the recombinant peptides was quantified using Waddell's method [17].

2.2.4 MALDI-TOF mass spectrometry

The formic acid cleavage of the recombinant proteins and the purity of MAG2 were evaluated by mass spectrometry at the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP). Samples were desalted and concentrated using microC18 ZipTips (Millipore, USA) accordingly to the manufacturer's protocol. The samples were eluted with the matrix α-cyano-4-hydroxycinnamic acid (5 mg/mL) in 50% acetonitrile/0.1% TFA and spotted onto a stainless steel 192-well MALDI plate. After sample crystallization, mass spectrum acquisition was performed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, USA). Mass spectra were acquired in positive linear mode for 1-15 kDa (m/z) mass window and in positive reflector mode for the ranges 700-7000, 4000-5000 and 4585-4600 Da (m/z). External calibration of the mass spectrum was performed using Applied Biosystems Calibration Mix 3 standards.

2.2.5 Antibacterial activity

The antibacterial activity of the pure peptides was quantified in 96-well polypropylene microtiter plates, as described by Vogt *et al.* [18], with some modifications. *E. coli* strain K12 was grown at 37ºC in LB medium until mid-logarithmic phase (OD_{600}=0.5). The cells were then resuspended in LB at 1×10^6 and 1×10^5 cells/ml; 100 µl of peptides, at different concentrations, were inoculated with 50 µl of bacterial suspension, incubated overnight at 37ºC and the bacterial growth was assessed by the measurement of optical density at 620 nm. Each assay was repeated three times and controls were made by adding sterile deionized water, instead of the peptide solutions.

2.3 RESULTS AND DISCUSSION

2.3.1 Expression and purification of recombinant proteins

The antimicrobial peptide MAG2 was successfully cloned at both the N- and C- termini of the LK-CBM3 in the expression vectors pET-21a and pQE-30. After transformation of the recombinant plasmids into E. coli BL21 (DE3) or M15 (pREP4), several fermentation conditions were tested, attempting to optimize the production of the recombinant proteins in the soluble form. The fusion protein MAG2-LK-CBM3 (~24 kDa) was successfully expressed in soluble form after induction at 37ºC with 1 mM IPTG (figure 2.2A). The optimal expression of the recombinant protein LK-CBM3-MAG2 (figure 2.2B) was achieved after induction with 1 mM IPTG at 25ºC.

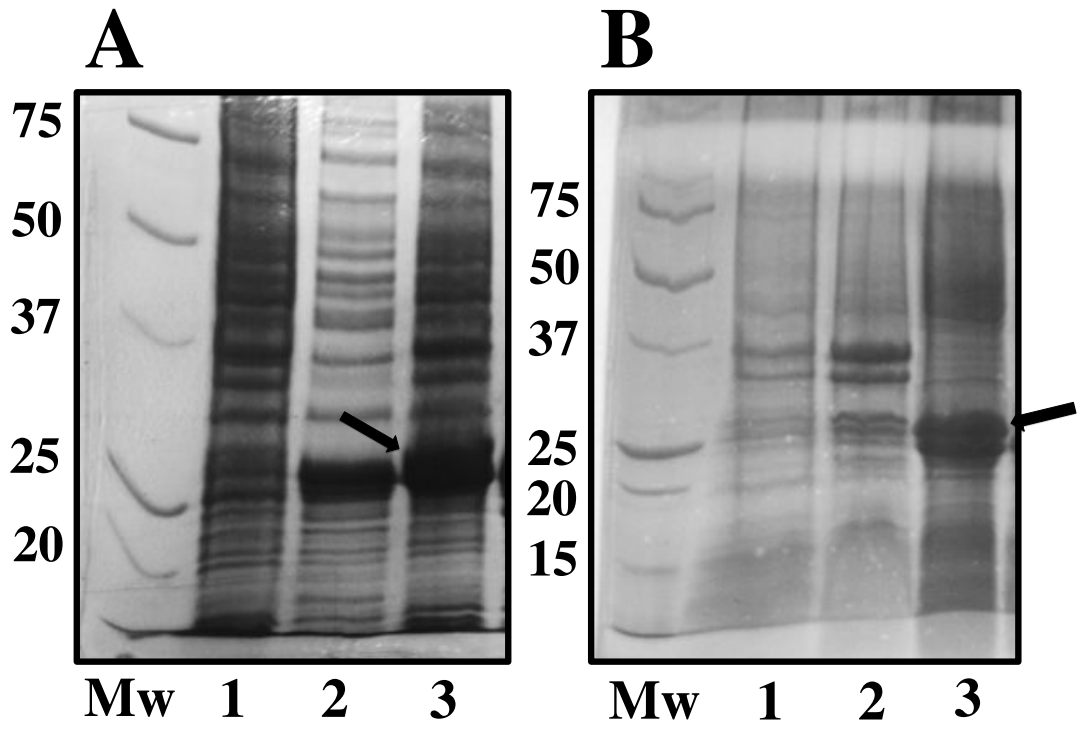

Figure 2.2. Expression of recombinant proteins MAG2-LK-CBM3 (A) and LK-CBM3-MAG2 (B). Mw, protein molecular weight marker; 1, before induction; 2, insoluble fraction; 3, soluble fraction.

There are two major problems in the expression of antimicrobial peptides in *E. coli*: the cytoxicity of the peptides to the host and the sensitivity of peptides with positively charged amino acids to proteases. The high toxicity of the peptides usually leads to expression in inclusions bodies or in aggregate form. To overcome this situation, the use of a fusion partner is highly recommended. Guerreiro *et al.* [16] reported the soluble expression of the family 3 CBM from *C. thermocellum* – also used in this work - fused to three different antimicrobial peptides: LKLLKKL, LKKLLKKLKKLLKK and porcine myeloid antibacterial peptide-23 (PMAP-23) RIIDLLWRVRRPQKPKFVTVWVR. Only the protein LKLLKKLLKLLKKLGGGK-LK-CBM3 was found to be insoluble when expressed by different *E. coli* strains, under a range of induction conditions. Therefore, the solubility of antimicrobial peptides fused to the CBM3 is rather unpredictable, but the expression of the fusion proteins was, in all cases, successful. In this work, the expression of soluble magainin-2, either at the N- or C-terminus of the LK-CBM3 protein, was achieved. The family 3 cellulose-binding module was chosen as fusion partner considering the previously demonstrated successful overexpression of several CBM-AMPs, and the simplicity of purification using cellulose as an affinity chromatography matrix.

The recombinant proteins MAG2-LK-CBM3 and LK-CBM3-MAG2 were purified trough IMAC and on cellulose CF11 fibers. Figure 2.3 illustrates the purification of MAG2-LK-CBM3 on cellulose (3A) and the purification of LK-CBM3-MAG2 by immobilized metal ion affinity chromatography (3B). The two recombinant proteins were purified using both methods and the results were similar.

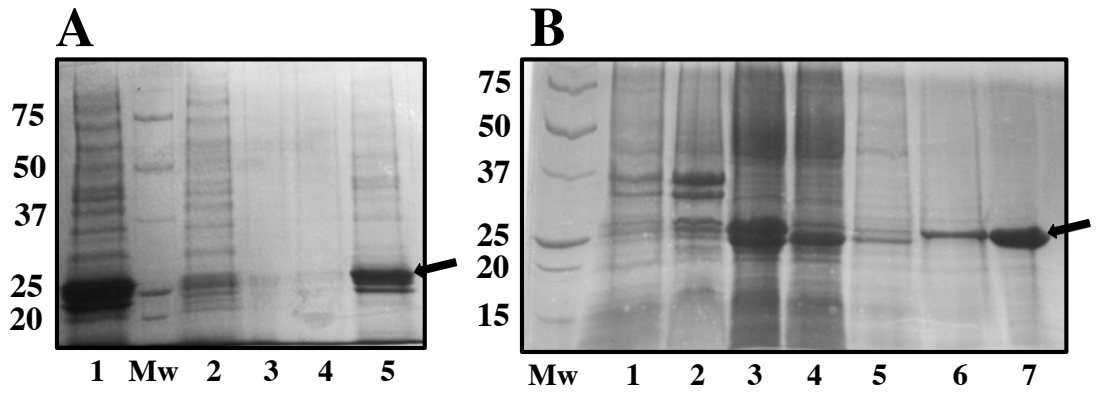

Figure 2.3. A: purification of MAG2-LK-CBM3 on cellulose fibers. Mw, protein molecular weight marker; 1, total fraction; 2, unbound protein; 3, first cellulose wash; 4, fifth cellulose wash; 5, bound protein eluted with 1%SDS. B: purification of LK-CBM3-MAG2 by IMAC. Mw, protein molecular weight marker; 1, uninduced cells; 2, insoluble fraction; 3, soluble fraction; 4, column filtrate; 5 and 6, column washes; 7, pure protein.

As demonstrated by Tomme *et al.*, CBM3 binds cellulose with high affinity. In fact, the equilibrium affinity constant (K_a) for Avicel was reported to be 7.7×10^6 M^{-1} [19]. Figure 2.3A shows that the fusion of the peptide MAG2 did not affect the binding to cellulose. However, the purification of the proteins was more efficient by IMAC. The MAG2-CBM and CBM-MAG2 proteins are expressed in the soluble form, which makes the purification on cellulose difficult to achieve due to unspecific binding of contaminating proteins.

2.3.2 Chemical cleavage of MAG2-LK-CBM3

Formic acid was chosen for hydrolysis instead of thrombin or factor Xa proteases, to reduce production costs. After purification, 50% formic acid was applied to cleave the AMP MAG2 from the fusion carrier, the CBM3. The results obtained for the two constructions were different. The formic acid cleavage of MAG2 (~2.5 kDa) from the fusion partner LK-CBM3 was successful, as illustrated in figure 2.4. After hydrolysis, the mixture was lyophilized to remove the formic acid. The proteins were resuspended in ultrapure water and centrifuged to remove proteins degraded by the formic acid.

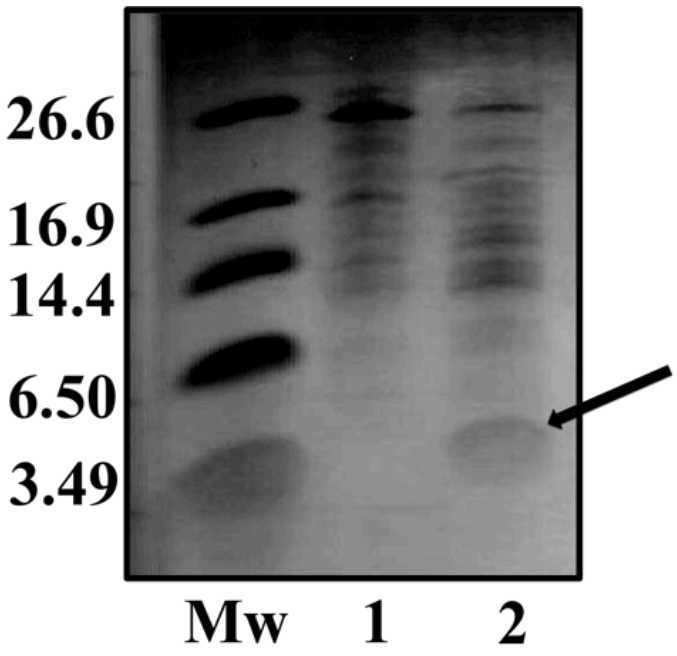

Figure 2.4. Formic acid cleavage of the recombinant protein MAG2-LK-CBM3. Mw, protein molecular weight marker; insoluble fraction of cleaved MAG2-LK-CBM3; 2, supernatant of cleaved MAG2-LK-CBM3;

The figure 2.4 shows that a significant number of contaminants were present in the supernatant after the formic acid cleavage. Consequently, the supernatant containing the MAG2 peptide was purified by reverse-phase HPLC. The fractions corresponding to the major peaks (figure 2.5A) were collected and the purity of the peptide was checked on 16.5% Tris–Tricine gel (figure 2.5B).

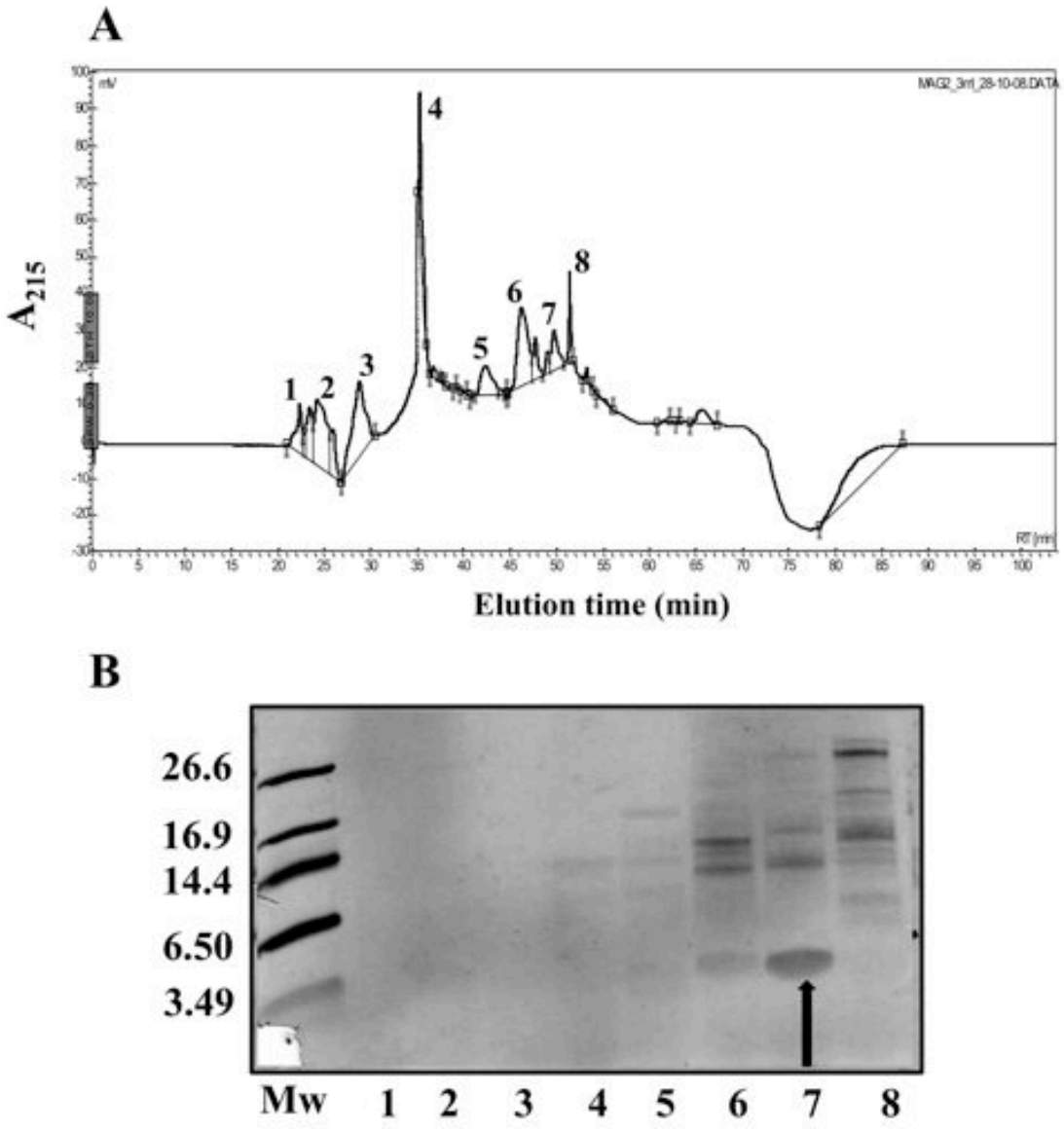

Figure 2.5. A: Reverse-phase HPLC chromatogram of MAG2-LK-CBM3. B: RP-HPLC peak fractions. Mw, protein molecular weight marker; lanes 1–8, peak fractions of cleaved MAG2-LK-CBM3.

The fraction with a retention time of about 50 minutes (peak 7) contained the mostly pure antimicrobial peptide MAG2. Some contaminant proteins were not efficiently removed by RP-HPLC and remained present in low amounts. To confirm the purity and molecular weight of the peptide, MALDI-TOF analysis was performed (figure 2.6).

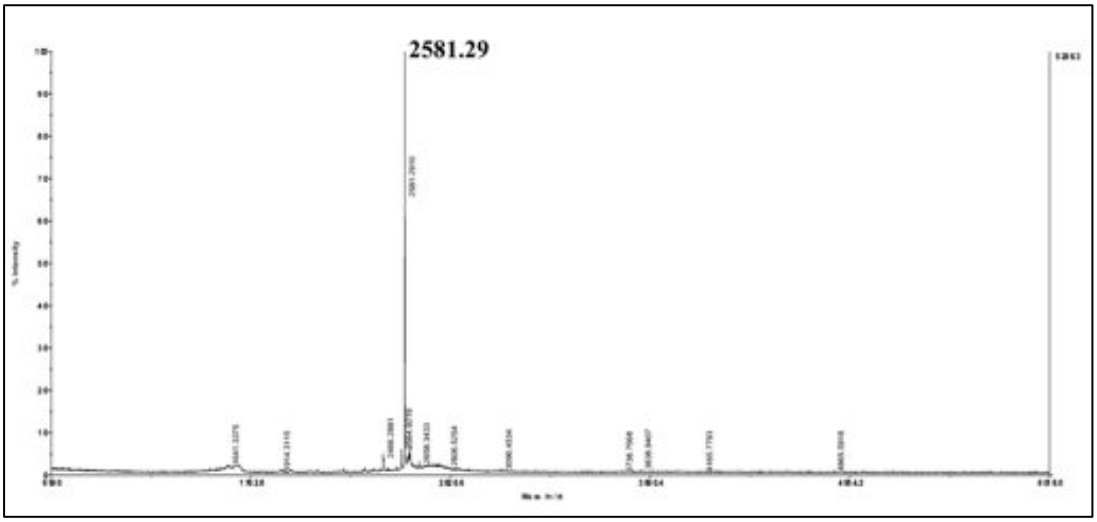

Figure 2.6. MALDI-TOF MS analysis of purified MAG2. The measured molecular weight is very close to the predicted theoretical value (2582 Da).

The peptide resulting from the hydrolysis has 24 amino acids with a C-terminal aspartic acid (MAG2-D), left by the chemical cleavage and a theoretical molecular weight of 2582 Da. The MALDI-TOF analysis confirmed the molecular weight of the recombinant peptide as well as its purity. This molecular weight indicates that the N-terminal methionine from the translation process has been cleaved. Giglione *et al.* [20] reported that 55–70% of mature proteins are subjected to N-terminal Met excision and this was the case with MAG2-LK-CBM3.

2.3.3 Antibacterial Activity of MAG2-LK-CBM3

After the successful expression and purification of magainin-2, its activity was tested against *E. coli* K12. The evaluation of the concentration of MAG2 was attempted using the Waddell's method [17] but no satisfactory results were obtained. The presence of other contaminants in the sample could cause an incorrect determination of the protein

concentration. Nevertheless, since the presence of MAG2 was confirmed by SDS-PAGE and MALDI-TOF its antibacterial activity was tested. However the peptide showed no effect against *E. coli* K12. The sample was concentrated by a factor of about 3 fold by lyophilization, but no antibacterial effect was detected. We strongly believe that the negatively charged C-terminal aspartic acid, left from acid cleavage, is the cause for the lack of activity. AMPs interact by electrostatic forces with the negatively charged bacterial membrane phospholipids, leading to its disruption. The lower net charge of the MAG2-D (+2) may suppress the antibacterial activity. Also, no bacterial activity was detected for the fusion protein MAG2-LK-CBM3. This result was somehow expected due to the mode of action of AMPs. In order to disrupt bacterial membranes, the peptides must reach a high peptide/lipid ratio to orientate perpendicularly and insert into the bilayer, forming transmembrane pores [21]. Gabriel *et al.* were able to prepare titanium surfaces with antimicrobial properties by grafting LL37. The application of a flexible hydrophilic poly(ethylene-glycol) spacer and selective N-terminal conjugation of LL37 resulted in a surface peptide layer which was capable of killing bacteria on contact [22]. However, since the CBM3 size is much larger than the linked AMP, the peptide/lipid ratio is probably unsuitable.

2.3.4 Chemical cleavage of LK-CBM3-MAG2

Given the negative results obtained with the MAG2-D peptide, a new construction was engineered. This construct made on the vector pQE-30, keeping a terminal His6-tag, was successfully expressed as shown above on section 2.3.1. After the purification of the fusion protein either on cellulose or by IMAC, the formic acid cleavage was performed as previously described and the samples were analyzed on 16.5% Tris-tricine gels. However, it was not possible to detect the antimicrobial peptide in the gels. Larger fermentations were made in order to increase the amount of fusion protein produced. After the chemical cleavage, the mixture was ultrafiltrated in a 5 kDa molecular weight cut-off Centricon to separate the peptide from the CBM3 and concentrated by lyophilization. Even so, we could not detect the peptide by SDS-PAGE.

In order to better evaluate the cleavage process, MALDI-TOF analysis of the samples before and after hydrolysis were performed. The fusion protein LK-CBM3-MAG2 has a theoretical molecular weight of 24690 Da (including the C-terminal His6-tag). The MALDI-

TOF analysis revealed a protein with molecular weight of 23974Da, below the theoretical expected value (figure 2.7).

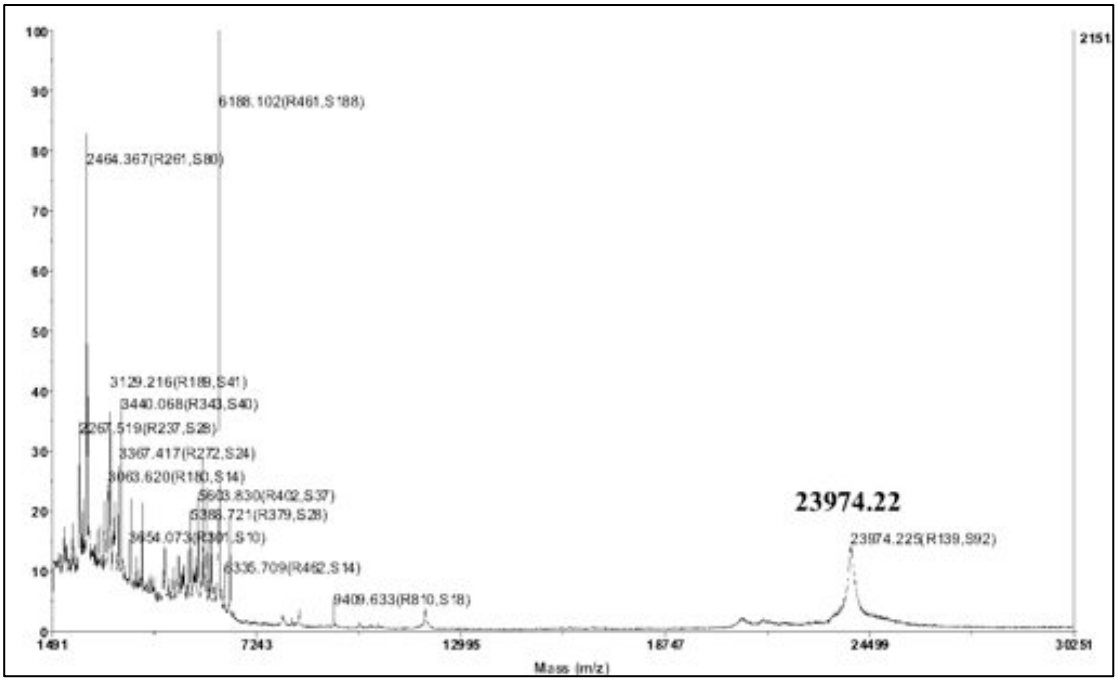

Figure 2.7. MALDI-TOF MS analysis of the fusion protein LK-CBM3-MAG2 before formic acid cleavage.

Considering this result and the lack of detection of the peptide by SDS-PAGE analysis, we assume that the antimicrobial peptide MAG2 might have suffered proteolysis. Several fermentations conditions were tested; phenylmethanesulfonylfluoride (PMSF), a serine protease inhibitor, and protease inhibitor cocktail tablets (Roche) were used but similar results were obtained.

To date, the successful cloning, expression and purification of the AMP magainin-2 in bacteria has not been reported. Barrell *et al.* reported the successful expression of MAG2 fused to the well-known thioredoxin for raising antibodies [23]. However, they did not cleave and purify MAG2 from its partner. A magainin analogue (MSI-344) was expressed and purified in a good yield, but from *E. coli* inclusion bodies. The recombinant peptide kept its antibacterial activity [24, 25]. In a different approach, Fan and Li successfully expressed MAG2 in a mammalian cell system at high productivity and efficiency [26].

2.4 CONCLUSIONS

The protein MAG2-D was successfully expressed cleaved and purified; however, the protein bears no antibacterial activity. On the other hand, when MAG2 was fused to the C-terminal of the CBM3 and transformed into *E. coli* M15 (pREP4) cells, proteolysis of the AMP was apparently observed. As already stated, the expression of AMPs in bacteria is challenging because they are sensitive to proteolytic degradation. Potent analogues of MAG2 have been obtained by substitutions of glycine or serine with alanine residues, and an amidation at the C-terminus, which resulted in enhanced α-helical structure and antimicrobial activity. Studies with pronase digestion suggested that the lower antimicrobial of MAG2 activity, when comparing to analogues, may be due to higher susceptibility to proteolysis in the presence of membranes [27]. The very few works reporting the expression and purification of MAG2 in *E. coli* may indicate that it is very difficult to produce MAG2 in bacteria. Interestingly, no such difficulties were observed in BL21 (DE3) in this work and by Guerreiro *et al.* [16].

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CHAPTER 3

ESCHERICHIA COLI EXPRESSION AND PURIFICATION OF LL37 FUSED TO A FAMILY III CBM FROM *CLOSTRIDIUM THERMOCELLUM*


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Escherichia coli expression and purification of LL37 fused to a family III carbohydrate-binding module from *Clostridium thermocellum*

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ABSTRACT

The cathelicidin derived human peptide LL37 has a broad spectrum of antimicrobial and immunomodulatory activities. The large variety of biological activities makes LL37 a very promising candidate for clinical applications. The production of biologically active LL37 in large amounts with reduced costs can only be achieved using recombinant techniques. In this work, LL37 has been cloned to the N- and C-termini of a family III carbohydrate-binding module fused to the linker sequence (LK-CBM3) from *Clostridium thermocellum*; both constructions (LL37-LK-CBM3 and LK-CBM3-LL37) were cloned into the pET-21a vector. A formic acid recognition site was introduced between the two modules, allowing the isolation of LL37 after chemical cleavage.

The recombinant proteins were expressed in Escherichia coli BL21 (DE3) and solubilized with Triton X-100. The purification was achieved using cellulose CF11 fibers, taking advantage of the CBM3 specific affinity for cellulose; after hydrolysis with formic acid, LL37 was further purified by reverse-phase HPLC, as confirmed by MALDI-TOF mass spectrometry. The production and purification methodology developed in this work compares advantageously to other protocols previously described, having fewer purification steps. Only the recombinant LL37 obtained from the C-terminally fused protein (LK-CBM3-LL37) showed antibacterial activity against *E. coli* K12, with a MIC of 180 μg/ml.

3.1 INTRODUCTION

The increasing resistance of microorganisms against common antibiotics has become a growing threat for the public health. Antimicrobial peptides (AMPs) are part of the innate immune system and have a large spectrum of antimicrobial activities against Gram-positive and Gram-negative bacteria, fungi [1] and viruses [2]. AMPs are generally defined as cationic, amphipathic peptides, with less than 50 amino acids, including multiple arginine and lysine residues [3] and show inhibitory concentrations (MIC) as low as 0.25–4 μg/ml [2]. They are widely distributed in nature, from in- sects to plants and animal species; over 1440 AMPs have been identified so far (http://aps.unmc.edu/AP/main.php; July 2009).

In mammals, defensins and cathelicidins represent the two major types of AMPs. Cathelicidins share a highly conserved N-terminal cathelin domain, flanked by a rather variable antimicrobial peptide on the C-terminus [4]. The hCAP-18/LL37 is the only human cathelicidin. The antimicrobial peptide is referred to as LL37, since it has a 37 amino acids sequence starting with two leucines. It is a 4.5 kDa, cationic (+6), amphipathic a-helical peptide, with a broad spectrum of antimicrobial activity. LL37 and the precursor protein, hCAP-18, can be found at different concentrations in many different cells, tissues and body fluids. Durr *et al.* [5] summarized the various tissues where LL37 expression has been detected. Besides its protective effect against infections, a variety of other biological activities have been described. In fact, LL37 induces chemotaxis of mast cells [6], monocytes, T lymphocytes and neutrophils [7], promotes wound healing [8], angiogenesis and arteriogenesis [9].

Many bacterial and fungal enzymes that hydrolyze insoluble carbohydrates share a common structure composed of a catalytic domain linked to a carbohydrate-binding module (CBM). CBMs that are specific for insoluble cellulose (cellulose binding domain – CBD) represent the predominant category. The CBMs can be grouped into distinctive families on the basis of amino acid sequence similarities [10]; 53 families of CBMs have been recognized so far (http://www.cazy.org/fam/acc_CBM.html; July 2009).

Family III CBDs normally comprise 150 amino acids residues, have been identified in many different bacterial enzymes, and also in non-hydrolytic proteins [11]. *Clostridium thermocellum* produces a multi-enzyme complex of cellulases and hemicellulases, termed the cellulosome, which is assembled by the scaffoldin protein CipA. Binding of the cellulosome to the plant cell wall is driven by the action of the CipA family 3 CBM

(CBM3), which presents high affinity for crystalline cellulose [12]. CBM3 belongs to the all-β family of proteins and is arranged in two antiparallel β sheets that form a β sandwich with jellyroll topology [11].

In this work, we describe the successful cloning, expression and purification of LL37 using the CBM3 from *C. thermocellum* as fusion partner. The CBM3 is overexpressed in *Escherichia coli* and it is possible to take advantage of its affinity properties to purify recombinant proteins on cellulose fibers, reducing significantly the costs of purification.

3.2 MATERIALS AND METHODS

3.2.1 Construction of expression vectors

The gene encoding CBM3 fused to the endogenous CipA N-terminal linker sequence (LK) has previously been cloned in the expression vector pET21-a (Novagen), in our laboratory [12]. Here, two expression vectors were constructed with LL37, either at the N-terminus (LL37-LK-CBM3) or C-terminus (LK-CBM3-LL37) of the pET21-LK-CBM3 vector (Figure 3.1).

The DNA fragment encoding LL37 was amplified by polymerase chain reaction (PCR) from hCAP-18/pET15b generously provided by Dr. Ole Sorensen, Lund University, Lund, Sweden. For the LL37-LK-CBM3 construction, the following primers were used: 5'- GGA ATTC **CAT ATG** CTG CTG GGT GAT TTC TTC-3' (forward primer) and 5'- CTA **GCT AGC** *CGG ATC* GGA CTC TGT CCT GGG TAC-3' (reverse primer). For the LK-CBM3-LL37 construction, the primers were: 5'- CCG **CTC GAG** *GAT CCG* CTG CTG GGT GAT TTC TTC-3' (forward primer) and 5´-CCG **CTC GAG** TTA GGA CTC TGT CCT GGG TAC-3´(reverse primer). *Nde*I, *Nhe*I (LL37-LK-CBM3) and *Xho*I (LK-CBM3-LL37) recognition sites are shown in bold. In order to allow the chemical cleavage with formic acid, proline and aspartate residues were introduced (in italic). PCR reactions were performed using the DNA Polymerase VENT (Stratagene). The PCR reactions were performed as follows: preheating at 95ºC for 2 min, 30 cycles at 95ºC for 30s, 30s at 52ºC and 30s at 72ºC, followed by a final elongation stage at 72ºC for 10 min. The amplified products were recovered from 1% agarose gel, digested with *NdeI* and *NheI* or *Xho*I and ligated into pET21-LK-CBM3 plasmid and sequenced to ensure that no

mutations had occurred during the PCR. Figure 3.1 represents LL37-LK-CBM3 and LK-CBM3-LL37 constructions. The construction LL37-LK-CBM3 kept the C-terminal His6-tag even though this tag was not used for purification.

Figure 3.1. Schematic representation of LL37-LK-CBM3 (A) and LK-CBM3-LL37 (B) constructions. The AspPro site for chemical cleavage with formic acid is shown in black; H, His-tag.

3.2.2 Expression and purification of fusion proteins

Figure 3.2 summarizes the strategy followed for the production and purification of recombinant LL37. The recombinant plasmids were transformed into the *E. coli* strain BL21 (DE3) (Novagen) for protein expression. The bacterial strains were grown in Luria-Bertani broth (LB) containing 100 µg/ml of ampicillin at 37ºC to mid-exponential phase (OD_{595}=0.6). Expression of the fusion proteins was initiated by adding isopropyl-β-D-thiogalactopyranoside (IPTG) at a final concentration of 1 mM and the culture was incubated overnight at 37ºC. The cells were centrifuged at 6000 rpm for 15 min at 4ºC, resuspended in Tris–HCl pH 7.0, 20 mM NaCl, 5 mM $CaCl_2(2H_2O)$ buffer (CBM buffer) and sonicated in ice for 6 min. After centrifugation at 12000 rpm, for 30 min at 4ºC, the cells were resuspended in CBM buffer with 1% Triton X-100. The cells were centrifuged again at 12000 rpm, for 30 min at 4ºC and the soluble fraction was collected.

The recombinant proteins were purified on cellulose CF11 (Sigma), exploiting the CBM3 cellulose-binding properties, as follows: 20 ml of cell-free extracts were mixed with 2 g of cellulose and incubated, with agitation, for 1h at 4ºC. The cellulose with bound proteins was then centrifuged and washed 5 times with CBM buffer.

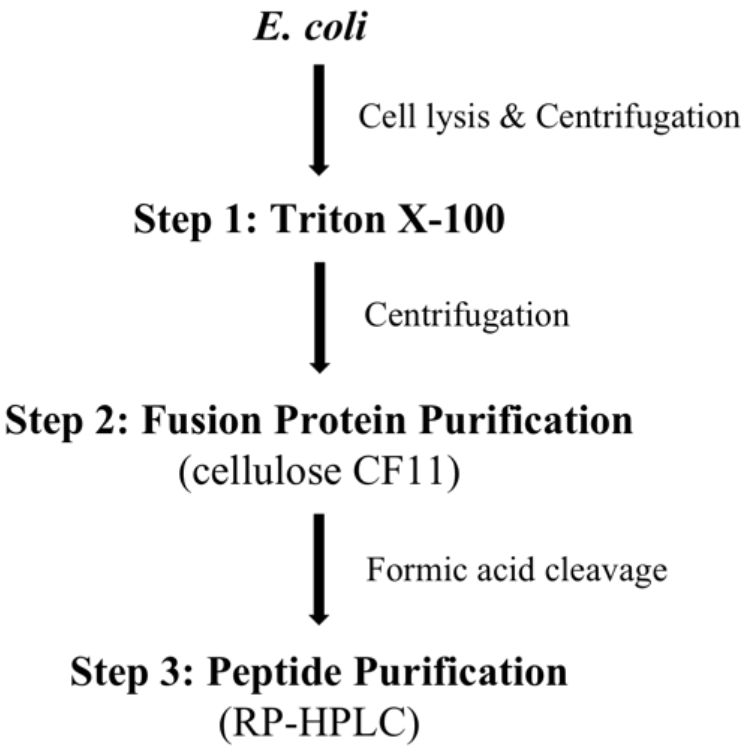

Figure 3.2. Flow chart for the expression and purification of LL37 from E. coli.

3.2.3 Formic acid cleavage and LL37 purification

In order to cleave LL37 of the recombinant protein, a 50% formic acid solution (20 ml of distilled water and 26 ml 88% formic acid) was applied directly to cellulose with the adsorbed fusion proteins. The mixture was then incubated for 24h at 50ºC. The supernatant was separated after centrifugation at 12000 rpm, for 30 min, and lyophilized to remove formic acid. The cleavage was confirmed by SDS-PAGE using a 16.5% Tris-Tricine gel.

The purification of LL37 was achieved by reverse-phase HPLC, using an YMC C18 preparative column (250×30 mm) equilibrated with aqueous acetonitrile (5%)/0.1% trifluoracetic acid (TFA). The peptide was eluted using a linear gradient of acetonitrile from 5% to 90% at a flow rate of 5 ml/min. The elution was monitored at 215 nm. Several peaks were collected and analysed by SDS-PAGE using a 16% Tris-Tricine gel. The fraction corresponding to pure LL37 was lyophilized. The concentration of the recombinant peptides was quantified using Waddell's method [13].

3.2.4 MALDI-TOF mass spectrometry

The purity of LL37 was confirmed by mass spectrometry at the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP). Samples were desalted and concentrated using microC18 ZipTips (Millipore, USA) accordingly to the manufacturer's protocol. The samples were eluted with the matrix α-cyano-4-hydroxycinnamic acid (5 mg/mL) in 50% acetonitrile/0.1% TFA and spotted onto a stainless steel 192-well MALDI plate. After sample crystallization, mass spectrum acquisition was performed using a 4700 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, USA). Mass spectra were acquired in positive linear mode for 1-15 kDa (m/z) mass window and in positive reflector mode for the ranges 700-7000, 4000-5000 and 4585-4600 Da (m/z). External calibration of the mass spectrum was performed using Applied Biosystems Calibration Mix 3 standards.

3.2.5 Antibacterial activity

The antibacterial activity of the pure peptides was quantified in 96-well polypropylene microtiter plates, as described by Vogt *et al.* [14], with some modifications. *E. coli* strain K12 was grown at 37ºC in LB medium until mid-logarithmic phase (OD_{600}=0.5). The cells were then resuspended in LB at 1×10^6 and 1×10^5 cells/ml; 100 µl of peptides, at different concentrations, were inoculated with 50 µl of bacterial suspension, incubated overnight at 37ºC and the bacterial growth was assessed by the measurement of optical density at 620 nm. The results were confirmed by plating 25 µl aliquots of the mixture from each well in LB plates and incubating for 16h at 37ºC, followed by colony counting. Each assay was repeated three times and controls were made by adding sterile deionized water, instead of the peptide solutions. The minimal inhibitory concentration (MIC) was determined as the lowest peptide concentration that inhibited bacterial growth.

3.3 RESULTS AND DISCUSSION

3.3.1 Expression and purification of recombinant proteins

The protein LL37 was successfully cloned at both the N- and C-terminus of the LK-CBM3 in the expression vector pET21-a. After transformation of the recombinant plasmids into *E. coli* BL21 (DE3), several fermentation conditions were tested, attempting to optimize the production of the recombinant proteins in the soluble form. The following operational parameters and *E. coli* strains were varied or tested: concentration of IPTG, induction temperature and time, fermentation in M9 minimal medium, transformation into *E. coli* Tuner (DE3) and Origami (DE3) and co-expression with chaperones GroEL-GroES and *E. coli* trigger factor (TF) [15]. Only using the nonionic detergent, Triton X-100, the production of the proteins (~27 kDa) in the soluble form was achieved (figure 3.3).

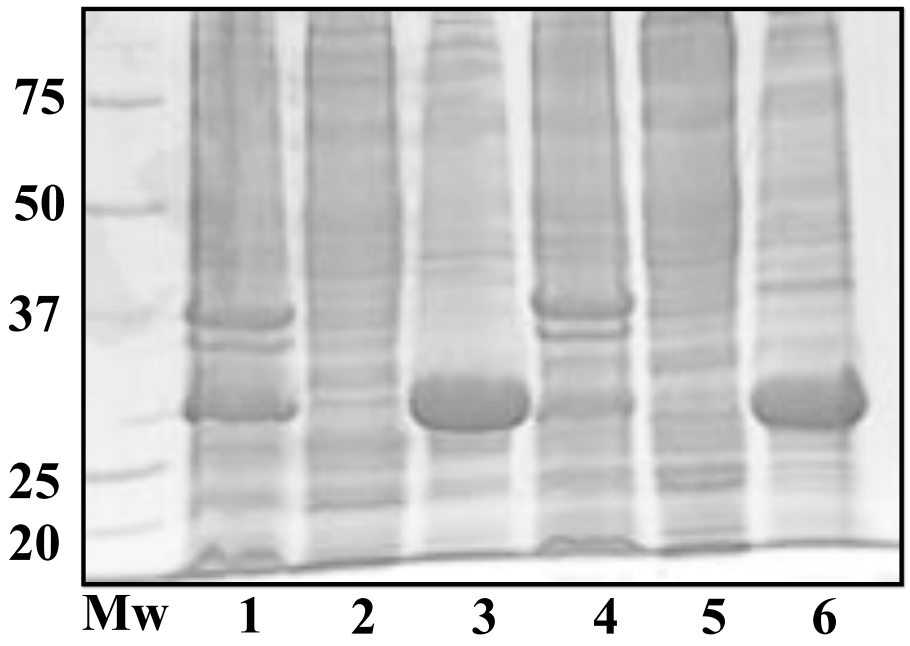

Figure 3.3. Expression of recombinant proteins LL37-LK-CBM3 and LK-CBM3-LL37. Mw, protein molecular weight marker; 1, LL37-LK-CBM3 insoluble fraction; 2, LL37-LK- CBM3 soluble fraction; 3, LL37-LK-CBM3 Triton X-100 solubilized fraction; 4, LK- CBM3-LL37 insoluble fraction; 5, LK-CBM3-LL37 soluble fraction; 6, LK-CBM3-LL37 Triton X-100 solubilized fraction.

In previous work, Guerreiro *et al.* [12] reported the expression of the same CBM3 from *C. thermocellum* used in this work. The CBM, fused or not with small antimicrobial peptides, was overexpressed in the soluble form. Only the protein LKLLKKLLKLLKKLGGGK-LK-CBM3 was found to be insoluble when expressed by different *E. coli* strains, under a range of induction conditions. In this work, the CBM3 fused to LL37 could only be solubilized with detergents. The use of CBM3 as fusion partner had two major goals: (1) to express and purify the peptide LL37 by a novel, fast and inexpensive method, using cellulose for purification and (2) to modify the surface of cotton fabrics with LL37, aiming at obtaining textiles with antibacterial and bioactive properties. As already stated, LL37 is an antimicrobial peptide but it also has a broad spectrum of biological activities. Thus, one main objective was to modify cellulose for biomedical applications. However, in this work, we focus on the production of the isolated LL37. For this purpose, the CBM is still useful, allowing the separation of the recombinant protein through inexpensive affinity-based separation, using cellulose fibers.

The purification of the LL37-LK-CBM3 and LK-CBM3-LL37 recombinant proteins was performed on cellulose CF11 fibers. The family III CBM from *C. thermocellum* is well studied. This CBM adsorbs to cellulose with high affinity constants [16]. The fusion with LL37 and the presence of residual detergent did not affect the binding of the CBM3 on CF11 cellulose. Figure 3.4 shows a binding assay of the LK-CBM3-LL37 recombinant protein, performed as described previously [12]. The recombinant proteins bind very efficiently to cellulose and only desorb using SDS.

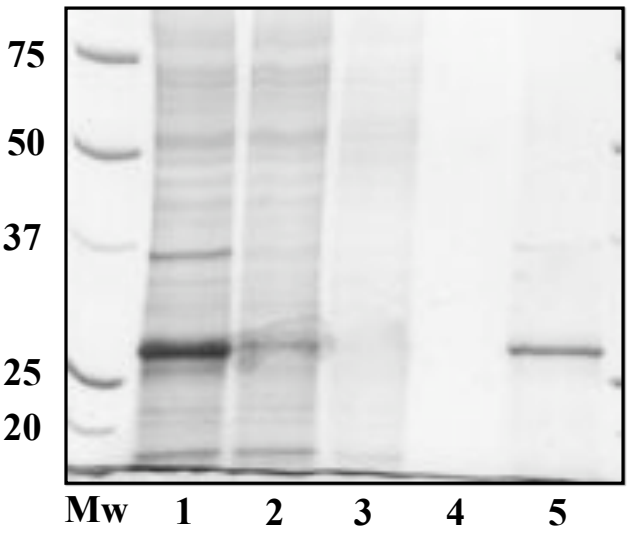

Figure 3.4. Binding assay of LK-CBM3-LL37 on CF11 fibers. Mw, protein molecular weight marker; 1, Triton solubilized supernatant; 2, unbound protein; 3, first cellulose wash; 4, fifth cellulose wash; 5, bound protein eluted with 1% SDS.

To date, few groups have reported the recombinant expression of LL37 either in the *E. coli* expression system [17-19] or in the *Pichia pastoris* system [20]. In these publications, the purification of the recombinant proteins is always performed through affinity chromatography techniques, using expensive matrixes (sepharose, cobalt). Although very effective, chromatography is a slow process with high costs. LL37 is a peptide with a large potential for biomedical applications. Thus, the production of large amounts of LL37 at low costs is of great importance, purification being generally the most critical step in this regard. Cellulose is cheap and available in many forms. Therefore, the expression system using the CBM3 as the expression and purification module is very attractive. As shown previously [12] and in this work, the CBM3, when fused to antimicrobial peptides, is overexpressed in *E. coli* conserving its binding properties. The adsorption of CBM3 on cellulose is quite fast, allowing the recombinant proteins to be purified in a quick, inexpensive method. Moreover, no columns and chromatography units are required. Compared with previous publications, this method is clearly the best for large-scale purification of LL37.

3.3.2 Chemical cleavage and purification of LL37

Several methods, both enzymatic (factor Xa and thrombin proteases) and chemical (CNBr and formic acid) are widely used to cleave proteins from fusion partners. Formic acid is a good option for large-scale production, due to its low cost. The chemical cleavage of the recombinant proteins was performed, in this work, with 50% formic acid, at 50ºC for 24h. LL37-LK-CBM3 and LK-CBM3-LL37 proteins were first purified on cellulose. Then, formic acid was applied directly on the CF11 fibers with the bound protein. After centrifugation, the supernatants were lyophilized to completely remove the acid and analyzed by 16.5% Tris-tricine gel (figure 3.5).

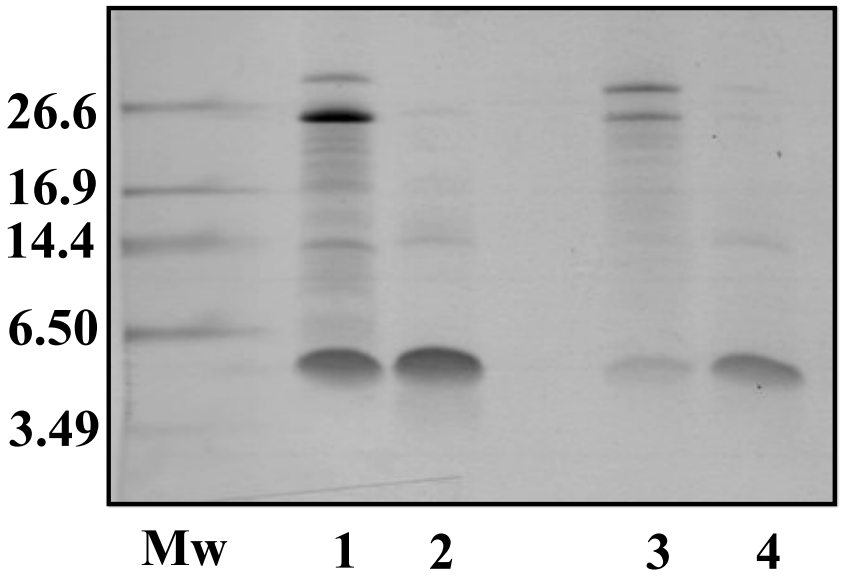

Figure 3.5. Formic acid cleavage and release of LL37. Mw, protein molecular weight marker; 1, insoluble fraction of cleaved LL37-LK-CBM3; 2, supernatant of cleaved LL37-LK-CBM3; 3, insoluble fraction of cleaved LK-CBM3-LL37; 4, supernatant of cleaved LK-CBM3-LL37.

After acid hydrolysis and lyophilization, part of the protein (mostly CBM) precipitates, probably degraded by the formic acid. On the other hand, most of the peptide (~4.5 kDa) remains soluble, as shown in Figure 3.5 (lanes 2 and 4). The precipitation of the CBM3 facilitates the purification. In fact, LL37 is separated from most of the CBM and other contaminating proteins by centrifugation. The soluble supernatants, with a large proportion of LL37, were then subjected to RP-HPLC to achieve higher purity (figure 3.6a). In this step, the fractions corresponding to the major peaks were collected and the purity of the peptides was checked on 16.5% Tris-tricine gel and confirmed by MALDI-TOF. Figure 3.6b presents the peak fractions from RP-HPLC for the two fusion proteins. Lanes 4 and 8 demonstrate the purity of the two peptides.

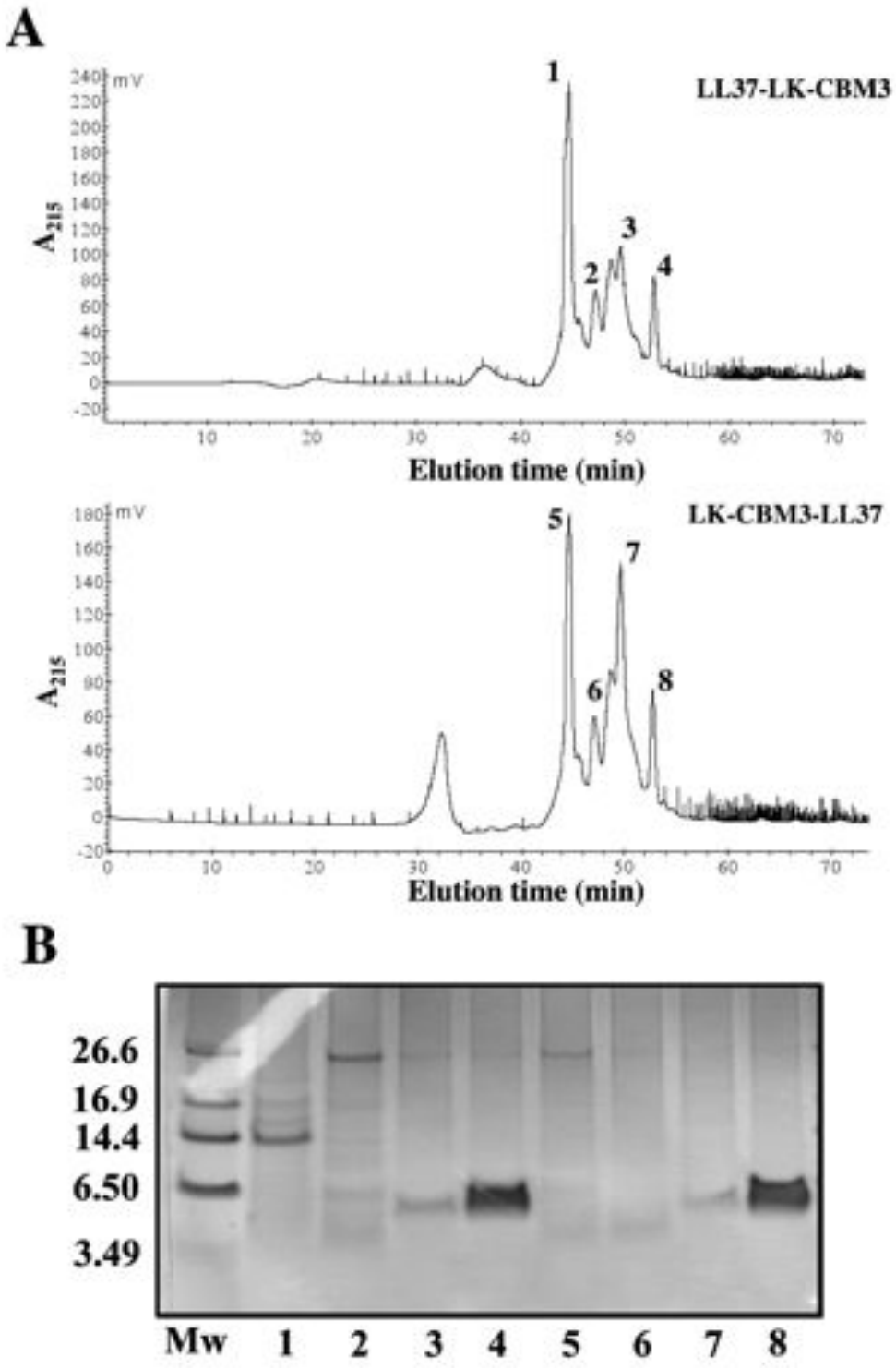

Figure 3.6. A: Reverse-phase HPLC chromatogram of fusion proteins LL37-LK-CBM3 and LK-CBM3-LL37. B: RP-HPLC peak fractions. Mw, protein molecular weight marker; lanes 1–4, peak fractions of cleaved LL37-LK-CBM3; lanes 5–8, peak fractions of cleaved LK-CBM3-LL37.

The two different proteins, both including the LL37, were obtained after the formic acid cleavage of the CBM constructs. The first peptide, obtained by the cleavage of the LL37-LK-CBM3 protein, has 39 amino acids with a theoretical molecular weight of 4739.6 Da. It is flanked with the N-terminal methionine, remaining from the translation process and a C-terminal aspartic acid, left by the chemical cleavage (M-LL37-D). Although 55-70% of mature proteins are subjected to N-terminal Met excision [21], M-LL37-D conserved the initial amino acid. Indeed, as demonstrated by Sherman *et al.* [22] most proteins retain the initial Met when the N-terminus residue is leucine. The second peptide, cleaved from the LK-CBM3-LL37 protein, is 38 amino acids long, with a theoretical molecular weight of 4590.4 Da, having a proline residue at the N-terminus (P-LL37). Mass spectrometry (figure 3.7) confirmed the purity of LL37 and the predicted molecular weights.

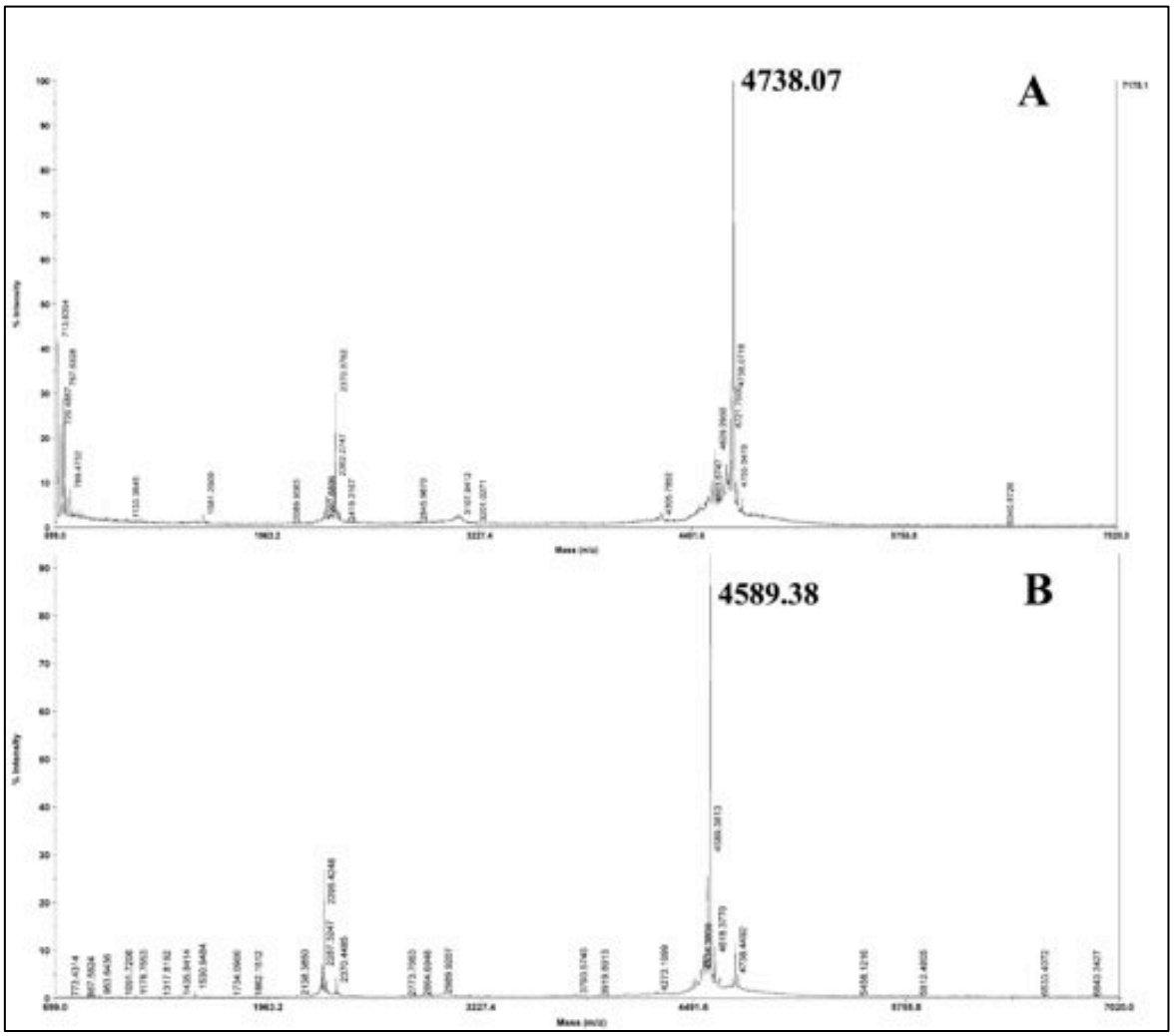

Figure 3.7. MALDI-TOF MS analysis of purified LL37. A: M-LL37-D. B: P-LL37. The measured molecular weights are very similar to the theoretical values (4739.6 and 4590.4 Da).

The production and purification of LL37 using CBM3 as the fusion partner only requires one chromatographic step. All previous publications refer to at least two, and use enzymes to cleave LL37. Li *et al.* [17, 23] also use formic acid for chemical release of LL37, however the purification scheme used is more complex. In this work, it was possible to obtain 1 mg of pure P-LL37 and 2 mg of pure M-LL37-D from 1 liter of bacterial culture. This yield is comparable to the ones obtained in previous studies [17-19]. Li *et al.* [23] also described a novel method to purify LL37 utilizing its property of aggregation. The authors were able to produce 2.6 mg of recombinant LL37 from 1 liter of culture. However, this method required two cleavage steps with thrombin and formic acid and three purification steps.

3.3.3 Antibacterial activity

M-LL37-D and P-LL37 antibacterial activities were tested against *E. coli* K12. The concentration of the peptides was quantified by UV spectroscopy using the Waddell's method [13]. Figure 3.8 demonstrates that P-LL37 has antimicrobial activity, with a MIC of about 180 µg/ml (40 µM), similar to the values found in the literature [5, 17]. At lower concentration, P-LL37 inhibited bacterial growth only partially. The peptide (M-LL37-D) obtained from the N-terminal fusion construct (LL37-LK-CBM3) showed no antibacterial activity. According to Nagaoka *et al.* [24], the N-terminus and C-terminus of LL-37 do not contribute to the formation of α-helical structures, being less important for its antimicrobial activity. However, the C-terminal aspartic acid reduces the global charge of the peptide. Furthermore, Giglione *et al.* [21] suggest that an N-terminal Met may act as a destabilization signal. This peptide has a total charge of +5, so the second hypothesis seems to be more likely. In fact, other AMPs with lower net charge still exhibit antibacterial activity. As already stated, recombinant LL37 has already been expressed and purified and *E. coli* but only two groups tested the antibacterial activity of the pure peptides. Yang *et al.* [18] produced the peptide GSLL-39 with a MIC of 12.5 µg/ml against *E. coli* DH5α and 25 µg/ml against *S. aureus*; Li *et al.* [17] obtained the peptide P-LL37 with a MIC of 180 µg/ml (40 µM) against *E. coli* K12. Durr *et al.* [5] report that the MIC of LL37 against *E. coli* and *S. aureus* is higher than 144 µg/ml (32 µM), similar to the P-LL37 expressed previously and in this work (figure 3.8).

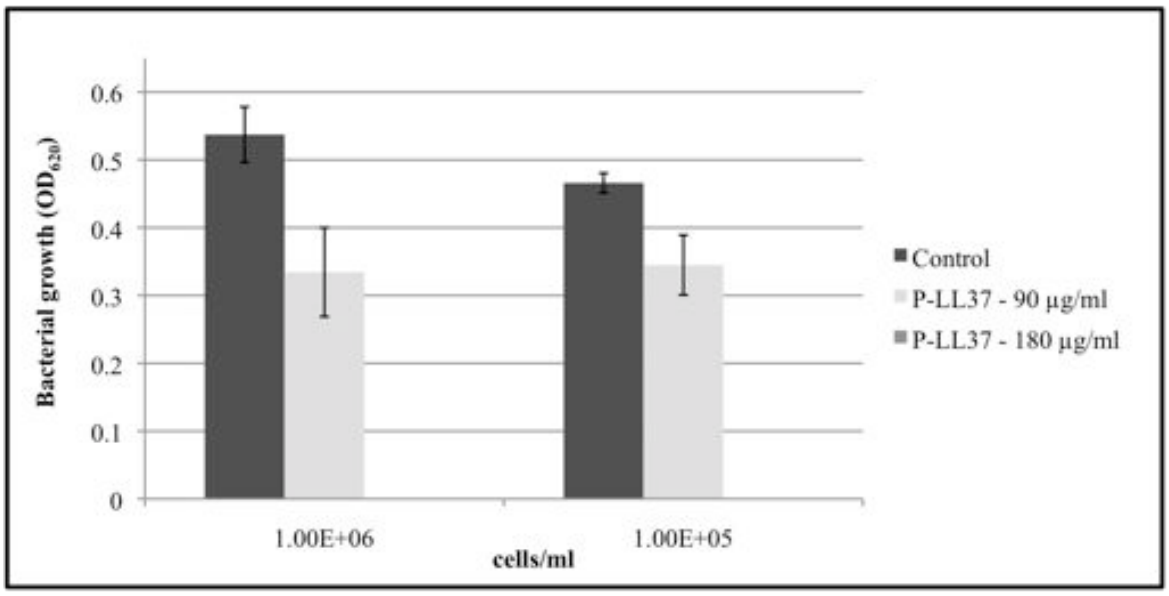

Figure 3.8. Antibacterial activity of P-LL37 against E. coli K12. Bacterial growth was inhibited at a concentration of 180 µg/ml of P-LL37.

3.4 CONCLUSIONS

LL37 has been extensively studied in recent years. The variety of immunological and antimicrobial activities makes LL37 a very promising candidate for clinical applications. For these purposes, high amounts of affordable LL37 can only be obtained using recombinant techniques. Major advances in solid-phase chemical synthesis of peptides, over the last five years have rendered the large- scale synthesis of long chain-length bioactive peptides possible, but this technique is too expensive for the production of large quantities of pure peptides with more than 30 amino acids. In this work, the family III CBM from *C. thermocellum* was used as fusion partner, taking advantage of its cellulose-binding properties for purification. The antibacterial assays showed that only P-LL37 was active. The methodology described in this work appears to be very effective for production and purification of AMPs. The use of cellulose for purification and formic acid for chemical cleavage make this method the more cost effective when comparing to previous publications.

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CHAPTER 4

WOUND HEALING ACTIVITY OF THE HUMAN AMP LL37


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Wound healing activity of the human antimicrobial peptide LL37

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ABSTRACT

Antimicrobial peptides (AMPs) are part of the innate immune system and are generally defined as cationic, amphipathic peptides, with less than 50 amino acids, including multiple arginine and lysine residues. The human cathelicidin antimicrobial peptide LL37 can be found at different concentrations in many different cells, tissues and body fluids and has a broad spectrum of antimicrobial and immunomodulatory activities. The healing of wound is a complex process that involves different steps: haemostasis, inflammation, remodeling/granulation tissue formation and re-epithelialization. Inflammation and angiogenesis are two fundamental physiological conditions implicated in this process.

We have recently developed a new method for the expression and purification of recombinant LL37. In this work, we show that the recombinant peptide P-LL37 with a N-terminus proline preserves its immunophysiological properties *in vitro* and *in vivo*. P-LL37 neutralized the activation of macrophages by lipopolysaccharide (LPS). Besides, the peptide induced proliferation, migration and tubule-like structures formation by endothelial cells. Wound healing experiments were performed in dexamethasone-treated mice to study the effect of LL37 on angiogenesis and wound regeneration. The topical application of synthetic and recombinant LL37 increased vascularization and re-epithelialization. Taken together, these results clearly demonstrate that LL37 may have a key role in wound regeneration through vascularization.

4.1 INTRODUCTION

Cationic antimicrobial peptides (AMPs) represent the first line of defense against many invading pathogens. These small amphipathic peptides are part of the innate immune system and have a broad-spectrum activity against bacteria, fungi [1] and viruses [2].

However, direct antimicrobial action is not the only, and probably not even the most important task of mammalian AMPs. In fact, these peptides present low antimicrobial activities under serum and tissue conditions [2, 3]. Nevertheless, they have affinity for lipopolysaccharide (LPS) and can prevent from lethal endotoxemia by suppressing cytokine production [4]. Moreover, these AMPs appear to be involved in the orchestration of many aspects of innate immunity and the inflammatory response [2].

In mammals, at least two distinct groups of AMPs are found. Defensins are the more representatives and cathelicidins form the second group of mammalian AMPs. Cathelicidins share a highly conserved N-terminal cathelin domain, flanked by a rather variable antimicrobial peptide on the C-terminus [5]. The hCAP-18/LL37 is the only known human cathelicidin. Upon stimulation, hCAP-18 is cleaved extracellularly by proteinase 3 to generate the peptide LL37 [6]. The antimicrobial peptide is referred to as LL37, since it has a 37 amino acids sequence starting with two leucines. It is a 4.5 kDa, cationic (+6), amphipathic a-helical peptide, with a broad spectrum of antimicrobial activity. LL37 and its precursor hCAP18, were first described in leucocytes and testis, but were soon found in a large variety of cells, tissues and body fluids, as summarized by Durr *et al*. [7]. Upon injury or infection, there is a strong upregulation of hCAP18/LL37, indicating that LL37 assists the immune system. The downregulation of LL37 has been associated to several diseases: a deficiency of LL37 in the skin of patients with atopic dermatitis [8] or chronic ulcers [9] may account for the increased risk for skin infections. Nizet *et al*. [10] showed that mice with disrupted *Cnlp*, the gene coding for CRAMP (cathelin-related antimicrobial peptide), showed increased susceptibility to skin infections.

Besides its antimicrobial properties LL37 plays a central role in innate immune responses and inflammation. It has been identified as a potent chemoattractant for mast cells [11], monocytes, T lymphocytes and neutrophils [12] through formyl peptide receptor–like 1 (FPRL-1). The fact that LL37 can attract leukocytes contributes to host defense against infections. LL37 also promotes wound healing [9], angiogenesis and arteriogenesis [13] and acts as immune adjuvant [14].

Acute wounds normally heal in a very orderly and efficient manner characterized by four distinct, but overlapping phases: hemostasis, inflammation, proliferation and remodeling. All these phases are strictly connected and play a key role for a complete and proper restoration of the injured tissue [15]. The inflammatory phase is initiated immediately upon injury. Several inflammatory cells migrate to the wound site to fight infection and remove debris. The proliferative phase is characterized by the formation of granulation tissue, epithelialization, and angiogenesis. Then, the new collagen matrix becomes cross-linked and organized during the final remodeling phase. Numerous cell-signaling events are required to generate this efficient and highly controlled repair process.

In this work, we demonstrate that P-LL37 produced by recombinant techniques in our laboratory [16] preserves its immunophysiological properties and promotes wound healing through re-epithelialization and vascularization in dexamethasone-treated mice.

4.2 MATERIALS AND METHODS

4.2.1 Animals

C57BL/6 mice experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals).

4.2.2 Reagents

All chemicals, media and reagents were purchased from Sigma-Aldrich except stated otherwise. Synthetic LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was purchased from Bachem, Switzerland. Recombinant P-LL37 (PLLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) was expressed and purified as described previously [16]. Briefly, the DNA encoding the fusion protein LK-CBM3-LL37 was successfully cloned in pET21a and expressed in E. coli BL21 DE3 (Novagen) in Luria-Broth (LB) medium. The fusion protein was purified on cellulose CF11 and formic

acid was applied to cleave LL37 from the fusion carrier (LK-CBM3). The resulting recombinant P-LL37 was finally purified by reverse-phase HPLC.

4.2.3 Culture of murine bone marrow-derived macrophages (BMDM); suppression of TNF production by LPS-activated macrophages

Macrophages were obtained from mouse bone marrow as follows: mice were sacrificed and femurs and tibias removed under aseptic conditions. Bones were flushed with Hanks' balanced salt solution. The resulting cell suspension was centrifuged at 500g and resuspended in RPMI 1640 medium supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum (FBS), 60 μg/ml penicillin/streptavidin, 0.005 mM β-mercaptoethanol (complete RPMI [cRPMI]), and 10% L929 cell conditioned medium. To remove fibroblasts or differentiated macrophages, cells were cultured on cell culture dishes (Sarstedt, Canada), overnight at 37°C in a 5% CO_2 atmosphere. Then, nonadherent cells were collected with warm cRPMI, centrifuged at 500g, distributed in 96-well plates (Sarstedt, Canada) at a density of $1x10^5$ cells/well, and incubated at 37°C in a 5% CO_2 atmosphere. Four days after seeding, 10% of L929 cell conditioned medium was added, and the medium was renewed on the seventh day. After ten days in culture, cells were completely differentiated into macrophages. This method allows for the differentiation of a homogenous primary culture of macrophages that retain the morphological, physiological and surface markers characteristics of these phagocytic cells [17-19]. LPS was then incubated with the macrophages culture. At the same time, synthetic LL37 and P-LL37 were added at different concentrations. After 24h, the supernatants were removed and tested for TNF-α production by ELISA (eBioscience).

4.2.4 Cell culture experiments

Human microvascular endothelial cells (HMECs) and human umbilical vein endothelial cells (HUVECs) were used in the experiments. HMECs were cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin/streptomycin, 4.76 g/l of HEPES, 1 ml/L of epidermal growth factor (EGF) and 1 mg/l of hydrocortisone. HUVECs were cultured in medium 199 (M199) supplemented with 0.1 mg/ml of heparin, 20% FBS, 1% penicillin/streptomycin and 2 µl/ml endothelial cell growth supplement (ECGS). Rat bone-

marrow mesenchymal stem cells (MSCs) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS and 1% penicillin/streptomycin. The cells lines were maintained at 37ºC in a humidified 5% CO2 atmosphere.

4.2.5 MTS toxicity assay

HMECs (2×10^5 cells/ml) were grown until 70-90% confluence and incubated with different concentrations of synthetic and recombinant LL37 for 24h in 96-well plates. Toxicity was confirmed using 10% DMSO. Cells were washed twice with PBS and their viability was assessed using Cell Titer 96®Aqueous ONE Solution Reagent (MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo-phenyl)-2H-tetrazolium] colorimetric assay (Promega, Madison, EUA), according to the instructions provided by the manufacturer. Optical density was measured at 492 nm. Results are expressed as percentage of control, which was considered to be 100%.

4.2.6 BrdU proliferation assay

HMECs and HUVECs (6×10^4 cells/ml) were cultured in 24-well plates to adhere overnight. The cells were then incubated for 48h with synthetic and recombinant LL37, in the presence of 5-bromodeoxyuridine (BrdU) solution at a final concentration of 0.01 mM. Proliferation was quantified using In-Situ Detection Kit (BD Biosciences Pharmigen, USA), according to manufacturer's instructions. The results are given as mean ± SEM and are expressed as percentage of control, which was considered to be 100%.

4.2.7 Migration analysis

Migration assays were performed in transwell BD-matrigel basement membrane matrix inserts (BD-Biosciences, Belgium). The chemotactic capacity of LL37 was evaluated by counting the cells that migrated through the membranes. Transwell inserts containing an 8 µm pore-size PET membrane coated with a uniform layer of matrigel basement membrane were used. HUVECs (5×10^4cells/ml) were seeded on inserts in serum-free medium, and placed on wells containing medium complemented with FBS 2% and

recombinant or synthetic LL37 at a concentration of 5 μg/ml. For MSCs (5×10^4cells/ml), the inserts were placed on wells containing DMEM with BSA 0.1% and recombinant LL37 at a concentration of 5 μg/ml. After incubation for 24 h membranes were removed from inserts, stained with DAPI-methanol for 5min and visualized under a fluorescence microscope. The invading cells of each membrane were counted and the results are expressed as percentage of control, which was considered to be 100%.

4.2.8 Angiogenesis

The ability of LL37 to promote the formation of capillary-like structures was examined in Matrigel assay. HMECs and HUVECs (3×10^4 cells/well) were incubated with 5 μg/ml of recombinant and synthetic LL37 for 24h on matrigel-coated 96-well plates. The capillary-like structures in each well were then counted and results are expressed as percentage of control, which was considered to be 100%.

4.2.9 Wound healing assay

C57BL/6 mice (purchased at Charles River, Wilmington, MA) with 8-12 weeks old were used in this study. After general anesthesia, hair on the dorsal side of the animals was shaved and the skin was cleaned with 70% ethanol. A 5 mm skin biopsy punch was used to create full thickness cutaneous wounds under aseptic conditions. Two wounds were created on the dorsal surface with one on each side of the midline. LL37 (recombinant or synthetic) and sterile water were applied topically twice a day (2×10 μg in 50 μl). Wounds were left open and not covered by any type of dressing. To impair healing, mice were treated daily with dexamethasone (0.25 mg/kg body weight, intramuscularly). Mice were examined daily for wound-healing progression. After 7 days, the animals were euthanized and wound tissue was collected for histological and immunohistochemistry studies. Skin wound tissue specimens were fixed in 10% neutral-buffered formalin solution and paraffin-embedded. Histological analyses were performed in 5-μm tissue sections.

4.2.10 Immunohistochemistry

Microvessel density (MVD) was evaluated in each formalin-fixed paraffin-embedded wounded tissue section by immunohistochemistry. Tissue slides were incubated overnight with an anti-CD31 antibody. Briefly, six areas with the highest visible blood vessel density (marked by the vessel marker) per section were selected, and the number of blood vessels was counted per high-power field.

4.3 RESULTS

4.3.1 Recombinant P-LL37 neutralizes the activation of macrophages by LPS

Murine macrophages are known to induce several cytokines, including tumor necrosis factor α (TNF-α), upon LPS activation. LPS was added to macrophage cultures to a concentration of 10 ng/ml, either alone or in the presence of 20 µg/ml of recombinant P-LL37 or synthetic LL37. Cultures were then incubated for 24h. The absence of LPS in the recombinant protein was confirmed using the limulus amoebocyte lysate test (data not shown). As expected, the recombinant P-LL37, as well as the synthetic peptide, neutralized the LPS-mediated activation of macrophages (figure 4.1).

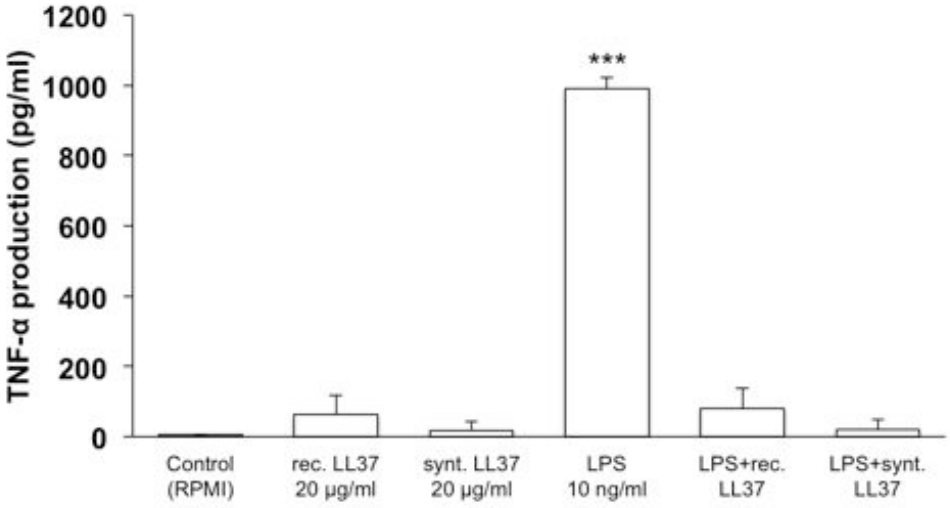

Figure 4.1. Recombinant and synthetic LL37 inhibit the TNF-α production by LPS-activated macrophages. *** P ≤ 0.001 versus control

4.3.2 Toxicity

In order to evaluate the cytotoxicity of recombinant P-LL37, HMECs were cultured for 24h in the presence of the peptide and the cell viability was evaluated by MTS. DMSO 10% was used as cytotoxic agent, a positive control. As shown in figure 4.2, P-LL37 did not affect viability of HMECs at any of the concentrations used. Actually, with 50 and 500 ng/ml of P-LL37, a slight increase of viability was observed, indicating that the recombinant peptide P-LL37 might have promoted proliferation, as previously shown by Koczulla *et al.* [13].

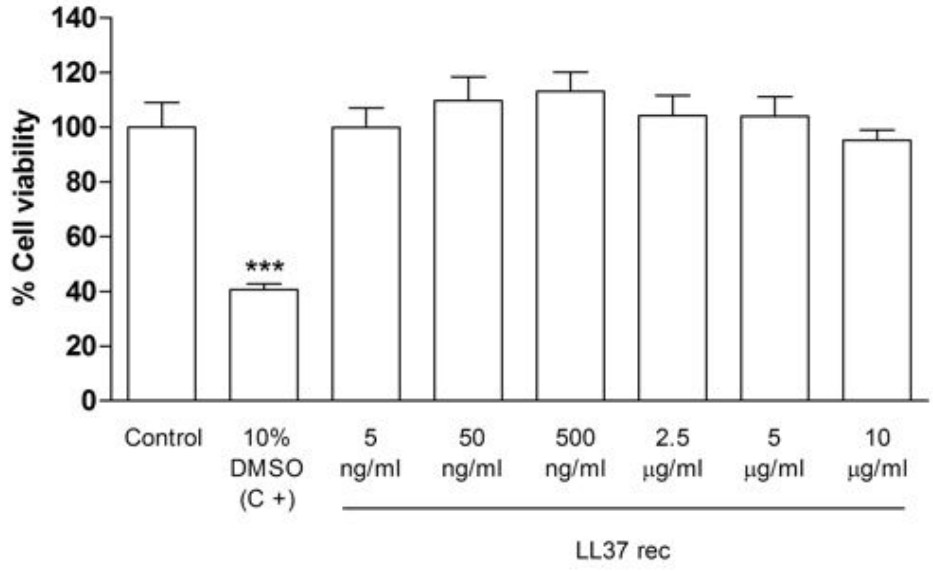

Figure 4.2. Cytotoxicity of P-LL37 in confluent HMECs using MTS assay. Three independent experiments were performed in duplicate. *** P ≤ 0.001 versus control

4.3.3 P-LL37 promotes proliferation, migration and tubule-like structures formation by endothelial cells

Proliferation of HMECs and HUVECs was evaluated using the BrdU incorporation assay. Synthetic LL37 (5 µg/ml) was used as a positive control and 100 µM of deoxycholic acid (DCA), an antagonist of FPRL-1, as negative control. DCA attenuates the activation of FPRL-1, by binding to the cell membrane and interfering with the access of the receptor to its agonists [20]. Figure 4.3 demonstrates that P-LL37 promoted proliferation with

concentrations as low as 50 ng/ml in HMECs (a) and the effect at 5 µg/ml was very similar to the one obtained using the synthetic LL37. The proliferation of HUVECs (b) was inferior but once again there were no significant differences between synthetic and recombinant peptides.

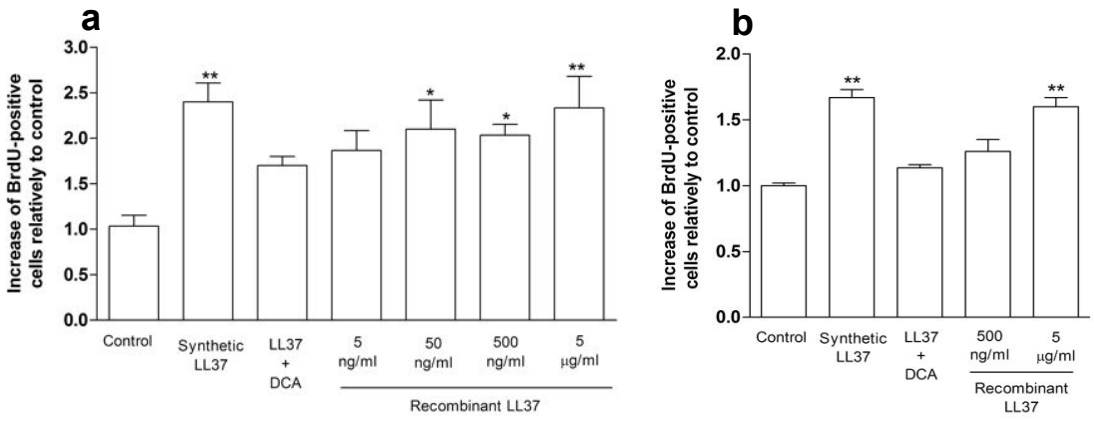

Figure 4.3. Effects of synthetic and recombinant LL37 in HMECs (a) and HUVECs (b) proliferation assessed by BrdU assay. P-LL37 and synthetic LL37 promoted similar proliferation of endothelial cells. Three independent experiments were performed in duplicate. * $P \leq 0.05$ and ** $P \leq 0.01$ versus control.

Next, the chemotactic and angiogenic activities of P-LL37 were evaluated. Figure 4.4 illustrates the invasion assays performed with HUVECs (a) and MSCs (b). Again, P-LL37 was able to increase cell invasiveness capacity both in HUVECs and in MSCs. The capacity of LL37 to promote the formation of capillary structures was assessed with HUVECs and HMECs using matrigel as three-dimensional scaffold (Figure 4.5). The use of LL37 had a strong effect in angiogenesis. In fact, it almost doubled the number of capillaries formed by HUVECs and HMECs.

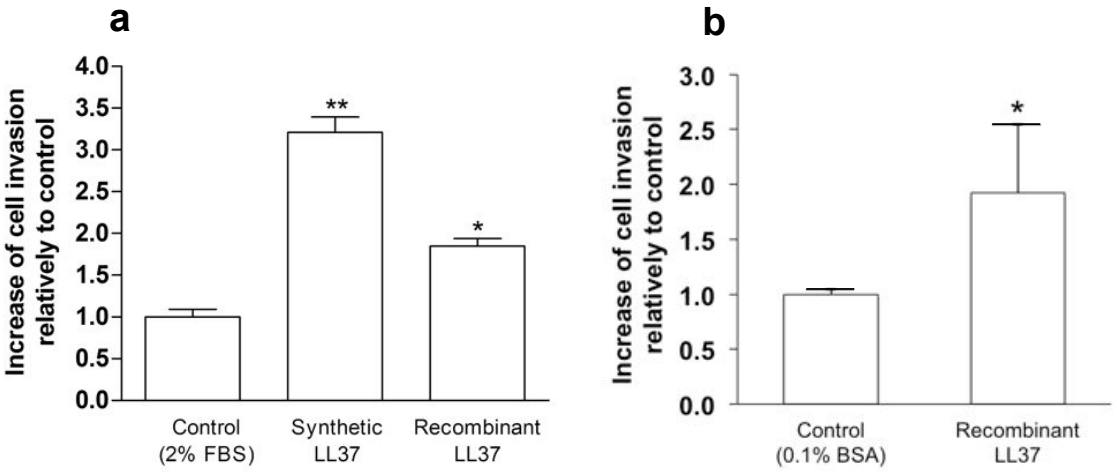

Figure 4.4. Invading cells relative to the initial amount of HUVECs (a) and MSCs (b) cultured using a double-chamber assay. Results are means ±SEM of 3 independent experiments performed in duplicate and expressed relatively to control. * P ≤ 0.05 and ** P ≤ 0.01 versus control.

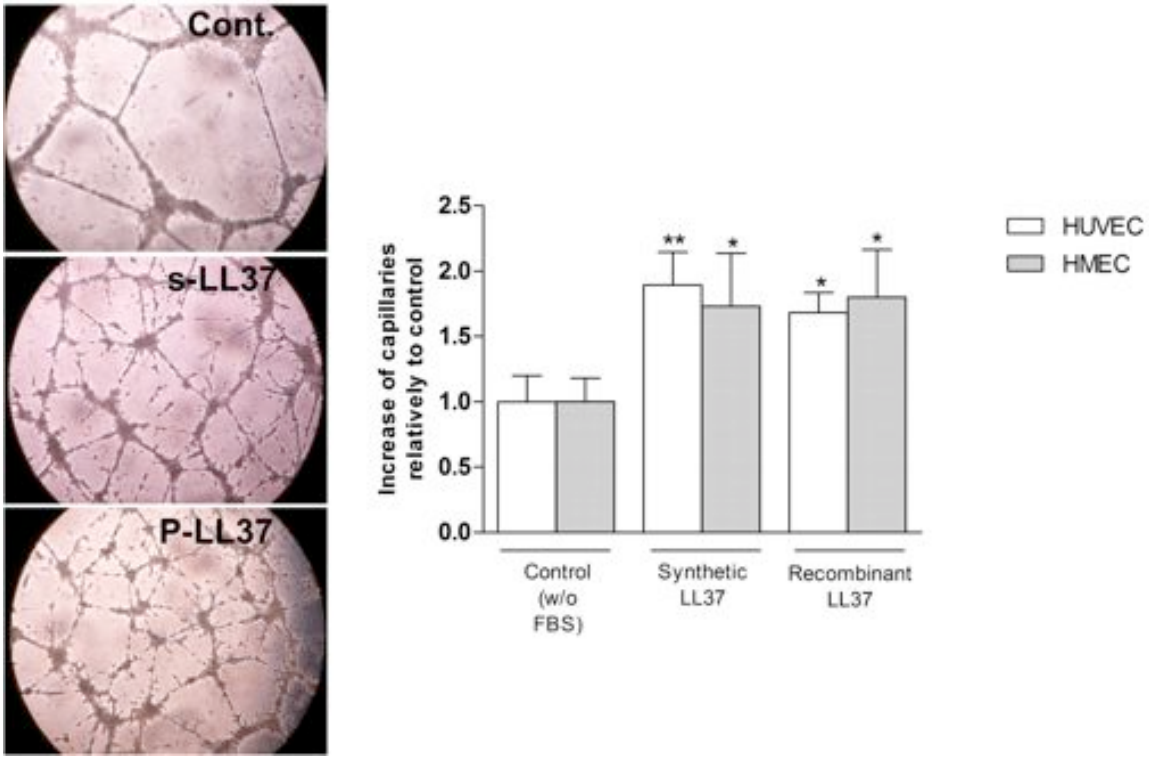

Figure 4.5. Recombinant and synthetic LL37 promote angiogenesis in vitro. Left: Capillary-like structures formation of HMECs, after treatment with 5 μg/ml of LL37, visualized under a phase-contrast microscope. Figures are representative of the whole cultures. Right: Quantification of capillary-like structures assembly. Results are means ±SEM of 3 independent experiments performed in triplicate and expressed relatively to control. * P ≤ 0.05 and ** P ≤ 0.01 versus control.

4.3.4 Wound healing assays

To further examine the effects and biological activities of synthetic and recombinant LL37, wound healing experiments in dexamethasone-treated mice were performed. The peptides or vehicle were topically applied daily for seven days in the 5 mm skin wounds. Dexamethasone was used to impair healing. Figure 4.6 illustrates the wounds after daily treatment with recombinant LL37 (two applications of 10 μg each) or vehicle, on day seven. The effect of the peptide on wound closure is very strong when comparing to control.

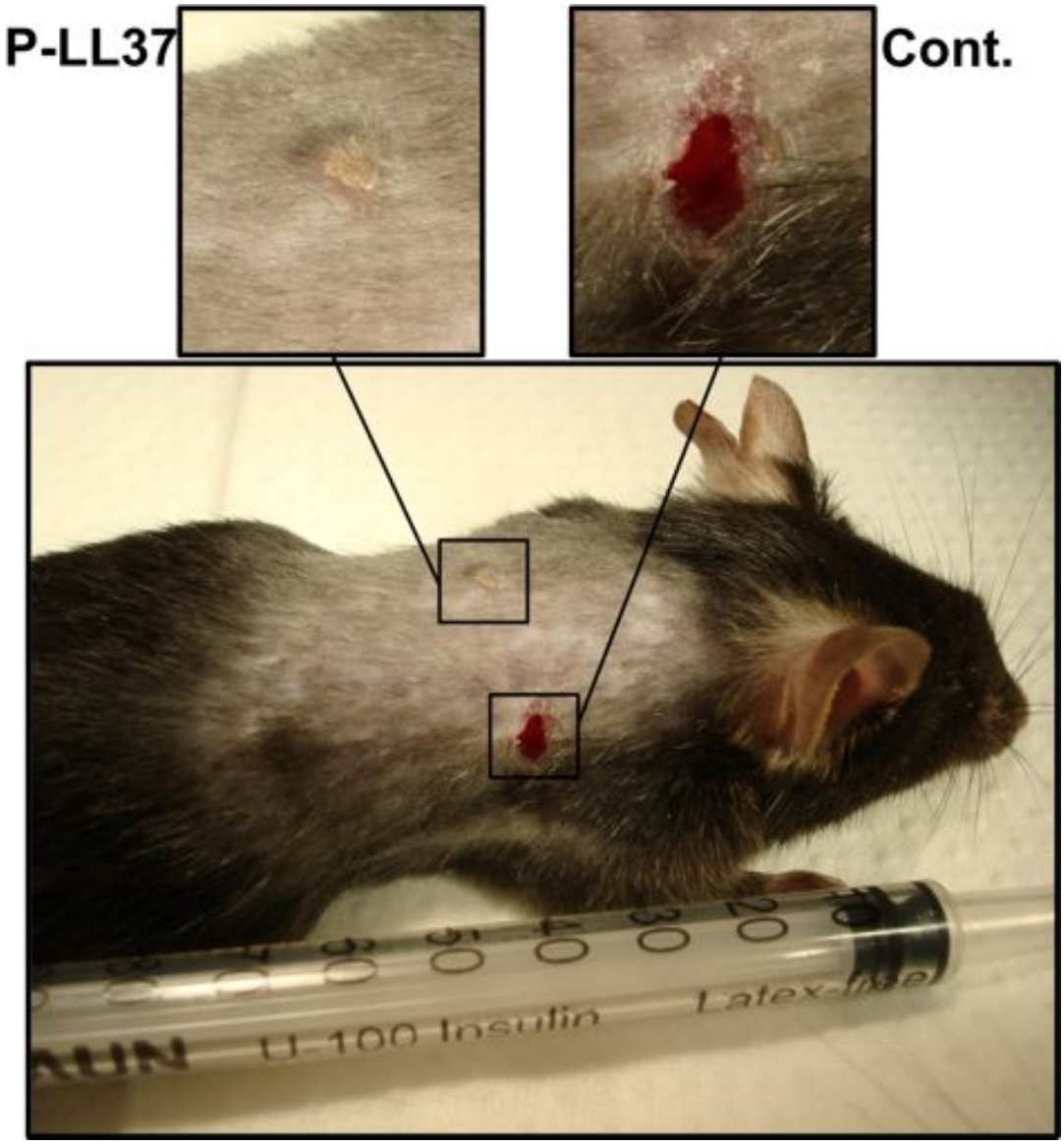

Figure 4.6. Macroscopic examination of the wounds at day seven.

The wound sections stained with hematoxylin and eosin are shown in figure 4.7. No significant differences are found in the granulation tissue with a high number of granulocytes in both cases. However, regarding re-epithelialization, the lesions treated with control or synthetic and recombinant LL37 present large differences. In the control, it is clear that the keranocytes layer is incomplete. On the contrary, in the wounds treated with LL37, the re-epithelialization is almost complete, demonstrating that the healing process is accelerated. More, a high number of blood vessels can be identified in the wounds treated with LL37 (pointed by the arrows), indicating that synthetic or recombinant LL37 strongly promote angiogenesis in vivo. These results were verified by immunohistochemistry (figure 4.8). The angiogenic process was confirmed, in our experimental model, by the augmented micro vascular density evaluated by the means of newly formed vessels counted after immunostaining. The wounds treated with either synthetic or recombinant LL37 possess a significantly higher number of new blood vessels. Moreover, the vessels in the tissues treated with the peptide are much larger. Several experiments were performed with both recombinant and synthetic LL37 and similar results were observed. These results clearly demonstrate the ability of LL37 to promote wound healing is a very promising result.

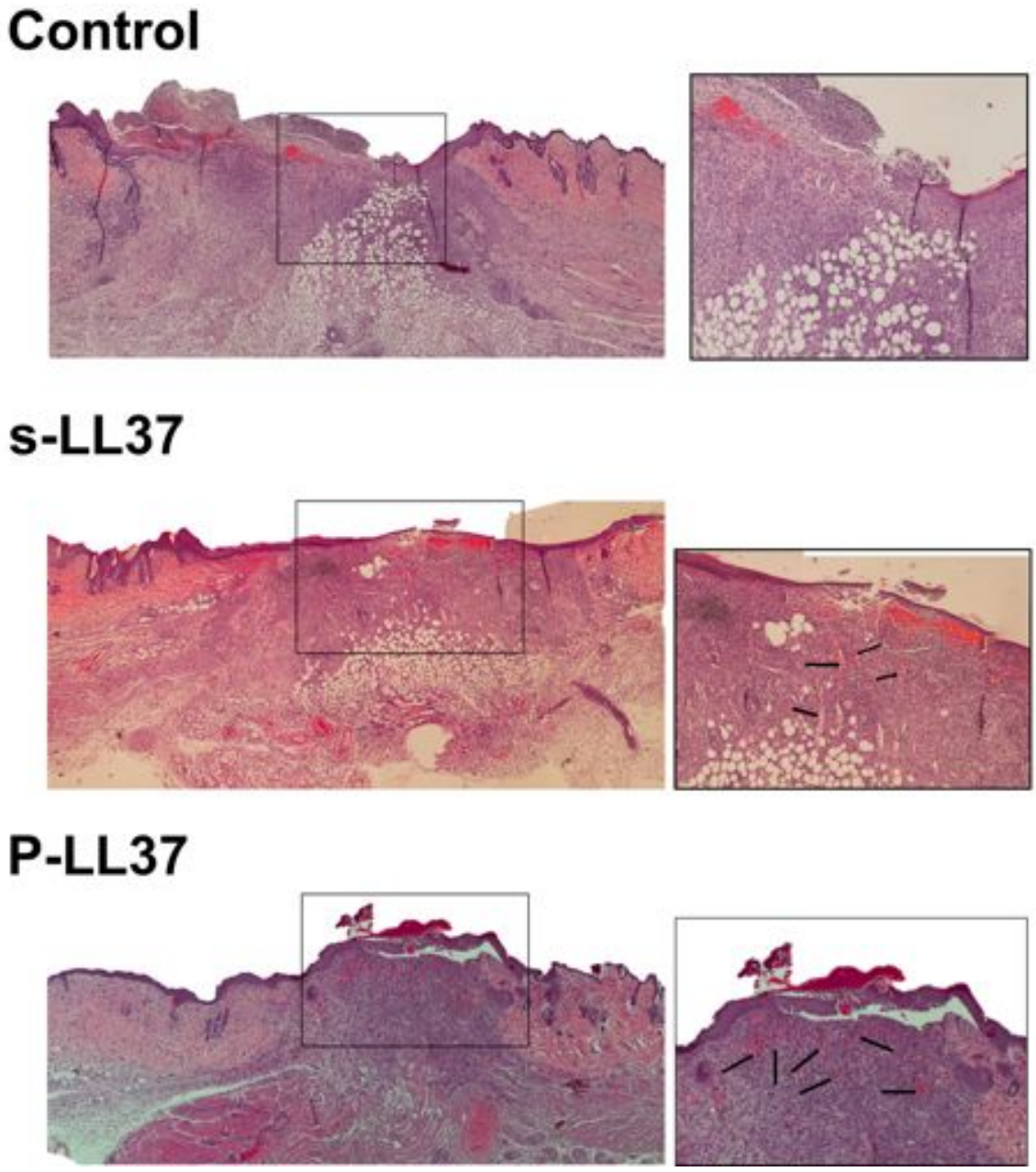

Figure 4.7. Hematoxylin and eosin stained micrographs of wound tissue sections treated with control, synthetic LL37 and recombinant P-LL37. The rectangle identifies an area further investigated in a higher magnification picture. At day 7, re-epithelialization is almost complete in the wounds treated with LL37 and a high number of blood vessels are present (pointed by arrows).

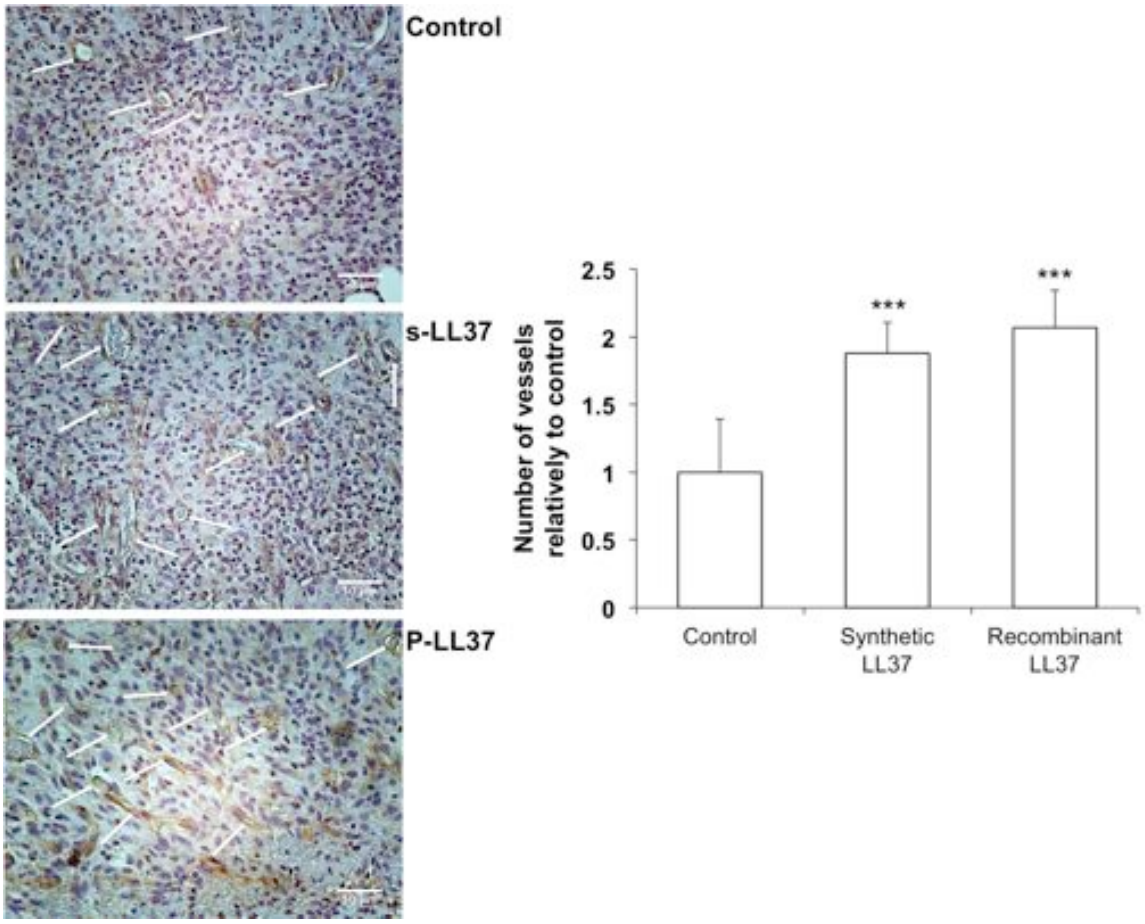

Figure 4.8. Left: representative immunostaining of skin wounds samples treated with vehicle or LL37 at day 7. Arrows indicate the blood vessels. Right: Quantification of blood vessels. Results are means ± SEM of 3 independent experiments and are expressed as percentage of control. $^{***}P \leq 0.001$ versus control.

4.4 DISCUSSION

A variety of endotoxins released by the outer membrane of Gram-negative bacteria, namely the lipopolysaccharide (LPS)-protein complex, play an important role in pathogenesis of many exogenous respiratory diseases. The massive release of LPS can lead in extreme cases to endotoxic shock and therefore, death [21]. Many antibiotics release endotoxin by disrupting the cell wall [22, 23], and patients cured of bacterial infections can suffer from endotoxic shock. The activation of macrophages is started when LPS binds the LPS-binding protein (LBP). In turn, LBP binds the macrophage surface protein CD14, the primary receptor of LPS, and is 100-fold more potent than LPS alone [24]. The antimicrobial activity of the peptide LL37 results from the disruption of the bacteria cell wall; however, LL37 can also neutralize LPS activity by forming high affinity complexes [25, 26].

The TNF-α production was very low in the presence of either the recombinant or the synthetic peptides, indicating that recombinant P-LL37 has affinity for LPS. The recombinant peptide only differs from the synthetic in one amino acid, a neutral proline resulting from the formic acid hydrolysis [16]. Therefore, the global net charge (+5) remains unaltered. Since electrostatic and hydrophobic interactions are the predominant forces driving the binding between antimicrobial peptides and LPS, these results were expected. However, Rosenfeld *et al.* [24] described that a strong binding of AMPs-LPS might not be sufficient to neutralize LPS-induced macrophage activation. In fact, those authors showed that the AMP magainin, although binding LPS with high affinity, did not reduce the production of TNF-α. Some AMPs, including LL37, can compete with LPS on its binding site within the CD14 receptor, modulating the TLR signaling pathway involving NFkB [27]. Therefore, the inhibition of TNF-α production by LL37 is not only caused by the interaction with LPS. Overall, these results demonstrate that the recombinant peptide P-LL37 is biologically active.

The human antimicrobial peptide LL37 was initially recognized for its antimicrobial properties. In fact, it has a broad spectrum of antimicrobial activity against bacteria, fungi, and viral pathogens, as summarized by Durr *et al.* [7]. Several studies reported that AMPs loose their antimicrobial activity in elevated salt concentrations and under serum conditions [28-30]. This inhibition is less pronounced in the case of LL37, probably due to its α-helical structure [31]; however, the peptide becomes inactive against certain

organisms - susceptible under low salt conditions - in media with 100 mM NaCl [32]. Furthermore, LL37 had no killing activity against *Staphylococcus aureus* or *Salmonella typhimurium* in the presence of tissue-culture medium [33]. Therefore, the relevance of antimicrobial activity in vivo is not clear. However, AMPs are imperative in innate immunity, since animals lacking gene expression of these peptides were more susceptible to infections [10]. Besides its antimicrobial properties, many other activities have been reported for the human peptide LL37. As already stated, LL37 is involved in many aspects of innate immunity, like chemotaxis, angiogenesis or wound healing. Although not completely understood, these activities have been associated to some cells receptors like FPRL-1, $P2X_7$ and epidermal growth factor receptor (EGFR) [34-36]. Among these, LL37 only binds directly to FPRL-1 [12], inducing cellular signaling and Ca^{2+} flux [13]. So, we have tested the effects of P-LL37 in proliferation, migration and angiogenesis of endothelial cells to ascertain whether the recombinant peptide binds the receptor FPRL-1. The results confirmed that P-LL37 promotes proliferation, migration and tubule-like structures formation by endothelial cells.

The effect of P-LL37 in the migration of HUVECs and MSCs is significant, with an increase of almost 100% invading cells being observed in both cases. In the experiments with HUVECs, synthetic LL37 promoted a very strong increase in invading capacity, much superior than the one obtained with P-LL37. This result was surprising, since no significant differences were found in the proliferation assays. P-LL37 is obtained by recombinant techniques and the batch used in this experiment could not be as pure as expected, misleading the concentration quantification. A smaller concentration of the recombinant peptide might have been used, which would explain this result. In fact, as illustrated in figure 4.5, the formation of capillary structures was very similar using both peptides. The invasion assay with MSCs was only performed with control and recombinant LL37. Coffelt *et al.* reported that LL37 was able to chemoattract MSCs through the activation of FPRL-1 [37]. Considering all the results obtained, we clearly demonstrate that the recombinant LL37 produced in our laboratory is fully active in vitro. The N-terminus proline, result of the formic acid hydrolysis, does not interfere with the binding of LL37-LPS and to the receptor FPRL-1.

Glucocorticoids affect almost every phase of wound healing because of their inhibitory effect on gene expression in various cells [38]. Beer *et al.* [39] showed that dexamethasone inhibits recruitment of various inflammatory cells and gene expression of

many key wound healing cytokines and growth factors. During the proliferative and remodeling phases, dexamethasone can inhibit the synthesis of several dermal extracellular matrix (ECM) proteins, and delay re-epithelialization and fibroplasia. The healing of a skin wound is a complex process requiring the collaborative efforts of many different tissues and cell lineages and involves the formation of new extracellular matrix, cell infiltration, and tissue remodeling. Inflammation and angiogenesis are two fundamental physiological conditions implicated in this process. In fact, the formation of new blood vessels is a prerequisite of tissue repair and wound healing [40]. As already stated and confirmed in this work, LL37 promotes angiogenesis. Moreover, LL37 appears to have a very important role in wound healing. In fact, Heilborn *et al.* [9] showed that the use of LL37 antibody inhibited re-epithelialization in a concentration-dependent manner in an organ cultured full-thickness ex vivo wound model. Moreover, Carretero *et al.* [41] transferred an adenoviral vector containing LL37 via intradermal injection to excisional wounds in ob/ob mice, improving re-epithelialization and granulation tissue formation. Considering all these results, we decided to study the effects of the topical application of LL37 on wounds. Synthetic and recombinant LL37 strongly accelerated the healing process with a re-epithelialization almost complete after 7 days. Moreover, a significant number of new blood vessels were present in the wounds treated with the peptide.

The importance of LL37 in wound healing has already been presented, but the topical application of LL37 on skin lesions with impaired healing has not been studied so far. In this work, we demonstrate that LL37 can be used topically to support wound healing. The topical application of the peptide allows a better regulation of the process. The applied concentrations can be easily controlled and the administration can be stopped at any moment. Moreover, delivery systems can be used to obtain a regulated administration. The tight regulation of AMPs is very important since elevated amounts may lead to a chronic inflammatory process, as it has been demonstrated for psoriasis and rosacea [42, 43]. In order to better understand the role of LL37 in wound healing, further experiments will be performed, namely with diabetic mice. Diabetic mice have impaired healing and it is well known that the healing treatments of diabetic ulcers did not deliver satisfactory results. In this type of lesions, angiogenesis seems to be the most compromised phase with impairment in VEGF synthesis [44]. Regarding the positive results obtained in this work, we believe that the treatment of diabetic ulcers with LL37 will improve wound healing.

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CHAPTER 5

LL37: ADJUVANT ACTIVITY AND WOUND HEALING IN A DIABETIC MICE MODEL

ABSTRACT

LL37 was initially recognized for its antimicrobial properties. Nevertheless, it has been found to have additional defensive roles such as regulating the inflammatory response, chemo-attracting cells of the adaptive immune system to wound or infection sites, and promoting wound closure. An adjuvant effect for LL37 has also been described.

We have recently developed a new method for the expression and purification of recombinant P-LL37 in *E. coli*. P-LL37 preserves its immunophysiological properties *in vitro* and *in vivo.* In this chapter, we tested the adjuvant activity of CRAMP and P-LL37 after simultaneous administration with *Candida albicans* Sap2. However, both peptides showed no adjuvant effect.

We demonstrated in previous work that LL37 accelerated the wound healing process in dexamethasone-treated mice. Therefore, we decided to study the wound healing effects of topically added LL37 in diabetic mice (db^+/db^+). The effects of LL37 on diabetic mice were weaker than the obtained in our previous work. However, histological analysis after 13 days showed that the wounds treated with LL37 were smaller and presented several new skin annexa.

5.1 INTRODUCTION

There are two major groups of antimicrobial peptides in humans: defensins and cathelidicins. All cathelicidins share a similar structure characterized by a highly conserved N-terminal domain of about 100 amino acid residues. This cathelin-like domain is flanked by a signal peptide (approximately 30 residues long) on its N-terminus, and by an antimicrobial peptide region on its C-terminus [1]. The only member of the cathelicidins family found to date in humans is LL37/hCAP18, an 18 kDa peptide encoded by the gene *CAMP.* The mature antimicrobial peptide has 37 amino acid residues starting with two leucines, hence being named LL37 and is derived by extracellular proteolysis of proteinase 3 from the C-terminal end of hCAP18 [2]. It is a 4.5 kDa, cationic (+6), amphipathic α-helical peptide, with a broad spectrum of antimicrobial activity [3]. The down and upregulation of LL37 have been identified in many diseases. A higher concentration of the peptide is more common in the presence of infections. In fact, in pulmonary infections the levels of LL37 are augmented two or three fold [4]. A low expression of LL37 is associated with skin disorders like atopic dermatitis [5] or chronic ulcers [6]. Nizet *et al.* [7] showed that mice with disrupted *Cnlp*, the gene coding for CRAMP (cathelin-related antimicrobial peptide), showed increased susceptibility to skin infections. These mice also exhibited insufficient and delayed wound closure.

LL37 has been identified as a potent chemoattractant for mast cells [8], monocytes, T lymphocytes and neutrophils [9] through formyl peptide receptor–like 1 (FPRL-1). LL37 also promotes wound healing [6], angiogenesis and arteriogenesis [10]. Kurosaka *et al.* reported that CRAMP could act as an immune adjuvant. The simultaneous administration of OVA with CRAMP to mice promoted both humoral and cellular antigen-specific immune responses [11].

Secreted aspartic proteases (Saps) have been described as virulence factors implicated in the mechanisms of host colonization by *Candida albicans* indifferent types of candidiasis. Saps degrade a number of cellular substrates, including proteins related to immunological and structural defenses, such as IgG heavy chains, α_2-macroglobulin, C3 protein, β-lactoglobulin, lactoperoxidase, collagen and fibronectin [12]. Vilanova *et al.* demonstrated that Sap2 can be successfully used as a vaccination target [13]. In this work, the adjuvant activity of LL37 was analyzed, namely the humoral reaction in mice to LL37-Sap2 mixtures.

Wound healing, or wound repair, is an intricate process in which the skin (or another organ-tissue) repairs itself after injury. Because the skin serves as a protective barrier against the environment, any break sets in motion the wound healing process, characterized by four distinct, but overlapping phases: hemostasis, inflammation, proliferation and remodeling [14, 15]. As the blood components spill into the site of injury, the platelets release clotting factors as well as essential growth factors and cytokines. Following hemostasis, several inflammatory cells migrate to the wound site to fight infection and remove debris as well as releasing more growth factors. Once the wound site is cleaned out, fibroblasts migrate in to begin the proliferative phase and deposit new extracellular matrix. The proliferative phase is characterized by the formation of granulation tissue, epithelialization, and angiogenesis. The new collagen matrix then becomes cross-linked and organized during the final remodeling phase. In order for this efficient and highly controlled repair process to take place, there are numerous cell-signaling events that are required [15].

Diabetic foot ulcers, a leading cause of amputations, affect 15% of people with diabetes mellitus. Diabetes is the prototypical model of impaired wound healing and several factors contribute to wound healing deficiencies [16]. Angiogenesis seems to be the most compromised phase in diabetes [17]. Therefore, the restoration of the angiogenesis process seems fundamental to reduce impaired healing in diabetes.

The other objective of this work was to study the wound healing effects of topically added LL37 in diabetic mice.

5.2 MATERIALS AND METHODS

5.2.1 Animals

C57BL/6 and diabetic mice (db^+/db^+) experiments were conducted according to accepted standards of humane animal care (Declaration of Helsinki, European Community guidelines (86/609/EEC) and Portuguese Act (129/92) for the use of experimental animals).

5.2.2 Reagents

Synthetic LL37 (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES) and CRAMP (GLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPEQ) were purchased from Bachem, Switzerland. Recombinant P-LL37 (PLLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPR-TES) was expressed and purified as described previously [18]. Briefly, the DNA encoding the fusion protein LK-CBM3-LL37 was successfully cloned in pET21a and expressed in *E. coli* BL21 DE3 (Novagen) in Luria-Broth (LB) medium. The fusion protein was purified on cellulose CF11 and formic acid was applied to cleave LL37 from the fusion carrier (LK-CBM3). The resulting recombinant P-LL37 was finally purified by reverse-phase HPLC. Sap2 was purified from *C. albicans* as described by Vilanova *et al.* [13]. *C. albicans* was grown in Winge medium for 48 h at 37ºC in an orbital incubator. Culture supernatant proteins were concentrated by ultrafiltration with a 10 000-MW cut-off membrane in a VivaFlow system (Vivascience, Hanover, Germany), dialysed and separated by ion-exchange chromatrography on a DEAE-cellulose (DE52; Whatman, Maid-stone, Kent, UK) column. Fractions enriched in Sap2 were concentrated by vacuum dialysis using a 14 000-MW cut-off membrane (Sigma, St Louis, MO). Mannoside constituents were removed from this fraction by affinity chromatography in a concanavalin A-sepharose column (Amersham Pharmacia, Uppsala, Sweden). Sap2 was finally purified by a Pepstatin A affinity-chromatography column (Sigma).

5.2.3 Immunizations

Two assays have been carried out. In the first one, eight-week-old C57BL/6 mice (purchased from Charles River, Barcelona, Spain) were injected intradermically (i.d.) twice, with a 3-week intervening period, with 100 µl of the following preparations: 5 µg of native Sap2 in PBS; 5 µg of Sap2 in a 1:1 PBS/alum suspension ($Al(OH)_3$ gel; Sigma-Aldrich); 5 µg of Sap2 plus 80 µg (20 nmol) of CRAMP in the first injection and 5 µg of Sap2 plus 60 µg (15 nmol) of CRAMP in the second injection.

In the second experiment, mice were injected intraperitoneally (i.p) twice, with a 3-week intervening period, with 100 µl of the following preparations: 5 µg of Sap2 in PBS; 5 µg of Sap2 in a 1:1 PBS/alum suspension ($Al(OH)_3$ gel; Sigma-Aldrich); 5 µg of Sap2 plus 150 µg (38 nmol) of P-LL37 in the first injection and 5 µg of Sap2 plus 100 µg (25 nmol) of P-LL37 in the second injection.

Alum was used as a positive control adjuvant. On days 21 and 42 (21 days after the second injection), blood samples were collected from each mouse. Serum was separated from each blood sample and used for measurement of the anti-Sap2 immunoglobulin.

5.2.4 Antibody detection

The quantitation of total Sap2-specific Immnunoglobulin G (IgG) in mouse serum was performed by enzyme-linked immunosorbent assay (ELISA) as previously described by Vilanova *et al.* [13]. Briefly, polystyrene microtitre plates (Nunc, Roskilde, Denmark) were coated with 5 µg/ml of Sap2 and incubated at 37ºC for 1 hour. Wells were then saturated for 1 hour at room temperature with 1% BSA in Tris-saline Tween 20 (TST) buffer, pH 8.0. Serial dilutions of the serum samples were then plated and incubated for 2 hours at room temperature. After washing, alkaline phosphatase-coupled monoclonal goat anti-mouse IgG (Southern Biotechnology Associates, Birmingham, AL) was added and incubation continued for 30 min at room temperature. After washing, the bound antibodies were detected by development with substrate solution containing p-nitrophenyl phosphate (Sigma) and the reaction was stopped by the addition of 0.1 M EDTA, pH 8.0. The absorbance was measured at 405 nm. The levels of anti-Sap2 IgG are illustrated as the reciprocal logarithmic value of the highest dilution that gave an absorbance ≥0.1 above the control value.

5.2.5 Wound healing assays

Diabetic female mice (db^+/db^+) (purchased at Charles River, Barcelona, Spain) with 8-10 weeks old were used in this study. After general anesthesia, hair on the dorsal side of the animals was shaved and the skin was cleaned with 70% ethanol. A 5 mm skin biopsy punch was used to create full thickness cutaneous wounds under aseptic conditions. Two wounds were created on the dorsal surface, one on each side of the midline. Synthetic LL37 and sterile water were applied topically twice a day (2×10 µg in 10 µl). Wounds were left open and not covered by any type of dressing. Mice were examined daily for wound-healing progression. After 6 and 13 days, the animals were euthanized and wound tissue was collected for histological studies. Skin wound tissue specimens were fixed in 10% neutral-buffered formalin solution and paraffin-embedded. Histological analyses were performed in 5-µm tissue sections. The 5 µm-thick sections were mounted

on glass slides, dewaxed, rehydrated with distilled water, and stained with hematoxylin and eosin according to routine procedures for light microscopy. As part of the histological evaluation, all slides were examined by a pathologist without knowledge of the previous treatment.

5.3 RESULTS AND DISCUSSION

5.3.1 Adjuvant activity of CRAMP and P-LL37

Kurosoka *et al.* reported a novel activity for CRAMP, the cathelin-related AMP in mice. This is the only work that describes the adjuvant activity of this AMP. Compared with immunization with ovalbumin (OVA) alone, the immunization with OVA plus CRAMP markedly increased the production of OVA-specific serum IgG antibody (Ab). CRAMP enhanced the anti-OVA IgG Ab response in a dose-dependent manner, with higher titers of serum anti-OVA IgG Ab induced by 40 than 10 nmol of CRAMP [11]. The authors also reported that CRAMP augmented both Th1- and Th2-type antigen-specific immune responses (helper T cell responses**)**. In fact, there was no obvious selective enhancement of either IgG1 or IgG2a and the splenocytes from mice immunized with OVA plus CRAMP at 40 nmol produced both Interleukin-4 (IL-4) and interferon-gamma (IFN-γ) in response to OVA stimulation. The production of the IgG2a subclass of Ab and IFN-γ is associated with Th1-type cytokine production while IgG1 Abs and IL-4 are associated with Th2-type cells. These responses should be fully functioning but none should be dominant. In fact, in the case of autoimmune diseases and severe allergies, one response has gained control of the system.

To better understand and characterize the adjuvant activity of CRAMP and LL37, immunizations studies were performed on mice using the *C. albicans* Sap2 as antigen. Vilanova et *al.* showed that Sap2 incorporated in alum adjuvant provided efficient immune protection against candidiasis [13]. The anti-Sap2 IgG concentrations were significantly higher in mice immunized with Sap2 than in the controls.

In this work, we first tested the adjuvant activity of CRAMP after i.d. injection. Then, we tested the adjuvant effect of i.p. injected P-LL37. CRAMP is similar to LL37 in structure, tissue distribution and antimicrobial activity. In fact, both peptides have been used in mice

models with similar results Alum was used as positive control. The results are illustrated in figure 5.1.

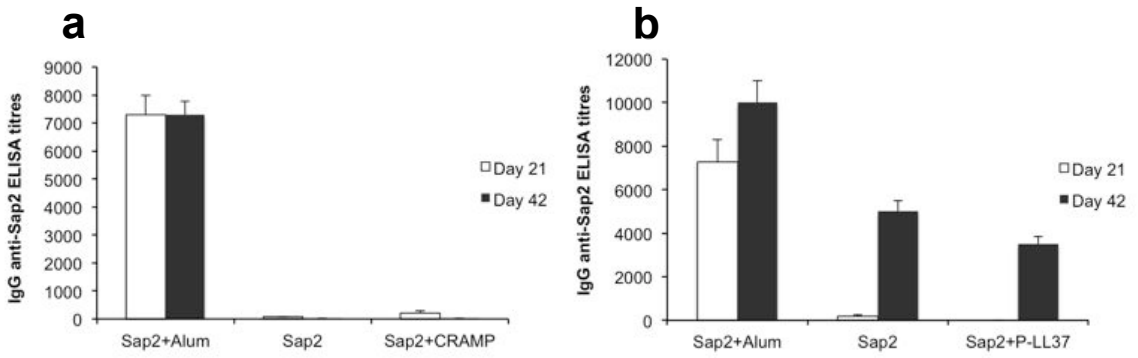

Figure 5.1. Serum IgG enzyme-linked immunosorbent assay (ELISA) titres of anti-Sap2 IgG in sera collected 21 days after the first immunization or when the mice were killed, 21 days after the second immunization (day 42). a: first experiment with CRAMP (i.d injections); b: second experiment with P-LL37 (i.p injections).

Figure 5.1 shows that CRAMP and P-LL37 had no adjuvant activity. The AMPs did not enhance the anti-sap2 IgG Abs when comparing with Sap2 alone or with Sap2 plus the positive control Alum. The use of different administrations routes (i.d. and i.p. injections) as well as different peptide concentrations, did not deliver better results. In the first experiment 80 µg (20 nmol) of CRAMP were injected intradermally in the first day and 60 µg (15 nmol) after 21 days. A very slight increase of anti-sap2 IgG Abs was seen after 21 days, however these were quite below the quantities obtained with Alum. A new experiment was performed, using higher concentrations of P-LL37: 150 µg (38 nmol) were injected intraperitoneally in day one and 100 µg (25 nmol) were injected on day 21. Once again, no adjuvant activity was detected.

LL37 and CRAMP can chemoattract mast cells [8], monocytes, T lymphocytes and neutrophils [9]. Moreover, LL37 is a potent modifier of dendritic cells differentiation [19]. Kurosaka *et al.* suggested in their work that these properties contribute to the enhancement of antigen-specific immune responses [11]. OVA and Sap2 are both negatively charged at physiological pH (pI=4.7 and pI=4.25 respectively); so, the electrostatic interactions of CRAMP and LL37 with OVA or Sap2 should be very similar. Therefore, the *in vivo* biological activities like chemotaxis or DCs differentiation that may contribute to the adjuvant activity should also be similar. Further experiments using OVA

and other antigens should be performed to clarify the actual adjuvant activity of LL37 and to better understand the associated mechanisms of action.

5.3.2 Wound healing experiments on diabetic mice

We reported in chapter 4 that the topical application of LL37 accelerated the wound healing process in dexamethasone-treated mice. The experiments showed that LL37 greatly increased the vascularization of the wounds. As already stated, diabetes is the prototypical model of impaired wound healing and angiogenesis seems to be the most compromised phase [17]. In fact, therapeutic interventions aiming at restoring the impaired VEFG production have succeeded in ameliorating the wound healing process in experimental model of diabetic mouse [20-23]. Considering these reports and our previous positive results we tested the effect of topically added LL37 in diabetic mice (db^+/db^+). Two applications of 10 µg in 10 µl each were applied daily. Two wounds were created on the dorsal surface of the animals. LL37 was applied on the left wound and sterile water on the right one. After 6 days the first group of 4 mice was euthanized. The treatment was continued for the second group until day 13 and the last 4 mice were then euthanized. Figure 5.2 illustrates the wounds after 6 and 13 days. After 6 days the wounds were still wide open but after 13 days the healing process was almost complete (the wounds were barely visible and are pointed with arrows). The macroscopic examination of the wounds shows no significant differences between the treatment with LL37 and sterile water.

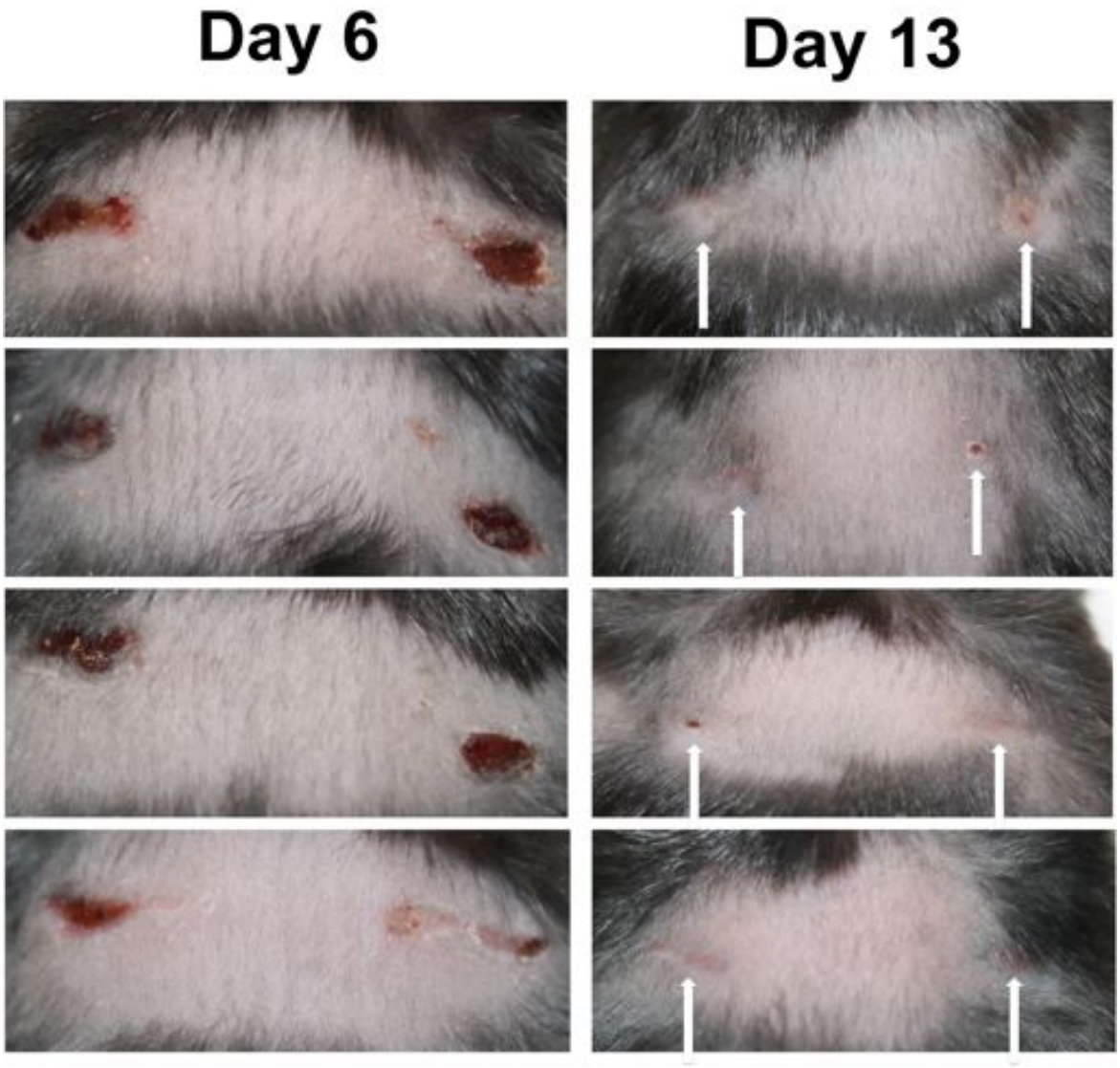

Figure 5.2. Macroscopic examination of the wounds at day 6 and day 13. LL37 was applied on the left wounds and sterile water on the right. After 13 days the wounds are almost healed (pointed by white arrows).

We decided to apply both solutions on each animal to avoid misleading conclusions and to better compare and control the evolution of the wounds. Indeed, some animals regenerate wounds faster than others. For example, the fourth mouse euthanized after 6 days was healing faster than the other three (figure 5.2). If this animal were only treated with LL37, the conclusion would have been incorrect.

The wound tissue was collected for histological studies. The wound sections stained with hematoxylin and eosin are shown in figure 5.3. After 6 days, no significant differences can be identified comparing the treated and control wounds. Both wounds in each animal present very large scabs and the healing process is still at the beginning. In fact, the keratinocytes layer is still very incomplete. The large scabs difficulted the processing of the skin wound sections. At the end of the process, the 5-µm tissue sections were very damaged.

After 13 days the healing process is almost terminated. The epidermis is complete and a small number of granulocytes are still present, indicating that the inflammatory phase is finished. However, there are now visible differences between the wounds treated with LL37 and the ones treated with control. The control wounds are still much larger than the ones treated with LL37. The micrographs shown (figure 5.3) were taken from the same animal with a 4X magnification. The picture that shows the control wound treated after 13 days is a merge of two photographs. This way, the entire wound is displayed. Comparatively, the wound treated with LL37 is much smaller. The limits of the wounds are pointed with black arrows. Moreover, the wounds treated with LL37 present what appears to be new skin annexa. These annexa, pointed with white arrows, could originate new hair follicles. Similar results were consistently obtained in the 4 animals. We cannot conclude whether these annexa will really generate new hair. Experiments should be performed over a longer time frame (e.g. 20 days or a month) to ascertain whether this hypothesis proves to be correct.

The healing process of dexamethasone-treated mice (chapter 4) was faster with an almost complete re-epithelialization after 7 days. The hematoxylin and eosin stained micrographs taken at day 6 using the diabetic mice were rather damaged, not enabling a proper comparison of the wounds vascularization and the effect of LL37. Differences can be seen after 13 days but LL37 did not accelerate the healing process very significantly, as judged from the macroscopic/external inspection.

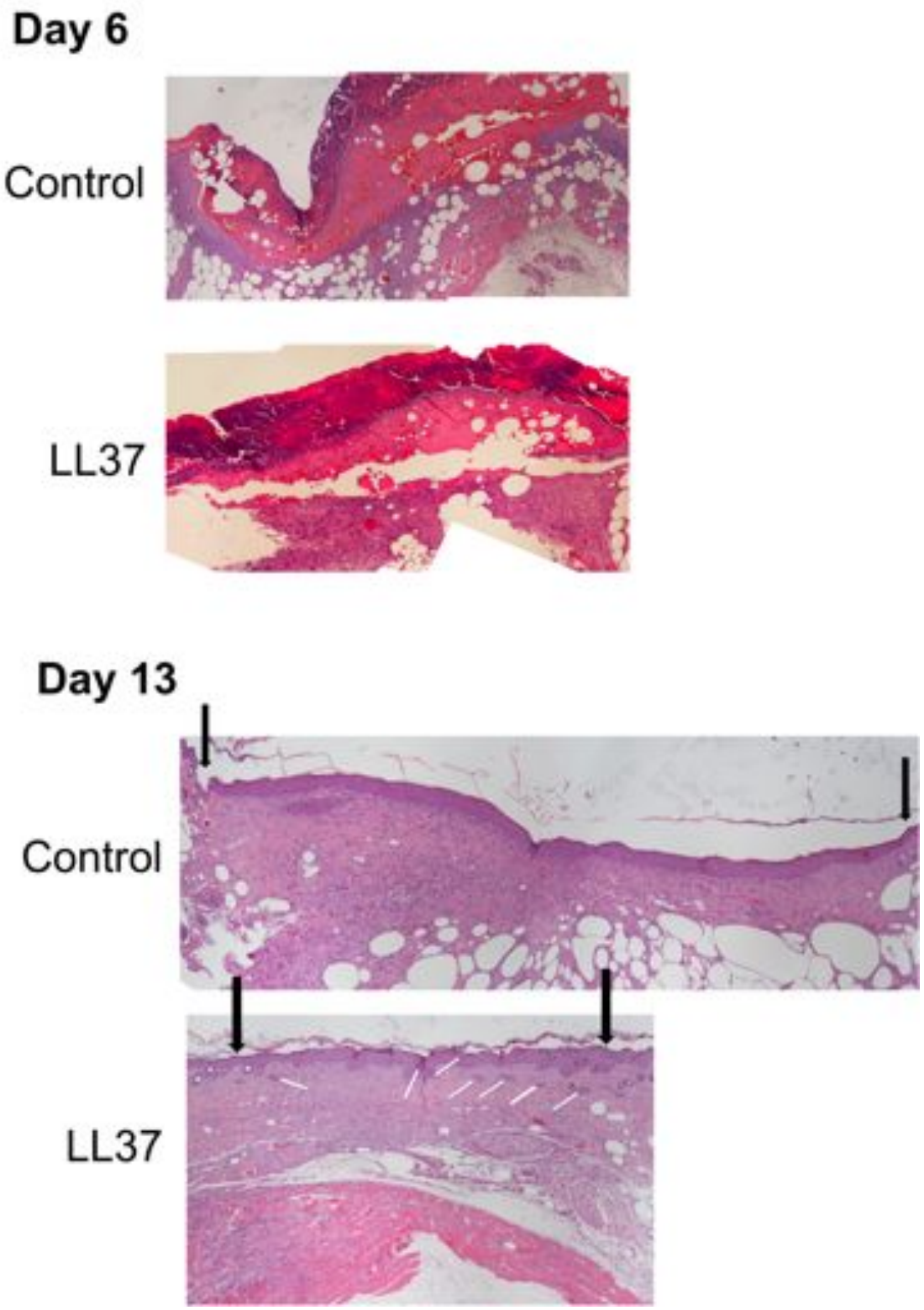

Figure 5.3. Hematoxylin and eosin stained micrographs of wound tissue sections treated with control, or synthetic LL37 after 6 and 13 days. After 13 days, the wound treated with LL37 is much smaller and presents several skin annexa (pointed with white arrows). The limits of the wounds are pointed with black arrows. The wound treated with control after 13 days is a merge of two micrographs.

5.4 CONCLUSIONS

The application of LL37 in the wounds of diabetic mice did not deliver an effect as strong as the one obtained on dexamethasone-treated mice. However, the histological analysis of wound sections after 13 days revealed encouraging results. In fact, the wounds treated with LL37 were smaller and presented many more skin annexa.

Wound healing is a complex process that involves many different cells and growth factors. To better understand the effect of LL37, further experiments need to be performed and a better characterization of the process is required. The expression and quantification of angiogenic factors associated to wound healing, such as VEGF, bFGF and angiopoietins, as well as the effect of LL37 on the expression of proteins in the wounds must be evaluated. Also, it is important to assess whether LL37 induces systemic angiogenic and inflammatory responses.

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CHAPTER 6

FINAL CONCLUSIONS AND PERSPECTIVES

Two antimicrobial peptides, magainin-2 and LL37, were fused to the family 3 CBM from *C. thermocellum* for recombinant expression in *E. coli*, aiming the affordable production and purification using cellulose as affinity matrix. Both AMPs were cloned at either the N- or C-terminus of the CBM3. The N-terminal AMP MAG2-D was sucessfully expressed, cleaved and purified from the fusion partner LK-CBM3 but showed no antibacterial activity against *E. coli* K12. The expression of C-terminal MAG2 from the construction LK-CBM3-MAG2 was not successful. The peptide may have suffered proteolysis during the recombinant expression. The human AMP LL37 was sucessfully cloned, cleaved and purified from the N- or C-terminus of the fusion partner LK-CBM3. However, only the recombinant P-LL37 obtained from the C-terminally fused protein (LK-CBM3-LL37) showed antibacterial activity against *E. coli* K12, with a MIC of 180 µg/ml. Regarding these results we can conclude that the negatively charged aspartic acid, resulting from the formic acid cleavage, is the cause for the non-activity of MAG2-D and M-LL37-D.

The expression of AMPs in *E. coli* is challenging because they are toxic to the host and sensitive to proteolytic degradation. In this work, different results were obtained. MAG2 was overexpressed in *E. coli* BL21 but suffered proteolysis in *E. coli* M15. LL37 was successfully expressed in *E. coli* BL21 but only in the insoluble form. The insoluble expression of LL37 can protect the host from the toxicity of the AMP but would also protect LL37 from proteolysis.

Overall, the methodology used in this work for the recombinant expression of AMPs compares advantageously to other protocols previously described, having fewer purification steps and using cellulose for purification. In fact, it allowed the successful expression and purification of MAG2-D, M-LL37-D and P-LL37. In future work, the expression of MAG2 at the C-terminus of LK-CBM3 will be attempted in different *E. coli* strains to avoid proteolysis and to finally achieve the production of the AMP with antimicrobial activity.

The cathelicidin derived human peptide LL37 has a broad spectrum of antimicrobial and immunomodulatory activities. The recombinant peptide P-LL37 with a N-terminus proline, produced using our new method, preserves its immunophysiological properties *in vitro* and *in vivo*. P-LL37 neutralized the activation of macrophages by lipopolysaccharide (LPS). Besides, the peptide induced proliferation, migration and tubule-like structures formation by endothelial cells.

The adjuvant activity of LL37 was also assessed using Sap2 as antigen. However, nor LL37 nor synthetic CRAMP showed adjuvant activity. The AMPs did not enhance the anti-sap2 IgG Abs when comparing with Sap2 alone or with Sap2 plus the positive control Alum. In future work, similar experiments will be performed using OVA and other peptides as antigen, to conclude whether LL37 has indeed adjuvant activity or whether this is specific to some antigens.

The results obtained in this work using LL37 in wound healing experiments may have therapeutic relevance. We showed that the topical application of recombinant or synthetic LL37 in dexamethasone-impaired wound healing mice increased vascularization and re-epithelialization, accelerating the wound healing process. This is the first work that describes the successful improvement of wound healing through the topical administration of LL37. These experiments simulate what might be a therapeutic application under a clinical setting. Similar experiments were performed in impaired healing diabetic mice. The topical application of LL37 on diabetic mice wounds did not accelerate the healing process very significantly. However, histological analysis after 13 days showed encouraging results. In fact, they revealed that the wounds treated with LL37 were smaller and presented several new skin annexa that could originate new hair. Further experiments need to be performed and a better characterization of the process is required. A more comprehensive characterization of the the role played by LL37, using different models of tissue regeneration, is thus required to estimate the potential of this peptide for for therapeutic applications. The expression and quantification of angiogenic factors associated to wound healing, as well as the effect of LL37 on the expression of proteins in the wounds must be evaluated. Also, it will be important to assess whether LL37 induces systemic angiogenic and inflammatory responses.

The major results of this thesis are:

- We developed a new, fast and cost-effective method for the production of AMPs using the CBM3 as fusion partner;
- The recombinant peptide P-LL37 maintains all its biological activity *in vitro* and *in vivo.* We demonstrated that LL37 strongly accelerated wound healing in dexam*ethasone*-trea*ted mice.* Encouraging results were obtained with diabetic mice.

Suggestions for future work include the following main topics:

- To test different constructions to achieve the expression of recombinant magainin-2;
- To further improve the productivity of LL37 using the *E. coli* expression system and the CBM3 as a fusion partner;
- To comprehensively estimate the therapeutic potential of LL37 by testing the effect of the application of the peptide in different systems of wound and tissue regeneration;
- To develop a delivery system for the topic controlled release of LL37 for wound regeneration.