Cathelicidin regulates homeostasis of innate immune responses

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Abstract

The human antimicrobial peptide cathelicidin acts as effector molecule of the innate immune system with direct antimicrobial and immunomodulatory functions. The aim of this study was to test whether the cathelicidin LL-37 modulates the response of neutrophils to microbial stimulation. Furthermore we wanted to investigate whether the presence of cathelicidin reduces pulmonary emphysema and enhances of pulmonary epithelial repair after acute lung injury induced by naphthalene.

Human neutrophils were stimulated with LPS, *Staphylococcus aureus* and *Pseudomonas aeruginosa* following incubation with LL-37. Cytokine release was measured by ELISA. Reactive Oxygen Species (ROS) production of neutrophils was determined by luminometric and a flow cytometric methods. Peritoneal mouse neutrophils isolated from CRAMP deficient and wildtype animals were treated with LPS and TNF-α was measured in the supernatant by ELISA. Antimicrobial activity of neutrophils was detected by incubating neutrophils isolated from CRAMP knockout and wildtype mice with bacteria. Pulmonary emphysema was induced in mice by intratracheal instillation of elastase and induction of emphysema was evaluated depending on morphological parameter like mean linear intercept (Lm).

To test whether cathelicidin enhances lung tissue repair, a selective injury was induced to mouse nonciliated bronchiolar epithelial cells (clara) with naphthalene. The repair of clara cells were determined by immunohistochemical staining for CC10 protein. Incubation with LL-37 significantly decreased the release of proinflammatory cytokines from human neutrophils stimulated with TLR ligands or whole bacteria. LL-37 induced the production of ROS and the increased engulfment of bacteria into neutrophils. Neutrophils from CRAMP deficient mice released significantly more TNF-α after LPS stimulation and showed decreased antimicrobial activity as compared to cells from wildtype animals.

Absence of cathelicidin in CRAMP deficient mice decreases significantly the repair of airway epithelium and increases the induction of pulmonary emphysema-induced by application of elastase. In conclusion, LL-37 modulates the response of various innate immune mechanisms involved in tissue homeostasis and inflammation. Cathelicidin controls the release of inflammatory mediators while increasing neutrophils antimicrobial activity.

Zusammenfassung

Das menschliche antimikrobielle Peptid Cathelicidin fungiert als Effektor-Molekül des angeborenen Immunsystems mit direkten antimikrobiellen und immunmodulatorischen Funktionen. LL-37 ist das einzige Cathelicidin des Menschen. Ziel dieser Studie war es zu prüfen, ob Cathelicidin die Antwort neutrophiler Granulozyten auf mikrobielle Stimulation moduliert. Wir untersuchten außerdem, ob bei akutem Lungenversagen, dass durch Naphthalin induziert wurde, die Anwesenheit von Cathelicidin das Auftreten eines Lungenemphysems reduziert und die Regenerationsfähigkeit pulmonaler Epithelzellen erhöht. Neutrophile Granulozyten wurden mit LPS, *Staphylococcus aureus* und *Pseudomonas aeruginosa* nach Inkubation mit LL-37 stimuliert. Die Zytokin-Produktion wurde per ELISA gemessen. Die Produktion reaktiver Sauerstoffspezies (ROS) von Neutrophilen wurde über Luminometrie und ein Flowzytometrsche Methoden bestimmt. Neutrophile wurden aus dem Peritoneum von CRAMP defizienten und Wildtyp-Mäusen isoliert und mit LPS stimuliert. TNF-α wurde per ELISA im Überstand gemessen. Die Inkubation mit LL-37 führt zu einer deutlich verringerten Freisetzung von proinflammatorischen Zytokinen durch humane Neutrophile, die mit TLR-Liganden oder ganzen Bakterien angeregt wurden. Ein Lungenemphysem wurde in Mäusen durch intratracheae Installation von Elastase induziert und die Induktion des Emphysem über morphologische Parameter wie den mittleren linear Intercept (Lm) analysiert. Um zu testen, ob Cathelicidin die Regenerationsfähigkeit von Lungengewebe erhöht, wurde eine selektive Schädigung muriner bronchiolärer Clara-Zellen durch Naphthalin induziert. Die Regenerationsfähigkeit von Clara-Zellen wurden durch Bestimmung der Zellzahl immunhistochemische Färbungen für CC10-Protein bestimmt. LL-37 induziert die Produktion von ROS und die zunehmende Phagozytose von Bakterien in Neutrophile. Neutrophile aus CRAMP-defizienten Mäusen gegeben deutlich mehr TNF-α nach LPS-Stimulation frei und weisen eine verringerte antimikrobielle Aktivität im Vergleich zu Neutrophilen aus Wildtyp-Tiere auf. Das Fehlen von Cathelicidin in CRAMP defizienten Mäusen führt zu signifikant verringerter Regenerationsfähigkeit von Epithelzellen der Atemwege und begünstigt die Entstehung eines durch Elastase induzierten Lungenemphysems. Zusammenfassend ist festzustellen, dass LL-37 die Reaktion verschiedener Mechanismen des angeborenen Immunsystems moduliert, die an der Gewebshomöostase und der Entstehung von Entzündungen beteiligt sind. Cathelicidin steuert die Freisetzung von Entzündungsmediatoren bei gleichzeitiger Erhöhung der antimikrobiellen Aktivität neutrophiler Granulozyten antimikrobielle Aktivität.

Contents

1 Introduction

1.1 General features of innate immunity

The human immune system differentiates between self and non-self, and this ability protects the body from the invasion of different pathogens and helps to eliminate altered cells. The immune system has been classified into two different branches titled adaptive and innate immunity (Figure 1.1.1).

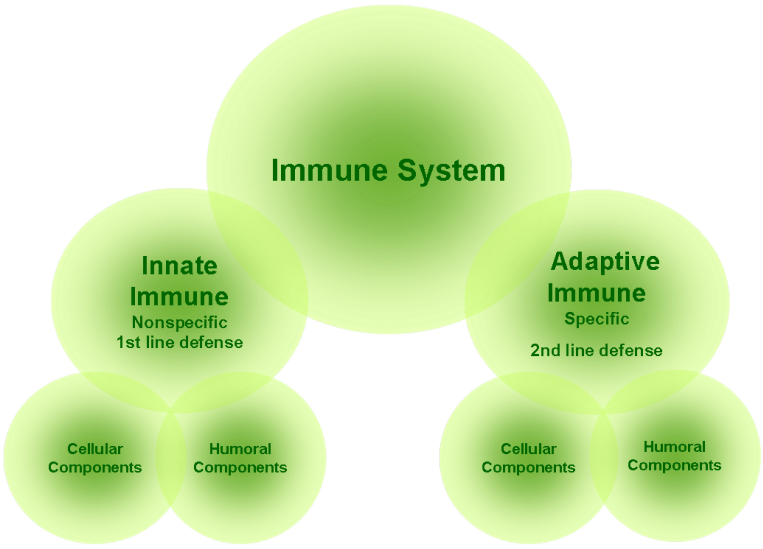


Figure 1.1.1: **Classical classification of human immune system.** Human immune system was classified depending on its specificity responses into innate and adaptive immune. Each of these two different branches has own humoral and cellular components. Innate immune exists in the human body from birth and is found in variable plants and animals. Innate immune is considered the first line defense responses to pathogens.

The first line of defense which is also called nonspecific defense is provided by different natural factors such as skin, bactericidal gland secretions and the mucociliary elevator of the mucosal surfaces (Figure 1.1.2) . This defense is essential and protects the body against dangers regardless of their nature. However, this type of defense mechanism is not sufficient, when the microbe escapes from it, therefore a second line of defense is needed to immediately recognize the pathogen after it crosses the barriers. This recognition system must also have the ability to discriminate pathogens from self. This kind of defense response can be called innate immunity because it exists in the body from birth and is based on discrimination of "nonself" from "self", that is a classical property of immune system [103]. Adaptive immunity distinguishes itself from the innate immunity by being acquired, based on individual experiences, induced by a pathogen, antigen-specific and long lasting. Adaptive immunity contains highly specialized cells (lymphocytes) able to recog-

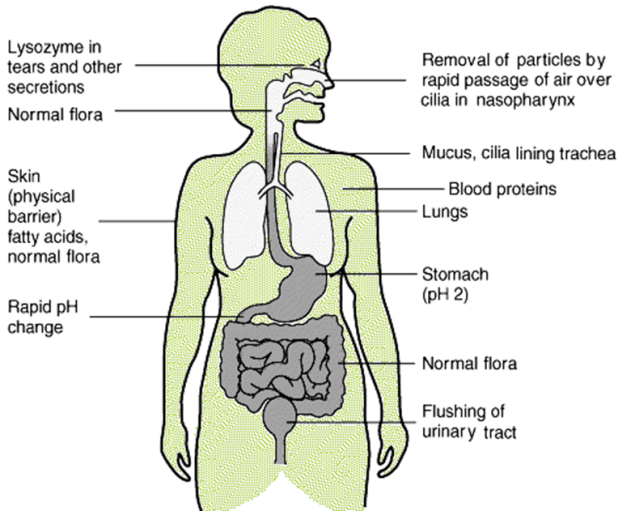


Figure 1.1.2: **Nonspecific barriers as a first line of defense.** Several natural barriers protect human body from infection. These barriers are classified by their nature into mechanical, chemical and biological barriers. Skin and membranes for example are from mechanical barriers in another hand stomach PH and antimicrobial peptides secreted in tears, saliva, semen, respiratory tract and breast milk are considered as chemical factors. And commensal flora serve as biological barriers.

nize the antigen, generate antibodies and activate cytotoxic cells, and induce the long-lasting immunological memory.

In addition, the adaptive immune system needs several days to initiate antigen-dependent processes (Figure 1.1.3). In contrast, the innate immunity has the ability to react immediately when microbes are present, and provide signals necessary of the development of the adaptive immune response to antigens. It is now widely accepted that antigen-specific immune responses may be achieved only if costimulatory molecules and cytokines are provided together with antigen, and these molecules are induced when the pathogen is first recognized by the cells of innate immune system [77].

The mechanism generated following the recognition of dangerous signals, results in the rapid mobilization of an inflammatory cascade. The components of innate immunity can be divided to:

- Soluble molecules and membrane receptors
  - Cytokines
  - Complement proteins
  - Antimicrobial peptides
- Cells (Table 1.1.1).

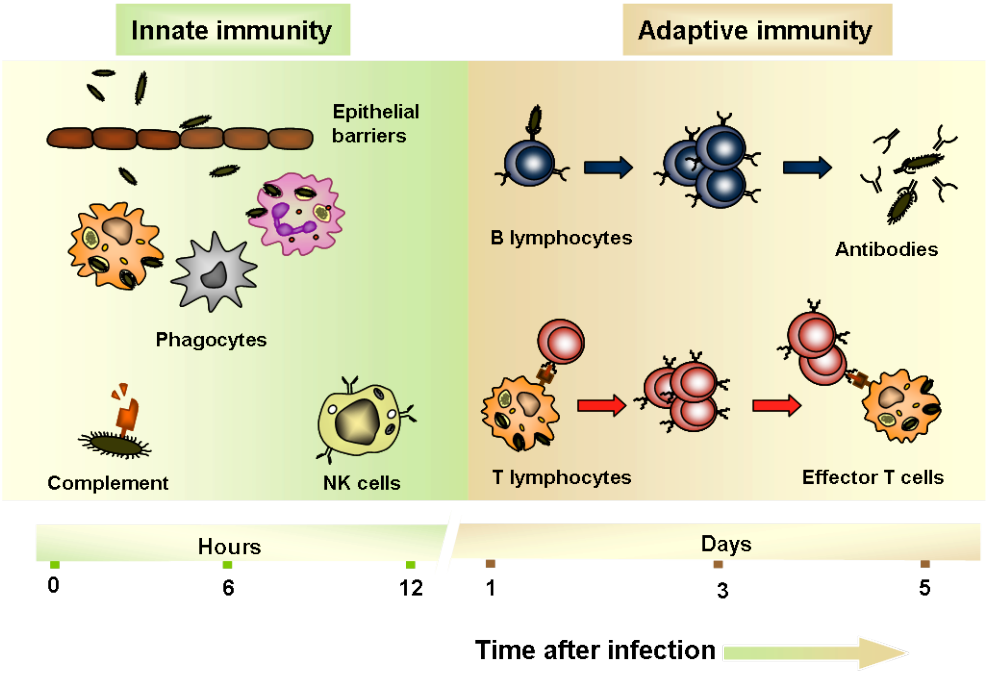


Figure 1.1.3: **General features of innate and adaptive immunity.** Although there are many differences between innate and adaptive immunity, they show high cooperation. Innate immunity reacts immediately and earlier than adaptive immunity in response to pathogens and this nonspecific responses gives time to adaptive immunity to prepare for specific responses. Innate immunity takes hours to eliminate pathogens but adaptive immunity takes days. Innate immunity is antigen- independent in opposite of adaptive immunity (adapted from cellular and molecular immunology 5 e. Abbas & Lichtman, 2005).

- Neutrophils
- Macrophages
- Dendritic Cells
- Natural killer (NK) Cells

Cell type	Neutrophil 	Macrophage 	Dendritic cell 	Natural killer cell 
	Phagocytosis	Phagocytosis	Antigen presentation	Lysis of viral-infected cells
	Reactive oxygen and nitrogen species	Inflammatory mediators	Costimulator signals	Interferon
	Antimicrobial peptides	Antigen presentation	cytokines	Macrophages activation
	Cytokines	Cytokines	interferon	
		Reactive oxygen and nitrogen species	Reactive oxygen species	
		Complement proteins		

Table 1.1.1: Cellular components of innate immunity. Adapted from Kuby immunology, sixth edition 2007.

1.2 Neutrophils

Phagocytes were discovered by Ilya Ilyich Mechinikov [102] and since then it was believed that neutrophils are short lived cells with no programmed instructions [22]. Two decades ago this whole idea was changed when it was discovered that neutrophils orchestrate complex arrays of adhesion-, chemoattractant-, and cytokine-driven signals and are able to synthesize cytokine in response to inflammatory stimuli [170].

Neutrophils are the first cells recruited and thus are an essential component of the acute inflammatory response. Neutrophils are found circulating in the blood stream, but they migrate out of the vasculature in response to inflammation or bacterial attack; neutrophil migration is initiated as a result of process called chemotaxis. Pus, which is hallmark of acute inflammation contains mainly neutrophils accounting for its whitish/yellowish appearance.

Neutrophils are the most abundant white blood cells in humans accounting for 70%. The average half-life of a non-activated neutrophil in the circulation is about 4-10 hours. Upon activation, neutrophils marginate, and undergo selectin dependent capture followed by integrin dependent adhesion in most cases, after which they migrate into tissues, where they survive for 1-2 days.

Neutrophils are phagocytes, internalizing and destroying microbes by the forma-

tion of a phagosome into which reactive oxygen species ROS and hydrolytic enzymes are secreted.

Neutrophils can also release an assortment of proteins in three types of granules by a process called degranulation as well as extrude neutrophil extracellular traps (NETs), a web of fibers composed of chromatin and serine proteases that trap and kill microbes extracellularly. It is suggested that NETs provide a high local concentration of antimicrobial components to bind, disarm, and kill microbes independent of phagocytic uptake [24].

1.2.1 Granule Biogenesis and Granule Proteins

Neutrophil-derived microbicidal molecules are packed in granules that are released upon cell activation [90]. Granule biogenesis follows the granulocyte differentiation pathway [21]. The azurophilic granules first emerge at the stage of promyelocytes and contain myeloperoxidase, serine proteases, and antibiotic proteins [58].

Later in differentiation, at the metamyelocyte stage, specific granules containing lactoferrin and collagenase emerge, followed by the tertiary granule population containing gelatinase, and a fourth type of granule, called the secretory vesicles, appears at the stage of mature neutrophil [21].

Neutrophils use an array of antimicrobial peptides and proteins to destroy invading microorganisms [90]. The azurophilic granules contain the majority of the antimicrobial proteins that are released into the phagolysosome.

One of the most active participants in host defense against Gram-negative bacterial infections is bactericidal/permeability increasing protein (BPI), a 50 kDa protein stored in azurophil granules but also expressed at the plasma membrane of neutrophils [49].

Another important group of antimicrobial peptides is the group of beta-sheet defensins that comprises four members: human neutrophils peptides HNP1 to HNP4 [65]. Specific compounds also have these antimicrobial molecules which destined for extracellular release, among them is hCAP-18 one of the cathelicidins.In neutrophils, they are stored in specific granules in an inactive form [149].

Neutrophil-derived proteases are packed in azurophil granules and have the ability to degrade the majority of extracellular matrix components; as a result, they play fundamental roles in physiological processes [123]. Serine proteases are a large family of enzymes characterized by their active site, the so-called “catalytic triad” composed of histidine, aspartic acid, and serine.

The group of the neutral serine protease homologs stored in the azurophilic granules of the neutrophil includes cathepsin G, elastase, proteinase 3, and the enzymatically inactive azurocidin or CAP-37 [109], which are cationic glycoproteins of similar size (25–29 kD).

Among purified neutrophil-derived proteases, cathepsin G [140], PR3, and azurocidin [30] appear to have significant in vitro antimicrobial potential independent of their enzymatic action.

Their antimicrobial activity is widely distributed because they are active against Gram-positive, -negative bacteria, and fungi. Although in vitro studies did not reveal antimicrobial activity of elastase, knock-out elastase mice show an impaired host defense against Gram-negative bacterial sepsis [18].

Neutrophils contain metalloproteinases such as collagenase (MMP-8), which specifically cleaves type I collagen, and (MMP-9), which degrades native type V collagen. In addition, neutrophil metalloproteinases have been involved in the modulation of neutrophil functions such as the shedding of Fcγ receptor [105].

Further regulation of the activity of metalloproteinases in the extracellular milieu is achieved by specific inhibitors tissue inhibitors of metalloproteases have been characterized (TIMP1, TIMP2 and TIMP3) [110] interacting with the activated enzymes.

1.2.2 Oxidative molecules of neutrophils

The antimicrobial efficiency of human neutrophils depends on two coincident events occurring in the nascent phagolysosome of stimulated neutrophils: the generation of ROS by assembly and activation of the NADPH-dependent oxidase and the release of enzymatic or antimicrobial protein content in the granules.

These responses are triggered by numerous agonists promoting adhesion or by phagocytic targets (Figure 1.2.1).

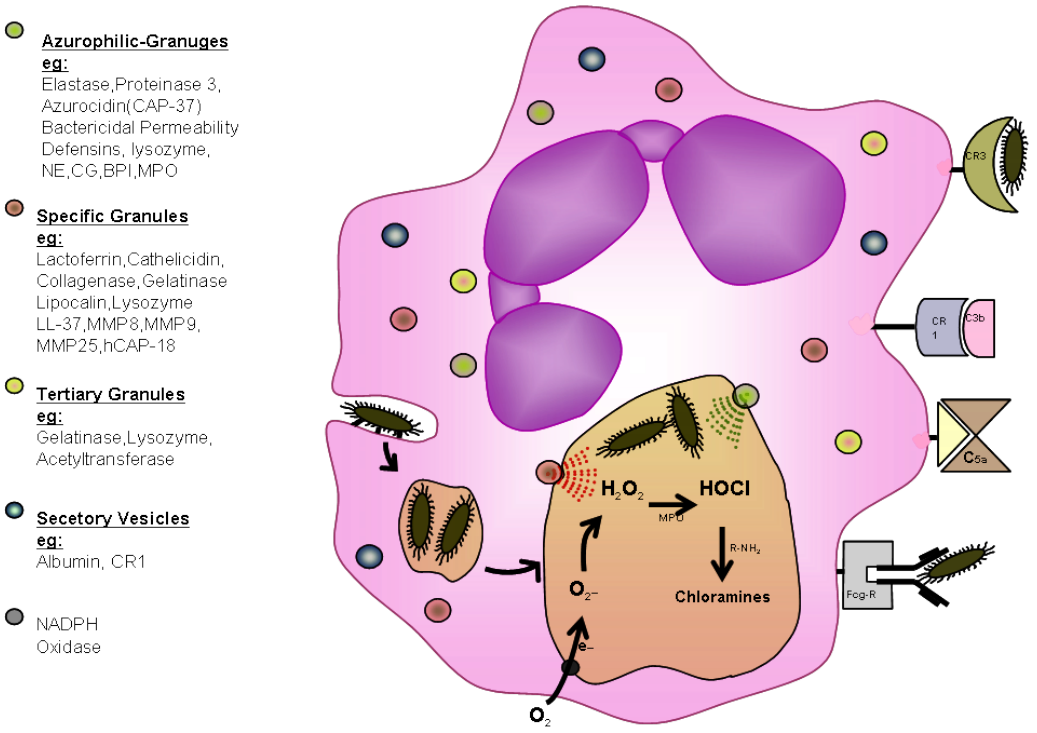


Figure 1.2.1: **Neutrophil effector systems are mobilized following phagocytosis.** Complement opsonins C3b and C4b are recognized by CR1 and CR3. IgG opsonins are recognized via the immunoglobulin receptors (FcgR). The first microbicidal pathway is the oxidative reponse, which consists of the production of radical oxygen species following NADPH-oxidase complex activation, including superoxide anion (O2-), hydrogen peroxide (H2O2), and, via myeloperoxidase, hypochlorous acid (HOCl) and chloramines. The second microbicidal pathway is non-oxygen–dependent and consists of the release in the phagolysosome or in the extracellular medium of preformed proteins stored in granules. Serine proteases, antibiotic proteins, as well as myeloperoxidase are contained in azurophilic granules. Metalloproteinases (collagenase and gelatinase) and antimicrobial proteins (lactoferrin and cathelicidin) are contained in specific granules. Gelatinase is also contained in tertiary granules, also called gelatinase granules. (Adapted from Witko-Sarsat and Descamps-Latscha, 1994.)

The activation of the oxidative metabolism, known as the respiratory burst, first involves NADPH oxidase, an enzymatic complex composed of cytosolic (p40phox, p47phox, and p67phox) and membrane proteins (p22phox and gp91phox), which constitute a heterodimeric flavohemoprotein known as cytochrome b558 [7].

Two low-molecular weight guanine nucleotide-binding proteins are involved: Rac2, which is located in the cytoplasm in a dimeric complex with RhoGDI (Guanine nucleotide Dissociation Inhibitor), and Rap1A, which is located in membranes.

Upon activation of neutrophils, p47phox becomes phosphorylated and cytosolic components migrate to the plasma membrane where they associate with cytochrome b558 to assemble the active oxidase. This enzymatic complex is thus able to generate superoxide anion (O_2^-), which can dismutate into H_2O_2 [115].

There are three intermediates in the reduction of O_2 to H_2O_2, namely O_2^-, H_2O_2, and the hydroxyl radical ($OH^\bullet$), which are formed by successive one electron additions. Despite numerous studies, the formation of $OH^\bullet$ in phagocytes is still controversial [25]. The formation of singlet oxygen appears to be an important event in the microbicidal potential of neutrophils.

The generation of superoxide anion via the activation of NADPH oxidase is the starting material for the production of a vast assortment of reactive oxidants, including halogenated oxidants generated through the myeloperoxidase (MPO) pathway (Figure1.2.2) [84]. MPO is a heme protein present in azurophil granules of neutrophils and monocytes, which is released upon cell activation into the phagolysosome or into the extracellular space.

MPO amplifies the toxic potential of H_2O_2 by producing reactive intermediates. At plasma concentrations of chloride ion, the major product of MPO is hypochlorous acid (HOCl). This potent oxidant chlorinates electron-rich substrates and oxidatively bleaches heme proteins and nucleotides[135].

MPO has a wide range of substrates leading to a wide variety of products. Amino acids, especially taurine, can be chlorinated to yield chloramines, the so-called long-lived oxidants. Interestingly, MPO can utilize nitrite and hydrogen peroxide as substrate to catalyze tyrosine nitration in proteins [135].

MPO-derived oxidants have also been implicated in other processes unrelated to host defense, including carcinogenesis [94], atherosclerosis, and chronic renal failure[169].

NO-synthases are unique among eukaryotic enzymes in being dimeric, calmodulin-dependent or calmodulin-containing cytochrome P450-like hemoproteins that combine reductase and oxygenase catalytic domains in one monomer.

Reactive nitrogen intermediates include nitric oxide (NO), which can react with oxygen to form much stronger oxidants such as nitrogen dioxide (NO_2). The direct toxicity of NO is modest, but is greatly enhanced by reacting with superoxide to form peroxynitrite ($ONOO^-$) (Figure1.2.2) [17].

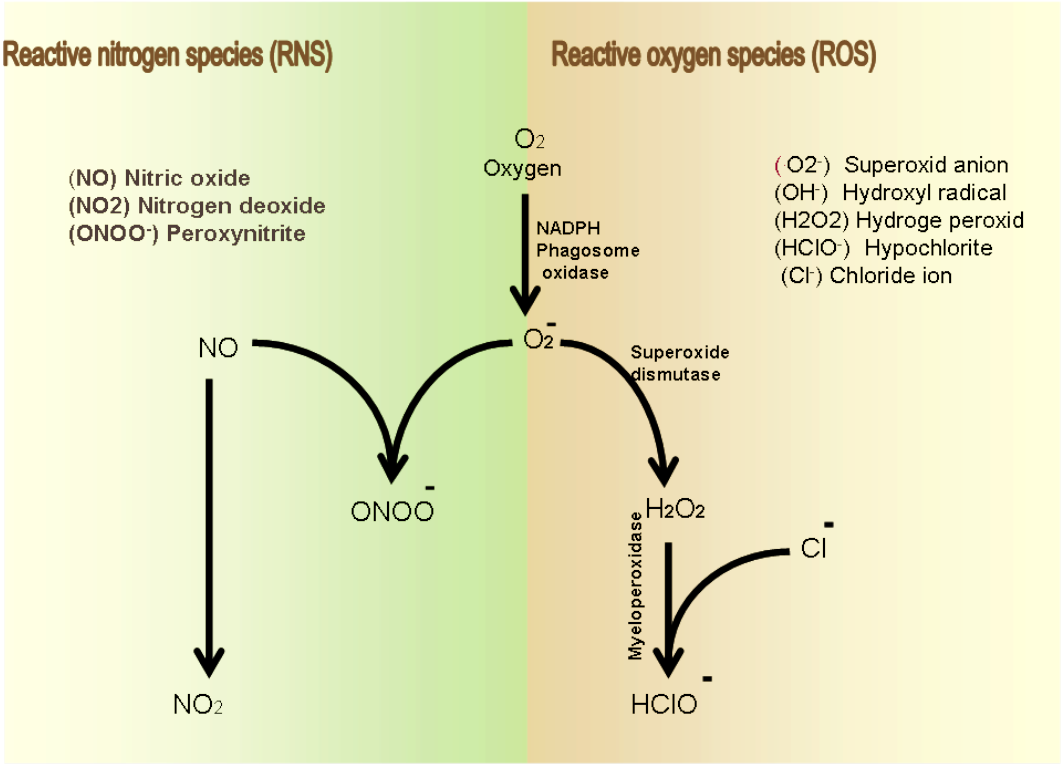


Figure 1.2.2: **The generation of RNS and ROS via the activation of NADPH oxidase.**

1.2.3 Neutrophil functions

1.2.3.1. Signaling, transepithelial migration and diapedesis toward inflammatory stimuli

Neutrophils display multiple structurally related receptors for chemoattractants that can trigger adhesion and direct cell migration and promote degranulation and oxidative responses. These G-protein-coupled seven transmembrane glycoproteins, also called "serpentines," include receptors for complement C5a, formylpeptides, PAF, leukotriene B4 [175], and receptors for C-X-C [131]. Ligation of chemoattractants to such receptors activates phospholipases via heterodimeric G proteins, resulting in intracellular Ca^{2+} release, Ca^{2+} channel opening and activation of conventional protein kinase C isoforms [131].

Tyrosine kinases and the GTP-binding protein Ras are also activated. Ras activation triggers the MAPK/ERK cascade, which appears to be involved in various chemo attractant-induced neutrophil functions [88]. Activation of small GTP-binding proteins of the Ras, Rac, and Rho families regulate actin-dependent processes such as membrane ruffling, formation of filopodia and stress fibers, mediating cell adhesion and motility [19].

The chemoattractant receptors, via their coupled G-protein heterodimers, activate PI3-Kinase, which is involved in the pathways leading to degranulation and NADPH-oxidase activation [156]. In many inflammatory diseases (gastrointestinal, respiratory, urinary) neutrophils finally transmigrate across a polarized epithelium to accumulate within a lumen [125].

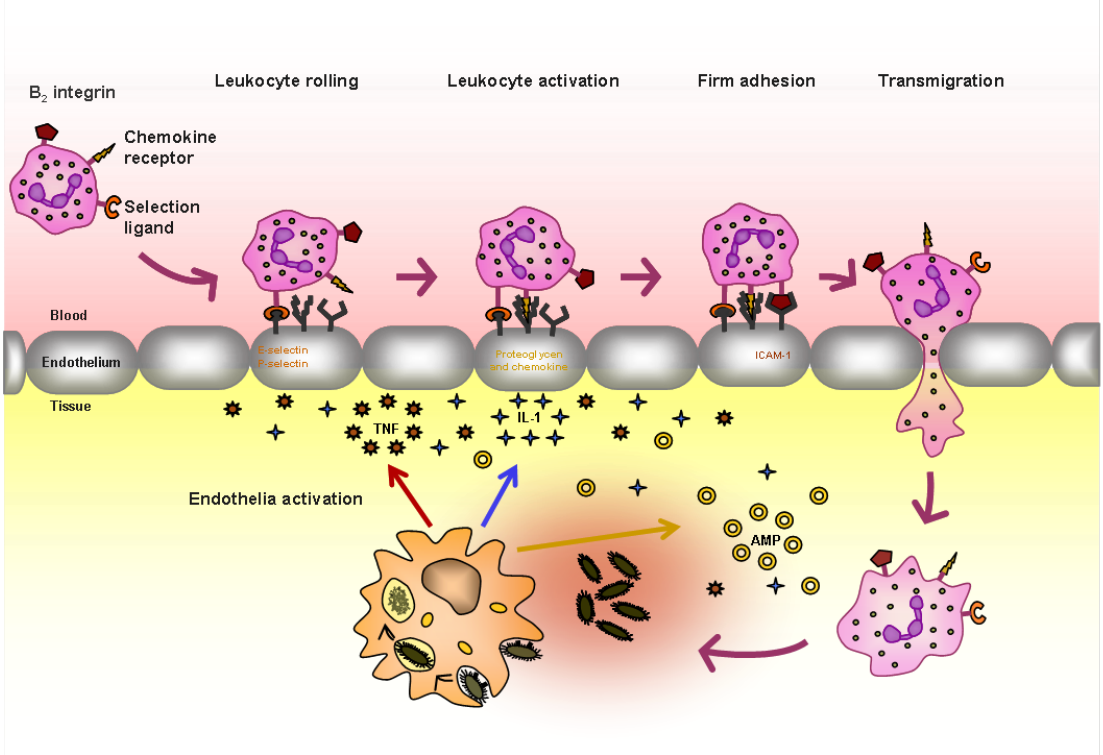


Figure 1.2.3: **Neutrophils adhesion and transmigration to site of inflammation.** Innate immune defence of neutrophils, in response to local infection or injury, neutrophils attach to the activated endothelium via a series of interactions among adhesion molecules and their corresponding receptors. Attachment in combination with locally secreted chemokines direct neutrophil migration to the site of infection or inflammation, where they become activated and execute a cascade of defence mechanisms to protect the host against infections. Finally, neutrophils commit apoptosis, resulting in phagocytosis by macrophages and subsequent resolution of inflammation. Abbreviations: CAM-1, cellular adhesion molecule1; AMP, antimicrobial peptide.

Neutrophil transepithelial migration is mediated by b2 integrin CD11b/CD18 interaction with unknown epithelial ligand(s) distinct from ICAM-1, which might include members of the proteoglycan family [125].

The rolling step is mediated by neutrophil L-selectin and by E- and P-selectins

newly expressed on inflamed endothelial cells, (Figure 1.2.3), P-selectin, readily mobilized in a few minutes to the endothelial cell surface following stimulation by thrombin, histamine, or oxygen radicals, interacts primarily with a mucin-like ligand PSGL-1 (P-selectin glycoprotein ligand-1), located at the tip of leucocyte microvilli [107].

During the initial rolling on endothelial cells, integrin "activation" signals are given by chemoattractants displayed on the endothelial membrane and presumably also by the engagement of selectins and their counter-receptors (Figure 1.2.3).

Neutrophils integrate these signals of integrin engagement and those delivered simultaneously by inflammatory cytokines or chemoattractants to activate a cascade of intracellular events resulting in cell spreading, locomotion, degranulation, and oxidative burst. These outside-in transduction pathways include the activation of various tyrosine kinases [96].

Integrins behave as promiscuous transducers mediating signals triggered by these GPI-linked receptors [126]. FcγRIIIb interaction with CD11bCD18 promotes antibody dependent phagocytosis [159], while CD14 interaction with CD11bCD18 only occurs in the presence of LPS and LPS-binding protein and may play a role in the generation of proinflammatory mediators [182].

Neutrophils migrate in tissues by haptotaxis. In particular, signals delivered by "end target-derived" chemoattractants-such as formyl peptides, released by bacteria or by mitochondria from dying cells, or complement C5a, produced in their immediate surrounding-are dominant and override "regulatory cell-derived" attractants, such as bioactive peptides (LTB4) or chemokines (IL-8) (Figure 1.2.4) [59].

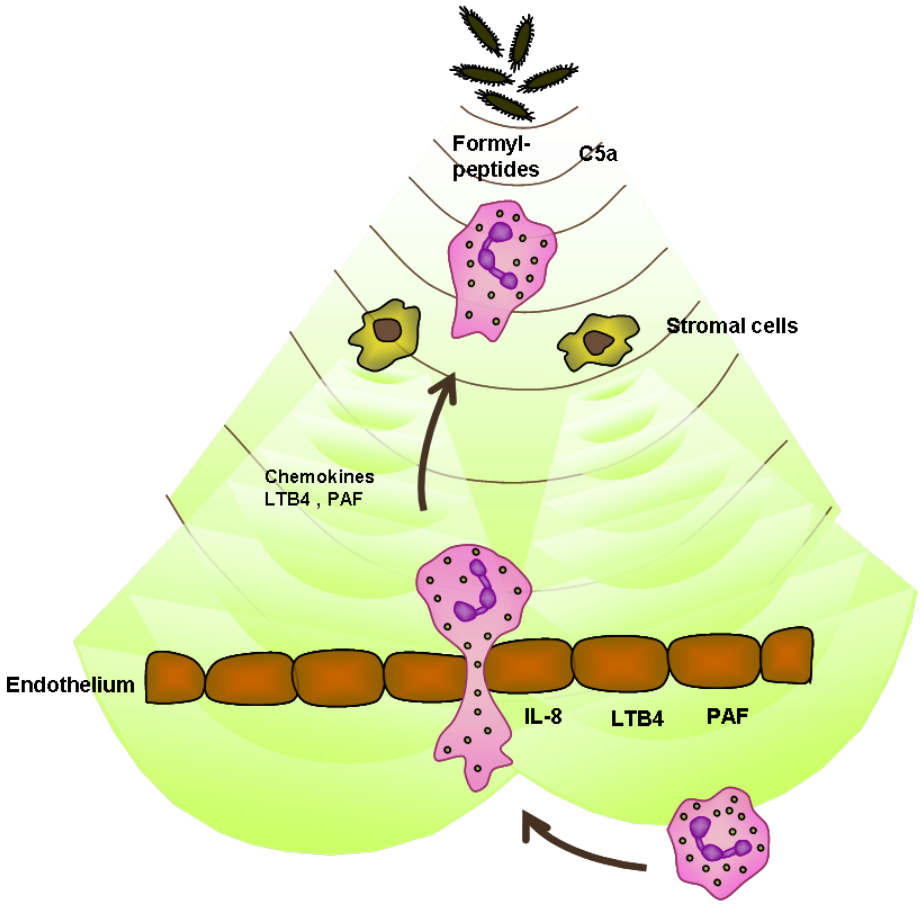


Figure 1.2.4: **The chemotactic migration of neutrophils towards an inflammation site.** Neutrophils move through the endothelium and within tissues by responding to successive combinations of chemoattractant gradients. Chemoattractants are released by endothelial cells, by activated stromal cells (macrophages, epithelial cells. . .), and by the inflammatory targets, ie, bacteria or dying cells. The direction of neutrophil movement is first guided by the steepest local chemoattractant gradient and is then regulated by successive receptor desensitization and attraction by secondary distant agonists. Finally, end-target attractants are dominant over regulatory cell-derived agonists (adapted from Foxman et al, 1999).

1.2.3.2. Phagocytosis, degranulation, and bacterial killing

Neutrophil phagocytosis involves two different receptor classes (Figure1.2.1), Fcγ receptors-FcγRIIA (CD32), and FcγRIIIB (CD16) - as well as complement receptors CR1 (CD35) and CR3 (or CD11b/CD18 integrin). Among these, the functional phagocytic receptors are FcγRII and CR3, while CR1 and FcγRIIIB appear mostly as co-receptors facilitating the function of the former receptors. Signaling pathways triggered by these two classes of receptors are different, as are the phagocytic processes themselves.

The ingestion of IgG-coated targets is promoted by the aggregation of FcγRII receptors and the phosphorylation of their cytoplasmic ITAMS (immunoreceptor

tyrosine-based activation motifs) via the activation of Src-tyrosine kinases [40]. Phosphorylated ITAMS indeed serve as docking sites for SH2 domains of Syk tyrosine kinase, which triggers various pathways involving the activation of PI3-kinase and of Rho proteins [101].

RhoA appears to be involved in the early F-actin recruitment and phagocytic cup formation, but may not be absolutely required for FcR-mediated phagocytosis [32]. PI3-kinase is indeed involved in the myosin-induced "purse-string-like" contraction of pseudopods that closes phagosomes [153]. CDC42 would regulate the extension of membrane over the particle edges, and Rac1, together with PI3-kinase, would allow membrane fusion and the final closure of the phagocytic cup [101].

Phagocytosis of C3bi-opsonized targets by complement receptor 3 (CR3) involves a different process: complement-opsonized targets sink into the cell, which produces little protrusions. CR3-mediated phagocytosis has been shown, in macrophages, to involve Rho but neither Rac nor Cdc42 [32]. Adhesion of neutrophil CR1 and CR3 to particles exclusively coated with C3b/iC3b is not sufficient to promote phagocytosis, unless neutrophils are activated by PMA or by formyl-peptides and a contact with fibronectin or laminin [171].

These stimuli result in the phosphorylation of CR1 and trigger the "inside-out" signaling that activates CR3 binding capacity. Cooperativity between Fcγ- and complement-receptors occurs when C3b/iC3b-bearing targets are also opsonized by antibodies or display glycosylated CR3 ligands [48].

Cross-talks between phagocytic receptors are suggested by the observation that neutrophils from CR3-deficient (CD18-deficient) patients display an impaired antibody dependent phagocytosis [42]. And that FcγRIIIB interacts in cis with CR3, via a lectin carbohydrate interaction [159]. Complex signaling pathways promoted by the engulfment of opsonized targets lead to the fusion of protease-rich granules with the phagosome and the triggering of the oxydative burst.

1.2.3.3. Cytokine synthesis

Human neutrophils are both a target and a source of various proinflammatory cytokines, chemokines, and growth factors. Neutrophils are targets of proinflammatory cytokines (IL-1 and TNF-α), chemokines (IL-8) and growth factors (granulocyte colony stimulating factor G-CSF and granulocyte monocyte colony stimulating factor GMCSF). Indeed, these cytokines have been shown to amplify several functions of neutrophils, including their capacity to adhere to endothelial cells and to produce ROS.

Neutrophils were previously considered to be devoid of transcriptional activity and capable of performing no or little protein synthesis. However, convincing molecular evidence has now shown that neutrophils can synthesize and release a wide range of cytokines and growth factors either constitutively or in an inducible manner (Table 1.2.1). Though neutrophils produce many types of cytokines, this production remains much lower than that produced by monocytes [33]. The production of cytokines is also largely influenced by the stimulating agents and among these, cytokines and bacterial endotoxins (LPS) are the most potent inducers.

TNF-α was originally described as a product of activated monocytes and macrophages displaying tumoricidal activity. It is a highly pleiotropic cytokine belonging to the superfamily of membrane-anchored and soluble cytokines that are notably involved in T cell-mediated immunity. Although it inhibits the growth of tumor cells, it has an enhancing effect on the proliferation of certain normal cells and has a great variety of nontumoral target cells, such as other white blood cells.

Cytokines that are Expressed by Neutrophils In Vitro	
TNF-α	Vascular endothelial growth factor (VEGF)
IL-1-α, IL-1-β	Hepatocyte growth factor (HGF)
IL-12	Macrophage-CSF (M-CSF), IL-3, GRO-β
IL-1 receptor antagonist (IL-1Ra)	IL-18 (IFN-γ inducible factor)
IL-8	TGF-α
Growth-related gene product-α (GRO-α)	Oncostatin (OSM) and neurotrophins
Macrophage infiltrating protein-1α (MIP-1α), MIP-1β	**Secretion still debated:**
Cytokine-induced chemoattractants (CINC)	IL-6, monocyte chemotactic protein-1 (MCP-1),
Interferon-α (IFN-α), IFN-β	granulocyte-macrophage CSF (GM-CSF), stem cell
Granulocyte colony-stimulating factor (G-CSF)	factor (SCF), and IFN-γ
Fas ligand (FasL), CD30 ligand (CD30L)	
Cytokines that are not Expressed by Neutrophils In Vitro	
IL-10	MCP-2
IL-13	MCP-3

Table 1.2.1: **Cytokine Expression by Neutrophils In Vitro.** Adapted from Cassatella, 1999

TNF-α is involved in septic shock, cachexia, autoimmunity, and inflammatory diseases. Its potent proinflammatory effects mainly result from its capacity to increase expression of endothelial cell adhesion molecules and subsequently promote neutrophil adherence to vascular endothelium. Finally, TNF-α is also a priming agent for neutrophils that notably increases their phagocytosis, degranulation, and oxidative responses. However, activated neutrophils have been shown to have the capacity to express TNF-α mRNA [93].

Using GM-CSF as a stimulus, no secretion of the related TNF protein was de-

tected. Soon after, the dual observation of expression of TNF-α mRNA and protein secretion was reported with LPS as a stimulating agent. This observation was substantiated by other reports showing that Candida albicans also induces a potent extracellular release of TNF-α [99].

Neutrophils express the mRNA of both IL-1α and IL-1β and release the related IL-1 proteins. The stimuli listed in (Table 1.2.2) are in general all capable of inducing IL-1 production by neutrophils. Some of these cytokine stimuli such as IL-1β and TNF-α appear to induce IL-1 production by neutrophils. Interestingly, anti-neutrophil cytoplasm autoantibodies (ANCA) have been shown to induce IL-1β mRNA expression in neutrophils.

Agents able to trigger cytokine production by neutrophils	
Cytokines and growth factors	ANCA
TNF-α, IL-1α, IL-1β	Matrix protein (fibronectin, laminin)
IL-4, IL-13, IL-10	Bacteria and related products
GM-CSF	LPS
TGF-β	*Staphylococcus aureus*
Chemoattractants	*Yersinia enterocolitica*
fMLP	*Listeria monocytogenes*
Surface molecule	*Fungi and related products*
Anti-CD32 (FcgRII) and anti-CD16 (FcgRIII) antibodies	*Candida*
Particulate agents	*Saccharomyces cerevisiae*
Calcium microcrystals	*Zymosan*
Urate microcrystals	*Protozoa*
Other agents	*Plasmodium falciparum*
Calcium ionophores	*Viruses*
PMA, Concanavalin A	*Epstein-Barr virus*

Table 1.2.2: **Agents induces cytokines production from neutrophils.** Adapted from Cassatella, 1999

IL-1 is usually released in concert with TNF-α and exerts similar effects on neutrophils (see above). Two classes of IL-1 receptors (IL-1RI and IL-1RII) expressed on a wide variety of cells have been described. IL-1Ra is a 23- to 25-kDa protein made by the same cells as those that produce IL-1. It exerts its inhibitory action on IL-1 by binding to IL-1 receptors without triggering any signal transduction or biological activity.

The expression of IL-1Ra mRNA in LPS-treated neutrophils appears to be greater than that of LPS-treated monocytes and an almost 100-fold excess of IL-1Ra over IL-1 is usually produced by activated neutrophils. It has thus become evident that neutrophil-derived IL-1Ra could contribute to modulate the IL-1 induced inflammatory and immune responses.

Chemokines are usually classified as C-X-C or C-C chemokines on the basis of the position of the first two cysteine residues, and IL-8 is a prototype of the C-X-C family. IL-8 was first described as a potent neutrophil chemoattractant and activator [8] and is expressed in response to LPS. IL-8 is secreted by a variety of cells including T lymphocytes, epithelial cells, keratinocytes, fibroblasts, endothelial cells, and neutrophils. Interestingly, IL-8 is the most abundantly secreted cytokine by neutrophils, and neutrophils are the primary cellular target of IL-8 [62].

Concerning other cytokine producing cells, cytokine expression by neutrophils can easily be modulated by the T-cell– derived regulatory cytokines, i.e., positively by Th1 type cytokines such as IFN-γ, and negatively by Th2 type cytokines such as IL-10, IL-4, and IL-13, Given the pathophysiological importance of such regulatory pathways [34].

1.2.3.4. Apoptosis and the resolution of acute inflammation

Most acute inflammatory responses resolve spontaneously due to endogenous "stop programs" that switch off inflammation and limit destruction of host tissues. These include the elimination of infectious agents by phagocytosis, the progressive decrease of leucocyte recruitment promoted by endogenous "braking signals," and finally, the apoptosis and clearance of leukocytes [92].

Neutrophil apoptosis and subsequent ingestion by macrophages is the major mechanism for clearing neutrophils that have been recruited to the inflamed sites and thus for resolving inflammation. The constitutive apoptosis of senescent neutrophils involves proteolytic cascades-caspases, calpains, and the proteasome-that activate kinases, e.g., caspase 3-mediated activation of protein kinase C-d [130].

Inflammatory mediators, such as LPS or GM-CSF, delay the apoptosis of neutrophils by increasing mitochondrial stability and reducing caspase 3 activity [168], and by down-regulating the gene expression of Bax, a pro-apoptotic member of the Bcl-2 family [46].

Macrophages can trigger neutrophil apoptosis by expressing cell surface Fas ligand (FasL) and releasing soluble FasL that reacts with the Fas "death receptor" on neutrophils. Ingestion of opsonized particles or of apoptotic neutrophils indeed promotes the release of soluble FasL by macrophages and the killing of bystander neutrophils [28]. This may represent a negative feedback loop accelerating the resolution of inflammation by eliminating recruited leukocytes by apoptosis. This active suppression of inflammatory mediator production is presumably an important step in the resolution of inflammation.

1.2.4 Neutrophils in diseases

Tissue damage after acute bacterial infection is partly due to excessive neutrophil infiltration and activation in the infected tissue [155]. Two inherited defects affect neutrophil granule structure [98]. The first is the specific granule deficiency, which is a rare congenital disorder marked by frequent and severe bacterial infections. In this disorder, neutrophils are characterized by a lack of specific granules and defensins, abnormalities in neutrophil migration, and impaired bactericidal activity. The second inherited granule deficiency is the Chediak-Higashi syndrome (CHS), which is a rare autosomal recessive disorder associated with an immune deficiency leading to increased susceptibility to infection and a life-threatening lymphoma-like syndrome. In CHS a lack of natural killer cell function and a neutropenia may be found, with a prominent defect in formation of neutrophil granules [113].

Cystic fibrosis is a hereditary disorder caused by mutations of the cystic fibrosis transmembrane conductance regulator (CFTR), the product of which is a membrane protein thought to function as a chloride channel. The lethal clinical manifestations are clearly related to the thick, infected mucous and chronic neutrophil-dominated airway inflammation [47]. Neutrophils are considered responsible for the early onset and the promotion of the inflammatory process in CF, which starts within the first year of a CF patient's life [6]. Regarding neutrophil functions, myeloperoxidase dependent oxygenation activities appear to be significantly higher not only in CF homozygotes, but also in heterozygote parents of CF patients [132].

1.3 Antimicrobial peptides (AMPs)

Innate immune system does not only recognize pathogens, but also inactivates them using antimicrobial peptides (AMPs) and proteins. The natural ability of normal tissues to possess antibacterial activity has been revealed in 1922 by Fleming, who has isolated the first tissue-derived bacteriolytic substance (now known as lysozyme) [57]. Nowadays there about 900 different AMPs and proteins have been described [27].

Antimicrobial proteins are present at relatively high concentrations in host defense cells of myeloid origin, especially neutrophils, but also in epithelial cells (ECs) and tissue secretions. Some of them (like lysozyme, secretory phospholipase A2 or cathepsin G) are enzymes that lyse various microbial components. For example, lysozyme, which is produced by phagocytes and ECs, degrades bacterial PGNs by cleaving the glycosidic bond of N-acetyl glucosamine [56].

Other antimicrobial proteins use nonenzymatic strategies. For example lactoferrin, a highly abundant component of milk and mucosal secretions, binds to iron, an essential survival factor for many microbes, and also possesses bactericidal activity at the N-terminus [63].

Another example is the bactericidal/permeability-increasing protein (BPI) selectively exerts multiple activities against gram-negative bacteria: cationic N-terminal peptide causes direct cytotoxicity and neutralizes LPS, while C-terminal domain binds to phagocytes and, therefore, may act as an opsonin [50].

AMPs are endogenous polypeptides of fewer than 100 amino acids. They have antimicrobial activity at physiological concentrations under conditions prevailing in the tissues of origin or elevating there during pathologic conditions [64]. Unlike the commonly called antibiotics, which are in most cases synthesized by special metabolic pathways, the amino acid sequence of AMPs is naturally encoded in the genetic material of the host organism [27].

There are many different groups of AMPs, however the most active group is the cationic amphipathic peptides, which are able to accumulate and interact with and subsequently damage negatively charged microbial membranes [183].

In addition, some AMPs can alter bacterial metabolic pathways, reduce cell-wall, nucleic acid, and protein synthesis, and inhibit enzymatic activity [27]. In humans and other mammals, defensins and cathelicidins constitute the two main AMP families [174]. Neutrophils and epithelial cells (ECs) are the major sources of mammalian AMPs [183].

1.3.1 Defensins

Defensins are cysteine-rich, cationic peptides with β-sheet structures that are stabilized by three intramolecular disulphide bonds between the cysteine residues. Mammalian defensins are classified into three subfamilies, α-, β- and ϑ-defensins, which differ in their distribution of and disulphide links [64]. Human α-defensin-1, -2, -3 and -4 are mainly expressed by neutrophils and, for this reason, are also called human neutrophil peptides (HNPs) [66].

hBD-1 is constitutively expressed, whereas hBD2 and hBD3 can be induced by microbes or inflammatory cytokines [64]. Defensins are small cationic, antibiotic peptides that contain six cysteines in disulfide linkage. They are active against Gram-positive and Gram-negative bacteria and act by inducing microbial membrane permeabilization. They also appear to regulate the inflammatory process through

binding to protease inhibitors such as α 1-antitrypsin and α1-antichymotrypsin [124]

1.3.2 Cathelicidins

Cathelicidin family contain an N-terminal signal peptide (preregion), a conserved cathelin-like domain (proregion), and a C-terminal microbicidal domain (hence the name "cathelicidin") (Figure 1.3.1) [180]. Humans generate only one cathelicidin, called LL-37 [89].

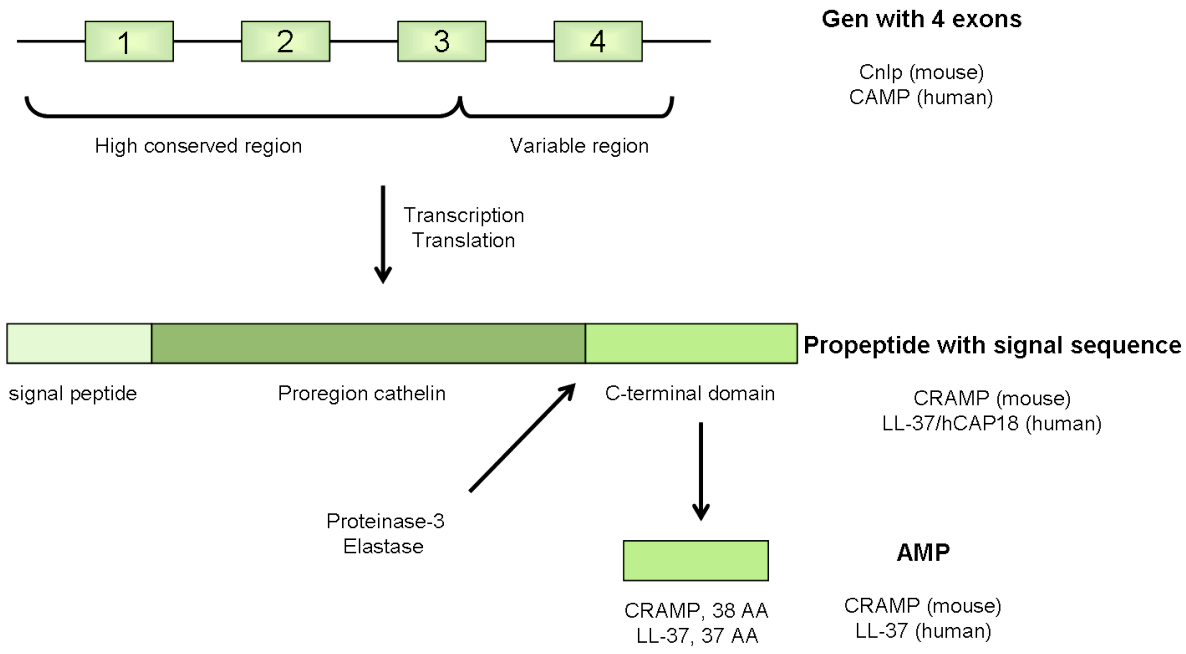


Figure 1.3.1: **Basic structure of cathelcidins LL-37/hCAP18 for human and CRAMP mice.** Humans and mice each express a single cathelicidin, which are encoded by similar genes and have similar alpha-helical structures, spectra of antimicrobial activity. Cathelicidin is synthesized as an inactive precursor protein with an amino-terminal signal sequence, a central cathelin domain, and an inactive carboxy-terminal antimicrobial peptide (AMP) domain. Serine proteases, including stratum corneum tryptic enzyme (SCTE), cleave the AMP domain to generate the active antimicrobial peptide.

They are usually stored in the granules of neutrophils as an inactive form and undergo processing to mature peptide during or after secretion by appropriate proteases.

For example, hCAP18 is cleaved by proteinase 3 [150] or elastase [69] to liberate its C-terminal antimicrobial domain, which is called "LL-37" because this peptide begins with two leucine residues and has 37 amino acid residues. hCAP18/LL-37 has also been found in various epithelia [11].

Because neutrophil secondary granules readily degranulate to the extracellular space, cathelicidins can be found in inflammatory fluids at relatively high concentrations [36]. Moreover, accumulation of neutrophils within mucosal tissues may activate epithelial cathelicidin by a proteolytic process mentioned above.

The C-terminal AMPs of cathelicidins are microbicidal against a broad spectrum of microorganisms, including bacteria, fungi, and parasites [183].

Similar to other cationic AMPs, the mechanism of cathelicidin-mediated microbial killing depends on the formation of ion channels or pores in the microbial cell membrane. LL-37 also possesses a potent endotoxin-neutralizing activity due to interaction with a negatively charged lipid A portion of the LPS molecule [89].

There is evidence for other host defense and immunoregulatory functions of AMPs. It has been shown that some of the AMP's may act as chemoattractants for inflammatory and immune cells. LL37 attracts neutrophils, monocytes and T cells via formyl-peptide receptor-like 1 (FPRL1), eosinophils via formyl-peptide receptor (FPR). AMPs may also be involved in tissue homeostasis: HNPs have been shown to increase airway epithelial repair [1], LL-37 can induce angiogenesis [88].

Recent data suggest that some AMPs may play a regulatory role during inflammation [106].

1.4 Inflammation as a host defense response

Inflammation is a tissue response to infection, injury or irritation. The principal features of inflammation are (1) the presence of damage as a trigger of host response; (2) activation of host defense and immune mechanisms; and (3) healing of damaged tissues [114].

Inflammation starts with tissue injury [102] (Figure 1.4.1).

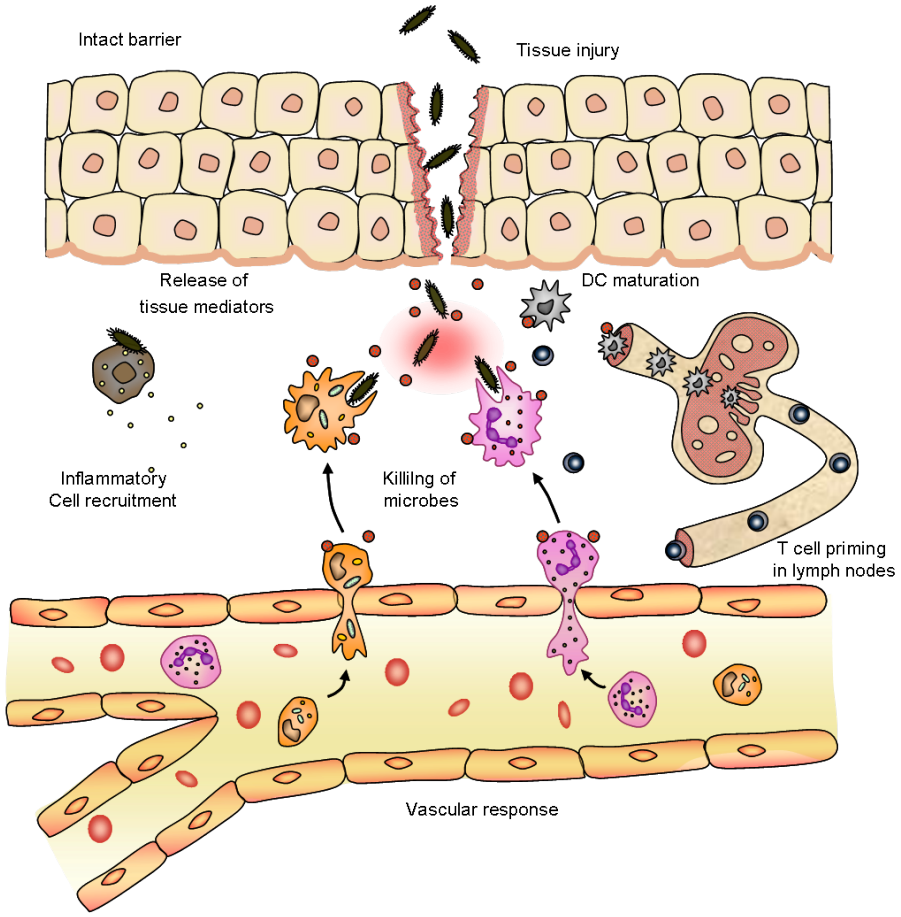


Figure 1.4.1: **Inflammatory process.** Inflammation is initiated by tissue injury, caused by physical damage to the tissue barrier or infection. Various mediators (including chemokines and vasoactive amines) are released by tissue cells (epithelial cells and mast cells) to increase vascular permeability and attract inflammatory cells from blood (neutrophils, monocytes or eosinophils), that migrate to the site of injury and kill microbes. Dendritic cells, matured in the presence of pathogens, migrate into regional lymph node, where they present antigen to T cells and thereby prime specific immune response.

Injury can be recognized at all levels of biological organization [74]. Following injury, tissue cells release mediators with different defense functions; AMPs to kill microbes immediately; vasoactive substances (for example, histamine of mast cells) to increase the local blood flow and vascular permeability, and cytokines to attract and activate inflammatory cells of hematopoietic origin. For example, IL-8 is a major chemoattractant for neutrophils; GM-CSF increases survival of granulocytes; TNF-α activates virtually all cells involved in inflammatory process [151].

Leukocyte migration into damaged tissue is an essential feature of inflammation. Neutrophils (also called polymorphonuclear /PMN/ leukocytes) are the most abundant hematopoietic cells during the early stages of nonspecific inflammation [114].

Once neutrophils have migrated into the tissue, their primary role is to recognize, phagocyte and kill pathogens. Following binding to opsonized pathogens, neutrophils extend pseudopods that engulf the particle and take them up into the maturing phagosome [139].

Killing by neutrophils occurs intracellular by AMPs, like α-defensins [66], and serine proteases, degranulate into phagosome, or reactive oxygen compounds generated upon activation of neutrophil membrane-bound NADPH oxidase enzyme complex [139] or extracellularly through a release of the AMPs (like cathelicidins) of specific granules [183]. At the end of their phagocytosis role they die and then are removed and/or replaced by mononuclear inflammatory cells. Following migration into tissues, monocytes can differentiate to become macrophages or DCs[5]. Being localized in tissues, these cells play a key role in inflammation as sensors of danger, initiators and regulators of host defense responses. They express particularly high levels of PRRs [78].

Phagocytosis of microbes is a fundamental function of macrophages, discovered by Mechnikov more than 100 years ago [102]. Immature DCs are also potent phagocytes [14]. An important consequence of phagocytosis by macrophages and, especially, DCs is processing the antigens for presentation on major histocompatibility complex (MHC) molecules to T cells, providing a link between inflammation and immunity (innate immunity and adaptive immunity) [20].

Conversion of the host defense response from antimicrobial tissue-damaging processes to the anti-inflammatory processes is necessary to promote tissue repair at the late stages of inflammatory reaction [114]. During the progression of inflammation, platelet-leukocyte interactions elicit the formation of lipoxins A4 and B4, which serve as “stop signals” by blocking the further recruitment of PMNs from the circulation [137].

Following phagocytosis of apoptotic cells generated in inflamed tissue, macrophages produce TGF-β, a potent anti-inflammatory cytokine [74]. On the other hand, prolonged and excessive local inflammation is associated development of chronic autoimmune diseases, tissue injury[108], pathologic angiogenesis, fibrosis and cancer [9].

1.5 Lung immunity and airway epithelium

The lung, like the skin and the gut, has a large surface area that is constantly exposed to the environment and is also a metabolically active organ with a large

blood-air interface designed for both gas exchange and capillary endothelial cell processing of a myriad of endogenous substances.

This dual function of gas exchange and metabolism can only be maintained throughout adult life if the structure of the organ is homeostatically maintained and defended against the external and internal environment. A program of lung structure maintenance has a critical importance during lung development. Later in life, elements of this developmental program are utilized to protect the lung against destruction, antioxidants, antiproteases, and attack by the innate immune system.

Airway epithelium represents a barrier not like any other barriers in the body. First, it serves as a mechanical and chemical filter of the inhaled air, providing sterility and safety of the alveolar compartment of the lung. The gas exchange process in alveoli requires the ventilation of a large volume of environmental air that potentially contains pathogens and dangerous particles. The latter must be captured and neutralized in the airways; otherwise, pulmonary infection will develop.

Second, intact airway epithelium is a primary regulator of the mucosal homeostasis, preventing the access of pathogens to the subepithelial and submucosal compartments that may result in excessive inflammation and / or generalized infection. Airway epithelium is covered on its apical surface by a thin liquid layer called airway surface liquid (ASL) (Figure 1.5.1).

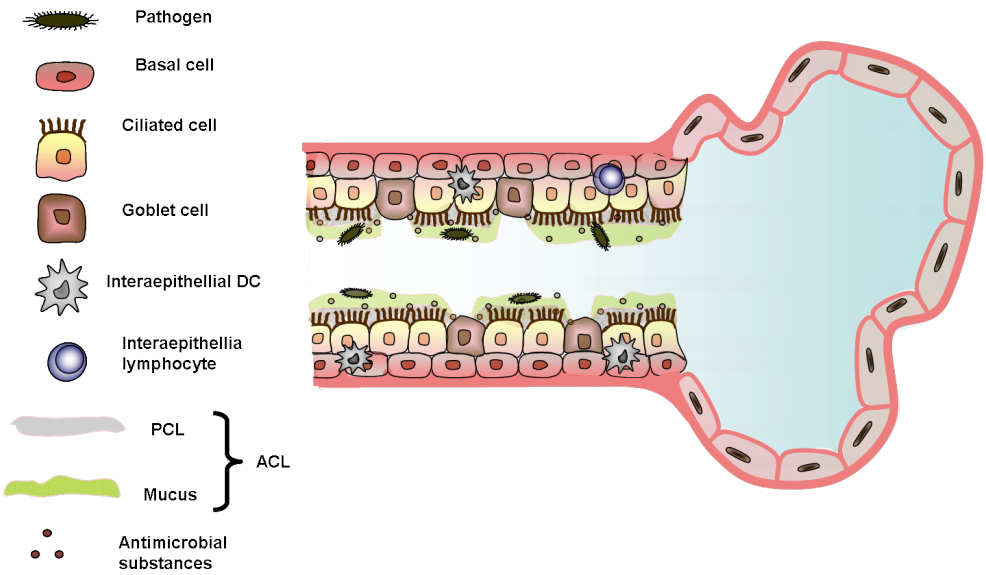


Figure 1.5.1: **Airway epithelial barrier.** represents a physical border of ECs that resists damage and prevents pericellular diffusion of exogenous factors due to TJs which link the neighboring ECs at the apical zones. Defense function is maintained by the mucociliary escalator and antimicrobial substances present in the ASL that consists of mucus, produced by goblet cells and other secretory cells, and periciliary liquid layer (PCL). Intraepithelial DCs and lymphocytes provide a local sentinel function and are involved in the regulation of tissue homeostasis.

The ASL is the first line of defense against inhaled pathogens and is important for effective mucociliary clearance [86]. Almost one century ago, Fleming observed that respiratory secretions possess bactericidal properties [57]. The ASL consists of two layers, a mucus layer and a periciliary liquid layer (PCL), that are propelled upward by coordinated ciliary beating [23].

1.5.1 Airway antimicrobial proteins

Antimicrobial proteins and peptides (AMPs) represent a first-line innate effector mechanism to kill microbes directly due to interaction with their membranes or disrupting their metabolic status as described earlier. In the airway mucosa, antimicrobial factors from submucosal gland cells, ECs and neutrophils are accumulated within the ASL [163].

Among them, the proteins lysozyme, lactoferrin and SLPI are the most abundant factors of airway secretions in health and in lung diseases such as asthma, chronic bronchitis, and CF [26].

Surfactant proteins A and D arising from alveolar ECs bind bacteria and fungi, as well as microbial factors, and enhance pathogen clearance [172].

Cationic AMPs of the defensin and cathelicidin families contribute considerably to the lung defense [63]. The production of other AMPs increases dramatically during infection and inflammation: for example, hBD2 can be induced via (TLR2)-MyD88-NFkappaB pathway by microbial factors and inflammatory cytokines [75].

The levels of LL-37 increase mainly via activation resulting from proteolytic cleavage of the mature peptide from inactive precursor hCAP18/LL-37 by proteinase 3 [150]or neutrophil elastase [69]. However, the exact mechanism of LL-37 activation in the airway epithelium remains unknown.

Although transcriptional induction of LL-37 expression in the skin has been reported [60], there is no data supporting the existence of a similar mechanism in the lung ECs. The levels of LL-37 were found to be increased in the sputum [148] and BALF [36] in CF lung disease, characterized by chronic neutrophil inflammation and colonization of airways with P. aeruginosa. Thus, activation of the peptide by proteases from neutrophils and bacteria is possible.

However, the major source of LL-37 in the airways is likely neutrophils recruited in the lung during inflammation. AMPs have a broad spectrum of activity against Gram-positive and Gram-negative bacteria as well as against fungi and enveloped viruses [10].

The minimal inhibitory concentrations of the peptides are in the range 0.1–100 μg/ml [10]. AMPs differ in their killing activity against particular pathogens: LL-37 is more potent than HNP1 against a variety of bacteria including P. aeruginosa [11], and HBD-2 is more effective than HBD-1 in killing Escherichia coli [146].

Many AMPs work synergistically. Three factors found in human airways - lactoferrin, SLPI and LL-37 - have synergistic activity with lysozyme [11].

The information about the activities of AMPs in the lung in vivo is limited. Cathelicidins are the only AMPs extensively studied in vivo. It has been shown that mice treated with intratracheal LL-37/hCAP-18 vector had a lower bacterial load and a smaller inflammatory response than did untreated mice following pulmonary challenge with P. aeruginosa [12].

CRAMP, a mouse analog of LL-37, protects mice against necrotic skin infection caused by Group A *Streptococcus* [118] and urinary tract E.coli infection [38].

The latter study provides evidence that EC-derived cathelicidin contributes substantially to mucosal protection from bacteria. Moreover, cathelicidin protects from sepsis due to the binding to and neutralization of endotoxin [12, 39].

1.5.2 Clara cells

Clara cells are epithelial cells lining the pulmonary airways (Figure 1.5.2), which are distinct from mucous and secretory cells in morphology and their secretory products. Clara cells are non-ciliated, non-mucous secretory cells localized mostly in the bronchiolar surface epithelium, and they are one of the most multifunctional and heterogeneous cell types in the mammalian lung [100].

Clara cells are also the principal site of xenobiotic metabolism by the cytochrome P450 mono-oxygenase system within the lung [127]. Studies have shown that naphthalene causes severe selective destruction to clara cells in the mouse [128].

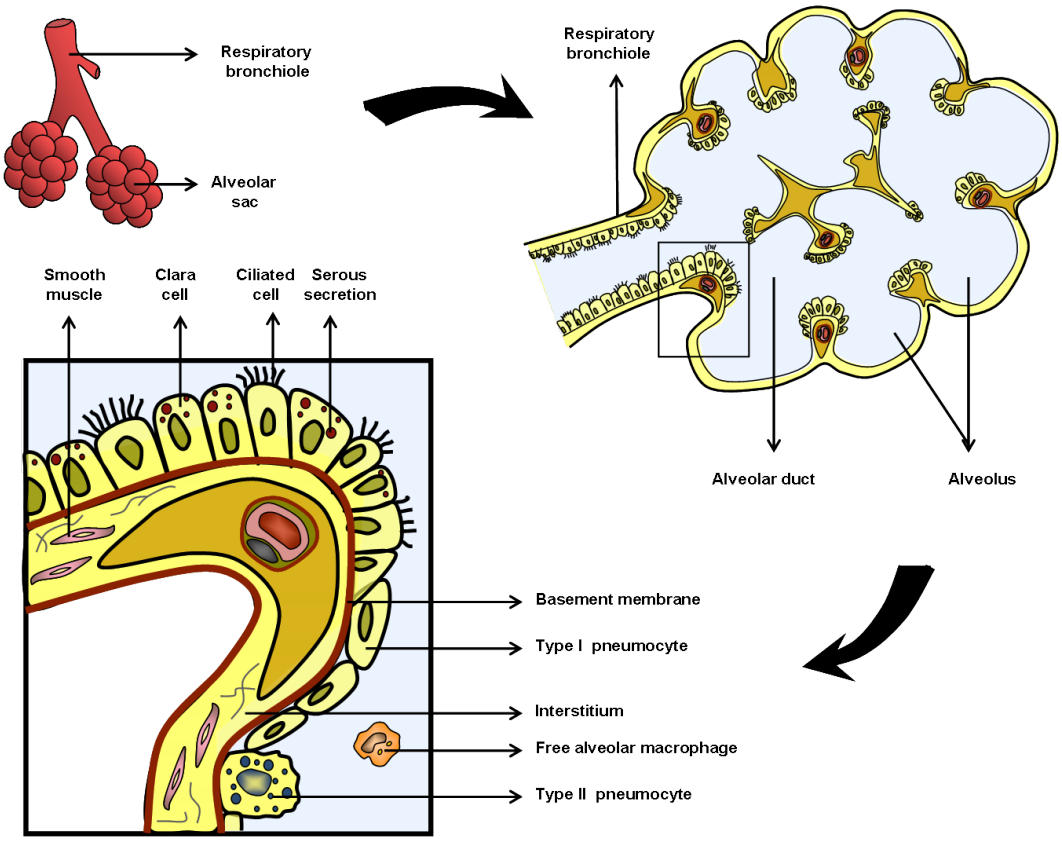


Figure 1.5.2: **Respiratory bronchiole and clara cells.** Alveolar ducts are small ducts leading from the respiratory bronchioles to the alveolar sacs.The respiratory bronchiole epithelium consists of ciliated cuboidal cells and clara cells.

Toxicity of the clara cells is due to the metabolism of naphthalene by cytochrome P450 mono-oxygenase and a toxic intermediate that causes distal airway clara cell swelling, vacuolization, and exfoliation into the lumen of the airways 24 h after injury is initiated [164].

One of the major secretory products of human pulmonary clara cells is the 10-kD protein (CC 10) [145]. This protein is a homodimer consisting of 70 amino acid subunits connected by two disulfide bonds [144]. CC10 possesses varied biochemical and biological properties including phospholipase A2 (PLA2)-inhibitory activity [143], its ability to bind methylsulfonyl polychlorinated biphenyls [120] and possess also anti-inflammatory activity [31].

1.5.3 Pathogenesis of pulmonary emphysema

Emphysema comes from the Greek word that means "inflation". It is a pathologic condition of the lung which is defined as airspace enlargement of the adult lung [147]. It is often caused by exposure to toxic chemicals, including long-term exposure to

smoke. Emphysema is characterized by loss of elasticity of the lung tissue, from destruction of structures supporting the alveoli. Thus the small airways collapse during exhalation, as alveolar collapsibility has increased. This impedes airflow and traps air in the lungs.

Emphysema symptoms include shortness of breath on exertion and later at rest, hyperventilation, and an expanded chest. There is no emphysema-specific treatment. Mortality and morbidity from COPD (chronic obstructive pulmonary disease) is an increasingly serious global health problem; COPD ranked sixth among the causes of death globally in 1990 but is expected to be the third most common cause of death in 2020 [35].

The destruction of alveolar-capillary (epithelial and endothelial) cells by proteolytic enzymes has been generally accepted as one of the principle mechanisms of destructive airspace enlargement based on the demonstration that intratracheal instillation of the cysteine protease papain (a meat tenderizer) caused emphysema in rats [68] and on an association between emphysema and a genetic deficiency in the neutrophil elastase inhibitor α1-antitrypsin (AAT) [52].

The pathology of COPD relates to inflammatory changes in the small airways and to the loss of alveolar septal structures and small vessels. The alveolar septae are infiltrated by neutrophils, clusters of CD68+ macrophages, and lymphocytes. Neutrophils elastase and MMP-12 released by activated macrophages enzymatically destroy the elastin scaffold of the alveolar spaces. However, the production of proteases is not restricted to inflammatory cells; structural cells such as epithelial and endothelial cells are also capable of producing proteases [104].

Elastin fragments in turn are chemotactic [76] and attract inflammatory cells to sites of injury, thus suggesting a joint role of inflammatory cells and proteases. Strong experimental support of this concept is provided by data showing that elimination of alveolar macrophages in rats [122] and knockout of MMP-12 in mice [71] protects these animals against cigarette smoke–induced emphysema.

2 Hypotheses and goals

The aim of the present study was to analyze the effect of cathelicidin on the interaction between neutrophils and microbial patterns. Stimulation experiments were used applying various microbial stimuli and characterized the effect of cathelicidin. Also the production of ROS was determined. Further, the role of endogenous cathelicidin were determined by using neutrophils isolated from CRAMP deficient mice. The role of cathelicidin as endogenous (physiological form) and exogenous (treatment form) presence in modulating some immunological functions of neutrophils (cytokines release, ROS release, phagocytosis and bactericidal activities) , enhancing airway epithelia regeneration and reducing induction of pulmonary emphysema in mice are essential findings of this work. In this project the following hypotheses were tested:

1. Whether cathelicidin LL-37 modulates inflammatory reaction of human neutrophils in response to LPS and whole bacteria. Although it is known that LL-37 neutralizes endotoxin [89] and modulates activation of macrophages and monocytes by LPS [106], the data regarding neutrophils are limited [185].

2. Whether mouse endogenous cathelicidin antimicrobial peptide CRAMP, expressed by mouse neutrophil has a role in increasing neutrophils antimicrobial activity.

3. Whether increasing of bactericidal of neutrophils in the presence of cathelicidin related to the improvement of neutrophil phagocytosis..

4. If endogenous CRAMP secretion is sufficient to reduces an inflammatory reaction of mouse neutrophils in response to bacterial stimulant. It has been shown that LL-37 may prevent sepsis and be useful in lower doses for treating sepsis in rats [61].

5. Whether the presence of cathelicidin can enhance airway repair after acute lung injury, in addition to its direct bactericidal effect. There are published data regarding defensins which can enhance and regulate airway repair [1].

6. Whether the presence of cathelicidin protects from the development of pulmonary emphysema. No data currently exist in literature regarding this possibility.

3 Materials and methods

3.1 Analysis of inflammatory innate immune reaction in response to bacterial stimulants

In present work neutrophils were isolated either from human blood or mouse peritoneal cavity and different methods and protocols were applied as described below.

3.1.1 Isolation and preparing of murine neutrophils

CRAMP-deficient mice in a 129/SVJ background [118] and their wildtype controls were used for neutrophil isolation. The animals were kept under specific pathogen free conditions at the animal center of the University of Marburg. The animal experiments were approved by the responsible authorities (Regierungspräsidium Giessen).

Mice were intraperitoneally injected with 1 ml of sterile 4% thioglycollate broth (BD Difco, Heidelberg, Germany), the animals were euthanized after 4 hours and the peritoneal cavity was lavaged twice with 10 ml of PBS (PAA, Cölbe, Germany).

The peritoneal lavage was centrifuged at 1200 rpm for 10min. and red blood cells were lyzed by RBC lysis buffer (NH4Cl 4.14 g, KHCo3 0.5 g, EDTA 0,1 ml and adjustment PH= 7.2-7.4 with Hcl 1N for 500 ml PBS)[95, 37].

After washing cells with PBS, the cells were resuspended in RPMI 1640 medium (Gibco, Grand Island, NY). The purity of neutrophils was determined using cytospin preparations stained with Giemsa.The peritoneal cell populations were consistently composed of >97% neutrophils. Viability of the cells was tested using trypan blue staining and was consistently >95%.

3.1.2 Isolation of human neutrophils

Buffy coats made from 500 ml blood of healthy volunteers donor were obtained from the blood bank of the University hospital Marburg and diluted 1:1 with PBS containing 2 mM of EDTA.

Human neutrophils were isolated using dextran sedimentation and Ficoll-Paque gradient centrifugation protocol[176]. Blood 9 ml of diluted Buffy coat was layered onto 15 ml of Ficoll 400 (Sigma-Aldrich, Schnelldorf, Germany), and centrifugated

at 1500 rpm for 30 minute and stopped without braking.

The cell pellet, containing granulocytes and red blood cells was resuspended in 25 ml dextran 4% and incubated at room temperature for 40 min. The neutrophils in the upper phase were collected and red blood cells lyzed with RBC lysis buffer which contains (NH4Cl 4.14 g, KHCo3 0.5 g, EDTA 0,1 ml and adjustment PH= 7.2-7.4 with HCl 1N for 500 ml PBS).

Cells were washed two times with PBS and the pellets resuspended in RPMI medium containing 0.1 % serum albumin. The neutrophils were counted, the purity of the cell preparation was determined by cytospin preparations and Giemsa staining, and the viability was tested using Trypan blue staining. The purity and viability of isolated neutrophils were more than 95%.

3.1.3 Preparation of bacteria

Staphylococcus aureus 113 wt (Dr. A. Peschel, University of Tubingen, Germany) and *Pseudomonas aeruginosa* NH57388A and NH57388C (Dr. Niels Hoiby, University of Copenhagen, Denmark) were used for stimulation experiments [11], One day before experiment a loop from bacteria stock (-80 °C) is inoculated into 50ml Luria-Bertani (LB) broth (Roth, Karlsruhe, Germany) and incubated with shaking overnight at 37 °C, then the next day the bacteria was streaked onto LB agar plates , and the plate were incubated at 37 °C.

Colonies were harvested, suspended and washed three times in Dulbecco's PBS with Ca2+ and Mg2+. The number of bacteria in suspension was adjusted based on measurements of the OD 600 nm and using a reference dilution. The medium was replaced with PBS. Complement from heat- inactivated human serum (95°C for 1 h) was used to opsonize the bacteria (37 °C, 30 minute, 60%).

3.1.4 Neutrophil stimulation

The LL-37 peptide (`LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES`) and its scrambled form sLL-37 (`RSLEGTDRFPFVRLKNSRKLEFKDIKGIKREQFVKIL`), were chemically synthesized (Charité, Humboldt-Universität, Berlin, Germany). Isolated neutrophils were stimulated with bacteria as following, 10^4 CFU / ml bacteria were heat-inactivated (95°C for 1 h) and opsonized with inactivated human serum (37 °C, 30 min., 60%) and incubated with human neutrophils (1 x 10^6 cell / ml).

The supernatants were collected after centrifugation at 1200 rpm for 10 min.

For LPS stimulation, LPS 100 ng/ml (Sigma-Aldrich Chemie GmbH, Munich, Germany) incubated with neutrophils suspensions were pre-incubated with different concentrations of LL-37 (5, 10, 15, and 20 µg / ml) for 30 minutes and others neutrophils were not pretreated with LL-37. After the time of incubation (12h) the supernatants were collected and kept for futher detection in -20 °C.

3.1.5 Bacterial killing assay

Neutrophils were isolated from the peritoneal cavity of CRAMP-deficient and their wildtype mice in a 129/SVJ background as described above. To analysis the neutrophils' antimicrobial activity, opsonized bacteria and mouse neutrophils (1 x 10^6 cell / ml) were incubated together in one 1.5 ml eppendorf tube at a ratio of 1:1 (shaking at 200 rpm, 37 °C). 20 µl aliquots were removed in deferent time points incubation 0, 30, 60, and 90 min and neutrophils were lyzed by adding Triton 0.1 %. Dilutions in PBS were plated onto LB agar plates and bacterial colonies were counted after 24 hrs of incubation in 37 °C.

The rate of dead bacteria was evaluated and used as a parameter to compare between neutrophils isolated from WT and CRAMP-KO mice [91]. opsonization was done by incubating the bacteria suspension with 60 % of inactive human serum at 37 °C for 30 minutes.

The isolated neutrophils were resuspended in PBS-G-SA contains 5 mM glucose and 0.1% serum albumin (Sigma-Aldrich, Schnelldorf, Germany) in order to reduce the adherent affinity of neutrophils. The serum used in opsonization was inactivated by heating 65 °C for 1h. The bacterial mediums were used are LB broth and LB agar (Roth, Karlsruhe, Germany). Controls were performed by incubating bacteria without neutrophils in the same medium and all condations.

3.1.6 Enzyme-linked immunosorbent assay (ELISA)

Cytokine levels in culture supernatants were determined using a commercially available DuoSet ELISA Development kits for IL-1ß,IL-6, IL-8 and TNF-α according to the manufacturer's instructions (R&D Systems).

In brief, 96-well microplates were precovered with capture antibody and incubated overnight. Then after washing and incubation with a blocking reagent, 100 µl per well of sample or standard were added and incubated overnight at 4 °C.

After washing and subsequent incubation with detection antibodies during 2 h

and Streptavidin-POD Conjugate (Roche Diagnostics) during 20 min at room temperature, 100 µl of TMB+Substrate-Chromogen (Dako Deutschland GmbH, Hamburg, Germany) was added. The reaction was stopped by adding 3N H2SO4, and the absorbance was measured using ELISA reader at 450 nm or 490 nm.

3.1.7 Cytotoxicity assays

The cytotoxic effect of different concentrations of LL-37 (0.5-50 µg/ml) on isolated neutrophils was assessed by colorimetric quantification of the lactate dehydrogenase (LDH) in cell supernatants using the Cytotoxicity Detection Kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, after 24 h of stimulation, cell-free supernatant was collected and incubated with the substrate mixture from the kit.

LDH activity was determined in a coupled enzymatic reaction, in which tetrazolium salt is reduced to formazan. The content of formazan dye was then quantitated by measuring the absorbance at 490 nm using ELISA reader.

3.1.8 Western-blot

To test whether CRAMP was released from neutrophils during LPS stimulation, western-blot analysis was performed to detect CRAMP release in supernatants . After stimulation, samples were centrifugated 1200 rpm for 5 min. and the supernatants were separated and stored in -20 °C.

Supernatants were mixed with Roti-Load® loading buffer (Roth, Karlsruhe, Germany) and separated on a 10 - 20% tris-tricine gel [136](Anamed, Darmstadt, Germany) according to the manufaturers instructions. The seperated samples were blotted on a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) using a tank-blotting system (Bio-Rad Laboratories, Hercules, CA, USA) at a constant power of 20 W for 1.5 hours using a standard towbin-buffer system [162].

After transfer the membrane was blocked for one hour in a blocking solution containing 5% poor fat dry milk (Sigma, Steinheim, Germany) and PBS. For autoradiographic detection a polyclonal CRAMP antibody (Pineda Antibody Service, Berlin, Germany) and a horseradish peroxidase (HRP)-labeled secondary rabbit-IgG antibody (GE-Healthcare, Little Chalfont UK)) were used.

The CRAMP antibody, raised against a synthetic CRAMP-Peptide (`ISRLAGLLRKGGEKIGEKLKKIGQKIKNFFQKLVPQPE`) (Dr. Peter Henklein, Charité Berlin) was

used in 500-fold dilution in 5% poor fat dry milk-PBS and incubated at 4 °C with mild agitation overnight (Roth, Karlsruhe, Germany). After 3 washing steps with PBS the species specific HRP-labeled secondary antibody was used in a 1000-fold dilution in 5% poor fat dry milk-PBS and incubated with mild agitation for 1 hour at room temperature.

After 3 final washing steps with PBS the peptides were detected using the SuperSignal® West Pico chemiluminsecent substrate (Pierce Biotechnology, Rockford, USA) on Kodak scientific imaging films (Eastman Kodak Company, Rochester, N.Y., USA).

3.2 Detection of neutrophilic reactive oxygen species (ROS) production

To measure cellular productions of reactive oxygen species in present measurement, luminometry and fluorometry methods were used because of their highest sensitivity and specificity among other [41].

3.2.1 Luminometric analysis of neutrophil reactive oxygen species generation

Luminol was used for chemiluminescence assay to determine the production of reactive oxygen species (ROS)[41]. Krebs-ringer buffer was prepared by dissolving one bottle of KRB buffer (Sigma-Aldrich, Schnelldorf, Germany) in 1 L distilled water and adding 1.26 g sodium bicarbonate and serum albumin (1 g / 100 ml) and this buffer was used to resuspend neutrophils were isolated from human buffy coat by dextran-sedimentation and Ficoll-Paque gradient centrifugation protocol.

Luminol was dissolved in 10 ml DMSO, then added to 1 L of 0.1 M of NaOH. In brief, 96-well microplate was used. In each well was pipetted 200 µl reaction mixture as fowloing:

1. 100 µl luminol solution (0.5 mM) included SOD (5000 U/ml) and catalase (200 000 U / ml).
2. 100 µl Neutrophil cells suspension (10^6 cells /ml).

Neutrophils were pretreated with different concentrations of LL-37 (5, 10, 20, and 30 µg / ml) and incubated at 37 °C for 30 minutes with shaking, some samples were not pretreated with LL-37 used as control and blank was performed by applying all

reagents without neutrophils cells. The emission of light was recorded for 1 hour (one measurement each 3 minutes) by a luminometer (Magellan; Chantilly, VA, USA) after triggering ROS production by adding PMA (100 nM) (Sigma-Aldrich, Taufkirchen, Germany) or *Staphylococcus aureus* (10^4 CFU/ml).

3.2.2 Detection of intracellular ROS from human neutrophils by flow cytometry

Human neutrophils isolated from buffycoat were gated depending on their SSC and FSC characters and labeled with two different antibodies FITC-labeled mouse anti-human CD66 and PreCP-labeled mouse anti-human CD45 (Becton-Dickinson, Heidelberg, Germany) for compensation and remove false light detections.

The cells were resuspended in Krebs-ringer buffer containing 100 µM 2´,7´ dichlorofluorescin diacetate (DCFDA; Fluka, Steinheim, Germany) and cells suspension 10^6cells/ml was incubated without/with various concentrations of LL-37 or sLL-37 for 30 minutes[185]. The ROS products were triggered by PMA (100 nM) and their fluorescence analyzed by flow cytometry (Becton-Dickinson, Heidelberg, Germany).

The mean fluorescence intensity of at least 10^5 neutrophil cells was calculated by the CELLQUEST software (Becton-Dickinson, Heidelberg, Germany). For each experiment, unstained cells and cells treated with sLL-37 served as controls.

3.2.3 ROS Production by mouse neutrophils

Neutrophils were isolated from mouse peritoneal cavity and resuspend in Krebs-ringer buffer included 1,26g sodium bicarbonate and serum albumin (1 g / 100 ml) after red blood cells were lysed and cells were washed for several times in PBS. Luminol solution (0,5 mM) was prepared by dissolving luminol in 10 ml DMSO which was added into 1 l of 0.1 M NaOH[41].

In each well of 96 well- plate 100 µl of neutrophils suspension (6 x 10^6 cell/ml) was mixed with 100 µl of luminol solution (0,5 mM). The ROS production was triggered with *Staphylococcus aureus* 10^4 CFU/ml. The emission of light was recorded for 1 hour (one measurement each 3 minutes) using a luminometer under shaking and 37C°.

3.3 Evaluation of neutrophil phagocytic activity

To investigate the role of cathelicidin in modulating phagocytosis activity of neutrophils, the uptake of bacteria by neutrophil was evaluated depending on the basic principle of phagocytosis assay is to incubate neutrophils with the target particle, and then monitor the rate of either target ingestion or its loss from the medium.

Microscopy is the most direct approach when measuring phagocytosis. The percentage of neutrophils ingesting bacteria can be determined using microscopy and flow cytometry [70].

3.3.1 Evaluation of neutrophil phagocytic activity by microscopic method

Human neutrophils were isolated using dextran sedimentation and Ficoll-Paque gradient centrifugation protocol as mentioned above. The isolated cells were washed with PBS two times and resuspended (10^6/ml) in Krebs-Ringer phosphate buffer (pH 7.3) containing glucose (10 mM), Ca2+ (1 mM), and Mg2+(1.5 mM), and 0.3% BSA (to minimize cellular aggregation) and then preincubated at 37 °C for 10 min in the presence of different concentrations of LL-37 (1, 5, 10 and 20µg/ml). The opsonized *Staphylococcus aureus* were incubated with neutrophils 10^6 CFU/ml at ratio of 1: 25 (neutrophil: bacteria). Adding bacteria took place in polypropylene tubes with slowly tumbling at 37 °C for 1 hour.

After incubation the cells were subjected to repeated washes in PBS (110 g, for 10 min, to remove nonattached bacteria) and resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS.

Cytospin was performed to determined microscopically the uptake of bacteria by neutrophils. After cytospin, slides were air dried overnight, fixed in methanol (5min), Giemsa stained, and mounted. Slides were studied using oil immersion and 1000 magnification, and the number of bacteria/cell was counted for the first 50 positive cells (Figure 3.3.1) [29].

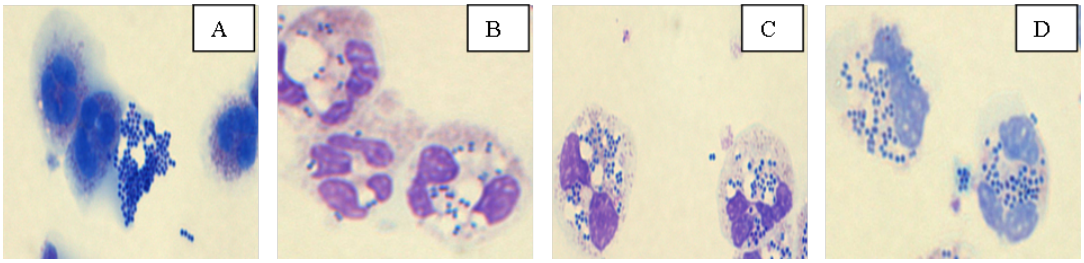


Figure 3.3.1: **Giemsa-stained slides of neutrophils after 1 h. incubation with LL-37 resistant opsonized *Staphylococcus aureus*.** (A) Negative control, human neutrophils were inactivated by formalin before incubation. (B) Positive control, bacteria were incubated with active human neutrophils. (C) stimulated human neutrophils with 5µg/ml LL-37 were incubated with bacteria. (D) stimulated human neutrophils with 10µg/ml LL-37 were incubated with bacteria.

Phagocytosis is expressed as mean number of bacteria (bound or ingested)/cell [29]. Positive and negative controls were performed (negative control = inactive neutrophils were fixed with 3.7% formaldehyde before incubation with bacteria, positive control = active neutrophils were incubated with bacteria without preincubation with LL-37).

3.3.2 Evaluation of neutrophil phagocytic activity by flow cytometric method

Human neutrophils isolated from buffycoat and cathelcidin-resistant *Staphylococcus aureus* were used for this measurment. *Staphylococcus aureus* 10^5CFU/ml were labeled with FITC and opsonized with heat-inactivated human serum.

The neutrophils were preincubated with or without variant concentrations of LL-37 or sLL-37 used to exclude a nonspecific effect of LL-37 served as control. Human serum opsonized and FITC-labeled bacteria were incubated with human neutrophils at 37 °C for 30 minute (shaking 200rpm) at ratio of 1: 25 (neutrophil: bacteria).

Then cells were resuspended in CellFix (BD PharMingen, Erembodegem, Belgium) and treated with 500µL of Trypan blue (400 mg/mL prepared in 0.85% saline solution) to quench the fluorescence of the bacteria bound on the surface.

Efficient quenching was confirmed on negative controls. The percentage of phagocytosing cells (bearing green fluorescence) was determined based on the negative control and autofluorescence and analyzed by flow cytometry (Becton-Dickinson, Heidelberg, Germany)[79].

Neutrophil cells were gated depending on their SSC and FSC characters and labeled with two different antibodies FITC-labeled mouse anti-human CD66 and

PreCP-labeled mouse anti-human CD45 (Becton-Dickinson, Heidelberg, Germany) to make compensation and remove false light detections. A 488 nm argon laser beam was used for excitation.

The mean fluorescence intensity of at least 10^5 neutrophil cells were calculated by the CELLQUEST software (Becton-Dickinson, Heidelberg, Germany). Each assay was performed in duplicate and all results were expressed as the mean + standard error of the mean of four to eight independent assays.

3.4 Assessment of lung tissue repair and emphysema induction in presence of cathelicidin

To study the role of cathelicidin in reducing emphysema induction and enhancing airway epithelium healing, two models were performed: the elastase-induced pulmonary emphysema and naphthalene-induced lung injury models.

3.4.1 Induction of specific lung injury and evaluation of airway epithelium regeneration

Clara cells were ablated in mice by cytoselective toxicity of the metabolic products of naphthalene produced by cytochrome P450 mono-oxygenase [128].

Acute lung injury was induced in murine lungs by intraperitoneal injection of naphthalene. Plopper and colleagues [128] reported that intraperitoneal administration of naphthalene is a specific toxicity to Clara cells and does not cause changes in other type epithelial cells, indicating that the toxicity of naphthalene to Clara cells is cytoselective. Stripp et al. [152] reported that treatment with naphthalene at a concentration of 300 mg/kg results in the ablation of clara cells in the bronchiolar region for at least 20 days in mice [152].

To investigate whether the endogenous cathelicidin enhances lung tissue healing, an acute lung injury induced in mice by naphthalene and the growth of destroyed airway epithelium was observed and evaluated during time for 15 days after lung injury was induced (first, fifth and fifteenth day) .

Clara cells or ciliated cells of cell-specific 10-kD a protein (CC10) density was determined during time by immunohistochemistry after specific epithelial injury by naphthalene.

Naphthalene was purchased from Fisher (Aschaffenburg, Germany) and dissolved in corn oil by overnight shaking 80 rpm at room temperature. Wild type

and CRAMP deficient SVJ129 mice (Pathogen-free, male, weighing 25–29 g and aged 8–10 weeks) were intraperitoneally injected with naphthalene 200 mg/kg body weight or corn oil alone with equivalent volume as vehicular control and after injection; mice were maintained in pathogens-free condition. On 1, 5 and 15 days later, four groups from mice: WT control, WT naphthelene, CRAMP-KO control and CRAMP-KO naphthelene were sacrificed (n =6 in each group).

The tracheas were exposed and cannulated, the lungs were removed and fixed with 6% paraformaldehyde perfusion with steady flow for 20 minute. The lungs were cut in the same orientation after they were embedded in agarose to keep the distance between septa of airway space. Then lung tissue sections were dehydrated and embedded in paraffin. This Sections of airways with 3 µm in thickness were deparaffinised in xylene and rehydrated in ethanol and PBS.

Endogenous peroxidase activity was inactivated using 1% hydrogen peroxide in methanol (Roth, Karlsruhe, Germany; pH 7.2) for 30 minute. Antigen retrieval was performed by microwave treatment in 3% citrate buffer (Roth; pH 6.0).

After washing in PBS, the sections were incubated in PBS containing 1% bovine serum albumin (Serva, Heidelberg, Germany) for 30 min followed by incubation with a polyclonal rabbit antibody (courtesy of J. Klug, Justus Liebig University, Marburg, Germany) directed against (CC10) diluted 1:3,000 in the same solution for 1 hour at 37 °C.

Sections were then incubated with an anti-rabbit secondary antibody diluted 1:10 for 30 minute at room temperature. This was visualised using 3,3' diaminobenzidine as chromogen according to the ABC method (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA, USA) and following the manufacturer's instructions. All sections were counterstained with hematoxylin and eosin (H&E).

A quantitative analysis was performed by drawing manually a line of 1000µm over basement membrane using the computer mouse, and stained Clara cells were counted. The index of CC10/ml basement was used to compare between CRAMP deficient as parameter for regeneration (Figure4.5.1) .

3.4.2 Elastase-induced pulmonary emphysema in mouse

All mice used in this study were male, from SVJ129 background, 6- to 8-weeks old and maintained in our animal facilities under specific pathogen-free conditions. The CRAMP-deficient and their wildtype mice were anesthetized and given an intratracheal instillation of 70 U/Kg elastase (Sigma, Steinheim, Germany) in 0.08 ml

of sterile PBS or 0.08 ml of PBS alone served as controls.

This dose of elastase was repeated after 10 days, this two doses of elastase were given to both CRAMP deficient and their wildtype mice (n=6-8 mice in each group). On the day 30th from the first dose of elastase administration, mice were anesthetized by i.p. injection of 50 mg/kg body weight ketamine hydrochloride (Ketanest, Parke Davis; Berlin) and scarified.

Then tracheas were exposed and cannulated, then the cannulated tracheas with lung were removed together and inflated with 6% paraformaldehyde perfusion with steady flow for 20 minutes. The lungs were cut in the same orientation after they were embedded in agarose to keep the distance between septa of airway space without change.

Then lung tissue sections were dehydrated and embedded in paraffin. Sections of 2-µm thick were stained with H&E. Air space enlargement was quantified by the mean linear intercept (Lm) in 20 randomly selected fields of lung tissue sections [134].

Calculation of mean linear intercepts (Lm), an estimate of the average distance between the opposing walls of a single alveolus was briefly performed as following: the photomicroscopic images were taken using digital sight (Olympus, Germany).

Horizontal and vertical lines were drawn in the images, and intercepts of airway walls with these lines were measured and length of mean linear intercept was estimated by software (Figure 3.4.1).

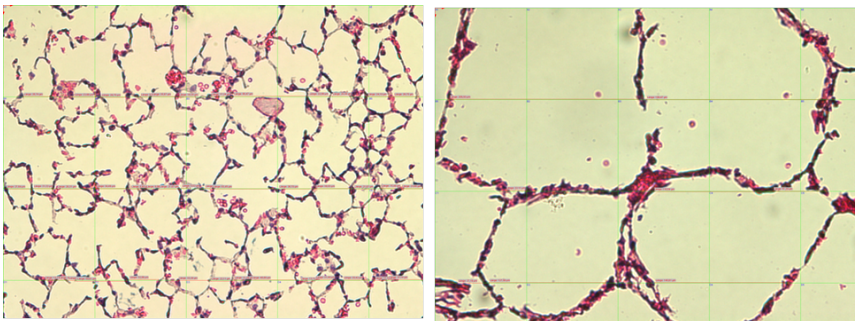


Figure 3.4.1: **Air space enlargement was quantified by the mean linear intercept (Lm).** Representative histological sections hematoxylin/eosin-stained (x 40). Left: mouse lung treated with PBS. Right: mouse lung treated with elastase. Emphysematous lung (right) shows air space enlargement.

3.4.3 Bronchoalveolar lavage of elastase-induced pulmonary emphysema in mice

After variant times 12 hours, 3, 6, 30 days of elastase administration in mice, the bronchoalveolar lavage fluid (BALF) was performed.

In brief, the tracheas were exposed and cannulated, then lungs were lavaged with 3 ml (three times with 1ml each time) of PBS, and BALF was centrifuged at 1500 rpm for 15 minutes, and the supernatants were stored at -20°C until ELISA analysis.

The cells pellets were resuspended with 3 ml PBS and total cells in BALF were counted by cells counter (GAZY, Germany). Cytospin specimens were obtained by centrifugation at 500 rpm for 10 minutes and cytospin slides were stained with Giemsa and the inflammatory cell fractions were evaluated by standard light microscope.

3.4.4 Statistical analysis

For all experiments, at least triplicate determinations were made for each experimental condition. In indicated cases, the results of representative experiments were shown. All data are expressed as mean and standard deviation (SD).

Comparisons between experimental groups were performed using Student's t test and ANOVA test. Results were considered statistically significant for P values less than 0.05.

4 Results

4.1 LL-37 decreases the release of proinflammatory mediators from activated neutrophils

Cathelicidin modulates the response of monocytes / macrophages to endotoxin [138]. To investigate whether this effect of cathelicidin is also effective on neutrophils, we added 100 ng / ml LPS to human neutrophils perincubated with the human cathelicidin LL-37 in different concentrations.

The release of the proinflammatory cytokines IL-1ß, IL-6, IL-8, and TNF-α was significantly decreased in presence of LL-37 as compared to the samples stimulated with LPS in absence of LL-37 (Figure 4.1.1) This effect of LL-37 was dose dependent.

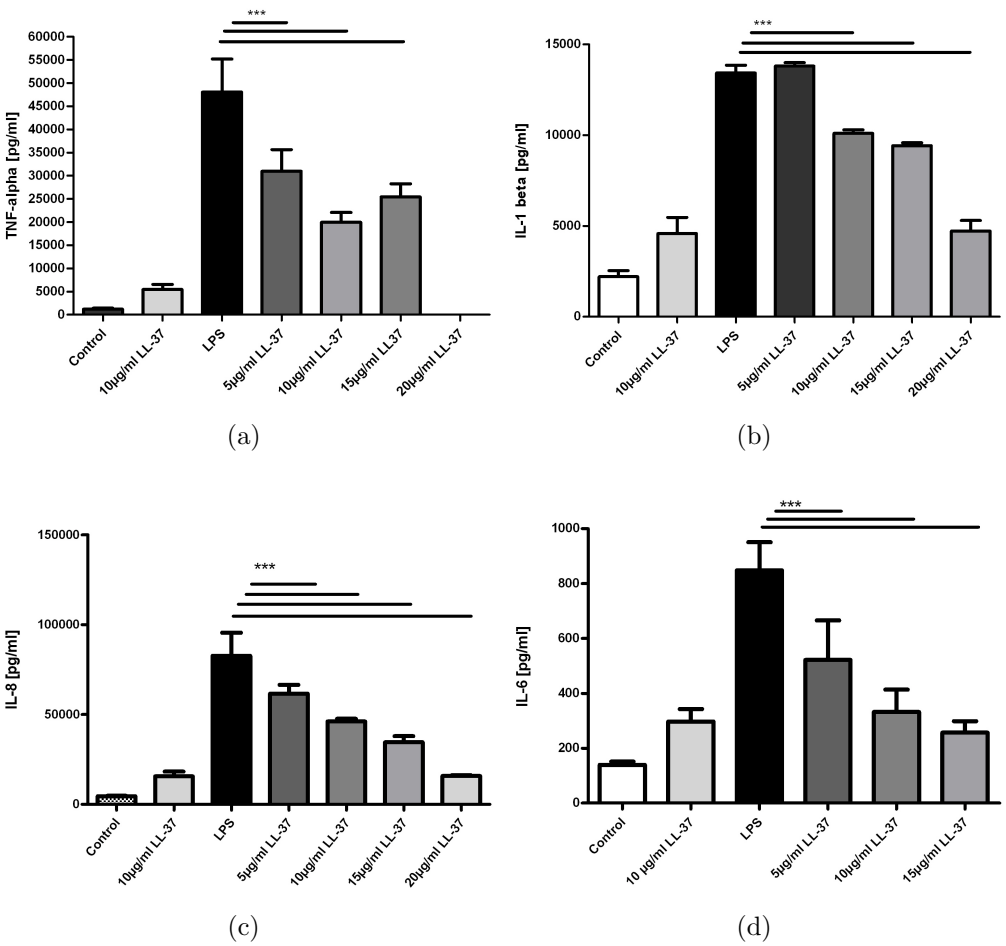
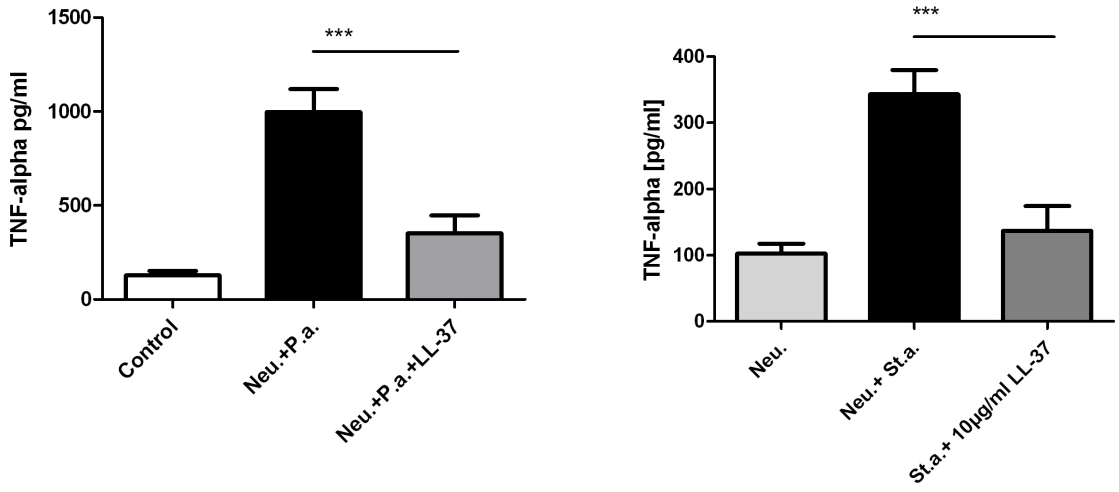


Figure 4.1.1: **LL-37 modulates inflammatory reactions of neutrophils in response to LPS.** LL-37 at the indicated concentration reduces the release of TNF-α (A), IL-1ß (B), IL-8 (C), and IL-6 (D) after stimulation with 100 ng / ml LPS. Cytokine concentrations were determined 12 hours after stimulation. * = $p < 0.05$, n = 6.

These data show that LL-37 modulates the response of neutrophils to microbial patterns. To investigate whether the effect of LL-37 is also effective when whole bacteria are used to stimulate neutrophils, we incubated cells with heat inactivated gram-negative *Pseudomonas aeruginosa* or gram-positive *Staphylococcus aureus*. TNF-α in the supernatant was quantified by ELISA Figure 4.1.2 shows that the secretion of this cytokine was significantly decreased in presence of LL-37.



(a) (b)

Figure 4.1.2: **LL-37 decreases the release of proinflammatory mediators in response to whole bacterial.** (A) Neutrophils were incubated with 104 CFU/ml of *Pseudomonas aeruginosa* (P.a.) with or without 20 µg / ml LL-37 and TNF-α was measured after 12 hours. * = $P < 0.05$, n = 6. (B) Neutrophils were incubated with *Staphylococcus aeurus* (Sta. a.) with or without 20 µg / ml LL-37 and TNF-α was measured after 12 hours. * = $P < 0.05$, n = 6.

4.2 LL-37 amplifies neutrophil ROS production

It has been demonstrated that LL-37 triggers ROS production in human neutrophils [12]. We assessed ROS production in response to PMA using luminometry and flow cytometry. Time course of ROS levels were performed by chemiluminescence (Figure 4.2.1) and the ROS levels at 30 minutes were chosen for comparsion.

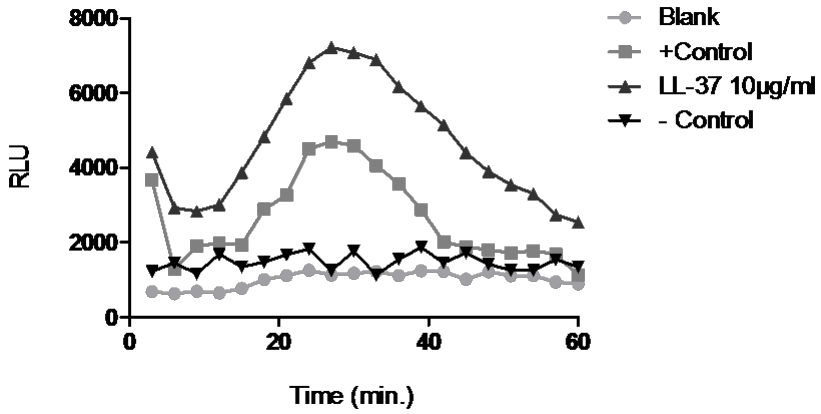


Figure 4.2.1: **Time course of ROS levels in human neutrophils trigged by PMA.** ROS levels were measured in human neutrophils (2 x 10^6cells/ml) by the luminol chemiluminescence method after PMA application at 37 °C for 60 minutes. The respiratory burst was triggered by application of 100 nM PMA. (Blank = no cells, – Control = cells without PMA triggering; + Control = cells + PMA). Time course revealed that the highest levels of ROS were at 30 min.

LL-37 significantly increased the ROS production in neutrophils triggered by PMA in a dose dependent way as determined by luminol chemiluminescence (Figure 4.2.4). The results of this assay were perform at 37°C.

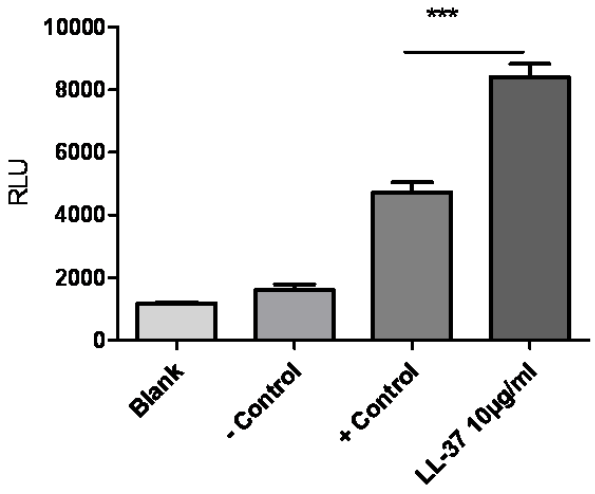


Figure 4.2.2: **LL-37 amplifies ROS production in human neutrophils by chemiluminescence method.** ROS levels were measured in human neutrophils (2 x 10^6 cells/ml) by the luminol chemiluminescence method after PMA application. The respiratory burst was triggered by application of 100 nM PMA. Blank = no cells, – Control = cells without PMA triggering; + Control = cells + PMA . Result reveals that the production of ROS triggered with PMA was significantly amplified with LL-37 and this activity was dose-dependent. ANOVA with Tukey's test; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$..

The amplification of ROS by LL-37 was also demonstrated using flow cytometry measuring the fluorescence intensity of DCFDA-loaded neutrophils for 30 minutes. The levels of ROS were significantly increased in the presence of LL-37. This effect of the peptide was dose-dependent. sLL-37 was used to exclude a nonspecific effect of LL-37 and which had no effect on ROS production (Figure 4.2.3, 4.2.4).

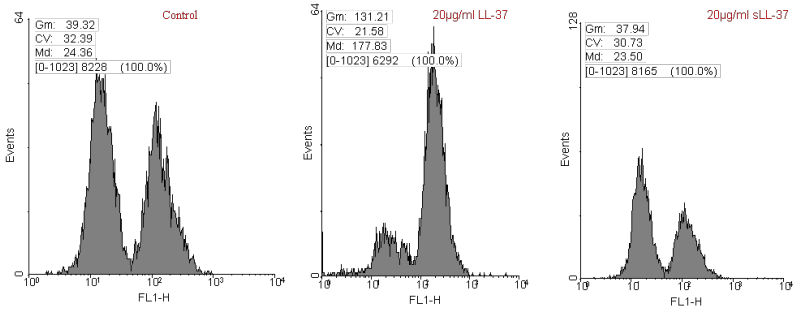


Figure 4.2.3: **ROS release detected by Flow cytometry; representative histograms.** FACS analysis shows differences in fluorescence intensity between neutrophils without LL-37 treatment (control) and neutrophils pretreated with LL-37 and both were treated with PMA to trigger ROS release.

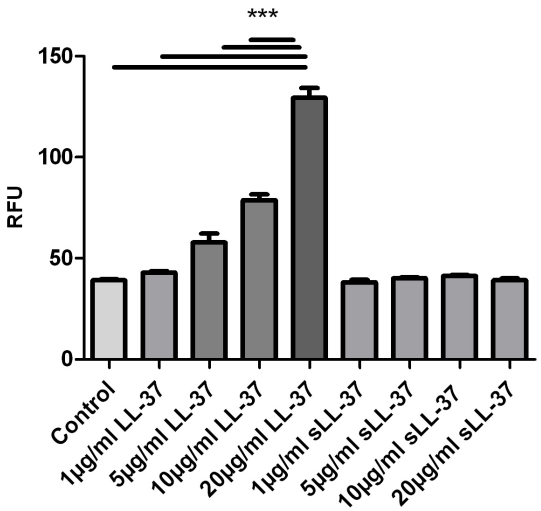


Figure 4.2.4: **LL-37 amplifies ROS production in neutrophils measured by flow cytometry.** ROS levels were measured in 2 x 10^6cells / ml neutrophils by flow cytometry after incubating neutrophils with DCFDA (1 hour after PMA application). LL-37 amplified significantly the triggered respiratory burst of human neutrophils induced by 100 nM PMA and this amplification was dose-dependent. sLL-37 had no effect. Control = cells without pretreatment with LL-37 + PMA. * = $P < 0.05$, n = 6.

The presence of serum (fetal calf serum) partly inhibited the effect of LL-37

(Figure 4.2.5). PMA is a stimulus that needs to pass the plasma membrane in order to activate PKC.

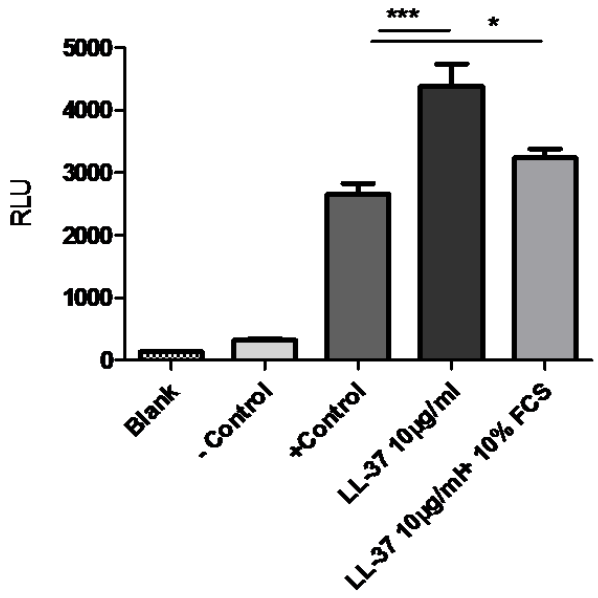


Figure 4.2.5: **The effect of serum on LL-37 activity as ROS amplifier.** ROS levels were measured in 2 x 10^6 cells / ml human neutrophils by luminol chemiluminescence method. ROS was triggered by opsonised 10^4CFU/ml *Staphylococcus aureus*. Results reveal that LL-37 amplified significantly ROS levels. n = 6; ANOVA with Tukey's test; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Thus LL-37 could potentially induce the uptake of PMA. In order to demonstrate that LL-37 also enhances ROS production induced by other agents, we exposed neutrophils to whole bacteria. LL-37 significantly increased the ROS production after bacterial stimulation (Figure4.2.5). To reveal the activity of endogenous cathelicidin we isolated peritoneal neutrophils from wildtype and CRAMP-deficient animals using the thioglycollate method as described in the method section. We found that after application of PMA, neutrophils from CRAMP-deficient animals had significantly lower levels of ROS (Figure 4.2.6).

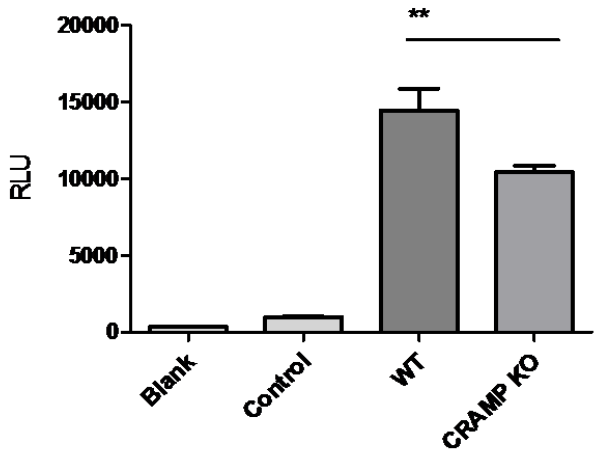


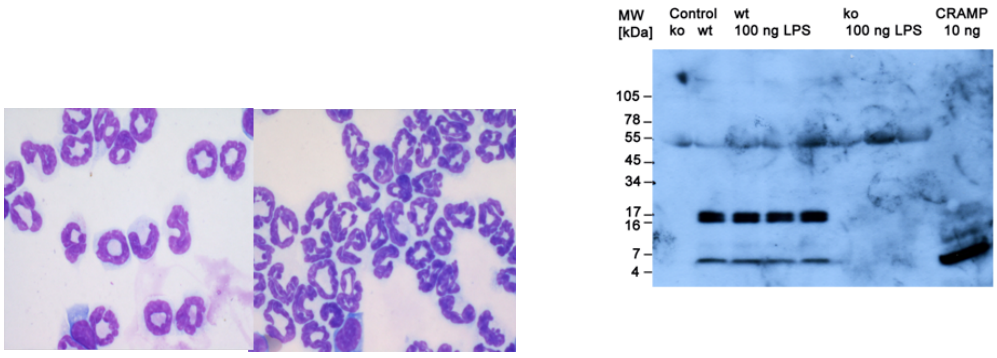
Figure 4.2.6: **Cathelicidin amplified ROS in mouse neutrophils.** Neutrophils were isolated from CRAMP-deficient animals and ROS levels were measured by the luminol chemiluminescence method after PMA application at 37 °C. results revealed that ROS in neutrophils from wild type mice was significantly higher than CRAMP-deficient animals. n = 6; ANOVA with Tukey's test; ** = $P < 0.01$.

LDH release was determined as marker of cytotoxicity and no increased levels were detected up to 30 µg/ml LL-37 (data not shown).

4.3 Endogenous murine cathelicidin CRAMP modulates neutrophil function

CRAMP is the homologous molecule of LL-37 in mice [61]. Based on the peptide's structure, expression pattern and biological activity, this gene and its encoded product CRAMP serve very similar functions as the human counterpart. To test whether endogenously expressed cathelicidin has similar effects as exogenously applied peptide, peritoneal neutrophils were isolated from CRAMP-deficient mice (Figure 4.3.1).

And to determine whether CRAMP is released from neutrophils, we performed Western blotting on cell supernatants and found the precursor and the cleaved peptide in supernatants from stimulated and native cells (Figure4.3.1). And after stimulation with LPS, neutrophils from CRAMP-deficient animals showed significantly increased release of TNF-α (Figure 4.3.2).



(a) (b)

Figure 4.3.1: **Neutrophils isolated from peritoneal cavities of mice.** (a) Cells isolated from mouse´s peritoneal cavity and cytospin was performed and Giemsa-stained slides shows that >95% was neutrophils. (b) western blot was performed for suppernatants form CRAMP-KO and wild type neutrophils suspensions, bands reveals that CRAMP-KO mice did not release CRAMP as wildtype mice did.

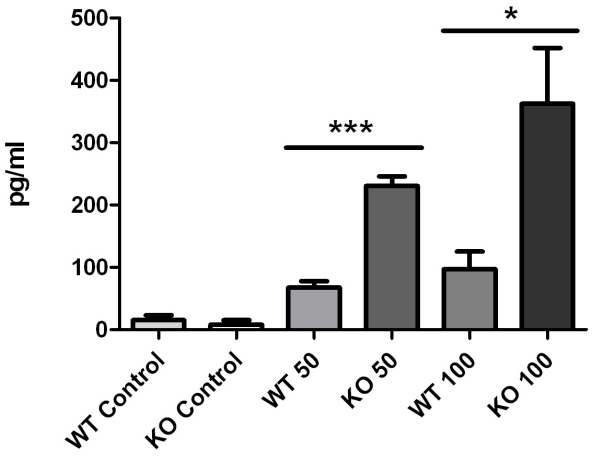
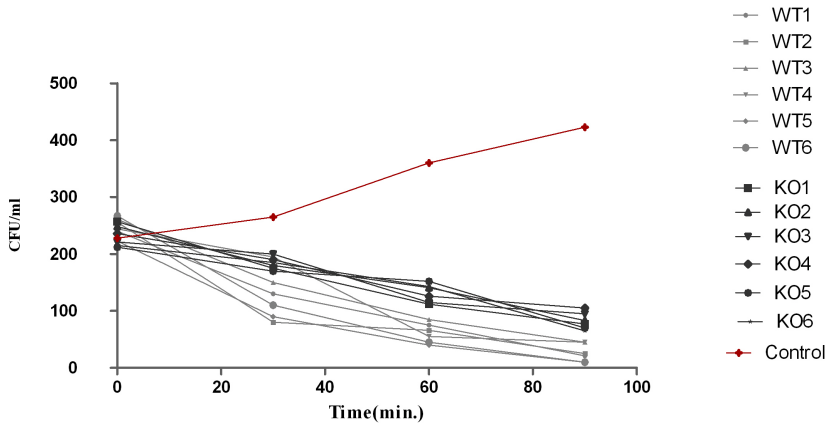


Figure 4.3.2: **Endogenous cathelicidin modulates the neutrophil innate immune reaction.** Neutrophils were isolated from CRAMP-deficient animals, stimulated with LPS (50 / 100 ng / ml), and the release of TNF-α was measured in supernatants. Neutrophils from CRAMP-knockout animals released significantly higher amounts of the cytokine as compared to the cells from wildtype animals.

To test whether this suppression of inflammatory activation is associated with a breach in antimicrobial activity, we performed bacterial killing assays applying viable P. aeruginosa to neutrophils isolated from CRAMP deficient and wildtype control mice.

We found that neutrophils from animals deficient in CRAMP have a significantly

decreased antimicrobial activity (Figure 4.3.3). These data show that endogenous cathelicidin modulates the response of neutrophils in responses of the innate immune system.



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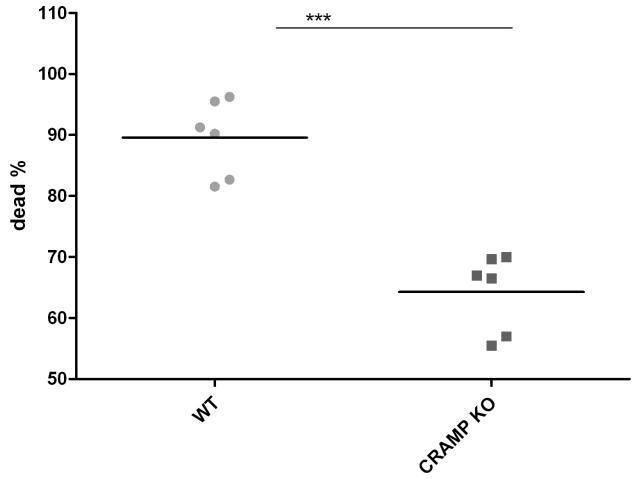


(b)

Figure 4.3.3: **Neutrophils from CRAMP-deficient animals showed a breach in their innate host defense.** (a) Neutrophils were incubated with *Pseudomonas aeruginosa* (1 neutrophil WT/KO : 1 bacterial cell) and the numbers of viable bacteria were analyzed by sampling 20 µl and plating on nutrient agar 30,60 and 90 min after bacterial inocculation. Control, bacteria incubated with all experiment reagents in absence of neutrophils.Control shows increasing of bacteria count (bacterial growth) in opposite of samples contain neutrophils. (b) neutrophils from CRAMP-KO mice shows significantly lower bactericidal activity than neutrophils from wildtype mice .* = $P < 0.05$, n = 6.

4.4 Cathelicidin improves phagocytosis of human neutrophils

As mentioned in 4.3, the physiological presence of CRAMP improved significantly the bactericidal activity of neutrophils. we wanted To test whether cathelicidin modulates the rate of phygocytotic uptake of bacteria into neutrophils, a critical step in killing of microorganisms [54]. The phagocytosis of neutrophil was investigated in presence of different concentrations of LL-37 by microscopic and flow cytometric methods. microscopic assays [29] were performed to investigate whether LL-37 modulates the uptake of bacteria into neutrophils. The cathelicidin peptide significantly increased the uptake of bacteria into neutrophil cells in a dose-dependent manner (Figure 4.4.1). These data showed that exposure to the cathelicidin peptide increases host defense activities such as ROS production and engulfment of bacteria. The result of the microscopic method showed that the mean numbers of bacteria (bound and ingested) in neutrophils treated with LL-37 was significantly higher than non treated neutrophils (Figure 4.4.1).

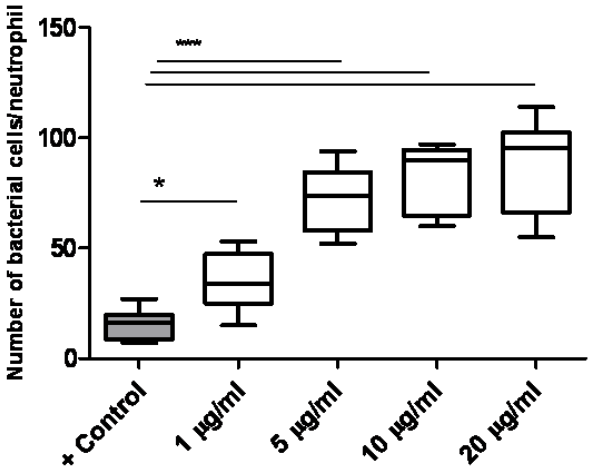


Figure 4.4.1: **Microscopic method to investigate the phagocytic activity of Neutrophils.** Microscopic evaluation in presence or absence of LL-37 is expressed as mean numbers of bacteria (bound or ingested)/ Neutrophil cell + SEM of three to six independent experiments.

Flowcytometric assay reveals also that LL-37 improves significantly phagocytic activity of human neutrophils (Figure 4.4.2, 4.4.3).

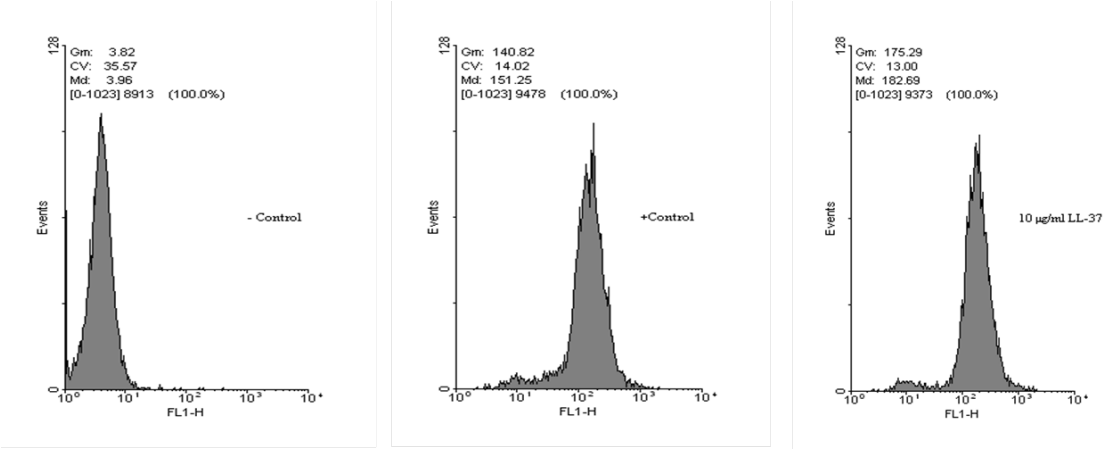


Figure 4.4.2: **Phagocytic activity of human neutrophils measured by FACS; representative histograms.** Human neutrophils were inactived by treatment with paraformaldehyde served as -control and active neutrophils without pretreatment with LL-37 served as +control.

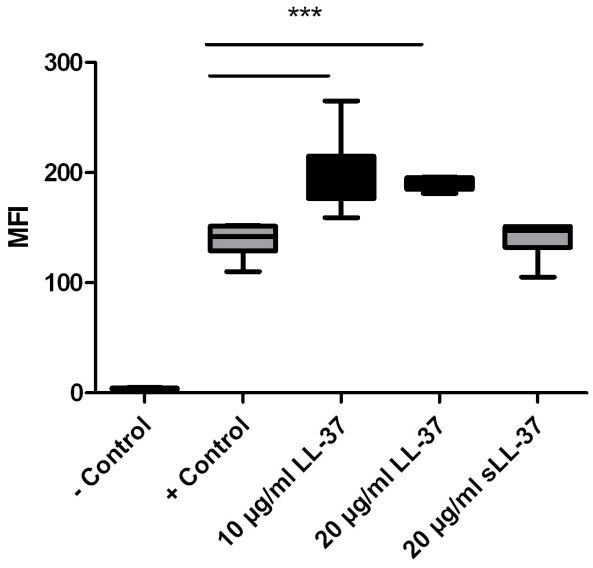


Figure 4.4.3: **Evaluation of neutrophil phagocytic activity by flow cytometric method.** Negative control, 10^5CFU/ml of opsonized and FITC-labeled *Staphylococcus aureus* were incubated with inactive human neutrophils. Positive control, 10^5 CFU/ml of opsonized and FITC-labeled bacteria were incubated with active human neutrophils. Three groups, pretreated human neutrophils with different concentrations of LL-37 (10 and 20µg/ml) incubated with 10^5 CFU/ml of opsonized and FITC-labeled *Staphylococcus aureus*. one group, pretreated human neutrophils with sLL-37 was used to exclude a nonspecific effect of LL-37 incubated with opsonized and FITC-labeled *Staphylococcus aureus.* Each assay was performed in duplicate and all results were expressed as the mean + standard error of the mean of fluorescence index. Four to eight independent assays were performed.

The results from both methods showed that the up take of bacteria by neutrophil

was increased by adding LL-37 and this function of LL-37 was dose-dependent.

4.5 CRAMP enhances lung tissue repair

To examine whether CRAMP in lung tissue enhances healing in airway, selective injury was induced to mouse nonciliated bronchiolar (Clara) epithelial cells with naphthalene 200 mg/kg body weight or corn oil alone with equivalent volume (control). 1, 5 and 15 days later, mice were sacrificed and histological sections were used for immunohistochemistry using antibodies directed against Clara (CC10 protein).

The proliferation was evaluated by counting Clara cells in one millimetre of basement membrane depending on the expression CC10 as marker for differentiated Clara cells. Sections showed that airway Clara cells were exfoliated after one day of naphthalene administration (Figure 4.5.1). After 5 and 15 days, the regeneration of lung tisssue was evaluated by measuring the density of Clara cells in one millimeter of basement membrane. The result showed that the Clara cells density in airway sections of CRAMP-knockout was significantly lower than wild type mice 15 days after naphthalene administration (Figure 4.5.2, 4.5.3), it means that the healing of lung tissue was signifigantly decreased in the absence of cathelicidin..

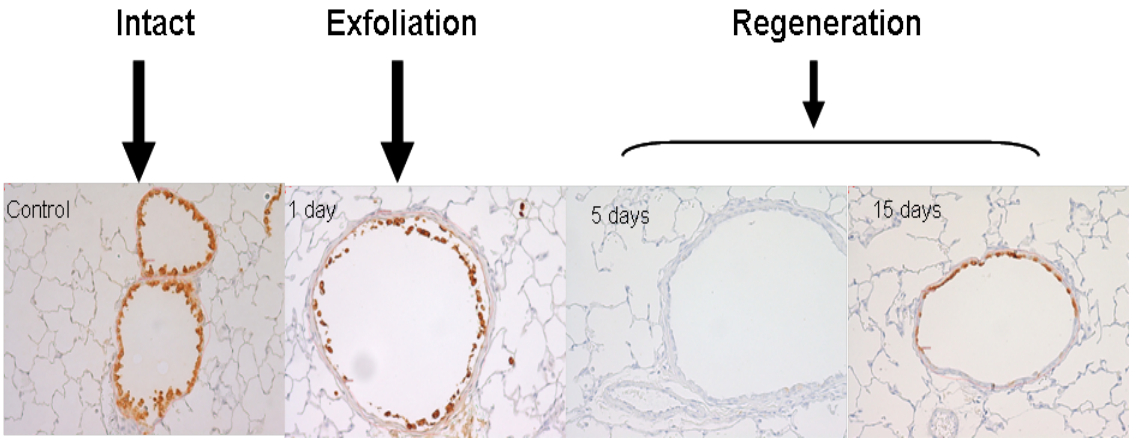


Figure 4.5.1: ***In vivo* naphthelene model and Clara cells regeneration.** Moue lung sections from naphthelene model were CC10 stained (immunohistochemistry) show how Clara cells are intact in control samples and after one day from naphthelene injection Clara cells were Exfoliated and regeneration started. adapted from experiments of this work.

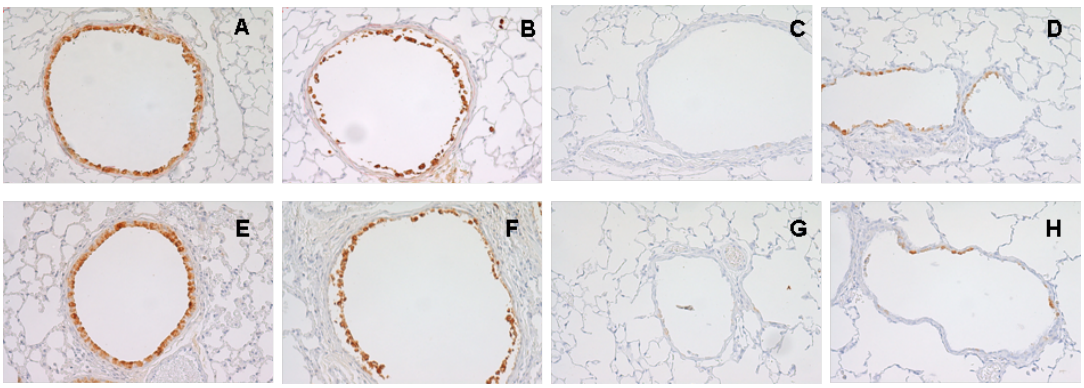


Figure 4.5.2: **Immunohistochemical staining with anti-CC10 antibody shows expression of the clara cell marker protein CC10 was observed in distal regions of the airways.** (A) Wild type control mice after intraperitoneal injection of only corn oil vehicle (WT Control). (B) clara cell-ablated WT mouse one day after intraperitoneal injection of naphthalene (C) clara cell-ablated WT mouse 5 days after intraperitoneal injection of naphthalene. (D) clara cell-ablated wild type mouse 15 days after intraperitoneal injection of naphthalene. (E) CRAMP knockout mice control after intraperitoneal injection of only corn oil vehicle (KO Control). (F) clara cell-ablated CRAMP- knockout mouse after one day intraperitoneal injection of naphthalene. (G) clara cell-ablated CRAMP- knockout mouse after 5 days intraperitoneal injection of naphthalene. (H) clara cell-ablated CRAMP- knockout mouse after 15 days intraperitoneal injection of naphthalene.

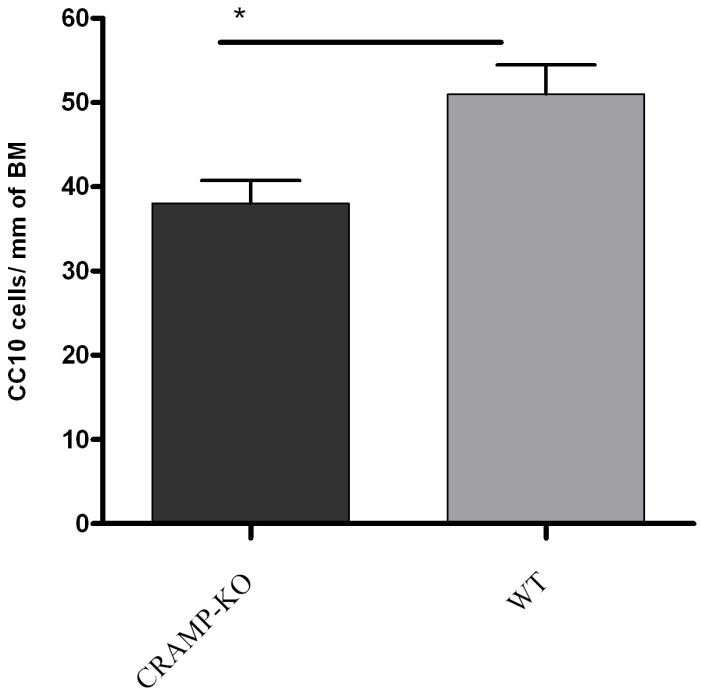


Figure 4.5.3: CRAMP enhances repair in mouse lung tissue. Morphometric analysis of CC10-positive cells in wild type and CRAMP knockout mice after 15 days of naphthalene intraperitoneal injection showed that CRAMP increased significantly regeneration of Clara cells in mice. Results are expressed as the means numbers of positive CC10/ 1000µm of bassment membrane mean and SD (n=6 animals per group and time period).

4.6 Cathelicidin protects from pulmonary emphysema induction

The elastase-induced emphysema model has been used in research for more than 30 years and has brought considerable insight into the pathogenesis of human disease [81]. Intratracheal instillation of elastase induced pulmonary emphysema in mice, which was recognized by lung appearance and microscopic measurement of alveolar erlargement (Figure 4.6.2).

Mice from the elastase groups (WT and CRAMP-KO) had emphysematous lung form (see photo Figure 4.6.2), and the mean linear intercept for WT mice was (85,51 ± 3,15µm, n=8) and for CRAMP-KO was (111,7 ± 6,850µm, n=8) markedly increased compared with the PBS groups WT (33,68 ± 3,117 µm, n = 6; P = 0.0001) and for CRAMP-KO (33,17 ± 1,5 µm, n = 6; P = 0.0001).

These results revealed that the presence of cathelicidin minimized significantly induction of pulmonary emphysema in mice. (Figure4.6.2).

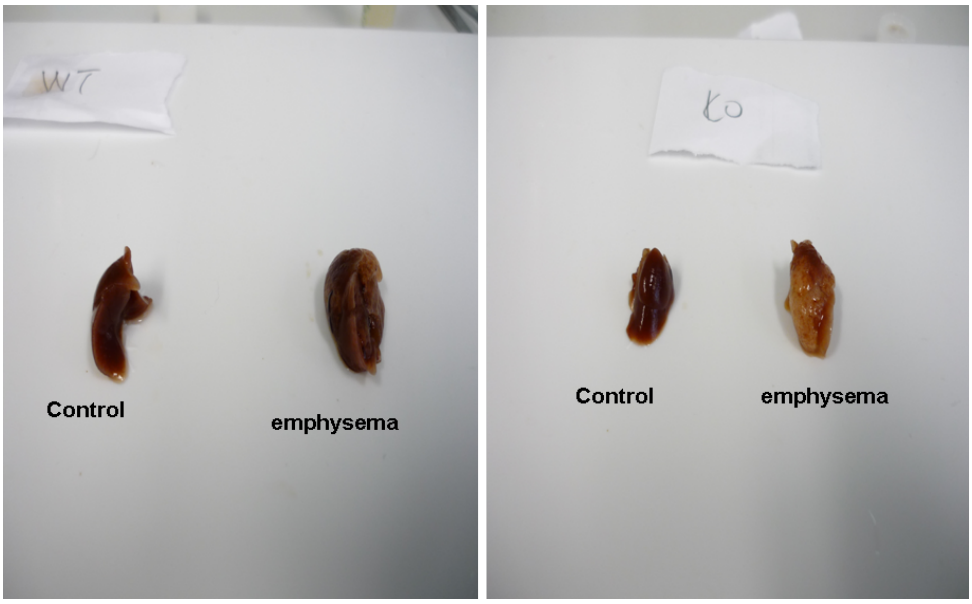
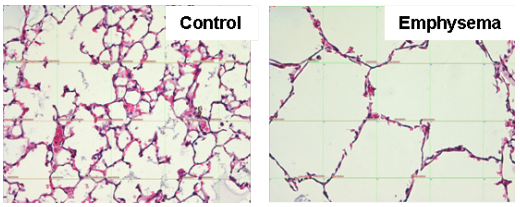


Figure 4.6.1: Picture of lungs from CRAMP-KO and WT mice. Some groups of mice were treated with PBS (control) and other groups were treated with elastase (emphysema) and these pictures showed that the lungs from groups were treated with elastase had emphysematous appearance.



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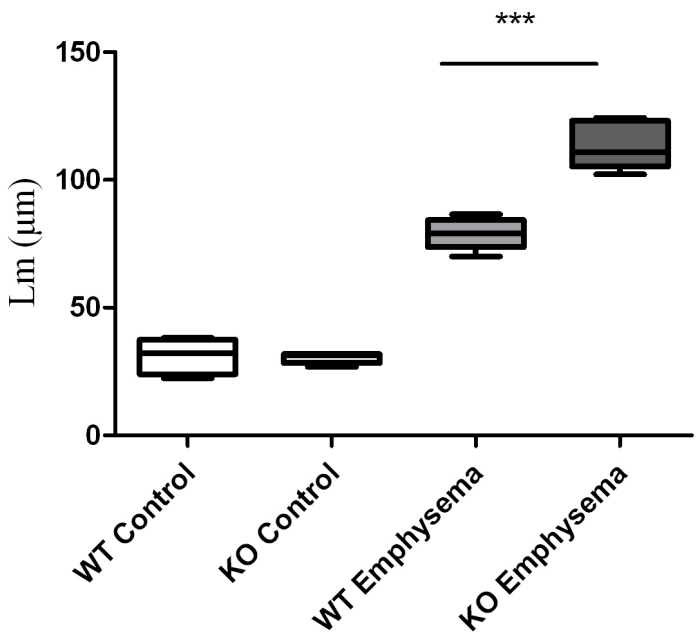

(b)

Figure 4.6.2: **Mean linear intercept (Lm) for emphysema and control mice reveals that cathelicidin presence protects from elastase- induced pulmonary emphysema in mice.** (a) Histological sections stained hematoxylin and eosin (H and E) shows mean linear intercept (Lm) for emphysema and control mice after 1 month of elastase or PBS treatment. Lm was measured at the same magnification x400 demonstrates airway spaces erlagement in lungs of elastase groups.(b) Lm expressed as mean and SD for the whole lungs. (n=8 animals per group and time period).

To investigate whether the anti-inflammatory activity of cathelicidin is responsible of reducing elastase- induced pulmonary emphysema. BALs were collected from (WT and CRAMP-KO) mice after 12 hours, one, 3, 6 days and one month from intratracheal instillation of elastase. Total count of cells per one milimetre and inflammatory cytokine TNF-α in BALs were measured. The results showed that the total number of cells and the concentration of TNF-α in response to intratracheal instillation of elastase were significantly higher in BAL of CRAMP-KO mice than WT mice (Figure 4.6.3).

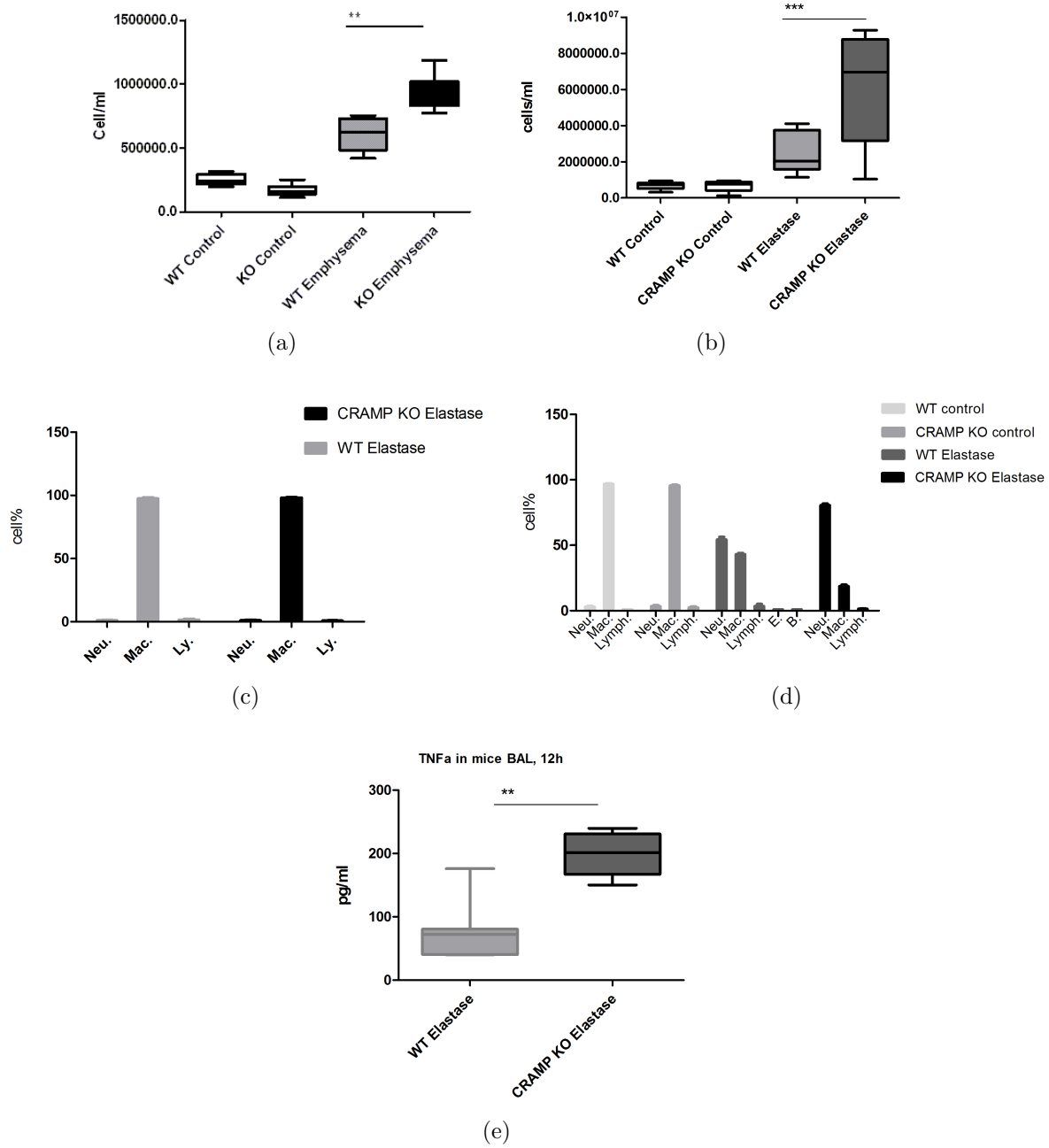

Figure 4.6.3: **The anti inflammatory activity of cathelicidin could be the reason of reducing the induction of pulmonary emphysema in mice.** (a) Total cells number in BAL of CRAMP-KO mice are significantly higher as compared to WT mice after 30 days of treatment with two doses of elastase. (b) The total cells number in BAL of CRAMP-KO mice are significantly higher than WT mice after 3 days of treatment with one dose of elastase. (c) Differential cell count of BALs from CRAMP-KO and WT mice after 30 days of treatment with two doses of elastase. Result revealed that the majoraty of cells was macrophages. (d) Cells differentiation of BALs from CRAMP-KO and WT mice after 3 days of treatment with one doses of elastase. Result revealed that the majoraty of cells was neutrophils and the neutrophils ratio in BALs of CRAMP-KO mice were significantly higher than WT mice. (e) shows that the release of TNF-α detected in BALs from CRAMP-KO mice teated with elastase to induce emphysema was significantly higher than WT mice. All results expressed as mean and SD. (n=6 animals per group).

5 Discussion

The main findings of the present study are: (1) modulating the inflammatory reaction of neutrophil to avoid the exaggerated reaction of neutrophils in response to bacteria or their commponents; (2) improvement of bactericidal activity of neutrophils by amplifying ROS release and phagocytosis; and (3) provide protective action against pulmonary emphysema and acute lung injury. In brief, this findings revealed cathelicidin as homeostasis of inflammation and repair processes.

5.1 Cathelicidin modulates inflammatory responses of neutrophils

One finding of the present study is the activity of the cathelicidin peptide LL-37 to modulate the activation of neutrophils stimulated by bacterial patterns. Exogenously applied peptide as well as endogenously, physiologically expressed peptide have this effect on neutrophils. These data support the role of cathelicidin peptide in the modulation of innate immune responses.

Cathelicidin peptides are known to impact on inflammatory mechanisms relevant in infection and sepsis [7, 161]. Peptide application and overexpression of the peptides' genes have been used in various sepsis models and demonstrated that cathelicidins inhibit overreactive responses to microbial patterns [138, 12, 161, 83].

The underlying mechanisms likely involve the modulation of the responses of host defense cells to microbial patterns. While it has been already known that cathelicidin modulates the response of macrophages [138, 106] and DCs [80] to TLR-ligands, the effect on neutrophil activation has been less clear. Our results show that cathelicidin modulates the response of neutrophils to endotoxin and to whole bacteria.

Releasing of a various proinflammatory mediators (IL-1ß, IL-6, IL-8, and TNF-α) was significantly decreased suggesting that the peptide protects from the development of a cytokine storm.

This effect was also detectable when whole Gram-positive and –negative bacteria were applied, indicating that the cathelicidin's effect was also effective in the interaction of neutrophils with biologically relevant microorganisms. Interestingly, also endogenous cathelicidin had this modulatory effect on cytokine release as shown in the experiments using neutrophils from CRAMP-deficient mice.

The study of Zheng et al. [185] found increasing IL-8 release at high concentration of LL-37 (20 and 40 µg/ml). This study did not investigate the combination

of LPS and LL-37. In the present study, we did not find an increased release of cytokines at concentrations of 5-20 µg/ml or evidence of cell lysis as measured by LDH release.

5.2 Cathelicidin presence amplifies bactericidal activity of neutrophil

In addition to the release of inflammatory mediators, cathelicidin modulates other cellular processes including cell death pathways and the production of ROS. Cathelicidin has a complex effect on neutrophil apoptosis. Cathelicidin modulates apoptosis via the receptors formyl peptide receptor like 1 (FPRL1) [112] and P2X7 [16], while different methodology revealed induction of secondary apoptosis [184].

Cathelicidin is known to interfere with the pathways that regulate the production of ROS in myeloid cells including macrophages [186] and neutrophils [185]. We confirmed these observations that application of LL-37 results in amplification of PMA-triggered ROS production. ROS are considered important effector molecules in the direct or indirect bactericidal activities of neutrophils [41]. The significant decreasing of ROS production in CRAMP-deficient animals emphasizes the role of the endogenous peptide in this process.

We therefore investigated whether the presence of cathelicidin is necessary for the antimicrobial activity of neutrophils and found that CRAMP-deficient neutrophils revealed significantly less antibacterial activity. While these data don't discriminate between different antimicrobial pathways of neutrophils, they show that cathelicidin increases antimicrobial activities of neutrophils while decreasing the release of proinflammatory mediators.

Phagocytosis assay also reveal increased uptake of bacteria into neutrophils as like as what was shown by Davidson study that LL-37 modified their phagocytic function in dentritic cells [43]. It might be that phagocytosis improvement of human neutrophils after addition of LL-37 was related to bactericidal of CRAMP or it could be possible that cathelicidin may prime neutrophils to face bacteria by increasing their phagocytic activity. ROS- production and antimicrobial activity were dependent on the endogenous peptide as demonstrated in the experiments with murine cells.

Since the precursor and active form are released by neutrophils it is difficult to speculate about the site of activity. In the living organisms, cathelicidin peptide from different sources could contribute to the described effects on neutrophils. Several

cell types produce the peptide, including epithelial [11] and immune cells [2]. Also peptide synthesized in and secreted from neutrophils likely contributes.

The peptide's effect of ROS production is partly inhibited by serum components. This could indicate that the described mechanisms is not active in the blood, however relevant for neutrophils that have immigrated into tissue and body surfaces.

The mechanism how cathelicidin modulates the function of myeloid cell types is largely unclear. The peptide is capable to bind to LPS and decreases the detection of this pattern by the receptor complexes [111, 133].

Additionally, the peptide likely interacts with cellular receptors, including FRPL1 [44], P2X7 [51], or epidermal growth factor receptor (EGFR) [157], with possible downstream effects on inflammatory pathways [106].

The effectiveness of an all-D-form of the peptide together with observations of altered cell membrane function and structure implies a more complex mechanisms including interaction with biomembranes and modulation of the function underscoreof membrane associated proteins [45].

Based on the current study and former data it is still difficult to shortly summarize the role of cathelicidin in host defense immunity. In vitro studies revealed a direct antimicrobial activity [3, 11] that is supported by the results of infection models in CRAMP-deficient animals [38, 118].

Cathelicidin interacts with a number of cell types such as endothelial cells [87], epithelial cells [157], mast cells [117], macrophages [138], and DCs [43, 80]. As shown in the present study, cathelicidin modulates neutrophil functions by suppressing the release of proinflammatory mediators and increasing antimicrobial activity.

Animal studies showed that cathelicidin protects from exaggerated inflammatory reactions [83], however, also has proinflammatory roles in skin [173]. Based on the current data, cathelicidin is an effector molecule of innate immunity with diverse functions, including antimicrobial activity and modulation of wound repair and inflammation.

5.3 Cathelicidin enhances repair of lung epithelial cells

Epithelium covers virtually all barrier organs. It is continuously exposed to external environment, where microbes and secreted microbial products, potential allergens, toxic substances and irritants are present. Epithelial injury is not a rare event. Restoration of the barrier integrity after injury represents a fundamental de-

fense function of epithelia. Epithelial repair process involves a stereotypical sequence of events - epithelial restitution and regeneration [129].

Epithelial restitution starts immediately after injury and is characterized by rapid migration of cells towards the wound area in order to re-establish surface epithelial continuity. An increased migratory capacity of the cells is a result of the de-differentiation process after injury [82] and may be explained by the loss of contact inhibitory signals at the damaged area [55]. Rapid restitution after injury limits fluid and electrolyte losses and prevents pathogens from the lumen getting into local and systemic immune compartments [129].

Various factors initiate the repair process. They include (a) epidermal growth factor (EGF) family members like EGF, transforming growth factor-alpha (TGF-α), heparin binding EGF or amphiregulin [85]; other growth factors like keratinocyte growth factor (KGF) [167], insulin-like growth factor-1 (IGF-1) [142], hepatocyte growth factor (HGF) [178]; (b) inflammatory cytokines like IL-1 beta [67]; (d) extracellular matrix proteins (fibronectin, fibrinogen) and matrix metalloproteinases [119]; and other factors.

Like in many other mucosal surfaces, epithelium of the airways has a great capacity to repair itself after injury, as demonstrated in vitro [82, 177] and in vivo [53].

Immediately (within 5-15 min) after injury, secretory, ciliated and (probably also) basal ECs at the wound edge de-differentiate, spread and migrate across the denuded basement membrane without any external stimulation or non-EC participation at a speed of about 2-3 microns/min [53].

Airway epithelium represents a physiological tissue barrier with two sides and two functions. First, it protects the respiratory compartment of the lung from pathogens by mechanical and chemical defense factors such as mucus, antimicrobial substances, or beating of cilia.

Second, intact airway epithelium protects from developing inflammation in response to microbes present in the airway lumen primarily by preventing the contact of bacteria with subepithelial inflammatory cells (macrophages and DCs) which are very sensitive to any kind of danger.

In addition to antimicrobial activity, several reports indicated that LL-37 is also able to mediate cellular responses that contribute to the immune response and might enhance the wound-healing process [13, 15]. LL-37 was shown previously to induce migration of epidermal cells by transactivating the EGFR [160]. It was also shown

to stimulate bronchial epithelial cell migration and proliferation [141].

It has been shown previously that re-epithelialization of organ-cultured skin wounds is inhibited by antibodies against LL-37 in a dose-dependent mode [73]. The result of this study showed that the presence of cathelicidin physiologically can enhance airway epithelium regeneration after lung injury in mice, which was induced by naphthalene administration.

5.4 Cathelicidin protects from pulmonary emphysema

The biological functions of LL-37 have been extensively studied during the last decade. As a result, many of its non-antimicrobial activities have been identified. The peptide was implicated in chemotaxis of inflammatory and immune cells including neutrophils, monocytes, lymphocytes [44], eosinophils [158] and mast cells [116]. LL-37 has been also shown to regulate tissue homeostasis: it stimulated regeneration of skin wounds [73], induced angiogenesis [87], increased growth of breast [72] and lung cancer cells [166].

For pathpogensis of pulmonary emphysema, it is possible that the destruction of the lung parenchyma in pulmonary emphysema may be a result of chronic unopposed oxidant stress, unopposed proteolysis, apoptosis, impaired clearance of apoptosed cell bodies [165], cellular senescence without adequate cell replacement [121], or a switch from an innate toward an autoimmune response [154].

An interesting finding of this study is that cathelicidin reduced elastase-induced pulmonary emphysema in mice, which was demonstrated using morphometric parameter called airway linear intercept (Lm) to compare between lungs from CRAMP knockout and wild type mice.

Cathelicidin could minimize pulmonary emphysema induction in mice through firs either its anti-inflammatory function [161, 138, 83], where it is known that inflammatory mechanism has essential role in inducing pulmonary emphysema [52, 4],or its anti-protease activity [179], so it inhibits elastase activity which in turn reduces lung injury- induced pulmonary emphysema. Third the cathelicidin activity enhances airway epithelium repair [73], as shown above (5.3) where the presence of cathelicidin enhanced healing of lung injury.

Taken all together we conclude that the reason of difference in the induction of pulmonary emphysema between wild type and CRAMP- deficient mice could be related to one or all of cathelicidin activities mentioned above which participated in reducing emphysema induction. according to previous findings we can hypothese

that the susceptibility toward pulmonary emphysema could be higher in absent of cathelicidin.

5.5 Conclusion

Inflammation is an integral part of reactions of the innate immune system. The innate immune system is responsible for maintaining a functional and physical barrier against microorganisms.

Overactive inflammatory responses often cause harm to the organism. Thus, several layers of regulatory mechanisms modulate the effectors of innate immunity to balance between host defense and inflammation. Neutrophils are one of the most prominent effector cells of the innate immune system [97].

They are recruited to the site of infection and are equipped with various antimicrobial effector mechanisms, whose role in host defense has been demonstrated for many pathogens. Antimicrobial peptides (AMPs) are effector molecules of the innate immune system with direct antimicrobial activity [183].

The defensins and the cathelicidins are the two prototypical families of AMPs expressed in mammals. AMPs are expressed in epithelial and professional host defense cells such as macrophages or neutrophils. In addition to their role as antibiotics, AMPs have diverse activities on various cell types [174]. LL-37/hCAP-18 is the only cathelicidin present in humans and has several regulatory activities [181].

LL-37 is the C-terminal cleavage product that carries the biological activity. The precursor hCAP-18 is found in the specific granules of neutrophils and is expressed in many immune and epithelial cells. Cathelicidin has an established role in host defense [38, 118].

In addition to their direct antimicrobial function, cathelicidin peptides are involved in the modulation of repair and tissue homeostasis: augmentation of angiogenesis by a direct effect on endothelial cells [87], epithelial wound healing [73], chemoattraction of immune cells [13], release of inflammatory mediators from epithelial cells by transactivation of the epidermal growth factor receptor (EGFR) pathway [157].

A number of reports showed that cathelicidins act on myeloid cells. LL-37 significantly up-regulates the endocytic capacity, modifies expression of phagocytic receptors, and increases secretion of Th1-inducing cytokines of dendritic cells (DCs) generated from blood monocytes [43].

LL-37 modulates the response of monocytes and DCs to TLR – ligands [80, 45]. Also effects of cathelicidins on neutrophils have been described. One study showed that LL-37 induces the generation of reactive oxygen species from human neutrophils [185].

Taken together, these findings which are discussed above let us to conclude that the cathelicidin could modulate and control many of the immunological reactions of neutrophils in response to bacteria and their components. In addition to reducing inflammation, cathelicidin improved bactericidal of neutrophils by amplifying reactive oxygen species and increasing phagocytosis.

The present study also showed that cathelicidin in physiological levels enhances airway epithelium repair after acute lung injury in mice induced by naphthalene and protectes from the induction of pulmonary emphysema in mice.

Further studies are undoubtedly necessary to reveal the physiological relevance of these data in order to translate the knowledge about "Cathelicidin as an immunological modulator" into clinically meaningful information.

This might be important for further progress in the development of novel therapeutic and diagnostic approaches to diseases (lung infection, acute respiratory distress syndrome and COPD), which are associated with concomitant immune dysregulation and epithelial tissue injury.

List of Figures

List of Tables

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List of Abbreviations

AMP	Antimicrobial peptide
ASL	airway surface liquid
BALF	Bronchoalveolar lavage fluid
BPI	Bactericidal/permeability increasing proteins
CC10	clara cell 10-kD protein
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
COPD	Chronic Obstructive Pulmonary Disease
CR3	Complement receptor 3
CRAMP	Cathelin related antimicrobial peptide
DC	Dendritic cell
ECs	Epithelial cells
EDTA	Ethylenediamine tetraacetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescent activated cell sorter
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FPRL1	Formyl peptide receptor-like 1
FSC	Forward Scatter
GM-CSF	Granulocyte macrophage colony-stimulating factor
hBD	Human beta-defensin
hCAP18	Human cathelicidin antimicrobial protein 18
HGF	Hepatocyte growth factor
HNP	Human neutrophil peptide (common name for alpha-defensins)
IFN	Interferon
IGF-1	Insulin-like growth factor 1
IL	Interleukin
KGF	Keratinocyt growth factor
KO	knockout
KRB	Krebs-ringer buffer
LB	Luria-Bertani
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES-COOH
Lm	mean linear intercept

LPS	Lipopolysaccharide (also known as endotoxin)
LTB4	Leukotrien B4
MPO	Myeloperoxidase
NADPH	Nicotinamide adenine dinucleotide phosphate
NETs	Neutrophils extracellular traps
NF-kB	Nuclear Factor kappa B
PBS	Phosphate-buffered saline
PKC	Protein kinase C
PMA	Phorbol Myristate Acetate
PSGL-1	P-selectin glycoprotein ligand-1
RBC	Red Blood Cells
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
sLL-37	scrambled LL-37
SLPI	Secretory leukoproteinase inhibitor
SOD	Superoxide Dismutase
SSC	Side Scatter
TCR	T cell receptor
Th	T helper lymphocyte
TJ	Tight junction
TLR	Toll Like Receptor
TNF	Tumor necrosis factor
WT	Wild Type

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Ehrenwörtliche Erklärung

Ich erkläre ehrenwörtlich, dass ich die dem Fachbereich Humanbiologie zur Promotionsprüfung eingereichte Arbeit mit dem Titel "Immunological Aspects of Cathelicidin As a Modulator of Innate Immune Responses" im Universitätsklinikum Gießen und Marburg, Standort Marburg, Innere Medizin, Schwerpunkt Pneumologie unter der Leitung von Herrn PD Dr. Dr. Robert Bals mit der Unterstützung durch die unten genannten Personen ohne sonstige Hilfe selbst durchgeführt und bei der Abfassung der Arbeit keine anderen als die in der Dissertation angeführten Hilfsmittel benutzt habe. Ich habe bisher an keinem in- und ausländischen Medizinischen Fachbereich ein Gesuch um Zulassung zur Promotion eingereicht noch die vorliegende oder eine andere Arbeit als Dissertation vorgelegt. Unterstützung der Arbeit fand durch folgende Personen statt:

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allgemeine technische Unterstützung

- Dr. Holger Garn

Unterstützung bei der FACS-Analyse

- Dr. Ali Önder

Unterstützung bei dem "Naphthalene"-Modell und "Elastase-induced emphysema in mice"-Modell

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