Human alpha-defensins neutralize anthrax lethal toxin and protect against its fatal consequences

vorgelegt von
Master of Life Science
Chun Kim
aus Berlin

von der Fakultaet III – Prozesswissenschaften
der Technischen Universitaet Berlin
zur Erlangung des akademischen Grades
Doktor der Naturwissenschaften
Dr. rer. nat.

genehmigte Dissertation

Promotionsausschuss:

Vorsitzender: Prof. Dr. Stefan H.E. Kaufmann
Berichter: Prof. Dr. Roland Lauster
Berichter: Prof. Dr. Ulf Stahl

Tag der wissenschftlichen Aussprach: 11.04.2005

Belin 2005
D 83

- CONTENT -

4. MATERIALS AND METHODS

4.1 Cationic peptides

4.2 Recombinant proteins

4.3 Spore experiments

4.4 Cell viability assay

4.5 Trypan-blue assay

4.6 Analysis of MKK3b in RAW 264.7 cells

4.7 *In vitro* MKK3b cleavage assay

4.8 MMP-1 inhibition assay

4.9 Furin inhibition assay

4.10 Analysis of activation of MAPKs in RAW 264.7 cells

4.11 Kinetic characterization

4.12 Mouse protection experiment

5. RESULTS

5.1 *B. anthracis* mediated cytotoxictity in the presence of HNP-1

5.2 LeTx mediated cytolysis in the presence of HNP-1.

5.3 Cell viability assay by Trypan blue-microscopy

5.4 Activities of HNP-1-3 against LeTx under serum supplemented conditions

1. ABSTRACT

(German) Anthrax, von *Bacillus anthracis* verursacht, stellt eine große bioterroristische Bedrohung dar. Letaltoxin (LeTx), die Kombination des Letalfaktors (LF) und des protektiven Antigens (PA), ist für die besondere Pathogenese und hohe Letalität des Anthrax entscheidend. Ich konnte zeigen, daß die humanen α-Defensine starke Inhibitoren des LF sind. Humanes Neutrophilen Protein-1 (HNP-1) schützte Maus-Makrophagen vor der durch *B. anthracis*-Sporen induzierten Zytotoxizität. HNP-1 vermittelte Schutz durch Hemmung der Anthrax LeTx-Aktivität, indem es die LeTx verursachte Spaltung einer Mitogen aktivierten Proteinkinase (MAPK) Kinase (MKK) blockiert. Dies stellte den unterbrochenen MAPK Kinase Signaltransduktionsweg in LeTx behandelten Makrophagen wieder her. Kinetische Analysen zeigten, daß HNP-1 in Form eines nicht-kompetitiven Inhibitors die Aktivität des Anthrax LF verhindert. HNP-1 und -2 schützten Maus-Makrophagen vor der durch LeTx verursachten Zell-Lyse. Darüberhinaus zeigten *In-vivo*-Versuche mit Mäusen, daß die Behandlung mit HNP-1-3 vor den tödlichen Folgen des Anthrax LeTx schützt.

(English) Anthrax caused by *Bacillus anthracis* represents a major bioterroristic threat. Lethal toxin (LeTx), the combination of lethal factor (LF) and protective antigen (PA), plays a central role in anthrax pathogenesis and is critical for its high mortality. I demonstrate that human neutrophil α-defensins are potent inhibitors of LF. Human neutrophil protein (HNP)-1 protected murine macrophages from *B. anthracis* spore induced cytotoxicity. HNP-1 achieved protection by inhibiting anthrax LeTx activity. HNP-1 inhibited LeTx induced cleavage of a mitogen activated protein kinase (MAPK) kinase (MKK) and restored impaired MAPK signaling in LeTx treated macrophages. Kinetic analysis revealed that HNP-1 is a noncompetitive inhibitor preventing anthrax LF activity. HNP-1 and -2 protected murine macrophages from LeTx induced cytolysis and in vivo treatment with HNP-1-3 protected mice against fatal consequences of anthrax LeTx.

2. INTRODUCTION

The recent dissemination of anthrax through the U.S. mail, has drawn considerable attention in both the medical and lay communities to the risks of bioterrorism. Robert Koch (1843-1910)[1], German scientist and Nobel laureate, who founded modern medical bacteriology, discovered several disease-causing bacteria, including the etiologic agent of anthrax, and discovered the animal vectors of a number of major diseases. Koch's first major breakthrough in bacteriology occurred in the 1876, when he demonstrated that the infectious disease anthrax developed in mice only when the disease-bearing material injected into the bloodstream of mice contained viable rods or spores of *Bacillus anthracis*. Koch's isolation of the anthrax bacillus was of momentous importance, because this was the first time that the causative agent of an infectious disease had been demonstrated beyond reasonable doubt.

Louis Pasteur (1822-1895)[2], French chemist and biologist, who founded the science of microbiology, proved the germ theory of disease, invented the process of pasteurization, and developed vaccines for several diseases. He also determined the natural history of anthrax. He proved that anthrax is caused by a particular bacillus and suggested that animals could be protected against anthrax by pretreating them with attenuated bacilli, thus providing immunity against potentially fatal attacks. In order to

test his theory, Pasteur inoculated 25 sheep; a few days later he inoculated these and 25 naive sheep with an especially strong inoculum, and he left 10 sheep untreated. He predicted that the second 25 sheep would all perish and concluded the experiment dramatically by showing protected 25 sheep by pretreatment and the carcasses of the 25 sheep.

2.1 *B. anthracis* and anthrax toxin

B. anthracis, the etiological agent of anthrax, is a Gram-positive, non-motile, aerobic, facultative anaerobic, spore-forming rod-shaped bacterium[3]. Anthrax is primarily a disease of herbivores, but all mammals including humans are susceptible. The disease is initiated by the entry of spores into the host body. This can occur via a minor abrasion, an insect bite, or by eating contaminated meat or inhaling airborne spores. There are three types of human infection: cutaneous, gastrointestinal and inhalational. Each form can progress to fatal systemic anthrax. Indeed, despite appropriate therapy, the disease may be rapidly fatal as a result of shock-like symptoms, sepsis, and respiratory failure[4].

Dormant spores are highly resistant to adverse environmental conditions and they are able to survive for long periods in contaminated soils. In a suitable environment, spores re-establish vegetative growth[5]. Spores are taken up by macrophages and

transported to the regional lymph nodes draining the inoculation site[6]. As the phagocytic capacity of the lymph node is overwhelmed, the infection extends to successive nodes and the bacilli will then enter the blood stream. Although *B. anthracis* is an extracellular pathogen, it nevertheless appears to require an intracellular step to initiate infection. It is possible that spore germination is triggered within the macrophage by host-specific signals[3].

Fully virulent strains of *B. anthracis* carry two large plasmids, pXO1 and pXO2, that encode the primary virulence factors: toxin production and capsule formation, respectively[5]. The capsule, a polymer of gamma-d-glutamic acid[7], contributes to pathogenicity by enabling the bacteria to evade the host immune defenses and provoke septicemia. The toxin consists of three proteins: lethal factor (LF), edema factor (EF) and protective antigen (PA). Individually none of these three proteins is toxic, but the combination of LF and PA (called lethal toxin; LeTx) can cause lethal shock and the combination of EF and PA (called edema toxin; ET) induces edema at the site of injection in experimental animals[8]. LF is a Zn^{2+}-protease that cleaves certain mitogen-activated protein (MAP) kinase kinases (MKK)[9], leading to death of the host via a poorly defined sequence of events. EF, a calmodulin- and Ca^{2+}-dependent adenylate cyclase, is responsible for the edema seen in the disease[10].

2.1.1 Overview of toxin action

LF, EF, and PA can form toxic complexes either on the surface of receptor-bearing cells or in solution. The toxin probably plays an important role early in infection. Assembly of the three toxin proteins is initiated when PA binds to a cellular receptor and is activated by a member of the furin family of cellular proteases[11]. This cleaves the molecule into two fragments: PA20 (20 kDa), corresponding to the N terminus of the protein, and PA63 (63 kDa), corresponding to the C terminus. PA20 slowly dissociates from PA63 and diffuses into the surrounding medium, leaving PA63 bound to the receptor. Receptor-bound PA63 then spontaneously self-associates to form ring-shaped, heptameric oligomers[12]. LF and EF bind competitively to high-affinity sites spanning the interface of the PA63 subunits, leading to formation of a series of toxic complexes containing 1 to 3 bound molecules of EF and/or LF per PA63 heptamer[13]

Oligomerization of PA63 induces endocytosis and trafficking of the complexes to an acidic compartment. There, the heptamer undergoes a conformational transition from a pore precursor (prepore) to an integral-membrane, ion-conductive pore[14]. Translocation of EF and LF across the membrane to the cytosol is linked to this transition, but the mechanistic relationship between pore formation and translocation is not well understood. Once within the cytosol, EF catalyzes the conversion of ATP to

cAMP, and LF acts proteolytically to cleave certain MKKs. EF and LF enter essentially all types of cultured cells (Fig.1).

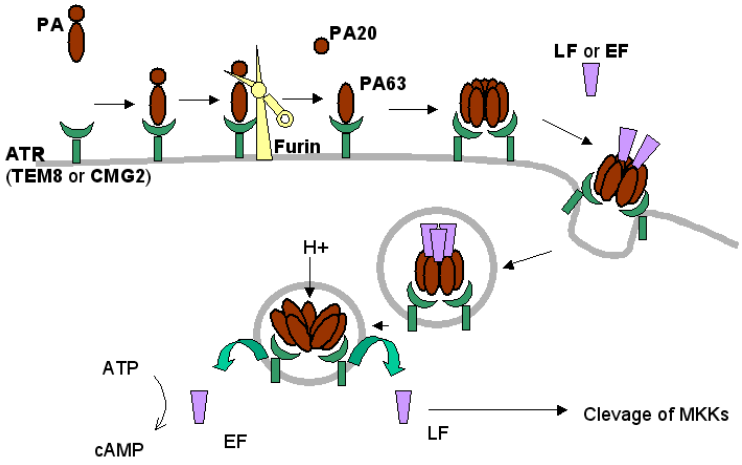

Fig. 1. Anthrax toxin action in cells
Protective antigen (PA) binds anthrax toxin receptors TEM8 or CMG2 and is cleaved by furin. Cleaved PA oligomerizes and provides binding sites for EF or LF. The complex is endocytosed and LF and EF are translocated to the cytosol after acidification of an intra cellular compartment. EF catalyzes the conversion of ATP to cAMP. LF cleaves members of MKK family.

2.1.2 Lethal factor

LF is a Zn^{2+} dependent metalloprotease. The only known substrates are MKK isotypes 1-4 and 6-7[15]. LF cleaves within the N-terminal proline-rich region that precedes the kinase domain of these proteins, disrupting a protein-protein interaction site involved in assembling signaling complexes, thus preventing MKK activation.

Another recent discovery is the inhibition of glucocorticoid receptor (GR) function by very low doses of LF[16]. The GR response is primarily associated with anti-inflammatory functions. GR inhibition may play a role in multiple stages of anthrax

infection: from promoting establishment of bacteremia to exacerbating subsequent molecular events induced by LF in various tissues. GR response differences between rat strains and among various species may define their relative abilities to resist the initial establishment of a *B. anthracis* infection or may alter their susceptibility to the effects of LF. The Fischer rat, for example, is known to be a hypersecretor of glucocorticoids in response to infection. At a later stage of infection, the control by glucocorticoids of the host response to hypoxia could be critical, because hypoxia was prominent in LeTx-treated mice[17].

How LeTx leads to death of the host is not clear. It has been reported that it suppresses proinflammatory cytokine production in mouse macrophages and that cleavage of MKKs reduces production of nitric oxide (NO) and TNF-α induced by bacterial lipopolysaccharaide (LPS) and IFN-γ[18]. These results suggest ways by which LeTx may impair the host innate immune system. They also cast doubt on earlier indications that lethality of the host is mediated by hyperproduction of cytokines by macrophages. LeTx has been reported to cause an increase in permeability to ions and rapid depletion of ATP in J774 macrophage-like cells, leading to colloid-osmotic lysis[19]. There is also evidence for a role for reactive oxygen intermediates[20]. Recently it was proposed that inhibition of the MKKs blocks induction of certain NF-κB target genes,

allowing apoptosis of activated macrophages[21]. Other reports indicate that protein synthesis and proteasome activity are required for expression of LeTx cytotoxicity and that Kif1C, a kinesin-like motor protein, mediates resistance to LeTx[22].

2.1.3 Protective Antigen

PA is a large protein consisting of four domains (I–IV), primarily involved in targeting the toxin to host cells by recognizing ATR2 or CMG2[23]. The crystal structure reveals that the high-affinity binding of PA with CMG2 is due partly to the involvement of a magnesium ion at the interface between PA and CMG2[24, 25]. A key aspartic acid residue in domain IV of PA works in conjunction with a metal-ion-dependent adhesion site (MIDAS) on CMG2 to coordinate the ion.

Once PA binds to CMG2 on the host-cell surface, a protease clips PA into two pieces. The smaller portion diffuses away, and the larger part remains bound to the CMG2 receptor, eventually forming a complex of seven PA–CMG2 modules, called a pre-pore. The edema factor and/or the lethal factor bind to this PA–CMG2 complex, triggering endocytosis. To inject the edema factor and the lethal factor into cells, the seven PA molecules must act together to form a pore bridging the endosome membrane and opening out into the cytosol. The pore transfers the edema factor and the lethal

factor to the cytosol, ultimately leading to cell death through the disruption of vital physiological processes[26].

The crystal structure elucidated by Liddington and colleagues reveals a molecular-switching mechanism in the complex that could control the formation of this pore (Fig. 2)[27]. The groove in CMG2, which interacts with PA domain II contains a crucial residue (histidine 121) that holds the PA in the right conformation until it is ready to insert into the endosomal membrane. Their model suggests that once the endosome is formed, the internal pH decreases and histidine 121 is protonated, becoming positively charged. This repels a nearby arginine on PA, reducing the affinity of the 3–4 loop of PA for CMG2. Consequently, the PA domain II undergoes a large conformational change, with the 2–3 strands adjacent to the 3–4 loop peeling away from PA like the skin of a banana peeling away from the fruit. The 2–3 strands are lined with several histidines, and protonation of these probably helps this unwrapping process. Once free of CMG2 and PA, the strands insert into the endosomal membrane and form the pore by twisting around the strands from the six neighboring PA molecules. Essentially, CMG2 acts as a pH-sensitive switch, holding the PA in the right shape until just the right time, before releasing it to form the pore.

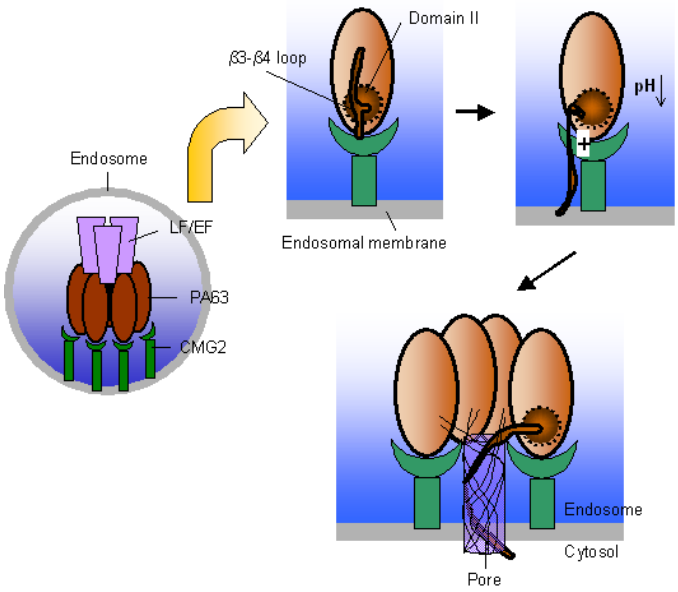

Fig. 2. Action mode of PA

When PA binds to CMG2, a loop of PA domain II is gripped in a groove on the CMG2 surface. Inside the endosome the pH decreases, generating a positive charge in the CMG2 groove. This repels the β3–β4 loop, resulting in a conformational change in the PA domain II. The loop and some neighboring strands peel away and insert into the endosomal membrane. They twist around strands from neighboring PA–CMG2 modules to form a pore.

2.1.4 Edema Factor

EdTx alone does not cause major tissue damage, and it is generally assumed that, consistent with functions of other toxins that elevate cAMP concentration, its main role in pathogenesis is to impair phagocyte function[10]. This hypothesis is supported, for example, by results showing that EdTx inhibits phagocytosis of spores by human polymorphonuclear leukocytes[28]. EF is a potent, calmodulin (CaM)-dependent, and Ca^{2+}-dependent adenylate cyclase, with a 1000-fold higher catalytic rate than that of mammalian CaM-activated adenylate cyclase. It is related to adenylate cyclases produced by *Bordetella pertussis* and *Pseudomonas aeruginosa*, but not to those of mammals.

2.1.5 Cellular Receptors

Anthrax toxin receptor (ATR), encoded by the tumor endothelial marker 8 (TEM8) has been identified[23]. TEM8 was initially defined as a gene expressed at elevated levels in tumor vasculature and in the vasculature of the developing mouse embryo. However, cDNA expression analysis and in situ hybridization experiments have revealed that this protein has a broad tissue distribution that includes brain, heart, intestine, lung, skeletal muscle, and pancreas. The normal physiological function of ATR/TEM8 is not known, yet.

It was recently shown that the related capillary morphogenesis protein 2 (CMG2) is also an anthrax toxin receptor[25]. CMG2 was originally identified on the basis of its increased expression levels in human umbilical vein endothelial cells (HUVECs) undergoing capillary formation in vitro. Although RT-PCR analysis had suggested that this gene is expressed only in human placenta, subsequent expressed sequence tag analysis has demonstrated CMG2 expression in a broad range of different tissue types including heart, lung, liver, and skeletal muscle. Although the physiological function of CMG2 is not yet known, one domain of this protein binds selectively to collagen IV and to laminin, making it likely that these two proteins are natural ligands for the receptor *in vivo*.

2.2 Human alpha-defensins

Among diverse mammalian antimicrobial peptides, defensins are a family of cationic peptides which characteristically comprise three intramolecular disulfide bridges to stabilize them in a complex, folded beta-sheet configuration[29]. There are three defensin subfamilies: The α-, β- and θ-defensins, which differ in the length of peptide segments between the cysteins and in the pairing of the cysteins that are connected by disulfide bonds. The six known human α-defensins include human neutrophil protein (HNP)-1-4 and two intestinal α-defensins, human defensin (HD)-5 and -6 (Fig.3). HNP-1-4 are expressed primarily by granulocytes[30] and certain lymphocytes[31]. The other two, human defensin (HD) -5 and -6, are expressed mostly by intestinal Paneth cells[29].

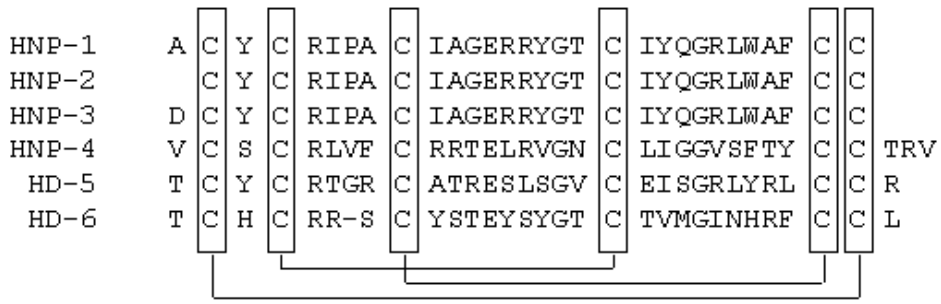

Fig. 3. Primary amino acid sequences of human α-defensins.
The pattern of cysteine-disulfide pairing is shown by boxed residues and lines.

HNP-1-3 are the most abundant peptides in azurophilic granules of neutrophils and constitute about 5% of the total protein in human neutrophils[32]. Notably, mice lack HNP-1-3 homologues in their neutrophils[33]. The other human neutrophil defensin, HNP-4, is approximately 100-fold less abundant and may perform functions other than host defense[34].

2.2.1 Defensin Genes

Human α-defensin genes encoding HNP-1 and HNP-3 are located on chromosome 8, band p23. Since the gene for HNP-2, a peptide that lacks the N-terminal alanine of HNP-1 and the N-terminal aspartic acid of HNP-3, has not been found, it is likely that HNP-2 is generated by differential posttranslational processing from preproHNP-1 and/or preproHNP-3[35].

2.2.2 Synthesis and Posttranslational Processing of Human α-defensins

HNP-1-3 mRNA are present at high levels in bone marrow. Although mature human PMNs contain about 5 μg of HNP-1-3 per 10^6 cells[32], the RNA is not detected in these cells, indicating that defensin synthesis is restricted to the bone marrow precursors of granulocytes. PreproHNP-1 is posttranslationally processed to inactive proHNP-1, then

to mature HNP-1 (Fig.4). The first HNP-1 intermediate contains 75 amino acids and arises by removal of the preproHNP-1 signal peptide. This intermediate is proteolytically processed over 20 hr via a 56 residue intermediate into the mature 29 and 30 residue HNP-1[36].

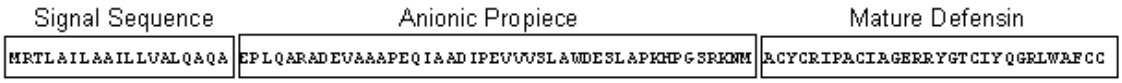

Fig. 4. Diagram of preproHNP-1.

A defensin-processing pathway removes the signal sequence, then over several hours cleaves the propiece leaving the mature defensin. The prodefensin (anionic propiece + mature defensin) contains a motif targeting the peptide into granules. The prodefensin lacks microbicidal activity, presumably because the anionic propiece keeps the mature cationic peptide latent.

2.2.3 Three Dimensional Structures

X-ray crystallography revealed that HNP-3 is an elongated ellipsoidal molecule whose structure is dominated by a three stranded, cysteine-stabilized antiparallel beta-sheet. No α-helical domains are present. HNP-3 was crystallized as a dimer that configured a six-stranded β-sheet stabilized by hydrophobic interactions and hydrogen bonds[37]. However, it is not clear whether this dimer is the biologically relevant form.

2.2.4 Antimicrobial activity of Human Neutrophil α-defensins

Despite their abundance in human polymorphonucleated neutrophils (PMN), α-defensins are not found in murine PMN. *In vitro* studies identified HNP-1-3 as natural peptide antibiotics that displayed antimicrobial activity against bacteria, fungi, and viruses[34]. In general, they exhibit greater potency against gram-positive bacteria than against gram-negative bacteria. However, HNP-1-3 show reduced antimicrobial activities in the presence of physiological concentrations of salt or serum[29]. Thus, the direct microbicidal effect of HNP-1-3 *in vivo* is likely to occur mainly in the phagocytotic vacuoles of phagocytes and on the surface of skin and mucosal epithelia, where there is low ionic strength[38].

The current model of antimicrobial mechanism is based on the cationic property of HNP-1-3 (Fig.5)[29]. Bacterial membranes are organized in such a way that the outermost leaflet of the bilayer, the surface exposed to the outside, is heavily populated by lipids with negatively charged phospholipid headgroups. In contrast, the outer leaflet of the membranes of the mammalian host is composed principally of lipids with no net charges; the majority of lipids with negatively charged head groups are segregated into the inner leaflet, facing the cytoplasm. On the basis of this different membrane composition, cationic HNP-1-3 specifically target microbes[39].

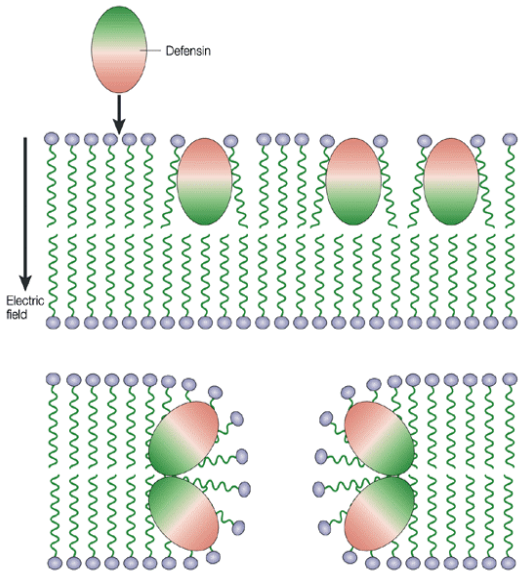

Fig. 5. The carper-wormhole model of action of defensin

Defensins are amphipathic molecules that have clusters of positively charged amino acid side chains and hydrophobic amino acid side chains. Electrostatic attraction and the transmembrane bioelectric field pull the peptide into the membrane. As peptides accumulate in a 'carpet', the membrane is strained and the peptide transition into another arrangement that lowers the strain but results in the formation of membrane 'wormholes'.

Following the eletrostatic adsorption of defensins to sites near the target cell membrane, individual defensin molecules or dimers enter the energized cell membrane under the influence of its electromotive force[40] and disrupt its integrity, probably by forming voltage regulated pores. Unless these lesions are repaired, irreversible target cell injury occurs.

2.2.5 Other activities of HNP-1-3

HNP-1-3 may participate in other aspects of innate immunity and adaptive immunity[38]. HNP-1-3 can stimulate bronchial epithelial cells to produce interleukin (IL)-8. Because IL-8 is a potent neutrophil chemotactic factor, HNP-1-3 might indirectly promote the

accumulation of neutropihls at inflammatory sites. HNP-1-3 also directly act on immune cells. HNP-1-3 are selectively chemotactic for immature dendritic cells (DC) and $CD4^+/CD45RA^+$ naive and $CD8^+$, but not $CD4^+/CD45RO^+$ memory T cells[41]. This chemotactic activity can be inhibited by pertussis toxin, suggesting that they are mediated by G protein coupled receptors. These capacities of HNP-1-3 predict that they play a role as natural adjuvants in the initiation of adaptive immune responses. Indeed, HNP-1-3, when simultaneously administered with ovalbumin (OVA) intranasally into mice, enhance the production of OVA-specific serum IgG and the generation of IFN-γ, IL-5, IL-6 and IL-10 by OVA-specific $CD4^+$ T cells[42]. Furthermore, intraperitoneal injection of HNP-1-3 with keyhole limpet hemocyanin (KLH) or B-cell lymphoma idiotype Ag into mice not only augment the levels of Ag-specific IgG but also enhance the resistance of immunized mice to tumor challenge[43].

3. AIM OF THE STUDY

The natural antibiotic peptides have emerged as important molecules of innate immunity. Human α-defensins are small cationic peptides that are composed of 29 to 35 amino acids. Of human α-defensins, HNP-1−3 are generally considered to be direct effectors of innate antimicrobial immunity since they show the capacity to kill a particular spectrum of bacteria, fungi, and enveloped viruses. Although it is well known that HNP-1−3 act as natural peptide, their ability against *B. anthracis* has not been demonstrated. I, therefore, examined the effect of HNP-1−3 in *B. anthracis* infection.

Specific aims were:

i) To examine the effect of HNP-1−3 on *B. anthracis*

ii) To investigate the inhibition mode of anthrax LeTx by HNP-1−3

iii) To evaluate the capacity of HNP-1−3 as a therapeutic candidate against anthrax.

4. MATERIALS AND METHODS

4.1 Cationic peptides

Synthetic LL-37 was generously provided by Dr. Hubert Kalbacher (University of Tübingen). Synthetic Magainin I was purchased from Sigma. Synthetic HNP-1 and -2 were obtained from Bachem. For mouse experiments, HNP-1-3 was purified from human buffy coats (Deutsches Rotes Kreuz) following the published procedure[44] with minor modifications. In brief, 1 volume of 3% Dextran in HBSS/10mM HEPES were added to 2 volumes of buffy coat, mixed by inverting. After 30 min incubation, top phase (leukocyte rich plasma) was taken and spun at 220g for 10 min. The pellet was resuspened in 10 ml PBS, and red blood cells were lysed by adding 30 ml water (hypotonic lysis of RBC). One min after hypotonic lysis, it was stopped by adding 3 ml of 10X PBS. Prepared white blood cells were resuspended in 15 ml of 0.34M sucrose (pH7.4) and homogenized with a glass homogenizer (tight pestle) until all cells were disrupted. Cell debris was removed by centrifugation at 1,600g for 10 min. and the granule rich supernatant was collected. To sediment the granules, the supernatant was spun 27,000g for 30 min. The collected granule pellet was resuspended in 20 ml ice-cold 5% acetic acid and sonicated. This granule extract was diluted with 30 ml 5%

acetic acid and stirred at 4 °C overnight. After spinning at 27,000g, the defensin-rich supernatant was colleted. The defensin rich extracts were loaded on Superdex peptide column equilibrated with 5% acetic acid and HNP-1-3 were eluted with 1.5 CV of 5% acetic acid and 1.5 CV of 50% acetic acid. HNP-1-3 containing fractions were further purified by C18 column with water-acetonitrile gradient that contains 0.1% trifluoro acetic acid (TFA).

4.2 Recombinant proteins

Recombinant LF and PA were purchased from Calbiochem or purified from the nonsporogenic, protease deficient, avirulent strain *B. anthracis* BH445 (pXO1-, pXO2-, CM^r) containing an LF[45] or PA[46] expression vector, kindly provided by Dr. Stephen H. Leppla (NIH). The modified FA medium was composed of 35 g of tryptone, 5 g of yeast extract and 100 ml of 10× salts per liter. The 10× salts solution (pH 7.5) was prepared from 60 g of Na_2HPO_4-$7H_2O$, 10 g of KH_2PO_4, 55 g of NaCl, 0.4 g of L-tryptophan, 0.4 g of L-methionine, 0.05 g of thiamine-HCl, and 0.25 g of uracil per liter. The solution was filter sterilized before it was added to the medium. Twenty ml overnight culture was inoculated to 6 liter of modified FA supplemented with 20 μg/ml kanamycin and 20 μg/ml chloramphenicol. This was cultured for 18 h in a fermentor in the condition of

Oxygen 30% saturation, 37°C, and pH 7.5. The supernatant was collected by centrifugation and filtered through 0.2 μm filter. After adding 60 ml 0.5M EDTA to inhibit proteolysis, the filtered broth was concentrated to 300 ml using ultrafiltration (Pellicon-2). Two hundred g $(NH_4)_2SO_4$/ liter was added gently and stirred in cold room for 1 h. Precipitated proteins were removed by spinning at 6,000g, 4°C, 30 min and filtering. The filtered supernatant was loaded to Phenyl Sepharose Fast Flow equilibrated with 1.5M $(NH_4)_2SO_4$/10mM HEPES/5mM EDTA and LF or PA was eluted with a 30-CV linear gradient from 1.5 M to 0 M $(NH_4)_2SO_4$ in 10 mM HEPES/5mM EDTA. Dialyzed LF/PA fractions from Phenyl Sepharose were loaded onto Q-sepharose equilibrated with 20 mM Tris-Cl/5 mM EDTA and eluted with a 15-CV linear gradient from 0 to 500 mM NaCl. Purified LF or PA was further cleared by Superdex 75 equilibrated in PBS containing 5 mM EDTA and the confirmed fractions were dialysed against 10 mM HEPES/10% glycerol.

4.3 Spore experiments

B. anthracis (Sterne) spores were prepared as described[47]. In brief, 2× SG medium was inoculated with one colony from the over night Brain heart infusion (BH) agar plate culture and incubated at 37°C on a shaker. An aliquot of 1 ml of this preculture was

added to 50 ml 2× SG medium and cultured with shaking. After 25 h, 200 ml of sterile H_2O was added to the culture and incubation was continued with shaking for additional 40 h. The culture was then spun down, washed with PBS and resuspended in 50 ml of sterile PBS. After heating the culture to 70°C for 40 min, the spores were evaluated by microscopy. 2× SG medium was prepared as followed. 16.0 g Difco™ Nutrient broth, 2.0 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$ were dissolved in 800 ml of distilled water and the pH was adjusted to 7.0 by addition of 1 M NaOH. After autoclaving, 1.0 ml 1 M $Ca(NO3)_2$, 1.0 ml 0.1 M $MnCl_2 \cdot H2O$, 1.0 ml 1 mM $FeSO_4$, 2.0 ml 50% (w/v) glucose were added and the final volume was adjusted to 1 liter with H_2O.

RAW264.7 cells were seeded in a 96 well plate at a density of 4×10^4 cells per well in RPMI 1640 medium containing serum without antibiotics. For the assay, 2×10^5 spores per well and described amounts of HNP-1 were added to the cells in serum-free RPMI 1640. Eight h after infection, cytotoxicity was determined by CytoTox 96® cytotoxicity assay (Promega).

For *in vitro* killing assay, 2×10^5 spores in serum-free RPMI 1640 were incubated in the presence or absence of 1 μM HNP-1. At each time point, colony forming units (CFU) were determined.

4.4 Cell viability assay

One day before the assay, RAW264.7 cells were seeded in a 96 well plate at a density of 3×10^4 cells per well in RPMI medium containing FCS. For the assay, 400 ng/ml LF, 1,600 ng/ml PA and described amounts of HNP-1-3 were added simultaneously to cells in serum-free RPMI or RPMI supplemented with 5% FCS. Five h after treatment, cell viability was determined by methyl thiazole tetrazolium (MTT) assay. In brief, 20 μl of MTT solution (2 mg/ml in PBS) was added to each well containing cells. The plate was incubated in a CO_2 incubator at 37ºC for 1 h. After removing media, 100 μl of DMSO was added to each well and pipetted up and down to dissolve the crystals. The plate was further incubated at the 37ºC for 5 min. The absorbance was measured at 510 nm by an ELISA reader (Reference wavelength 690 nm).

4.5 Trypan blue Assay

RAW 264.7 cells were seeded in a well of a chamber slide at a concentration of 6×10^4, one day before the experiment starts. On the experiment day, the medium was exchanged with serum free RPMI. Cells were treated with LeTx (400 ng/ml LF and 1600 ng/ml PA) in the presence of 7 μM HNP-1 or LL-37. Four hours later, the cells were stained for 10 min by adding final concentration of 0.1% Trypan blue to culture

medium. After staining, cells were washed with PBS, and fixed with 4% formaldehyde for microscopy.

4.6 Analysis of MKK3 in RAW 264.7 cells

RAW 264.7 cells were seeded in a well of a 6 well plate at a concentration of 5×10^5, one day before the experiment. LeTx (400 ng/ml LF and 1600 ng/ml PA) was added to culture medium in the presence of 10 μM HNP-1 or LL-37. One or 2 h after the treatment, cells were lysed by 100 μl 1× SDS PAGE loading buffer per well. Twenty μl of the each lysate was loaded to a well in 10% SDS polyacrylamide gel. For immunoblotting, a specific antibody against C-terminal of MKK3 (Santa Cruz) was used. This experiment was done under serum free condition.

4.7 *In vitro* MKK3b cleavage assay

S^{35} labelled MKK3b was *in vitro* translated from pcDNA-MKK3b (with kind help of Dr. Jiahuai Han, the Scripps Research Institute) using TNT® quick coupled transcription/translation systems (Promega) following the provided protocol. *In vitro* translated MKK3b was incubated at 37 °C for 1 h in reaction buffer (20 mM HEPES, 1 mM $CaCl_2$, pH 7.2) with indicated amounts of LF and either HNP-1, Magainin I or LL-

37. The reactions were loaded on a 10% SDS-PAGE gel and after running, analyzed by autoradiography.

4.8 MMP inhibition assay

Ten μg of native collagen (Type I) was incubated at room temperature for 1 h with 100 ng of MMP-1 (Sigma) in the presence of described amount of HNP-1 or MMP inhibitor III ($C_{19}H_{29}N_3O_4$, Calbiochem). The reaction was performed in 30 μl of reaction buffer consisting of 50 mM HEPES, 200 mM NaCl, 10 mM CaCl2 and 0.05% Brij-35 and analyzed by 7% SDS-PAGE.

4.9 Furin inhibition assay

PA (10 μg) was incubated in a volume of 30 μl with recombinant furin (New England Biolab) in 50 mM HEPES, 200 mM NaCl, 10 mM $CaCl_2$ and 0.05% Brij-35. 0-10 μM of HNP-1 or Furin inhibitor II (Calbiochem) were added to the reaction. The reaction was analysed by 7% SDS-PAGE

4.10 Analysis of activation of MAPKs in RAW 264.7 cells

RAW 264.7 cells were seeded in a well of a 6 well plate at a concentration of 5×10^5, one

day before the experiment. On the day of the experiment, cell culture medium was changed to 5% FCS containing RPMI. Cells were treated with 200 ng/ml LF and 1600 ng/ml PA for 2h and subsequently activated by 10 μg/ml *Bacillus subtilis* lipoteichoic acid (LTA) (Sigma). Thirty min after LTA treatment, cells were lysed with 100 μl SDS-PAGE loading buffer per well. The lysates were analyzed by immunoblotting with antibodies specific to different MAPKs and their phosphorylated (activated) forms. All antibodies in this experiment were purchased from Cell Signaling.

4.11 Kinetic characterization

Chromogenic substrate was synthesized by Jerini Peptide Technology as descibed[48]. In order to determine IC_{50} value and the inhibition type, we measured initial enzyme rates. To ensure initial kinetics, proteolysis was followed only 5% toward completion. For IC_{50} determination, 10 nM LF was preincubated with 0-10 μM HNP-1 for 30 min at room temperature and the reaction was started by adding substrate, to a final concentration of 100 μM. Competition assay was performed without preincubation of LF and HNP-1. To examine the effect of DTT, HNP-1 was treated with 20 mM DTT at room temperature for 1 h and then dialyzed against 20 mM HEPES and 1 mM $CaCl_2$ using a 1 kDa cut-off dialysis membrane. LF (10 nM) was preincubated with DTT

treated or untreated HNP-1 at room temperature for 15 min and the relative activity was measured by monitoring pNA release from the substrate. In all kinetic experiments, the reaction buffer was 20 mM HEPES, 1 mM $CaCl_2$.

4.12 Mouse protection experiment

Seven to 8 weeks old female BALB/c mice were treated with LeTx (50 µg of LF and 50 µg of PA in 0.2 ml PBS) i.v. into one tail vein, immediately followed by i.v. injection with the indicated doses of purified HNP-1-3 or synthetic LL-37 diluted in 0.2 ml PBS into the other tail vein. Survival of mice was monitored for 10 days after toxin treatment. Experiments were conducted according to the German animal protection law.

5. RESULTS

5.1 *B. anthracis* mediated cytotoxicity in the presence of HNP-1

It has been shown that a toxin producing *B. anthracis* strain (Sterne) kills murine macrophages[49, 50]. To determine whether HNP-1 protects macrophages from toxin producing *B. anthracis*, we examined the cytotoxicity of anthrax spores in HNP-1 treated RAW 264.7 cells. As shown in Fig. 6A, as little as 1 μM of HNP-1 inhibited the anthrax spore induced cytotoxicity almost completely. Surprisingly, this protective effect was independent from HNP-1's well-known microbicidal activity because HNP-1 at this low concentration of 1 μM did not show any significant sporicidal effect (Fig. 6B).

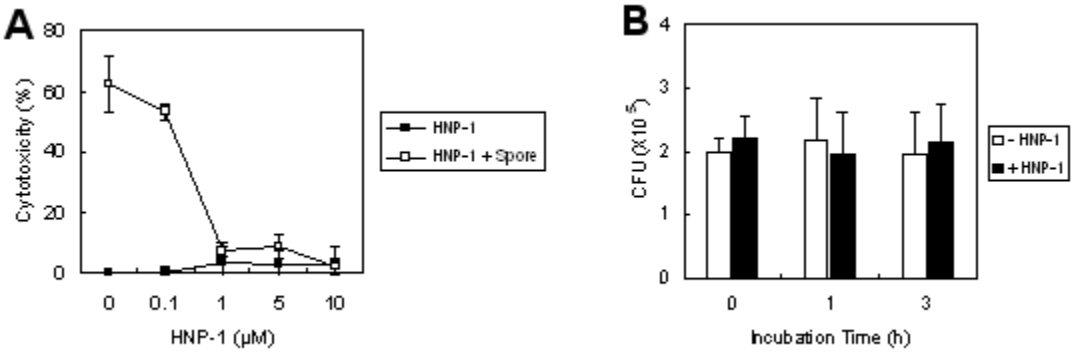

Fig.6. HNP-1 rescues macrophages from anthrax spore induced cell death

a. RAW 264.7 cells were infected with *B. anthracis* spores and followed by treating with indicated amounts of HNP-1. Cytotoxicity was determined by measuring released lactate dehydrogenase (LDH) levels. **b.** *In vitro* killing assay was performed against spores in the presence or in the absence of 1 μM HNP-1. After indicated incubation times, CFU were determined.

5.2 LeTx mediated cytolysis in the presence of HNP-1.

Since *B. anthracis* Sterne mediated macrophage cytotoxicity is largely determined by LeTx, we examined whether HNP-1−3 have any effect on this anthrax toxin. The murine macrophage cell line, RAW 264.7, is commonly used for LF assays because it is highly sensitive to cytolysis caused by LeTx. When these cells were treated with LeTx, they succumbed to the toxin within few h. In marked contrast, the addition of HNP-1 completely abolished cytotoxicity (Fig. 7A). This HNP-1 mediated protection was observed even 24 h after LeTx treatment (Fig. 7D). HNP-2 (Fig. 7B) whereas LL-37, another neutrophil cationic peptide with a similar size and net charge like HNP1-3, did not display any significant effect (Fig. 7C).

5.3 Cell viability assay by trypan blue

Consistent with the above results from MTT assay, microscopy using trypan blue staining showed that LeTx treatment caused cell death resulting in staining by trypan blue. In constrast, HNP-1 treated cells were not stained by the dye even in the presence LeTx, suggesting HNP-1 protect cells from LeTx induced cell lysis. LL-37 and LeTx cotreated cells showed the same phenotype as LeTx treated cells (Fig. 8).

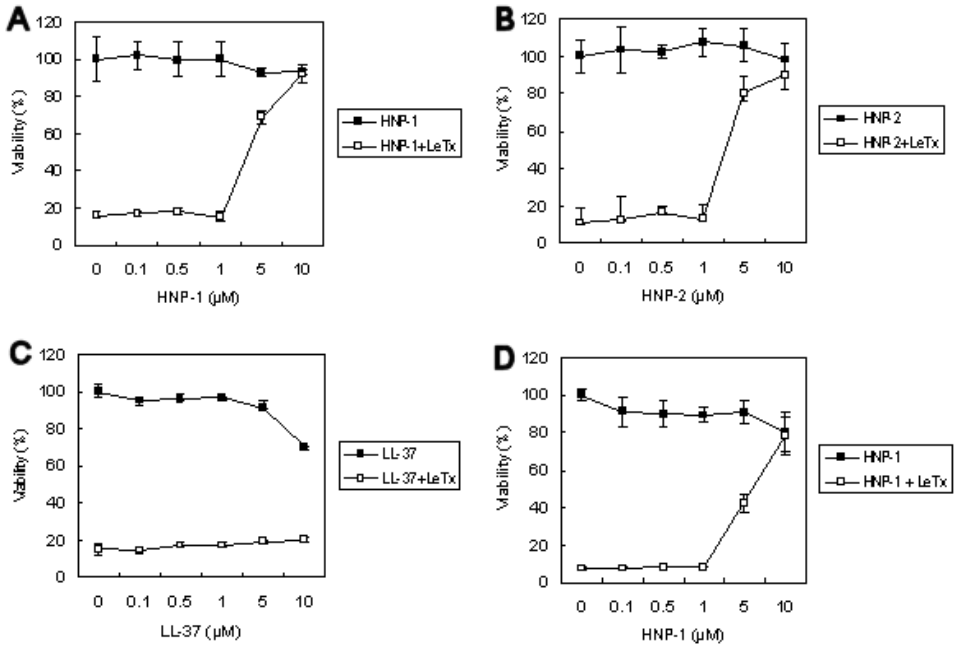

Fig. 7. Inhibition of LeTx cytotoxicity by HNP-1 and -2

RAW264.7 cells were treated with LeTx and various concentrations of HNP-1 (A), HNP-2 (B) or LL-37(C). Five h after treatment, cell viability was determined by MTT assay. Panel (D) shows HNP-1 mediated cell protection 24h after LeTx treatment.

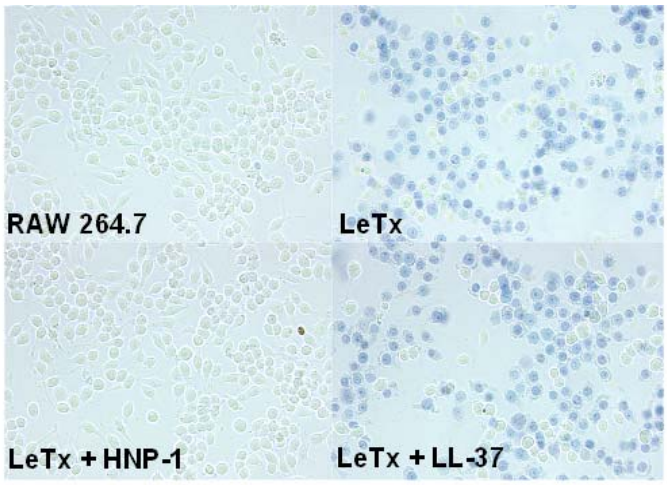

Fig. 8. Trypan blue cell assay

RAW 264.7 cells were treated with LeTx (400 ng of LF AND 1600 ng of PA/ml) in the presence of 7 μM HNP-1 or LL-37. Four hours after treatment, cells were stained with Tryphan blue.

5.4 Activity of HNP-1-3 against LeTx under serum supplemented conditions

To examine whether this phenomenon is physiologically relevant and to assess potential effects of serum components, I performed the same assay under serum-supplemented conditions. In the presence of 5% fetal calf serum (FCS), HNP-1 still protected cells from LeTx induced cytotoxicity, although a higher amount of HNP-1 was needed (Fig. 9A). Purified natural HNP 1-3 mixture from human leukocytes (Fig. 9B) showed similar protection.

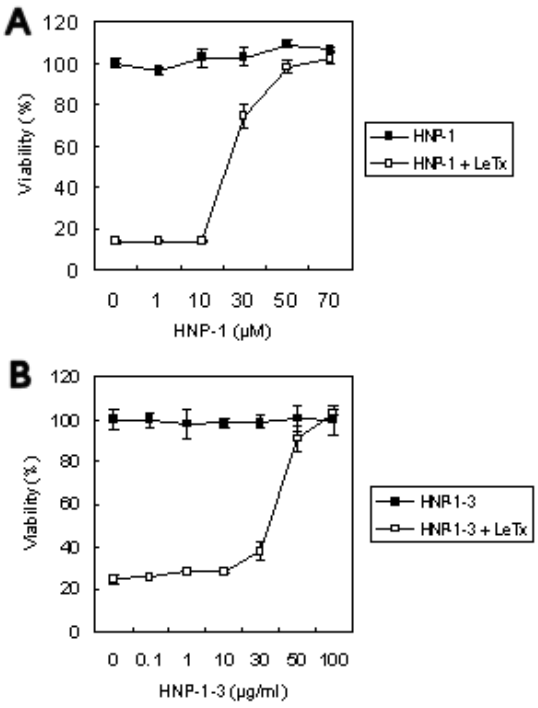

Fig. 9. Inhibition of toxin action in the presence of serum

RAW264.7 cells were treated with LeTx and indicated concentrations of synthetic HNP-1 (A) or natural HNP-1-3 (B) in 5% FCS supplemented medium. Five h after treatment, cell viability was determined by MTT assay.

5.5 The effect of HNP-1 on LeTx induced MKK cleavage.

Because LF is a protease cleaving the N-terminus of MKKs[9], I investigated whether HNP-1 inhibited cleavage of MKK3b in LeTx treated cells. RAW 264.7 macrophages were treated with LeTx (400 ng/ml LF and 1600 ng/ml PA) and HNP-1 (7 μM) for 1 or 2 h, and the cell lysates were analyzed using an antibody directed against the C-terminal end of MKK3. Within 2 h of LeTx treatment, MKK3b was almost completely converted to its cleaved form but this cleavage was efficiently inhibited by HNP-1 (Fig. 10).

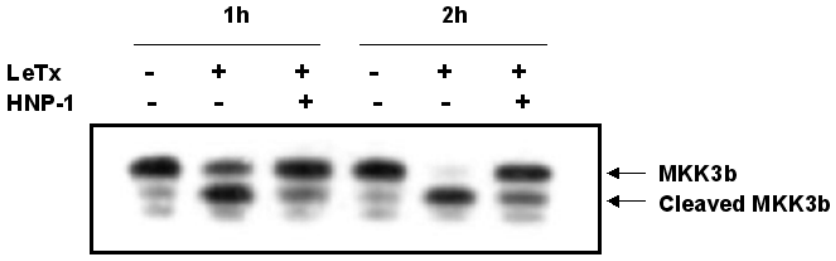

Fig. 10. Inhibition of MKK3 cleavage in macrophages
RAW 264.7 macrophages were either untreated (-) or treated (+) with anthrax LeTx (LF 400 ng/ml and PA 1600 ng/ml) and HNP-1 (7 μM) for 1 h or 2 h. The cell lysates were analyzed by immunoblotting with anti- MKK3 antibody.

5.6 Analysis of a MKK cleavage by LF in the presence of HNP-1

To verify whether HNP-1 directly inhibits the endoprotease activity of LF, we performed *in vitro* cleavage assay with S^{35}-labeled LF substrate (Fig. 11). *In vitro*

translated MKK3b was almost completely cleaved within 1 h by 500 ng of LF. In contrast, in the presence of 10 μM HNP-1, proteolysis was efficiently inhibited, suggesting that HNP-1 inactivates the catalytic activity of LF. Other cationic antimicrobial peptides, Magainin I (Fig. 11 upper panel) and LL-37 (Fig. 11 lower panel) did not prevent cleavage of MKK3b mediated by LF.

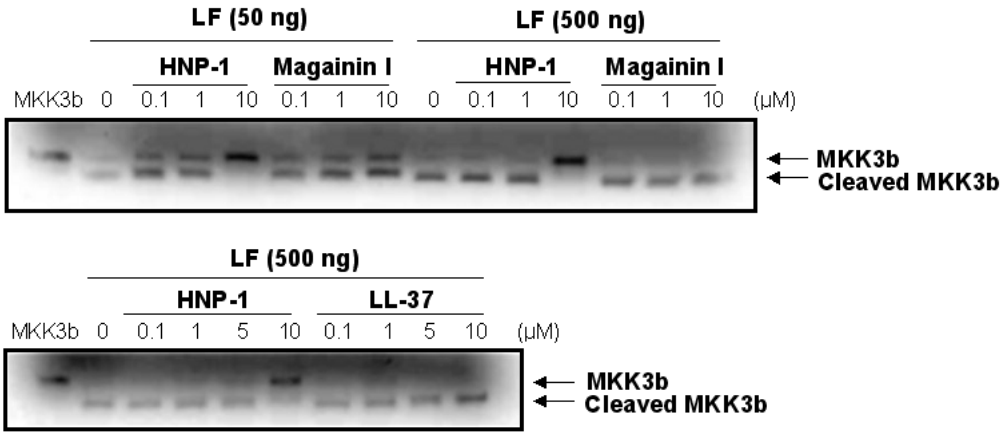

Fig. 11. Inhibition of LF mediated proteolysis

In vitro translated MKK3b was incubated for 1h with indicated amounts of LF and either HNP-1, LL-37 (lower panel) or Magainin I (upper panel).

5.7 MMP inhibition assay

To determine the specificity of HNP-1 against LF, a metalloprotease, I assessed whether HNP-1 showed inhibitory effects on host matrix metalloprotease (MMP)-1. As shown in Fig.12, the established MMP-1 inhibitor prevented MMP-1 from producing cleavage

products of collagen type I, a known substrate of MMP-1. However, at the same range of concentration, HNP-1 did not show any significant effect on this reaction.

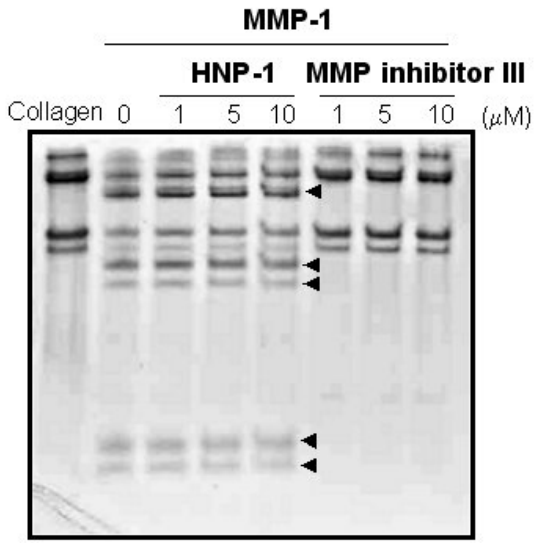

Fig. 12. HNP-1 has no effect on a host metalloprotease

Native collagen (Type I) was incubated with 100 ng of MMP-1 in the presence of described amounts of HNP-1 or MMP inhibitor and analyzed by 7% SDS-PAGE. Arrowheads indicate cleavage products.

5.8 Furin inhibition assay

Cationic characteristics of HNPs are determined mainly by poly-arginine residues in their amino acid sequences. Since poly-arginine containing peptides represent potent inhibitors of furin[51], which cleaves RXXR motif in PA[11], I assessed whether HNP-1 also has furin inhibitor activity. Hexa-D-arginine (D6R) was shown to inhibit the endoprotease activity of furin. As expected, 1-10 μM D6R inhibited PA cleavage by furin in a dose dependent way. In contrast, HNP-1 failed to inhibit the cleavage of PA at the same range of concentration (Fig. 13).

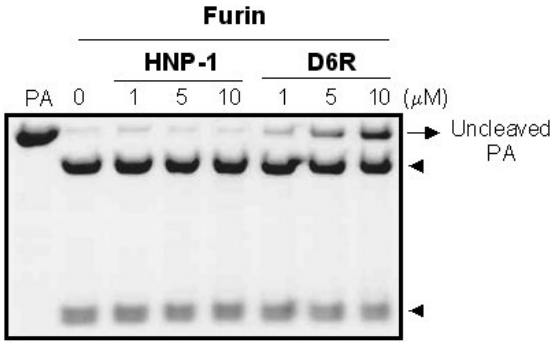

Fig. 13. HNP-1 has no effect on host furin

Protective antigen was incubated with furin in the presence of HNP-1 or Furin inhibitor. Arrowheads indicate cleavage products.

5.9 Effect of internalized HNP-1 on LeTx induced cell death

Potential LF inhibitors are expected to enter cells to exert their activity against LF, and HNP-1−3 can, indeed, be internalized into host cells. Given the described effects of HNP-1 on LeTx, I investigated whether HNP-1 can inhibit LF inside cells. RAW 264.7 cells were incubated with HNP-1 at 37ºC for 1h, washed extensively to remove free HNP-1 and subsequently treated with LeTx (400 ng/ml LF and 1600 ng/ml PA) at 37ºC for 5 h. As shown in Fig. 14, treatment of macrophages with HNP-1 prevented LeTx toxicity in a HNP-1 dose dependent manner, indicating that HNP-1 acts on LF inside cells.

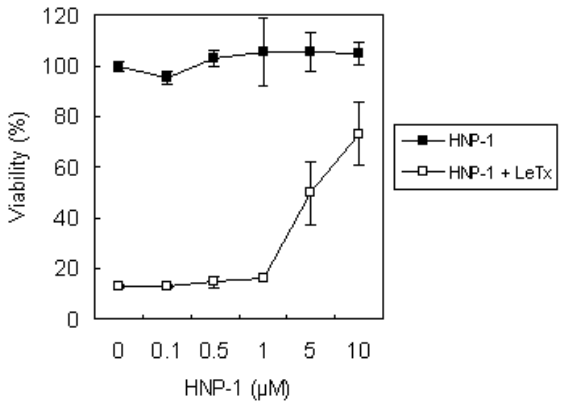

Fig. 14. Intracelluar protection
RAW 264.7 cells were incubated with HNP-1 at 37ºC. After 1 h, the medium was removed and replaced with fresh medium containing LeTx. Cells were incubated further at 37ºC for 5 h.

5.10 Restoration of LeTx mediated impairment of MKK signaling by HNP-1

LF inhibits extracellular signal-regulated kinase (ERK) and p38 MAPK signaling through cleavage of members of MKK family in activated macrophages[21]. To characterize the effects of HNP-1 on LeTx mediated impairment of MAPK signaling, macrophages were incubated with LeTx (200 ng/ml LF and 1600 ng/ml PA) and HNP-1 (30 μM) for 2 h, followed by stimulation with *B. subtilis* LTA. This experiment was performed under 5% FCS supplemented conditions to achieve efficient stimulation of Toll-like receptors (TLR) by LTA[52]. LeTx strongly inhibited ERK and p38 activation in macrophages and phosphorylation of these two MAPKs was restored by HNP-1 (Fig. 15).

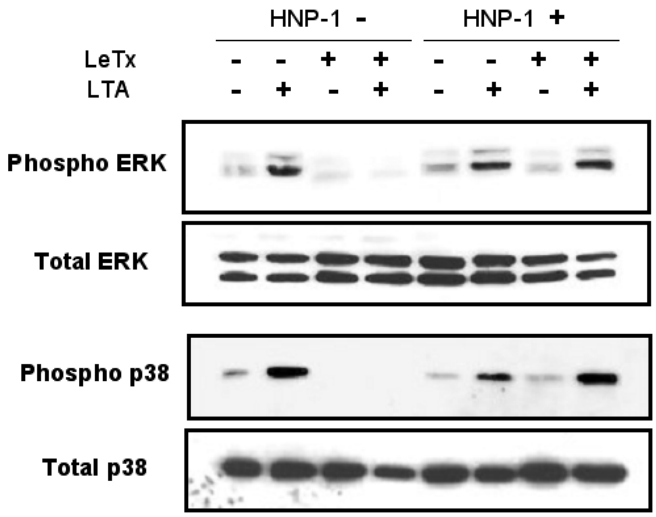

Fig. 15. Restoration of impaired MAP signaling

RAW 264.7 cells were treated(+) with LeTx (200 ng/ml LF and 1600 ng/ml PA) and HNP-1 (30 μM) in 5% FCS containing medium. Two h after treatment, cells were stimulated with 10 μg/ml *B. subtilis* LTA for 30 min and the lysates were assessed with antibodies against MAPKs (Total) and their phosphorylated forms (Phospho).

5.11 Kinetic characterization

To elucidate the inhibition mode, we performed kinetic characterization using a chromogenic peptide substrate[48]. The initial rates of enzyme reaction in the presence of various concentrations of HNP-1 provided IC_{50} values of 190 ± 33 nM (Fig. 16A). In the competition assay (Fig. 16B), HNP-1 acted noncompetitively. When V_{max} versus [E], where [E] represents concentration of enzyme, was plotted, HNP-1 was identified as a reversible noncompetitive inhibitor showing smaller slopes than the control curve (Fig.16C). Reversibility testing using ultrafiltration confirmed that inhibition is

reversible.

Since HNP-1−3 have intramolecular disulfide bridges, I determined whether these disulfide bonds are important for the inhibitory capacity. As shown in Fig. 17D, DTT treated HNP-1 did not show any significant inhibition suggesting that the unique structure of HNP-1 determined by disulfide bridges is a critical requirement for inhibition.

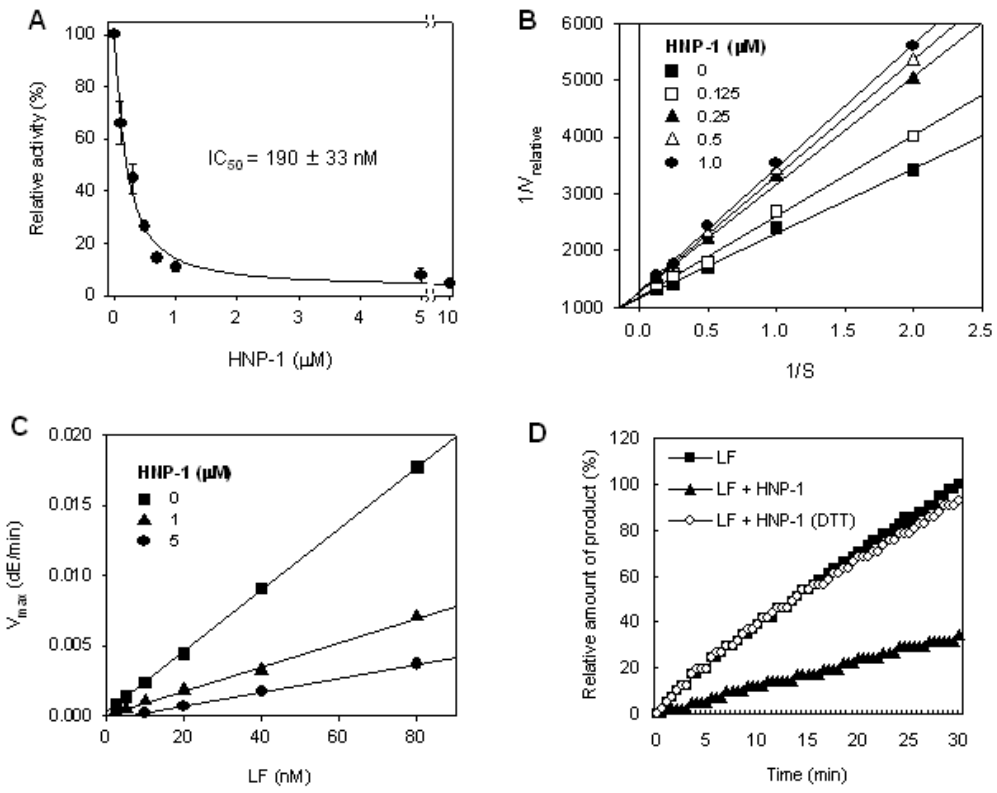

Fig. 17. Characterization of LF inhibition by HNP-1

A. HNP-1 inhibited 50% of LF activity at a concentration of 190 ± 33 nM. B. Lineweaver-Burk plot indicates noncompetitive inhibition mode. C. A plotting of V_{max} versus concentrations of LF confirms that HNP-1 is a reversible noncompetitive inhibitor. D. DTT treated HNP-1 did not show any significant effect on LF.

5.12 *In vivo* protection experiment

Having identified LeTx neutralization as a novel biological function of HNP-1-3, I decided to exploit this activity for therapeutic intervention against anthrax. To this end, LeTx sensitive Balb/c mice received LeTx (50 µg LF and 50 µg PA) intravenously (i.v.), immediately followed by the indicated amounts of purified HNP-1−3 i.v. (Fig. 18A and B). Within 2 days, mice succumbed to the toxin. In striking contrast, 500 µg of HNP-1-3 protected mice from intoxication of LeTx. LL-37, a control antimicrobial peptide, had no effect on LeTx toxicity (Fig. 18B).

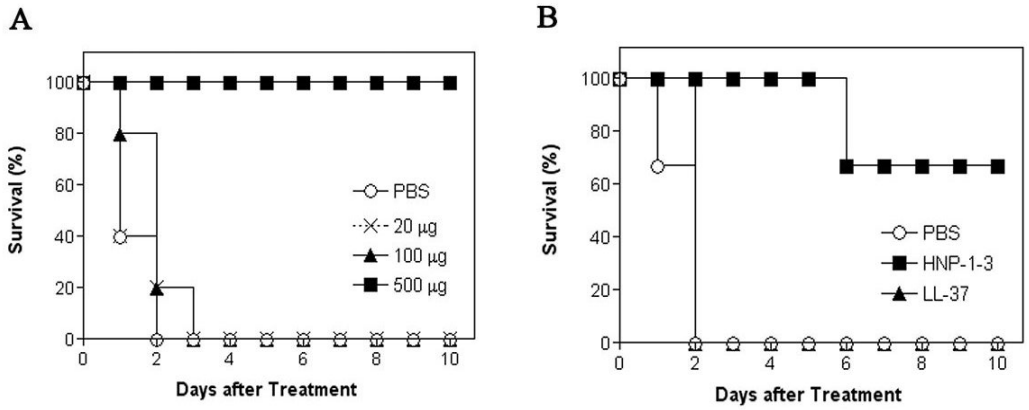

Fig. 18. Protection of mice from LeTx intoxication by HNP-1–3

LeTx sensitive Balb/c mice received LeTx (50 µg LF and 50 µg PA) intravenously (i.v.), followed by PBS, purified HNP-1−3, or LL-37 i.v. A: 5 mice per group and B: 3 mice per group were monitored for 10 days

6. DISCUSSION

6.1 Beyond antimicrobial activities of defensins

HNP-1-3 have well established capacity to kill a variety of microbial pathogens. However, the antimicrobial activity is often extrapolated from in vitro measurements at high concentrations and under non-physiological, low ionic strength conditions and serum free conditions. By contrast, divalent cations such as Mg^{2+} or Ca^{2+}, which are present at concentrations of 1-2 mM in almost every body fluid, will reduce or completely eliminate the antimicrobial activity of HNP-1-3[29, 38]. Interestingly, beside their potential antimicrobial activity, these peptides show other functions in physiological conditions; e.g., immunopotentiating activities of defensins have been reported[38]. Human neutrophil α-defensins show chemotactic activities for T cells and dendritic cells[41]. They enhance the production of antigen specific antibodies and certain cytokines by immune cells[42]. In addition to these functions, my data reveal a novel biological function of HNP-1−3; namely neutralization of a secreted bacterial enzyme.

The structural characteristics of HNP-1−3 such as overall dimensions, positive charge, β-sheet, and disulfide bonds are reminiscent of various snake, scorpion, and spider toxins[37]. Since many venom peptides act as enzyme inhibitors, the structural characteristics of HNP-1−3 prompted me to assess the potential of HNP-1−3 to

neutralize secreted bacterial enzymes. Supporting my finding that HNP-1−3 inhibit LF activity, a recent report showed that HNP-1−3 neutralize fibrinolytic activity exerted by staphylokinase secreted by *Staphylococcus aureus*[53]. In addition, I recently identified several bacterial ADP-ribosyltransferases including Diphtheria toxin and Pseudomonas exotoxin A, which are also inhibited by HNP-1−3 (data not shown). Many bacterial pathogens secrete enzymes into the host environment to avoid destruction by the immune system. Because recruited human neutrophils release abundant HNP-1−3 locally in the infection regions, I assume that HNP-1−3 achieve efficient neutralization of bacterial enzymes *in vivo*.

6.2 Restoration of impared MAPK cascade

Activation of TLRs by LTA[21] and Anthrolysin O[54] from *B. anthracis* results in activation of an apoptotic signaling cascade in macrophages and this apoptosis process is usually inhibited by NF-κB and p38 induced survival genes. However, LeTx renders macrophages sensitive to LTA or Anthrolysin O-induced apoptosis by preventing activation of the p38 MAPK pathway[21]. LF cleaves MKKs between their NH_2-terminal extension and the catalytic domain[9]. Because the NH_2-terminal extension is required for interactions with both MAPKs and MKK kinases (M3Ks), this cleavage prevents

MAPK activation.

In addition, the inactivation of the MAPK pathway by LeTx in both macrophages and dendritic cells[55] leads to inhibition of proinflammatory cytokine secretion, downregulation of costimulatory molecules such as CD80 and CD86, and ineffective T cell priming. The net result is an impaired innate and adaptive immune response. Endothelial cells of the vascular system undergo apoptosis upon LT exposure, also likely due to inactivation of the MAPK pathway[56]. The activity of various hormone receptors such as glucocorticoids, progesterone and estrogen is also blocked, due to inhibition of p38 MAPK phosphorylation, thus affecting the body's response to stress[16]. So far, there has been no study showing how our immune system could protect against this detrimental weapon of *B. anthracis*. Here, my data show that human neutrophil released HNP-1−3 neutralize LF and this results in restoration of impaired MAPK cascade, proposing our immune system has developed HNP-1−3 as central innate defense molecules against distinct toxins.

6.3 Fatal consequence of inhalation anthrax

The concentration of HNP-1−3 in plasma under normal circumstances is around 40 ng/ml but this rises up to 0.9-170 μg/ml during severe infections[57]. The local

concentrations of HNP-1−3 are expected to be much higher. In this regards, it is unclear why inhalation anthrax often causes fatal consequences in humans even in presence of profound natural inhibitors of LF. It is possible that the endogenous expression levels of HNP-1−3 are not sufficiently high to neutralize the lethal effects of LeTx during infection, particularly after inoculation of high doses of *B. anthracis*. It is also possible that *B. anthracis* suppresses secretion of HNP-1−3 by human leukocytes via unknown mechanisms.

6.4 Mode of action of HNP-1–3

HNP-1−3 contain three intramolecular disulfide linkages to maintain its stable and compact conformation. It has been shown that the cyclic analogs of HNP-1 with different S-S pairings exhibit antimicrobial activity whilst the linear HNP-1 is inactive[58]. Thus, it is of interest to address whether these S-S bonds are essential for its function as LF inhibitor. My data suggest that the unique structure of HNP-1 determined by disulfide bonds is a critical requirement for inhibition since reduced HNP-1 did not show anti-LF activity.

According to LF structure analyses, the cluster of acidic residues in the active center shows a preference for basic residues in the substrates and the substrates should

bind with antiparallel β-sheet formation to LF[59]. Complying with these requirements, HNP-1-3 are β-sheet dominant cationic peptides containing the LF recognition motif (Fig.19). Considering this notion, it was not expected that kinetic studies would indicate noncompetitive inhibition mode. However, as shown in Fig. 17, kinetic analysis revealed that HNP-1 binds to a remote region from the active site of LF and causes a conformational change in the active center, preventing the enzyme from converting the bound substrate to its product. It is unclear whether this motif in HNP-1–3 is important for the function as LF inhibitors at this moment. Future investigations are aimed at determining how the disulfide-bond dependent structure of HNP-1 influences its binding to undefined site(s) of LF. It would be of great interest to analyze the crystallized co-complex of HNP-1 and LF.

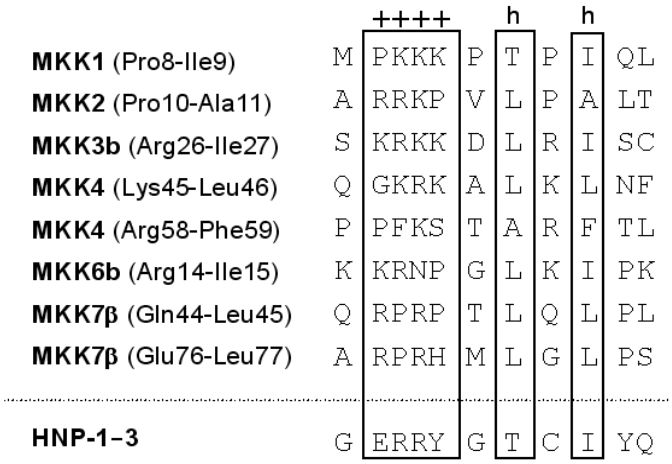

Fig. 19. Alignment of MKKs and HNP-1–3

Known LF substrates, MKK, and HNP-1–3 are aligned according to common physiochemical properties: + indicates a basic residue and h stands for hydrophobic amino acid. The amino acids in blanks indicate the cleavage site of each LF substrate. In the first 'h' position, predominantly hydrophobic residues come, in second 'h' invariably hydrophobic amino acids exist, and in +++ motif, at least one basic residue is present.

6.5 Pharmacological potential of HNP-1–3

My data not only reveal that the human immune system produces potent inhibitors for LF, but also demonstrate a potential of HNP-1–3 for therapy of anthrax. Although *B. anthracis* itself can be treated by antibiotics, this frequently fails if not initiated promptly after infection, because even after bacterial eradication, secreted toxins remain active. These obstacles underscore the need for novel intervention strategies against anthrax. Indeed, recently, based on recent progress in understanding the mechanisms of anthrax toxin, new therapeutic candidates have been designed, such as recombinant

antibodies against the toxin[60], peptide[61, 62] or small chemical inhibitors of LF[48, 63], and polyvalent inhibitors of PA-LF interactions[64]. HNP-1−3 have several therapeutic advantages over other candidates. HNP-1−3 are multifunctional peptides. Besides their well-established capacity to kill a variety of microbial pathogens, immunoenhancing capabilities have also been reported in HNP-1−3. Human neutrophil α-defensins show chemotactic activities for T cells and dendritic cells[41]. They enhance the production of antigen specific antibodies and certain cytokines by immune cells facilitating the initiation of adaptive immune responses[42]. Hence, LeTx neutralization by HNP-1−3 in combination with antibiotic eradication of *B. anthracis* should be exploited for efficient prevention of fatal anthrax incidences.

7. REFERENCES

1. Bird, R. & Allen, G. E. Robert Koch. Microsoft® Encarta® Online Encyclopedia (http://encarta.msn.com) (2004).

2. Bird, R. & Allen, G. E. Louis Pasteur. Microsoft® Encarta® Online Encyclopedia (http://encarta.msn.com) (2004).

3. Mock, M. & Mignot, T. Anthrax toxins and the host: a story of intimacy. Cell Microbiol 5, 15-23 (2003).

4. Turnbull, P. C. Anthrax vaccines: past, present and future. Vaccine 9, 533-9 (1991).

5. Mock, M. & Fouet, A. Anthrax. Annu Rev Microbiol 55, 647-71 (2001).

6. Lincoln, R. E. et al. Role of the lymphatics in the pathogenesis of anthrax. J Infect Dis 115, 481-94 (1965).

7. Zwartouw, H. T. & Smith, H. Polyglutamic acid from Bacillus anthracis grown in vivo; structure and aggressin activity. Biochem J 63, 437-42 (1956).

8. Collier, R. J. & Young, J. A. Anthrax toxin. Annu Rev Cell Dev Biol 19, 45-70 (2003).

9. Duesbery, N. S. et al. Proteolytic inactivation of MAP-kinase-kinase by anthrax lethal factor. Science 280, 734-7 (1998).

10. Leppla, S. H. Anthrax toxin edema factor: a bacterial adenylate cyclase that increases cyclic AMP concentrations of eukaryotic cells. Proc Natl Acad Sci U S A 79, 3162-6 (1982).

11. Klimpel, K. R., Molloy, S. S., Thomas, G. & Leppla, S. H. Anthrax toxin protective antigen is activated by a cell surface protease with the sequence specificity and catalytic properties of furin. Proc Natl Acad Sci U S A 89, 10277-81 (1992).

12. Milne, J. C., Furlong, D., Hanna, P. C., Wall, J. S. & Collier, R. J. Anthrax protective antigen forms oligomers during intoxication of mammalian cells. J Biol Chem 269, 20607-12 (1994).

13. Mogridge, J., Cunningham, K. & Collier, R. J. Stoichiometry of anthrax toxin complexes. Biochemistry 41, 1079-82 (2002).

14. Beauregard, K. E., Collier, R. J. & Swanson, J. A. Proteolytic activation of receptor-bound anthrax protective antigen on macrophages promotes its internalization. Cell Microbiol 2, 251-8 (2000).

15. Vitale, G., Bernardi, L., Napolitani, G., Mock, M. & Montecucco, C. Susceptibility of mitogen-activated protein kinase kinase family members to proteolysis by anthrax lethal factor. Biochem J 352 Pt 3, 739-45 (2000).

16. Webster, J. I. et al. Anthrax lethal factor represses glucocorticoid and progesterone receptor activity. Proc Natl Acad Sci U S A 100, 5706-11 (2003).

17. Moayeri, M., Haines, D., Young, H. A. & Leppla, S. H. Bacillus anthracis lethal toxin induces TNF-alpha-independent hypoxia-mediated toxicity in mice. J Clin Invest 112, 670-82 (2003).

18. Pellizzari, R., Guidi-Rontani, C., Vitale, G., Mock, M. & Montecucco, C. Anthrax lethal factor cleaves MKK3 in macrophages and inhibits the LPS/IFNgamma-induced release of NO and TNFalpha. FEBS Lett 462, 199-204 (1999).

19. Hanna, P. C., Kochi, S. & Collier, R. J. Biochemical and physiological changes induced by anthrax lethal toxin in J774 macrophage-like cells. Mol Biol Cell 3, 1269-77 (1992).

20. Hanna, P. C., Kruskal, B. A., Ezekowitz, R. A., Bloom, B. R. & Collier, R. J. Role of macrophage oxidative burst in the action of anthrax lethal toxin. Mol Med 1, 7-18 (1994).

21. Park, J. M., Greten, F. R., Li, Z. W. & Karin, M. Macrophage apoptosis by anthrax lethal factor through p38 MAP kinase inhibition. Science 297, 2048-51 (2002).

22. Watters, J. W., Dewar, K., Lehoczky, J., Boyartchuk, V. & Dietrich, W. F. Kif1C, a kinesin-like motor protein, mediates mouse macrophage resistance to anthrax lethal factor. Curr Biol 11, 1503-11 (2001).

23. Bradley, K. A., Mogridge, J., Mourez, M., Collier, R. J. & Young, J. A. Identification of the cellular receptor for anthrax toxin. Nature 414, 225-9 (2001).

24. Petosa, C., Collier, R. J., Klimpel, K. R., Leppla, S. H. & Liddington, R. C. Crystal structure of the anthrax toxin protective antigen. Nature 385, 833-8 (1997).

25. Scobie, H. M., Rainey, G. J., Bradley, K. A. & Young, J. A. Human capillary morphogenesis protein 2 functions as an anthrax toxin receptor. Proc Natl Acad Sci U S A 100, 5170-4 (2003).

26. Chaudry, G. J., Moayeri, M., Liu, S. & Leppla, S. H. Quickening the pace of anthrax research: three advances point towards possible therapies. Trends Microbiol 10, 58-62 (2002).

27. Santelli, E., Bankston, L. A., Leppla, S. H. & Liddington, R. C. Crystal structure of a complex between anthrax toxin and its host cell receptor. Nature 430, 905-8 (2004).

28. O'Brien, J., Friedlander, A., Dreier, T., Ezzell, J. & Leppla, S. Effects of anthrax

toxin components on human neutrophils. Infect Immun 47, 306-10 (1985).

29. Ganz, T. Defensins: antimicrobial peptides of innate immunity. Nat Rev Immunol 3, 710-20 (2003).

30. Ganz, T. et al. Defensins. Natural peptide antibiotics of human neutrophils. J Clin Invest 76, 1427-35 (1985).

31. Agerberth, B. et al. The human antimicrobial and chemotactic peptides LL-37 and alpha-defensins are expressed by specific lymphocyte and monocyte populations. Blood 96, 3086-93 (2000).

32. Ganz, T. Extracellular release of antimicrobial defensins by human polymorphonuclear leukocytes. Infect Immun 55, 568-71 (1987).

33. Eisenhauer, P. B. & Lehrer, R. I. Mouse neutrophils lack defensins. Infect Immun 60, 3446-7 (1992).

34. Lehrer, R. I., Lichtenstein, A. K. & Ganz, T. Defensins: antimicrobial and cytotoxic peptides of mammalian cells. Annu Rev Immunol 11, 105-28 (1993).

35. Ganz, T. Biosynthesis of defensins and other antimicrobial peptides. Ciba Found Symp 186, 62-71; discussion 71-6 (1994).

36. Valore, E. V. & Ganz, T. Posttranslational processing of defensins in immature human myeloid cells. Blood 79, 1538-44 (1992).

37. Hill, C. P., Yee, J., Selsted, M. E. & Eisenberg, D. Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. Science 251, 1481-5 (1991).

38. Yang, D., Biragyn, A., Kwak, L. W. & Oppenheim, J. J. Mammalian defensins in immunity: more than just microbicidal. Trends Immunol 23, 291-6 (2002).

39. Zasloff, M. Antimicrobial peptides of multicellular organisms. Nature 415, 389-95 (2002).

40. Lehrer, R. I. et al. Interaction of human defensins with Escherichia coli. Mechanism of bactericidal activity. J Clin Invest 84, 553-61 (1989).

41. Yang, D., Chen, Q., Chertov, O. & Oppenheim, J. J. Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. J Leukoc Biol 68, 9-14 (2000).

42. Lillard, J. W., Jr., Boyaka, P. N., Chertov, O., Oppenheim, J. J. & McGhee, J. R. Mechanisms for induction of acquired host immunity by neutrophil peptide defensins. Proc Natl Acad Sci U S A 96, 651-6 (1999).

43. Tani, K. et al. Defensins act as potent adjuvants that promote cellular and humoral immune responses in mice to a lymphoma idiotype and carrier antigens. Int Immunol 12, 691-700 (2000).

44. Harwig, S. S., Ganz, T. & Lehrer, R. I. Neutrophil defensins: purification, characterization, and antimicrobial testing. Methods Enzymol 236, 160-72 (1994).

45. Park, S. & Leppla, S. H. Optimized production and purification of Bacillus anthracis lethal factor. Protein Expr Purif 18, 293-302 (2000).

46. Ramirez, D. M., Leppla, S. H., Schneerson, R. & Shiloach, J. Production, recovery and immunogenicity of the protective antigen from a recombinant strain of Bacillus anthracis. J Ind Microbiol Biotechnol 28, 232-8 (2002).

47. Lyons, C. R. et al. Murine model of pulmonary anthrax: kinetics of dissemination, histopathology, and mouse strain susceptibility. Infect Immun 72, 4801-9 (2004).

48. Min, D. H., Tang, W. J. & Mrksich, M. Chemical screening by mass spectrometry to identify inhibitors of anthrax lethal factor. Nat Biotechnol 22, 717-23 (2004).

49. Guidi-Rontani, C., Levy, M., Ohayon, H. & Mock, M. Fate of germinated Bacillus anthracis spores in primary murine macrophages. Mol Microbiol 42, 931-8 (2001).

50. Dixon, T. C., Fadl, A. A., Koehler, T. M., Swanson, J. A. & Hanna, P. C. Early

Bacillus anthracis-macrophage interactions: intracellular survival survival and escape. Cell Microbiol 2, 453-63 (2000).

51. Cameron, A., Appel, J., Houghten, R. A. & Lindberg, I. Polyarginines are potent furin inhibitors. J Biol Chem 275, 36741-9 (2000).

52. Schroder, N. W. J. et al. Lipoteichoic Acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus Activates Immune Cells via Toll-like Receptor (TLR)-2, Lipopolysaccharide-binding Protein (LBP), and CD14, whereas TLR-4 and MD-2 Are Not Involved. J. Biol. Chem. 278, 15587-15594 (2003).

53. Bokarewa, M. & Tarkowski, A. Human alpha -defensins neutralize fibrinolytic activity exerted by staphylokinase. Thromb Haemost 91, 991-9 (2004).

54. Park, J. M., Ng, V. H., Maeda, S., Rest, R. F. & Karin, M. Anthrolysin O and Other Gram-positive Cytolysins Are Toll-like Receptor 4 Agonists. J Exp Med 200, 1647-55 (2004).

55. Agrawal, A. et al. Impairment of dendritic cells and adaptive immunity by anthrax lethal toxin. Nature 424, 329-34 (2003).

56. Kirby, J. E. Anthrax lethal toxin induces human endothelial cell apoptosis. Infect Immun 72, 430-9 (2004).

57. Panyutich, A. V., Panyutich, E. A., Krapivin, V. A., Baturevich, E. A. & Ganz, T.

Plasma defensin concentrations are elevated in patients with septicemia or bacterial meningitis. J Lab Clin Med 122, 202-7 (1993).

58. Mandal, M. & Nagaraj, R. Antibacterial activities and conformations of synthetic alpha-defensin HNP-1 and analogs with one, two and three disulfide bridges. J Pept Res 59, 95-104 (2002).

59. Pannifer, A. D. et al. Crystal structure of the anthrax lethal factor. Nature 414, 229-33 (2001).

60. Maynard, J. A. et al. Protection against anthrax toxin by recombinant antibody fragments correlates with antigen affinity. Nat Biotechnol 20, 597-601 (2002).

61. Tonello, F., Seveso, M., Marin, O., Mock, M. & Montecucco, C. Screening inhibitors of anthrax lethal factor. Nature 418, 386 (2002).

62. Turk, B. E. et al. The structural basis for substrate and inhibitor selectivity of the anthrax lethal factor. Nat Struct Mol Biol 11, 60-6 (2004).

63. Panchal, R. G. et al. Identification of small molecule inhibitors of anthrax lethal factor. Nat Struct Mol Biol 11, 67-72 (2004).

64. Mourez, M. et al. Designing a polyvalent inhibitor of anthrax toxin. Nat Biotechnol 19, 958-61 (2001).

8. ACKNOWLEDGEMENT

I would like to express my thanks to Prof. Dr. Stefan H.E. Kaufmann, who offered me the opportunity to work in his department at the Max Planck Institute for Infection Biology in Berlin. I thank also Prof. Dr. Roland Lauster, who was kindly willing to be my mentor and who was generous in finding time for my thesis. Thank you also to Dr. Nadesan Gajendran and Dr. Hans-Willi Mittruecker for all the time, and stimulating discussion

9. RESUME

PERSONAL DETAILS

Name: Kim, Chun

Address: Max-Planck Institute for Infection Biology

Department of Immunology

Schumannstrasse 21/22

D-10117 Berlin, Germany

Phone: +49 (0)30 28460 562

E-mail: ckim@mpiib-berlin.mpg.de

EDUCATION

● Philosophy of Doctor of Science Degree, Max-Planck Institute for Infection Biology and University of Technology, Berlin, Present

● Master of Science Degree in Life Science, Sogang University, February 1999

● Bachelor of Science Degree in Life Science, Sogang University, February 1997

EXPERIENCE

1. Aug 2001 - Current

Prof. Stefan HE Kaufmann in Max-Plank Institute for Infection Biology and Prof. Roland Lauster in Technischen Universitaet Berlin, Berlin, Germany

- Identification of a novel function of human neutrophil defensin
- STAT-JAK signaling study in *Salmonella, Listeria,* and *Mycobacteria* infected hosts
- Establishment of liposome based *in vivo* DNA delivery

2. May 1999 - June 2001

Dr. Jeongshil Kim-Ha in Samsung Biomedical Research Institute, Seoul, Korea

- Search of target transcripts for RBP9, an RNA binding protein, in Drosophila
- Characterization of interacting proteins with RBP9
- Functional analysis of Femcoat, a novel gene

3. December 1996 - March 1999

Dr. Jaimyung Yang in Department of Life Science at Sogang University, Seoul, Korea

- Development of a stable cell line producing high level of human erythropoietin

-

PUBLICATIONS

1. Proc Natl Acad Sci U S A. 2005 Mar 16

Human alpha-defensins neutralize anthrax lethal toxin and protect against its fatal consequences.

Kim C, Gajendran N, Mittruecker H, Weiwad M, Song YH, Hurwitz R, Wilmanns M, Fischer G and Kaufmann SH

2. Mechanisms of Development 2002 Jan; 110 (1-2): 61-70

Femcoat, a novel eggshell protein in Drosophila: functional analysis by double stranded RNA interference.

Kim C, Han K, Kim J, Yi JS, Kim C, Yim J, Kim YJ, Kim-Ha J

3. Biotechnology and Applied Biochemistry 2000 Dec; 32 (Pt 3): 167-172

Efficiency of promoter and cell line in high-level expression of erythropoietin.

Park JH, **Kim C**, Kim WB, Kim YK, Lee SY, Yang JM.

PATENT

α-defensins as anthrax immuno therapeutics (European Patent Application No. 04 01 7392.4)

- End of Resume -