COMPARATIVE STUDIES ON

Cardiac Innate Immunity

by

ANNIKA LINDE

D.V.M., University of Copenhagen, Denmark, 1999

AN ABSTRACT OF A DISSERTATION

Submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy & Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2008

Abstract

Background

Cardiovascular disease (CVD) impacts the lives of millions, and ranks as the number one killer world-wide. Despite significant research efforts, CVD remains a major burden on the national health care system, and novel therapeutic modalities to effectively and curatively fight many debilitating diseases of the heart and vasculature are urgently needed. The role of inflammation in the development of CVD has been increasingly in focus through the past decade. Elucidating upon the plethora of innate immune mechanisms likely involved in CVD therefore becomes of immediate interest. Host defense peptides (HDPs) are central elements of innate immunity, encompassing molecules (including the defensin peptides) with wide-reaching biological effects, including immunomodulation and antimicrobial activity.

Hypothesis & Specific Aims

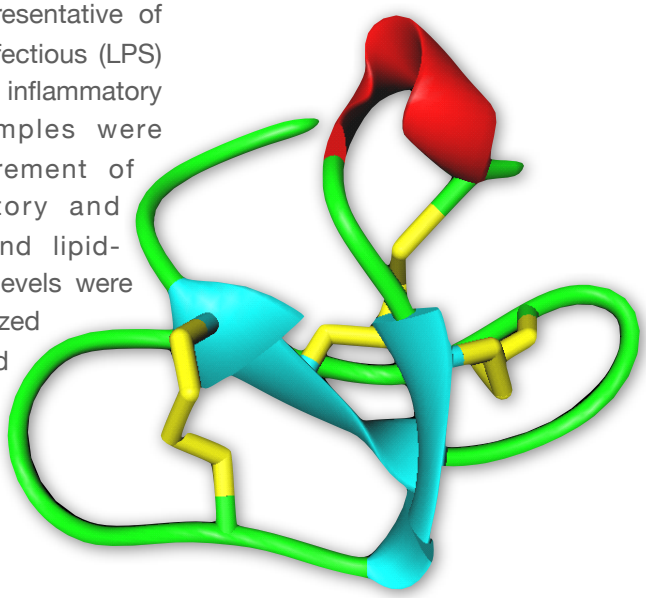
The study's main hypothesis relies upon the basic concept that the heart possesses a local innate defense system, which actively aids in fighting off a variety of "danger signals", and that a disarray in this defense contributes to development of CVD. The heart expresses beta-defensin peptides (BDs), and we theorized that these HDPs act as a local defense system within the myocardium - or in other words as "guardians of heart health". The specific aims of the experimental studies were to 1) Evaluate expression of cardiac BDs in response to inflammatory mediators, and 2) Assess the functional properties (including antimicrobial activity and immunomodulation) of synthetic BD peptides *in vitro*.

Design & Methods

To test our hypothesis, we studied myocardial beta-defensin expression (rBDs) in a rat model, comparing levels among two experimental and one control group. Animals were exposed to lipopolysaccharide (LPS) or high-fat diet (HFD) intake - representative of exposure to either an infectious (LPS) or non-infectious (HFD) inflammatory mediator. Serum samples were collected for measurement of cytokines, inflammatory and cardiac biomarkers and lipid-profiling. Beta-defensin levels were assessed using customized Superarray assays and qRT-PCR, and all amplicon sizes on the PCR products were subsequently confirmed using agarose gel electrophoresis. Serum levels were assessed on commercial ELISA kits. Functional assessment of select rBDs included computational modeling as well as *in vitro* antimicrobial and cell migration assays.

Results & Conclusion

Exposure to high-fat diet feeding for a period of three weeks resulted in a multifold-increase in cardiac mRNA expression of select rBDs, while short-term LPS exposure resulted in a smaller, but statistically non-significant, elevation in the myocardial expression of rBDs. Synthetic analogues of two naturally occurring cardiac rBDs were evaluated for *in vitro* activity. The synthetic rBD11 peptide exhibited antimicrobial activity against *Staph aureus*, and both rBDs exhibited chemoattraction of rat leukocytes. Our data suggests that rBDs might play a central role in the intrinsic immune mechanisms of the cardiovasuclar system, and possibly act as protectors of heart health.



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Approved by;

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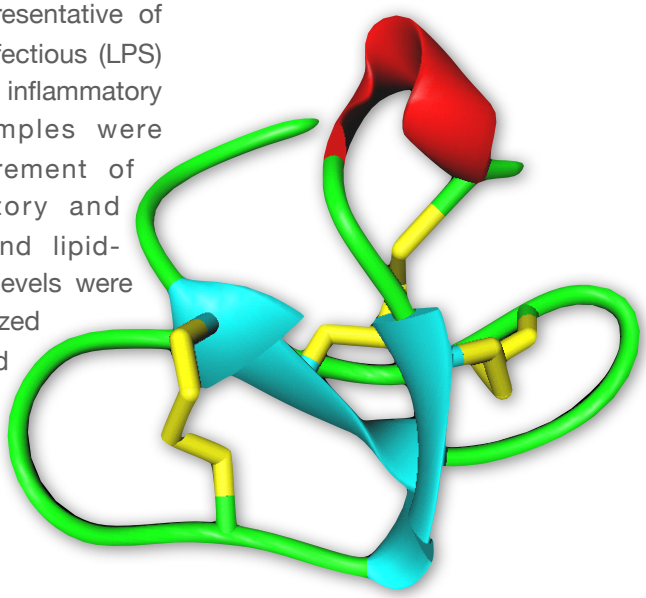

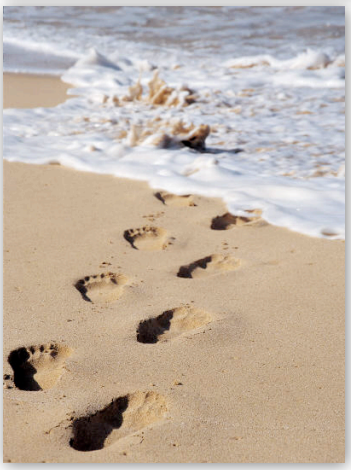

In search of new therapies to effectively fight heart disease

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"Research is the act of going up alleys to see if they are blind"

PHOTOGRAPHY

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To Nicky, who patiently walked this road with me for so long (1993-2006)

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....a few more words of gratitude

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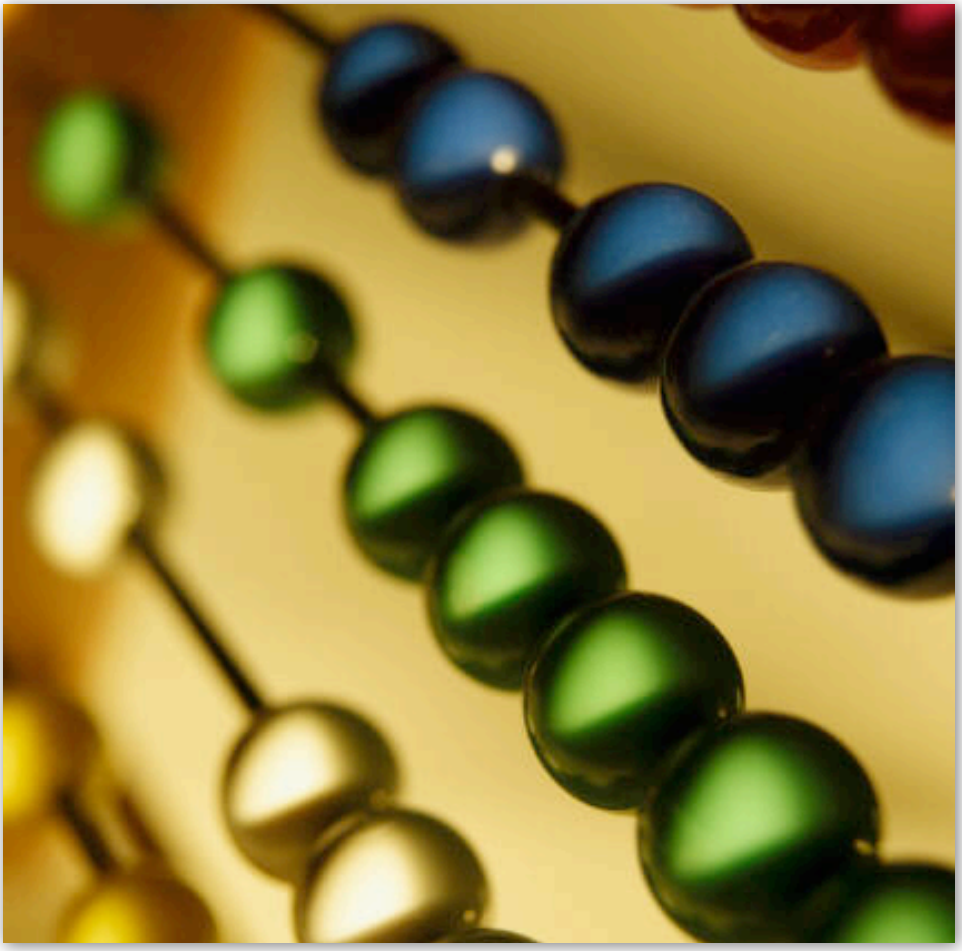
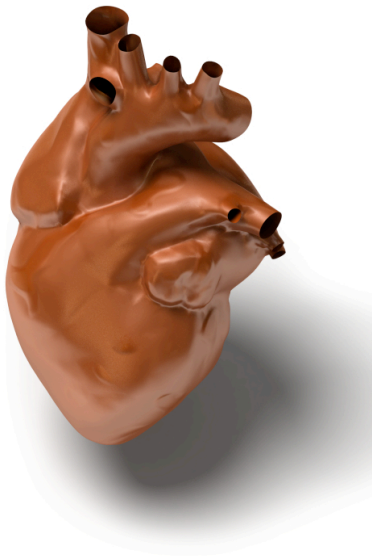
On a final note, I would in closing like to also state my sincere gratitude to Mrs. Elouise & Dr. John Junkins, College Station, TX. Without your help I would never have made it through in one piece – you have my deepest respect.

Preface





Cardiovascular disease is the leading cause of mortality world-wide, and responsible for almost 1/3 of all deaths.

Nature (Feb. 2008) Vol. 451 (7181): 903

Why Cardiac Immunology?

The basic idea of the immune system as a conductor that orchestrates all cellular events within and between organs across species is essentially what renewed my interest in the field of immunology. Veterinary medicine seemed a natural path early on, and cardiology was the subspecialty that fascinated me most during vet school, logically leading me to post-graduate residency training in small animal clinical cardiology.

Focus Directed by Statistics

A cardiologist will naturally claim that the heart is the single most important organ in the body - and more or less gracefully ignore when colleagues try to suggest that the kidneys, liver, lungs and so on are equally important for survival. The more stubborn of specialists would then of course argue that cardiovascular disease is the number-one killer, and that keeping the heart healthy therefore has to be the central theme in modern medicine. Setting aside all differences in perceived importance of any individual organ, my professional loyalty will however remain with the heart, based on the simple fact that no other organ has the same degree of complexity associated with it on such a multitude of levels - at least in my humble opinion

Clinical Applications

While on clinics, I experienced the basic frustration of dealing with terminal types of disease processes and frequently having only palliative measures to offer our heart-patients – an unfortunately all too common scenario in veterinary as well as human medicine. I suspect I am far from alone in wondering why we as clinicians/scientists cannot offer more to our patients – other than perhaps

....how cardiology and immunology match up....

extending life for a few weeks or months. Despite vast sums invested in R&D throughout the developed world, heart disease continues to take millions of lives every year.

Immunity & Heart Disease

Most answers to why an organism develops disease logically hide at a molecular level, and few diseases are truly "fixable" on a purely macroscopic level. Additionally, more and more pathologies seem linked to a mishap in the immune system, and inflammation has in the past decade or so been in focus in the field of cardiovascular medicine. To further pursue new answers and help identifying novel and possibly curative measures, a logical step therefore seemed to focus my attention on the role of innate immune mechanisms in the development of diseases affecting the heart and vasculature.

In Perspective

The field of cardiovascular immunopathology is as such still in its infancy, and consequently much basic (not to mention clinical) research remains to be done. By means of this dissertation, I hope to have made a small, yet valuable, contribution to a field still in its most early stages. Undoubtedly, a significant amount of work still lies ahead before the complex immune mechanisms underlying heart disease have been unraveled, and novel drugs for the heart hopefully are identified.

The Thesis in Short

The thesis initially presents a background section on host defense peptides (HDPs) and their general relevance in veterinary medicine (Chapter 1), followed by an overview of innate immune mechanisms in comparative cardiovascular pathology (Chapter 2). The experimental studies on cardiac expression of HDPs in infectious and non-infectious inflammatory processes are presented subsequently in Chapter 3. Finally, the thesis looks at HDPs from an evolutionary viewpoint (Chapter 4). In essence, the "evolutionary shortcut theory" (which created the foundation for this section) focuses on studies aimed at the identification of novel antimicrobial and immunomodulatory compounds derived from special species surviving extreme conditions, and importantly these molecules' potential relevance as templates for development of pharmaceutical compounds to treat inflammatory disease processes, including select types of heart disease.

Future Directions

Naturally, such early studies inevitably raise a plethora of additional research questions – primarily focusing on the distinction between causative mechanisms in cardiac pathology versus mere coincidental events. I am excited, however, about the immense prospects that this field offers, and hope that this will be an important scientific route towards improving heart health and quality of life for animals and humans alike. I hope you will enjoy the reading!

Annika Linde

Chapter 1
Introduction to Innate Immunity

This introductory chapter of the thesis contains a literature review focused on the current scientific knowledge pertaining to host defense peptides and related elements of innate immunity, and most importantly the role that these molecules play in fighting off disease and maintaining health.

The section, as such, includes a recently published review article entitled ***"Innate Immunity and Host Defense Peptides in Veterinary Medicine"*** from the *Journal of Veterinary Internal Medicine* (JVIM 2008;22:247–265). In this paper we discuss the main types of host defense peptides (HDPs) and their characteristics - including chemical structure and biological function.

The overall field of HDP-research does not trace back very far, and articles focused on these small peptides were practically non-existing prior to the mid-eighties, when the first defensin peptides were reported from the laboratories of Drs. Ganz, Selsted and Lehrer at UCLA School of Medicine as well as Dr. Boman's group at Karolinska Institutet in Stockholm, Sweden.

Since then the scientific world has been presented with a significant amount of work on HDPs in various exotic, and less exotic, species - covering representative from virtually all major clades of animals. According to the "Defensin Knowledgebase" - which is an online tool maintained by the Bioinformatics Institute and the Singapore Eye Research Institute - the number of primary literature on defensins alone exceeded 350 papers just in 2006 (perhaps this will amount to a new saying along the line of: *"...a defensin-paper a day keeps the doctor away..."*). Suffice to say, however, that the scientific interest and amount of basic research conducted on these small cationic peptides has been steadily increasing since 1985.

The paper which follows is focused primarily on the role of HDPs (also known as "antimicrobial peptides", or AMPs) in small and large animal (domestic) species of direct relevance to veterinary medicine.

Review

J Vet Intern Med 2008;22:247–265

Innate Immunity and Host Defense Peptides in Veterinary Medicine

A. Linde, C.R. Ross, E.G. Davis, L. Dib, F. Blecha, and T. Melgarejo

Recent years have witnessed a surge in interest directed at innate immune mechanisms. Proper conceptualization of the key elements of innate immunity, however, is still a work in progress, because most research in immunology traditionally has been focused on components of the acquired immune response. The question of why an animal stays healthy in a world filled with many dangers is perhaps as interesting as why it sometimes surrenders to disease. Consequently, studies with an increased focus on inborn mechanisms of animal host defense may help further the development of appropriate preventative and therapeutic measures in veterinary medicine. Host defense peptides (HDPs) are central effector molecules of innate immunity, and are produced by virtually all living species throughout the plant and animal kingdoms. These gene-encoded peptides play a central role in multiple, clinically relevant disease processes. Imbalances in the expression of HDPs can lead to overt pathology in different organ systems and cell types in all species studied. In addition, HDPs are an ancient group of innate chemical protectors, which are now evaluated as model molecules for the development of novel natural antibiotics and immunoregulatory compounds. This review provides an overview of HDPs and is aimed at veterinary practitioners as well as basic researchers with an interest in comparative immunology involving small and large animal species.

Key words: Antimicrobial peptides; Danger-associated molecular patterns; Natural antibiotics; Pathogen-associated molecular patterns; Pattern recognition receptors; Toll-like receptors.

In a world filled with microorganisms, survival without the inherent protection of innate immunity would be virtually unattainable. The clear success of survival based on innate defense mechanisms alone is solidly evident in plants, fungi, and invertebrates – all of which completely lack acquired immune mechanisms.[1] Innate immunity as such constitutes an evolutionarily ancient scheme founded on a relatively generic, but nevertheless quite effective defense strategy. In addition to the immediate anatomical barriers of the organism, this intrinsic resistance system relies primarily on pattern recognition receptors and associated signaling pathways, specialized chemical mediators (cytokines), the complement cascade, leukocytes, and importantly host defense peptides (HDPs).[2] The list of natural compounds with antimicrobial activities is extensive, but largely includes 3 functional groups: (1) digestive enzymes targeting microbial structures (eg, lysozyme), (2) peptides that bind essential elements such as zinc or iron (calprotectin and lactoferrin, respectively), and (3) peptides that disrupt the microbial membrane (eg, defensins and cathelicidins, as discussed below).[3] At the end of the 1920s, Alexander Fleming identified lysozyme as the 1st peptide with antimicrobial activity. It is, however, only in the past 2 decades that developments in molecular biology techniques have allowed isolation and identification of individual peptides, and the establishment of their structural and functional features.[4,5] This review is aimed at providing an overview of the current understanding of HDPs, with special emphasis on defensins and cathelicidins and their role in immunological defense in companion and production animals. Defensins and cathelicidins are highlighted because these currently are the most studied vertebrate HDPs. In addition, the potential application of natural antimicrobial compounds as templates directed at the development of novel antibiotics and immunoregulatory drugs for use in veterinary medicine is discussed.

The Importance of an Innate Host Defense

An immediate nonspecific defense system aimed at controlling potential infectious as well as noninfectious dangers efficaciously is vital to ensure animal health. The term ''danger'' is used here in reference to the ''Danger-Model'' concept,[6] which entails activation of an immune response not only in response to microorganisms (nonself), but also as a reaction toward all other types of insults (or ''danger signals''), including physical trauma, ionizing radiation, oxidative stress, ischemia, and extreme temperatures. Innate immunity thus ensures an immediate mode of defense in virtually all living organisms. A group of multifunctional antimicrobial peptides (ie, the HDPs) comprise the core of this innate immune response.[7]

In animals higher on the evolutionary ladder (eg, mammals being more ''evolved'' than insects), the initial interaction between microbial intruders and their prospective host takes place on the cutaneous surface or on the epithelial lining of the gastrointestinal, reproductive, respiratory, or urinary tract.[8,9] Thus, it is not surprising that epithelial cells of vertebrates produce HDPs as components of this 1st line of defense. Because inflammation comprises an initial reaction in the innate immune cascade, it is reasonable that HDPs are also produced by inflammatory cells such as neutrophils, and tissue phagocytes, including macrophages.[3,4,10] Perhaps not immediately logical, however, is that HDPs are expressed by less typical cell types (at

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least from a purely immunological point of view) such as endotheliocytes and myocytes, thus suggesting a universal function of innate immune mechanisms.[11–13] This would further expand the common view of the immune system as an entity of professional immune cells surveying the body for potential intruders to that of an integrated and inclusive entity of cells communicating and collaborating to ensure maintenance of homeostasis.[14]

Broadly defined, HDPs have the capability of targeting any organism with a cholesterol-free, negatively charged membrane. The functional capacity of different HDPs thus includes broad-spectrum antimicrobial activities against Gram-positive and Gram-negative bacteria, mycobacteria, fungi, intracellular parasites, and enveloped viruses.[4,15] Importantly, HDPs are able to kill transformed or cancerous cells, a cytotoxicity that tends to be neither species-specific nor selective.[2,16,17] A linkage to initiation of an adaptive immune response has been observed for defensins, which act as direct chemoattractants for immature dendritic cells.[2,17,18] Some defensins are opsonic (ie, they enhance phagocytosis) and also have the capability to modify hormonal reactions.[16] Thus, HDPs are far more than "simple natural antibiotics." As such, HDPs seem to play a central role in a number of clinically relevant disease processes, including low grade inflammation, obesity, diabetes, and hyperlipidemia.[19–22] Table 1 outlines clinically relevant disease processes (and associated pathogens) in which HDPs most likely play a role. The physiological properties and regulation of these molecules therefore may hold a key to explaining many complexities in veterinary medicine.

Structural Characteristics of Defensins and Cathelicidins

Natural antimicrobial substances are numerous and, as a group, rather heterogeneous, varying in size from relatively large protein complexes (eg, the complement cascade) to small inorganic molecules (eg, hydrogen peroxide).[8] To date, approximately 900 different HDP sequences have been identified (cataloged in the Italian Trieste Database at http://www.bbcm.univ.trieste.it/). The conventional definition of "antimicrobial peptides" (synonymous with HDPs), however, includes only gene-encoded, ribosomally synthesized polypeptide antimicrobial substances 100 amino acid residues in length.[8] Because the majority of fungal and bacterially derived peptide antibiotics are nonribosomally synthesized peptides incorporating atypical amino acids, the above definition separates HDPs from this category.[8] Two major classes of conventional HDPs are the defensins and cathelicidins. A large number of other HDP families are present in invertebrates, most notably a wide variety of different insects, yet these peptides do not fall within the scope of this paper.

Four broad structural groups of folded HDPs have been described, including α-helical peptides (eg, cathelicidins), β-sheet peptides with 2–4 disulfide bonds (eg, α- and β-defensins), loop peptides with 1 disulfide bond (eg, bactenecin), and extended peptide structures rich in arginine, glycine, histidine, praline, tryptophan, or some combination hereof (eg, indolicidin).[23] The biological effect of these cationic peptides is primarily dependent on their (tertiary) structure, and thus their structural characteristics are of direct interest.[3]

Classical defensin molecules encompass a family of small amphipathic[a] variably arginine-rich cationic peptides (typically 30–40 amino acid residues in length) characterized by 6 disulfide-paired cysteines (linked Cys [1–6], Cys [2–4], and Cys [3–5], for α-defensins, and Cys [1–5], Cys [2–4], and Cys [3–6], for β-defensins—see descriptions below).[2,4,11,16] Some defensins are particularly abundant in mammalian phagocytes, where they can comprise up to 50% of total protein in azurophil[b] granules.[4,16] Defensins have, however, also been identified in other cell types, including tissue macrophages, small intestinal epithelial cells, and cardiomyocytes.[13,24,25] The overall structure of the defensin peptides has been compared with a bent paperclip, because of the characteristic chemical composition consisting of a triple-stranded β-sheet structure and a connecting loop that creates a base from which a β-hairpin hydrophobic structure extends almost perpendicularly[4] (Fig 1). To date, 3 different categories of vertebrate defensins have been described (in addition to the insect and plant defensins) based on size and structural differences in the cysteine linkage (secondary structure).[7,26]

α-defensins are the classical "neutrophil defensins," which were first described in the mid-1980s, whereas the slightly larger β-defensins were reported initially in the early 1990s.[9] The Trieste Database[c] contains 90 β-defensins and 55 α-defensins. More recently, θ-defensins[d] have been described. α- and β-defensins are widely distributed across species, but θ-defensins are expressed only in granulocytes of the rhesus macaque and some other primates, including other Old World monkeys and orangutans.[27] Other great apes (including humans) and New World monkeys do not express θ-defensins.[28–30] θ-defensins are double-stranded small circular molecules, in contrast to α- and β-defensins, which are flat triple-stranded β sheets.[7]

A unifying feature of the cathelicidin peptides is a marked homology termed the cathelin[e] domain at the 5′ region, and a variable C-terminal antimicrobial domain.[5,31,32] Cathelicidins are found in varying numbers in numerous different species, including domestic animals.[15,33] They are stored as inactive propeptides and processed only upon stimulation, thus resulting in the release of active HDPs into the extracellular fluid.[15] Cathelicidins typically are expressed by myeloid precursor cells, but expression also has been reported in mature circulating neutrophils and neonatal lymphoid tissue in some animal species.[15,34] Moreover, the number of cathelicidin antimicrobial peptides varies among species, which most likely leaves different animals with varying levels of resistance toward specific types of infections.[35] Interestingly, cathelicidins and defensins exhibit synergism,[36] implying their combined role in the orchestration of the innate host defense, as further discussed below.

Host Defense Peptides – Synthesis, Expression, and Mechanism of Action

HDPs can be either constitutively expressed or induced in response to specific stressors such as

Table 1. Host defense peptides in veterinary medicine.

Species	Peptide	In vitro Antimicrobial Activity	Clinical Disease (examples)
Dogs	K9CATH cBDs	*Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Candida albicans*	Urinary tract infections
		Salmonella enteritidis, S. typhimurium, Escherichia coli	Gastroenteritis
		Listeria monocytogenes	Meningitis, abortion
		Staphylococcus aureus	Dermatitis
		Candida albicans	Stomatitis, spondylitis, dermatitis
Horses	eNAPs	*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus zooepidemicus*	Endometritis
	eCATHs	*Streptococcus equines, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens*	Inflammatory airway disease
	eBD	(*Corynebacterium* sp. and *Staphylococcus intermedius*)[a]	Otitis
Cattle	Epithelial BDs	*Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, Staphylococcus aureus, Candida* spp.	Mastitis
	BNBDs		
	LAP	*Mannheimia hemolytica, Mycobacterium paratuberculosis*	Shipping fever, paratuberculosis
	TAP	*Aspergillus* and *Candida* spp.	Systemic mycosis
	Bactenecins	*Escherichia coli, Klebsiella pneumoniae, Salmonella typhimurium, Enterobacter cloacae, Leptospira interrogans* and *L. biflexa*	Mastitis, enterocolitis, meningitis, leptospirosis
	BMAPs	*Staphylococcus aureus, Pasteurella multocida*	Mastitis, pasteurellosis
Sheep	sBDs	*Mannheimia hemolytica*	Shipping fever
	SMAPs OaBac5α	*Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Staph epidermitis, Candida albicans*	Mastitis, enterocolitis
Goats	ChBac5	*Escherichia coli, Pseudomonas aeruginosa, Listeria monocytogenes*	Mastitis, listeriosis
Pigs	pBDs	*Escherichia coli, (Salmonella typhimurium), Listeria monocytogenes, Candida albicans*	Gastroenteritis, listeriosis
	PR-39	*Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Actinobacillus pleuropneumoniae*	Gastroenteritis, listeriosis, wound healing, pleuropneumonia
	PMAPs	*Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa, Staphylococcus aureus, Candida albicans*	Gastroenteritis, wound infections, systemic mycosis
	Protegrins	*Staphylococcus aureus, Pseudomonas aeruginosa*	Wound infections
Poultry	Gallinacins THPs	*Haemophilus/Avibacterium paragallinarium, Salmonella Spp., Escherichia coli, Staphylococcus aureus*	Infectious coryza, enteritis, septicemia, dermatitis
	Fowlicidins	*Escherichia coli, Klebsiella pneumoniae, Listeria monocytogenes, Staphyloccocus aureus*	Enteritis, airsacculitis, septicemia, cellulites, tracheitis, encephalitis, gangrenous dermatitis

The table provides an overview of clinically relevant diseases in which HDPs are likely to play a role. It should be noted that this is a non-comprehensive list, because a dysfunctional host defense peptide response in all probability contributes to infectious and inflammatory disorders in general. See text for references and abbreviations.

[a]Pathogens associated with the clinical disease. However, the antimicrobial activity of the listed HDP(s) remains to be tested.

infection and inflammation.[3,31,37–39] α-defensins tend to be produced constitutively, whereas the majority of β-defensins are inducible.[7,15] Moreover, α-defensins have evolved to operate mainly from within phagosomes, whereas β-defensins are produced primarily by epithelial cells.[7]

Lipopolysaccharide (LPS) and the proinflammatory cytokines IL-1β and TNF-α promote HDP synthesis.[3] Their production resembles that of peptide hormones, involving sizable precursor molecules and tissue-specific sequential proteolytic processing.[4] After removal of the signal sequence, the proregion is disposed of, yielding the mature HDP.[15] Defensin molecules are produced as neutral preprodefensins (approximately 95 amino acids), which are not cytotoxic to the cell.[16] The antimicrobial and cytotoxic functional properties of the mature defensins (and other HDPs) generally are thought to be associated with their pore-forming activities as multimers in biological membranes leading to self-promoted uptake,[15,16] a mechanism that has been further described by the Shia-Matsuzaki-Huang model[40–42] (Fig 2). The HDPs target the ''Achilles heel'' of the microbial membrane (ie, the absence of cholesterol and negatively

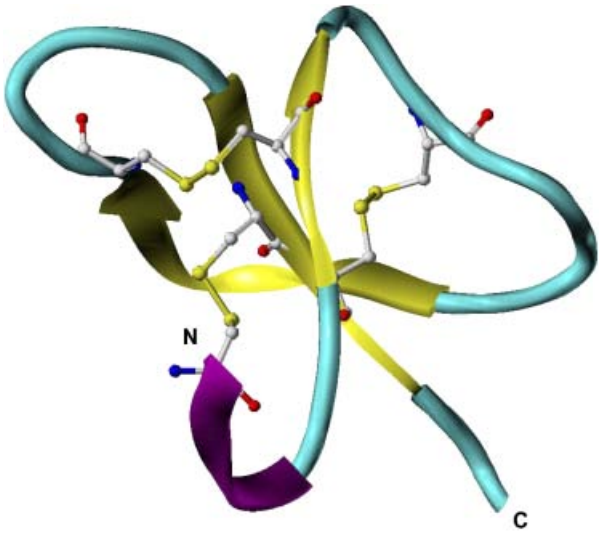

Fig 1. Model of a β-defensin molecule from dog. The structure displays the characteristic 3 disulfide bonds (ball and sticks), an α-helix (purple) and three β-sheets (yellow).

charged phospholipids on the outer leaflet of the cytoplasmic membrane).[43] The positive net charge (+2 to + 7 because of an excess of basic over acidic amino acids)[15] facilitates binding of an increasing number (1–10 billion molecules) of HDPs to the phospholipids on the bacterial surface until the bacterial membrane collapses completely.[7,44] Cholesterol prevents membrane damage, and because this lipid is an essential part of eukaryotic membranes, it explains why normal concentrations of HDPs do not cause host-damage.[7] The membrane potential of eukaryotic cells (−15 mV) also is low compared with the bacterial transmembrane potential (−140 mV), which also minimizes interaction.[15] Resistance to HDPs is rare, because it is exceedingly difficult for any microorganism to change its structural organization of surface phospholipids.[26] Some HDPs target intracellular sites in addition to the bacterial membrane.[45] Also, defensins have been implicated as a link between the innate and adaptive immune responses (Fig 3).

Various tissues and cell types in the body contain gene-encoded pattern recognition receptors (PRRs) and can mandate a number of different signaling pathways in

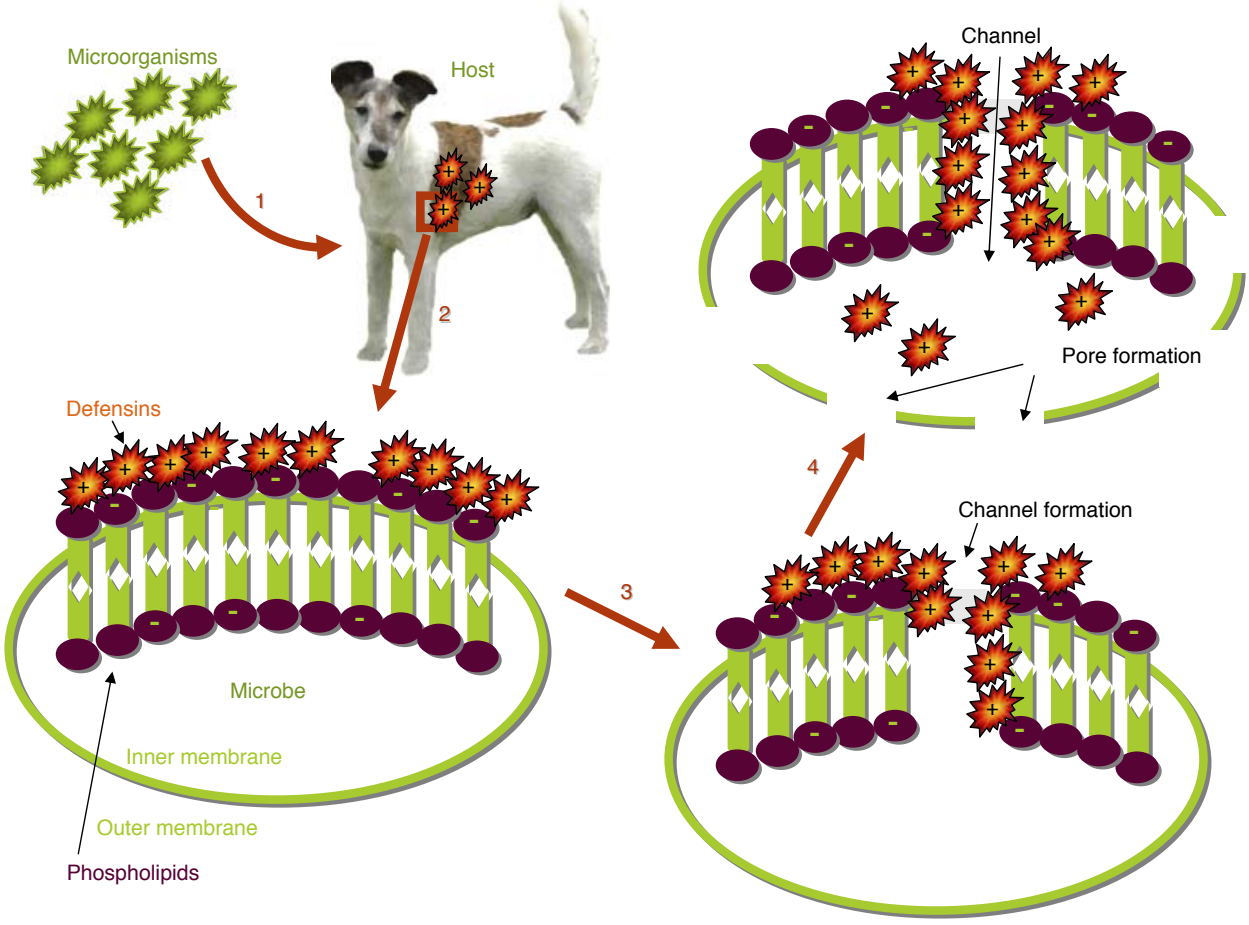

Fig 2. Shia-Matsuzaki-Huang model. The model displays the general consensus for HDPs' antimicrobial mode of action (other possible theories for membrane disruption by AMPs have been published also).[40–42] 1: Host is initially exposed to microorganisms. 2: The innate immune response involves recruitment of cationic HDPs, which are immediately attracted toward the anionic microbial membrane. 3: The HDPs form a carpet-like structure on the microbial membrane, instituting channel formations. 4: The channels lead to pore-formation membrane destabilization and microbial demise. HDP, host defense peptides.

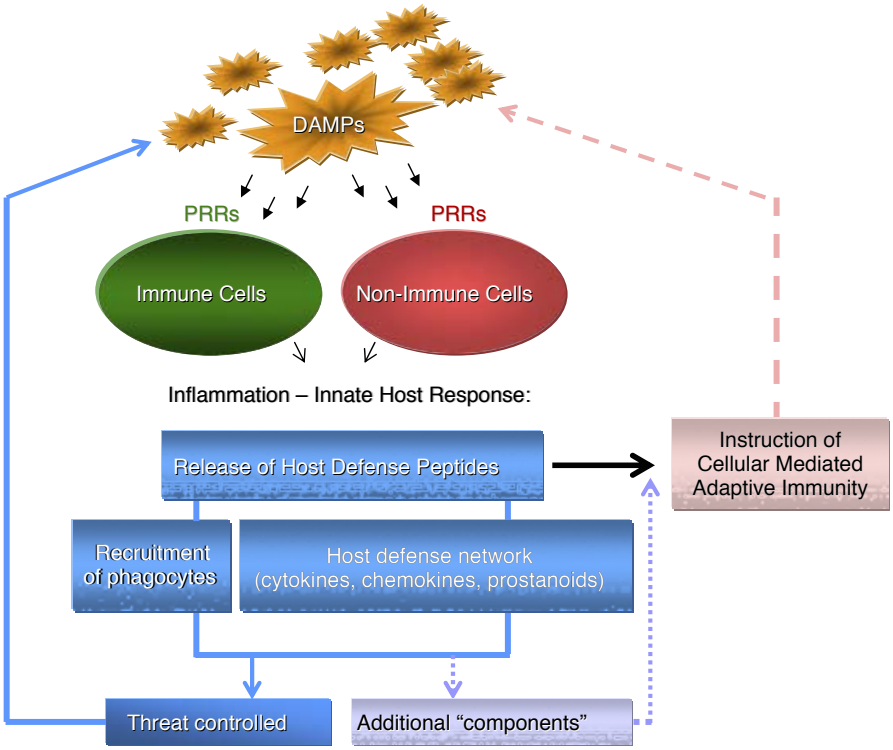

Fig 3. Innate defense mechanisms and host defense peptides (HDPs) linkage to adaptive immunity. The schematic displays the key components of an innate immune response induced by danger-associated molecular patterns' (DAMPs) interaction with pattern recognition receptors (PRRs) on professional as well as nonprofessional immune cells (in the context of the figure "non-immune cell" indicates a nonprofessional immune cell type such as epithelium/endothelium or myocytes). In addition to their immediate actions within the frame work of an inborn immune response, HDPs also create a biological link between innate and acquired immunity, thus orchestrating an appropriate overall host defense.

response to stress, ultimately ensuring production of all necessary signaling and effector molecules required for an appropriate and immediate host defense. Host PRRs generally are surface proteins that immediately identify conserved molecular structures associated with microbial pathogens or other impending dangers. The repertoire of PRRs capable of regulating gene expression encompasses the Toll-like receptors (TLRs) and the virus-sensing RIG-I and Mda5 helicases.[f,47] Other non-TLR recognition molecules, however, also have been described. The structures identified by a given PRR are classified either as pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs). Classical PAMPs include LPS and lipoteichoic acid (LTA) from Gram-negative and Gram-positive bacteria, respectively, viral double-stranded RNA (dsRNA), and fungal β-glucans.[48] The term DAMPs is used here as a common name referring to PAMPs as well as endogenous alarm signals released by dying or injured cells.[14,49] Matzinger's Danger Model defines "dangers" as anything (exogenous or endogenous) that has the potential to cause tissue stress or destruction[6,14] (Fig 4).

Also in the category of innate sensors are the intracellular Nod-like receptors (NLRs), which present a powerful combined defense at the plasma membrane (ie, TLRs) as well as from within the cell (ie, NLRs).[48] Both TLRs and Nod[g] proteins can trigger the nuclear factor κB (NF-κB) transcription factor, thus activating a highly stereotypical signaling pathway responsible for a range of different cellular responses[48] including production of HDPs. The NLRs have been linked to recognition of bacterial components as well as endogenous danger signals.[48] TLRs initially received considerable research interest, and consequently this group of PRRs is most well-described. More than a dozen different members have been reported in 6 major families, with each member recognizing different PAMPs. LPS is the classical ligand for TLR4, whereas LTA and CpG oligodeoxynucleotides are recognized by TLR2 and TLR9, respectively.[50]

NF-κB signaling is one of the main down-stream pathways responsible for HDP production, although other signaling routes (including MAPK[h] and JAK/STAT[i] signaling) have been implicated in their synthesis.[51] NF-κB is a transcription factor involved in the integration of numerous parallel signaling pathways and a variety of cellular responses central to an immediate and functional immune response, including the production of cytokines and cell adhesion molecules.[15] Signaling through these pathways leads to transcriptional activation and subsequent production of HDPs. The TLRs and NLRs also result in activation of the inflammatory caspases,[j] which

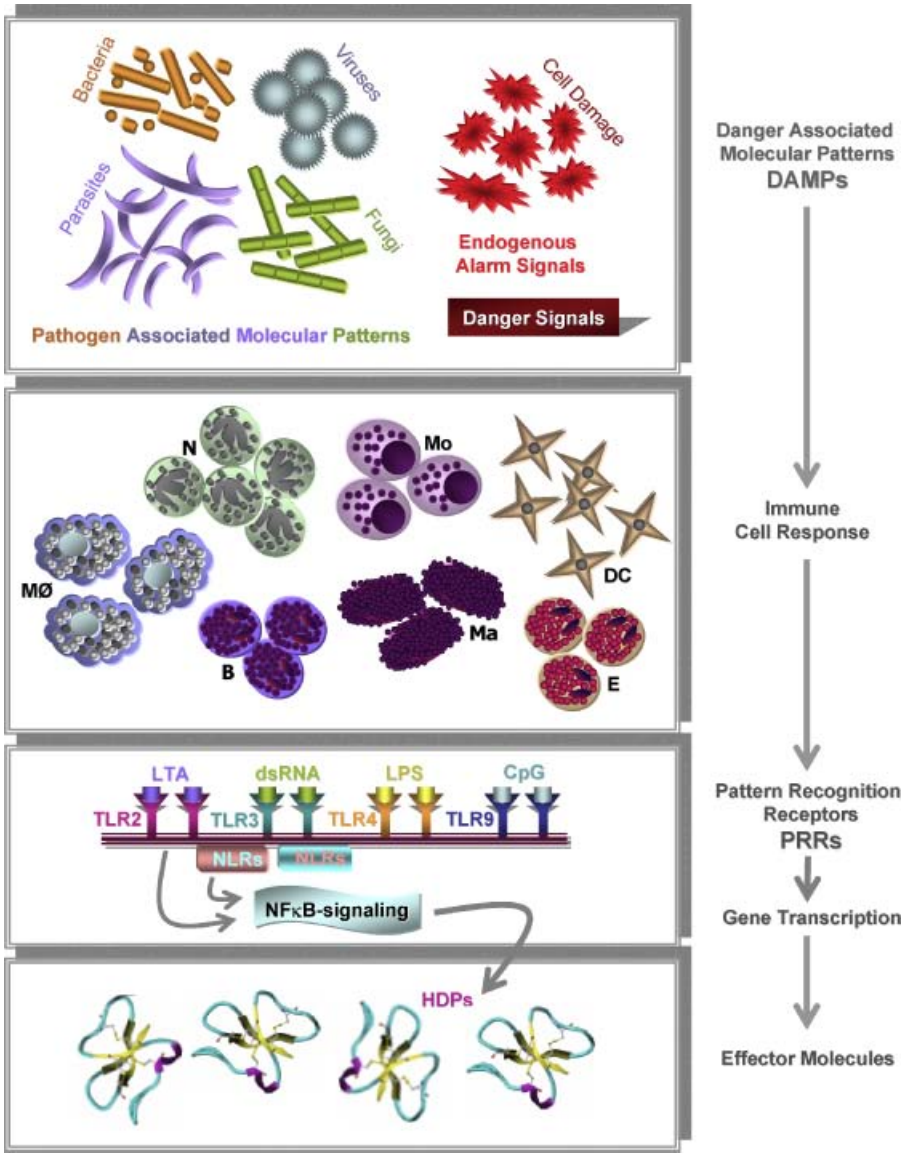

Fig 4. Danger Model of Innate Immunity. Different infectious and noninfectious molecular structures (PAMPs and endogenous alarm signals, respectively) constitute indicators known as danger associated molecular patterns (DAMPs). The DAMPs activate the innate immune system through pattern recognition receptors (PRRs) and NFκB-signaling, leading to production of host defense effector molecules (HDPs). N, neutrophils; E, eosinophils; B, basophils; Mo, monocytes; DC, dendritic cells; Ma, mast cells; MØ, macrophages; LPS, lipopolysaccharide; LTA, lipoteichoic acid; CpG, DNA with cytosine and guanine separated by a phosphate; TLR, toll-like receptor; NLR, nod-like receptor; NF-κB, nuclear factor κB; HDPs, host defense peptides; PAMP, pathogen-associated molecular patterns.

comprise a field of research beyond the scope of this paper.[52,53]

Biological Activity of HDPs

HDPs are the frontiers of inborn immunity in virtually all living species (Fig 3), and the central importance of these peptides is evident by their abundance in circulating neutrophils.[15] HDPs participate in the inflammatory response by acting as chemoattractants for immune cells (including neutrophil recruitment by induction of IL-8 production and mobilization of immunocompetent T-cells[54,55]) as well as enhancers of cellular adhesion and the subsequent cellular transepithelial migration. Furthermore, studies suggest that defensins can enhance the cytotoxicity of NK-cells.[15] The versatile nature of HDPs also includes roles in wound healing (possibly by induction of syndecan[k] synthesis[56]) as well as modulation of the inflammatory response by inhibiting the activation of the classical complement pathway through C1q.[57]

Given the ubiquitous production of HDPs in the organism, it is not surprising that many of these peptides can be found in various types of body fluids and secretions.[3] Plasma α-defensin concentrations of

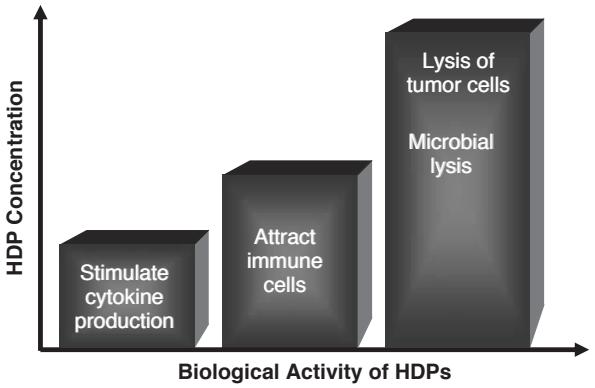

Fig 5. Biological activity of host defense peptides (HDPs) The graph shows that the breadth of activity for HDPs is dependent upon peptide concentration.

40 ng/mL have been measured in normal human subjects, increasing in concentration to $>1\,\mu g/mL$ during infections.[4] Also, plasma concentrations of 170 μg/mL have been measured in sepsis,[58] as have concentrations of $>1600\,\mu g/mL$ in sputum from cystic fibrosis patients.[59] The antimicrobial activity of α-defensins in vitro generally relies on peptide concentrations between 10 and 100 μg/mL, although their contribution to tumor cell lysis occurs at higher concentrations[4] (Fig 5). HDPs are most likely secreted at higher concentrations in infected or otherwise diseased tissue, but local peptide concentrations have yet to be investigated.[4] Certain HDPs act as anti-inflammatory compounds in sepsis because of their LPS- and LTA-binding capacity,[15] and, in addition to neutralizing endotoxin, some cathelicidins act directly to decrease the release of TNF-α.[60]

A number of HDPs are known to be inactivated by salt, and some have decreased antimicrobial activity even at physiological salt concentrations (approximately 150 mM NaCl).[7,17] Current research suggests that extracellular release of certain defensins yields inactive peptides, whereas concomitant release of cathelicidins ensures active HDPs working synergistically.[36] Synergy has also been described between lysozyme and other HDPs.[15] Some HDPs promote angiogenesis and epithelial growth, and some act as chemokines attracting circulatory or migrating cells.[26,61–63] Defensins possess chemotactic features toward monocytes, and can act as "corticostatins" by reversibly interacting with the receptor for adrenocorticotropic hormone (ACTH).[4] Defensins can modify a number of signaling pathways and cellular functions in the body by potent inhibition of protein kinase C.[64] A role of β-defensins in sperm maturation also has been suggested.[65]

Versatile Host Defense Peptides in Companion and Production Animals

Host defense peptides are produced throughout the animal kingdom. Many HDPs have been identified in domestic animals, but a striking interspecies variation exists with regard to the expression of these peptides.[1,10] A given species may have a dozen or more different HDPs, presumably with some overlap in their antimicrobial and immunomodulatory activities, although some peptides tend to function preferentially in only 1 of the these 2 biological roles.[23] The importance of HDPs as microbicidal compounds versus their role as immunomodulators is somewhat controversial.

Owing to the ease of access to material from production animals, cattle, sheep, goats, and pigs have been used widely in the field of HDP research. However, information on HDPs in companion animals is sparse. Studies in horses have focused on defensins and cathelicidins[66,67] and a few reports on canine HDPs are available.[68,69] The need for in vivo experiments in the area initially led to an increased focus on the mouse as an animal model. The mouse possesses a single cathelicidin and a number of enteric α-defensins (cryptidins).[7] Mouse granulocytes, however, lack α-defensins completely, making its usefulness as an animal model questionable.[7] Most species contain a wide range of HDPs with varying expression levels in different tissues, which ensures a broad range of antimicrobial coverage and immunomodulatory regulation throughout the organism.[15] The following section provides an overview of the data available on HDPs in different species of relevance to veterinary medicine, including companion and production animals (Fig 6) and selected other species (Table 2).

Companion Animal Species

Dogs and Cats. The literature on innate immune mechanisms of the dog and cat is limited. Thus far, 3 β-defensins (cBD-1, cBD-2, and cBD-3) and 1 cathelicidin (K9CATH) have been identified in the dog[68,70] whereas none has been reported in the cat. By means of computational analysis only, sequences for 43 β-defensin genes and pseudogenes have been identified in the canine genome.[69] Recently, canine hepcidin (an acute phase protein with antimicrobial and iron regulatory capacity) was extracted from canine liver, which is of interest because hepcidin is thought to be a key mediator in chronic anemia.[71]

Most studies on the immunophysiology of cats have focused on the acquired immune response to infectious disease.[72,73] Although specific HDPs have not yet been identified in the cat, 1 study focused on feline TLR expression.[74] Normal cat lymphoid tissue expresses TLR1–TLR9, an expression that is altered by feline immunodeficiency virus (FIV).[74] Because TLRs are involved in the synthesis of HDPs, these findings suggest that the cat, like virtually all other species, also has a range of natural antimicrobial peptides. Select TLR expression (TLR2, TLR4, and TLR9) has also been reported in different tissues and cells from the dog,[75–79] which would similarly indicate wide spread expression of different canine HDPs. Predicted sequences for canine TLR5 and TLR7 have in addition been generated by automated computational analysis (GeneID: 488605 and 491743, respectively). Functional studies on canine TLRs are lacking, but 1 suggestion has been that dysregulation of TLR2 and TLR4 in intestinal epithelium may contribute to the pathogenesis of canine inflammatory bowel disease.[80]

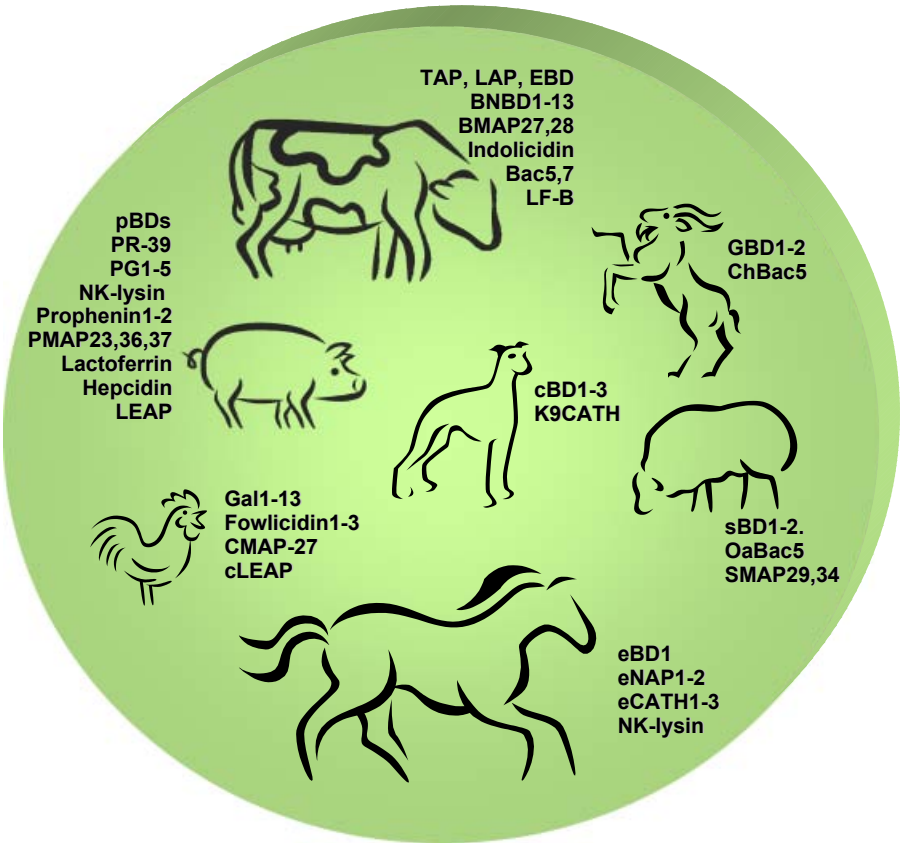

Fig 6. Overview of host defense peptides in veterinary medicine. Cow – TAP and LAP, tracheal and lingual antimicrobial peptide, respectively; EBD, enteric β-defensin; BNBD, bovine neutrophil β-defensin; BMAP, bovine myeloid antimicrobial peptide; Bac, bactenecins; LF-B, bovine lactoferricin B. Goat – GBD, goat β-defensin; ChBac, caprine analog to bovine bactenecin. Sheep – sBD, sheep β-defensin; OaBac, ovine analog to bovine bactenecins; SMAP, sheep myeloid antimicrobial peptide. Pig – pBD, porcine β-defensin; PR, proline rich; PG, protegrin; PMAP, porcine myeloid antimicrobial peptide; LEAP, liver-expressed antimicrobial peptide. Horse – eBD, equine β-defensin; eNAP, equine neutrophil antimicrobial peptide; eCATH, equine cathelicidin. Dog – cBD, canine β-defensin; K9CATH, canine cathelicidin. Chicken – Gal, gallinacin; CMAP, chicken myeloid antimicrobial peptide; cLEAP, chicken liver-expressed antimicrobial peptide.[q]

Table 2. Host defense peptides in special species.

Species	Host Defense Peptide		Place of Expression	Reference
Chinchilla	cBD1	β-defensin	Epithelia	236
Guinea Pig	GPNP1	α-defensin	Neutrophils, bone marrow	237
	GPCSIII	α-defensin	Neutrophils, bone marrow	238
	CAP11	cathelicidin	Neutrophils, bone marrow	239
Hamster	HaNP1-4	α-defensin	Neutrophils	240
	Crp1-6	α-defensin	Paneth cells	23
Mouse	mBD1-15	β-defensin	Epithelia	241
	mBD34-40	β-defensin	Epithelia	241
	CRAMP	cathelicidin	Neutrophils, bone marrow	242
Rabbit	NP1-3a	α-defensin	Neutrophils	243
	NP3b-5	α-defensin	Neutrophils	243
	CAP18	cathelicidin	Granulocytes	244
	rNP1-2,4	α-defensin	Neutrophils, tissue	245
Rat	42 rBDs	β-defensin	Epithelia	69, 246
	rCRAMP	cathelicidin	Granulocytes, tissue	247

BD, β-defensin; NP, neutrophil peptide; Crp, cryptdins; CRAMP, cathelin-related antimicrobial peptide; CAP, cationic antibacterial polypeptide. The data provided in the table are intended as an overview only of some of the defensin and cathelicidin HDPs, which have been reported in small mammals, including examples of their tissue/cellular expression. The majority of the 42 rat β-defensins is based on information from the Rat Genome Database (http://rgd.mcw.edu/), and a recent paper on cross-species analysis of mammalian β-defensins.[69]

Our group recently identified 3 β-defensins in dog testes, with selective expression of the 3 isoforms among different testicular cell types.[68] The most active and longest of the isoforms, canine β-defensin-1 (cBD-1), is expressed more ubiquitously, whereas the relatively shorter peptides (cBD-2 and -3) appear to be testes-specific storage HDPs.[68] The antimicrobial effect of canine β-defensin includes activity against a wide spectrum of Gram-positive (*Listeria monocytogenes* and *Staphylococcus aureus*) and Gram-negative (*Escherischia coli, Klebsiella pneumoniae*, and *Neisseria gonorrhoeae*) bacteria, yeast (*Candida albicans*), and *Ureaplasma* in a salt-dependent fashion.[68] We have also identified a more potent canine HDP, K9CATH (canine cathelicidin), in myeloid bone marrow cells and circulating neutrophils.[70] This peptide has broad-spectrum activity and also exhibits unprecedented antimicrobial potency against *N. gonorrhoeae* and *Ureaplasma* in a salt-independent manner. Because this peptide is expressed in circulatory cells, it has the inherent capability to act not only as a potent antimicrobial compound but also as a potential immunomodulator. These findings may explain why dogs apparently are resilient to sexually transmitted disease pathogens. Consequently, synthetic forms of these canine-derived peptides may provide novel therapeutic options for treating sexually transmitted disease in humans as well as urinary tract infections in dogs. The use of synthetic peptides derived from heterospecifics has proven successful previously (eg, use of the moth-derived synthetic cecropin to treat naturally acquired canine leishmaniasis).[81]

Horses. The existence of antimicrobial compounds in equine neutrophils was first reported nearly 20 years ago.[82] Later, these peptides were characterized as equine neutrophil antimicrobial peptides (eNAP-1 and -2),[83,84] followed by the identification of 3 equine cathelicidins (eCATH-1, -2, and -3).[67,85] Another antimicrobial compound, equine NK-lysin, produced by lymphocytes was recently found in the horse.[86] α-defensins have thus far not been reported in the horse, but expression of 1 β-defensin (eBD-1) was described recently.[66] Eight potentially functional β-defensin genes and an α-defensin-like sequence have been reported in the horse based on computational sequence analysis.[87] Equine β-defensin 1 (eBD-1) appears to be constitutively expressed in several different tissue types and organs, including liver, kidney, spleen, heart, and the intestine.[66] Functional characteristics of eBD-1 have yet to be established, but examination of the peptide sequence indicates similarities to other known β-defensins.[66] A recent study reported on β-defensin production in cerumen, where it most likely acts as a natural antimicrobial to safeguard the equine auditory canal.[88]

Cathelicidins from the horse are stored in the classical unprocessed form (pro-eCATHs) in secretory granules, and released only upon neutrophil activation.[67] Of the 3 cathelicidin genes identified in the horse, only 2 (eCATH-2 and -3) seem to be able to encode a protein.[67] eCATH-1 is expressed at fairly low levels, and the gene is present in only half of the examined horses. The mature eCATH-1 protein has yet to be detected.[85] Common equine neutrophil-dominated inflammatory disorders such as acute bronchiolitis and recurrent airway obstruction result in measurable concentrations of mature eCATH-2 and -3 as well as their respective propeptides in tracheobronchial lavage, findings that are consistent with active processing of these HDPs in equine inflammatory processes.[85] The antimicrobial activity and potency of eCATH-1, -2, and -3 generally is broad, intermediate, and low, respectively.[85] The eCATH-1 peptide (synthetic form) has the strongest antimicrobial capacity and exhibits virtually no hemolytic activity in vitro, whereas eCATH-3 has fairly modest antimicrobial activity in low-salt medium only.[85] It is therefore possible that the eCATH-1 peptide is induced only under specific and different conditions than what has been investigated so far, and based on studies involving a modified version of eCATH-3, the amphipathicity and biological activity of this peptide seem to be highly interdependent.[85] The known versatility of HDPs also leaves open the question of what additional role the eCATHs may play in vivo.

The antimicrobial peptides eNAP-1 and -2 are structurally unrelated to the family of defensins found in neutrophil granules of other species, and substantial internal differences exist between the 2 peptides.[84] The antimicrobial activity of both eNAPs has been tested against pathogens commonly involved in clinical endometritis in mares, including *E. coli, K. pneumoniae, Pseudomonas aeruginosa*, and *Streptococcus zooepidemicus*.[83,84] The content of eNAP-1 in neutrophils is fairly low compared with eNAP-2,[l] but the peptides appear to have comparable antimicrobial activities against typical uterine pathogens in the horse.[84] The bactericidal activity of eNAP-1 and -2 (after 2 hours and 100 μg/mL concentration for both) against *S. zooepidemicus* seems most pronounced (>99.8% and 94% decrease in colony forming units [CFU]/mL, respectively), with a relatively lower efficacy against *E. coli* and *P. aeruginosa* (mean decrease of 87% in CFU/mL for eNAP-1, and 90% and 78% decrease, respectively, for eNAP-2 after 2 hours, and 200 μg/mL concentration for both). eNAP-2 also exhibits bacteriostatic activity against *K. pneumoniae* at 200 μg/mL.[83,84] In addition to direct antibacterial activity, a selective microbial serine protease inhibition[m] has been reported for eNAP-2.[89] It is thus likely that eNAPs play a central role in the innate uterine defense mechanisms of the horse.

Large Animal Species

Cattle. In the mid-1980s, a group of researchers from University of Trieste initially reported the presence of broad-spectrum antibiotic polypeptides in bovine granulocytes.[90] In the following years, different bovine neutrophil antimicrobial peptides have been isolated, including members of the defensin and cathelicidin families. Cattle possess at least 38 HDPs, including different defensins and cathelicidins (BMAPs [bovine myeloid antimicrobial peptides], bactenecins [loop peptides], and indolicidin [extended peptide]).[15] Bovine oligosaccharide-binding protein (bOBP) is a peptidoglycan recognition protein found in bovine neutrophils and

eosinophils, suggesting that this peptide may contribute to antiparasitic activity.[91] Furthermore, antimicrobial compounds from milk (eg, lactoferricin, LF) have received considerable research attention.[92,93]

Epithelial β-defensins have been isolated from the bovine trachea (tracheal antimicrobial peptide, TAP),[94] tongue (lingual antimicrobial peptide, LAP),[9] intestine (enteric β-defensin, EBD),[95] and mammary gland (bovine β-defensin-1, bBD-1, and others).[96,97] Bovine neutrophil dense granules[n] also contain β-defensins (bovine neutrophil β-defensins, BNBD-1 to -13),[o] some of which also are expressed in alveolar macrophages (predominantly BNBD-4 and -5, in addition to the 2 epithelial β-defensins, TAP and EBD).[98–100] Cattle, on the other hand, do not have α-defensins in neutrophils and the intestinal epithelium.[23] The bovine epithelial and neutrophil β-defensins are different gene products, but share a high degree of structural similarity.[98,101] mRNA expression of some BNBDs can be observed in cells of different tissues, including trachea, lung, spleen, and intestine.[98,101]

Bovine β-defensins possess antimicrobial activity against *E. coli, K. pneumoniae, P. aeruginosa, Staph. aureus* and *Candida* spp.[94] TAP is expressed throughout the bovine airway,[102,103] and is an example of a β-defensin that is inducible by various infectious agents and proinflammatory mediators, including TNF-α, IL-1β, and LTA.[38,39] Incubation of primary cultures with *E. coli* LPS results in a substantial increase in mRNA levels encoding TAP.[37] Synthetic TAP has a rapid and potent bactericidal effect as well as antifungal activity against *Aspergillus* and *Candida* spp.[104] Contrary to TAP, LAP expression is more widespread, involving epithelium of the alimentary tract as well as the respiratory system, mammary glands, and cornea.[101,105] Induction of LAP expression is observed in acute infection with *Mannheimia* (*Pasteurella*) *hemolytica* as well as in chronic paratuberculosis-infected (*Mycobacterium paratuberculosis*) tissue.[101] It also has been suggested that LAP plays a role in the innate immune response against bovine mastitis pathogens, because LAP expression is increased in infections of the udder.[105] Similarly, local expression of BNBD5 as well as that of PRRs TLR2 and TLR4 is upregulated in mastitis.[106,107] Importantly, a recent study reported that steroid-treated cattle have lower expression levels of LAP and TAP, which suggests that stress and exogenous corticosteroid administration can lead to an impaired innate immune response in the lung.[108]

The bovine alimentary tract expresses low levels of a number of different HDPs (including LAP, TAP and BNBD-3, -4, and -9), but the main enteric β-defensin in the gut is EBD.[95] mRNA levels of EBD are increased in experimental cryptosporidiosis in calves, suggesting that this HDP plays an active role in the host response to parasitic infection.[95] The broad spectrum of antimicrobial activity and inducible expression in inflammation strongly support a central role for β-defensins in bovine mucosal host defense.[9,37,95]

Cathelicidins were first reported in cattle myeloid bone marrow cells, and include the bactenecins Bac 5 and Bac 7,[p] which are bactericidal against *E. coli, Salmonella typhimurium*, and *K. pneumoniae*, and bacteriostatic toward *Enterobacter cloacae*.[109–112] Selected antiviral activity also has been noted,[109] as well as killing of spirochetes (*Leptospira interrogans* and *Leptospira biflexa*).[113] A 3rd bactenecin, Bac2S, shows activity against *P. aeruginosa* and some Gram-positive bacteria.[114] Although traditionally associated with myeloid precursor cells, neutrophils also are capable of de novo synthesis of cathelicidin peptides at sites of inflammation.[115] BMAP-27 and BMAP-28 are synthetic bovine cathelicidins with broad-spectrum activity against bacteria, including methicillin-resistant *Staph. aureus*, and fungi,[116] yet exhibiting some cytotoxicity. The synthetic BMAP-34 peptide, however, exhibits a similar breadth of antimicrobial activity, but without any adverse effects on eukaryotic cells. At a sufficiently high dose almost all AMPs may exhibit toxicity toward eukaryotic cells.[117,118] BMAP-28 also has activity against tumor cells in vitro.[119] Moreover, BMAP-27 (as well as Bac7) can effectively bind LPS, and thus may have potential use in treatment of endotoxin-induced septic shock.[120,121] BMAP-28 also exhibits broad-spectrum activity against *Pasteurella multocida* isolates.[122] The role of bovine HDPs as immunomodulatory molecules has received some attention.[123] Indolicidin is one of the shortest cathelicidin peptides (13 amino acids), exhibiting potent and wide antimicrobial activity against Gram-negative (*E. coli*) and Gram-positive (*Staph. aureus*) bacteria[92] as well as fungi,[124] and it also has antiendotoxic and chemokine-inducing properties.[93,123,125,126] Moreover, modified synthetic versions of indolicidin (eg CP-11C) have improved in vitro antibacterial and antifungal activity combined with less cytotoxicity.[125,127]

A range of bioactive peptides has been identified in bovine milk, such as synergistically acting probiotics and antimicrobial compounds, including casecidin and lactoferrin.[128–132] Lactoferrin is also present in other body secretions such as saliva, tears, and bronchoalveolar lavage (BAL) fluid and in leukocyte granules.[133] The peptide LF-B is generated by gastric pepsin degradation of lactoferrin and has a wide range of antimicrobial and antifungal activity as well as immunomodulatory properties.[128,134–138] Lactoferrin is a much larger molecule (80 kDa) than free LF (3 kDa), possibly explaining the peptide fragment's higher degree of activity because of the ease with which it can penetrate the bacterial membrane.[133] Partial synergism with penicillin G against *Staph. aureus* also has been reported.[130] Lactoferrin has in addition been suggested as a good candidate for a novel natural antiviral compound.[139,140] Importantly, it is economically feasible to obtain protein fractions from milk and generate active peptides for use as nutraceuticals and as templates for development of new pharmaceutical compounds.[128,141,142] Oral administration of LF produces a host-protective effect in a number of different animals (including humans), and a pepsin hydrolysate of lactoferrin is already used in infant formulas.[143]

The vast majority of naturally occurring HDPs are cationic peptides. A unique finding, however, is a group

of anionic antimicrobial peptides in the bovine lung that are constitutively expressed and distinctly different from most HDPs with regard to size and polarity.[144] These small peptides (unlike the majority of HDPs) have increased their activity at higher NaCl concentrations.[144] The anionic peptides are not inducible by pathogens or microbial byproducts, but their expression in the lung suggests a role in innate host defense of the bovine respiratory system.[144]

Sheep and Goats. Smaller ruminants have attracted some research attention because of their potential use as animal models to study HDP regulation in epithelial tissue and importantly the impact of pharmaceutical intervention on peptide expression patterns.[145] Two sheep β-defensins (sBD-1 and sBD-2) with differential expression have been identified in the ovine gastrointestinal and respiratory tract epithelium.[145,146] However, unlike cattle, sBDs are not found in neutrophils.[145] In goats, 1 study has reported the expression of β-defensin precursors (preproGBD-1 and preproGBD-2), principally in the caprine respiratory and gastrointestinal tracts.[147] Goat milk also contains lactoferrin, which exhibits antimicrobial properties.[148] Proline-rich antimicrobial peptides are highly conserved HDPs in ruminants, and caprine (ChBac5) and ovine (OaBac5α) analogs to the bovine Bac5 have been described, both exhibiting potent antimicrobial activity.[149] Sequence analysis has determined that there potentially are 8 ovine cathelicidins,[150] but only 2 peptides have actually been isolated from ovine neutrophils.[151,152] The sheep myeloid antimicrobial peptides (SMAP29 and SMAP34) are cathelicidins with broad-spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria, and fungi.[122,153–156] SMAP29 binds LPS with high affinity,[157] and maintains its potent activity under high-salt conditions.[153] This peptide (including synthetic derivatives) may find clinical application in the treatment of respiratory infections.[158,159]

Pigs. Porcine HDPs include more than a dozen different peptides, primarily with representatives from the cathelicidin family (including the prophenins, protegrins, PR-39, and the PMAPs).[160,161] Thus far, no α-defensins have been isolated from the pig, but at least 12 different porcine β-defensins (pBD-1 to pBD-12) have been reported.[162,163] Other HDPs, including NK-lysin and porcine LF, also have been reported in this species.[164–166] Recently, another porcine AMP (ie liver-expressed antimicrobial peptide, LEAP) was described, along with porcine hepcidin (an iron-regulating hormone with antimicrobial effects).[167]

Thirty-nine residue proline-arginine–rich peptide (PR-39) was originally isolated from pig intestine,[168] and later identified in porcine bone marrow cells[169] and neutrophils.[170] In addition to its antimicrobial activity, PR-39 has been implicated in tissue repair[171] and as a chemoattractant of neutrophils[172] as well as an inhibitor of apoptosis.[173] Expression of PR-39 is constitutive in myeloid cells and present in pigs of all ages.[34] The PR-39 peptide is upregulated in the presence of bacterial products,[174,175] and its antimicrobial action relies on a non–pore-forming mechanism.[176] It has a potency against Gram-negative bacteria similar to that of tetracycline.[177] A smaller synthetic peptide (PR-26) derived from PR-39 has been shown to have at least as much potency as its parent molecule.[178] Importantly, PR-39 also has been suggested as a novel biomarker of porcine respiratory health.[179]

The protegrin family of HDPs was first identified in porcine leukocytes,[180] and 5 protegrin sequences (PG-1 to -5) have been identified.[181–183] The protegrins are elastase-activated cathelicidin polypeptides with potent microbicidal activities.[180,184–186] PG-1 exhibits a wide spectrum of in vivo activity against Gram-negative and Gram-positive bacteria, and the synthetic peptide thus has potential for use as an antimicrobial agent in the treatment of clinically relevant antibiotic resistant pathogens.[181,187] PG-1 also exhibits in vitro activity against certain spirochetes.[188] In addition, the peptide has attracted interest because of its potent activity against human STD pathogens, including human immunodeficiency virus (HIV),[189–192] periodontal pathogens,[193,194] and *Mycobacterium tuberculosis*.[195] Importantly, PG-1 also exhibits powerful antimicrobial activity against *P. aeruginosa*, and substantially reduces bacterial growth in established porcine wound infections.[196]

Other members of the cathelicidin family include the prophenins (prophenin-1 and -2)[197,198] and the porcine myeloid antimicrobial peptides (PMAP-23, PMAP-36, and PMAP-37).[199–202] Prophenin-1 has been purified from porcine leukocytes and is substantially more active against Gram-negative bacteria,[197] whereas the PMAPs are broad-spectrum highly potent HDPs derived from pig myeloid cells. Their spectrum of activity includes Gram-negative and Gram-positive bacteria[199] as well as fungi and nematodes.[203,204] Novel peptide analogs of PMAP-23 have shown promising effects against fungi (*C. albicans*), and may act as templates for design of novel antifungal pharmaceutical compounds to treat clinical fungal infections.[205]

Porcine β-defensin-1 (pBD-1) is particularly abundant in tongue epithelium and expressed at only low mRNA levels in other epithelial tissues.[162,206] The expression pattern of the peptide appears to be developmentally regulated,[207] and antimicrobial effects include activity against *E. coli, L. monocytogenes, S. typhimurium*, and *C. albicans* under low-salt conditions.[162,206] pBD-1 acts synergistically with some of the porcine cathelicidins, ensuring antimicrobial activity at higher salt-concentrations.[206] The expression of pBD-1 may be regulated by the recently identified porcine peptidoglycan recognition proteins (pPGRP-L1 and -L2 [ie long-isoforms]).[208] Using bioinformatics and expression analysis, an additional 11 porcine β-defensins have been identified.[209] The main gene expression sites for pBD-2 are liver and kidney, and the peptide is the most highly expressed defensin in the ileum.[167,210] The expression of pBD-1 and pBD-2 has been studied with the porcine intestinal cell line IPI-21[163] as well as the porcine small intestinal segment perfusion (SISP) technique.[210] In vitro, *Salmonella enteritidis* and *S. typhimurium* increase pBD-1 and pBD-2 mRNA levels, respectively, whereas neither of the two is affected by *S. typhimurium* exposure

using the SISP model.[163,210] Despite the common notion of pBD-1 as a constitutively expressed AMP, upregulation of the peptide by *S. typhimurium* (ie, enterocolitis) exposure does seem possible under some circumstances.[163,210]

The expression pattern and activity of porcine HDPs also become important in reference to using porcine organs and tissues in xenotransplantation.[162] Finally, porcine antimicrobial peptides can be of interest in the development of novel functional foods, because digestion of protein of porcine origin may lead to the release of latent bioactive peptides with potential impact on human health.[211]

Special Species

Small Mammals. Very little research has focused on HDPs in small mammals with the exception of the mouse. An exhaustive review of murine HDPs is beyond the scope of this paper, and we have presented an overview of currently reported HDPs in special mammalian species in Table 2. A wide range of AMPs has been discovered in other exotic species and food animals, including amphibians, fish, and other aquatic vertebrates.[212] These studies are also beyond the scope of this paper.

Birds. Avian heterophil antimicrobial peptides of the β-defensin family initially were reported in the chicken (CHP-1 and -2/aka Gal-1 and -2) and turkey (THP-1 and -2).[213–216] A total of 13 different β-defensins (gallinacin-1 to -13) and 3 cathelicidins (fowlicidin-1 to -3) are encoded by the chicken genome according to computational analysis.[217,218] The chicken genome does not, however, code for any α-defensins.[217] Furthermore, TLR expression has been reported in chicken heterophils.[219] Based on tissue expression analysis, gallinacin-1 to -7 are found primarily in the respiratory tract and bone marrow, whereas the remaining genes are restricted to the urogenital tract and liver.[217] Gallopavin (GPV-1) and gallinacin-3 are epithelial β-defensins from the turkey and chicken, respectively, and the latter is inducible by experimental infection with *Haemophilus paragallinarium*.[220] Mature fowlicidin peptides exhibit potent LPS-binding and broad antimicrobial activity in a salt-independent manner, features that make them attractive as candidates for novel antimicrobial and antisepsis compounds.[218,221] Another cathelicidin, chicken myeloid antimicrobial peptide (CMAP-27), has been identified in chicken bone marrow cells,[222] and a liver-expressed epithelial antimicrobial peptide (cLEAP-2) also has been reported in the chicken with activity against different *Salmonella* strains.[223,224] In addition to chicken and turkey, avian HDPs have thus far been isolated from ostrich circulatory cells, and from king penguin stomach content, where the peptides are believed to ensure long-term preservation of stored food.[225,226] Description of the avian antimicrobial profile is of interest to identify novel compounds aimed at fighting infectious diseases in avian species, but also because birds act as asymptomatic carriers and thus major reservoirs for bacteria that are known human enteropathogens.[223,227]

Therapeutic Potential in Veterinary Medicine

One of the major problems in modern medicine is an alarming increase in antibiotic resistance to conventional antibiotics, which has created an obvious need to search for novel compounds to maintain a functional armamentarium aimed at fighting pathogens. From an evolutionary viewpoint, HDPs are ancient yet widely successful endogenous biochemical weapons.[43] Contrary to classical antibiotics, which are made in a sequential fashion involving different enzymatic steps, HDPs are all gene-encoded peptides originating from an RNA template.[228] Their consistency in efficacy throughout evolution would furthermore speak against the common belief that microorganisms inevitably will develop resistance against any imaginable antimicrobial compound over time.[43] The structure of naturally occurring antimicrobial compounds from higher eukaryotes is distinctly different from conventional bacterial and fungal types of antibiotics,[8] which makes them highly attractive as potential templates for new therapeutic agents in the continuous search for novel antimicrobials to fight progressively more resistant microbial pathogens.[8] Interestingly, natural HDPs can act synergistically with certain conventional antibiotics targeted at Gram-negative as well as Gram-positive bacteria.[45,229,230] Figure 7 summarizes the main features that warrant consideration of HDPs as a desirable new class of antibiotics. It is furthermore of interest that certain HDPs adopt amphipathic structures only on contact with biological membranes or when exposed to a membrane-mimicking environment.[23]

As a group, HDPs also are of medical interest as possible future model molecules for novel immunoregulating drugs because of their natural capacity to act as immune response modifiers. Pharmaceutical compounds of tomorrow therefore may be designed as immunomodifiers, aimed at optimizing HDP synthesis in a chosen organ or tissue type, which is of the utmost importance because the regulation of innate immunity is organ-specific.[7,231] Isoleucine is an example of 1 such compound, which can induce synthesis of β-defensin production in enteric cells using TLR2 and NF-κB-signaling.[7,43,232,233] Also the use of corticosteroids in certain inflammatory diseases may lead to iatrogenic complications, because these are synthetic compounds that suppress endogenous HDP synthesis.[26]

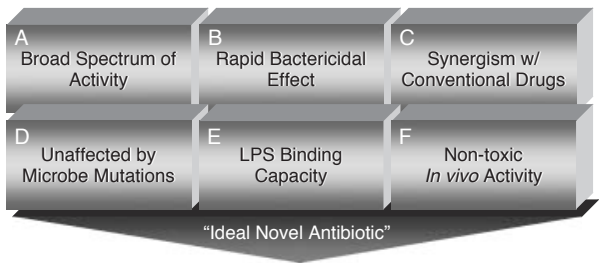

Fig 7. Elemental features of an ideal novel antimicrobial agent. **A.** Broad spectrum of antimicrobial activity (against bacteria, viruses, fungi, parasites). **B.** Rapid bactericidal effect. **C.** Synergy with conventional antibiotics. **D.** Activity unaffected by classical microbial mutations.[r] **E.** Endotoxin neutralizing effect. **F.** Nontoxic activity maintained in animal models.

Four cationic peptides have progressed to Phase 3 trials, of which half have demonstrated clinical efficacy.[23] Pexiganan (a frog HDP derivative) has been used to treat diabetic foot ulcers and Omiganan (a cattle HDP variant) has been used for the prevention of catheter-associated infections). Despite a 90% efficacy, Pexiganan has not obtained FDA approval for clinical use, but Omiganan currently is in Phase 3b trials to confirm initial findings of its clinical usefulness.[23] More than a dozen other peptides and peptidomimetics are currently in commercial development, but the 1st clinically approved cationic peptide aimed primarily at catheter-associated infections is likely to be available within the next few years.[23] Thus, cationic antimicrobial peptides have important potential as model molecules for design of urgently needed novel pharmaceutical compounds. Much research is, however, still warranted to assess the practicality and clinical usefulness of selected compounds from a plethora of naturally occurring HDPs.

Concluding Remarks

The vast majority of species (invertebrates and plants in particular) rely on innate immunity exclusively to effectively fight off potentially lethal pathogens and maintain overall health, whereas immune memory is somewhat of a biological luxury granted only to species higher in the evolutionary hierarchy.[234] The last 10 years in particular have placed innate immunity at the forefront of immunological research as a rapidly developing field, continuously leading to novel ideas together with new discoveries. Increasing antibiotic resistance is a well-known phenomenon in modern medicine, and novel natural antibiotics therefore become immediately attractive. The importance of a well-balanced immunologic response has become evident in a variety of different disease processes (including cardiovascular disease and cancer),[19,235] and the immediate need of novel immunomodifying compounds is consequently obvious. Hopefully much of the ongoing research will translate into original therapeutics for different immunological disease processes, potentially opening up exciting new avenues for immune intervention in veterinary medicine.

The past decade has produced a remarkable amount of new knowledge on the tissue expression and in vitro activity of animal HDPs. Still, these are the formative years for the investigation of innate immune defense mechanisms as they pertain to animal disease with the ultimate goal of elucidating the intricate roles of these versatile peptides in naturally occurring disease affecting small and large animal species.

Footnotes

[a] Amphipathic: Molecules that have both hydrophilic and hydrophobic parts

[b] Azurophil: Primary lysosomal granule found in neutrophil granulocytes. Contains a wide range of hydrolytic enzymes and is released into the extracellular fluid

[c] http://www.bbcm.univ.trieste.it/

[d] A schematic of the molecular motif of these defensins resembles the Greek letter "theta" (θ)

[e] Cathelin domain: so called because it is also present in cathelin, a porcine cysteine protease inhibitor

[f] RIG-I: retinoic acid inducible gene I. Mda5: Melanoma differentiation associated gene 5. The RNA helicases play an essential role in double-stranded RNA-induced innate antiviral responses[46]

[g] Nod: nucleotide-binding oligomerization domain

[h] MAPK: mitogen-activated protein kinases

[i] JAK/STAT: Janus kinase/signal transducer and activator of transcription signaling pathway

[j] Caspases are cysteinyl aspartate-specific proteinases, known for their role in cytokine maturation and apoptosis[52]

[k] Syndecans: cell surface heparan sulfate proteoglycans[56]

[l] The concentration of eNAP-2 in equine neutrophil granulocytes is approximately 4.5–9.0 mg/mL[84]

[m] Microbial exoproteases have the potential of acting as virulence factors, and select anti-proteinase activity may therefore benefit the host[89]

[n] The dense granules distinguish ruminant neutrophils from leukocytes of nonruminant mammals[98]

[o] The BNBDs were initially numbered from 1 to 13 based on their increasing retention time on reversed-phase high performance liquid chromatography (RP-HPLC)[99]

[p] The two bactenecins (from the Latin words "bacterium" and "necare" [to kill]) have molecular masses of approximately 5 and 7 kDa, respectively[109,110]

[q] Animal drawings are clip art images (http://office.microsoft.com/en-us/clipart/default.aspx?lc=en-us)

[r] Relatively invariant bacterial structures have a low frequency of mutations, which may explain why resistance to HDPs (which recognize PAMPs, highly conserved structures) is rare[3,248]

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Chapter 2
The Heart of Innate Immunity

My very first encounter with immunology - as a scientific subject that is, since I did have my share of common colds as a kid also - was during vet school in the mid-nineties. Admittedly, quite a few things can happen in a decade or so, but it is probably somewhat of an understatement to say, that when I decided to begin my doctoral studies around five years ago - and thereby direct my attention towards the field of cardiac immunophysiology - I had to partially modify my perception of how the immune system actually works; and most importantly, how influential the players of immunity actually seem to be in a wide range of different disease processes that were not classically considered "inflammatory" in origin.

One of the "few novelties" that caught my attention the most, was probably Janeway & Matzinger redefining the basic idea of what actually triggers immunity. So, if it is "dangers", and not necessarily mere "foreignness" that awakens a response in our body, then the door is left wide open for a plethora of things that immunity might take part in.

After having spent around four years on clinics at that time specializing in cardiology, I found it quite fascinating that the "missing link(s)" in the current understanding of many cardiovascular pathologies, might be localized somewhere within the interplay of the numerous molecular components of innate immunity.

So, like with anything else that is "broken", identifying the actual source of the problem is likely to also offer a solution. There seems to be an overall consensus in the field of cardiovascular medicine as to the importance of inflammation in CVD - and atherosclerosis in particular. Furthering the knowledge on innate immunity and inflammation in CVD is consequently a central theme. The following article, ***"Innate immunity and inflammation - New frontiers in comparative cardiovascular pathology"***, is a review on this important topic within cardiology. The paper was published in *Cardiovascular Research* in 2006.

"Danger" broadly defined represents any exogenous or endogenous component that alerts the immune system. All while, the "Janus-face" of inflammation represents a bipolar quality, which can either lead to resolution of e.g. an infectious process, or in other instances progress to a stage of hyper-inflammation, causing significant damage in the body - and to the heart

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Innate immunity and inflammation — New frontiers in comparative cardiovascular pathology

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Abstract

Innate immunity and inflammation play key roles in a wide range of pathology — including heart disease and vasculopathies. Current thinking suggests "damage" rather than "foreignness" as the actual trigger of the immune system, which has caused a dramatic change in how we tend to view the etiopathology of most types of heart disease. The future potential of certain anti-inflammatory therapeutic strategies in addressing heart disease is intriguing. Still, the Janus face of immunity/inflammation cannot be over emphasized as adverse manipulation of these systems may prove ineffectual or worse, damaging. Knowledge on functional characteristics of individual immune mediators is undoubtedly a central theme, but in depth understanding of the multiple biological actions of these molecules, as well as their contextual function, is the corner stone in deciding on potential future targets for pharmacologic manipulation. Animal models of human heart disease are currently being investigated and clinical trials conducted to gain further knowledge in this essential area of cardiovascular research, but the scarcity of cardiovascular research focusing on signaling molecules and pathways of innate immunity is still evident. Genomic and proteomic research in heart disease is going through its formative years, and much is still unknown about the complex pathway dynamics utilized by the innate immune system. This review will provide an overview of the current literature focusing on innate immunity and the heart, and hopefully will spark an interest in further basic as well as clinical research. As more information on cardiovascular immunity becomes available, this will provide a better understanding and thus act as the foundation for potential development of new treatment strategies for treatment of cardiovascular disorders.

Keywords: Toll like receptors; Antimicrobial peptides; Nuclear factor kappa beta; Innate immunity

1. Innate immunity and inflammation in heart disease

Virtually all organisms live under constant exposure to a variety of infectious as well as non-infectious environmental microorganisms. External and internal surfaces, including the cutaneous epithelia and the mucosal linings constitute an immediate and proficient barrier towards potential pathogens [1]. The vast majority of microorganisms that succeeds in passing this first-line-of intrinsic defense will be eliminated by an array of other defense mechanisms of the innate immune response, including accumulation of macrophages and phagocytic neutrophils at the infection site [1]. In addition to this cellular innate immune response, plasma proteins, including complement components, join at the site of infection, constituting humoral innate immunity [1]. Modern perspectives recognize inflammation as a reaction involved in a variety of diseases including those of non-pathogenic (i.e. without microbial involvement) origin [2]. A key feature of inflammation in non-pathogenic disease is the more recent recognition that sentinel cells of all tissues can elicit as well as participate in the inflammatory response along with classically circulating leukocytes and cells of the lymphoid organs [2]. Classically, the term sentinel cells has been used in reference to dendritic cells

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qua their localization along the major routes of entry for microorganisms and their capacity to link innate and adaptive immune responses [3,4]. Recently, however, the term "sentinel" has been applied more widely to include different cell types which continuously sense the environment and produce mediators which may act to either activate or silence particular immune functions [5–8]. As such, sentinel cells can thus also include endothelial cells, cardiomyocytes, fibroblasts and mast cells (which are found copiously in the heart) [2]. The endothelium in particular figures prominently in any consideration of the role of inflammation as endothelial cells are now recognized for their ability to switch from an anti-inflammatory function into a pro-inflammatory mode [2]; just as inflammation at large has been analogized with a "Janus Face" — qua its capacity to heal as well as destroy [2]. Inflammation is a fundamental reaction instigated against virtually all injury types. Not surprisingly many forms of cardiovascular diseases therefore involve cells and mediators of the inflammatory system [2]. Even though cardiomyocytes do not fall within the category of traditional immune cells, they do respond to injury by producing some of the mediators (including different cytokines) that are classically associated with cells of the innate immune system [9]. The innate immune response, including inflammation, may therefore play an important role – on a local as well as systemic level – in a range of heart diseases that were not traditionally considered immunologic in etiology. Mediators of an innate immune and inflammatory response affect the cardiovascular system: 1) through direct immune and inflammatory reactions within the heart; including pathology such as cardiac remodeling, heart failure, ischemia and reperfusion injury, and 2) by constituting a central role in atherosclerosis and other types of vascular diseases; a role which has been reported in recent reviews [10,11]. Adding to the outlined local innate response mechanisms within the myocardium comes an activation of the immune system on a systemic level, as is seen in chronic heart failure patients with increased levels of pro-inflammatory cytokines in both plasma as well as the failing myocardium [12,13]. As such, the resulting overall innate immune response defending the heart against any given danger is therefore likely an orchestration between "non-immune derived" mediators operating within the myocardium as an "immediate first-line-of-defense" and a systemic reaction including recruitment of traditional bone marrow derived professional immune cells.

2. Toll-like receptors are expressed in the heart

Toll-like receptors (TLRs) are classic pattern recognition receptors (PRRs), which are gene-encoded proteins used by the innate immune system to recognize largely invariant pathogen-associated molecular patterns (PAMPs) that are shared by pathogen groups, but at the same time not present in the host [14]. Examples of PAMPs are lipopolysaccharides of bacteria and double-stranded RNA of different viruses [14]. TLRs are characterized by an extracellular leucine-rich repeat domain and a cytoplasmatic toll/interleukin-1 receptor homology domain (TIR). Thirteen different transmembrane proteins of the TLR family have been identified so far, with a number of specific ligands associated whose binding result in activation and transcription of appropriate host-defense genes [15]. Virtually all cells of the heart express TLRs [16]. TLR-2 to -4, and TLR-6 are readily detectable in cardiomyocytes, while TLR-1 to TLR-6 are found in endotheliocytes, smooth muscle cells and macrophages of the vasculature [16]. The ligands for TLR-2, -3, -4 and TLR-9 are the most well-characterized to date. A variety of pathogens, including different bacteria and yeast, is recognizable by TLR-2, whereas TLR-3 is the PRR for viral double-stranded RNA [17]. The first mammalian TLR described was TLR-4, and hence this is the best described receptor of the family [18]. TLR-4 is the PRR for LPS, which is the major component of the outer-membrane of Gram-negative bacteria and a compound that can induce a robust increase in the pro-inflammatory cytokines TNF-α and IL-1β within the myocardium (Fig. 1 — Immediate Effects) [19]. In addition, TLR-4 recognizes a variety of PAMPs from other invading pathogens as well as additional non-pathogenic ligands, including some chemotherapeutic agents [19]. TLRs can set off a complete immune response including innate immune activity through macrophages activated by induction of pro-inflammatory cytokines and antimicrobial molecules (e.g. nitric oxide), which enables macrophages to fight invading pathogens, as well as an adaptive immune response by activating dendritic cells, which can stimulate T-cell expansion and differentiation [16]. TLRs are furthermore able to maintain this adaptive immune response by providing the necessary co-stimulatory molecules [16]. The TLR family can initiate an immune response that is both cell and pathogen specific. Members of the TLR system are expressed differentially among immune and parenchymal cells, and many TLRs are activated by more than a single class of PAMPs [16]. In addition, distinct cell signaling pathways are triggered by different TLR classes, such that TLR-2 responses involve IL-8, IL-12 and IL-23 secretion, whereas TLR-4 responses include release of cytokines such as IL-10, IFN-β and IL-12. This allows for some degree of specificity even for the TLR-dependent innate immunity signaling pathways, which permit different immune responses for various PAMPs [16]. In the case of TLR-4, LPS initially binds to LPS binding protein (LBP) which transfers LPS to CD14 (Fig. 2A). It has, however, been reported that LPS-induced activation of signal transduction likely occur via a CD-14 independent mechanism in cardiomyocytes [20], even though CD14 and LBP expression have been reported on cardiomyocytes [20,21]. Fig. 2A shows the CD14/LBP-dependent LPS-mediated NF-κB-signaling via TLR4, since less is known about the specific details of the CD14-independent pathway. Fig. 2B is a schematic of the reported pathways for LPS-mediated CD14/LBP-independent signaling in cardiomyocytes [22]. LBP and CD14 are both expressed on the plasma membrane and activate TLR-4 after associating with MD2 [16]. Once activated, signaling continues via the intracytoplasmatic TIR homology domain, following either a "MyD88

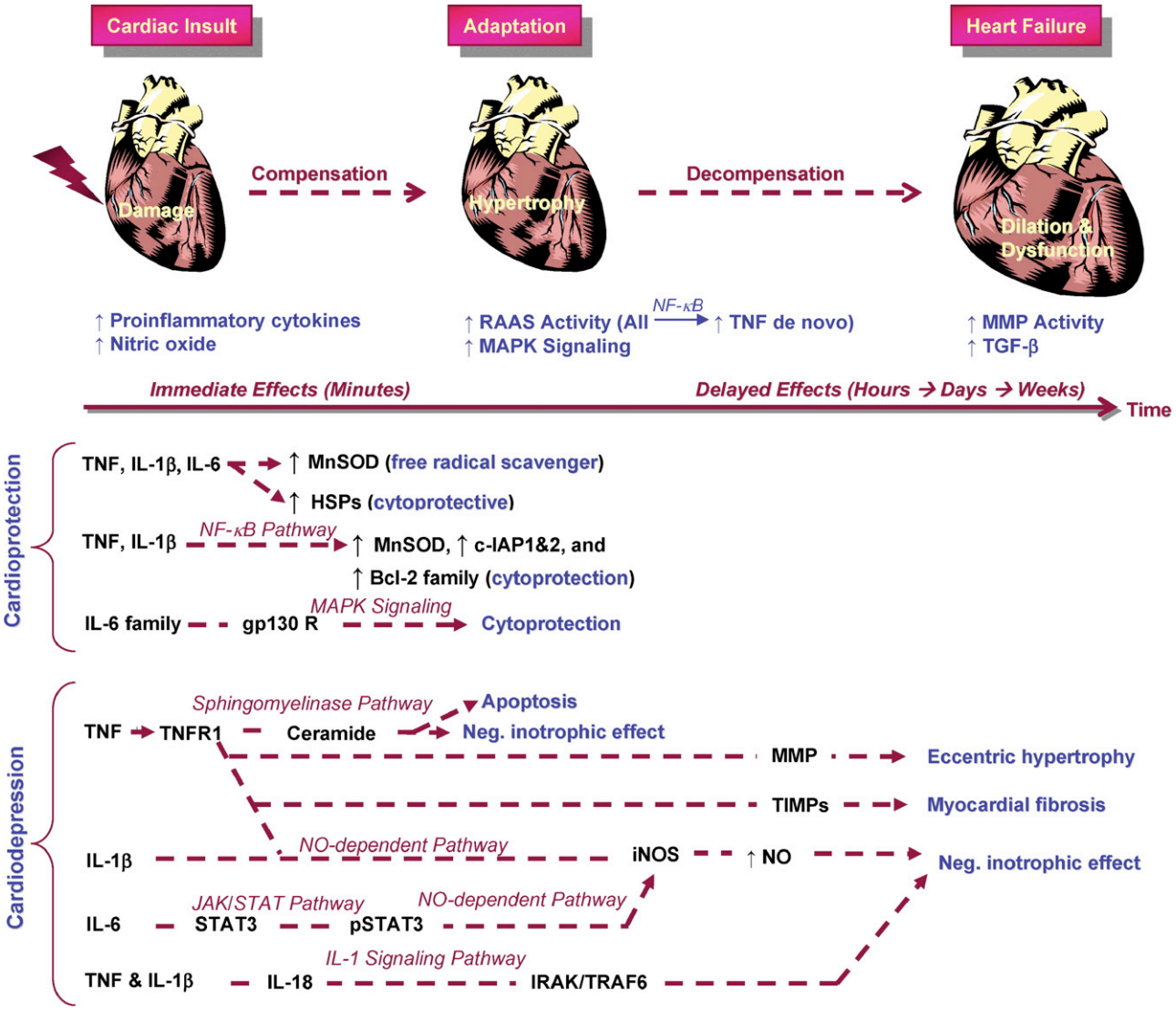

Fig. 1. Different types of myocardial injuries can activate an inherent cardiac stress response, which includes the expression of pro-inflammatory cytokines. The innate stress response plays a central role in instigating and orchestrating homeostatic responses within the heart. The effect of different cytokines upon the heart is depending on factors such as time of exposure and concentration. Abbreviations — RAAS: renin–angiotensin–aldosteron system; AII: angiotensin-2; NF-κb: nuclear factor kappa B; TNF: tumor necrosis factor; MAPK: mitogen-activated protein kinase; MMP: matrix metalloproteinases; TGF-β: tissue growth factor beta; MnSOD: manganese superoxide dismutase; HSPs: heat shock proteins; IL-1β: interleukin-1-beta; IL-6 family: incl. interleukin-6 (IL-6), leukemia-inhibitory factor (LIF), cardiotrophin-1 (CT-1), ciliary neurotrophic factor (CNTF), interleukin-11 (IL-11), and oncostatin M (OSM); c-IAP1 and 2: cellular inhibitors of apoptosis 1 and 2; Bcl-2: B-cell lymphoma/leukemia-2 gene; gp130 R: signal-transducing glycoprotein 130 receptor; TNFR1: tumor necrosis factor receptor-1; TIMPs: tissue inhibitors of matriz metalloproteinases; iNOS: inducible nitric oxide synthase; NO: nitric oxide; STAT3: signal transducer and activator of transcription 3; pSTAT3: phosphorylated STAT3; TRAF6: TNF receptor associated factor-6; IRAK: IL-1 receptor associated kinase; IL-18: interleukin-18 [Figure content based on information from Wilson et al. J Mol Cell Cardiol 37 (2004):801-11)].

dependent" or "MyD88 independent" pathway [23]. The MyD88 dependent route involves recruitment of the cytoplasmic adapter protein MyD88 and IRAK (interleukin receptor associated kinase), which is associated with TOLLIP (toll interacting protein). IRAK consequently becomes autophosphorylated and dissociates from the receptor complex after which TRAF-6 (TNF receptor-associated factor 6) is recruited. This activates downstream kinases including the inhibitory κB kinase complex that directly phosphorylates IκBα leading to nuclear translocation of NF-kB and initiation of gene-transcription (Fig. 2A) [24]. NF-κB activation is among the best-characterized pathways, but studies strongly suggests that several other yet unidentified pathways may exist as well [25].

3. Nf-κB signaling in the heart

NF-κB (nuclear factor kappa-B) was first discovered in 1986 [26], and found to contain subunit proteins involving Rel-homology domains (i.e. Rel family members), which are well preserved central components of the innate immune response [27]. To date, five mammalian NF-κB subunit

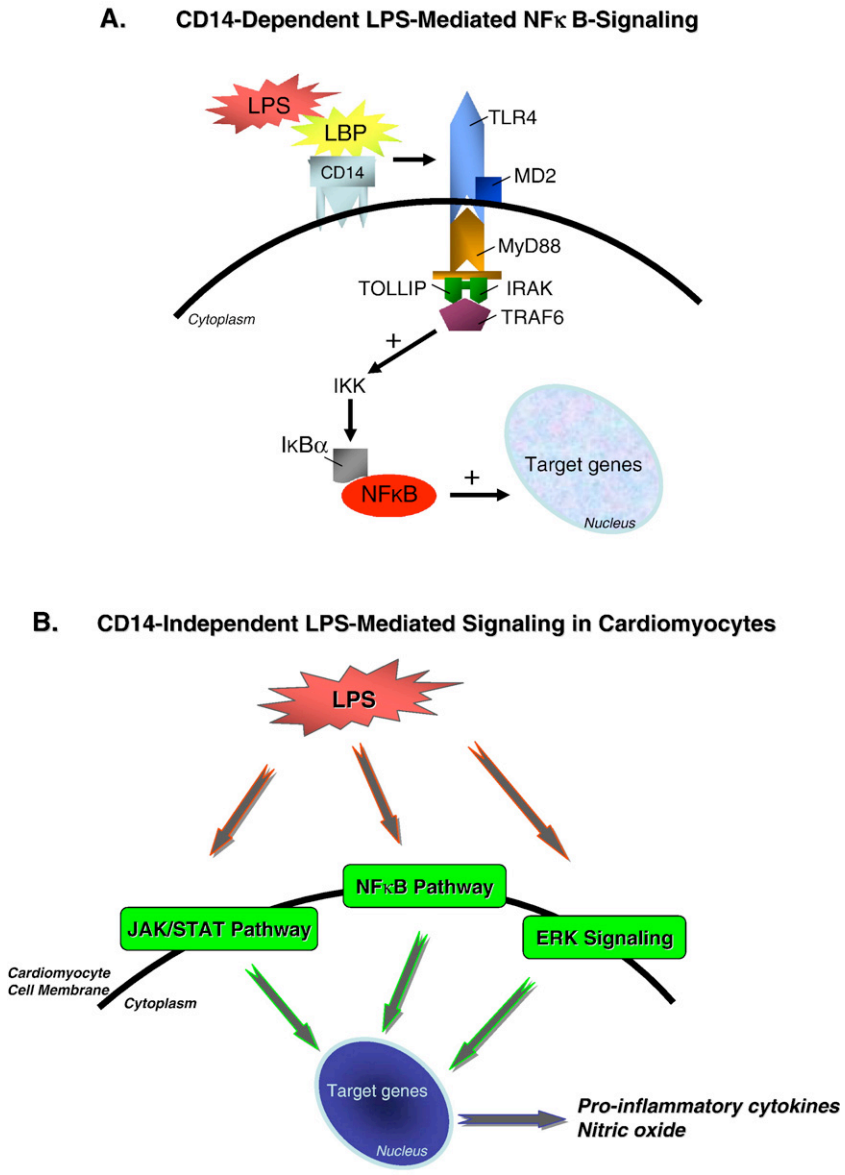

Fig. 2. A. Nuclear factor kappa B CD14-dependent signaling pathway. See text for details. Abbreviations — LPS = lipopolysaccharide; LBP: LPS binding protein; CD-14 (LPS-R) = Cluster of Differentiation 14 (LPS receptor); TLR-4 = Toll-like Receptor 4; MD-2 (LY96) = lymphocyte antigen 96; MyD88 = myeloid differentiation factor 88; IRAK = IL-1 receptor-associated kinase; TOLLIP: toll-interacting protein; TRAF6 = TNF receptor-associated factor 6; IKK: IκB kinase complex; IκB = inhibitory k B kinase; NF-κB = nuclear factor kappa B. B. Pathways for CD14-independent signaling in cardiomyocytes. See text for details. Abbreviations — ERK = extracellular signal-regulated protein kinases; JAK/STAT = Janus-kinase/signal transducer and activator of transcription; LPS = lipopolysaccharide; NF-κB = nuclear factor kappa B.

genes have been identified (RelA, cRel, RelB, NF-κB1, and NF-κB2) and NF-κB exists as dimers of these subunit proteins [27]. NF-κB dimers are complexed to one of seven known members of the IκB (inhibitory kappa-B) family which interacts with the Rel-homology domain of NF-κB [27]. The most common of the IκB proteins are the IκBα and IκBβ which are both expressed in the heart [28]. Contrary to the common perception, evidence indicates that NF-κB and IκBα, as well as components of the upstream signaling cascade, shuttle between the nucleus and the cytoplasm [29]. Thus NF-κB activation may occur via IκB phosphorylation in both compartments (Fig. 2A) [29]. The prevailing model dictates that the IκB proteins regulate NF-κB activity by: 1) maintaining equilibrium between the nucleus and the cytoplasm, where NF-κB levels are quite low, in the absence of any stimuli, and 2) inhibition of NF-κB's DNA-binding activity via interaction between NF-κB and the C-terminal PEST[1] domain of IκBα [30]. NF-κB can, however, be activated by two pathways. Degradation of IκB inhibitors leads the way for the so called canonical (NF-κB1) signaling pathway, which is present in the heart [28,31]. A second non-

[1] Polypeptide sequences enriched in proline (P), glutamate (E), serine (S), and threonine (T) that are proposed to expedite protein degradation [101].

canonical (NF-κB2) pathway has been described for NF-κB activation, which is based on regulated NF-κB2 processing rather than IκB degradation [32]. The canonical pathway may be activated by an array of signaling cascades including the JAK/STAT pathways [27]. As with many transcription factors phosphorylation increases the transactivational activity of NF-κB. NF-κB is a multi-tasking transcription factor implicated in an array of normal biological phenomena as well as different states of pathology such as cell growth and cell death, atherosclerosis, and innate immunity including inflammation [27]. Activating stimuli, such as cytokines, that result in initiation of transcription of genes which would otherwise remain silent or become transcribed at a very low rate in the responding cell, is a central theme in immunology. NF-κB can be considered a prototype, when discussing transcription factors, since it plays a central role in a number of different immunological reactions. Synthesis of pro-inflammatory cytokines and adhesion molecules is principally mediated by NF-κB, which itself is activated in response to a wide range of stimuli including cytokines, pathogenic microorganisms, viruses, mitogens, oxidative stress and modified LDL [33]. One of the recently identified nucleotide-binding oligomerization domain proteins (aka caspase recruitment domain-containing proteins), Nod1, has also been associated with NF-κB activation, and Nod1 furthermore appears to play a key role in endothelial cell immunity [34]. Nod proteins are implicated in intracellular pattern recognition, and Nod1 and Nod2 mRNA expression have both been identified in the heart [35]. The recently established Nod1 and Nod2 knockout mice will therefore help shed light on the specific role played by these newly identified peptides within the intrinsic cardiac immune system and its potential interaction with other organs [34]. NF-κB signaling in the heart is quite complex, since this factor is positioned as an integrator of diverse signaling pathways at multiple levels [27]. NF-κB activity is normally undetectable in the myocardium, suggesting that it acts predominantly in response to various stimuli to the heart [27]. Activation of NF-κB can tip the normal homeostatic balance of opposing processes in the myocardium, but the precise effect on the cardiac physiology or pathophysiology depends largely on the specific cell type and the set of NF-κB-dependent genes that is activated as well as the immediate environment [27]. The functional ambivalence can be exemplified by the fact that NF-κB activity seems to be involved in angiogenesis after hypoxia [36], while activation of NF-κB after ischemia/reperfusion has been implicated in a pro-cell death effect [27]. Multiple biological processes can be affected by NF-κB in the heart [37]. NF-κB activates gene expression that affects processes such as cardiomyocyte growth, contractile function and death as well as extracellular matrix remodeling and inflammation (Fig. 1) [27]. A compendium of all identified NF-κB regulated genes is maintained by courtesy of Dr. Gilmore at Boston University[2]. It is critical to note, that NF-κB regulate genes that are involved in paradoxical responses given that this has profound implications for understanding the role of NF-κB in pathology [27]. Understanding how the output of numerous parallel signaling pathways is integrated by NF-κB (as well as other transcription factors) resulting in gene expression with specific effects on the heart is a fundamental first step towards developing rational molecular therapies to address the injurious while retaining the protective aspects of transcriptional signaling networks activated by NF-κB in different types of cardiovascular disease [27]. Also, NF-κB activation occurs in several different types of cells and organs and may be primarily protective in one location while injurious in another [27]. Consequently, knowledge about NF-κB gene activation from one organ- or cell type cannot be immediately extrapolated to the immunophysiological mechanisms in the heart.

[2] http://people.bu.edu/gilmore/nf-kb/lab/index.html.

4. Signaling via the JAK/STAT pathway in the heart

The Janus kinase/signal transducer and activator of transcription (JAK–STAT) signaling pathway plays a central role in cardiac pathophysiology, and it is now recognized that a large number of different cytokines exert their effect through binding to receptors that activate this pathway, thus utilizing a rapid and direct route to effect changes in gene expression in the nucleus [1]. The JAK–STAT pathway involves cytokines acting via receptors associated with cytoplasmic Janus kinases (JAKs), which have two symmetrical kinase-like domains and are thus named after the two-headed mythical Roman god Janus [1]. Upon activation, the JAKs phosphorylate the cytosolic "signal transducers and activators of transcription" (STATs) proteins, which lead to their dimerization and translocation to the nucleus where they activate a variety of genes, including those contributing to lymphocyte growth and differentiation and thus adaptive immunity [1]. A range of different JAKs and STATs exists and using different combinations hereof ensures the specificity of signaling in response to different cytokines [1]. Mammals have four members of the JAK family (JAK1–3, and Tyrosine kinase 2 [Tyk2]), and seven members of the STAT family (STAT1–4, STAT5A, STAT5B, and STAT6), which are all expressed in cardiac tissue [38]. The STAT factors can function both as modulators of cytokine signaling and as sensors responding to cellular stress [39]. JAK–STAT signaling has been implicated in pressure overload-induced cardiac hypertrophy and remodeling, ischemic preconditioning and ischemia/reperfusion-induced cardiac dysfunction, while also playing an important role in cytokine signaling in cardiomyocytes (Fig. 1) [40]. Furthermore, the promoter of the prohormone angiotensinogen gene acts as the target site for STAT proteins, thus linking the JAK/STAT pathway to activation of the autocrine angiotensin II loop within the heart [41]. In addition, stress-induced activation of matrix metalloproteinases (MMPs) is mediated by angiotensin II acting via the JAK–STAT pathway [42]. Activation of specific STAT proteins constitutes the primary signaling event in

the development of myocardial hypertrophy and ischemia [41]. Angiotensin II has been shown to activate STAT1, 3 and 5 through JAK2 in cardiomyocytes. Despite similar structural organization, STAT1 and STAT3 have opposing effects on the myocardium with STAT1 exhibiting pro-apoptotic effects while STAT3 is able to protect cardiomyocytes from apoptosis after ischemia/reperfusion [39]. Studies have also shown that STAT5A and STAT6 are activated during ischemia, whereas activation of STAT3 and STAT5A occurs in myocardial hypertrophy [41]. Much research is currently aimed at generating strategies for targeting different STATs, and a significant amount of attention is being paid to developing JAK inhibitors aimed at use in the area of transplantation and immunosuppressive treatments. A selective JAK3 inhibitor was recently generated, which may represent a novel class of effective immunosuppressants since it has proved effective in transplant rejection in animal models [43]. The JAK/STAT pathway is but one of the stress/stretch-activated signaling pathways which have been identified in cardiomyocytes exposed to diverse demands [44]. Other pathways include G-proteins (guanine nucleotide-binding proteins), MAPK (mitogen-activated protein kinases), PKC (protein kinase C), ERK (extracellular signal-regulated protein kinases), JNK (c-Jun NH2-terminal kinases), the protein phosphatase calcinuerin, intracellular Ca^{2+} regulation, and a number of autocrine and paracrine factors [45]. Not only do these stress-activated pathways initiate and maintain the phenotypical cardiac alterations, but they have also been implicated in affecting the cardiomyocytes in deciding whether to survive or undergo apoptosis (Fig. 1) [45]. Consequently further research aimed at exploring the function of these individual proteins might pave the way for novel therapeutic opportunities in treatment of heart disease.

5. Cytokines and their implications in heart disease

It is well-established that cardiac myocytes and fibroblasts produce cytokines locally [9]. Studies have shown that various pro-inflammatory markers, including the cytokines TNF-α (tumor necrosis factor alpha), IL-1 and IL-6 (interleukin 1 and 6 respectively), are activated in different types of pathophysiologic processes involving the heart (Fig. 1) [46,47]. TNF-α has furthermore been implicated in regulation of vascular functions related to atherosclerotic plaque stability and may as such be partially responsible for plaque rupture [48]. Similarly, IL-6 promotes expression of ICAM-1 (intercellular adhesion molecule 1) and synthesis of CRP (C-reactive protein), with potential significant implications in atherosclerotic plaque formation and progression [49]. Accumulating evidence indicates that proinflammatory cytokines negatively influence the inotrophic state of the heart as well as induce hypertrophy and promote apoptosis or fibrosis, thereby actively contributing to myocardial remodeling and thus development of heart failure [46]. TNFα is one of the proinflammatory cytokines that has been implicated in cardiac dysfunction [50] and recent studies have shown that NF-κB activation and increased TNFα production may play a central role in cardiac injury due to intracellular Ca^{2+} overload [51]. In addition, myocyte apoptosis can be triggered by TNFα and its second messenger sphingosine, which consequently is partially responsible for the cardiac cachexia seen in heart failure patients [52]; just as cytokine-induced depression of myocardial contractility reportedly results from production of sphingosine due to interference with myocardial Ca^{2+} handling (Fig. 1) [47]. TNFα and IL1β appear to act both separately as well as synergistically towards depressing myocardial function, and it is likely that sphingosine also participates in this synergistic signaling leading to injurious effects on the heart [53]. Studies on feline cardiomyocytes have additionally shown that agents which modulate sphingosine production also minimize cardiodepression thus providing a potential therapeutic benefit in clinical conditions of myocardial inflammatory injury [54]. Paradoxically, recent studies have shown that low doses of TNFα can induce cardiac preconditioning, furthermore concluding that there is evidence for a production and role of free radicals in TNFα-induced cardioprotection [55]. IL-6 is another proinflammatory cytokine involved in cardiodepression and plasma levels of IL-6 are typically elevated in heart failure patients and inversely correlated to left ventricular function [56]. In addition, growing experimental evidence suggests a role for IL-6 in mediating part of the deleterious cardiovascular modifications observed in heart failure [56]. It has furthermore been suggested that IL-6 is involved in a paracrine interaction between cardiac myocytes and fibroblasts resulting in cardiac fibroblasts enhancing myocyte hypertrophy and cardiac myocytes regulating fibroblast adhesion and proliferation [9]. Numerous endogenous mechanisms exist for negatively regulating cytokine signaling and whether novel therapies can be devised that exploit these mechanisms remains to be further elucidated [43]. Recent multi-center trials with the anti-TNF-alpha compounds etanercept (i.e. RENEWAL trial) and infliximab (i.e. ATTACH trial) have questioned the beneficial effect of targeting single cytokines [57,58]. Both trials concurred that no safety issues was seen with regards to infliximab or etanercept at lower dosages, but pointed out that high doses of anti-TNF therapy may be without effect in heart failure patients [59]. Other smaller trials (e.g. ENBREL) have, on the other hand, reported dose-dependent improvement in heart function with etanercept treatment [60], whereas adverse effects including toxicities secondary to TNF-alpha blocker therapy have been reported by others [13,61]. Despite the somewhat disappointing and rather controversial results thus far, the "cytokine hypothesis" remains an interesting concept in development of novel efficacious therapies for heart failure patients. The studies accentuate the complexity of the cytokine network, however, and have partially caused a redirection of the focus towards more general immunomodulating treatment modalities [62]. Interestingly, nevertheless, the pro-inflammatory cytokines are frequently induced

even before classical neurohormones such as angiotensin II and noradrenaline in patients with chronic heart failure, and so the overactive immune system still remains a promising target for therapeutical interventions aimed at slowing down cardiac disease progression. On the other extreme, a number of cytokines, including G-CSF (granulocyte colony stimulating factor), leukemia inhibitory factor and EPO (erythropoietin) have proven to have beneficial effects on cardiac remodeling after infarction [40,63]. Furthermore endogenous anti-inflammatory cytokines such as IL-2 and IL-10 have proved to protect the myocardium against injury induced by ischemia and reperfusion [64]. It has been shown that G-CSF activates the JAK/STAT pathway in cardiomyocytes thus protecting against cardiac remodeling by reducing apoptosis post infarction [40]. Rapid activation of potassium channels and protein kinases by EPO reportedly represents a central new mechanism for increased cardioprotection. Another recent study suggests that EPO has cardioprotective effects by preventing cardiomyocyte apoptosis [63]. EPO seems to instigate the immediate protection of the heart through multiple signal transduction pathways, among which the JAK/STAT is one of them [65]. Additional experiments have revealed that the rapid cardioprotective effect of EPO is associated with ATP preservation in the ischemic myocardium [66]. In view of the existing knowledge on the biological effects of cytokines on the heart, anti-cytokine therapy is likely to provide a new direction for management of heart failure. Even though a cytokine-mediated response initially may be beneficial, it might become deleterious when sustained. In addition, the same therapeutic approach has the potential of acting differently in a given patient depending on the disease state. Consequently future therapies likely need to aim at intracellular goals to inactivate signaling systems responsible for injurious effect in cardiac disease and heart failure [67].

6. Cardiac antimicrobial peptides

Antimicrobial peptides (AMPs) are "multi-tasking natural antibiotics" and ancient molecules of innate immunity with functions extending far beyond that of simple antibiotics — including anti-tumor and mitogenic activity, as well as immunomodulation and signal transduction characteristics [68]. The term antimicrobial is used because these peptides have extraordinary broad spectra of activity, including an ability to kill or neutralize Gram-negative and Gram-positive bacteria, fungi, yeast, cancer cells and some enveloped viruses [69]. The overall effectiveness of an innate immunity based host defense is shown by the clearly successful survival of plants and invertebrates, organisms which completely lack adaptive immunity. Defensins (including α-, β- and θ-defensins) and cathelicidins constitute the two major groups of AMPs in the majority of mammalian species [70]. Mammalian defensins are endogenous cysteine-rich peptide molecules classically produced by epithelial cells of the external and internal surfaces or by circulating cells, including granulocytes and macrophages. β-defensins are small (3.5–4.5 kDa) highly basic cationic peptides, structurally defined by a conserved cysteine-rich motif forming three disulphide bonds, which stabilize a β-sheet formation [71]. Their folding pattern is determined by the amphipathicity of these molecules, which is also believed to govern their antimicrobial effect. The mechanism of action is thought to rely on permeabilization of the microbial membrane and lysis of invading organisms, which is explained in theory by the Shai–Matsuzaki–Huang (SMH) model [72]. Epithelial β-defensins consequently represent a rapidly mobilized local defense against microbial intruders at the epithelial and mucosal surfaces, and several studies have shown induction of these defensins at sites of inflammation, injury, infection as well as other types of disease processes [73]. β-defensins are either constitutive or inducible, and their production can be elicited by ligands such as bacterial LPS through TLRs using the NF-kB pathway (Fig. 2A) [74]. Other pathways, including MAPK and JAK/STAT signaling, seem to be involved in β-defensin regulation [75]. β-Defensins have recently been identified in what may be considered as non-traditional tissue such as the heart; including HBD-3 (human beta-defensin 3) expression in adult human heart [76], pBD1 (porcine beta-defensin 1) in pigs [77], eBD1 (equine beta-defensin 1) in the horse [78], Defb1 (murine beta-defensin 1) in mice [79], and rBD1 (rat beta-defensin 1) in the rat [73]. Our laboratory has furthermore recently documented that at least seven different beta-defensins (i.e. rBD1/3/10/11/15/18 and 33) are expressed in the adult rat heart (unpublished data). These findings suggest that AMPs might participate as effector molecules of the innate immune system in the heart as in other types of tissue, but information on cardiac AMPs is still sparse and further research is needed to elucidate the actual role played by AMPs in heart disease and health.

7. The heart as an immunological organ

The heart possesses a gene-encoded intrinsic or innate stress system, which is activated in response to different types of injury (Fig. 3) [80]. This local innate stress response plays a key role in instigating and coordinating homeostatic responses in the heart, while the released inflammatory mediators at the same time possess the potential of producing cardiac decompensation if expressed at sufficiently high concentrations [80]. The quintessential feature of this innate immune system is that it serves as an immediate warning system thus constituting a first-line-of-defense allowing the host to discriminate self from non-self [80]. The "Danger Model of Immunity" suggests that cell damage rather than foreignness is what prompts an immune response (Fig. 3) [81], and current research also suggests that chronic heart failure in fact is a state of chronic inflammation, thus stressing the importance of a functionally intact innate immune system in the heart. Pro-inflammatory cytokines appear to play a central role in the orchestration and timing of the intrinsic cardiac stress response providing instantaneous

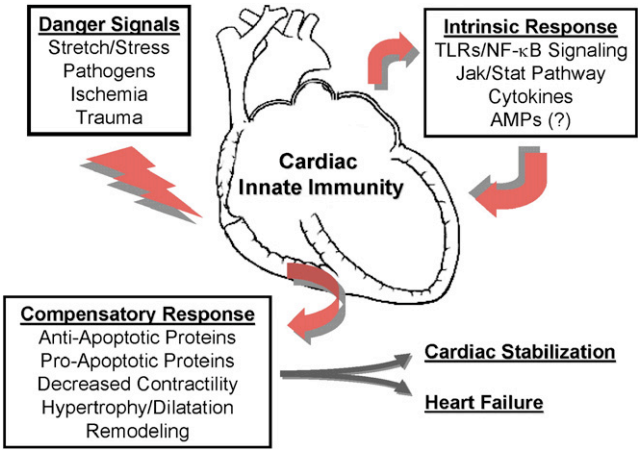

Fig. 3. Schematic danger model of intrinsic cardiac immunity illustrating factors which may initiate an innate immune response within the heart, leading to activation of intrinsic signaling pathways and production of certain immune mediators, which may have either protective or deleterious effects — ultimately resulting in cardiac stabilization or failure. Abbreviations — AMPs = antimicrobial peptides; JAK/STAT = Janus-kinase/signal transducer and activator of transcription; NF-κB = nuclear factor kappa B; TLRs = toll-like receptors.

anti-apoptotic cytoprotective signals, as well as delayed signals facilitating tissue repair and/or remodeling. The protective response may occur at the cost of unwanted injurious effects occurring when cytokines are elaborately expressed for sustained time intervals or at pathologic/supra-physiologic levels contributing to cardiac remodeling through different mechanisms involving cardiomyocytes as well as non-cardiomyocytes [80]. As in the case with TNFα, NF-κB and AP1 (activator protein 1) have been suggested as potential therapeutic targets, since these proteins are activated in heart failure patients [16]. Not only has NF-κB proved to play a key role in muscle wasting and cardiac cachexia in heart disease, but its activation also seems to play a role in proliferation of vascular smooth muscle cells and intimal hyperplasia in atherosclerosis [82]. Furthermore, NF-κB is both necessary and sufficient to elicit a hypertrophic response in isolated rat cardiomyocytes [83], and TNFα-induced hypertrophy is reported as being dependent on NF-κB activation [84]. The activities of NF-κB-dependent genes can in fact explain many of the actions of TNFα on the heart [27]. Results from studies involving transgenic mice show that TNFα over-expression can cause development of severe dilated cardiomyopathy with rapid progression to heart failure [85]. Interestingly, the cardiac-specific TNFα expression in these transgenic mice can lead to not only heart failure through activation of pro-apoptotic signaling pathways, but also cardioprotection through activation of anti-apoptotic proteins [86]. NF-κB-signaling is therefore likely a mixture of pro- and anti-apoptotic activity, as well as other effects such as inflammation and infiltration, as well as effects on Ca^{2+} handling and NO (nitric oxide) production etc. [27]. Moreover, cross-talk seems to exist between NF-κB and other signaling pathways [27]. Apoptosis has been implicated in different aspects of cardiac pathology, including cardiomyopathy, myocardial infarction and heart failure [87], thus making regulation of pro- and anti-apoptotic signaling a quite central topic when addressing heart disease. Apoptosis and necrosis differentially contribute to myocardial injury. Still, the extent to which apoptosis versus necrosis is mechanistically accountable for cell death in the intact heart post infarction/reperfusion is somewhat controversial [27]. Apoptosis appears to be the predominant mechanism for at least the first 24 h post infarction, whereas necrosis seems to be more predominant in the developing infarct [88]. Modification of TLR expression may prove another important therapeutic target. TLR1, 2 and 4 expressions are all enhanced in human atherosclerotic plaques [89], and studies in knock-out mice have shown that inhibitors of TLR4 may be helpful in treatment of atherosclerosis [90]. Studies have shown that inhibition of TLR2 may be advantageous after myocardial infarction since this TLR seems to play an important role in ventricular remodeling [91]. Much attention has clearly been paid to non-pathogenic insult affecting the heart, while infections with viruses, protozoa, fungi or bacteria clearly also can be associated with heart disease and thus responsible for eliciting an innate immune response and inflammation. The most common virus infection type identified in the human heart are due to the Coxsackie B group viruses, which can be detected in up to 50% of patients with dilated cardiomyopathy [92] — work which, however, remains controversial. As such one of the main etiological factors in DCM is persistence of cardiotrophic viruses (including enterovirus, adenovirus, human cytomegalovirus, parvovirus B19, and influenza virus). The significance of viral injury and inflammation in the etiology of certain cardiomyopathies is further recognized by the most recent WHO/WHF definition of inflammatory cardiomyopathy (DCMi) as a distinct entity adding to the already existing five major forms of cardiomyopathies [93,94]. Naturally occurring, as well as experimentally induced, cardiovascular disorders have been associated with a range of different causative pathogens [95]. It is worth keeping in mind that cardiovascular organs may also become “casualties” of inflammatory processes that have a focus outside the heart [2]. In sepsis, TNFα has also been implicated as a key factor in development of myocardial dysfunction [24]. The canine and human cardiovascular profile is remarkably similar in sepsis, and the sphingomyelin pathway is believed to play a central role in TNFα-induced myocardial depression in both species. In addition, these highly specialized immune processes are not surprisingly of outmost interest in thoracic surgery, as a systemic inflammatory response syndrome (SIRS) due to cellular and humoral defense reactions are activated in cardiac surgery using cardiopulmonary bypass [96]. A line of research has focused on the innate immune response associated with open heart surgery and cardiac transplantation. One study found that activation of an innate immune response through TLR4 contributes to development of chronic rejection after heart transplantation

[97]. In children with congenital heart disease, cardiopulmonary bypass elicits a prominent innate immune response, and cardiac operations are associated with increased oxidative stress, leukocyte activation and increased production of pro- and anti-inflammatory cytokines [98]. TLR2 and 4 are suggested as putative signaling receptors for heat shock proteins (HSP) in mediating synthesis of inflammatory cytokines in cardiac surgery [99]. In conclusion, evidence is rapidly accumulating that the effector-molecules and signaling pathways of the innate immune response have a marked impact on the heart in different types of cardiac diseases. Ultimately, the myocardium's ability to adapt to environmental stress determines if it will maintain health or decompensate and fail [80]. Still, further research aimed at elucidating the molecular mechanisms behind potential injurious and protective effects caused by the innate immune response is pivotal toward gaining a better understanding of many types of cardiovascular pathology.

8. Summary

Inflammation underlies the pathogenesis of a range of the most common cardiovascular diseases. Recent development and use of genetically manipulated murine models such as transgenic/knock-out mice have created an opportunity to pinpoint specific signaling pathways underlying heart disease as well as evaluate therapeutic strategies of candidates for clinical development. When formulating treatment strategies in inflammatory processes in any organ it is essential to understand tissue-specific pathways that may either intensify or dampen cells and mediators of the inflammatory system [100]. It is important to recognize that amplification and/or damping of the inflammatory reaction is likely to vary between organs, and that the primary cellular composition of an organ, as well as the matrix and mediators released by specialized cells will govern the overall inflammatory reaction [100]. Immunomodulatory therapy has emerged as a possible new treatment modality in CHF, as traditional cardiovascular drugs seem to have little if any effect on the overall cytokine network. Selective gene targeting to identify which PRRs play a central role in the heart secondary to injury will likely be one obvious area for future discovery. Overall, relatively little is still known about how the innate immunity response is modulated in the heart and it is therefore most likely that the learning curve will remain rather steep in the most immediate future [80]. In all considerations it is pivotal to keep in mind the "Janus Face" of innate immunity and inflammation as a system that aims to heal but holds the capacity to destroy.

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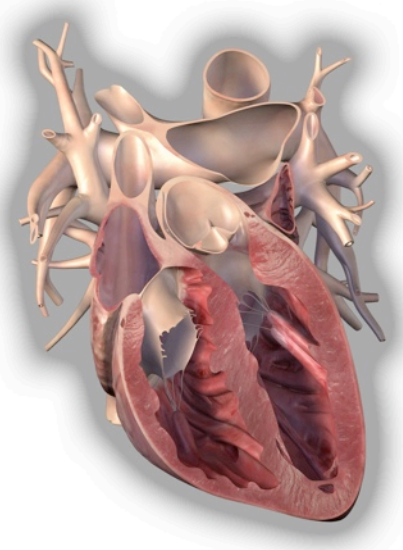
Chapter 3
Studies on Cardiac Innate Defense



The experimental studies presented in this chapter were conducted between 2004 and 2008 at Kansas State University, Departments of Anatomy & Physiology, and Human Nutrition

This third section of the thesis presents our experimental data, collected from the comparative studies on cardiac host defense peptides and pattern recognition receptors.

We here present three original articles, of which the first looks at Toll-like receptor expression in the dog heart. This paper was published in *American Journal of Veterinary and Animal Sciences* 2 (1) : 6-10, 2007 and titled "***Toll-Like Receptor (TLR) 2 and TLR4 Gene Expression in Canine Heart"***.

The second study is reflective of what was our initial findings on specific expression of beta-defensin-1 in rat cardiomyocytes. These findings have been published by *American Journal of Veterinary and Animal Sciences 3(1): 1-6, 2008*, in the paper titled ***"Rat Cardiomyocytes Express a Classical Epithelial Beta-Defensin"***.

The above study created the scientific foundation for our third manuscript, which presents the core of this work. The study was focused primarily on assessing the gene-expression levels of ten different beta-defensins in the rat heart secondary to exposure with either an infectious or a non-infectious inflammatory mediator. We also studied functional features of the cardiac rat beta-defensins. The resulting manuscript ***"Cardiac Beta-Defensins: Novel Antimicrobial and Immunomodulatory Host Defense Peptides with Enhanced Expression in Inflammation"*** will be submitted to a suitable journal, such as *Circ Res*.

We are very encouraged to here present data which in summary suggests, that the myocardium actually respond "by itself" to "dangers" - incl. a high fat (33%) content in the diet or exposure to a bacterial cell-wall component (LPS).

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Toll-Like Receptor (TLR) 2 and TLR4 Gene Expression in Canine Heart

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Abstract: Toll-like receptors (TLRs) are archetypal pattern recognition receptors of immediate importance for an efficacious innate immune response. TLRs exhibit marked differential tissue activity and their levels within a discrete cell type can be highly dynamic. Of 13 known mammalian paralogues, three TLRs have been identified in the dog. Although cardiac TLR expression has been reported in other species, this study is the first to present evidence that these innate immune receptors are expressed in the canine heart. Heart tissue samples from all four chambers were collected from healthy dogs immediately after euthanasia and stored at -80ºC until analysis. Total RNA was extracted with TRI Regent. Specific primers were designed for amplification of canine TLR2 and TLR4 based on previously reported sequences for these genes. Reverse transcription was performed with M-MLV reverse transcriptase. PCR amplification was performed and PCR products analyzed by agarose gel electrophoresis. Bands were excised from the gel and the DNA isolated and cloned using the TA Cloning® Kit. The correct sequence for each product was verified by nucleotide sequencing. TLR4 expression was detected in the left ventricle and right atrium; TLR2 was detectable at low levels in the right atrium only. Identity of the RT-PCR products was confirmed by sequencing. Our findings show that at least two TLR paralogues – namely TLR2 and TLR4 – are expressed in the canine heart. Additional studies are warranted to determine these immune receptors' potential implication in the development of naturally occurring heart disease in the dog.

Key words: Innate immunity, pattern recognition receptors, cardiac and dog

INTRODUCTION

Progress in the field of acquired immunity has historically overshadowed the field of immunology entirely[1]. More recent advances have, however, proven that innate defense mechanisms play key roles in a number of disease processes, and that several different so-called 'danger-signals' (infectious as well as non-infectious) may act as the trigger mechanism for an immune response[2]. Toll-like receptors (TLRs) are highly conserved classical pattern recognition receptors of the innate immune system, enabling the host to discriminate between pathogen associated molecular patterns (PAMPs) and self[1]. Detection of microbial components by TLRs also triggers activation of adaptive immunity, furthermore making this receptor family an important link between the two branches of the immune system[3,4]. The mammalian TLR family includes thirteen paralogues with differential tissue and cellular expression varying between species[1,3]. Different microbial patterns, such as lipopolysaccharide (LPS) from Gram-negative bacteria, lipotechoic acid (LTA) from Gram-positive bacteria and viral dsDNA, can act as ligands for different TLRs[1,3,5,6]. Following tissue damage, TLRs may also recognize certain host proteins, such as beta-defensins and heat shock proteins[1]. TLR-mediated signaling occurs through either a MyD88-dependent or MyD88-independent pathway, and it has been established that TLRs are complicit in not only immunological responses but also more general cellular homeostasis[1,5]. Toll-Like Receptor 4 (TLR4) was the first mammalian TLR identified, and it is consequently the best described of the family[3,7]. Information on TLRs in small animal species is still quite sparse. Recently, the full-length cDNA for TLR4 was cloned in dogs and cats[8], and TLR2 expression has been identified in canine blood phagocytes[9]. Furthermore, the expression of TLR9 has been investigated in canine tissues[10], and TLR expression has been reported in feline lymphoid tissues[11]. Additional studies have been conducted pertaining to TLR expression in other domestic animals, and TLRs have thus been identified in various organs of different species[12-17]. Increasing attention is paid to innate immunity and inflammation in heart disease and further identification of receptors and effector molecules of innate immunity is thus warranted. Virtually all cell types of the human and murine heart express TLRs[18]. TLR2-4, and TLR6 are readily detectable in cardiomyocytes[19,20], while TLR1-6 are expressed on endotheliocytes, smooth muscle cells and macrophages of the vasculature[18]. A recent

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study suggests that mRNA expression of all ten human TLRs can be identified in the adult heart in people[21]. It has been suggested that endogenous signals, such as oxidative stress and heat shock proteins, can activate TLR-mediated signaling pathways in isolated ventricular cardiomyocytes[19]. Furthermore, the activation of myocardial TLR-mediated signaling pathways in response to exogenous ligands can induce cardiac dysfunction[22,23]. TLR2 has also been implicated in ventricular remodeling after myocardial infarction in mice[24]. No studies to date have reported on TLR expression in the canine heart. Since TLRs are essential signaling molecules governing an innate immune response, and because mediators of inflammation and innate immunity with increasing certainty are proven to play key roles in different types of cardiovascular diseases, we hypothesized that the normal canine heart expresses TLRs as a natural part of its intrinsic defense system. Here we show that TLR2 and TLR4 are expressed in whole heart homogenate from normal dogs. Further studies pertaining to TLR function and regulation in the canine heart are needed to establish the significance of these receptors in the heart of dogs in health and disease.

MATERIALS & METHODS

Tissue preparation: Heart tissue samples were collected from healthy dogs immediately after euthanasia and snap-frozen in liquid nitrogen. Separate samples were obtained from both ventricles and atria. All animals were treated in strict accordance with university IACUC guidelines. Samples were temporarily stored at -80ºC until further analysis.

RNA extraction: Total RNA was extracted with TRI Regent (Sigma-Aldrich, St. Louis, MO) after grinding the frozen tissues in liquid nitrogen. Briefly, total RNA was treated with RQ1 RNase-free DNAse I (Promega, Madison, WI) to remove possible genomic DNA contamination.

Identification and cloning of TLR2 and TLR4 in the canine heart: To initiate our investigation of canine cardiac TLRs, specific primers were designed for amplification of canine TLR2 and TLR4 based on the previously reported sequences for these genes (GenBank No. NM_001005264 and NM_001002950). TLR2 forward and reverse primers sequences were 5′-ATGATTCCTACTGGGTGGAGAAC and 5′-CGCAGCTTACAGAATCGCTG, respectively. TLR4 forward and reverse primers sequences were 5′-CCTGGAAGGACTGTGCAATT and 5′-TGCTTCAGTCTGGTTGTCCC, respectively.

Using the designed primers and total RNA (from each heart tissue section) as template, reverse transcription was performed with M-MLV (Moloney Murine Leukemia Virus) reverse transcriptase (Invitrogen, Carlsbad, CA) following the instructions of the manufacturer, in a 20-μl reaction containing 500 ng of total RNA and two-base anchored oligo(dT) primers (5′-$(dT)_{14}$VN, where N is any base and V is G, A or C). The template cDNA (1 μl) was amplified by PCR in a volume of 20 μl containing 1 μM of each primer and 2.5 units of *Taq* DNA polymerase (Promega). PCR amplification was performed by 35 cycles consisting of template denaturation (94°C, 15 sec), primer annealing (55°C, 30 sec) and polymerization (72°C, 1 min). PCR products were analyzed by agarose gel electrophoresis. To confirm the specificity of the amplification products, bands were excised from the gel and the cDNA isolated and cloned using the TA Cloning® Kit (Invitrogen, Carlsbad, CA) and sequenced. Full cDNA sequences were compared with previously reported canine TLR2 and TLR4 sequences using the BLAST tool from the National Center of Biotechnology Information.

RESULTS AND DISCUSSION

Expression of TLR4 was detected in cardiac tissue samples originating from the left ventricle and right atrium; TLR2 expression was detectable at low levels in the right atrium only. No expression of either TLR2 or TLR4 was found either in the left atrium or right ventricle. Nucleotide sequences obtained from the RT-PCR products were compared to previously described canine toll-like receptor nucleotide sequences and displayed complete homology. Tissue expression of TLR2 and TLR4 mRNA is shown in Fig. 1.

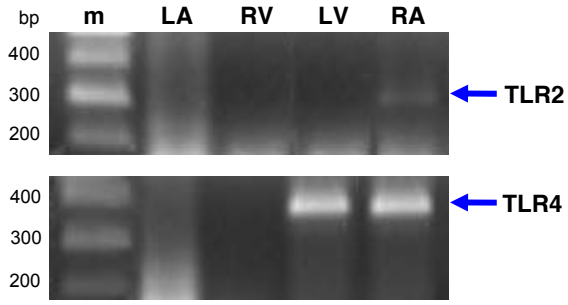

Fig. 1: Tissue expression of TLR2 and TLR4 mRNA. Total RNA (500 ng) was subjected to RT-PCR and 10 l of 35-cycle amplicons were separated on 2% agarose gel and stained with ethidium bromide. Top gel: TLR2, Bottom gel: TLR4. m: size markers; LA: left atrium, RV: right ventricle, LV: left ventricle, RA: right atrium. TLR2 and TLR4 amplicons (blue arrow) were 310 and 420 bp in size, respectively.

Innate immunity and inflammation play central roles in a variety of pathologies - including common cardiovascular disorders, as shown in humans as well as rodent models[25]. It is likely that specific effector molecules and signaling pathways governing an innate immune response are of key importance to the development of certain types of naturally occurring heart diseases in dogs and other domestic animals. TLRs are an ancient pattern recognition system central for signaling in innate immunity[6]. TLR2 expression has been reported in canine blood phagocytes[9], and TLR4 and TLR9 have been identified in selected dog tissues[8,10,26]. To date, no study has reported on TLR expression in the dog heart. Knowledge of the expression pattern of these receptors in the normal canine heart is, however, of immediate importance for further mechanistic studies pertaining to innate immunity and the heart. The present work provides novel evidence that at least two TLRs - TLR2 and TLR4 - are expressed in the canine heart. We also report that TLR2 expression was detectable in the right atrium only, while TLR4 could be identified in the left ventricle as well as the right atrium. The global expression of TLRs changes secondary to intrinsic factors such as age, as well as cellular exposure to environmental stressors including pathogenic microorganisms[1]. Distinct TLRs may also affect each other's expression levels, and functional dimerization of certain TLRs has been reported[5]. A key design feature entails the trafficking of certain TLRs (TLR1-2 and TLR4-6) to the cell membrane thus allowing interaction with extracellular pathogens, while other TLRs (TLR3 and TLR7-9) are found virtually only at intracellular sites – this division, however, is not absolute[1]. The heart possesses a germ-line encoded innate stress system, which is activated in response to different types of injury[27]. Since TLRs are ancient germ-line encoded receptors of an innate immune response, and the right atrium the first location reached by blood entering the heart from the systemic circulation, it may be theorized that the expression of TLRs in this chamber is constitutive and possibly more diverse compared to other cardiac sites to allow early detection of noxae. Similarly, constitutive expression of TLR4 in the left ventricle would be advantageous as an intrinsic measure of detection situated immediately prior to blood leaving the heart and entering the main coronaries and systemic circulation. Bacterial LPS is the classical ligand for TLR4 activation (although other endogenous substances have been suggested as possible TLR4 stimulants also), whereas TLR2 recognizes a variety of microbial components, including LTA from Gram-positive bacteria[3,6,28]. TLR2 has also been implicated as a mediator of staphylococcal induced cardiodepression by acting as a receptor for proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1β (IL-1β) (both of which are known contributors to cardiac decompensation in sepsis)[25]. In isolated coronary endothelial cells, TLR4 is required for LPS-signaling leading to an inflammatory response[29]. TLR4 is also a known contributor to development of chronic rejection after heart transplantation[30]. Interestingly, one study reports that cardiomyocytes and endotheliocytes have an equal amount of TLR4, but that only the latter responds to LPS, possibly making the need for cardiomyocytes as detectors obsolete[31]. However, TLR expression observed in cardiomyocytes may function as a back-up system that is activated after longer exposure to a pathogen or other noxia, thus serving as receptors in a "second line of defense" of an innate immune response. Further studies on the expression and function of TLRs within the frame work of an intrinsic cardiac immune response are needed as this may facilitate development of future novel treatment modalities in canine heart disease.

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Rat Cardiomyocytes Express a Classical Epithelial Beta-Defensin

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Abstract: Beta-defensins (BDs) are classical epithelial antimicrobial peptides of immediate importance in innate host defense. Since recent studies have suggested that certain BDs are also expressed in non-traditional tissues, including whole heart homogenate and because effector molecules of innate immunity and inflammation can influence the development of certain cardiovascular disease processes, we hypothesized that BDs are produced by cardiomyocytes as a local measure of cardioprotection against danger signals. Here we report that at least one rat beta-defensin, rBD1, is expressed constitutively in cardiomyocytes specifically isolated using position-ablation-laser-microdissection (P.A.L.M. Microlaser Technologies). RT-PCR analysis showed expression of a single 318 bp transcript in adult rat heart (laser-excised cardiomyocytes) and H9c2 cells (neonatal rat heart myoblasts). Moreover, the full length cDNA of rBD1 was established and translated into a putative peptide with 69 amino acid residues. The predicted amino acid sequence of the adult rat cardiac BD-1 peptide displayed 99% identity with the previously reported renal rBD1 and 88, 53, 53 and 50% identity with mouse, human, gorilla and rhesus monkey BD1 respectively. Furthermore, structural analysis of the cardiac rBD1 showed the classical six-cysteine conserved motif of the BD family with an alpha-helix and three beta-sheets. Additionally, rBD1 displayed a significantly greater number of amphoteric residues than any of the human analogs, indicating a strong pH functional dependence in the rat. We suggest that rBD1, which was initially believed to be a specific epithelium-derived peptide, may be also involved in local cardiac innate immune defense mechanisms.

Key words: Innate immunity, host defense peptides, heart, microdissection

INTRODUCTION

Defensins are master-players in innate immunity and one of the best characterized families of antimicrobial/host defense peptides identified in numerous species across the animal and plant kingdoms. To date, three categories of the defensin-family have been described in mammals, varying with respect to expression pattern and structural placement of six conserved cysteine residues[1]. Dissimilar to α-defensins-which are principally produced by circulatory neutrophils and Paneth cells in the gut-β-defensins (BDs) are classical epithelium-derived peptides, whereas θ-defensins thus far have been reported in non-human primates only (mini defensins). More recently, BDs have been identified in non-epithelial cell types also, including circulatory polymorph nuclear cells and tissues macrophages[1-3]. Defensins are commonly referred to as natural endogenous antibiotics. Still, these important molecules seem to function far beyond that of simple antimicrobial peptides, including features such as immunomodulatory and anti-tumor activities. Their expression can be either constitutive or inducible-classically instigated via Toll-like receptor mediated NF-κB (nuclear factor kappa-B) signaling secondary to pathogen associated molecular pattern recognition[3,4] .

Cardiomyocytes have not traditionally been considered significant role-players in the orchestration of an innate immune response. Inflammation and innate immunity have, however, proven to be of major importance in a number of different cardiovascular disease processes, including atherosclerosis and myocarditis[5-9]. The Danger-Model of Immunity furthermore defines damage-more than mere foreignness-as the actual trigger of an innate immune response[10]. Moreover evidence exist that various sentinel cell types, including endotheliocytes, cardiomyocytes, fibroblasts and mast cells, can partake actively in an immune response along side more classical immune cells[9]. Local effector molecules of an

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innate immune response (incl. cardiac BDs) may thus be central for cardioprotection in infectious as well as non-infectious disease processes. Recent studies have incidentally found concomitant mRNA expression of select beta-defensins in whole heart homogenate from different species, including dBD1 in mice, eBD1 in horses, pBD1 in pigs and hBD3 in humans[11-14]. Rat beta-defensin-1 (rBD1) mRNA expression has in addition been reported in a number of different organs, including the heart, based on screening of an array of whole tissue homogenates in rats with diabetic nephropathy[15].

Similar to other organs, the heart contains a number of different cell types. Even though BDs have been detected in heart tissue, no studies have conclusively documented that the cardiomyocytes are in fact the actual source of the BD expression. We thus surmised that the rBD1 expression in whole heart homogenate can be ascribed directly to cardiac myocytes and also that this can be identified in different life-stages. The presence of these key effector molecules of innate immunity may prove central to the heart's protective mechanisms towards an array of incoming dangers. This study provides evidence that laser-excised cardiomyocytes constitutively express at least one BD isoform.

MATERIALS AND METHODS

Whole heart tissue preparation: Whole hearts were excised from adult Wistar rats immediately after euthanasia, snap-frozen in liquid nitrogen and stored at -86°C. All animal care and usage were in accordance with the Kansas State University IACUC (Institutional Animal Care and Usage) guidelines. The cryopreserved hearts were transferred individually to a LEICA CM3050 S Cryostat and ½×½ cm tissue blocks of the frozen specimens were excised at -20°C. Each tissue block was covered with TBS™ Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC), sectioned at 10 m and transferred to PALM® MembraneSlides (Cat.No.1440-1000, P.A.L.M. Microlaser Technologies AG, Bernried, Germany) for a total number of 8-10 sections per slide. Tissue sections were fixed in 100% ethanol for 3 min at -20°C immediately prior to staining. H and E (hematoxylin and eosin) staining was performed using a modified H&E technique (IHC World) with all staining reagents kept on ice throughout the entire procedure.

H9c2 cell culture: A rat embryonic cardiomyocyte cell line H9c2 was purchased through American type culture collection (ATCC, Cat. No. CRL-1446). Cells were cultured in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 g mL^{-1} streptomycin and grown up to 75-80% confluence before being used for experiments.

Position-Ablation-Laser Microdissection (P.A.L.M. Technology): The P.A.L.M. technology was used to selectively excise rat cardiomyocytes for further use in down-stream applications as described below. After H & E staining on ice, each P.A.L.M. MembraneSlide was transferred individually to the P.A.L.M.® MicroBeam System (P.A.L.M. Microlaser Technologies AG, Bernried, Germany) for micro-dissection and pressure catapulting. Micro-dissection was used to collect cell clusters containing an average of 100 cardiomyocytes exclusively by catapulting cells into a collecting cap above the slide. To preserve RNA upon catapulting of selected cells the collecting cap was preloaded with 40 L of RLT buffer (Cat.No. 79216, QIAGEN, Valencia, CA). Once approximately 2,000 cells were catapulted, the cap was placed back into its original micro-vial and stored on ice for RNA extraction and further analysis.

RNA Extraction: RNA from laser-excised cardiomyocytes as well as H9c2 cells (75% confluency) was isolated using the RNeasy® Micro Kit 50 (Cat.No. 74004, QIAGEN, Valencia, CA) following manufacturer's directions. RNA yield and integrity were assessed using a NanoDrop Spectrophotometer (NanoDrop, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) respectively.

Identification and cloning of rBD1: To begin our identification of rat heart BD1, specific primers were designed using the cDNA sequence of the previously reported rat kidney β-defensin (GenBank No. AF093536): forward, 5'-TCT CTg CAC TCT ggA CCC TgA CTT-3' and reverse, 5'-CAA ACC ACT gTC AAC TCC TgC AAC-3'. Using the designed primers and total RNA (from the fetal and laser-excised cardiomyocytes) as template, a one-step RT-PCR reaction was performed using 2 µg of total RNA purified from the cultured cells and micro-dissected cardiomyocytes respectively (OneStep RT-PCR Kit (25) Cat.No. 210210, QIAGEN, Valencia, CA). After electrophoresis of the PCR products on an agarose gel, a single band corresponding to approximately 300 bp was excised from the gel and the DNA isolated and cloned using the TA Cloning® Kit (Cat.No. K2000-01

Invitrogen, Carlsbad, CA) and sequenced with an ABI 3700 DNA Analyzer at the K-State Sequencing and Genotyping Facility (Manhattan, KS). The correct sequence for the cloned product was verified by nucleotide sequencing. The efficiency of the RT procedure was standardized by assurance of comparable levels of the house-keeping gene GAPDH, amplified with PCR.

Three-dimensional structural analysis of rBD1: A structural model was generated for the rat BD via homology modeling. The target structure was predicted via alignment to a set of three human BD templates (isoforms 1,2 and 3 respectively) for which experimental structural characterization was available[19,22]. The rat BD sequence was aligned to the human templates via the CLUSTAL-W program[23], using the Blosum 30 substitution matrix, a gap-opening penalty of 10 and a gap-extension penalty of 0.1. The resulting alignment and the corresponding three-dimensional human BD peptide structures were then processed via the Modeller program[24] to yield a structural prediction for the rBD1 target. Modeller's default simulated annealing cycles were used for structural refinement. Analysis of the peptide secondary structure and surface characteristics was carried out on the resulting structure via SYBYL (SYBYL 6.9.2, The Tripos Associates, 2004, St. Louis, MO).

RESULTS AND DISCUSSION

Expression of rBD-1 in laser-excised cardiomyocytes and H9c2 cells: Expression of rBD1 in laser-excised normal rat cardiomyocytes as well as fetal rat heart myoblasts (H9c2) was confirmed by a single 318 bp transcript identified by gel electrophoresis on the RT-PCR products (Fig. 1). The nucleotide sequencing results from the rBD-1 amplicon are shown in Fig. 2.

Comparison of heart rBD-1 with beta-defensins from other species: The open reading frame of the cardiac rBD cDNA was translated into a putative peptide with 69 amino acid residues: MKTHYFLLVMLFFLFSQM ELGAGILTSLGRRTDQYRCLQNGGFCLRSSCPSH TKLQGTCKPDKPNCCRS. Using CLUSTAL-W, the predicted amino acid sequence of the rat heart BD1 peptide was aligned to five BDs from other species. The cardiac rBD1 displayed the strongest identity with the renal rBD1 (99%), mouse (88%), human (53%), gorilla (53%) and Rhesus monkey (50%) BDs (Fig. 3).

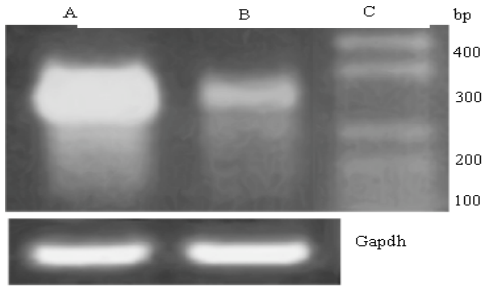

Fig. 1: Tissue expression of rBD1 mRNA. 2 µg of total RNA were subject to RT-PCR and 10 1 of 40-cycle aplicons were separated on 2% agarose gel and stained with ethidium bromide. RT-Expected products had a 318 bp in size. Laser-excised cardiomyocytes (line A), and fetal rat heart myoblasts; H9c2 (line B)

```
HrBD1          ----------TCTCTGCACTCTGGACCCTGACTTCACCGACATGAAAACTCATTACTTT 49
rBD1           GTCTCACGTCCTCTCTGCACTCTGGACCCTGACTTCACCGACATGAAAACTCATTACTTT 60
                          **************************************************

HrBD1          CTCCTGGTGATGTTATTTTTTCTCTTCTCCCAGATGGAGCTGGGTGCTGGCATTCTCACA 109
rBD1           CTCCTGGTGATGTTATTTTTTCTCTTCTCCCAGATGGAGCTGGGTGCTGGCATTCTCACA 120
               ************************************************************

HrBD1          AGTCTTGGACGCAGAACAGATCAATACCGATGCCTCCAAAATGGAGGATTCTGTCTCCGC 169
rBD1           AGTCTTGGACGCAGAACAGATCAATACCGATGCCTCCAAAATGGAGGATTCTGTCTCCGC 180
               ************************************************************

HrBD1          TCCAGCTGCCCATCTCATACCAAACTACAAGGAACATGTAAACCAGATAAGCCCAACTGT 228
rBD1           TCCAGCTGCCCATCTCATACCAAACTACAAGGAACATGTAAACCAGATAAGCCCAACTGT 240
               ************************************************************

HrBD1          TGCAGGAGTTGACAGTGGTTTG---------------------------------------- 250
rBD1           TGCAGGAGTTGACAGTGGTTTGAAGAATGGACATAAAGGACAAAGCAAGGGATTGTAAAA 300
               **********************

HrBD1          -
rBD1           T 301
```

Fig. 2: Full length cardiac rBD1 cDNA sequence showing the start and stop codons (bold and underlined). The cDNA sequence displayed a 99% homology with the BD previously described in rat kidney[15]

```
HrBD1     36 RCLQNGGFCLRSSCPSHTKLQGTCKPDKPNCCRS 69 Identity
rBD1      36 RCLQNGGFCLRSSCPSHTKLQGTCKPDKPNCCRS 69     100
dBD1      36 KCLQHGGFCLRSSCPSNTKLQGTCKPDKPNCCKS 69      88
DEFB-1    37 NCVSSGGQCLYSACPIFTKIQGTCYRGKAKCCK~ 68      53
DEF1-lp   37 NCVSSGGQCLYSACPIFTKIQGTCYGGKAKCCK~ 68      53
bD1       37 NCVRSGGQCLYSACPIYTRIQGTCYHGKAKCCK~ 68      50
```

Fig. 3: Amino acid alignment of the defensin-domain regions of the heart rat beta defensin (HrBD1) with other known beta-defensins. The conservative cysteine residues are framed. GenBank accession number: rBD1 (rat): AF093536, dBD1 (mouse): BC024380, DEF1 (Homo sapiens): BC047677, DEF1-like protein (gorilla): AAK61461, bD-1 (Macaca mulatta): AAK26258.

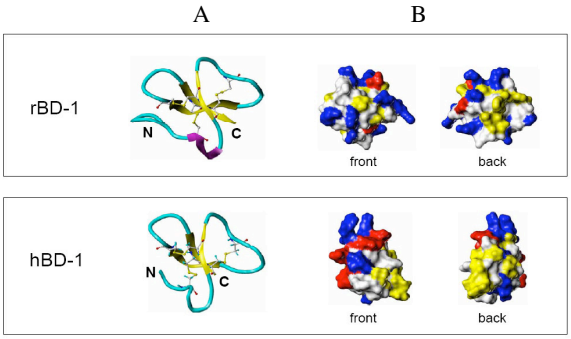

Fig. 4: Secondary structure assignment and surface analysis for rBD1 and hBD1. (A) Ribbon diagrams showing alpha-helix (purple), beta-sheet (yellow), and coil (turquoise) secondary structure elements for the two peptides. (B) Front and back views of peptide solvent accessible surfaces. Acidic (anionic) residues are red, basic (cationic) residues are blue, and hydrophobic residues are yellow.

Three-dimensional structural analysis of rBD-1: The structure of rBD1 (Fig. 4) was predicted through alignment with three NMR solution structures of human beta defensins 1-3 (PDB IDs: 1KJ5, 1FD4 and 1KJ6 respectively). The key six bridging cysteine motif was found to be conserved in the sequence alignment of rat and human defensins and no alignment gaps were observed in the core region, thus suggesting similar three-dimensional packing in all cases. In terms of functionality, the rat structure was found to be much more similar to hBD1 (53% identical, 86% homologous over the core 36 aligned residues) compared to either hBD2 (35% identical, 59% homologous over 37 core residues) or hBD3 (32% identical, 59% homologous over 37 residues). Within the core region, rBD1 and hBD1 displayed the same charge (+4 at pH = 7.0) comparable to that of hBD2 (+5), but significantly less than that of hBD3 (+8). rBD1 displayed a significantly greater number of amphoteric residues (6 histidines, asparagines and glutamines) than any of the human analogs (4 for hBD1, 2 each for hBD2 and hBD3). In place of these extra amphoteric residues were additional hydroxyl amino acids in the case of hBD1, nonpolar and hydrophobic species for hBD2 and bases for hBD3.

Innate immunity and inflammation have become central themes of investigations into a range of different disease processes, including common cardiovascular pathologies such as atherosclerosis and chronic heart failure[16]. Continual identification of specific effector molecules and immunological pathways potentially contributing to the development of such ailments is therefore indicated. The classical perception of the immune system as a complex but somewhat fixed entity of specialized organs, vessels and cells reacting in response to pathogens only has changed. A modernized view recognizes danger-more than foreignness-as the actual trigger mechanism of immunity, while also acknowledging the importance of non-immune cells and organs in host defense[9,10].

The heart possesses a germ-line encoded innate stress system, which is activated in response to different types of injury[6]. Recent studies have focused on the role of individual cytokines in development of cardiac disease and heart failure[7]. Attention has also been directed at identifying different toll-like receptors (TLRs) in the heart and their potential implication in cardiovascular pathology[17]. TLRs are classical pattern recognition receptors utilizing NFκB-signaling-a shared pathway for induction of pro-inflammatory cytokines as well as BDs[3,18]. BDs play a significant role in host defense and some are up-regulated in disease[1,3]. No studies have, however, focused on specifically identifying the expression pattern of heart BDs and their potential role in cardiac health and disease.

Our findings demonstrate the specific expression of a classically known epithelium-derived natural antibiotic peptide (β-defensin) in non-epithelial cells (rat cardiomyocytes). By using the P.A.L.M. MicroBeam System, we were able to specifically excise clusters of cardiomyocytes in a non-contact, non-heat-producing fashion. This allowed us to perform gene expression studies on one specific cell type from the rat heart. The local expression of cardiac BDs may play a central role within an intrinsic innate immune response of the heart. Contextual studies are warranted to investigate the actual role of beta-defensins in cardiomyocytes further. It may be speculated that cardiac BDs act as a second line of defense within the framework of an innate immune response providing cardiomyocytes with a self-preserving mechanism when the immediate protecting barriers of the endothelial lining become disintegrated by damage. The presence of at least one BD isoform in cardiomyocytes further supports the notation that the heart is capable of producing innate effector molecules locally, as has previously been reported for certain cytokines.

Different BD isoforms have been identified within single organs[1] and our preliminary data indicate that different BD isoforms are also present in the rat myocardium. Generally, the BD-1 isoform is known as a more ubiquitous and constitutively expressed isoform compared to other known BDs[19]. The antimicrobial activity of this isoform is, however, potentially limited as it is not inducible by LPS[19-21]. It is possible that

rBD1 act primarily as a local myocardial storage that can be mobilized and transformed into other BD isoforms with higher antimicrobial activities. A higher number of amphoteric sites in the rBD1 compared to hBDs would on the other hand indicate a strong pH functional dependence in rat relative to the human counterparts. Studies focused on tissues other than heart have shown that rBD1 is not directly up-regulated by the presence of certain bacterial components[20,21]. It has furthermore been suggested, that over-expression of rBD1 may play a role in diabetic nephropathy in rats[15]. Based on the structural analysis of rBD1 it may, however, be hypothesized that the acid-base status in tissues may play a central role for the functional properties of this antimicrobial peptide. Alternatively, this isoform may possess other yet to be identified specific functional characteristics of potential significance in cardioprotection against non-infectious insults.

The present study is the first to not only identify a classical epithelial BD (rBD1) in cardiomyocytes from rat specifically isolated using position-ablation-laser-microdissection, but also to demonstrate BD expression in embryonic cardiac tissue from rat. Investigation of BD expression in laser-excised cardiomyocytes is novel as it virtually eliminates the potential cross-contamination from other types of cells within the heart. Further investigations are warranted to identify the potential significance of cardiac BDs in heart disease.

ACKNOWLEDGEMENTS

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Cardiac Beta-Defensins: Novel Antimicrobial and Immunomodulatory Host Defense Peptides with Enhanced Expression in Inflammation

Annika Linde, Christopher Ross, Frank Blecha, Gerald Lushington,
Amy Hanson, Lea Dib, Tonatiuh Melgarejo

Abstract – Beta-defensins belong to the ubiquitous group of naturally occurring host defense peptides (HDPs), which cover a breadth of functions including antimicrobial, chemotactic, anti-carcinogenic and tissue healing properties. In mammals, three sub-groups of defensins are known (alpha, beta and theta-defensins). Beta-defensin expression in whole heart homogenate has been reported in different species, and plasma alpha-defensins have moreover been associated with cardiovascular morbidity and mortality. Still, the role of defensins in cardiac physiology remains widely unknown. Inflammation plays a central role in the development of cardiovascular pathology, and furthering the understanding of key-elements in innate immunity will expectedly lead the way for novel interventional strategies to address heart disease. Here we show that a subset of rat beta-defensins (rBDs) is constitutively expressed in the myocardium. We moreover establish that the gene-expression level of these cardiac rBDs is influenced by systemic exposure to known inflammatory mediators. Additionally, we show that a synthetic analogue of a select rBD peptide has antimicrobial activity. Finally, we present data indicating that select rBDs exhibit cell migratory activity towards circulatory cells. Combined, the results suggest that an intrinsic myocardial host defense peptide response involving rBDs is activated secondary to systemic inflammatory mediators possibly acting as a myocardial "first-line-of-defense" against danger signals of non-infectious as well as infectious origin.

Key Words: defensins, inflammation, heart

The article (incl. 11 pages, 2 tables and 8 figures) is currently pending submission and has been included as a general abstract only in this version. Once published, the full-length manuscript will be available from the author upon request.

Chapter 4
Innate Immunity in the Light of Evolution

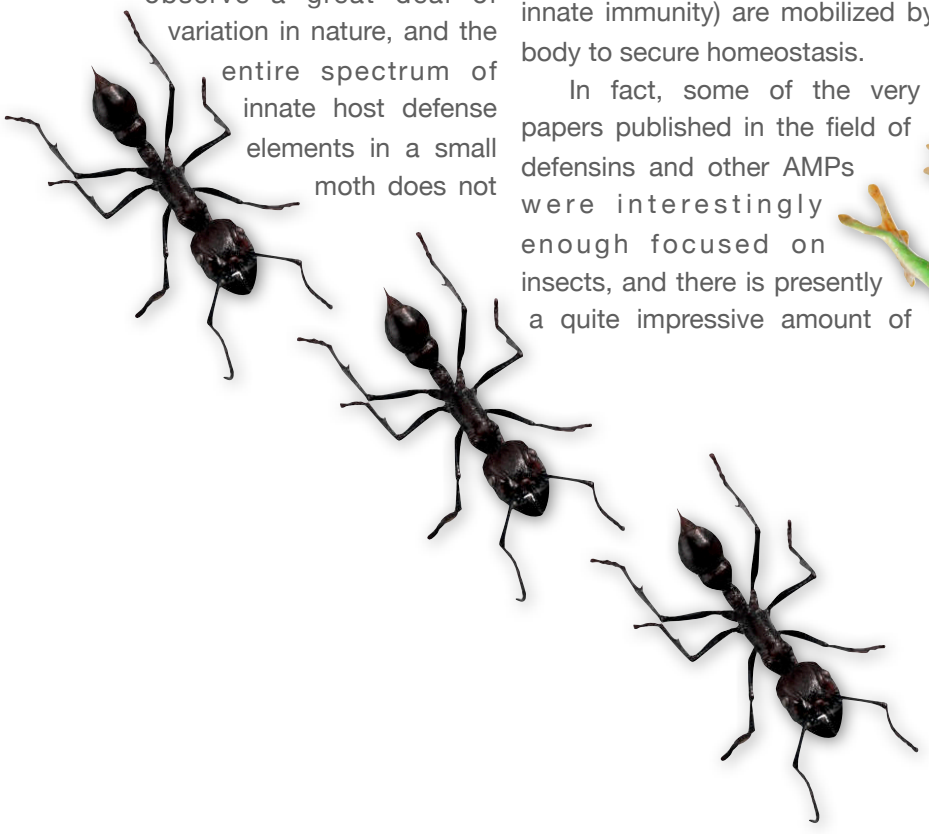
The distribution of HDPs in nature is rather ubiquitous, and it is probably safe to say that all species alive today - to one extent or the other - relies on innate immune host defense peptides to keep pathogens at bay. The central point to be made - and perhaps also the main reason for why "evolutionary immunology" as a topic caught our group's attention in the first place - is the fact that even though HDPs have been around for a very long time, they are to a large extent still conserved across different species.

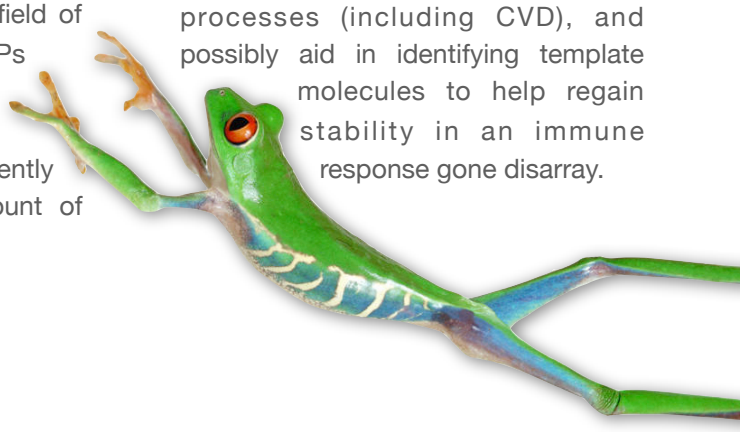
Yes, we are of course able to observe a great deal of variation in nature, and the entire spectrum of innate host defense elements in a small moth does not necessarily match that of an elephant perfectly. Nevertheless, the interesting part is that the core of innate immunity is maintained - and the most logical answer as to why that is, would in essence be, that it is simply a quite efficient defense system.

HDPs, in their very nature, do not possess the same extent of specificity when compared to what acquired immunity can mobilize. However, the key to why we all - for the most part at least - in fact manage to stay healthy lies within the potency of and immediateness with which HDPs (in combination with other elements of innate immunity) are mobilized by the body to secure homeostasis.

In fact, some of the very first papers published in the field of defensins and other AMPs were interestingly enough focused on insects, and there is presently a quite impressive amount of scientific articles on HDPs in various "special species" - ranging from a plethora of exotic frogs, to the king penguin, terrestrial salamander, hagfish, etc.

The paper which follows, ***"Natural History of Innate Host Defense Peptides: A Travel through Evolution in Search of Natural Antibiotics"***, has been submitted to a suitable journal in biology, and is currently in review. It is plausible, that some of the most "successful species" (from an evolutionary standpoint) possess HDPs with both unique potency and biological activity. If confirmed, this might help further our understanding of inflammatory processes (including CVD), and possibly aid in identifying template molecules to help regain stability in an immune response gone disarray.





MINI REVIEW

Natural History of Innate Host Defense Peptides: A Travel through Evolution in Search of Natural Antibiotics

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Abstract

Host defense peptides act on the forefront of innate immunity, thus playing a central role in the overall survival of animals and plants. Despite vast morphological changes in species through the evolutionary history, all animals examined to date share common features in their innate immune defense strategies, hereunder expression of host defense peptides (HDPs). Most experimental studies have focused on HDPs in humans domestic and laboratory animals, and close to 900 different sequences have been identified so far, while data on HDPs in wild-living animals is sparse. The biological functions of HDPs include broad-spectrum antimicrobial activity and immunomodulatory features. Natural selection and coevolutionary host-pathogen arms race theory suggest that the extent and specificity of the microbial load influences the spectrum and potency of HDPs in different species. It is therefore likely, that individuals of extant species that have lived for an extended period in evolutionary history and are still living in populations where processes of natural selection are intact possess the most powerful and well-adapted “natural antibiotic peptides”. Research on the evolutionary history of the innate defense system and the host in context of the consequences of challenges and the efficacy of the innate immune system under natural conditions is therefore of interest. This review focuses on evolutionary aspects of immunophysiology with specific emphasis on the effector molecules of innate immunity. Studies on host defense in wild-living animals may significantly enhance our current understanding of inborn immune mechanisms, and ultimately help identify molecules that may assist us to cope better with the increasing microbial challenges.

Keywords: innate immunity, defensins, cathelicidins, comparative genomics, vertebrate evolution,

The article (incl. 10 pages and 5 figures) is currently in review and has been included as an abstract only in this version. Once published, the full-length manuscript will be available from the author upon request.

Summary
What did we learn & where do we go from here?

The challenge of basic research is that it at times can feel a bit like opening a can of worms. Reality is, how do we keep track of what is actual "novel discoveries", and what is merely "background-noise" - and how do we ensure that what our statistical software program so effortlessly has declared "significant" is in fact also biological "relevant"?

The trend in biomedical research seems to be heading increasingly in the direction of seeking collaborations across different fields within the sciences - which is a positive step.

At the end of the day, this approach is likely the best route by which we can appropriately obtain and interpret scientific data. The world of science is becoming increasingly "specialized", and I am among the ones who have taken advantage of this - and very pleased I did. The last five years or so have taught me many valuable lessons about basic research - an awareness I would not have been able to obtain had I continued directly on clinics. So, did I miss out on anything in the process. Sure - but that's only to be expected, and being able to view problems from a clinical as well as a basic research aspect is for me a very valuable tool.

So, what **did** we actually learn? Simply put, the data appears in favor of our working hypothesis - the heart does seem to up-regulate its' local production of HDPs in response to inflammation, and synthetic analogues of the peptides exhibit activity *in vitro*, It is reasonable to think that the myocardium utilizes these peptides *in vivo* also. However, much work still lies ahead, before we can make more definite statements in regard to the putative biological role of cardiac beta-defensins in heart health.

"If we knew what it was we were doing, it would not be called research, would it?"

Albert Einstein

Appendix A

Bio Sketch

Annika Linde, DVM

Annika Linde *- born in Copenhagen Denmark. Conducted undergraduate studies with a focus on mathematics, biology and chemistry. Received a doctorate in veterinary medicine (DVM) in 1999 from the* ***University of Copenhagen*** *(RVAU), and finished a Master's thesis on canine heart diseases, followed by an internship in small animal internal medicine. Relocated to the USA in 2000, and worked in a cardiology residency, followed by a cardiology lectureship at the* ***University of Pennsylvania****, Philadelphia until 2003. Hired in 2003 as a DeBakey Scholar in cardiac physiology at* ***Texas A&M University****, and has since 2004 been focusing on basic research in the field of cardiovascular immunology, working towards a PhD in Anatomy & Physiology at* ***Kansas State University****. Research interests include studies on the role of innate immune factors in cardiovascular disease processes - specifically host defense peptides and Toll-like receptors. Clinical research projects have included studies on congenital cardiac defects in dogs, cardiac imaging in special species, and cardiac biomarkers in dogs with pericardial effusion. Professional member of the American Heart Association, International Society for Heart Research, European Society of Veterinary Cardiology, and the Phi Kappa Phi Honor Society. Has authored several review and original articles, scientific abstracts and proceedings in the field of veterinary cardiology & comparative cardiovascular immunophysiology, and has given presentations in national and international forums on different topics in cardiology.*

Appendix B
Letters of Permission

Letters of permission to include the published works are attached from the editors of the relevant journals, including *American Journal of Veterinary and Animal Sciences*, *Cardiovascular Research*, and *Journal of Veterinary Internal Medicine*.

Please note that the remaining two manuscripts are in review for publication, and are included in abstract format only in this version to avoid any risk of copyright infringement. Once published, the full-length manuscripts will be available from the author.

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To Whom It May Concern:

I am currently in the process of finalizing my PhD studies at Kansas State University and am writing for permission to include in my dissertation the article titled: Innate Immunity and Host Defense Peptides in Veterinary Medicine, by A. Linde, C.R. Ross, E.G. Davis, L. Dib, F. Blecha, and T. Melgarejo published in J Vet Intern Med 2008; 22:247–265.

The PhD dissertation will be made available online through the K-State Research Exchange (http://krex.ksu.edu), and will be microfilmed by UMI/ProQuest Information and Learning.

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Kind Regards,

Annika Linde, DVM
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Date: Wed, 9 Apr 2008 09:37:59 +0100
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To: "Dr. Annika Linde" <alinde@ksu.edu>
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-----Original Message-----
From: Dr. Annika Linde [mailto:alinde@ksu.edu]
Sent: 08 April 2008 22:23
To: Journals Rights
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To Whom It May Concern:

I am currently in the process of finalizing my PhD studies at Kansas State University and am writing for permission to include in my dissertation the article titled: Innate Immunity and Host Defense Peptides in Veterinary Medicine, by A. Linde, C.R. Ross, E.G. Davis, L. Dib, F. Blecha, and T. Melgarejo published in J Vet Intern Med 2008; 22:247-265.

The PhD dissertation will be made available online through the K-State Research Exchange (http://krex.ksu.edu), and will be microfilmed by UMI/ProQuest Information and Learning.

I would appreciate if you can provide me with a signed letter granting me permission to use the work. Please forward letter of permission to the contact information listed below.

Thank you for your assistance in this matter.

Kind Regards,

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Date: Thu, 14 Feb 2008 17:23:20 -0700
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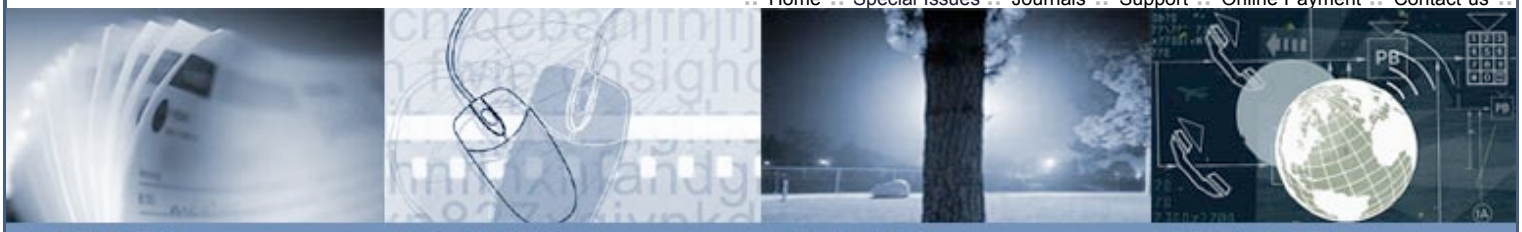

2008-02-14
Dr. Annika Linde

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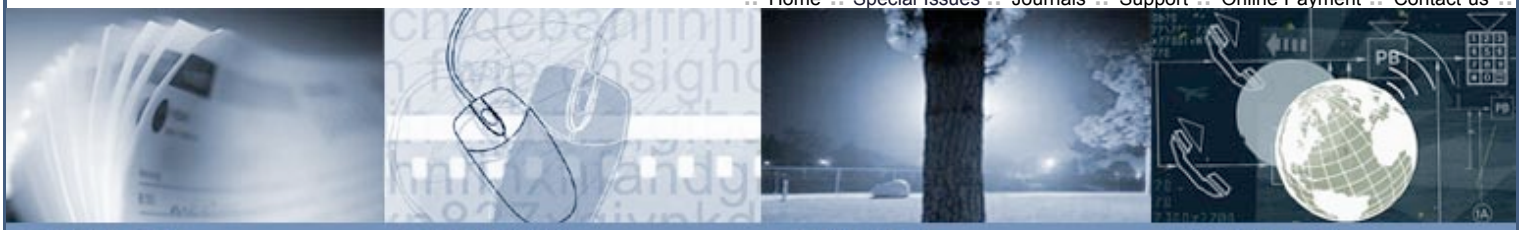

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Dr. Annika Linde

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