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IMMUNOMODULATORY EFFECTS OF ANTI-MICROBIAL PEPTIDES

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Anti-microbial peptides (AMPs) were originally thought to exert protecting actions against bacterial infection by disintegrating bacterial membranes. Upon identification of internal bacterial targets, the view changed and moved toward inhibition of prokaryote-specific biochemical processes. However, the level of none of these activities can explain the robust efficacy of some of these peptides in animal models of systemic and cutaneous infections. A rapidly growing panel of reports suggests that AMPs, now called host-defense peptides (HDPs), act through activating the immune system of the host. This includes recruitment and activation of macrophages and mast cells, inducing chemokine production and altering NF-κB signaling processes. As a result, both pro- and anti-inflammatory responses are elevated together with activation of innate and adaptive immunity mechanisms, wound healing, and apoptosis. HDPs sterilize the systemic circulation and local injury sites significantly more efficiently than pure single-endpoint *in vitro* microbiological or biochemical data would suggest and actively aid recovering from tissue damage after or even without bacterial infections. However, the multiple and, often opposing, immunomodulatory functions of HDPs require exceptional care in therapeutic considerations.

Keywords: angiogenesis, anti-inflammatory responses, chemokines, macrophages, wound healing

Introduction

Anti-microbial peptides (AMPs) are among the first line defense mechanisms of animals [1]. These gene-encoded 10–50 amino acid residue polyamides are either strategically deployed at barrier sites where bacterial infection can occur such as the skin and epithelial mucosa in mammals or rapidly delivered to the injury and infection sites in response of invading microorganisms [2]. The two major AMP families in humans are defensins and cathelicidins [3], both produced

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by neutrophils and epithelial cells and indeed share similar roles in lung and other tissue immunity [4]. In support, administration of a human neutrophil-derived defensin reduces bacterial load in the infected peritoneal cavity 24 h after infection in an experimental *Klebsiella pneumoniae* infection in mice [5]. Likewise, when administrating post-*Francisella tularensis* infection, the cathelicidin-derived peptide LL-37 protects against respiratory tularemia in a murine model [6]. As opposed to the mammalian immune system that activates both innate and adaptive immunities, lower animals such as insects rely almost solely on a panel of AMP against bacteria in environments where these pathogens strive and are abundant [7]. Their importance gained widespread recognition in 2011, when Jules Hoffmann received the Nobel Prize in physiology or medicine for his pioneering work on innate immunity mechanisms, including identification of AMP from insects and discovery of the genetic background of their synthesis [8]. Unfortunately neither native drosocin [9] nor pyrrhocoricin [10] that the Hoffmann group isolated from insects after pricking them with *Escherichia coli* shows efficacy against experimental *E. coli* infection in higher animals, such as mice [11]. Thus, it must be something else than direct killing of microbes that make mammalian-derived peptides active and insect-derived peptides inactive in animal models of bacterial infections.

Most AMPs are cationic at pH = 7, i.e., their sequence contains positively charged residues, arginine, lysine, and histidine, in excess of the negatively charged aspartic and glutamic acids. It appeared quite logical that the first explanation of their mode of action was accumulation on the negatively charged bacterial membrane surface up to a threshold level leading to membrane permeation/disintegration [12]. Apparently, the fine details of the membrane–peptide interaction are dependent upon the specifics of both players of the association, as while magainin, a frog skin AMP, lies in the plane of the bi-layer; M2 delta, a helical segment of the nicotinic acetylcholine receptor, spans the membrane, perpendicular to the bi-layer plane [13]. It slowly became clear that the biophysical assumptions of neither of the two main membrane-perturbing models (barrel-stave and Shai–Matsuzaki–Huang carpet) are reliable for reaching structure–function conclusions [14]. The last push for alternative modes of action came after the observations that many AMPs fail to modulate membrane permeability at their microbiologically active concentration [15].

Since the discovery of magainins, AMPs were considered viable drug candidates and entered many clinical trials. Pexiganan, a synthetic 22-amino acid version of magainin-2, shows activity in healing of diabetic foot ulcer wounds in Phase-III trials [16]; omiganan, a bovine peptide origin AMP, was in Phase-II clinical trials against mild-to-moderate acne [17], and novexatin, a cationic cyclic heptapeptide, is close to registration as a topical treatment for fungal infections of

the toenail [18]. The Holy Grail of all drug development platforms is finding a novel biopolymer target, specific for the given disease, in our case for bacterial survival, virulence or toxicity. In that regard, newly discovered activities of AMP include inhibition of RNA [19] or protein synthesis [20], attenuation of protein folding [21] or lipid complexation [22], and activation of microbial autolytic systems [23] or deactivation of bacterial toxins [24]. While these activities can explain some positive *in vivo* results that bacterial membrane disintegration cannot, they are unable to account for the success of many systemic and local infection treatments in the absence of *in vitro* killing of bacteria or inactivating the newly recognized target proteins.

The complexity of the effects of AMP in infection and inflammation suggests that these biopolymers play a strong role in activating the immune system of the hosts [25]. Just a single recent review lists many major functions for AMPs, such as direct anti-microbial effects, clearance of endotoxins, chemotaxis, modification of anti- and pro-inflammatory immune responses, wound healing and tissue repair, angiogenesis, and apoptosis [26]. Historically, lactoferricin analogs were found first to protect mice from infection through both anti-microbial activity and immunostimulatory effects [27]. The best example of the cooperation between microbiological and immunological effects came when a hyaluronic acid-binding peptide, preventing staphylococcal infections, was considered an immunological therapeutic agent rather than an antibiotic in spite of its sequence demonstrating striking similarity to cationic AMP [28]. Later research on AMPs was extended to activation of macrophage activity [29] and upregulation of anti-inflammatory cytokine production [30]. Because the true role of cationic peptides is to enhance chemotaxis or growth factor activation, it was suggested that these peptides were misclassified as anti-microbials [31]. Their newly coined name is host-defense peptides (HDPs) reflecting their complex functions in inflammatory responses, chemoattraction, cellular differentiation, and activation of the innate and adaptive immune systems [32]. Further activities, including angiogenesis, culminate in effects as far as adjuvant activities in animal models of both bacterial and viral infections [33]. This review intends to update the view on HDPs with the most important strategic results and concepts on the various immunomodulatory activities from the past few years.

Wide Spectrum of Immune Stimulatory Peptides

Cathelicidins and defensins activate a long series of receptors, signaling pathways, and transcription factors [34]. Some of the effects of the cathelicidin family of HDP will be detailed later. β-Defensin 131 enhances the expression of

cytokines IL-1, IL-6, and IL-12, as well as chemokines CCL20, CCL22, and CXL8 in human prostate cancer cells upon concomitant lipoteichoic acid treatment [35]. These activities proceed through the TLR2/NF-κB signaling pathway promoting strong immune responses in a cellular environment characteristic to gram-positive bacterial infections. β-Defensins are chemotactic for immature monocytes and memory T cells [36]. Human β-defensin-3 activates monocytes through both CD86 and CD80 upregulation signaling suggesting that autocrine activation of the ionotropic P2X7 receptor plays an important role in shaping the inflammatory microenvironment [37]. β-Defensins activate primary macrophages along with enhancing pro-inflammatory responses to support inflammatory responses initiated by TLR ligands [38].

Apidaecin is a honey bee originated proline–arginine-rich peptide [7]. At high concentrations, apidaecin upregulates the production of the T-cell co-stimulatory molecule CD80 as well as cytokines and chemokines in macrophages but not in monocytes [39]. Low concentrations (5–10 μM) of apidaecin partially inhibit lipopolysaccharide (LPS)-induced increase in class II MHC, and CD86 and release of IL-6 and in TNF-α in macrophages. Interestingly, at low concentrations, the peptide also inhibits IL-6, TNF-α, FGF, and eotaxin in monocytes. A derivative of apidaecin, Api88, also inhibits LPS-induced TNF-α production in a concentration-dependent manner in both monocytes and mast cells, although resting monocytes do not respond to Api88 [40]. Api88 does not induce migration or affects the phagocytic activity of monocytes. To add to the complexity of the issue, another insect-derived AMP, the cecropin-derived peptide HB-107, lacks anti-microbial activity but, when applied to wounds in mice, induces leukocyte infiltration and keratinocyte hyperplasia [41]. In addition, HB-107 stimulates IL-8 secretion from cultured endothelial cells, an effect that may explain the increase in leukocyte migration.

The frog antibiotic-based cationic peptide Tiger17 is one of the smallest HDPs containing only 11 amino acid residues [42]. Tiger17 promotes remarkable levels of wound healing in a mouse full-thickness skin injury model. The peptide recruits macrophages to the wound site during the inflammatory reaction stage, activates mitogen-activated protein kinase signaling pathways, aids tissue formation and tissue remodeling through the release of transforming TGF-β1 and IL-6 in macrophages, and of significance to the previous paragraph, promotes migration and proliferation of both keratinocytes and fibroblasts leading to re-epithelialization and granulation.

Dairy products and milk contain a large number of bioactive peptides many of which exhibit extra-nutritional functions. As evidenced by lactoferricin, in addition to the frequently cited anti-oxidant and anti-hypertension activities, these peptides do possess immunomodulatory, anti-inflammatory, and anti-microbial

activities [43]. Here, the sequence diversity moves from cationic peptides to downright negatively changed molecules and peptides with non-natural amino acid residues. While the main function of casein phosphopeptides is transporting calcium, they may also enhance its bioaccessibility [44]. Toothpaste made up of casein phosphopeptides and amorphous calcium can prevent tooth demineralization and improve enamel remineralization [45]. The question remains unsolved if the anti-cariogenic, cytomodulatory, and immunomodulatory effects of these peptides are related or these are independent, sequence-specific events. Another peptide containing post-translationally modified residues is nisin that has been approved by the US Food and Drug Administration for clinical use [46]. Nisin is a lantibiotic produced by *Lactococcus lactis* and is primarily used as a food preservative. Originally considered only as an antibiotic, new data document that nisin activates human polymorphonuclear neutrophils *in vitro* and additionally elevates neutrophil intracellular superoxide levels [47]. Nisin induces responses in both adaptive and innate immunities leading to cell death.

Stimulation of the immune system can be achieved with significantly lower drug doses than anti-microbial chemotherapy. When indolicidin, a bovine neutrophil-originated HDP containing 13 amino acid residues, was coupled to carbon nanotubes or gold nanoparticles, the resulting nanostructures elicited similar levels of stimulation of immune gene expression and protection from bacterial challenge at a 1000-fold less concentration than the free peptide [48]. Likewise, the *in vivo* anti-bacterial effects of the designer Chex-Arg20 proline–arginine-rich peptide are more extensive when embedded in a nanofiber formulation than after intramuscular administration using only one-tenth of the active pharmaceutical ingredient [49]. The various cutaneous and wound healing activities are likely linked to the ability of HDP to activate mast cells and enhance the production of cytokines (GM-CSF, TNF-α) and chemokines (IL-8-MCPs and MIPs) at inflammation and injury sites [50]. Along these lines, a polyelectrolyte multilayer film based on polyarginine and hyaluronic acid has a remarkable anti-microbial activity in a film format against *Staphylococcus aureus* and as well as inhibits the production of inflammatory cytokines released by human primary macrophages [51]. The polyarginine–hyaluronic acid film was embedded into a nanoscale silver coating and was further functionalized by embedding catestatin, a natural HDP. Indeed, this chimera film is efficacious as an anti-microbial coating against yeast and fungi. Self-assembling peptides can themselves assemble in nanofiber structures [52]. QL6, one of these self-assembled nanomaterial peptides, preserves spinal cord tissue upon injection into rats 24 h after spinal cord injury. The mode of action of QL6 is suggested to be a significant reduction in apoptosis, inflammation, and astrogliosis [53].

Pro- and Anti-Inflammatory Activities

As opposed to β-defensins, human α-defensins increase the expression of the pro-inflammatory cytokines TNF-α and IL-1 in human monocytes [54]. Likewise, intramuscular injection of synthetic human α-defensin into rainbow trout induces the transcript expression of genes encoding both pro-inflammatory cytokines (IL-1β, TNF-α, and IL-8) and CC chemokines (CK5B, CK6, and CK7A) [55]. CD14(–)/CD24(+) cell secreted α-defensins HNP1 (Table I) and HNP3 suppress the differentiation capabilities of monocytes [56]. This is likely a sequence-specific effect, as treatment of immortalized macrophages with a non-cationic AMP, epinecidin-1 from fish, fails to induce TNF-α production. Rather, epinecidin-1 inoculation into mice elevates the plasma level of the anti-inflammatory cytokine IL-10 [57]. Having said this, a C-terminal 15-residue fragment of human β-defensin exhibits anti-inflammatory activity in LPS-treated mouse macrophages [58]. Significantly, the hBD3-3 peptide downregulates NF-κB-dependent inflammation and, in a lung inflammation model, reduces interstitial infiltration of polymorphonuclear leukocytes. Os and Os-C are peptides derived also from the carboxy-terminal region, this time of a tick defensin [59]. Both peptides bind *E. coli* LPS and inhibit LPS/IFN-γ-induced NO and TNF production in murine macrophages identical to those used in the previous paragraph. In addition, they exhibit anti-endotoxin activities and protect macrophages from oxidative damage. Neutralization of LPS appears to be a cornerstone of the anti-inflammatory actions of HDP [60]. Production of inflammatory cytokines is inhibited by cationic peptides via neutralizing circulating LPS. These activities come on top of the promotion of immune cell proliferation, angiogenesis, and epithelialization.

Synthetic granulysin-derived peptides kill *Propionibacterium acne*s in human microcomedone preparations and show anti-inflammatory effects [61]. They suppress *P. acnes*-stimulated cytokine release that suggests their utility as topical therapeutic agents and competitors of current acne therapies. In other peritoneal macrophage and peripheral blood mononuclear cells (PBMC) models, HDPs induce both pro-inflammatory and anti-inflammatory cytokines indicating that clinical applications as anti-inflammatory, immunosuppressive, and immunostimulatory agents are possible [62]. Talactoferrin-α is a recombinant form of human lactoferrin, which has been shown to have a wide range of effects on the immune system [63]. In mice, talactoferrin enhances the production of key repair inflammatory mediators IL-8, IL-6, macrophage inflammatory protein-1α, and TNF-α [64]. Although talactoferrin failed in sepsis therapy, due to its ability to control, inflammation is currently being investigated in cancer studies [65]. A 20-residue N-terminal fragment of porcine lactoferrin prevents LPS-induced

Table I. Selected host-defense peptide families with immunomodulatory and inflammatory activities

Peptide name	Sequence	Origin	Typical immune response modulation	Role in inflammation
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	Cathelin	Macrophage activation	Either pro- or anti-inflammatory
IDR-1018	VRLIVAVRIWRR	Innate effectors	Monocyte chemotaxis	Anti-inflammatory
Pep19-2.5	GCKKYRRFRWKFKGKFWFWG	Synthetic anti-LPS	Not studied extensively	Anti-inflammatory
A3-APO	(Chex-RPDKPRPYLPRPRPPRPVR)$_2$-Dab	Designer proline–arginine rich	Macrophage activation	Anti-inflammatory
Human α-defensin	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	Human neutrophils	Inhibition of monocyte differentiation	Pro-inflammatory

infiltration of macrophages and leukocytes and reduces inflammation markers a well as IL-6, TNF-α, and IFN-γ levels in a porcine epithelial cell line [66]. By altering the NF-κB signaling pathway, the peptide downregulates the inflammatory response and tight junction structure impairment that occurs after exposure to LPS.

AMP can also induce chemokines and anti- or pro-inflammatory cytokines in the oral mucosa and such should be considered in the early events of oral inflammation [67]. The major allergen of house dust mite can induce pro-inflammatory cytokine production contributing to airway inflammation and allergic asthma [68]. A short, weakly cationic cell penetrating peptide derived from human eosinophil cationic proteins significantly upregulates the production of pro-inflammatory cytokines IL-1β, IL-6, and IL-8 in stimulated PBMC obtained from house dust mite allergic patients [69]. The upregulation of caspase-1 upon inflammasome activation in monocytes can explain why allergens induce chronic airway inflammation that can lead to allergic asthma. Apparently, the immuno-modulatory effect of the peptide proceeds through upregulation of IFN-α production and not through induction of autophagy and has the potential to be developed as a new anti-inflammatory agent for asthma therapy.

The amino acid sequence of a family of designer AMP is reduced to nothing else than alternating lysine and leucine residues, sometimes interspersed with tryptophans or glutamines [70]. These meant to assume amphipathic helical conformation in membrane-mimetic solvents, although when in contact with ligands, the truthfulness of this paradigm in real-life membranes is increasingly debated [71]. WALK11.3, one of these model peptides, inhibits the expression of inflammatory mediators, including NO, COX-2, TNF-α, INF-β, IL-1β, and IL-6 in LPS-stimulated mouse macrophages [72]. The presence of the peptide attenuates TLR4-mediated pro-inflammatory signals in the TRIF-dependent pathway, while no effect on downstream signaling in the MyD88-dependent pathway is observed. Remarkably, WALK11.3 specifically inhibits TLR4 endocytosis that is essential for triggering TRIF-mediated signaling in macrophages. When another lysine–leucine peptide, LLKKK18, is released from dextrin conjugates, it accelerates wound healing in rats, due to reduced oxidative stress and inflammation as presented by low neutrophil and macrophage infiltration and pro-inflammatory cytokine levels [73]. LLKKK18 induces rapid resolution of the inflammatory stage through early M2 macrophage recruitment and stimulates angiogenesis. Yet another HDP that exhibits potent anti-inflammatory activity by inhibiting LPS-induced NO and pro-inflammatory cytokine including TNF-α, IL-6, and IL-1β production is a cathelicidin from the sea snake *Hydrophis cyanocinctus* [74]. The mode of action is reported to be competitive binding to the Toll-TLR4/MD2 complex, preventing LPS attachment and subsequent activation of LPS-induced inflammatory response pathways.

Specific Peptide Families

Human cathelicidins

Like many HDPs, the human cathelicidin LL-37 (Table I) kills bacteria only in low-ionic strength solvents, and becomes inactive when tested in tissue culture media [75]. Actually, it is in the more physiological conditions when LL-37 exhibits a wide range of immunomodulatory properties *in vitro* and these activities can be recapitulated in animal models [76]. A 24-amino acid-shortened derivative of the 37-mer LL-37 shows LPS and lipoteichoic acid neutralization activities similar to the mother peptide but induces lower levels of pro-inflammatory effects [77]. LL-37 was widely studied and showed to have multiple immunomodulatory activities, including activation of macrophages, upregulation chemokines and chemokine receptor production, and promotion of wound healing [76]. Yet another shortened version (19 amino acid residues) of LL-37 reduces cellular infiltration in joints, prevents cartilage degradation, and suppresses pro-inflammatory cytokines in arthritic mice suggesting efficacy in autoimmune and inflammatory diseases [78]. Interestingly, the bovine cathelicidin-derived peptide IDR-1018 fails to exhibit similar beneficial effects in the same murine model.

Peptide LL-37 enhances phagocytosis of non-opsonized *E. coli* by human macrophages [79]. LL-37 consistently elevates expression of FcγRs on macrophages but not the complement receptors CD11b and CD11c. In addition, the expression of TLR4 and CD14 is increased on LL-37-treated macrophages. Daily treatment of an inbred laboratory rat strain that spontaneously develops autoimmune type-1 diabetes with LL-37 results in enhanced β-cell neogenesis and upregulation of beneficial gut microbes [80]. In general, by inducing migration of neutrophils, monocytes and macrophages, eosinophils, and mast cells as well as prolonging the lifespan of neutrophils, cathelicidins directly modulate epithelial cell and keratinocyte responses to infecting pathogens [81]. Moreover, they indirectly balance TLR-mediated responses of monocytes, macrophages, dendritic cells, epithelial cells, and keratinocytes.

Exogenous LL-37 decreases TNF-α and IL-17 while inducing anti-inflammatory IL-10 and TGF-β production in a monocyte-derived macrophage cell line infected with *Mycobacterium tuberculosis* [82]. However, the decreased production of anti-inflammatory cytokines does not reduce anti-mycobacterial activity supporting the concept that LL-37 modulation of cytokines is independent of the P2X7 receptor. In a grand scheme, LL-37 controls the expression of both pro- and anti-inflammatory cytokines during infection. In the presence of physiological concentrations of cyanate, LL-37 undergoes compositional changes

yielding a variety of carbamylated peptides [83]. As small structural changes as carbamylation have diverse effects on the biological properties of LL-37, most notably conversion from anti-inflammatory to pro-inflammatory properties. The capacity of a chicken-derived cathelicidin analog to modulate chemokine production and attenuate endotoxin-induced pro-inflammatory responses in immune cells suggests that cathelicidin-based peptides can serve as leads for the design of immunomodulatory anti-infectives [84].

Due to the extensive range, and frequently opposing immunomodulatory activities, a wide spectrum of diseases is suggested to be treated with cathelicidins and other HDPs, or the opposite, these peptides can serve as targets for therapeutic interventions. Most reports recommend the utilization of HDP for wound healing therapy because of the peptides' ability to harness the mast cell defense of the hosts, as well as to activate a G-protein-coupled receptor MrgX2 without being inhibited by LPS [85]. However, the modulation of keratinocyte pro-inflammatory responses by LL-37 suggests novel roles for this peptide in skin inflammation including promotion of IL-17/IL-22/IL-6-associated psoriasis and suppression of TSLP-associated atopic dermatitis [86]. On one hand, inhibition of LPS-induced cytokine and adhesion molecule expression in human corneal fibroblasts suggests that LL-37 can promote the resolution of corneal inflammation associated with bacterial infection [87]. On the other, treatment of various brain cells with LL-37 induces secretion of inflammatory cytokines IL-1β and IL-6, chemokines IL-8 and CCL2, and other materials toxic to human neuroblastoma SH-SY5Y cells [88]. The mode of action involves activation of intracellular pro-inflammatory pathways involving phospho-P38 MAP kinase and phospho-NF-κB proteins, leading to the conclusion that LL-37 may play a role in chronic neuroinflammation that can be observed in neurodegenerative diseases including Alzheimer's disease and Parkinson's disease.

Innate defense regulators

A family of 12–13 residue peptides, vaguely resembling the C-terminal half of LL-37 variants in sequence, are named innate defense regulators (IDRs) [33]. Two of these peptides, IDR-1 and IDR-1002 do not induce pro-inflammatory cytokines in human PBMC; rather they suppress pro-inflammatory responses in animal models. IDR-1002 enhances monocyte chemotaxis toward chemokines CCL3 and CCL5 [89], a phenomenon that correlates with selective upregulation of CCR5 surface expression. In human fibroblast-like synoviocytes, IDR-1002 suppresses the production of MMP-3 induced by IL-1β and monocyte chemotactic protein-1 [90]. The change in IL-1β-induced proteome is

evident from the alteration of the expression of NF-κB and JNK pathway members.

Two other analogs, IDR-HH2 and IDR-1018, fail to kill *M. tuberculosis in vitro* to any noticeable degree but reduce bacterium loads in drug-sensitive as well as drug-resistant *M. tuberculosis* infections [91]. In a mouse model, the leading drug candidate, IDR-1018 (Table I) also reduces lung inflammation as detected by reduced pneumonia. In human neutrophils, IDR peptides increase the release of the neutrophil-generated HDPs (anti-microbial), human α-defensins, and LL-37, and accelerate neutrophil-mediated killing of *E. coli* [92]. As a clue for the mode of action, these HDPs increase the expression of chemokines MCP-1, MP-3, and GRO-α, all implicated in anti-infective functions leading to immune cell recruitment and anti-infective actions [93]. Consistent with their ability to control inflammation, IDR peptides suppress LPS-mediated neutrophil degranulation, the release of ROS, and the production of pro-inflammatory cytokines, TNF-α and IL-10 [92].

Anti-lipopolysaccharide peptide

The synthetic AMP Pep19-2.5 (Table I) belongs to the class of synthetic anti-LPS peptide family. Treatment of murine cardiomyocytes stimulated with pathogen-associated or host danger-associated molecular patterns with peptide Pep19-2.5 decreases the inflammatory response [94]. The peptide also blocks a pro-inflammatory response induced by soluble heparan sulfate in serum from patients with gram-negative or gram-positive septic shock. Peptide Pep19-2.5 also interacts with heparanase, an enzyme that is elevated in mammalian sepsis and consecutively decreases the levels of circulating heparan sulfate fragments in systemic inflammation [95]. Due to these activities, peptide Pep19-2.5 was suggested to have a potential for pharmaceutical development as a broad-spectrum anti-inflammatory agent in sepsis-induced myocardial inflammation and dysfunction. Moreover, ibuprofen potentiates the anti-inflammatory activity of Pep19-2.5 both *in vivo* and *in vitro*, indicating that commonly used non-steroidal anti-inflammatory drugs might be useful to supplement future anti-sepsis therapies [96].

Designer proline–arginine-rich antibiotics

The peptide dimer A3-APO (Table I) and its monomeric *in vivo* metabolite are among the most active HDP in a wide spectrum of bacterial infection models in mice [97]. Although the APO peptides have only weak activity against

Acinetobacter baumannii in vitro, when administered either intravenously or intramuscularly to mice infected with a carbapenem-resistant *A. baumannii* strain, the peptides significantly reduce bacterial load and increase survival compared with mice treated with higher doses of imipenem [98]. While the dimer practically does not kill *S. aureus* or *Proteus mirabilis* strains *in vitro*, when administered intramuscularly or as an aerosol, it improves mouse survival and reduces bacterial counts at the infection site and in blood in wound infection or pneumonia models [99]. The wounds of treated animals lack pus or signs of inflammation while in human PBMC, A3-APO upregulates the expression of the anti-inflammatory cytokines IL-4 and IL-10 by 4–6-fold. One of the modes of actions of APO peptides might be the prevention of inflammation around bacterial infiltration. The efficacies of the A3-APO dimer and its monomeric metabolite were compared in mouse models of *P. acnes* intradermal ear infections following administration as intramuscular or topical treatments [100]. Once again, despite being inactive against the strain *in vitro*, *in vivo* the skin conditions of the experimental animals were dramatically improved upon peptide treatment regardless of administration mode or drug valency. APO peptides statistically significantly reduce ear thickness (a measure of inflammation) and ear bacterial counts when therapy success is correlated with the amount of ear connective tissue and level of epithelial macrophages.

HDPs excel in wound healing models even without bacterial infections and among the listed underlying immunological changes are macrophage recruitment, leukocyte infiltration, chemotaxis, vascularization, and re-epithelialization as well as reduced, or as opposite, elevated inflammation markers [101]. A3-APO improves full-thickness skin abrasion conditions probably due to macrophage activation [102]. In addition, A3-APO restricts the proliferation of *Bacillus anthracis* in infected macrophages by 40–45% compared to untreated cells both intra- and extracellularly [24]. The APO monomer retains its ability to close wounds when the peptide is applied to wounds in a polyvinyl alcohol nanofiber dressing [49]. APO-induced re-epithelialization of wounds looks very similar to those upon treatment with two peptides discussed earlier, the immune defense regulator IDR-1018 [103] or HB-107, a non-bacteriostatic fragment of cecropin B [41].

Conclusions

In addition to killing bacteria, HDPs invoke a long range of immune reactions in the host including macrophage and mast cell recruitment and activation, cytokine and chemokine release with ensuing signal transduction events,

and anti- and pro-inflammatory signaling. They also upregulate angiogenesis and downregulate toxin production. All these activities likely work independently; efficient AMPs are not necessarily active as anti-endotoxin therapies and vice versa [104]. In turn, the pleiotropic functions require a drug development design process that is more complex than that with drugs having only single functions (and minor side effects). To initiate human trials, small molecule antibiotics have to clear certain threshold in minimal inhibitory concentrations and exhibit better efficacy in animal models than existing therapy measures. Likewise, immunological agents have their own criteria set for moving ahead in the discovery process. For HDPs, drug developers have to identify a single *in vitro* function that explains the superior *in vivo* efficacy data and present the new molecular entity to regulatory agencies reflecting that function which unlikely will be bacterial killing. As a consequence, HDPs with similar sequences may end up in drug classification fairly distant from each other. The peptide research community faces an uphill battle educating regulators, investors, and the pharmaceutical industry about the advantages and specifics of these drug candidates and why and how we can manage the long and cruel clinical trial process [105].

As HDPs exert a variety of effects resembling cytokines and growth factors in maintaining normal immune homeostasis, unfortunately, they can also induce autoimmune diseases [106]. For example, pexiganan induces cross resistance to human defensin-1, a key component of the innate immune response to infection in *S. aureus* [107]. Preventing the ability of the innate immune system to act upon HDP therapies presents a serious potential risk of the use of cationic peptides in a clinical setting.

HDPs usually initiate anti-inflammatory responses, but sometimes pro-inflammatory signaling is observed. When one of the two activities is detected in any given assay, it likely depends upon the fine-tuned environment of the actual measures. The switch between receptor agonist and antagonist activities is a well-known phenomenon in peptide pharmacology [108] and can occur in minor sequence-specific changes and assay design [109]. Based on all available reports, while for activating the innate immune system and preventing deleterious effects on infections, both activities should be positively viewed, it is unlikely that HDP will end up in the treatment arsenal against rheumatoid arthritis or other inflammatory diseases, as single experiments would suggest. In fact, similar observations were made on human β-defensins, as the basic cellular functions associated with these HDPs demonstrate dual impact on health that might be advantageous under certain conditions and detrimental in others [110]. No doubt HDP will end up on pharmacy shelves [111]. The question is just when, against what conditions, and for what price.

Conflict of Interest

Dr. Otvos is the inventor of a composition of matter patent covering the A3-APO family of peptides. The patent is owned by Temple University.

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