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Immune modulation by multifaceted cationic host defense (antimicrobial) peptides

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Cationic host defense (antimicrobial) peptides were originally studied for their direct antimicrobial activities. They have since been found to exhibit multifaceted immunomodulatory activities, including profound anti-infective and selective anti-inflammatory properties, as well as adjuvant and wound-healing activities in animal models. These biological properties suggest that host defense peptides, and synthetic derivatives thereof, possess clinical potential beyond the treatment of antibiotic-resistant infections. In this Review, we provide an overview of the biological activities of host defense and synthetic peptides, their mechanism(s) of action and new therapeutic applications and challenges that are associated with their clinical use.

Cationic host defense peptides (HDPs) are small peptides that typically contain an abundance of positively charged and hydrophobic residues[1]. More than 2,000 natural peptides are abundant in eukaryotes and are also found in bacteria. Direct antimicrobial activities were originally considered to be the primary function of these peptides, hence the alternative name antimicrobial peptides. In this capacity, they exhibit variable, but often weak, direct cytotoxic activities toward bacteria, viruses, archaea, fungi, parasites and even cancer cells[1–5]. Moreover, it is noteworthy that these biological activities are often lost at physiologically relevant concentrations of salt, glycosaminoglycans and serum[2,4]. More recent studies have indicated that HDPs modulate immunity and immune-cell function under physiological conditions[2,4,5] and that these activities are the primary role of these peptides in the host (**Box 1**). Here we discuss their immune functions only, as direct antimicrobial activities were recently reviewed[2].

The importance of HDPs in immunity has been recognized in mouse models. The immunomodulatory properties of HDPs have been studied extensively over the last decade, and considerable effort has been made to generate synthetic peptides with enhanced immunomodulatory activities. As the majority of studies have reported immunomodulation at the level of innate immunity, we hereafter refer to these synthetic peptides as innate defense regulator (IDR) peptides. The immunomodulatory properties of HDPs and IDR peptides include (i) reduction in the levels of proinflammatory cytokines produced in response to microbial signature molecules; (ii) modulation of the expression of chemokines, reactive oxygen species and reactive nitrogen species (for example, nitric oxide); (iii) stimulation of angiogenesis; (iv) enhanced wound healing; (v) leukocyte activation; and (vi) macrophage and leukocyte differentiation (**Fig. 1**)[2,4–6]. For example, cathelin-related antimicrobial peptide (CRAMP)-null mice develop necrotic skin lesions after challenge with group A *Streptococcus* and are more susceptible to urinary tract infections[7,8]. In addition, HDP dysregulation in humans has been implicated in pathological conditions. For example, abnormally high levels of cathelicidin antimicrobial peptide (LL-37, also called CAMP) are associated with psoriasis[9,10]. In this capacity, it was proposed that LL-37 complexes with self DNA, which in turn activates plasmacytoid dendritic cells (pDCs) in a Toll-like receptor 9 (TLR-9)-dependent manner, causing interferon-γ (IFN-γ) production and autoimmune T-cell activation. LL-37 can also act as a vasodilator through the induction of histamine release from mast cells[11], but this property and its ability to cause apoptosis in epithelial cells are not observed in all peptides, including IDRs[12]. The absence of HDPs also contributes to human disease. Patients with specific granule deficiency, which is characterized by an increased susceptibility to pyogenic infections, lack defensins almost completely[13]. Similarly, patients with morbus Kostmann are deficient in LL-37, express reduced levels of human neutrophil peptide 1 (HNP-1) through HNP-3 (ref. 14) and are susceptible to severe periodontal disease, which can be reversed by bone marrow transplantation.

HDPs can be released from the granules of host leukocytes or produced locally (for example, induced at the site of infection) by a variety of cell types[4]. This explains how autologously produced HDPs can tailor the immune response at the infection site. Nevertheless, exogenously administered HDPs or IDR peptides can be delivered systemically and have shown considerable promise in animal models[15]. Studies on human and mouse cells have indicated a variety of targets, including monocytes, macrophages, DCs, epithelial cells, neutrophils, keratinocytes and others. The responses of these cells are somewhat distinct and are dependent on the peptide in question, the type of cells, their activation state and the pathogen and other host immune molecules that are coadministered. Collectively, the available data indicate that HDPs and IDR peptides are multifaceted mediators of the immune system. To further illustrate this point, we discuss the biological activities of these peptides below, with emphasis on the latest findings and *in vivo* efficacies.

HDPs and IDRs show anti-infective properties

The immunomodulatory activities of HDPs and IDR peptides explain their ability to treat microbial infections[2,4,5]. Thus, the addition of protease-labile L-amino acid peptides up to 48 h before initiating infection in a mouse leads to reduction of the infection relative to peptide-untreated animals[12,16]. Similarly, despite its very weak antimicrobial activity, as little as 0.4 ng of HNP-1 protects mice from *Klebsiella pneumoniae* and *Staphylococcus aureus* infections in a neutrophil-dependent manner[17]. In principle, anti-infective peptides can be biologically active because of their ability to manipulate immune-cell function, direct antimicrobial activities or a combination thereof. However, as mentioned above, their antibacterial properties are substantially lost under physiological conditions[2,4], which is consistent with the suggestion that these peptides are biologically active largely because of their immunomodulatory properties. This hypothesis was supported when the peptide IDR-1,

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Box 1 | Overview of the immune response to microbial infection

The immune response is divided into two branches: innate immunity and adaptive immunity. Innate immunity is the first line of defense against pathogenic organisms. Innate immunity is mediated by several cell types, particularly epithelial cells at mucosal and skin surfaces, which act as a barrier against pathogen entry, and phagocytic cells, which reside in tissues or are recruited from the blood to the site of infection. At the molecular level, innate immune cells sense pathogens, which is accomplished by an interaction between microbial signature molecules (sometimes referred to as pathogen-associated molecular patterns) and pathogen recognition receptors (for example, TLRs). Under pathological conditions (for example, psoriasis), TLRs can recognize host signature molecules, which might trigger an inappropriate immune response. The result of these interactions is the activation of multiple signal transduction pathways in the innate immune cell (for example, MAPK) and, among many responses, the subsequent production of cytokines (which alert other host cells to the presence of an infection) and chemokines (which drive the recruitment of other immune cells from the blood to the site of infection). The activation of innate immunity can also induce a systemic response to the pathogen, such as the development of a fever. Moreover, activated innate immune cells become directly microbicidal through the induction of reactive oxygen species, reactive nitrogen species (for example, nitric oxide) and antimicrobial peptides and proteins.

Adaptive immunity (acquired or learned immunity) is initiated in response to specific molecular shapes and/or sequences termed antigens. Adaptive immunity requires time (generally 3–7 d) to become activated and occurs through a number of complex signals between cells of the innate immune system, specifically APCs (macrophages or DCs) and cells of the adaptive immune system (T and B cells). T cells require antigen-interacting APCs to become activated. Although several varieties of T cells exist, the three major types are T_H cells, cytotoxic (killer) T (T_c) cells and regulatory T (T_{reg}) cells. After activation, T_H cells secrete cytokines that guide the evolution of the immune response. This may include recently characterized responses, such as T_H9, T_H17, T_H22, $T_{reg}1$ and induced T_{reg} (iT_{reg}) cell responses, but has traditionally been divided into either a T_H1 or a T_H2 cell response, which are simplistically described as cell-mediated or antibody-mediated immune responses, respectively. Activated T_H2 cells are required for B cell activation, and B cells are the cell type that produces antibodies. Again, the nature of the interaction between the T cell and the B cell determines which type of antibody will be produced. In contrast, T_c cells bind to and kill infected cells, such as those that are infected with viruses, or mutated host cells (i.e., cancer cells). T_{reg} cells generally modulate these processes. T cells cannot become activated without innate immune cells and specific antigens; therefore, the initial interaction between pathogens and cells of the innate immune system guides the evolution of the immune response.

which is a derivative of bovine bactenecin that does not possess antibacterial activities *in vitro*, was found to be protective in several mouse models of Gram-negative and Gram-positive infections[12]. Interestingly, IDR-1 is protective when delivered topically or systemically through intravenous, intraperitoneal and subcutaneous routes (as compared to clinically tested antimicrobial peptides that are only active topically[2]) and is effective when delivered before or after bacterial challenge. Indeed, IDR-1 promotes bacterial clearance by acting on the host innate immune response, specifically enhancing the production of chemokines that are involved in infection clearance (for example, monocyte chemotactic protein-1 (MCP-1, also called CCL2)) while suppressing potentially harmful proinflammatory cytokine production (for example, tumor necrosis factor-α (TNF-α)). IDR-1–induced anti-infective activities are dependent on monocytes and macrophages but not neutrophils.

Notably, further refinement of IDRs demonstrated three new peptides with different sequences that can be aligned (**Table 1**) and that have improved activity in *S. aureus* models[4,18,19], IDR-HH2, IDR-1002 and IDR-1018, suggesting a possible structure-activity relationship that should be further investigated. For example, IDR-1002 is more potent than IDR-1 at selectively

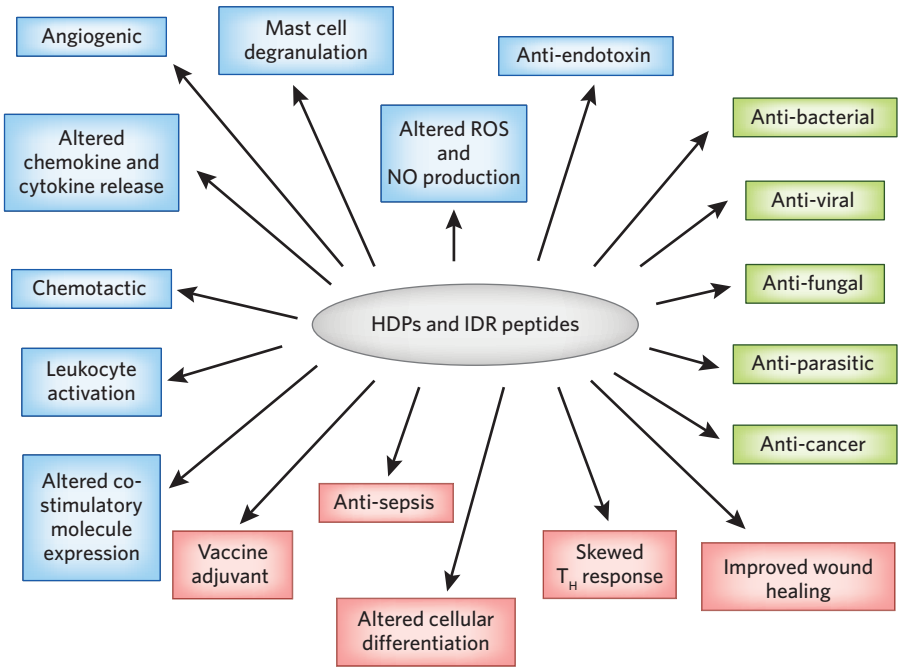

Figure 1 | Overview of the biological activities of HDPs and IDR peptides. Direct cytotoxic activities are shown in green, direct immunomodulatory properties are shown in blue and indirect immunomodulatory properties that are a consequence of direct immunomodulatory properties are shown in pink. ROS, reactive oxygen species; NO, nitric oxide.

Table 1 | Immunomodulatory activities of peptides that have been demonstrated *in vivo*

Peptide[a]	Sequence	*In vivo* activity	Reference
LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES	*S. aureus*, *E. coli* sepsis, anti-endotoxin, wound healing, adjuvant, angiogenesis	46,47,75,76,86–88
HNP-1[b]	ACYCRIPACIAGERRYGTCIYQGRLWAFCC	*K. pneumoniae*, *S. aureus*, adjuvant	17,89
IDR-HH2[c]	**VQL**R**I**R**V**AV**IR**A-NH_2	*S. aureus*, *M. tuberculosis*, adjuvant	4,18,60,63
IDR-1018[c]	**V**R**LIV**A**V**RIW**R**R-NH_2	*S. aureus*, *M. tuberculosis*, cerebral malaria anti-inflammatory, wound healing	4,18,19,46
IDR-1002[c]	**VQ**RW**LIV**WR**IR**K-NH_2	*S. aureus*, *E. coli*, adjuvant	4,16,61
IDR-1	KSRIVPAIPVSLL-NH_2	Methicillin-resistant *S. aureus*, vancomycin-resistant *Enterococcus*, *Salmonella typhimurium*	12

[a]Although each of these peptides except IDR-1 has some direct antimicrobial activity, these activities are strongly antagonized by physiological conditions, including monovalent and divalent cations, glycosaminoglycans (for example, heparin) and serum. In contrast, *in vitro* immunomodulatory activities are generally robust when these agents are added. [b]Defensin HNP-1 forms three disulfide bridges connecting Cys1-Cys6, Cys2-Cys4 and Cys3-Cys5. [c]Alignment of these sequences is demonstrated by the amino acids that align in these IDR peptides (shown in bold). There is no alignment for IDR-1 or LL-37, implicating that there are other immunomodulatory motifs.

inducing chemokine production, including MCP-1, MCP-3, growth-related protein-α (GRO-α, also called CXCL1) and interleukin-8 (IL-8), in human peripheral blood mononuclear cells[16]. Similarly to IDR-1, IDR-1002 does not induce the production of proinflammatory cytokines such as TNF-α and actually suppresses proinflammatory responses *in vivo*. IDR-1002 protects mice from invasive *S. aureus* and *Escherichia coli* infections by a mechanism that involves monocyte and neutrophil recruitment and, similarly to IDR-1, is monocyte and macrophage dependent. Subsequent studies demonstrated that IDR-1002 is not directly chemoattractive for monocytes but rather enhances monocyte migration by promoting β1-integrin–mediated interactions in a phosphatidylinositol 3-kinase (PI3K)-AKT–dependent manner[20]. Other studies demonstrated that IDR-1002 enhances neutrophil adhesion to endothelial cells in a β2-integrin–dependent manner, induces neutrophil migration, induces neutrophil chemokine production, increases the release of HDPs found in neutrophils (for example, LL-37) and enhances neutrophil-mediated bacterial killing[21]. It is likely that these biological activities contribute to the anti-infective properties of IDR-1002 *in vivo*.

Certain IDR peptides also protect mice against *M. tuberculosis* infections. In this capacity, IDR-HH2 and IDR-1018, but not IDR-1002, reduce bacillary loads in mouse models of drug-sensitive and multidrug-resistant *M. tuberculosis* infections despite having only modest *in vitro* activity against *M. tuberculosis*[18]. Moreover, IDR-1018 significantly reduced lung inflammation in treated mice, as evidenced by reduced pneumonia. These findings suggest that IDR peptides also hold potential as new agents for the treatment of infections.

Mechanisms of action

Systems biology, biochemical and immunological studies indicate the amazing complexity of the mechanism of action of HDPs and IDR peptides. Although mechanisms differ in various immune-cell types (for example, the mechanism in monocytes and/or macrophages is shown in **Fig. 2**), the peptides interact either with surface receptors (including G_i protein–coupled receptors, such as formyl peptide receptor 2 (FPR2) in leukocytes and MRGX2 (also called MRGPRX2) in mast cells, the tyrosine kinase receptor insulin growth factor 1R (IGF-1R) in cancer cell lines and the purinergic receptor P2X7 in multiple cell types) or the plasma membrane and then translocate across the plasma membrane in a manner similar to that of cell-penetrating peptides[12,19,22–24]. Translocation is essential for many but not all immunomodulatory activities[12,19]. Exceptions include direct chemokine activity. For example, LL-37 increases Ca^{2+} flux through chemokine (C-X-C motif) receptor 2 (CXCR2) and FPR2 (previously termed FPRL1) and chemoattracts human peripheral blood neutrophils and monocytes; FPR2 is also responsible for LL-37–induced chemotaxis in monocytes[25,26]. Analogously, human β-defensin 2 (hBD-2), hBD-3 and mouse hBD-4 chemoattract keratinocytes[27], and both human and β-defensins can also chemoattract monocytes through CCR2 (ref. 28).

After translocation, HDPs and IDR peptides bind to intracellular receptors, two of which were identified using stable isotope labeling by amino acids in cell culture (SILAC) proteomic approaches, glyceraldehyde 3-phosphate dehydrogenase (GAPDH)[23] and sequestosome 1 (SQSTM1)[29]. This binding leads to stimulation of multiple signal transduction pathways that are important in innate immunity, including p38, extracellular related kinases 1 and 2 (ERK1/2, also called MAPK3 and MAPK1, respectively), JNK mitogen activated protein kinases (MAPKs), nuclear factor-κB (NF-κB), PI3K, three Src family kinases, TRIF–interferon regulatory factor (IRF), TREM and others[12,16,30]. Downstream of these pathways, at least 11 transcription factors are mobilized into the nucleus and/or activated[30]. The result of transcription factor activation is the dysregulation of more than 900 genes in macrophages[12,30–32] (R.E.W.H., unpublished data.), which can be linked in part to the immunomodulatory activities observed. For example, most peptides increase the expression of multiple chemokines, including MCP-1, MCP-3 and GRO-α, which have been implicated *in vitro* and *in vivo* in anti-infective functions and lead to one of the hallmarks of HDP and IDR-peptide action, namely immune-cell recruitment[16].

Another consequence of these pathway modulation events is cellular differentiation, which is observed for macrophages[31], DCs[33] and neutrophils[21]. For example, macrophages display a range of functions depending on the conditions that are present during differentiation from monocytes and are often classified as M1 or M2 (**Box 2**). When present during monocyte-macrophage differentiation, IDR-1018 induces distinctive macrophage profiles that are intermediate between M1 and M2 (ref. 31). Although several of the features of IDR-differentiated macrophages are M2 like, with anti-inflammatory and wound-healing properties, the peptides are not locked into this state and can be reverted with IFN-γ[31].

Not only do peptides affect cellular differentiation, they also demonstrate distinct activities on different macrophage subsets, as has been shown for the human HDP LL-37 on mouse M1- and M2-polarized macrophages and on primary alveolar and peritoneal macrophages *in vitro* and *in vivo*[34]. Interestingly, this study showed that when bone marrow–derived macrophages are polarized to M1 in the presence of LL-37 for 20 h, there is a marked improvement in tumoricidal activity toward EL4 tumor cells in culture. Generally, LL-37 treatment mediates strong anti-inflammatory activity in M1 macrophages (assessed by decreased production of TNF-α and nitric oxide)[34]. Conversely, addition of LL-37 during the differentiation of macrophages into the M1 (using granulocyte macrophage

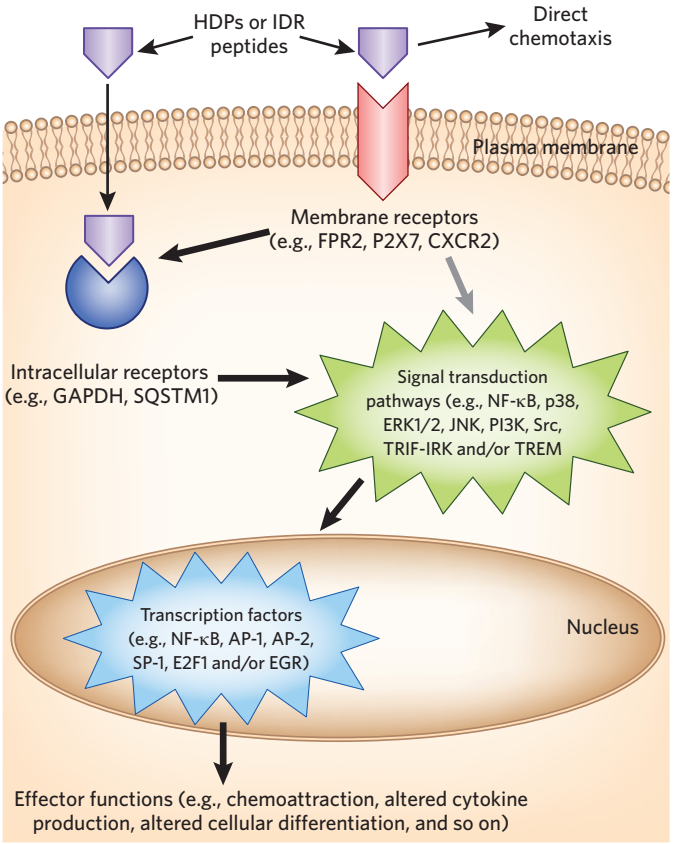

Figure 2 | A simplified schematic of common mechanisms of action of HDPs and IDR peptides in monocytes and/or macrophages. HDPs and IDR peptides can interact with G_i protein–coupled receptors on the cell surface or, alternatively, translocate through the membrane (likely through lipid rafts) into the cytosol, where they interact with intracellular receptors. Receptor binding triggers the induction of specific signal transduction pathways, which leads to the activation of the transcription factors that are responsible for the effector functions of HDPs and IDR peptides. Peptides with variations on this general scheme do exist in nature. A more thorough description of the mechanism of action of one immunomodulatory peptide (LL-37) is found elsewhere[30] and is summarized in the text. AP, activator protein; SP, specificity protein. EGR, early growth response factor EGR-1.

colony-stimulating factor (GM-CSF)) or M2 (using M-CSF) phenotypes led to increased levels of the inflammatory marker IL-12p40, whereas adding LL-37 to fully differentiated M1 macrophages produced no discernable changes in this marker[35]. However, as the species, differentiation methods and markers used as readouts were quite different, these data merit further study.

Although these findings broadly describe the stand-alone responses to peptides, more complex responses are observed in the presence of bacteria, their pathogen recognition receptor agonists (for example, the bacterial signature molecule lipopolysaccharide (LPS), CpG oligonucleotides, flagellin and lipoteichoic acid, among others) or endogenous host mediators (for example, IFN-γ, GM-CSF and IL-1β, among others)[2,5,36]. In these cases, peptides appear to modulate the inflammatory milieu, as discussed below.

HDPs and IDRs selectively alter inflammatory responses

The innate immune system is essential for human survival, yet the outcome of an overly robust and/or inappropriate immune response can paradoxically result in harmful sequelae. Abnormal inflammation is at the heart of a large number of diseases and disorders, including infection, cancer, atherosclerosis, ischemic heart disease, asthma, inflammatory bowel diseases, arthritis and vasculitis (many of which are now thought to have microbial triggers), and anti-inflammatory therapeutics often have clinical benefits (for example, statins in atherosclerosis). The reason why inflammation becomes chronic is still open to debate but might be different for each type of disease. Thus, inflammation cannot be considered a single syndrome but is rather a perturbation of regulatory networks that govern inflammatory (innate immune) processes, and these networks involve thousands of separate proteins, pathways, transcription factors and functional elements[5,37,38]. As HDPs and IDR peptides modulate innate immune pathways, it is predictable that they will have selective effects on inflammation that are dependent on the agonists involved and outputs measured, as has been shown for LL-37 with different TLR agonists, where it suppressed certain downstream responses and reinforced others[32].

As mentioned above, *in vivo* anti-infective studies usually show some evidence of selected anti-inflammatory activities, and to a greater or lesser extent, most HDPs and IDR peptides suppress proinflammatory cytokines in both mouse Gram-negative and Gram-positive bacterial infection models[12,16] and human primary cells[21,39,40] in response to various host and endogenous molecules; they also increase survival in rat sepsis models[41]. Indeed phase 2 clinical trials of CLS-001 (also known as MX-226) have indicated anti-inflammatory activity in humans in the context of severe acne and rosacea[2,5]. Other studies showed that IDR-1018 has potential as a new treatment option for severe malaria. Thus, when delivered with standard antimalarial agents, IDR-1018 increases the survival of treated mice by decreasing harmful neural inflammation that is associated with fatality but does not demonstrate antiparasitic activity[19].

The effects of HDPs and IDR peptides are complex, including both proinflammatory and anti-inflammatory effects, and have been observed in various cell types both *in vitro* and *in vivo*[2,4]. Mechanistically, the peptides act by multiple mechanisms. For example[32], LL-37 suppresses LPS-induced proinflammatory responses in human macrophages by (i) inhibiting LPS-induced translocation of the NF-κB subunits p50 and p65; (ii) selectively modulating gene transcription by completely or partly inhibiting certain proinflammatory genes while upregulating anti-inflammatory cytokines and pathways (for example, IL-10 and TNF-α–induced protein 3 (TNFAIP3)); (iii) triggering MAPK and PI3K pathways that can affect proinflammatory pathways; (iv) interacting directly with LPS to reduce its binding to LPS-binding protein (LBP), lymphocyte antigen 96 (MD2, also called LY96) or another component of the TLR-4 receptor complex, thus reducing activation of the downstream pathway; and (v) likely acting directly or indirectly to influence TNF-α protein translation, stabilization or processing.

In addition to its effects on macrophages, LL-37 decreases the levels of LPS-induced proinflammatory cytokines in primary mouse and human neutrophils[21,42], dendritic cells[43] and B lymphocytes[44] stimulated with TLR agonists such as LPS. Conversely, neutrophils from mice lacking CRAMP produce more TNF-α than do wild-type controls *ex vivo*[45]. IDR peptides have functions similar to those of HDPs—they often suppress proinflammatory cytokines, are indirectly chemotactic for neutrophils and monocytes, induce chemokine production and promote wound healing and monocyte differentiation[16,21,31,39,46].

HDPs and IDRs act as adjuvants in several mouse models

Vaccines are one of the most successful medical interventions for the prevention of infectious diseases. Vaccines are typically delivered as a formulation with a specific antigen and an appropriate adjuvant that functions to activate innate immunity and skew the adaptive immune response in favor of an enhanced antigen-specific immune response. In contrast, therapeutic adjuvants enhance the immune response, which leads to the resolution of infection in the

absence of a specific antigen. Because of their immunomodulatory properties, various HDPs and IDR peptides act as therapeutic adjuvants by modulating innate immunity, as discussed above. Similarly, they can act as vaccine adjuvants[5,47].

The precise nature of adjuvanticity is not well understood, but three mechanisms stand out, namely an ability to enhance recruitment of immune and antigen-presenting cells (APCs) to the site of vaccine deposition, the ability to activate those cells and the ability to form a depot or discrete compartment where the antigen concentration remains high. Whereas vaccine adjuvants may act on various immune cells, all adjuvants either directly or indirectly influence antigen presentation by APCs (for example, macrophages and DCs)[48]. Antigen presentation may be altered by (i) enhanced antigen uptake by APCs, which is partly dependent on recruitment of APCs to a focused depot of antigen; (ii) enhanced APC activation, i.e., signal 0; (iii) promotion of antigen presentation to T cells, i.e., signal 1; and (iv) an enhanced co-stimulatory signal, i.e., signal 2 (refs. 47,48). These actions involve altered cytokine production, skewed cellular differentiation and polarized immune responses, all of which promote the development of an effective immune response against the specified antigen[5,47,48].

Previous studies have shown excellent vaccine adjuvant properties in mouse models for a range of HDPs, including defensins and LL-37 (ref. 47). DCs, which are APCs that can be derived from monocytes, are chemoattracted to HNP-1 and hBD-1, which promote their subsequent activation and maturation[49]. Similarly, in mouse bone marrow–derived DCs, mouse β-defensin 2 acts through TLR-4 to promote DC maturation[50]. Conversely LL-37 polarizes DC maturation, favoring T helper type 1 (T_H1) cell responses[33]. HDPs then affect cytokine and maturation responses in ways that appear to depend on the differentiation state of, and other exposures to, DCs[33,43,49]. For example, when added during DC differentiation, LL-37 exposure without TLR agonists leads to a modest proinflammatory signature with increased levels of IL-6 and IL-12 (ref. 33). Conversely, LL-37 decreases the inflammatory response to TLR agonists in differentiated DCs, reducing the production of IL-6, IL-12p70 and TNF-α[43].

Although the effects of HDPs and IDR peptides have focused largely on cells of the innate system, there is also evidence that they can alter T- and B-cell responses[51]. Indeed naive and memory T cells can be mobilized with HNP-1, HNP-3 and HD-5 (ref. 52). LL-37 chemoattracts T cells through FPR2 (ref. 53). LL-37 also selectively induces granzyme-mediated apoptosis in cytotoxic T lymphocytes[54]. In B cells, LL-37 increases CpG sensing[55], whereas LL-37 decreases the inflammatory response in LPS-treated B cells[44].

Box 2 | M1 as compared to M2 macrophages

Macrophages display a range of signature expression patterns but are typically divided into the classically activated M1 and the alternatively activated M2 subsets, which are sometimes referred to as inflammatory and wound-healing macrophages, respectively. In humans (the details differ somewhat in mice), macrophages are skewed toward an M1 phenotype in response to TLR ligands (for example, LPS), T_H1 cytokines (for example, IFN-γ and TNF-α) and other immune stimulants (for example, GM-CSF). M1 macrophages produce high levels of proinflammatory cytokines (for example, TNF-α and IL-13, among others) and possess enhanced microbicidal and antigen-presentation activities. In contrast, macrophages are skewed toward an M2 phenotype in response to T_H2 cytokines (for example, IL-4 and IL-10) and other immune stimulants (for example, M-CSF). M2 macrophages enhance the expression of anti-inflammatory cytokines (for example, IL-10), which dampen the expression of proinflammatory cytokines, thereby maintaining immunological homeostasis, and exhibit enhanced phagocytic activity.

A recent study showed that hBD-2 and hBD-3 exhibit strong adjuvant activities[56]. Similar to LL-37, hBD-2 and hBD-3 form aggregates with DNA, including CpG DNA. Together the hBD-2–DNA and hBD-3–DNA aggregates induce TLR-9–dependent IFN-α production in pDCs. In mice, intravenous delivery of hBD-3–CpG complexes increases the concentration of inflammatory cytokines (for example, IFN-α and IFN-γ) in the blood, whereas subcutaneous injections enhance inflammatory cell recruitment to the skin at the injection site. Intraperitoneal injections of preformed hBD-3–CpG complexes in combination with ovalbumin cause a robust antiovalbumin immune response.

The adjuvant properties of synthetic IDR-HH2 and IDR-1002 have been well studied[57–61]. In these cases, as with hBD-3, coformulation with other molecules such as CpG oligonucleotides and/or depot-forming polyphosphazene are required for optimal activity. IDR-HH2–CpG complexes induce MCP-1 production in a synergistic manner[60] with minimal changes in TNF-α production. Moreover, IDR-HH2–CpG complexes augment IFN-α production in pDCs and increase co-stimulatory molecule expression on monocytes and DCs directly or indirectly *ex vivo*. *In vivo* studies have shown that intranasally administered detoxified pertussis toxin (PTd) in combination with HH2-CpG complexes leads to a 100-fold increase in total IgG levels (as compared to CpG alone) with balanced levels of IgG1 and IgG2a, the latter of which favors a T_H1 cell response. Collectively these data suggest that the IDR-HH2–CpG complex bridges the innate and adaptive immune response to create a balanced T_H1 and T_H2 cell response.

PTd coadministered with complexes of polyphosphazene, IDR-1002 and CpG results in increased levels of both IgG2a and IgG1 antibodies in mice and pigs[61]. These responses are very exciting immunologically, as high titers (≥10^6) that occurred even with a single dose[61] were observed in neonatal mice (and pigs) with no maternal interference[59] and were equally as protective against *Bordetella pertussis* as the commercial vaccine tetravalent Quadracel (alum adjuvanted). Moreover, the enhanced response was initiated earlier and lasted longer than the immune response that was generated by the PTd antigen alone, suggesting a potential use in neonates who are at increased risk of developing whooping cough, as they cannot currently be vaccinated effectively until they are 6–8 weeks of age[61,62].

IDR-HH2–CpG complexes also exhibit efficacy toward other antigens and enhance cellular immune responses to a prime-boost *Chlamydia* vaccine regimen comprising a recombinant adenovirus vector engineered to express the *Chlamydia* antigen CPAF (AdCPAF) followed by recombinant CPAF (rCPAF), both of which are formulated with IDR-HH2 and/or CpG[63]. Strong humoral and T_H1-biased cellular-mediated immune responses are observed using this regimen with the two-component adjuvant but not with IDR-HH2 or CpG alone. In contrast, priming and boosting with rCPAF formulated with HH2-CpG results in the generation of a weak humoral and potent mixed T_H1 and T_H17 cellular–mediated immune response. Despite these disparities, both regimens significantly protect mice from genital *Chlamydia muridarum* challenge when compared to AdCPAF alone.

Wound healing is accelerated by HDPs and IDRs

Cutaneous wound repair is a dynamic multistep process that involves three overlapping phases: (i) inflammation, including cell recruitment; (ii) formation of new granulation tissue (i.e., connective tissue formation and angiogenesis); and (iii) wound contraction and extracellular matrix reorganization[64]. Wounds provide an ideal breeding ground for microbes[65]. Therefore, proper wound healing is dependent on maintaining a manageable microbial burden whereby conditions that favor bacterial growth as biofilms may result in chronic wounds that require antimicrobial therapy for successful healing[66].

Considering the biological activities that HDPs and IDR peptides possess, it is perhaps not surprising that certain peptides exhibit wound-healing properties *in vitro* and *in vivo*. Thus, a variety of host defense peptides, especially human LL-37, mouse CRAMP and various defensins, are induced in human keratinocytes and wounds and mouse skin infection models by bacteria or by wound-healing growth factors such as TGF-α and IGF-1 (refs. 67–69). This fact has been exploited in experimental therapies, and in addition to the above-described HDPs that have weak antimicrobial activities, the synthetic cecropin B–derived peptide HB-107 is devoid of antimicrobial activity but promotes wound healing in a full-thickness mouse wound model[70]. A recent study compared the wound-healing activities of IDR-1018, LL-37 and HB-107 in diabetic and nondiabetic mice[46]. In comparison to LL-37 and HB-107, IDR-1018 is significantly less toxic to immortalized human keratinocytes and primary human fibroblasts and promotes dose-dependent wound closure in mice that surpasses wound closure mediated by LL-37 and HB-107. Interestingly, the wound-healing properties of all peptides are lost in diabetic mice, perhaps because of dysfunctional immune responses in the diabetic host[71]. IDR-1018 and LL-37 also promote wound healing in infected full-thickness wounds in pigs, although IDR-1018 exhibits higher rates of epidermal healing than LL-37 (ref. 46).

Regarding mechanism, various activities have been implicated, including enhanced migration of epithelial and influential immune cells because of the induced and nascent chemoattractant properties of peptides[46,72], increased cellular proliferation[46], alteration of the cytokine milieu (including dampening of potentially refractory proinflammatory cytokines and inflammatory neutrophils), increased synthesis of extracellular matrix proteoglycans (syndecans), improved angiogenesis (blood vessel growth) and anti-infective activities that suppress bacteria and antagonize wound healing[2,69,73,74]. Several of these features coincide with the mechanisms discussed above for other immunomodulatory activities of HDPs and IDR peptides. In addition to their activities on leukocytes, HDPs can also induce changes in other cells, including keratinocytes and endothelial cells. LL-37 increases angiogenesis in a rabbit ischemia model[75]. Another HDP, the porcine cathelicidin PR-39, also demonstrates a proangiogenic function[73]. In human bronchial epithelial cells, LL-37 promotes IL-8 release and wound healing through the epidermal growth factor receptor (EGFR) and MAPK signaling pathway[74,76]. LL-37 also increases the levels of IL-6, but not TNF-α or IL-1β, in epithelial cells, partially through NF-κB activation[77].

So far, the vast majority of studies evaluating the clinical potential of HDPs have involved topical applications, which are of limited clinical use because of cost of production and peptide degradation at the infection site[78]. To address this issue, researchers developed a cell-based approach for sustained delivery of agents such as HDPs. In this regard, NIKS keratinocytes have been engineered to constitutively express hBD-3 (ref. 79) and are then used to generate three-dimensional biological dressings for infected wounds. The resulting skin substitute reduces the growth of methicillin-resistant *S. aureus* both *in vitro* and in a mouse model of third-degree burns. Overall these findings clearly indicate that selected HDPs and synthetic peptides exhibit wound-healing properties that are in large part independent of their antimicrobial activities.

Therapeutic applications and challenges

Rising antibiotic resistance coupled with a lack of new treatments for bacterial infections threatens human medicine. HDPs and IDR peptides show considerable promise as new therapies for the treatment of infectious diseases, particularly those caused by multidrug-resistant organisms, and hyperinflammatory diseases (for example, cystic fibrosis[40]) because of their unique mechanism(s) of action and spectrum of biological activities[12,19,46,60]. Thus, there is growing interest in exploiting HDPs and IDR peptides for therapeutic use. Many peptides with antimicrobial and/or immunomodulatory properties have been studied clinically for efficacy against multidrug-resistant pathogens, although so far the majority of clinical tests have been conducted using topically applied peptides[1,2]. Despite considerable progress, certain limitations remain, including cost of production, stability and toxicity *in vivo* and appropriately exploiting the broad spectrum of biological activities.

Ideally, peptide therapeutics should have a low cost of production. Unfortunately, fluorenylmethoxycarbonyl (FMOC) chemical synthesis, which is the current method of peptide production, is quite expensive. One way to address this issue would be to create truncated derivatives with equivalent potencies, thus reducing production costs. To create biologically active peptides of minimal length, comprehensive structure-activity relationships must be conducted that involve high-throughput screening for various immunomodulatory activities. Alternatively, recombinant synthesis strategies for large-scale peptide production of such peptides are under development[2].

HDPs and IDR peptides are generally susceptible to proteolytic degradation, which reduces their half-life *in vivo*. Our own unpublished pharmacokinetic studies show that these peptides have a half-life of approximately 2 min in blood, although the peptides distribute rapidly to the tissues. Peptide stability can be enhanced through the use of D-amino acids, alternative backbones (peptidomimetics) or synthetic amino acids, all of which are resistant to proteolytic degradation[80]. However, each of these strategies increases the cost of production. Alternatively, appropriate peptide formulations, such as the use of lipid nanoparticles, may also contribute to improved biological stability *in vivo*, although this has not been studied.

Some cationic peptides are toxic to eukaryotic cells, which might explain why the majority of clinical trials have involved topically applied peptides. Toxicity to eukaryotic cells is the result of direct cell lysis or the induction of apoptosis in the target cell[81], whereas toxicity *in vivo* might also involve histamine release from mast cells[82]. However, some IDR peptides are protective in animal models of infection (for example, IDR-1) using various administration routes, including intravenous, with little or no associated toxicity[12]. Thus, it is imperative that the peptides be tested in animals and against normal human cells *ex vivo* to examine toxicity at an early development stage. These findings will allow the researchers to develop appropriate formulations to minimize toxicity and improve the biological activities of their lead peptides.

Concern has been expressed over the emergence of bacterial species that are resistant to HDPs and IDR peptides[83]. However, bacterial resistance is only a concern for peptides that are directly antimicrobial. Immunomodulatory peptides circumvent the issue of bacterial resistance because they target the immune system rather than the pathogen.

It is noteworthy that specific HDPs or IDR peptides are unlikely to possess all of the biological activities mentioned in this Review. For example, IDR-1002 and IDR-1018 are potent immunomodulators[16,39], and yet IDR-1018, but not IDR-1002, has anti-tuberculosis activity in mouse models[18]. Moreover, certain HDPs possess unexpected biological activities that may markedly affect their therapeutic use. For example, LL-37 exhibits angiogenic activities, which may contribute to the healing of infected wounds[75]. In contrast, lactoferricin, which is an immunomodulatory HDP that is found in milk[84], is a potent inhibitor of angiogenesis when isolated from bovine milk[85], which may affect its use as a therapeutic agent for the treatment of infected wounds. Collectively these data highlight the importance of thorough preclinical testing before beginning clinical trials.

HDPs and IDR peptides are multifaceted effectors of innate and adaptive immunity. HDPs and IDR peptides have a wide range of unique biological activities that define their therapeutic utility, and thus specific peptides show considerable promise as new therapeutic agents for the treatment of inflammatory and infectious diseases

and wounds. Although certain limitations are apparent, the clinical potential of this group of molecules will undoubtedly be revealed as thoughtfully designed studies further elucidate their mechanism(s) of action while simultaneously minimizing the cost of production and improving on currently available formulation strategies.

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Competing financial interests

The authors declare competing financial interests: details are available in the online version of the paper.

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