A Re-evaluation of the Role of Host Defence Peptides in Mammalian Immunity

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Abstract: Host defence peptides are found in all classes of life and are a fundamental component of the innate immune response. Initially it was believed that their sole role in innate immunity was to kill invading microorganisms, thus providing direct defence against infection. Evidence now suggests that these peptides play diverse and complex roles in the immune response and that, in higher animals, their functions are not restricted to the innate immune response. In *in vitro* experiments certain host defence peptides have been demonstrated to be potent antimicrobial agents at modest concentrations, although their antimicrobial activity is often strongly reduced or ablated in the presence of physiological concentrations of ions such as Na^+ and Mg^{2+}. In contrast, in experiments done in standard tissue culture media, the composition of which more accurately represents physiological levels of ions, mammalian host defence peptides have been demonstrated to have a number of immunomodulatory functions including altering host gene expression, acting as chemokines and/or inducing chemokine production, inhibiting lipopolysaccharide induced pro-inflammatory cytokine production, promoting wound healing, and modulating the responses of dendritic cells and cells of the adaptive immune response. Animal models indicate that host defence peptides are crucial for both prevention and clearance of infection. As interest in the *in vivo* functions of host defence peptides is increasing, it is important to consider whether in mammals the direct antimicrobial and immunomodulatory properties observed *in vitro* are physiologically relevant, especially since many of these activities are concentration dependent. In this review we summarize the concentrations of host defence peptides and ions reported throughout the body and compare that information with the concentrations of peptides that are known have antimicrobial or immunomodulatory functions *in vitro.*

Keywords: Cationic peptides, antimicrobial peptides, innate immunity, adaptive immunity, host defence, defensins, cathelicidins.

INTRODUCTION

Host defence peptides are of burgeoning scientific interest, and considerable therapeutic potential. Initially described as "antimicrobial peptides", these components of the immune system are conserved across plants, animals and insects. It is becoming increasingly evident that this label is misleading in some cases, relating more to a bias for *in vitro* antimicrobial testing at the point of discovery rather than their likely *in vivo* function. In mammals, conditions at many *in vivo* sites are such that several of these peptides probably have little if any direct microbicidal activity, but instead may have multiple immunomodulatory effects. Peptides initially isolated as and termed "antimicrobial peptides" have been shown to have more significant alternative functions *in vivo* (e.g. hepcidin [1]), while conversely a variety of other molecules with previously-established functions (e.g. anti-proteases like serum leukoprotease inhibitor [2] and elafin [3], and certain chemokines [4]) have been shown to have antimicrobial activity *in vitro* under low salt conditions.

Host defence peptides share many key features. In particular they are generally cationic and amphipathic. However, although related peptides and their derivatives may have very similar antimicrobial activity, their effects on mammalian cells can be quite different. We have recently shown (Bowdish, D. M. E., Davidson, D. J., Scott, M. G. and R. E. W. Hancock, manuscript in review) that despite similar antimicrobial activities, the bovine cathelicidin indolicidin can act as a chemokine for a monocyte-like cell line, whereas another small peptide derivative Bac2a (derived from the bovine cathelicidin bactenecin) does not. Similarly, although human neutrophil defensins HNP-1 and -2 are chemotactic for T lymphocytes, HNP-3 is not [5]. The converse holds true for certain chemokines that may or may not have antimicrobial activity in low salt conditions [6]. Hence, the future classification of host defence peptides might benefit from increased attention to their immuno-modulatory activities, as these become clear. A recent review proposed a novel index for theoretical protein binding potential of host defence peptides, to distinguish the potential for "hormone action" from the capacity for direct antibacterial activity [7]. Although this approach certainly attributed high index values to the well-characterised multifunctional immune modifiers PR-39 and LL-37, its predictive capacity remains to be demonstrated.

Many naturally occurring host defence peptides clearly have antimicrobial activities *in vitro* at concentrations generally substantially higher than those found *in vivo* and

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under non-physiological conditions. However, the significance of this activity *in vivo* is often harder to demonstrate. Although the protective effects of a large variety of peptides in animal models have been demonstrated [8], it is not clear that these are the result of direct microbicidal activity. Indeed, although synthetic derivatives can have substantially more effective antimicrobial activities than their natural counterparts, this does not necessarily correlate with their effectiveness in animal models. One argument that is often made is that peptides cause an additive or synergistic effect *in vivo*, as demonstrated for certain peptides *in vitro* [9] [10]. However, the levels of synergy tend to be quite modest, and the impact on synergy of physiological concentrations of ions has not been investigated to our knowledge. It seems reasonable to propose, therefore, that for many peptides the additional or alternative immunomodulatory activities are critical.

Studies of these additional effects are in their early stages, and have largely been performed *in vitro*. Innovative *in vivo* modelling approaches will be required to dissect the constitutive components of the host response that can be assigned to these peptides, and the significance of each component. Another important aspect that must be considered concerns the ionic conditions *in vivo*. Often addition of 100 mM NaCl is used as a surrogate for *in vivo* conditions in antimicrobial activity studies, but this ignores the more potent antagonism observed with much lower concentrations of divalent cations that are present in every body fluid.

Thus whether host defence peptides have meaningful microbicidal or immunoregulatory activities *in vivo* must be examined by considering two fundamental issues; i) the environment in which these activities are assessed *in vitro* compared to *in vivo* conditions, and ii) the concentrations at which such peptides are found *in vivo*.

ANTIMICROBIAL ACTIVITY OF HOST DEFENCE PEPTIDES

Overview

The classical description of cationic antimicrobial peptides includes molecules that are between 12 and 50 amino acids long (although there is a continuum of sizes of such molecules up to large proteins), with 2 or more positively charged residues provided by arginine, lysine or, in acidic environments, histidine, and a large proportion (generally >50%) of hydrophobic residues (see [11-13] for overviews). The secondary structures of these molecules follow 4 themes, including i) α-helical, ii) β-stranded due to the presence of 2 or more disulphide bonds, iii) β-hairpin or loop due to the presence of a single disulphide bond and/or cyclization of the peptide chain, and iv) extended. Many of these peptides are in fact unstructured in free solution, and fold into their final configuration upon partitioning into biological membranes. This tropism for membrane insertion tends to be a definitive feature of antimicrobial peptides [14] even though the peptides have a variety of antimicrobial activities ranging from membrane permeabilization to action on a range of cytoplasmic targets.

Although the above provides a general description of antimicrobial and host defence peptides, there are many variations on this theme. Peptides can contain a variety of modifications and vary dramatically in structure and sequence even within a single species and a single animal, e.g. the cow has more than two dozen individual peptides with antimicrobial activity [15]. However in our experience, virtually any cationic peptide can be demonstrated to have antimicrobial activity in dilute media; indeed a common method of testing antibiotic activity involves examining the ability to kill bacteria in 10 mM phosphate buffer [16]. One major argument that has been proposed as favouring the property of antimicrobial activity as being the most important function of host defence peptides is the relatively rapid sequence divergence of such peptides, i.e. sequence variation, even within a structural class, despite the fact that the up-stream regions of these genes (encoding the 5' untranslated and pre-pro regions) are relatively conserved. This sequence divergence of the regions encoding the mature peptides has been proposed to be driven by adaptive evolution due to the need to adapt such peptides to counter microbial adaptation/diversity [17]. However there are many examples of other proteins without direct antimicrobial functions that diverge rapidly, e.g. genes involved in reproduction, immunity and host defences [18], so it is by no means certain that antimicrobial activity drove evolution in this instance.

Physiological Conditions in the Human Body

Most attempts to mimic the conditions in the human body consider the major influence on antimicrobial activity to be salt in the form of 100 mM or more sodium chloride. Such salt conditions do in fact reflect the concentrations of Na^+ in the blood and probably the airway surface liquid (ASL) of the lung. However, we feel that another major influence is consistently overlooked, namely the divalent cations Mg^{2+} and Ca^{2+}. Most body fluids contain between 1 and 2 mM divalent cations, for example, sputum [19], milk [20], airway surface liquid [21], and serum/plasma [22]. Another factor that must be considered is the presence of anionic polysaccharides including glycosaminoglycans such as heparin and chondroitin sulphate, which could bind and segregate cationic antimicrobial peptides. Similarly the influence of trypsin-like proteases that abound in the body [23] and specifically digest proteins at basic residues, should be considered for both antimicrobial and non-antimicrobial activities.

Influence of Media Conditions on the Antimicrobial Activities of Peptides

A number of papers have defined an effect of salt in the form of NaCl, on the activity of antimicrobial peptides. These effects represent decreases in activity (increased MICs) ranging from severe, with many of the defensins [7, 12], to mild with other peptides [24]. However divalent cations are more antagonistic than monovalent cations [25]. For example with model optimized α-helical peptides, 1 mM $MgCl_2$ raised the MIC for *Pseudomonas aeruginosa* as much as did 200 mM NaCl [25]. This was explained by the peptides competing with Mg^{2+} for a specific binding site on the surface of cells (e.g. on lipopolysaccharide) rather than a

generalized ionic effect. Similarly it was demonstrated that as little as 0.05% (wt/vol) of sodium alginate could increase the MIC by 4 to 16 fold. Thus it is clear that future studies attempting to mimic the effects of physiological concentrations of ions should investigate the ability of at least 2 mM divalent cations to antagonize activity. Our general conclusion is that by not taking this into consideration the antimicrobial activity of cationic host defence peptides might well have been overemphasized in many cases.

Conversely, the non-antimicrobial, immunomodulatory activities of host defence peptides are generally studied in tissue culture media, containing more physiological levels of divalent cations. It is worth noting that the ability of human cathelicidin-derived peptide LL-37, to induce IL-8 release [26] and to neutralize the LPS- and LTA- induced stimulation of pro-inflammatory cytokines [27] has been demonstrated in whole human blood that contains nearly 2 mM ionized divalent cations. In addition, although serum, which contains 2 mM divalent cations (or the addition of 1 mM $MgCl_2$ to medium) will inhibit the microbicidal activity of LL-37, we have recently shown that it is required for this peptide to activate mitogen activated protein kinases (MAP kinase) signalling in human monocytes [28]. Nevertheless, further studies are required to investigate the immunomodulatory effects of host defence peptides in an ionic milieu, that mimics *in vivo* conditions, and at physiologically relevant peptide concentrations.

EXPRESSION AND CONCENTRATION OF HOST DEFENCE PEPTIDES *IN VIVO*

The Respiratory System

Innate immunity in the respiratory system is a complex and finely tuned mechanism by which the commensal microflora in the nasal passages are tolerated but controlled and the deeper regions of the lung remain essentially sterile. In the healthy individual the mucus layer provides a physical barrier to bacterial pathogens and the mucocilliary clearance pathway removes the majority of microbial intruders [29]. The underlying ASL contains host defence peptides and proteins including α and β-defensins and LL-37 as well as lysozyme, lactoferrin, secretory leukoprotease inhibitor, secretory phospholipases, complement, and immunoglobulin (Table **1**) [30]. These factors can be secreted from the submucosal glands or epithelial cells or may be released from resident macrophages and recruited neutrophils. A few components of the ASL have been clearly shown to have direct antibacterial activity under physiological conditions. This includes the biologically active protein lysozyme, which is antimicrobial in nasal secretions at physiological concentrations [31], although lysozyme by itself has a relatively narrow spectrum of activity. Although it has been proposed that host defence peptides play an important role in maintaining the sterility of the lung, the physiological relevance of their *in vitro* antimicrobial activities still remains unclear due to both their salt [32] and divalent cation sensitivity, as discussed above, and to the apparently modest concentrations in the ASL. This sensitivity to the ionic environment is also relevant to the antimicrobial activities of other biologically active proteins such as lysozyme, lactoferrin and SLPI [33].

Studying the *in vivo* significance of host defence peptides is complicated by the difficulties inherent in accurately assessing their concentrations in the tissues. The most common method to measure the concentration of host defence peptides and other soluble components in the lung is to perform bronchoalveolar lavage (BAL, reviewed in [34]) although other methods such as collecting sputum have also been utilized. The estimates published in the literature are confounded by technical inconsistencies. These include the target area of the lung, the volume and composition of washing fluid and the effects of repeated lavage procedures, removal of peptide rich leukocytes such as neutrophils and macrophages, and the method of peptide quantification (HPLC as compared to radial diffusion [35]). Of particular importance are the difficulties of accurately assessing the dilution factor for the ASL, which has an estimated depth of only 10μm - 20 μm, corresponding to as little as 1 μl per cm^2 of tissue [36], and of accounting for any secretory response to the lavage procedure. Despite differences in the methods used for detection of host defence peptides, various groups have found remarkably similar concentrations of host defence peptides (Table **1**).

Assuming that these estimated concentrations are roughly accurate, the question becomes one of physiological relevance; in light of *in vitro* studies are these *in vivo* concentrations of host defence peptides sufficient for direct antimicrobial activity, either in health or disease? Whereas constitutively expressed peptides are likely to play a more important role in homeostasis or prevention of infection in a healthy host [37], inducible peptides are presumed to constitute a component of an acute inflammatory response.

Role of Host Defence Peptides in Health

Numerous groups have compared the concentrations of host defence factors in the bronchoalveolar lavage of healthy patients. Human β-defensin-1 (HBD-1) is an example of a constitutively expressed defensin (Table **1**). HBD-1 mRNA is expressed by epithelial cells of the surface and submucosal glands, but is not up-regulated in cystic fibrosis (CF) lung disease [38], pulmonary tuberculosis [39] or inflammatory lung disease [38]. The role of HBD-1 in the healthy, adult lung is not entirely clear, especially since numerous groups have demonstrated that although the transcript is detectable, the protein does not appear to be present at readily detectable levels [38]. A salt sensitive antimicrobial activity was described in the ASL of primary epithelial cultures and bronchial xenografts, and initially attributed to HBD-1 [32, 40]. This peptide is antimicrobial *in vitro* at concentrations of 1 μg/ml, but only under low sodium (<50 nM) conditions [32, 38]. In contrast, the concentration of HBD-1 in bronchoalveolar lavage fluid is often not detected, but when detectable the concentration is estimated to be 0.1 μg/ml, which is 10 fold less than the antimicrobial concentrations in dilute medium. In addition, the estimated ion concentrations of the ASL are Na^+ 80-90 mM, Cl^- 70-80 mM, K^+ 25-30 mM at a pH of 7.0 [41] and under these conditions HBD-1 has no measurable antimicrobial activity *in vitro* [42]. Similar salt sensitivity has been reported for the mouse β-defensins [43]. Thus it appears as though the primary role of HBD-1, in the ASL cannot be direct antimicrobial activity.

Table 1. Concentrations of Host Defence Peptides Found in the Lung, Compared to the Cationic Proteins Lysozyme and Lactoferrin[1]

Peptide	Cellular Sources	Concentration (μg/ml)				
		Healthy (BAL)	CF (BAL)	Infection (BAL)	Inflammatory Lung Disease (BAL)	References
HBD-1	Neutrophils, epithelia	0 - 0.1	0 – 0.002	0 - 0.00007	0 – 0.2	[162, 39, 163, 164]
HBD-2	Neutrophils, epithelia	0 - 0.0004	0.0001 -0.01	0.0002	0.01 - 0.1	[38,39]
LL-37	Neutrophils, epithelia, submucosal glands	2.5 - 20*	-	2.5 - 30*	-	[65]
HNP-1-3	Neutrophils	0.2	300 - >1600‡	0.2 - 1.2	~10	[44, 165]
Lactoferrin	Submucosal glands, neutrophils	1.9	1.4 - 6.1	23	4.1	[166, 167]
Lysozyme	Submucosal glands, neutrophils, macrophages	0.8	-	13	0.9	[166]

[1]All values were determined for adult volunteers, except those indicated by an asterisk (*) which were determined from infants.
0 implies not detected in a particular assay or patient; "-" indicates no information available.
‡ These include concentrations found in sputum.

Role of Host Defence Peptides in Disease

CF, chronic obstructive pulmonary disease, bronchitis and chronic inflammation due to smoking or other types of environmental exposure provide interesting models of dysregulation of innate immunity in the lung. The bronchoalveolar lavage fluid from patients suffering from either inflammation or infection contains increased numbers of polymorphonuclear leukocytes [44] (reviewed in [34]) and host defence peptides compared to that of healthy individuals. This is probably because neutrophils in the lung undergo degranulation in response to bacterial or pro-inflammatory stimuli and in doing so release α-defensins (also called human neutrophil peptides; HNP). Thus, the concentration of neutrophil defensins generally correlates with that of IL-8, a potent chemoattractant for neutrophils [45], as well as the presence of other neutrophil components such as elastase [46]. In addition, epithelial cells themselves respond to either bacterial signalling molecules such as LPS, and to pro-inflammatory cytokines, with an increase in transcription and release of certain host defence peptides.

Some of the highest estimated concentrations of host defence peptides have been found in the bronchoalveolar lavage or sputum of CF patients. However, these estimates are complicated by changes in the volume and composition of the CF ASL [29] (reviewed in [36]). In particular, at high concentrations defensins precipitate plasma proteins [47] and it has been postulated that repeated freezing and thawing of samples of CF sputum leads to a loss of soluble immuno-reactive defensins [48]. The high peptide concentrations found in CF may be significant in this specific disease, which is characterised by high neutrophil influx and chronic bacterial colonization, and a consequent chronic inflammatory state, but care should be taken in extrapolating this situation to a normal response to infection.

Unlike the constitutively expressed HBD-1, HBD-2 expression at both the mRNA and protein-level is induced in primary airway epithelial cells and epithelial cell lines upon exposure to LPS or pro-inflammatory cytokines [49-51], as also are the murine beta-defensins –2 and –3 [52, 53]. HBD-2 mRNA co-localizes with HBD-1 in the surface and submucosal epithelia [38], and in the entire respiratory tract including nasal, tracheal and bronchial epithelia [50]. HBD-2 has been shown to be increased at the transcript level in response to either live rhinovirus or to synthetic double-stranded RNA, although in this case it was not possible to detect the HBD-2 protein [54]. HBD-2 mRNA and protein expression is increased in airway epithelial cells treated with IL-1β [38], TNF-α, mucoid *P. aeruginosa* [50], and LPS [49]. HBD-2 mRNA expression is greater in the epithelial cells of the CF lung upon stimulation with IL-1β [38]. It has therefore been proposed that HBD-2 is induced upon detection of infection to permit it to kill bacteria. Despite the fact that HBD-2 is a more potent antimicrobial than HBD-1 *in vitro*, the amount required to reduce the number of colony forming units of common pathogenic bacteria still remains in the μg/ml range at favourable concentrations of Na^+ [38, 50]. However, even in inflammation HBD-2 reaches an estimated concentration of only 100 ng/ml in the lung (Table **1**) [38] so again it appears unlikely that this is its main function. HBD-4 is a recently identified member of the defensin family with chemotactic properties which is up-regulated at the

transcriptional level in lung epithelial cells in response to live bacteria or phorbyl ester, but the protein product has not been identified in BAL or epithelial cultures [55].

HBD-3 expression and activity is not well characterised, however it has been demonstrated to be inducibly expressed at the transcriptional level in bronchial epithelial cell lines stimulated with TNF-α, bacteria, or live rhinovirus [54, 56]. As the antimicrobial activity of HBD-3 is relatively low (≤ 5 μg/ml) and is resistant to the presence of physiologically relevant concentrations of sodium ions it may have direct antimicrobial activity *in vivo*, however to date there are no published reports on concentrations found in ASL or BAL.

As mentioned above, α-defensins are found in the primary granules of neutrophils at concentrations as high as 10 mg/ml [57]. Upon ingestion of microbes these granules fuse with phagocytic vacuoles. This creates a protective niche in which these very high concentrations of host defence peptides almost certainly have a direct antimicrobial effect, although this is muted by the acidic pH of the phagolysosomes [58]. These peptides are also thought to be released upon degranulation of neutrophils in an attempt to resolve infections and are found at significantly higher concentrations in the bronchoalveolar lavage of patients suffering from a variety of infectious or inflammatory conditions (Table **1**). In patients suffering from inflammatory lung disease or infection HNPs are diluted in the ASL and the maximum concentration in the bronchoalveolar lavage has been estimated to be slightly greater than 1-10 μg/ml. This contrasts with a optimal antimicrobial activity *in vitro* at >10 μg/ml for *S. aureus* and *E. coli* [59], and reduction of the infectivity of adenoviruses in an airway epithelial model at concentrations of between 8-50 μg/ml [44].

LL-37 is derived from the cathelicidin hCAP18 [60]. hCAP-18 is constitutively expressed by neutrophils (~630 μg per 10^9 cells), lymphocytes, macrophages and a range of epithelial cells [61-64]. It can be detected at concentrations of 1 μM (5 μg/ml) in the bronchoalveolar lavage of healthy infants, and this is increased by 2 to 3 fold in bronchoalveolar lavage from infants with either systemic or pulmonary inflammation [65] (Table **1**). This compares unfavourably with the minimum inhibitory concentration (MIC) of LL-37, which is between 15-30 μg/ml under optimal *in vitro* conditions for a range of common bacteria [64].

Gastrointestinal System

The Gastrointestinal System in Health

The gastrointestinal system has a complicated and diverse range of mechanisms to prevent pathogenic organisms from gaining a foothold. These include antagonism by a rich and diverse normal flora, the physical barriers of the mucus layer, the constant shedding of the epithelial cells, the low pH of gastric acid, and the action of bile acids and pancreatic secretions. In addition host defence peptides, such as defensins, and proteins, such as lactoferrin and lysozyme, form a more specific defence. The innate immune response of the gut must distinguish between the large numbers of normal resident bacterial microflora and potentially pathogenic intruders that enter the gut.

The epithelial cells of the intestine, consisting of the absorptive enterocytes, goblet cells and Paneth cells play an important role in detecting pathogens and initiating an immune response. Paneth cells in particular play an important role in innate immunity. They contain antimicrobial peptides and proteins, such as α-defensins (HNP-1 to -4 and HD-5 and -6), lysozyme, and secretory phospholipase A2 which they release upon stimulation with conserved bacterial signalling molecules such as LPS or cholinergic agents (reviewed in [66]).

The dynamic nature of the gastrointestinal tract complicates *in vivo* assessment of host defence peptide patterns of expression and concentrations in epithelial lining fluids. As a result, host defence peptides are generally purified from acid extracts of epithelial cells from intestinal tissues, and consequently less information is available on the physiological concentrations encountered by bacteria. Nevertheless, immunohistochemical staining indicates that host defence peptides are expressed by the Paneth cells in the colon mucosa, and are present on the apical layer of the intestines. In contrast to the lung, where a clear correlation seems to occur between peptide concentration and infection, the induction of host defence peptides in the gut is less well defined.

Peptide Concentrations & Functions in the Gastrointestinal System

HBD-1 is presumed to be involved in immune surveillance and homeostasis [67] as it is constitutively expressed, at the mRNA and protein levels, in intestinal epithelial cells as well as in intestinal and colon cell lines and this expression is not altered by pro-inflammatory stimuli such as IL-1β or bacteria [68]. Generally the expression levels of HBD-1 throughout the gastrointestinal system appear to be quite low.

HD-5 is also constitutively expressed in Paneth cells of the small intestine and at the base of crypts (reviewed in [66]). HD-5 and HD-6 were found to be expressed at the mRNA level in human intestinal xenografts, and in contrast to HBD-2 were not up-regulated with exposure to *Salmonella* [68]. HD-5 expression was not up-regulated in patients suffering from *H. pylori* induced gastritis [69]. Despite the fact that HD-5 does not appear to be up-regulated upon detection of inflammation or infection, studies with transgenic mice expressing human HD-5 in their Paneth cells have provided the most convincing evidence that HD-5 is an important component of the innate immune response [67]. These mice were more resistant to oral *Salmonella* infection, resulting in a 10-fold reduction in bacteria recovered from their spleens. However, it is possible that this result may relate to the number of copies of the transgene, and the consequent levels of over-expression of this human peptide, in addition to their existing murine host defence peptide repertoire.

It has been proposed that the purpose of constitutively expressed host defence peptides such as HBD-1 and HD-5 is to regulate the numbers and composition of the luminal microbial flora and to provide immediate host defence against food and water borne pathogens. HBD-1 has been shown to have antimicrobial activity against Gram negative

bacteria *in vitro*, under conditions of optimal ionic environment and concentration [70] and HD-5 is antimicrobial *in vitro* towards *Lactobacillus monocytogenes*, *E. coli* and *Candida albicans* over a wide range of salt and pH conditions ([71] reviewed in [72]). However, it is unclear just how this antimicrobial activity could be specific for pathogenic and not for commensal organisms. Nonetheless by one estimate the steady state concentration of HD-5 in the human ileal mucosa might be between 50 and 250 μg/ml [73], at which concentration, HD-5 has antimicrobial activity, while concentrations as high as 100 mg/ml have been proposed in the environment of the mouse crypts and due to the similar architecture of the human crypts similar concentrations might be found in humans [73]. Other proposed functions for HD-5 include protection of crypt stem cells, the prevention of attachment and permanent colonization of undesirable microorganisms, or the reduction of the numbers of bacteria in the small intestine to increase nutrient absorption.

HBD-2 is inducible at both the mRNA and protein levels during the course of inflammation and infection in the gastrointestinal system. HBD-2 expression in intestinal and colonic epithelial cell lines is increased upon stimulation with IL-1β, flagellin or bacteria, in an NF-κB dependent manner [68] [74]. Interestingly other inflammatory mediators such as TNF-α and LPS do not induce HBD-2 up-regulation [68]. This may be due to a predominantly intracellular expression pattern of TLR4 in these cells, which has been suggested to be an evolutionary adaptation to the high bacterial load in the intestine [75, 76]. Increases in HBD-2 expression have been detected in inflamed intestinal and colon tissue by RT-PCR and immunohistochemistry in Crohn's disease and ulcerative colitis [77] and in the stomach of patients suffering from *Helicobacter pylori* induced gastritis [69]. However, the role of HBD-2 in these diseases, and the effect of its induction is not entirely clear. Patients with inflammatory bowel disease do not seem to be particularly prone to infections. Thus although HBD-2 is potentially antimicrobial, such an activity might only be physiologically relevant under these extreme circumstances.

In healthy intestinal tissue, α-defensins HNP 1-3 are expressed only in neutrophils of the lamina propria and not in Paneth cells or other intestinal epithelial cells. In tissues that are inflamed due to active ulcerative colitis and Crohn's disease, the epithelial cells themselves express HNP1-3 and lysozyme, and an increased number of neutrophils are found in the lamina propria [66,78]. As with HBD-2 it is not entirely clear what is the effect of HNP-1-3 expression in inflammatory bowel disease.

The intestinal epithelial cells of the intestines and colon in a healthy individual express a number of host defence peptides constitutively. LL-37 is constitutively expressed at the mRNA and protein level in human colon [79] and is not up-regulated by pro-inflammatory cytokines, although increases in expression do occur upon exposure to enteropathic strains of *E. coli* or *Salmonella* [80]. Consistent with this observation, LL-37 is antimicrobial for strains of *E. coli* and *Salmonella* at concentrations of less than 10 μg/ml even at high (100mM) salt concentrations *in vitro* [81]. However, it remains uncertain whether or not such high concentrations of this peptide occur in the intestines. Interestingly, LL-37 expression has been shown to be decreased in *Shigella* infection, suggesting a possible mechanism of evasion by this bacteria [82]. However, it is not clear whether this is a direct downregulation of expression, or a consequence of denuding of the epithelium, with reduced expression in the replacement cells.

The evidence that best demonstrates the importance of LL-37 as a host defence peptide in the alimentary canal comes from studies of its role in the mouth. LL-37 is expressed constitutively in the epithelial cells and salivary ducts and its expression is increased in inflammatory conditions [83]. It is believed that this constitutive production and deposition by neutrophils is of crucial importance to maintaining the immunological balance of the mouth. Patients, who suffer with morbus Kostman and are treated with G-CSF to restore neutrophils levels, do not express LL-37 in these cells. One of the manifestations of this disease is a chronic and severe periodontal condition [84]. It has been proposed that the absence of LL-37 may give a selective advantage to bacteria which, at low levels are commensal but at higher levels are responsible for periodontal disease. It is unclear, however, whether LL-37 is directly microbicidal towards common pathogens of the mouth. Although a number of oral bacteria are susceptible to LL-37 (<10 μg/ml) at 10 mM NaCl *in vitro*, in a physiologically more relevant isotonic environment far fewer bacteria are susceptible [85]. Although LL-37 has been detected in saliva, the concentration is unknown [86].

Skin

Skin Structure in Health

The skin, besides being the largest organ of the body, is also one of the more complex organs. It consists of the dermis, which is constructed of connective tissue, and the avascular epidermis, which is composed primarily of keratinocytes. The dermis is made of a complex mixture of collagen, elastin and glycosaminoglycans (collectively called the ECM) and fibroblasts. The dermis, due to its highly vascular nature also includes the sweat glands, adipose cells and a large number of effector cells of the innate immune response such as mast cells, dendritic cells and low numbers of neutrophils. Menon [87] has provided a comprehensive review of skin structure and function.

In contrast to other organs, skin samples may be collected more readily, both by performing biopsies of healthy tissues and by tape-stripping psoriatic skin. The samples may be homogenized for Western blot analysis or stained for immunohistochemistry. Estimates of peptide concentrations can then be obtained from these samples.

Peptide Function in the Skin

HBD-1 is generally believed to be constitutively expressed in many of the cell types that compose the skin [88, 89], although it may not be found in certain cell lines [89]. Although HBD-1 expression is not inducible by pro-inflammatory cytokines or bacteria, there are reports in which it is slightly up-regulated in acne biopsies [88]. It has been proposed that areas of the epidermis that are vulnerable to microbial invasion, such as the hair follicles, require basal

production of defensins to fend off infection. The transcript for HBD-1 is generally found at low abundance and it is not clear if the HBD-1 peptide is found at directly microbicidal concentrations [90].

There is no basal HBD-2 expression in healthy skin tissue although it may be detected in keratinocyte cell lines [91]. The differentiation state of the cells in question may affect HBD-2 expression. For example, non-differentiating keratinocytes express only very low levels of the HBD-2 transcript but cultivated keratinocytes grown as either monolayers or multilayers produce significantly more HBD-2 mRNA, while fully differentiated keratinocytes grown at an air-liquid interface produce substantial amounts of HBD-2 mRNA [90]. The HBD-2 peptide was only detected in the constructed epidermis [90]. This observation may explain why different groups report conflicting data concerning HBD-2 upregulation by LPS, IL-1β, TNF-α or certain bacteria [90, 92, 93]. Interestingly, different locations of the body may demonstrate contrasting expression patterns of HBD-2, with one study demonstrating that HBD-2 was found in all foreskin and facial samples but in only 50% of breast and abdomen samples [94]. This may reflect differences in the skin flora, cell turnover or other as-yet undefined differences [94]. It is believed that the immune cells, which exist in close contact with the skin, play an important role in regulating defensin expression in the skin. It has been demonstrated that although *E. coli* and LPS induces HBD-2 mRNA or protein, a far greater up-regulation occurs when epidermis is co-cultured with monocyte-derived cells. This is believed to be primarily because LPS causes the monocytes to produce IL-1 which is a potent stimulator of HBD-2 expression [95].

In IL-1α-stimulated keratinocytes or in psoriatic skin HBD-2 was localized to the lamellar bodies and the intercellular spaces as determined by immunostaining. The intercellular spaces stained especially strongly for HBD-2, indicating that HBD-2 may be highly concentrated in this area of the inflamed epidermis [91, 93, 96, 97]. Although the average concentration of HBD-2 in these stimulated tissues is 10 μg/ml, the concentration in the intercellular spaces may be much higher [96]. It has been proposed that HBD-2 mRNA is rapidly and strongly up-regulated since HBD-2 protein is stored only in small amounts in the lamellar body and must be generated *de novo* upon detection of infection [95].

The role of HBD-2 in preventing microbial infection is not entirely clear. The sweat and upper levels of the skin are believed to have Na^+ concentrations of 20-60 mM, which are below the concentration that antagonizes HBD-2 antimicrobial activity (>100 mM) [93]. HBD-2 is induced by the presence of numerous bacteria such as *E. coli*, *S. aureus* and *S. epidermidis* that are rarely implicated in skin infections, but only poorly induced by *S. pyogenes*, a common skin pathogen [98]. In addition, HBD-2 was found to be antimicrobial for *S. aureus* only at concentrations of 100 μg/ml or greater, *in vitro*, implying that induction of HBD-2 would not provide protection against this organism. However, HBD-2 expression is depressed in patients with atopic dermatitis who often present with cases of acute and chronic colonization by *S. aureus* [97]. This is consistent with the suggestion that any effect of HBD-2 in this condition may be indirect. In contrast to atopic dermatitis, HBD-2 expression is increased in psoriatic skin, a disease in which patients are fairly resistant to bacterial infection [99]. Interestingly, although it induces HBD-2, the commensal *S. epidermidis* is largely resistant to this peptide. This resistance may be an important factor in permitting colonization with this organism [98] and once again the importance of commensal organisms in the defence against pathogenic bacteria must be emphasized.

In normal skin HBD-3, a newly characterised member of the defensin family, is found at low levels, but in psoriatic lesions it is possible to isolate 10- to 30-fold higher amounts of HBD-3 [56]. This peptide displays salt insensitive antimicrobial activity without haemolytic activity at concentrations of approximately 5 μg/ml in media of low ionic strength [56]

LL-37 is found in psoriatic lesions but not in healthy skin [100]. It is up-regulated at the mRNA and protein levels upon exposure to Group A *Streptococcus* or by sterile incision [101]. Expression is also increased by various growth factors that are involved in wound healing, such as insulin-like growth factor. Consistent with this, LL-37 has been demonstrated to be involved in the re-epithelialization of skin wounds. Using a non-inflammatory *ex vivo* wound healing model of organ cultured human skin, upregulation of LL-37 expression has been demonstrated in the wound area, and antibodies specific for LL-37 have been shown to inhibit the re-epithelialization process in a concentration dependent manner [102]. Since LL-37 is probably not found at concentrations sufficient for antimicrobial activity [101] it is possible that one of the primary functions of LL-37 in the skin is to promote re-epithelialization.

Interestingly, both the pro-protein, hCAP18, and mature processed form of LL-37 are constitutively produced in the sweat [103]. The unprocessed pro-protein does not have antimicrobial activity ([104] until it is cleaved by proteinase 3. Thus LL-37 might remain inactive until it is cleaved on the skin surface. Conceivably this cleavage might only occur when the barrier function is breached or when microbial proteinases are present at the surface. Although the necessary concentration of LL-37 for direct microbicidal activity is not found in the sweat (1 μM), it has been shown that sweat has antimicrobial activity, indicating that there may be other host defence factors that work independently, in combination with or in synergy with LL-37 [103].

Other Sites

In addition to expression in the pulmonary system, alimentary canal and skin as described above, host defence peptides have been found throughout the body. It is quite conceivable that there is considerable variation at different body sites in the nature and significance of the effects that specific peptides might exert both in health and disease. Indeed, even broadly expressed peptides may have very specific roles in certain tissues or secretions. This hypothesis may help to explain the rather more subtle and narrow range of host defence defects seen in transgenic mouse models in which peptides have been knocked out [105-108]. Human β-defensins are found at concentrations of 10-100 μg/litre in

the urine and at increased concentrations in the urine of pregnant women [109]. Defensins, such as HNP 1-3 and HBD-1, are found at concentrations of approximately 12 mg/g and 1 mg/g respectively in the cervical plug where they are hypothesized to maintain the sterility required for pregnancy [110]. LL-37 has been found at high levels in seminal plasma (42-143 μg/ml) [111] and other fluids such as sweat (5 μg/ml) [103] and in its unprocessed form it is also found at low levels (1.2 μg/ml) in blood [112]. Low levels of defensins are found in blood. HBD-1 is constitutively present at concentrations of 5 ng/ml and HBD-2 is found at concentrations of 5-100 pg/ml in healthy volunteers but is increased by 5-10 fold in patients suffering from panbronchitis [42, 45]. The highest concentrations of host defence peptides are found in leukocytes. For example, in leukocyte granules the concentrations of defensins can be greater than 10 mg/ml [57, 113]. The significance of higher peptide concentrations at these sites may prove to be illuminating in determining their physiological relevance.

CYTOTOXICITY AND *IN VIVO* ACTIVITY OF HOST DEFENCE PEPTIDES

It seems clear that in most cases, the concentrations of host defence peptides found *in vivo* are low, and they are probably not present at levels, or under conditions, that would favour direct microbicidal activity (Table **1**). However, under certain conditions, or at certain sites, higher doses have been observed, and indeed higher doses are generally employed in therapeutic models. In this context, it is important to consider reports that at high concentrations, host defence peptides are cytotoxic to a variety of eukaryotic cell types (Table **2**). It has been suggested that the increased concentrations of host defence peptides at sites of inflammation and infection may actually be responsible for some pathology in certain diseases. For example, patients suffering from conditions such as inflammatory lung disease have elevated pulmonary levels of both alpha and beta defensins (Table **1**). *In vitro* cytotoxicity data indicates that human neutrophil peptides derived from bronchoalveolar lavage are cytotoxic towards alveolar macrophages [44] and epithelial cells [114]. To determine if a high concentration of defensins could induce lung injury, one study instilled a combination of HNP-1-3 into the lungs of mice at a concentration believed to be between 1 and 10 mg/ml [115]. Very little lung dysfunction occurred at lower concentrations, but at the higher concentrations there was marked decrease in peripheral arterial O_2 saturation, impairment of mitochondrial function, increased lung permeability, and a marked neutrophil influx (as measured by increased elastase activity). However, by immuno-histochemical analysis there was no difference between the lungs of mice treated with defensins versus the untreated group [115]. Thus, although concentrations as high as 1.2 μg/ml have been demonstrated in the bronchoalveolar lavage of patients suffering from active pulmonary infection, it is likely that this is a consequence of disease, rather than a contributing factor and that defensins do not reach concentrations sufficient to markedly affect lung function.

Another important factor in considering the physiological relevance of *in vitro* reports of the cytotoxic effects of host defence peptides is once again the conditions under which these peptides are tested. Many components of serum have been demonstrated to have an effect on antimicrobial activity. Apoplipoprotein 1, a common protein found in serum, has been demonstrated to block the antimicrobial activity of LL-37 [116]. The presence of physiologically relevant concentrations of albumin or fibronectin have also been demonstrated to impair HNP mediated killing of *S. aureus* [117]. In addition to these effects on microbicidal function, evidence in our laboratory indicates that the addition of human serum to *in vitro* studies also decreases the *in vitro* cytotoxicity of antimicrobial peptides for mammalian cells. In primary blood-derived monocytes cultured in the absence of serum, cytotoxicity becomes apparent after a short incubation with 50 μg/ml of LL-37 (Fig. **1**). In contrast, when these cells are cultured with serum

Table 2. Cytotoxic Concentrations of Various Host Defence Peptides

Peptide	Cytotoxic Concentration (μM)	Cell type	Presence of Serum	Reference
LL-37	50-100	Red blood cells	-	[168]
	13-50	T cell line	-	[169]
	<50	Primary blood derived monocytes	-	Fig. 1
	> 50	Primary blood derived monocytes	+	[28]
HNP1-3	7.5-30	Alveolar macrophage	-	[44]
	6-24	Epithelial cell line	-	[114]
	10-100	Monocytes	-	[170]
	>100	Monocytes	+	[170]
HNP1-3 ribosylated	>24	Epithelial cell line	-	[114]

this cytotoxic effect is abolished [28]**.** However, the presence of serum does not inhibit the ability of LL-37 to stimulate IL-8 secretion or MAP kinase phosphorylation [28], and indeed appears to be necessary for these effects. In addition, the composition of serum may have an important effect. We have recently observed that human serum is more protective against the cytotoxic effects of very high doses of host defence peptides in an airway epithelial cell line than is fetal bovine serum (Lau, E., Davidson, D. J., Hancock, R. E. W. unpublished data). Another intriguing possibility is that host defence peptides may be altered *in vivo* to reduce their cytotoxicity, without affecting their immunomodulatory properties. An arginine-specific ADP ribosyltransferases present on airway epithelial cells has been shown to be capable of modifying an arginine residue in HNP-1. This ADP-ribosylated defensin had decreased antimicrobial and cytotoxic activities but its ability to stimulate T cell chemotaxis and IL-8 release from A549 cells was unchanged

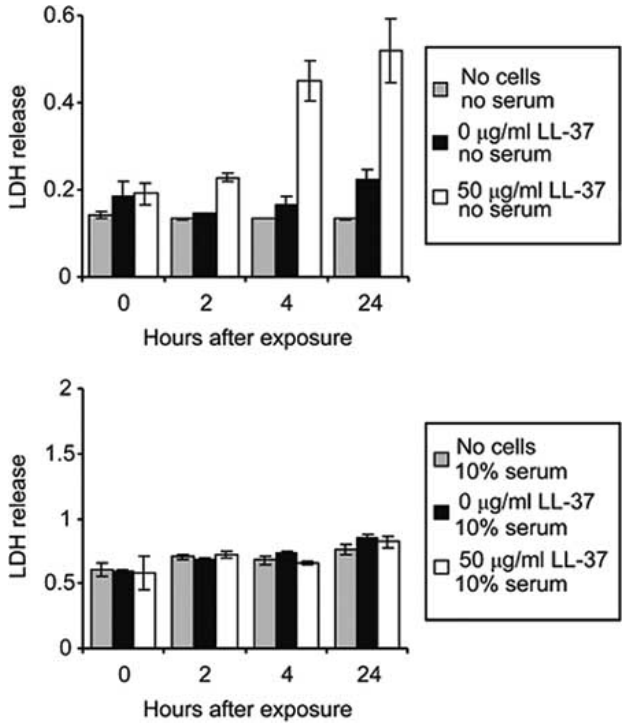

Fig. (1). Serum decreases the cytotoxicity of antimicrobial peptides for mammalian cells.

Human blood-derived monocytes were cultured at 37°C in RPMI medium, with 50 μg/ml of the host defence peptide LL-37, a) in the absence of serum, b) in the presence of 10% foetal calf serum. Cytotoxicity was determined by quantifying the cellular release of lactate dehydrogenase (LDH) at the time points indicated, and compared to control cells treated with endotoxin free water (carrier control for the peptide), or media incubated without cells. The cytotoxicity observed is abolished by the addition of serum. In the presence of serum there is no substantial release of LDH in response to 50 μg/ml of LL-37, even up to 24 hours after exposure, in comparison to cells cultured in the absence of LL-37, or even media alone. The addition of serum in the media resulted in a low level positive signal even the absence of cells, as shown. Each condition was studied in triplicate. Error bars indicate the range of mean responses between two donors.

[114]. This indicates that methods may exist to modulate cytotoxicity induced by host defence peptides *in vivo.*

Thus, whether by virtue of low physiological concentrations, protective host factors or peptide modifications, it seems unlikely that host defence peptides cause significant cytotoxicity of mammalian cells *in vivo*.

THE *IN VIVO* SIGNIFICANCE OF HOST DEFENCE PEPTIDES

It is clear that although some directly microbicidal components of the innate immune system probably play important roles in resisting infection, many of the best-studied host defence peptides may be present in concentrations that are too low, or in environments that would inhibit such activities. Nevertheless, *in vivo* evidence suggests that such peptides can have significant effects. In humans, an increased susceptibility to recurrent bacterial infections has been observed in cases of neutrophil deficiency of α-defensins [118]. Morbus Kostmann is a severe congenital neutropenia, typified by low concentrations of LL-37 and other cationic peptides in the mouth, and recurrent oral infections. Interestingly however, G-CSF can restore neutrophil numbers, although these cells have no LL-37 and decreased levels of α-defensins [84]. In transgenic mice, a deficiency in matrilysin (required for production of mature intestinal α-defensins) led to increased susceptibility to infection with oral *S. typhimurium* [105], while mice generated with a deficiency in the cathelicidin peptide CRAMP were somewhat compromised in their ability to combat skin infections by group A *Streptococcus* [106]. In mice in which the murine β-defensin-1 gene was knocked-out, the defects were even more subtle, with more *Staphylococcus* species harboured in the bladder in one model system, and delayed pulmonary clearance of *H. influenzae* in another [107, 108]. The very specific and subtle nature of these observations might be due to redundancy amongst the many murine β-defensins, or knocking out a constitutively expressed, rather than inducible, member of the family. Nevertheless, alterations in the ability of these animals to counter microbes were evident. Transgenic mice expressing the human α-defensin HD-5 had increased resistance to *S. typhimurium* [67], while expression of LL-37 in the murine lung was shown to enhance protection against infection with *P. aeruginosa* and endotoxaemia [119]. These examples are consistent with a role for host defence peptides in protecting against infection. However, we propose that their immunomodulatory interactions with host cells, rather than direct microbicidal activity, may play a significant role in this protection.

THE ROLE OF HOST DEFENCE PEPTIDES IN INNATE IMMUNITY

In addition to their direct antimicrobial potential, a number of properties that would modulate the innate immune response have been attributed to host defence peptides in mammals (Fig. **2**). These include epithelial cell proliferation, enhanced wound healing, angiogenesis, the stimulation of chemokine production, inhibition of pro-inflammatory cytokines, direct chemotaxis of many types of leukocytes, mast cell degranulation, and modulation of host cell gene

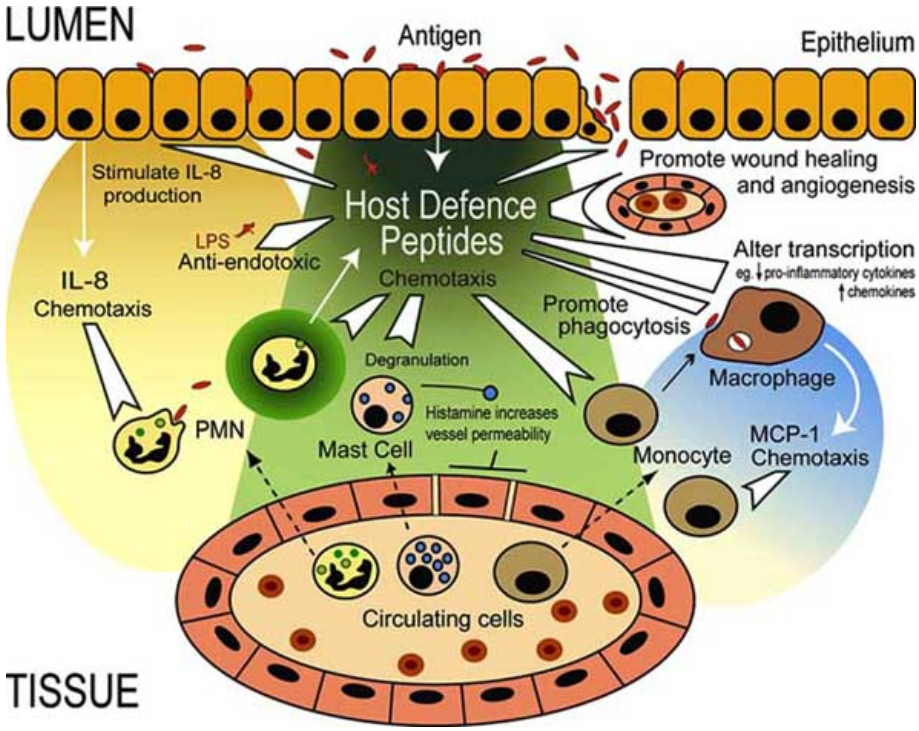

Fig. (2). The role of host defence peptides in innate immunity.
In addition to their potential for direct microbicidal activity and anti-endotoxic capacity, a variety of innate immune functions have been proposed for certain host defence peptides. These include the chemotaxis of neutrophils, mast cells, and monocytes, promotion of phagocytosis, wound healing and angiogenesis. Many peptides are induced at sites of infection and inflammation, produced primarily by neutrophils and epithelial cells, and interact with host cells to alter gene transcription. The resultant modulations can include the increased production of chemokines such as IL-8 and MCP-1 from epithelial cells and macrophages, inducing further chemotaxis of neutrophils and monocytes. Host defence peptides can also stimulate mast cell degranulation, which can further increase blood vessel permeability. The range of these activities exhibited is peptide specific. In this manner, these peptides can orchestrate an innate immune response and enhance both clearance of pathogens and subsequent healing.

expression (Reviewed in [120-122]). Whereas the antimicrobial functions of host defence peptides are most often studied in media of low ionic strength, the immunomodulatory properties of these peptides are studied in tissue culture media that contains more physiologically relevant concentrations of ions and may thus provide a better system for studying physiologically relevant functions.

It has been known for some time that many cationic peptides, including LL-37, are able to neutralize endotoxin, both *in vitro* (inhibiting LPS-induced TNF-α production in macrophages) and *in vivo* (protecting animals against lethal endotoxaemia/sepsis) [123, 124]. This was at first assumed to be entirely due to the ability of these cationic amphipathic peptides to bind and sequester the anionic glycolipid LPS, although CD14 binding has also been proposed [125]. However further studies indicated that peptides actually interacted with cells to neutralize pro-inflammatory responses; (i) peptides could neutralize the ability of LPS to stimulate TNF-α production even when added one hour after the LPS (at which time the LPS would have been internalized), (ii) peptides selectively suppressed the expression of subsets of LPS-induced genes in macrophages, whereas binding and neutralization would be anticipated to result in global suppression [26], and (iii) peptides themselves caused direct up-regulation of macrophage gene expression, including certain anti-inflammatory genes [26]. The ability of LL-37 to neutralize both LPS and LTA responses occurred at concentrations as low as 5 μg/ml and could be demonstrated in whole human blood [22, 26]. This indicates that peptides have anti-inflammatory properties, especially since the phenomena of inflammation, sepsis and endotoxaemia are highly related. However, peptides also stimulate the production of selected pro-inflammatory molecules such as the chemokines IL-8 and MCP-1, and certain chemokine receptors. Thus they appear to stimulate a mixed response. One possibility is that induction of innate immunity leads to stimulation of natural cationic host defence peptide synthesis leading to suppression of pro-inflammatory cytokines as a feedback mechanism to try to limit the induction of septic levels of such cytokines, while permitting other pro-inflammatory mechanisms involved in resolution events, such as repairing tissues that have been damaged during infection.

LL-37 induces multiple responses in host cells and thus a direct interaction of cationic peptides with host cells can be inferred. Consistent with this, three groups have found evidence of LL-37 receptors on host cells. Yang *et al.* [126] demonstrated that LL-37 bound to the FPRL-1 receptor in inducing chemotaxis of monocytes and T-cells. FPRL-1 is an orphan receptor and a promiscuous G-protein-coupled

receptor that has no *bona fide* ligand, but studies have shown that it may be activated by numerous ligands including bacterial formyl peptides, T21 (an ecodomain of HIV gp41), W-peptide, serum amyloid A and lipoxin A4 (a lipid derivative of arachidonate metabolism) leading to cell signalling [127]. Niyonsaba *et al.* [128] demonstrated that LL-37 bound to both a high affinity (Kd = 2.3 μM) and a low affinity, G-protein-coupled receptor (Kd = 112 μM) in inducing mast cell chemotaxis, but that neither of these were the FPRL-1 receptor. Hiemstra *et al.* [129] conversely suggested that LL-37 induced cell signalling in an epithelial cell line occurred via the epidermal growth factor receptor. To rationalize these data one must conclude that LL-37 can bind to a variety of relatively moderate affinity receptors present on different cell types. This may also explain why the effects of LL-37 can also be seen in animal models, for example, mast cell chemotaxis in rats [128], or angiogenesis in rabbits [130], where the receptors have clearly not co-evolved with the peptide.

Subsequent to binding, certain cell signalling cascades are activated, including ERK 1/2 and p38, both of which are MAP kinase pathways involved in growth and differentiation. This occurs in both a human epithelial line [28,129] and primary blood monocytes [28]. Suppressing these pathways also causes suppression of IL-8 upregulation by LL-37, indicating that activation of these pathways was directly linked to downstream gene regulation and its functional consequences. Another interesting observation is that signalling in human monocytes is serum dependent (cf. antimicrobial activity that is serum antagonized), and much elevated in the presence of GM-CSF [28].

Host defence peptides have also been implicated in wound healing in a number of tissue types and in animal models. Another proposed function of host defence peptides is to accelerate wound healing or closure by stimulating epithelial cell proliferation. Breaches of the physical defences provided by epithelial surfaces represent a serious threat to the host, and an invasive opportunity to many pathogenic bacteria that would otherwise not normally bind exposed extracellular matrix components. Therefore it is of crucial importance that any such damage is promptly repaired. It has been demonstrated that growth factors such as epidermal growth factor are released upon infection and stimulate both wound closure and epithelial cell proliferation [131]. Antimicrobial peptides may also be involved in these processes. In *in vitro* models of airway epithelial cell proliferation, a mixture of human neutrophil peptides (HNP-1 to -3) at concentrations of 4 to 10 μg/ml was effective at increasing epithelial cell proliferation [132]. Similar effects on proliferation were observed using a variety of different peptides [102, 133]. The presence of low (4 to 10 μg/ml) concentrations of HNP1-3 increased the rate of wound closure using an airway epithelial cell line model, and also induced gene expression of the two genes in the mucin family, which are also involved in regeneration of the intact epithelial layer [134]. These concentrations are moderately higher than those described in the lung (Table **1**), but might well be observable in inflammatory situations. However peptides may also operate at physiological concentrations. For example, it has been demonstrated that mice deficient in CRAMP, a mouse homolog of LL-37, are deficient in wound neo-vascularization [130]. Also LL-37 has been demonstrated to induce angiogenesis, a process essential for host defence, wound healing, and tissue repair, at concentrations believed to be of physiological relevance (50-500 ng/ml) [130].

Certain host defence peptides have been shown to induce chemotaxis under conditions of physiological salt concentrations. LL-37 is directly chemotactic for monocytes, T cell, neutrophils and mast cells at concentrations between 10 ng/ml and 50 μg/ml [5,126]. The α-defensins have been shown to be chemotactic for $CD4^+$ and $CD8^+$ T cells [5,135], monocytes and immature dendritic cells [135,136] at concentrations of 10 ng/ml or less, an effect unaltered by the presence of 10% serum [5]. The α-defensins have a complementary spectrum of chemotactic activity, with HBD-2 capable of inducing chemotaxis of memory T cells and immature dendritic cells through the CCR6 receptor [137]. The concentrations required for chemotaxis are generally more consistent with concentrations known to occur in health or disease.

In addition to these direct chemotactic effects, host defence peptides may also stimulate chemotaxis indirectly by inducing chemokine production in a variety of cell types. LL-37 is capable of modulating the expression profile of chemokines, chemokine receptors and additional genes in a macrophage cell line, and induces MCP-1 upon installation into the mouse lung and in whole human blood [26]. Both LL-37 and the α-defensins have also been demonstrated to induce IL-8 production in epithelial cells [26, 138], at concentrations which are only moderately higher than those found *in vivo*.

Finally there are a variety of additional effects of host defence peptides on the effector cells of the innate immune response. These include the capacity of both LL-37 and the human neutrophil peptides to induce mast cell degranulation [139, 140] and a range of interesting activities demonstrated for the porcine cathelicidin PR-39 (reviewed in [122]), including the inhibition of IkBα degradation, abolishing the induction of NF-κB-dependent gene expression [141].

THE ROLE OF HOST DEFENCE PEPTIDES IN ADAPTIVE IMMUNITY

It is clear that host defence peptides have the potential to modulate the innate immune response through a wide variety of mechanisms, but additional studies suggest that these peptides may also play a role in modifying the adaptive immune response (Fig. **3**). When ovalbumin (OVA), was delivered intranasally to mice, the co-administration of human α-defensins HNP1-3, led to enhanced production of the IgG antibodies specific to OVA. In addition, OVA-specific $CD4^+$ T cells were generated, which produced significantly more IFNγ, IL-5, IL-6, and IL-10 [142]. This suggested the capacity of these host defence peptides to act as adjuvants. In a more recent study using a very similar approach, HNP-1, and the human β-defensins HBD-1 and -2, were each capable of enhancing the production of OVA-specific IgG, when only 1 μg of peptide was administered intranasally with OVA [143]. Furthermore, OVA-stimulated splenic lymphoid cell cultures were found to produce significantly decreased levels of IFN-γ, when taken from

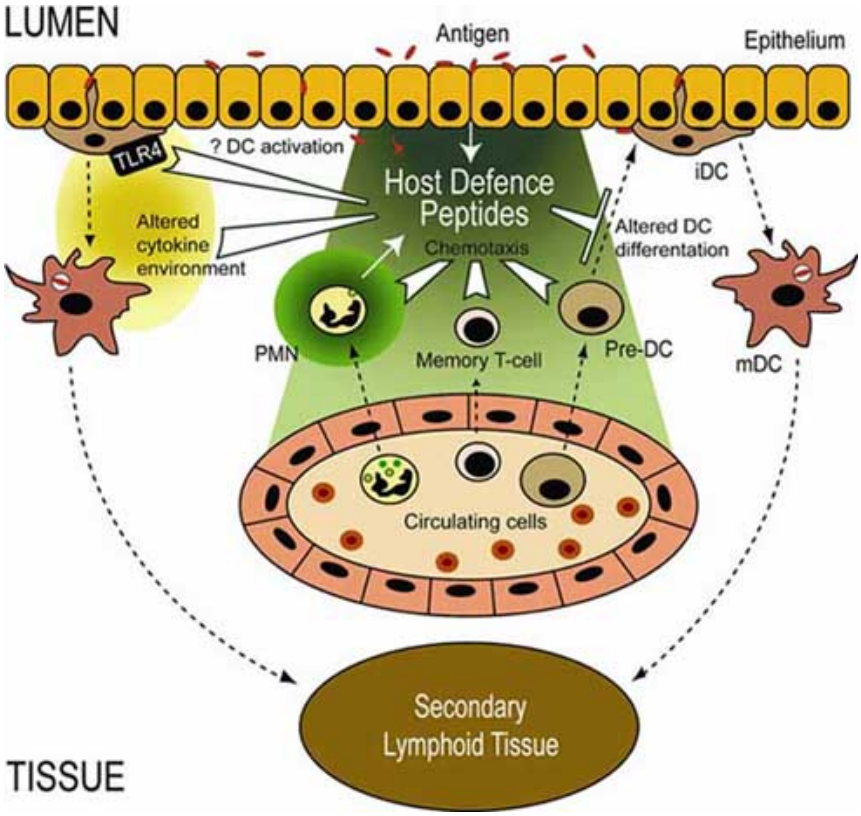

Fig. (3). The role of host defence peptides in adaptive immunity.
In addition to their potential for direct microbicidal activity, anti-endotoxic capacity, and effects on innate immunity, a variety of adaptive immune functions have been proposed for host defence peptides. These include the chemotaxis of lymphocytes (including memory T cells) and monocytes (with the potential for differentiation to iDC), the capacity to act as mitogens for lymphocytes, and the potential to modulate DC differentation and/or maturation. Many peptides are induced at sites of infection and inflammation, produced primarily by neutrophils and epithelial cells, and interact with host cells. These peptides can alter the production of stimulatory cytokines by epithelial cells and leukocytes, with the potential to induce DC maturation, or might directly activate iDC via TLR4. In addition, peptides can alter the differentiation of iDC from precursor cells, generating enhanced iDC with altered antigen capture, presentation and T cell priming capacity. The range of these activities exhibited is peptide specific. In this manner peptides have the potential to modulate the adaptive immune response, functioning as potent adjuvants and generating a more robust response to pathogens.

HNP-1 and HBD-2 treated animals, and significantly more IL-10 after co-exposure of OVA and HBD-1. An additional report, in which mice were treated with an intraperitoneal vaccination combining B-cell lymphoma idiotype antigen and daily 1 μg injections of human α-defensins, also observed increased levels of antigen-specific IgG antibodies [144]. This study demonstrated the capacity for defensins to enhance IFN-γ production and proliferation by concanavalin A-stimulated murine splenic cells at levels as low as 10 ng/ml, and a significant increase in the number of splenic B cells in defensin treated mice. This observation of mitogenic properties could provide a partial explanation for host defence peptide enhancement of the humoral response, but this study also showed an increased resistance to challenge with tumour [144]. This raises the possibility that an antigen-specific cytotoxic T cell response was being generated in addition to a humoral response.

Each of these studies demonstrated that several host defence peptides, when simply co-administered with relatively non-immunogenic antigens, are capable of altering and enhancing the host's adaptive immune response to these antigens. This indicates that these host defence peptides have adjuvant capabilities, perhaps by acting to modulate lymphocyte responses directly, behaving as endogenous danger signals, or functioning indirectly by altering the cytokine environment in which antigen was encountered by key host cells, such as immature dendritic cells (iDC). The physiological significance of the doses used in these studies for *in vivo* homeostatic processes is unclear, distributed over unknown volumes, but might be within relevant ranges and are clearly of interest from an immunotherapeutic standpoint. The published characterizations of transgenic mice with defective production of host defence peptides have not described defects in the adaptive immune responses. However, one mBD-1 knockout model was found to display a defect in generating antibodies to the carbohydrate capsule of pneumococci (Moser, C., personal communication). This is consistent with an *in vivo* role for this constitutively expressed defensin in generating components of the humoral response.

In a study that took an alternative DNA-vaccine approach, mice were immunized with plasmids encoding non-immunogenic lymphoma antigens fused to murine beta-defensins, traditional chemokines, or control plasmids [145].

Cells that were successfully transfected *in vivo* should then express these peptide/lymphoma antigen fusion proteins. This strategy aimed to target antigen to iDC, using the affinity of the peptide portion of these fusion proteins for the iDC-expressed chemokine receptor CCR6. In this case IgG responses were only observed when the plasmids encoded fusions of the antigen and peptides and not after simple co-administration. Interestingly, anti-tumour activity generated in these mice (more effective with murine β-defensin 2) did not correlate with the amplitude of the humoral response (superior with murine β-defensin 3), and indeed could be transferred to other mice with delivery of splenocytes, but not serum, from vaccinated animals. This indicated the generation of cytotoxic T cells in response to non-immunogenic antigens when fused to peptides. The mechanisms underlying these observations have not been fully elucidated. An additional report suggested that murine β-defensin 2 fusion proteins were capable of activating iDC directly in a Toll-like receptor-4 dependent manner, to produce T helper (Th-1) polarized responses [146]. In this context, stimulation of the innate pattern recognition pathways through Toll like receptors would occur in conjunction with interaction with the otherwise non-immunogenic antigen.

These animal studies all suggest that host defence peptides have the potential to act directly on cells of the adaptive immune response, and/or modulate responses at the interface between the innate and adaptive immune systems. The most obvious mechanisms for such a role are the potential for these peptides to directly modulate lymphocyte activity and proliferation [144] as discussed above, and an ability to act directly as chemokines, or to stimulate the production of key classical chemokines. The production and release of peptides by epithelial cells and neutrophils at sites of infection and inflammation has the potential to result in the chemotaxis of monocytes, neutrophils, macrophages, iDC, mast cells and T lymphocytes [5, 126, 136, 137], and the enhancement of chemokine receptor expression on these cells [26]. The additional release of peptides by both neutrophils and mast cells could amplify these gradients, and stimulate the release of potent traditional chemokines (such as IL-8) from epithelial cells [147], while mast cell degranulation would enhance vascular permeability (Fig. **2**). The effect of this positive feedback-loop amplifying the inflammatory response, and of direct chemotaxis, would be to bring key cells of the adaptive immune response to the site of infection. In addition to recruiting memory T cells to the infection site to induce a more rapid cellular response to previously encountered antigens, the recruitment of monocytes and iDC is likely to be critical (Fig. **3**).

The adaptive immune system can be considered to be directed by dendritic cells. These sentinel leukocytes capture antigen in the peripheral tissues and then initiate and orchestrate T-cell helper (Th-1) responses, the nature of which determines the character of the adaptive immune response [148]. This process is critical to generating a successful defence against harmful microbial non-self antigens while maintaining tolerance to self, and is dependent upon the antigen-capturing capabilities of iDC, and antigen-presenting capabilities of mature dendritic cells (mDC). iDC are derived from circulating haematopoietic precursor cells and preDC populations (monocytes and plasmacytoid cells) under the influence of specific cytokines and growth factors [149, 150]. In the tissues these cells encounter and take up antigen. Stimulation of iDC by conserved structures on these antigens, acting via the Toll-like receptors of the innate immune system [151] or by signals from host cytokines, results in DC activation. These activated cells mature to become effective antigen-processing and presenting mDC, migrate to the secondary lymphoid organs and interact with naïve T-lymphocytes [152]. The characteristics of the mDC determine the nature and consequences of this interaction, resulting in proliferation and differentiation, or deletion of T cells, and determine the polarization of the Th response [153]. Whereas steady-state trafficking of non-activated iDC carrying self-antigen is thought to help maintain tolerance, it has been proposed that sustained trafficking of large numbers of highly stimulatory mDC to the T cell areas is necessary for the generation of an effective T cell proliferative response [153]. This would require extensive, repeated recruitment of circulating preDC to the site of infection, with rapid differentiation to replace the "first-line" resident iDC. Thus, at the simplest level, it is conceivable that the *in vivo* effects of host defence peptides on the adaptive immune response are the result of direct and indirect chemotaxis of iDC and monocytes to the site of inflammation. However, this hypothesis must be incomplete, as it fails to explain the generation of both humoral and cytotoxic T lymphocyte responses to non-immunogenic antigens. In these examples, an increase in the number of DC encountered should make no difference to the response in the absence of an activating signal. Indeed, theoretically, this might serve to increase host tolerance to these antigens.

The capacity of host defence peptides to generate an adaptive immune response to non-immunogenic antigens is not understood. However, three hypotheses are supported by the current literature. The first two theories propose that these peptides directly or indirectly provide an activating signal to differentiated iDC concurrent with these cells encountering antigen, while the third proposes peptide modulation of DC differentiation from precursor cells (Fig. **3**).

As described above, lymphoma antigen fused to murine β-defensin 2 has been proposed to activate iDC directly in a Toll-like receptor-4 dependent manner [146], suggesting the capacity of host defence peptides to function as endogenous ligands of innate pattern recognition receptors. However, neither murine β-defensin 3 fusion proteins, nor murine β-defensin 2 in the absence of fusion to lymphoma antigen, has the capacity to do this. In addition, studying a range of peptides at or above proposed physiological concentrations, we have seen no evidence of an ability to directly mature human monocyte-derived DC *in vitro* (Davidson, D. J., Currie, A. J., Hancock, R. E. W., Speert, D. P., unpublished data). These data suggest that direct activation of iDC may not be an inherent property of host defence peptides, or that the temporal coordination of TLR4 ligation and chemokine receptor directed antigen uptake by the same cell is critical.

An alternative mechanism would view the effect of host defence peptides as indirect. Defensins have been shown to increase expression of various cytokines, including IL-8, IL-6, MCP-1 and GM-CSF [154], in different airway epithelial cells, while LL-37 can induce IL-8 and MCP-1 expression in epithelial and monocytic cells [26]. These changes in the cytokine environment may induce a myriad of effects, from the chemotactic activities of MCP-1 and IL-8, and cellular differentiation effects of GM-CSF, to the enhancement of B-cell proliferation and blockade of the suppressive effects of regulatory T cells by IL-6 [155]. Possibly other factors are induced that might activate iDC even in the presence of non-immunogenic antigens. Although this has the potential to explain some *in vivo* observations and support the therapeutic use of peptides as adjuvants, an ability to modulate the responses in the presence of danger is more likely to be of physiological significance. In this regard, following activation with *Staphylococcus aureus* or phorbol myristate, human α-defensins at concentrations as low as 1 nM, can increase the expression of TNF-α and IL-1β by monocytes [156]. These cytokines have the potential to directly induce DC maturation, sharing components of activating pathways with Toll-like receptors, and thus potentially enhancing the generation of highly stimulatory mDC.

The third hypothesis results from our recent observations indicating that the human cathelicidin LL-37 can modulate the differentiation of iDC from precursor cells, with consequent impact upon Th cell polarization [157]. As discussed, the generation of an effective T cell proliferative response has been proposed to require repeated recruitment of circulating preDC to the site of infection, with rapid differentiation to replace the "first-line" resident iDC [153]. These "second-line" DC must also be capable of sustained antigen sampling and highly stimulatory presentation. To date, the host factors involved in differentiation of these cells have not been defined. However, it is clear that the stimulatory nature of mDC is subject to dynamic temporal regulation [158] and can be modified by precursor cell lineage, the specific antigen captured, the receptors engaged, and the microenvironment for both differentiation and maturation [149, 150, 159, 160]. We have demonstrated that LL-37 is a potent modifier of DC differentiation from monocytic precursors [157]. LL-37-derived DC displayed significantly upregulated endocytic capacity, modified phagocytic receptor expression and function, upregulated co-stimulatory molecule expression, enhanced secretion of Th-1 inducing cytokines, and promoted Th-1 responses *in vitro*. Thus this host defence peptide has the capacity to function as a bridge between the innate and adaptive immune systems; indirectly facilitating the generation of an enhanced Th1 response by inducing the differentiation of "primed" second line iDC. We propose that these LL-37-derived iDC may be generated at sites where LL-37 is upregulated in response to infection or inflammation, to promote a more robust adaptive immune response. Although many of these studies were performed with a higher dose of LL-37 (50 μg/ml), changes were observed at doses as low as 5 μg/ml, and in the presence of serum, thus well within the ranges observed *in vivo* during inflammation (Table **1**). The potential for other host defence peptides to function in a similar manner remains to be determined, although preliminary data indicates that related peptides such as the murine homologue CRAMP and the bovine cathelicidin indolicidin may also have the potential to modulate DC differentiation, whereas the human β-defensins HBD-1 and -2 appear to be less effective (Davidson, D. J., Currie, A. J., Hancock, R. E. W., Speert, D. P., unpublished data).

Overall the potential for host defence peptides to modulate the adaptive immune response is evident, but remains largely undescribed. In addition to further exploration of the effects *in vitro*, innovative *in vivo* modelling is a priority. Such studies must seek to dissect the mechanisms underlying these observations and to separate the direct microbicidal activities from the more complex immunomodulatory effects. A clear understanding of the extent of immunomodulatory effects of host defence peptides will be fundamental to their future development as novel therapeutic agents.

CONCLUSIONS

It is clear that at physiological concentrations most human host defence peptides are not antimicrobial in the majority of extracellular sites within the body. Although we cannot rule out the possibility that some direct antimicrobial activity might be observed due to the synergistic antimicrobial properties of peptides working in combination [161] (indeed certain body fluids appear to have innate antimicrobial activity [31]), we feel that the bulk of current evidence argues that this is not the primary function of these host defence peptides. At higher concentrations, or in situations in which the ionic environment is low, such as in the phagosomes of leukocytes, there is no doubt that host defence peptides reach concentrations high enough to be antimicrobial. This may initially appear to be a dichotomy but is consistent with the redundant and efficient nature of evolution. Animal models have demonstrated that host defence peptides are required to reduce bacterial load and inhibit infection, but there is as yet no definitive evidence that these are mediated by direct microbicidal activity as opposed to the myriad of other functions attributed to these peptides. Reports of the immunomodulatory properties of host defence peptides are becoming increasingly frequent and although there is not yet a cohesive picture of their role in the innate and adaptive immune response it is becoming increasingly apparent that they are an enigmatic and essential component of the immune response.

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ABBREVIATIONS

HBD = Human beta defensin

MAPK = Mitogen activated protein kinase

HNP = Human neutrophil peptide

CF = Cystic fibrosis

ASL = Airway surface liquid

BAL = Bronchial alveolar lavage

G-CSF = Granulocyte colony stimulating factor

TNF-α = Tumour necrosis factor alpha.

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