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Immunomodulatory Properties of Defensins and Cathelicidins

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Abstract Host defence peptides are a conserved component of the innate immune response in all complex life forms. In humans, the major classes of host defence peptides include the α- and β-defensins and the cathelicidin, hCAP-18/LL-37. These peptides are expressed in the granules of neutrophils and by a wide variety of tissue types. They have many roles in the immune response including both indirect and direct antimicrobial activity, the ability to act as chemokines as well as induce chemokine production leading to recruitment of leukocytes and lymphocytes to the site of infection, the promotion of wound healing and an ability to modulate adaptive immunity. It appears

that many of these properties are mediated though direct interaction of peptides with the cells of the innate immune response including monocytes, dendritic cells, T cells and epithelial cells. The importance of these peptides in immune responses has been demonstrated since animals defective in the expression of certain host defence peptides show greater susceptibility to bacterial infections. In the very few instances in which human patients have been demonstrated to have defective host defence peptide expression, these individuals suffer from frequent infections. Although studies of the immunomodulatory properties of these peptides are in their infancy, there is a growing body of evidence suggesting that the immunomodulatory properties of these small, naturally occurring molecules might be harnessed for development as novel therapeutic agents.

Abbreviations

PBMC	Peripheral blood mononuclear cells
HNP	Human neutrophil peptide
HBD	Human beta defensin
TLR	Toll-like receptor
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
TNF-α	Tumour necrosis factor alpha
MHC1	Major histocompatibility complex class 1
BAL	Bronchoalveolar lavage

1 Overview

1.1 Host Defence Peptides in Humans

Host defence peptides are small (generally less than 50 amino acids), positively charged peptides that are an evolutionarily conserved component of the innate immune response. Originally characterised as natural antimicrobial agents, it is becoming increasingly apparent that these peptides have a wide range of immunomodulatory properties that are either complementary to, or independent of, antimicrobial activity. Interest in the immunomodulatory functions of these peptides is increasing, and indeed many peptides and proteins with similar characteristics to host defence peptides have been found to have either antimicrobial or immunomodulatory properties, in addition to their primary functions.

In humans the best-characterised host defence peptides are the defensins and the sole cathelicidin, hCAP-18/LL-37. The amino acid sequences of these peptides are summarised in Table 1. In general, the defensins are between 29 and 30 amino acids long (approximately 3.5 kDa) and contain three conserved

Table 1 Sequences of major human host defence peptides

		Sequence
α-Defensins	HNP1	ACYCRIPACIAGERRYGTCIYQGRLWAFCC
	HNP2	CYCRIPACIAGERRYGTCIYQGRLWAFCC
	HNP3	DCYCRIPACIAGERRYGTCIYQGRLWAFCC
	HNP4	VCSCRLVFCRRTELRVGNCLIGGVSFTYCCTRV
	HD5	ATCYCRHGRCATRESLSGVCEISGRLYRLCCR
	HD6	AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL
β-Defensin	HBD1	DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKACCK
	HBD2	TCLKSGAICHPVFCPRRYKQIGTCGLPGTKCCKKP
	HBD3	GIINTLQKYYCRVRGGRCAVLSCLPKEEQIGKCSTRGRKCCRRKK
	HBD4	MQRLVLLLAVSLLLYQDLPVRSEFELDRICGYGTARCRKKCRSQE YRIGRCPNTYACCLRKWDESLLNRTKP
Cathelicidin	hCAP-18/ LL-37	LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES

disulphide bridges (White et al. 1995). The genes for all human defensins are clustered on chromosome 8 (Sparkes et al. 1989; Maxwell et al. 2003). They are further subdivided to include the α- and β-defensins, a distinction based on the organisation of the three characteristic cystine disulphide bonds.

The canonical sequence of the α-defensins is $x_{1-2}CxCRx_{2-3}Cx_3Ex_3GxCx_3Gx_5CCx_{1-4}$, where x represents any amino acid residue. These peptides are rich in cysteine, arginine, and aromatic residues (Selsted et al. 1985). The cysteines are linked 1–6, 2–4, and 3–5. Initially these peptides were isolated from the neutrophils and are thus called human neutrophil peptides (HNP)-1 to -3 (Ganz et al. 1985). The HNPs are expressed at the transcriptional level in the bone marrow, spleen and thymus, where they co-localise with peripheral blood leukocytes (Zhao et al. 1996). The three HNPs are highly homologous, differing by only one amino acid at the NH_2 terminus. Because of the high sequence similarity and difficulties in purifying the individual peptides as well as the high degree in functional similarity, the HNP1–3 are often studied as a group, although certain studies have demonstrated differences in their antimicrobial (Lehrer et al. 1988) and immunomodulatory activities (Chertov et al. 1996). HNP4 was identified as a HNP due to its structural homology to the HNP1–3 (Wilde et al. 1989). This gene differs from the other genes of this family by an extra 83-base pair segment that is apparently the result of a recent duplication within the coding region (Palfree et al. 1993). As with the other HNPs, HNP4 is found in the neutrophils,

but is also called corticostatin because it exhibits corticostatic activity and inhibits corticotrophin-stimulated corticosterone production (Singh et al. 1988). Despite its variation from the conserved sequences of HNP1–3, it appears to have much more potent antimicrobial activity (Wilde et al. 1989).

Two other α-defensins, HD5 and HD6, are found solely in the intestinal tract. HD5 and HD6 were found to be expressed at the transcriptional level solely in the small intestine and in situ hybridisation demonstrated that this expression occurs in the Paneth cells (Jones and Bevins 1992, 1993). Southern blot analysis using a nucleotide probe for the conserved signal sequence of the defensins indicated that a number of genes with high homology to HNPs exist within the human genome.

The β-defensins are expressed in a variety of tissue types, including epithelial cells from the trachea and lung, in the salivary and mammary glands, in a variety of organs such as in the plasma and skin (Bensch et al. 1995; Zhao et al. 1996; Harder et al. 1997; reviewed in Lehrer and Ganz 2002). The expression of certain β-defensins is inducible upon stimulation with bacterial components or pro-inflammatory cytokines and thus these peptides are presumed to be an important component of host defence to infection or inflammation. The canonical sequence for the beta defensins is $x_{2-10}Cx_{5-6}(G/A)_xCX_{3-4}Cx_{9-13}C_{x4-7}CCx_n$. The best characterised members of the β-defensin family are HBD1–3; however, the antimicrobial properties of HBD4 have been recently published (Garcia et al. 2001) and over 20 potential β-defensin homologues have been identified in the human genome based on sequence similarity to HBD1–4 (Schutte et al. 2002).

The cathelicidins are an evolutionarily conserved family of host defence peptides which are found in cows, sheep, guinea pigs, rabbits, mice, and primates (reviewed in Zanetti 2004) and are characterised by having an evolutionarily conserved N-terminal domain called the cathelin domain. In addition, these peptides have a signal sequence, which is believed to target their delivery to the secondary granules of neutrophils. The C terminal domain, which is released by cleavage of proteases, has both antimicrobial and immunomodulatory properties. Despite the conserved nature of the cathelin domain, its function remains unclear, although it has been proposed to block the antimicrobial activity of the cleaved product, presumably as a mechanism which allows storage of the peptide in its inactive form (Zaiou et al. 2003), and there is some evidence that it has anti-protease activity (Zaiou et al. 2003). The sole human cathelicidin LL-37/hCAP-18 is found at high concentrations in its unprocessed form in the granules of neutrophils and is processed upon degranulation and release (Sorensen et al. 2001). Consequently, it is found at sites of neutrophil degranulation. It is also produced by epithelial cells and is found in a number of tissues and bodily fluids, including gastric juices, saliva,

semen, sweat, plasma, airway surface liquid and breast milk (Bals et al. 1998c; Murakami et al. 2002b; Hase et al. 2003; Murakami et al. 2005). Generally epithelial cells have been shown to produce the hCAP-18 form. Although the hCAP-18 has been shown to be cleaved by the neutrophil protease, protease 3, when released from neutrophils, it is not entirely clear how or when hCAP-18 is cleaved when it is produced by epithelial cells. There are a variety of processed forms of hCAP-18 that result from as-yet uncharacterised cleavage processes. For example, a 6-kDa form is found in gastric juice (Hase et al. 2003), while numerous cleavage products are found in the sweat (Murakami et al. 2002b). As well, hCAP-18 from semen is cleaved to a 38-amino acid antimicrobial peptide ALL-38 in the vagina, thus potentially providing some antimicrobial protection after sexual intercourse (Sorensen et al. 2003). There appears to be some overlapping, complementary and possibly even enhanced antimicrobial activity of these isoforms (Murakami et al. 2004); however, to date there is no information about their immunomodulatory properties.

1.2 Induction of Host Defence Peptides

Due to the high homology of HNP1–3, they are often classed as a group. The HNPs are found exclusively in leukocytes and at their highest concentrations in neutrophils where they are localised to azurophilic granules. The recently described HNP4 is also found in azurophilic granules but at much lower concentrations (Wilde et al. 1989). To date there has been no indication that their expression levels can vary substantially. Neutrophils stimulated with IL-8, FMLP or phorbol 12-myristate 13-acetate causes degranulation and release of HNP1–3 (Chertov et al. 1996), thus it is believed that relatively high concentrations of these peptides are present at sites of infection or inflammation. Interestingly, in the course of infection HNP levels increase systemically. Although the mechanism is not entirely clear, the plasma levels of defensins increase significantly in patients experiencing septicaemia or meningitis (from approximately 40 ng/ml to as much as 170,000 ng/ml). The concentration of neutrophil defensins generally correlates with that of IL-8, a potent chemoattractant for neutrophils (Ashitani et al. 2002), as well as the presence of other neutrophil components such as elastase (Zhang et al. 2002). Presumably this occurs due to neutrophil degranulation in response to bacterial or pro-inflammatory stimuli as concentrations decrease after antibiotic treatment (Panyutich et al. 1993).

HD5 and HD6 are expressed throughout the gastrointestinal tract, with the highest levels of expression occurring in the jejunum and ileum (Dhaliwal et al. 2003). Specifically, immunohistochemical studies have shown that

HD5 is expressed by Paneth cells, some villous epithelial cells and in the terminal ileal mucosa (Cunliffe et al. 2001). Although there is great variability between individuals, levels of these defensins are increased in certain disease states such as acute coeliac sprue (Frye et al. 2000b) and are decreased in others such as HIV-related cryptosporidiosis (Kelly et al. 2004). In patients with Crohn's disease or ulcerative colitis, but not in healthy individuals, HD5 immunoreactive cells are present in the crypt region of a large proportion of colonic samples, indicating that expression in these disease states might be dysregulated (Cunliffe et al. 2001). Limited studies have demonstrated that HD5 and HD6 may also be present at low levels in airway epithelial cells (Frye et al. 2000a) or the female reproductive tract (Quayle et al. 1998).

The patterns of expression of the β-defensins are markedly different. In general HBD1 is not up-regulated during the course of infection or inflammation or by stimulation with pro-inflammatory cytokines or bacterial components and in many cases the presence of HBD1 is detectable at the transcriptional level but is undetectable at the protein level (O'Neil et al. 1999; Frye et al. 2000b). However its constitutive presence in airway surface liquid, in intestinal and colon cell lines and in other tissues and bodily fluids implies that it may be involved in maintenance and homeostasis of these areas (Salzman et al. 2003). The expression levels of HBD1 appear to be quite low, ranging from the pg/ml to ng/ml levels in most bodily fluids.

HBD2 is an inducible host defence peptide whose expression is altered under both infectious and inflammatory conditions. It has been found to be up-regulated by pro-inflammatory stimuli in oral epithelial cells and keratinocytes (Krisanaprakornkit et al. 2000), in intestinal and colonic epithelial cell lines (O'Neil et al. 1999; Ogushi et al. 2001; Vora et al. 2004) and in various lung epithelial cell lines (Singh et al. 1998; Becker et al. 2000). HBD2 is believed to be an important component of host defences, since HBD2 expression is depressed in patients with atopic dermatitis who often present with cases of acute and chronic colonisation by *Staphylococcus aureus* (Ong et al. 2002) but is increased in psoriatic skin, a disease in which patients are fairly resistant to bacterial infection. It has been demonstrated to be up-regulated by both commensal and pathogenic bacteria in the oral mucosa and keratinocytes, although by different mechanisms (Krisanaprakornkit et al. 2002; Chung and Dale 2004). HBD2 is inducible during the course of inflammation and infection in the gastrointestinal system, as observed at both the mRNA and protein levels. HBD2 expression in intestinal and colonic epithelial cell lines is increased upon stimulation with IL-1α, flagellin or bacteria, in an NF-κB-dependent manner (O'Neil et al. 1999; Ogushi et al. 2001), and by either lipopolysaccharide (LPS) or lipoteichoic acid (LTA) in a TLR4 and TLR2-dependent manner (Vora et al. 2004). Interestingly, other inflammatory

mediators such as TNF-α and LPS do not induce HBD2 up-regulation (O'Neil et al. 1999). This may reflect 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 (Naik et al. 2001; Hornef et al. 2002). Increases in HBD2 expression have been detected in inflamed intestinal and colonic tissues by RT-PCR and immunohistochemistry, in Crohn's disease and ulcerative colitis (Fahlgren et al. 2003), and in the stomach of patients suffering from *Helicobacter pylori*-induced gastritis (Wehkamp et al. 2003). In the lung, HBD2 has been found to be down-regulated in a variety of infectious and inflammatory diseases including cystic fibrosis and infections (Chen et al. 2004). In lung transplant patients, it has been found to be present at greater than ten times the concentration in patients suffering from bronchiolitis obliterans syndrome, a consequence of rejection of the transplant, compared to transplant patients without signs of rejection (Ross et al. 2004).

Less is known about the newly characterised HBD3. HBD3 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 (Harder et al. 2001; Duits et al. 2003). In addition, this peptide can be induced in amnion cells in response to bacterial components and is found at high concentrations in human fetal membranes, by immunohistology, indicating that it may be involved in maintaining the sterility of the intra-amniotic environment (Buhimschi et al. 2004).

The pro-cathelicidin hCAP18 is found at high concentrations in the granules of neutrophils and is produced by epithelial cells. The CAMP-gene promoter that directs the expression of this peptide contains many transcription factor binding sites (Larrick et al. 1996), including a vitamin D response element (Wang et al. 2004). Consistent with this, hCAP18, and/or its processed product LL-37, has been shown to be up-regulated in sinus epithelial cells, at the transcriptional level, by the bacterial products LPS and LTA (Nell et al. 2004). It is also increased in bronchial airway cells by IL-α (Erdag and Morgan 2002). In other cell types, pro-inflammatory cytokines do not increase the expression of this peptide, implying that other signalling pathways may be involved (Hase et al. 2003). Its expression is increased in gastric epithelial cells upon stimulation with a wild-type strain of *H. pylori*, but not a type IV secretion mutant (Hase et al. 2003), and is found at increased levels in other forms of bacterial infection. It is not entirely clear under what circumstances neutrophils release LL-37.

1.3 Antimicrobial Properties In Vitro

The antimicrobial activity of all host defence peptides is highest in media of low ionic strength, and the activity of most peptides is sensitive to the presence of physiological concentrations of ions such as Na^+, Mg^{2+} and Ca^{2+}. The antimicrobial properties of the β-defensins, for example demonstrate profound salt sensitivity, and in some cases their antimicrobial activity is completely lost at concentrations of 100 mM NaCl (Bals et al. 1998a, 1998b; Garcia et al. 2001). For example, HBD1 is antimicrobial towards Gram-negative bacteria at concentrations of 1–10 μg/ml (Singh et al. 1998), but its antimicrobial activity is almost completely abrogated by the presence of 100 mM sodium ions. Although HBD2 is slightly less sensitive to the presence of sodium ions, its antimicrobial activity is reduced from 0.001–0.1 μg/ml in conditions of low ionic strength to 0.5 μg/ml or more in the presence of 100 mM sodium (Singh et al. 1998). Of the human β-defensins, HBD3 is the most potent antimicrobial. This peptide is more basic, has a broader spectrum and stronger bactericidal activity against Gram-positive and Gram-negative bacteria, as well as yeast, and is salt-insensitive at concentrations less than 200 mM Na^+ ions (Harder et al. 2001).

Most biological fluids, including sputum (Halmerbauer et al. 2000), airway surface liquid (Baconnais et al. 1999) and serum/plasma (Hoshino et al. 2003) contain Mg^{2+} and Ca^{2+} at free concentrations between 1 and 2 mM, and the presence of these ions is generally more detrimental to antimicrobial activity than Na^+ alone. The α-defensins are susceptible to concentrations of Ca^{2+} and Mg^{2+} as low as 0.5 mM (Lehrer et al. 1988). In the presence of 100 mM Na^+ ions, the antimicrobial activity of LL-37 is decreased two- to eightfold (Turner et al. 1998), and in the presence of standard tissue culture media, which contains 150 mM NaCl and 1–2 mM of Mg^{2+} and Ca^{2+}, LL-37 has no killing activity against *S. aureus* or *Salmonella typhimurium* even at concentrations as high as 100 μg/ml (Bowdish et al. 2004).

In some cases, for example in the granules of neutrophils, the concentrations of host defence peptides are estimated to be as great as 10 mg/ml, and there is no doubt that upon ingestion of bacteria these concentrations are sufficient to cause direct antimicrobial activity, despite the presence of divalent cations or other inhibitory substances. However, it is questionable whether these concentrations are reached at mucosal surfaces. For example, in patients suffering from inflammatory lung disease or infection, the concentration of HNPs in the bronchoalveolar lavage (BAL) has been estimated to be 0.7–1.2 and 10 μg/ml in two different studies (Cole et al. 2001; Spencer et al. 2003). This contrasts with antimicrobial activity in low salt medium in

vitro at greater than 10 μg/ml for *S. aureus* and *Escherichia coli* (Nagaoka et al. 2000), and reduction of the infectivity of adenoviruses in an airway epithelial model at concentrations of between 8–50 μg/ml (Spencer et al. 2003).

Interestingly, the antimicrobial activity of the host defence peptides may also be inhibited by components of serum. For example, HNP1 has also been demonstrated to possess antiviral activity towards enveloped viruses, but this activity is abrogated by the presence of serum or albumin (Daher et al. 1986). It is believed but has not been conclusively shown that the high concentrations of α-defensins in neutrophils would overcome any localised serum effects (Daher et al. 1986). Moreover, the antibacterial activity of LL-37 has been demonstrated to be abrogated by the presence of apolipoprotein (Wang et al. 1998).

In contrast to the antimicrobial activity of these peptides, their immunomodulatory properties are generally studied in the presence of standard tissue culture media, which contains physiologically relevant concentrations of ions and serum proteins. Under these conditions, host defence peptides have been demonstrated to induce chemokine production, reduce pro-inflammatory cytokine production, alter transcription, induce proliferation and angiogenesis, induce chemotaxis and alter dendritic cell differentiation. Thus the immunomodulatory properties of host defence peptides are unaffected by physiological ion concentrations and it seems possible that they may be the predominant function of these peptides in vivo. This perspective is quite controversial, and will remain so since it is extremely difficult to discriminate between direct and indirect (i.e. through stimulation of innate immunity) mechanisms of killing. One often used argument for the likely antimicrobial function of these peptides is their substantial variation over evolution (e.g. the mouse CRAMP and human LL-37 peptides share only 67% homology), which could have arisen from the evolutionary pressure of dealing with different pathogens. However, not all antimicrobial proteins are as divergent, and we note that a number of proteins involved in immunity and reproduction show similar "rapid evolution" to the antimicrobial peptides (Emes et al. 2003).

1.4 Evidence for Antimicrobial and Immunomodulatory Properties In Vivo

There has been some debate about whether host defence peptides might be directly antimicrobial in vivo. Certainly, neutrophil granules and the crypts of the lumen contain sufficiently high concentrations of peptides to ensure substantial antimicrobial activity; however, it is less clear that antimicrobial activity occurs at the lower concentrations in such sites as mucosal surfaces,

and it is worth noting that such sites are often heavily colonised by a rich and diverse collection of commensal bacteria. The evidence for antimicrobial activity in certain body sites is in our opinion inconclusive. On the one hand, certain bodily fluids such as sinus fluid (Cole et al. 1999) and gastric fluids (Hase et al. 2003) can directly kill certain micro-organisms, and this antimicrobial activity is ablated or reduced by removal of proteins or immunodepletion with a peptide-specific antibody. However, in certain animal models in which peptides and bacteria are instilled simultaneously, bacterial counts are often not significantly different from mice treated with bacteria alone, despite improved outcome or reduced pro-inflammatory responses (Sawa et al. 1998; Giacometti et al. 2004). The difficulties in assessing the role of host defence peptides in vivo are profound, as it is almost impossible to account for synergistic interactions between peptides and other factors, to assess the actual concentrations at the sites of infection and to discriminate the direct antimicrobial activity of peptides from other less direct effects such as enhancement of inflammatory mechanisms (chemotaxis and recruitment of effector cells, enhancement of nonopsonic phagocytosis, etc.). Nonetheless, creative experiments and animal models have begun to elucidate the roles of these peptides in vivo.

In transgenic mouse model studies in which the expression of certain host defence peptides is ablated, these mice are somewhat more susceptible to infection and carry increased bacterial loads when challenged (Wilson et al. 1999; Nizet et al. 2001). Although this was interpreted as being due to direct antimicrobial activity, other components of host defences must be considered. For example, in a mouse model of peritoneal *Klebsiella pneumoniae* infection, small doses of HNP1 (4 ng–4 μg) caused an increase in leukocyte accumulation. In this model, it was the leukocyte accumulation which was linked to HNP1 induced antimicrobial activity, as the reduction in bacterial counts was significantly diminished in leukocytopenic mice. Similar results were observed in *S. aureus* thigh infections (Welling et al. 1998).

Gain of function studies have found that introducing or increasing the expression of a host defence peptide can reduce bacterial loads in certain animal models of infection. For example, adenovirus transfer of LL-37/hCAP-18 into the lungs of mice that were subsequently challenged with *Pseudomonas aeruginosa* led to a reduction in both the bacterial load and in production of the pro-inflammatory cytokine, TNF-α (Bals et al. 1999), and intriguingly, similar gene therapy decreased susceptibility to sepsis induced by LPS in the complete absence of bacterial infection. In other models, the simultaneous instillation into the mouse lung of *P. aeruginosa* and either of HBD2 or a LL-37 derivative led to reduced lung damage and pro-inflammatory cytokine production, but did not affect bacterial counts (Sawa et al. 1998).

There are very few human diseases that are characterised by defects in host defence peptide production, perhaps emphasizing their importance. However, the neutrophils of individuals with specific granule deficiency, a disease characterised by frequent and severe infections, have a reduction in the size of the peroxidase positive, defensins-containing granules (Parmley et al. 1989) and are deficient in defensins (Ganz et al. 1988). However, it is difficult to assess the extent to which these infections result from the lack of defensins, as these patients are also deficient in other neutrophil components. It is believed that the constitutive production and deposition of neutrophils is of crucial importance to maintaining the immunological balance of the mouth. Patients who suffer from morbus Kostman, a severe congenital neutropenia, and are treated with G-CSF to restore neutrophil level, do not express LL-37 in these cells. One of the manifestations of this disease is frequent and severe infections and periodontal disease (Putsep et al. 2002). It has been proposed that the absence of LL-37 may give a selective advantage to bacteria that at low levels are commensals 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 or marshals other defences. Although a number of oral bacteria are susceptible to LL-37 (<10 μg/ml) at 10 mM NaCl in vitro, far fewer bacteria are susceptible in physiologically more relevant isotonic environments (Tanaka et al. 2000). Although LL-37 has been detected in saliva, the actual concentration was not determined (Murakami et al. 2002a).

Other indirect evidence for the in vivo antimicrobial activity of host defence peptides is that a decreased level of expression often correlates with frequency or severity of disease. For example, HBD2 and LL-37 expression is depressed in patients with atopic dermatitis who often present with cases of acute or chronic colonisation by *S. aureus* (Ong et al. 2002). In contrast to atopic dermatitis, HBD2 expression is increased in psoriatic skin, a disease in which patients are fairly resistant to bacterial infections (Harder et al. 2001; Nomura et al. 2003).

Whether host defence peptides are directly or indirectly antimicrobial, it is apparent that it is of advantage for bacterial pathogens to subvert their expression or activity. For example, *Streptococcus pyogenes* binds to α_2-microglobin and secretes a small proteinase which inhibits LL-37 from interacting with the bacteria and thus prevents LL-37 mediated killing (Nyberg et al. 2004). LL-37 expression has been shown to be decreased in *Shigella* infection, consistent with a proposed mechanism of evasion by this bacterium (Islam et al. 2001). However, it is not clear whether this is a direct down-regulation of expression, or a consequence of denuding the epithelium, with reduced expression in the replacement cells.

2
Host Defence Peptides in the Innate Immune Response

2.1
Role of Host Defence Peptides in Wound Healing

2.1.1
Re-epithelisation and Proliferation

Early experiments with host defence peptides demonstrated that many of these peptides have mitogenic effects on a variety of cells and cell lines. Since modest to high concentrations of host defence peptides are found at sites of infection and inflammation, it has been hypothesised that this proliferative effect might be involved in wound healing and re-epithelisation. Consistent with this hypothesis, both the human and mouse cathelicidins are up-regulated at sites of incision or wounding, even if the wound is sterile. The appearance of cathelicidins in the skin has been ascribed to both synthesis within epidermal keratinocytes, and deposition from granulocytes that migrate to the site of injury (Dorschner et al. 2001). Upon incision, hCAP-18 (the precursor to LL-37) has been shown to be up-regulated in the epidermis bordering the wound. This increase in expression at both the RNA and protein levels was clearly evident at the migrating front of the wound during re-epithelialisation. Levels of hCAP-18 decreased following wound closure and eventually returned to baseline levels when the wound was intact and re-epithelisation was complete. hCAP-18 was found to be an active component in the process of re-epithelisation since antibodies specific for the peptide decreased the rate of re-epithelisation in a concentration-dependent manner (Heilborn et al. 2003). Consistent with this observation, low levels of LL-37 (as low as 50 ng/ml) have been demonstrated to increase proliferation in an endothelial cell line (Koczulla et al. 2003). The importance of this peptide in re-epithelisation has been further inferred from its presence in wounds which are healing normally, but its absence in chronic ulcers (Heilborn et al. 2003).

HNPs are potent mitogens for epithelial cells, squamous cell carcinoma cell lines and fibroblasts in vitro at low concentrations (Murphy et al. 1993; M. Nishimura et al. 2004). Interestingly, in one of the earliest studies of these effects it was demonstrated that the HNPs acted synergistically with insulin to induce proliferation (Murphy et al. 1993). In general it has been hypothesised that the mitogenic properties of the neutrophil defensins on non-myeloid cells is an important component of the healing process. However, certain tumours and tumour cell lines have been demonstrated to inappropriately express neutrophil defensins, and in such cases it is believed that this expression might lead to inappropriate proliferation. For example, in a renal carcinoma

cell line, the α-defensins HNP1–3 are expressed at both the transcriptional and protein levels. At moderate levels (i.e. ≤12 μg/ml), the defensins had mitogenic activity on a subset of these cell lines. By influencing tumour cell proliferation, α-defensins could potentially modulate tumour progression of renal carcinoma cells (Muller et al. 2002).

Moderate concentrations (e.g. ≤10 μg/ml) of neutrophil defensins (HNP1–3) induce proliferation of a lung epithelial cell line in vitro (Aarbiou et al. 2002). Consistent with these observations, a combination of HNP1–3 caused a dose- and time-dependent increase in cell migration and wound closure of an airway epithelial cell line, possibly due to an ability to induce the expression of genes involved in proliferation (Aarbiou et al. 2004). The mitogenic activity of the HNPS and cathelicidins does not appear to be shared by the β-defensins. Although HBD2 has been demonstrated to be up-regulated in chronic ulcers (Butmarc et al. 2004), it has not been demonstrated to be involved in re-epithelisation. In addition, the β-defensins investigated did not increase the proliferation of epithelial cells, squamous cell carcinoma cell lines or fibroblasts (M. Nishimura et al. 2004).

2.1.2 Angiogenesis and Vasculogenesis

An interesting phenomenon which has been observed to occur in response to two cathelicidins, human LL-37 and its mouse homologue CRAMP, is the induction of angiogenesis, which is the process of blood vessel formation and/or growth. The formation of new blood vessels results in restoration of tissues increasing the oxygen supply and the provision of blood substances and cells to these tissues. As such it is a requirement for tissue repair and wound healing as well as for the marshalling of innate immunity. Thus this function is consistent with a role for host defence peptides in the maintenance and repair of tissues. In a chorioallantoic membrane assay, 5 μg of LL-37 induced an increase in blood vessel growth, while in a rabbit hind limb model of angiogenesis, collateral vessel growth and blood flow were increased (Koczulla et al. 2003). Interestingly however, despite the known chemotactic properties of this peptide, no inflammatory infiltrate was detected. The angiogenic properties of LL-37 appear to stem from its direct interaction with endothelial cells rather than induction of growth factors. These data are consistent with the observation that CRAMP knockout mice have reduced vascular structures at the wound edge at the site of injury (Koczulla et al. 2003).

A mouse β-defensin, DefBD29, has been shown to be involved in vasculogenesis, which is the differentiation of endothelial cells from progenitor cells during blood vessel development, leading to the de novo formation of blood

vessels and tubes. Tumours expressing DefBD29 recruit dendritic cell (DC) precursors via CCR6 and result in enhanced vascularisation and growth in the presence of the cytokine Vegf-A (Conejo-Garcia et al. 2004). Interestingly, these DCs differentiate to express both DC and endothelial cell markers in response to Vegf, indicating that these cells undergo endothelial cell-like specialisation after or during migration to newly formed vessels. This implies that host defence peptides may play important roles in vascular development.

2.2 Role of Host Defence Peptides in Chemokine Production and Chemotaxis

It has been observed that there are similarities between chemokines and host defence peptides. Indeed, many chemokines have modest antimicrobial activity (Hieshima et al. 2003; Yang et al. 2003), while a derivative of the highly active antimicrobial peptide, horseshoe crab polyphemusin is a potent antagonist of CXCR4 (Tamamura et al. 1998). Indeed it has been proposed that certain host defence peptides have evolved from duplication of chemokine genes, although this connection is controversial (Durr and Peschel 2002; Yang et al. 2002); consistent with this, certain peptides have chemotactic activity. Interestingly, unlike the chemokines characterised to date, many host defence peptides appear to have chemotactic activity over a wide range of species, and generally speaking these activities are often observed at concentrations 100-fold or more higher than observed with the classical chemokines.

HNP1 and -2 have been demonstrated to induce chemotaxis of T cells in vitro at concentrations of between 0.1–100 ng/ml, with maximal activity occurring at less than 10 ng/ml (Chertov et al. 1996). HNP1 is a more potent chemoattractant of monocytes that HNP2, with optimal activity at concentrations of 10^{-8}–10^{-9} M, while HNP3 failed to induce significant chemotaxis (Territo et al. 1989). Conversely, these peptides were not chemotactic for neutrophils (Territo et al. 1989), and indeed a subsequent study demonstrated that HNP1 actually suppressed polymorphonuclear (PMN) migration to formyl-methionyl-leucyl-phenylalanine but not to interleukin 8 (Grutkoski et al. 2003). In BALB/c mice, 4 h after subcutaneous injection, a mixture of HNP1–3 was demonstrated to induce infiltration of PMNs and mononuclear cells, while in huPBL-SCID mice the defensins-induced infiltrate consisted of modest numbers of $CD3^+$ cells (Chertov et al. 1996). Interestingly, in contrast to in vitro results, in this animal model study, the infiltration of PMNs was observed. However, it is unclear whether PMN infiltration was caused by direct chemotaxis or indirect effects of the peptide treatment. Further studies demonstrated that these peptides specifically lead to chemotaxis of immature dendritic cells and naïve, but not memory, T cells (Yang et al. 2000a).

Collectively, these data indicate that neutrophil granules contain important chemotactic factors which promote the infiltration of cells of both the innate and adaptive immune responses.

The β-defensins HBD1 and HBD2 are chemoattractants for immature dendritic cells and memory T, cells with peak activities occurring at 1 μg/ml (Yang et al. 1999). These activities are mediated through the chemokine receptor CCR6, which also binds the chemokine LARC. HBD2, but not HBD1, has also been demonstrated to be a chemotactic agent for TNF-α treated human neutrophils (Niyonsaba et al. 2004), a response that is also mediated through CCR6.

LL-37 has been demonstrated to be chemotactic for rat mast cells (Niyonsaba et al. 2002b), mouse mononuclear cells and PMNs (Chertov et al. 1996), as well as human neutrophils, monocytes and T cells (De et al. 2000). As LL-37 has been demonstrated to induce a number of chemokines, there has been some debate as to whether it induces chemotaxis directly or indirectly by induction of classical chemokines. In the rat mast cell model, it appears as though this chemotaxis is a direct effect: as when mast cells are cultured with LL-37 and the supernatants are used for the chemotaxis assay, chemotaxis can be blocked by anti-LL-37 antiserum (Niyonsaba et al. 2002b).

Host defence peptides may also indirectly enhance chemotaxis by inducing the production of chemokines from a variety of different cell types, including epithelial cells and monocytes. The HNPs, for example, have been demonstrated to induce IL-8 from lung epithelial cells and cell lines (Van Wetering et al. 1997; Sakamoto et al. 2004) and to induce the production of IL-1β and IL-8 mRNA production (Sakamoto et al. 2004) from a lung epithelial cell line.

It is unclear whether the β-defensins have similar chemokine-inducing activities. HBD2, for example, does not induce IL-8 expression in bronchial epithelial cells (Sakamoto et al. 2004). However, in BAL from patients with diffuse panbronchiolitis, the HBD2 concentration correlated significantly with the numbers of cells recovered from the BAL fluid (total cells, neutrophils, and lymphocytes) (Hiratsuka et al. 2003), implying that there might be a link between this peptide and cellular infiltration to the site of infection.

LL-37 has been demonstrated to induce MCP-1 and IL-8 release in a mouse macrophage and a human bronchial epithelial cell line, respectively, and both chemokines were increased upon stimulation with LL-37 in whole human blood (Scott et al. 2002). LL-37 has also been demonstrated to induce chemokine transcription (IL-8, MCP-1, MCP-3) and release (IL-8) in a mitogen-activated protein kinase (MAPK)-dependent manner in human peripheral blood derived monocytes (Bowdish 2004). Both LL-37 and the HNPs are neutrophil-derived peptides which are released upon neutrophil degranulation. These peptides induce the transcription and release of chemokines,

specifically IL-8, which preferentially attract neutrophils. Consequently the presence of these peptides correlates well with that of IL-8, a potent chemoattractant for neutrophils (Ashitani et al. 2002), as well as the presence of other neutrophil components such as elastase (Zhang et al. 2002).

It appears that host defence peptides induce chemotaxis in two ways: first through direct chemotactic activity of PMNs and mononuclear cells mediated through CCR6 and other as yet to be identified receptors and second through inducing chemokine production which would hypothetically increase the numbers of neutrophils and monocytes at sites of infection. This then would have the net effect of promoting or marshalling cells important in innate immunity to the sites of excessive production (through induction) or deposition (through neutrophil degranulation) of these host defence peptides. This then begs the question as to whether host defence peptides are overtly pro-inflammatory.

2.3
Anti-inflammatory (Anti-endotoxin) Roles of Host Defence Peptides

Early experiments determined that a number of host defence peptides from various sources bound to LPS from diverse Gram-negative bacteria and reduced LPS-induced release of pro-inflammatory cytokines (e.g. TNF-α, IL-1, IL-6) and nitric oxide from monocyte or macrophages and protected mice from LPS lethality (Larrick et al. 1994, 1995; VanderMeer et al. 1995; Kirikae et al. 1998).

Initial studies focussed on the unprocessed form of cathelicidin, hCAP-18 (Kirikae et al. 1998); however, it was later found that the LPS-binding properties of the peptide were contained within the processed 37-amino acid C-terminal domain, LL-37 (Turner et al. 1998). It has been proposed that the anti-endotoxic properties of these peptides are the result of the inhibition of binding of LPS to CD14 (Nagaoka et al. 2001) and lipopolysaccharide-binding protein (LBP) (Scott et al. 2000), and/or indirect effects on cells (Scott et al. 2002). LL-37 has been shown to block a number of LPS-induced inflammatory responses, including contractility and (nitric oxide) NO release in aortic rings (Ciornei et al. 2003), pro-inflammatory cytokine production in a macrophage cell line and in animal models (Scott et al. 2002) (Ohgami et al. 2003), suppression of leukocyte infiltration in a model of endotoxin-induced uveitis (Ohgami et al. 2003) and lethality in animal models of sepsis (Scott et al. 2002). These effects occur at concentrations in the physiological range for LL-37 (1–5 μg/ml) and may reflect a natural role for LL-37 in the body (e.g. balancing of the potential stimulus by endotoxin from commensals). This anti-endotoxin activity appears to correlate with an ability to dampen the

pro-inflammatory effects of the Gram-positive surface molecule lipoteichoic acid (Scott et al. 2002).

It appears that there may be marked differences in the ability of LL-37 and the defensins to inhibit the pro-inflammatory effects of endotoxin. For example, HNP1 and HBD2 are not potent inhibitors of LPS–LBP binding (Scott et al. 2000). In ex vivo whole blood experiments, HNP1 was approximately 1,000-fold less potent than BPI at reducing TNF-α in response to Gram-negative bacteria and is much less potent in blocking endotoxin activity, as assessed by a surrogate assay, the *Limulus* amoebocyte lysate assay, or in priming PMN for arachidonate release or stimulating leukocyte oxidase activity (Levy et al. 1995). Thus, the ability to bind to and neutralise endotoxin-induced activity in humans may be more evident for LL-37 and other proteins such as bacterial permeability-inducing protein (BPI) and LBP (Weiss 2003).

2.4 Interactions with Effector Cells of the Innate Immune Response

2.4.1 NK Cells

Natural Killer cells are $CD56^{-}CD3^{-}$ lymphocytes that are an important component of the innate immune response. They kill transformed and infected cells, but unlike T cells they are active against cells that have decreased or ablated expression of major histocompatibility complex class 1 (MHC1) molecules. The cytolytic properties of NK cells are increased in the presence of cytokines produced by cells of the innate immune response. NK cells themselves produce cytokines, such as IFN-γ, which are involved in the enhancement of both the innate and adaptive immune responses. NK cells contain a wide variety of cytotoxic peptides of which granulysin (NK-lysin) is considered to be the most important (Kumar et al. 2001). Recently NK cells have also been demonstrated to express the transcripts for LL-37 and HNP1–3, and these peptides were found in the supernatants of IL-2-treated cells consistent with an involvement in the cytotoxic properties of these cells (Agerberth et al. 2000), or alternatively an immunomodulatory role. Consistent with these observations, it has been shown that both TLR2 and TLR5 agonists induce the release of HNP1–3 from NK cells into the supernatant and that this release is increased synergistically in the presence of other cytokines found at the site of inflammation (Chalifour et al. 2004).

It is not entirely clear what the role of defensins may be in modulating NK-induced cytotoxicity. In one study it was found that NK mediated cytolysis of the transformed cell line KN62 is decreased in the presence of HNP1–3 in a dose-dependent manner. As well, NK cells treated with HNPs

had a decreased expression of both CD16 and CD56 (Zhang et al. 2004). This study also demonstrated that there are high concentrations of HNPs due to the infiltration of neutrophils in colorectal tumours, but not in surrounding healthy tissue. Thus the authors hypothesised that the presence of HNPs might actually protect cancerous cells from NK cytolysis. A conflicting study demonstrated that PBMCs, treated with opsonin-coated zymosan particles, induced the release of substances that enhanced NK-mediated cytotoxicity. These substances were identified as neutral serine proteases and HNPs. Of the peptides tested, HNP1 was the most potent, increasing NK-mediated cytolysis optimally at a concentration of 1.25 μg/ml (Lala et al. 1992). Clearly, further studies are required to fully elucidate the role that host defence peptides have on NK mediated cytoxicity.

2.4.2 Monocytes and Macrophages

Monocytes and macrophages do not express high levels of defensins or cathelicidins unless stimulated by LPS or pro-inflammatory mediators (Agerberth et al. 2000; Duits et al. 2002). However, when thus stimulated, they secrete as yet unidentified factors that stimulate epithelial cells and keratinocytes to produce host defence peptides (Liu et al. 2003). Monocytes and macrophages are, however, quite responsive to stimulation with these peptides and both LL-37 and the defensins have been demonstrated to induce chemotaxis (Territo et al. 1989; De et al. 2000). It has been noted that host defence peptides are strong inducers of chemokine activity in monocytes (Chaly et al. 2000; Bowdish 2004). Interestingly it has been demonstrated that the HNPs are able to prevent HIV replication in monocytes and monocyte-derived macrophages and that this property may be due to their ability to induce chemokine production and/or receptor antagonism (Guo et al. 2004). HNP1 and HNP2 were both demonstrated to induce production of MIP-α and MIP-1β, the ligands for CCR5 in monocyte-derived macrophages and to prevent replication of a CCR5 tropic strain of the virus, presumably by blocking virus binding to CCR5 (Guo et al. 2004).

There has been some evidence that host defence peptides might work as opsonins (Fleischmann et al. 1985; Sawyer et al. 1988). Although this property would be predicted to generally enhance that antimicrobial activity associated with these peptides, one study demonstrated that an LL-37 derivative actually promoted infectivity of *Coxiella burnetii*, an intracellular pathogen of macrophages (Aragon et al. 1995).

Generally, host defence peptides are thought to possess anti-inflammatory properties, as described above. However, in some cases, they may actually

enhance some aspects of a pro-inflammatory response. LL-37, for example, has been demonstrated to enhance IL-1β processing and release in LPS-primed primary human monocytes (Elssner et al. 2004). This property appears to be conserved across a range of host defence peptides from a number of different species (Perregaux et al. 2002).

2.4.3 Mast Cells

Mast cells are distributed throughout the body and are also found in low amounts in the blood. These cells rapidly accumulate at sites of infection, and upon encountering certain bacterial components or pro-inflammatory stimuli they promote the inflammatory response by releasing histamine, which causes vasodilation and thus assists in the recruitment of cells and substances from the blood. Two host defence peptides, LL-37 and HBD2 have been demonstrated to be chemotactic for rat mast cells, although they may work by different mechanisms (Niyonsaba et al. 2002b, 2003). Thus mast cells may accumulate at sites of high concentrations of host defence peptides such as at sites of neutrophil degranulation or at epithelial surfaces in vivo. HBD2 and LL-37 as well as the HNP1–3 and HNP homologues from rabbits and guinea pigs have also been demonstrated to induce histamine release (Befus et al. 1999; Niyonsaba et al. 2001). This property may be especially important in the development of host defence peptides as drugs, as mast cell degranulation is a potentially detrimental side effect.

2.4.4 Epithelial Cells

Host defence peptides have been demonstrated to interact with epithelial cells. Neutrophil peptides have been demonstrated to induce proliferation (M. Nishimura et al. 2004), induce chemokine production (Van Wetering et al. 1997) and stimulate cell signalling pathways (Bowdish 2004). LL-37 has been demonstrated to bind to a lung epithelial cell line in a manner which suggests that it may have more than one receptor (Lau et al. 2005). It has also been demonstrated that binding and subsequent internalisation is required in order to induce IL-8 production (Lau et al. 2005).

3
Host Defence Peptides in the Adaptive Immune Response

3.1
Adjuvant Activity

In addition to apparently having multiple roles in innate immunity, it is becoming clear that host defence peptides can modulate the adaptive immune response, and several studies have now demonstrated adjuvant activities of host defence peptides in vivo. The mechanisms involved remain unclear, although these activities could reflect the innate immunity modulating activity of host defence peptides and the fact that there appears to be a strong interconnection between innate and adaptive immunity.

The relatively non-immunogenic model antigen ovalbumin (OVA) is widely used to study adaptive immune responses. Intranasal co-administration of human α-defensins HNP1–3 with OVA was shown to enhance the production of OVA-specific IgG antibodies and OVA-specific $CD4^+$ T cells, which produced significantly more IFNγ, IL-5, IL-6, and IL-10 (Lillard et al. 1999). This indicated the capacity of α-defensins to alter the host response to OVA, acting as adjuvants to promote a mixed T helper (Th) cell response. In two other recent studies, HNP1, the human β-defensins HBD1 and HBD2 (Brogden et al. 2003), and a simple synthetic peptide $KLKL_5KLK$ (Fritz et al. 2004) were also demonstrated to be effective adjuvants. The observation that such effects are observed with the model peptide $KLKL_5KLK$ suggests the possibility of a relatively non-specific mechanism, and that such activities may therefore be seen with a broad range of host defence peptides. However, the nature of the enhanced responses may depend both on the antigen and the peptide used. In contrast to the mixed Th-1/Th-2 response enhanced in the HNP1–3-treated animals (Lillard et al. 1999), OVA-stimulated splenic lymphoid cell cultures were found to produce significantly decreased levels of IFN-γ, when taken from HBD2-treated mice (Brogden et al. 2003). On the other hand, although $KLKL_5KLK$ induced a strong Th-2 type response when co-administered with OVA, it enhanced a mixed response when the trivalent influenza split-vaccine FLUVIRIN was used as antigen, with the production of both IgG_1 and IgG_2 antibodies (Fritz et al. 2004). Interestingly, this report also demonstrated that a peptide could markedly enhance antigen association with a monocytic cell line in vitro, and that co-administration in vivo could result in the formation of a transient depot of antigen at the site of injection. These observations indicate that antigen uptake by antigen-presenting cells (APCs) might be enhanced in the presence of the peptide, and thus influence responses in the presence of $KLKL_5KLK$ in vivo. Although these studies clearly showed altered humoral and Th responses to

antigens, the functional consequences of these alterations were not clearly demonstrated.

In another study, mice were given an intraperitoneal vaccination combining a B-cell lymphoma idiotype antigen and daily 1 μg injections of human α-defensins. This study also demonstrated adjuvant activity, whereby the defensins led to increased levels of antigen-specific IgG antibodies and enhanced IFN-γ production by splenic cells (Tani et al. 2000). Moreover, defensins showed mitogenic properties (with a significant increase in the number of splenic B cells) and led to an increase in resistance to tumour challenge. The latter observation raised the possibility that an antigen-specific cytotoxic T cell response was being generated in addition to a humoral response.

These studies collectively demonstrate that co-administration of host defence peptides with antigens can enhance and perhaps alter the nature of the host's specific adaptive immune responses in vivo. This raises the question of whether host defence peptides might naturally act as endogenous adjuvants to enhance normal immunological responses, since many peptides can be up-regulated or secreted at sites of infection and inflammation. It is unclear whether the doses used in such studies to assess in vivo immunological processes are within relevant physiological ranges. The physiological significance should be addressed by examining transgenic mice with defective production of host defence peptides, although the issue of possible functional redundancy amongst the many murine defensins must be considered when examining single gene knockouts. The published characterisations of such mice have concentrated on innate responses and have generally not described defects in adaptive immune responses (Nizet et al. 2001; Morrison et al. 2002; Moser et al. 2002). However, one mBD-1 knockout model was found to display a defect in generating antibodies to the carbohydrate capsule of pneumococci (C. Moser, personal communication). While this is consistent with an in vivo role for this constitutively expressed defensin in generating an effective humoral response, it is clearly an area requiring further study. Regardless of possible physiological significance, the adjuvant effects of host defence peptides are clearly of interest from an immunotherapeutic and vaccinology perspective.

In contrast to the studies that have co-administered host defence peptides and antigens, other groups have taken an alternative DNA-vaccine approach. This methodology involved immunizing mice with DNA plasmids encoding non-immunogenic lymphoma antigens fused to murine β-defensins (Biragyn et al. 2001). Successfully transfected cells of an undefined nature should then express the peptide/lymphoma antigen fusion proteins. This strategy represents an attempt to target antigen to immature dendritic cells (iDCs), by exploiting the affinity of the β-defensin portion of the fusion proteins for the

chemokine receptor CCR6, expressed on iDCs. This approach also demonstrated an adjuvant capacity for host defence peptides; however, IgG responses were only observed when the plasmid encoded a fusion of the antigen and peptide, and not observed after simple co-administration of peptide and antigen. Interestingly, anti-tumour activity was also generated in these mice (most effectively with murine β-defensin 2), but did not correlate with the amplitude of the humoral response (superior with murine β-defensin 3). Furthermore, this anti-tumour activity could be transferred to other mice with the delivery of splenocytes, but not serum, from vaccinated animals, indicating the generation of cytotoxic T cells in response to non-immunogenic antigens when fused to peptides. In another recent study, using a similar approach, immunisation of mice with a plasmid fusing the human cathelicidin LL-37 to M-CSFR (acting as a tumour antigen in this model) also generated enhanced antigen-specific humoral and cytotoxic responses, and prolonged survival in a tumour model (An et al. 2004). LL-37 fusion plasmids were found to be significantly more effective than the M-CSFR plasmid alone, or co-administration of separately encoded M-CSFR and LL-37 plasmids.

These animal studies all demonstrate the adjuvant capacity of host defence peptides in vivo, but the mechanisms underlying these observations have not been fully elucidated. A variety of hypotheses can be proposed, including direct modulation of lymphocyte responses, mitogenic effects, chemotactic capacity, increased APC antigen uptake and consequently enhanced presentation, activity as endogenous danger signals, alterations to the APC cytokine environment, or direct modulation of APC function (Fig. 1).

The most obvious mechanisms might include altered antigen uptake (Fritz et al. 2004) and direct modulation of lymphocyte activity and proliferation (Tani et al. 2000), boosting APC presentation, cellular and humoral responses. This could be further enhanced by direct chemotactic effects of host defence peptides, resulting in the chemotaxis of monocytes, neutrophils, macrophages, iDCs, mast cells and T lymphocytes (Territo et al. 1989; Chertov et al. 1996; Yang et al. 1999, 2000a, 2000b; Niyonsaba et al. 2002a, 2002b), and the enhancement of chemokine receptor expression on these cells (Scott et al. 2002). In addition, host defence peptides could act indirectly to stimulate the release of potent traditional chemokines (such as IL-8) from epithelial cells (Van Wetering et al. 1997), and/or cause mast cell degranulation (Niyonsaba et al. 2001), enhancing vascular permeability. These direct and indirect chemotactic effects could amplify the inflammatory response and bring key cells of the adaptive immune response to the location of the antigen. While recruiting memory T cells to an infection site may induce a more rapid cellular response to previously encountered antigens, the recruitment of monocytes and iDC is likely to be critical to generating the initial response.

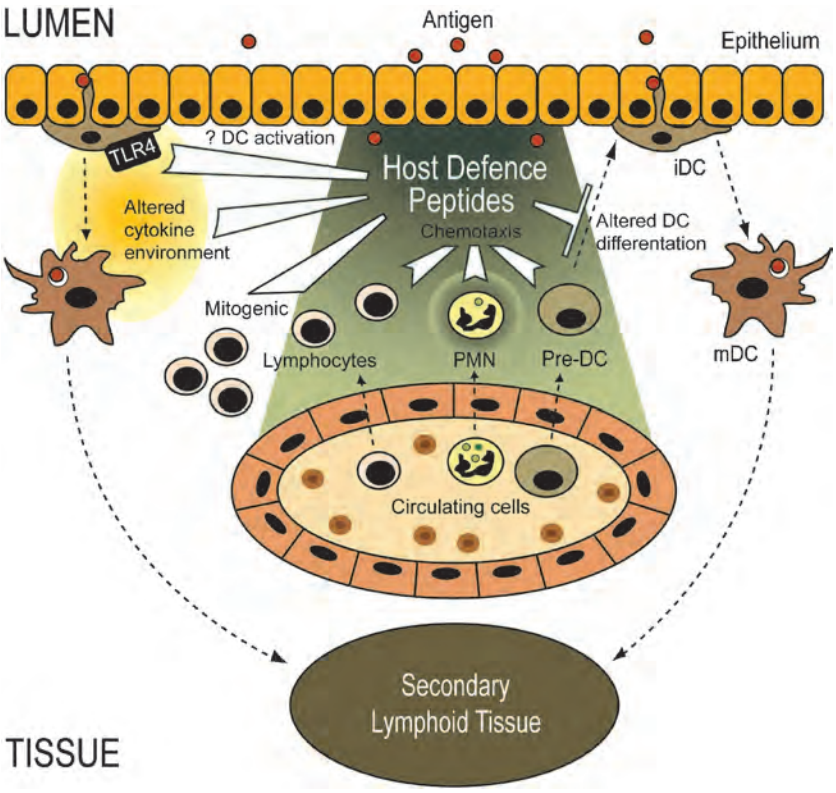

Fig. 1 Cationic host defence peptides modulate the adaptive immune response. Cationic host defence peptides, produced by epithelial cells, neutrophils (*PMN*), transfected cells, or added exogenously, might alter the adaptive immune response to antigen by inducing: (a) chemotaxis of immature dendritic cells (*iDC*), monocytes/Pre-DCs, PMN and T cells, (b) modulation of lymphocyte activity and/or proliferation, (c) alteration of the local cytokine environment, (d) direct iDC activation via TLR4, or (e) generation of primed iDCs with enhanced antigen uptake and presentation capacity. (Reproduced with kind permission from *Leukemia Research*)

Dendritic cells are sentinel leukocytes that 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 (Moser and Murphy 2000). This process is critical to generating a successful defence against harmful microbial non-self antigens while maintaining tolerance to self. It is dependent upon the antigen-capturing capabilities of iDCs, and antigen-presenting capabilities of mature dendritic cells (mDCs). iDCs

are derived from circulating haematopoietic precursor cells and preDC populations (monocytes and plasmacytoid cells) under the influence of specific cytokines and growth factors (Liu 2001; Pulendran et al. 2001). In the tissues, these cells encounter and take up antigen. Stimulation of iDCs by conserved structures on certain microbial antigens, acting via the Toll-like receptors (TLRs) of the innate immune system (Medzhitov and Janeway 2000) or by signals from host cytokines, results in DC activation. These activated cells mature to become effective antigen-processing and presenting mDCs, migrate to the secondary lymphoid organs and interact with naïve T-lymphocytes (Banchereau et al. 2000). The characteristics of the mDCs determine the nature and consequences of this interaction, resulting in proliferation and differentiation, or deletion of T cells, and determine the polarisation of the Th response (Lanzavecchia and Sallusto 2001). Whereas steady-state trafficking of non-activated iDCs carrying self-antigen is thought to help maintain tolerance, it has been proposed that sustained trafficking of large numbers of highly stimulatory mDCs to the T cell areas is necessary for the generation of an effective T cell proliferative response (Lanzavecchia and Sallusto 2001). This would require extensive, repeated recruitment of circulating preDCs to the site of infection, with rapid differentiation to replace the "first-line" resident iDCs. 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 iDCs and monocytes to the site of inflammation.

3.2 Mechanisms

Thus, chemotaxis, altered antigen uptake, and mitogenic effects on lymphocytes offer potential mechanisms by which host defence peptides may enhance responses to immunogenic antigens. However, these explanations do not account for the generation of humoral and cytotoxic T lymphocyte responses to non-immunogenic antigens observed in vivo. In these examples, an increase in the number of DCs encountered and the amount of antigen taken up 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. Despite this, host defence peptides clearly enhance an adaptive immune response to non-immunogenic antigens in vivo.

On the basis of current literature, we propose three hypotheses that might explain these observations. The first two theories propose that these peptides directly or indirectly provide an activating signal to differentiated iDCs concurrent with these cells encountering antigen, while the third proposes peptide modulation of DC differentiation from precursor cells.

In one intriguing report, it was demonstrated that murine β-defensin 2 fusion proteins were capable of activating iDCs directly in a TLR-4 dependent manner, to produce T helper (Th-1) polarised responses (Biragyn et al. 2002). In the context of these DNA plasmid vaccines, stimulation of the innate pattern recognition pathways through TLR would occur in close spatial and temporal conjunction to an otherwise non-immunogenic antigen. This suggests that host defence peptides might be capable of functioning as endogenous ligands of innate pattern recognition receptors. However, activation of iDCs was not observed with murine β-defensin 2 in the absence of fusion to lymphoma antigen. Although this appears to make it improbable that this mechanism is responsible for most of the above-described in vivo observations, it is possible that peptide and antigen concentrations are much higher in co-administration studies, than when relying on DNA plasmid expression. Thus, if the temporal coordination of TLR4 stimulation and antigen presentation are critical, this may be achieved by high concentration co-administration and depot formation at the site of delivery but, when utilizing a DNA vaccine approach, require peptide fusion. However, despite proving effective as an adjuvant for humoral responses (Biragyn et al. 2001), murine β-defensin 3 fusion proteins did not have TLR-4 dependent iDC-activating capabilities (Biragyn et al. 2002). Furthermore we have seen no evidence of an ability to directly mature human monocyte-derived DC in vitro when studying a range of peptides at or above the putative physiological concentrations, (DJ Davidson, AJ Currie,, REW Hancock, DP Speert, unpublished data). These data indicate that direct activation of iDCs may not be an inherent property of host defence peptides. Thus, although direct activation of iDCs is unlikely to be the basic mechanism underlying host defence peptide adjuvant activities, we cannot rule out a peptide-specific effect in which temporal coordination of TLR4 ligation and chemokine receptor-directed antigen uptake by the same cell are critical.

An alternative mechanism to explain altered iDC activation is to suggest that the effects of host defence peptides might be indirect, acting to alter the milieu in which these cells encounter antigen. Defensins have been shown to increase expression of various cytokines, including IL-8, IL-6, MCP-1 and GM-CSF (van Wetering et al. 2002), in different airway epithelial cells, while LL-37 can induce IL-8 and MCP-1 expression in epithelial and monocytic cells (Scott et al. 2002). Changes to 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 (Pasare and Medzhitov 2003). Possibly other factors are induced that might activate iDCs even in the presence of non-immunogenic antigens. Following activation of monocytes with *S. aureus* or phorbol myristate, human α-defensins at con-

centrations as low as 1 nM, can increase the expression of TNF-α and IL-1β (Chaly et al. 2000). These cytokines have the potential to directly induce DC maturation, sharing components of activating pathways with TLR, and thus potentially enhancing the generation of highly stimulatory mDCs. However, while such mechanisms might therefore be proposed at sites of inflammation, similar activities in the presence of non-immunogenic antigens are only speculative.

The third hypothesis relates to our recent discovery that the human cathelicidin LL-37 can modulate the differentiation of iDCs from precursor cells, with consequent impact on Th cell polarisation (Davidson et al. 2004). The stimulatory nature of DCs is subject to dynamic temporal regulation (Langenkamp et al. 2000) and can be modified by precursor cell lineage, the specific antigen captured, the receptors engaged, and the microenvironment for both differentiation and maturation (Liu 2001; Pulendran et al. 2001; de Jong et al. 2002; Boonstra et al. 2003). We demonstrated that LL-37 has the potential to act as an endogenous environmental modifier of DC differentiation (Davidson et al. 2004). LL-37-primed DCs displayed significantly upregulated endocytic capacity, modified phagocytic receptor expression and function, up-regulated co-stimulatory molecule expression, enhanced secretion of Th-1-inducing cytokines, and promoted Th-1 responses in vitro. These results suggest the potential for host defence peptides to exert effects on the adaptive immune system by priming newly differentiating DCs to enhance their antigen uptake and presentation capabilities and influence the nature of the response they will subsequently generate. According to this hypothesis, host defence peptides would not simply affect "first-line" resident iDCs, but act upon the differentiating "second-line" DCs that can sustain highly stimulatory presentation of antigen to generate an effective T cell proliferative response. In the context of a physiological role, LL-37-primed iDCs might be generated at sites where LL-37 is up-regulated in response to infection or inflammation, be matured by immunogenic antigens, and promote a more robust adaptive immune response. However, LL-37-primed iDCs also have increased expression of the co-stimulatory molecule CD86 in vitro in the absence of activating stimuli and any other signs of maturation. If such cells were generated in vivo in the presence of a high concentration depot of host defence peptides at the site of vaccination, or in an area of peptide overexpression by host cells transfected with a DNA vaccine, they might be capable of presenting non-immunogenic antigen in a stimulatory context. Although enhanced humoral and cytotoxic T-lymphocyte (CTL) responses in DNA vaccinated mice are dependent on the fusion of LL-37 and the antigen (An et al. 2004), this might again relate to issues of local concentration and co-presentation to the same cells. Interestingly however, enhanced splenocyte IFN-γ responses were

observed not only in mice vaccinated with the LL-37 fusion plasmid, but also in those given separately encoded LL-37 and antigen plasmids. This might reflect the enhanced IFN-ɣ responses of T cells stimulated with LL-37-primed DCs in vitro. However, further research is required to establish the in vivo significance of the LL-37-priming of DCs observed in vitro. Furthermore, the effects of other host defence peptides on DC differentiation have not been described, raising uncertainty about this hypothesis in the context of the in vivo studies using defensins, or synthetic peptides as adjuvants.

In conclusion, 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 to dissect the mechanisms underlying these observations. A clear understanding of the extent and mechanisms of the immunomodulatory effects of host defence peptides will be fundamental to their future development as novel therapeutic agents. However, these early in vivo studies demonstrate great potential for targeting tumours, recalcitrant, antibiotic-resistant pathogens, infections for which effective vaccines do not exist, and vaccines, which generate suboptimal responses of an inappropriate nature.

4 Conclusion

Mammalian host defence peptides were originally discovered as components of the non-oxidative killing mechanisms of neutrophils. In the granules of neutrophils, these peptides are found at sufficiently high concentrations to be antimicrobial. However, it is less clear that this is the case at mucosal surfaces or in other body fluids, especially at sites that already support a rich and diverse normal flora. Certain body fluids, including sinus fluid and gastric juices, have innate antimicrobial activity against certain bacteria, and the components that appear to contribute to this include a variety of antimicrobial proteins (e.g. lysozyme, secretory phospholipase A_2), as well as peptides (e.g. defensins) (Cole et al. 2002). However, the specific contributions of each of these components to overall antimicrobial activity has not been determined, and given the moderate levels of peptides often found in these fluids, synergy between individual agents working in combination may be important, as been demonstrated for some peptides, and combinations of lysozyme and peptides in vitro (Singh et al. 2000; Yan and Hancock 2001).This is still further complicated at sites such as the mucosa when considering the abilities of host defence peptides to modulate innate immunity, as discussed extensively in this review.

One approach to trying to resolve these mechanisms is to use genetic strategies using either knockout models in animals or specific genetic deficiencies in humans (Ganz et al. 1988; Nizet et al. 2001; Putsep et al. 2002; Salzman et al. 2003). Such studies have clearly demonstrated that defensins and cathelicidins are integral components of host defence in mammals, and that these peptides are required to reduce bacterial load and inhibit infection. However, they do not always permit the discrimination between the various potential mechanisms of host defence peptides, namely direct antimicrobial activity, synergistic activity with other antimicrobial components and/or the broad range of abilities to modulate immunity. Indeed, these distinctions may be unimportant, as they all have the same net result, namely the control of potentially dangerous microbes. We hypothesise that all of these mechanisms operate in the body of mammals, and that any given peptide may have different roles in anti-infective immunity according to the body site it is found, its local concentration, the prevailing physiological conditions, and the other antimicrobial and cellular components of immunity at that site. A clear illustration of this complexity is provided by a study of the use of human defensin 1 (HNP-1) to treat experimental peritoneal *K. pneumoniae* infections (Welling et al. 1998). In this model, HNP1 injection was shown to markedly reduce bacterial numbers, but the antibacterial effect was associated with an increased influx of leukocytes into the peritoneal cavity, and this was strongly related to the antibacterial effect, as no such activity was observed in leukocytopenic mice.

A further challenge to our thinking, and possibly the most profound question in innate immunity, is how mammals manage to support a complex normal flora while retaining the ability to respond to potentially dangerous pathogens. The Toll-like receptors (TLRs), which represent one of the major "triggers" of innate immunity, do not really distinguish between the conserved surface molecules from pathogens and commensal organisms. Thus it is of interest as to whether host defence peptides may play a role in this delicate dance between symbiosis and pathogenesis. As shown by E. Nishimura et al., many commensal bacteria from the oral cavity are quite susceptible to HBD2, while Chung and Dale indicated that both commensals and pathogenic bacteria can induce this defensin (Chung and Dale 2004; E. Nishimura et al. 2004). Conversely, Putsep et al. compared germ-free and normal mice to conclude that an intestinal microflora does not have a major influence on the production or processing of defensins (Putsep et al. 2000). However, we consider at least one activity of these peptides might play a role for host defence peptides in homeostasis, namely anti-endotoxic activity, which in our experience is expressed at lower concentrations of LL-37 than other immunomodulatory activities. It seems possible that this would provide a mechanism for balancing the po-

tential stimulation of TLRs by surface molecules from commensal organisms. Innate immunity would then be triggered by local perturbations of peptide concentrations though mechanisms such as degranulation of phagocytes, or specific up-regulation by certain cytokines. In addition, the efficiency of these peptides might the increased by other local factors, for example phagocytes that enter the local site (Welling et al. 1998) or local cytokines such as GM-CSF that has been shown to enhance MAPK signalling by LL-37 (Bowdish 2004).

The full spectrum of the immunomodulatory properties of these peptides is not yet known and each new report demonstrates that the range and importance of these immunomodulatory effects is greater than initially suspected. Most likely both antimicrobial and immunomodulatory activities are to some degree involved, as this is consistent with the redundant and efficient nature of evolution, and with the concept of innate immunity as a network of overlapping mechanisms. Understanding the interplay between host defence peptides and innate and adaptive immunity will expand our knowledge of immunity in general and allow us to develop anti-infective therapies adapted from nature's design that will enhance the efficiency of immune defences.

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