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REVIEW

Mammalian defensins in the antimicrobial immune response

Michael E Selsted & Andre J Ouellette

Defensins are peptidic components of the innate immune system of plants and animals. In mammals, defensins have evolved to have a central function in the host defense properties of granulocytic leukocytes, mucosal surfaces, skin and other epithelia. This review focuses on the biological functions of three structural subgroups of mammalian defensins and the evidence for their involvement as effectors of antimicrobial innate immunity.

The efficacy of antimicrobial host defense in animals can be attributed to the ability of the immune system to recognize and neutralize microbial invaders quickly and specifically. It is evident that innate immunity is fundamental in the recognition of microbes by the naive host[1]. The efficient sensing of microbes is mainly the result of binding of pathogen-associated molecular patterns by Toll-like receptors (TLRs) and other pattern-recognition molecules on host cells or in plasma[2]. After the recognition step, an acute antimicrobial response is generated by the recruitment of inflammatory leukocytes or the production of antimicrobial substances by affected epithelia. In both cases, the host's cellular response includes the synthesis and/or mobilization of antimicrobial peptides that are capable of directly killing a variety of pathogens[3–5]. For mammals, there are two main genetic categories for antimicrobial peptides: cathelicidins[6] and defensins[7,8]. This review will discuss the present state of knowledge regarding the host defense functions of defensins in vertebrates, with an emphasis on mammalian defensins.

Three defensin structural motifs

Three defensin subfamilies, α-defensins, β-defensins and θ-defensins, are expressed in vertebrates (**Fig. 1**). The mature peptides share several features, including short polypeptide sequences (ranging from 18 to 45 amino acids), three intramolecular disulfides, cationic net charge (ranging from +1 to +11), lack of glycosyl or acyl side-chain modifications, and tertiary structures that are dominated by turn-linked β-strands. All defensins are synthesized as 'prepropeptides' and are processed to various degrees depending on the site of expression (discussed further below). The α- and β-defensins are products of distinct gene families that evolved from an ancestral β-defensin gene that is expressed in species at least as ancient as venomous snakes[9]. The divergence of the α- and β-defensin genes gave rise to distinct clusters that are adjacent on the chromosomal maps of all mammals that express both families[10–12]. It is apparent that there has been rapid evolution of defensin genes in mammals through duplication and diversification. This is postulated to reflect an evolutionary response of the immune system to the ever-changing 'microbial ecology' of the host's habitat[13].

The α- and β-defensins are distinguished structurally by the linear spacing and disulfide pairings of their six conserved cysteine residues (**Fig. 1**). Despite their differing covalent structures, the tertiary structures of α- and β-defensins are very similar. The θ-defensins are structurally dissimilar to α- and β-defensins, as they are backbone-cyclized peptides that derive from mutated α-defensin genes[14,15]. The θ-defensin precursors (of which three have been identified) are α-defensin paralogs that are truncated by a stop codon corresponding to residue 4 on the carboxyl side of the third cysteine in full-length α-defensin. A nine–amino acid segment is excised from the resulting truncated α-defensin precursor and is then spliced, head-to-tail, to another identical or similar nonapeptide (**Fig. 1**). The resulting octadecapeptide is a cyclic molecule stabilized by three disulfides, the only known cyclic polypeptide motif in animals. Three rhesus macaque θ-defensin precursors can theoretically pair to generate six different cyclic peptides. So far, three of these have been isolated from granulocytes and have been characterized[16]. The θ-defensins are expressed in several species of Old World monkeys and in orangutans (an ape) but not in humans or New World primates[17]. Although humans express mRNA encoding θ-defensin orthologs, mutations that introduce stop codons into the otherwise open reading frame of the θ-defensin precursors abolish peptide production[18].

Sites of defensin expression

Before their structural characterization (and naming), α-defensins were identified as antimicrobial proteins purified from extracts of cytoplasmic granules of polymorphonuclear leukocytes[19]. Subsequent structural characterization of the bioactive molecules showed them to be cationic, tridisulfide peptides, which were then called α-defensins. So far, leukocyte α-defensins have been isolated from leukocytes of primates (humans and rhesus macaques), rabbits, and several rodentia (rats, guinea pigs and hamsters). Notably, leukocytes of mice lack

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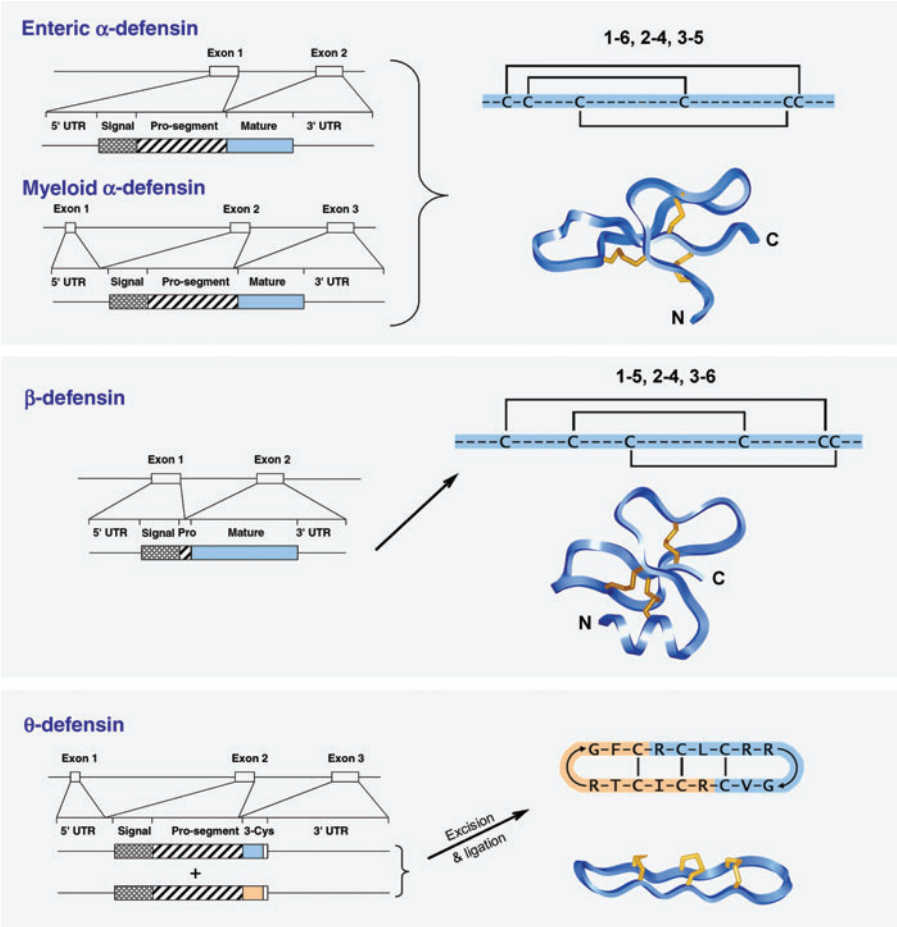

Figure 1 Defensin genes and peptides. Left, alignment of α-defensin, β-defensin and θ-defensin genes. Crosshatching, signal peptides (Signal) and propieces (Pro-segment; Pro); blue, residues present in the mature defensin. Right, three different disulfide 'schemes'. Numbers above diagrams indicate the disulfide connections in each. The three-dimensional structures are of rabbit α-defensin RK-1 (top), human β-defensin-1 (middle) and θ-defensin RTD-1 (bottom).

defensins[20]. Indeed, expression of α-defensins in different leukocyte lineages varies considerably among species. For example, rabbits are the only animals with appreciable amounts of α-defensins in their alveolar macrophages. It has been shown that human monocytes[21] and natural killer cells[22] express human neutrophil α-defensin peptides (HNP1–HNP3) that were thought to be unique to polymorphonuclear leukocytes. In rhesus monkeys, θ-defensin expression is detected only in neutrophils and monocytes[15].

Although mice lack leukocyte α-defensins, Paneth cells that populate the crypts of Lieberkühn throughout the mouse small intestine express many antimicrobial α-defensins called 'cryptdins' (crypt defensins). The crypts invaginate the intestinal mucosa, opening into the lumen of the small bowel (**Fig. 2**). Cryptdin peptides, packaged in apically oriented granules of Paneth cells, are secreted into the crypt by degranulation that is both constitutive and inducible. Six unique α-defensins isolated from Paneth cells of outbred Swiss mice have been characterized structurally and functionally, but genetic evidence has identified many more enteric α-defensins, as 17 unique cryptdin-encoding mRNAs were detected in a single mouse small intestinal crypt. Mouse cryptdins are processed to their active form during granulogenesis, and the convertase responsible for their activation is matrix metalloproteinase 7 (MMP-7, also called matrilysin)[23]. HD-5 and HD-6 are enteric α-defensins expressed in human Paneth cells, and processing of 'pro-HD-5' is mediated by one or more isoforms of Paneth cell trypsin[24]. Although the main sites of human α-defensin production are leukocytes and Paneth cells, the human female reproductive tract expresses HD-5, and α-defensins are also present in rabbit kidney[25].

Biosynthesis of 'prepro-α-defensins' involves the rapid cleavage of the signal peptide producing 'pro-α-defensins' that have little or no microbicidal activity *in vitro*[26–28]. Activation of the peptide requires proteolytic removal of an anionic 'propiece' of about 40 residues. The propiece is a characteristic of all known α- and θ-defensins and generally confers charge balance to the propeptide, potentially minimizing autotoxicity in the producing cell. The β-defensins, however, have very short amino acid sequences separating the signal and mature peptide regions. Thus, it seems that the structural requirements for the biosynthesis and intracellular trafficking of the α- and β-defensin peptide families are very different.

It had been assumed that the tridisulfide structure of mature α- and β-defensin peptides was essential for the microbicidal activity of the folded molecule, in part based on the finding that linearized human neutrophil α-defensins were inactive against viral or bacterial targets that were effectively neutralized by the native molecule[29,30]. However, data now indicate that the main function of the disulfides may be to protect the backbone from proteolysis during biosynthesis and in protease-containing microenvironments where they function as effector molecules. Mutagenesis of disulfide bonds in cryptdin-4, a mouse Paneth cell α-defensin, produced analogs with *in vitro* bactericidal activities equal to or greater than that of the native peptide[31]. Similar results have been obtained by elimination of the three disulfides of human β-defensin 3 (hBD-3)[32]. Cryptdin-4 disulfide analogs but not native cryptdin-4 are degraded by MMP-7, indicating that the disulfide-stabilized conformation confers protease resistance to the endogenous pro-cryptdin-4 convertase[31].

Unlike mouse enteric α-defensins, which exist in their mature form in secretory Paneth cell granules, the human ortholog HD-5 is packaged as a pro-α-defensin that is subsequently processed by one or more trypsin isoforms during or after exocytosis[24]. Within the propeptide, the mature α-defensin segment is in the oxidized state, and this protects it from internal digestion by trypsin. Human myeloid α-defensins are similarly protected by their 'pro-segment' during neutrophil granulogenesis in the bone marrow[27].

The first β-defensin described was isolated from tracheal epithelium of cattle[33], in which its expression is inducible by lipopolysaccharide (LPS)[34]. Like α-defensins, bovine β-defensins have evolved rapidly, as indicated by the 13 β-defensins isolated from bovine neutrophils[35] and by bioinformatics approaches that have identified about 30 human β-defensin genes and about 45 β-defensin genes in mice[36]. Although many of the human β-defensins are located adjacent to the α-defensin

locus on chromosome 8p23, several newly identified β-defensins map to chromosomes 6 and 20, and those clustered on chromosome 20 have open reading frames encoding C-terminal tails extending more than 120 residues beyond the last conserved cysteine[36]. *In situ* hybridization studies have shown that several of these genes are expressed at distinct sites along the epididymis[37], and there is evidence for their involvement in sperm maturation[38,39].

The first human β-defensin peptide isolated, hBD-1, was purified from hemodialysis fluid[40]; it is present in plasma, and several N-terminally truncated forms have been found in urine[41]. Two additional β-defensins, hBD-2 and hBD-3, were subsequently isolated from the scales of psoriatic skin[42,43] and have been characterized structurally and functionally[44] (discussed below). Expression of a fourth β-defensin, hBD-4, has been characterized at the mRNA level, and a synthetic form has been produced and functionally characterized, but isolation of natural hBD-4 has not yet been reported[45].

These human β-defensins (hBD-1–hBD-4) are widely expressed in epithelium and leukocytes, and their expression is constitutive and/or inducible depending on the site of expression (**Figs. 2** and **3**). The expression of hBD-1 seems to be constitutive in most tissues but may also be upregulated in some circumstances (discussed below). The antimicrobial activities of various hBD-1 isoforms have been characterized, demonstrating that the N-terminally truncated variants are bactericidal or bacteriostatic against several bacterial species *in vitro*[41]. The mRNAs of hBD-2 and hBD-3 are differentially expressed in many tissues and the peptide concentrations of hBD-2 and hBD-3 are substantially increased in bronchoalveolar inflammation[46,47] and in certain skin diseases such as psoriasis[44]. Psoriatic lesions, rich in β-defensins, rarely become infected. Conversely, expression of hBD-2 and hBD-3 and the cathelicidin LL-37 is diminished substantially in atopic dermatitis, a skin condition often accompanied by bacterial, fungal or viral infection[48]. In atopic dermatitis, expression of hBD-2 and hBD-3 mRNA is suppressed by upregulation of T helper type 2 cytokines, which may explain the increased susceptibility to skin infection of affected patients[49].

Regulation of defensin expression and mobilization

Bone marrow and Paneth cell expression of α-defensin seems to be constitutive (**Fig. 2**). Therefore, upregulation of α-defensin activity occurs mainly by peptide mobilization within cells (phagocytes) or by induction of their secretion (**Fig. 3**). Neutrophil α-defensins (HNPs) accumulate in azurophil granules (**Fig. 2**), which fuse with phagosomes during phagocytosis of microbes. HNPs are not secreted appreciably unless neutrophils are stimulated by suprapharmacological concentrations of secretagogs such as phorbol myristate acetate[50], indicating that they are targeted for intracellular functions as are other azurophil granule components. This seems to be the case for myeloid θ-defensins as well. In contrast, enteric α-defensins are secreted constitutively by Paneth cells and secretion is stimulated by cholinergic agonists and prokaryotic (but not eukaryotic) microbial antigens. Paneth cell degranulation is modulated by mIKCa1, a calcium-gated potassium channel that controls the uptake of extracellular calcium[51]. Paneth cells may also be induced to degranulate by exposure to bacterial DNA (CpG oligonucleotides)[52]. Natural killer cells, which have been shown to express HNP1–HNP3, are activated to release these α-defensins when stimulated by bacterial antigens such as OmpA or flagellin[22].

In contrast to expression of α and θ-defensins, β-defensin expression is inducible at the transcriptional level in many tissues, often involving responses to TLR-mediated production of proinflammatory cytokines (**Fig. 3**). The mRNA of hBD-1 is constitutively expressed in various epithelia, but expression of all four human β-defensins is inducible in

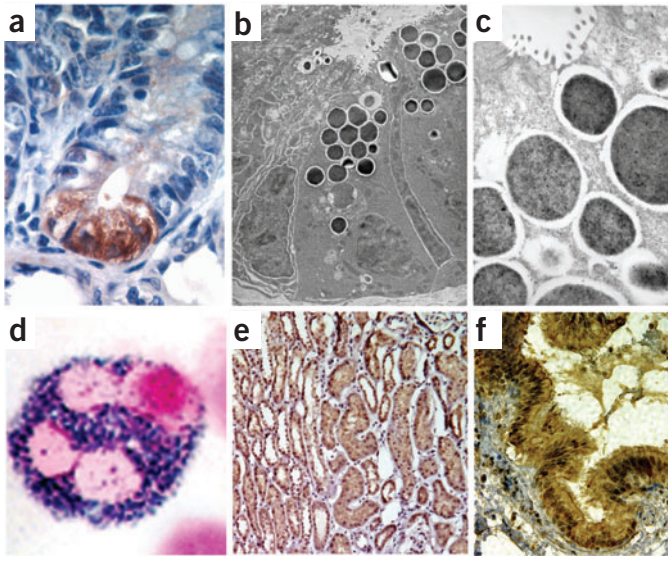

Figure 2 Constitutive expression of α- and β-defensins. (**a**) Mouse enteric α-defensin (cryptdin) expression visualized by immunoperoxidase staining of ileal crypts. (**b**) The peptide is packaged in apical dense granules that are discharged into the lumen. (**c**) Immunogold labeling showing the cryptdin contained in apical granules destined for secretion. (**d**) Neutrophil α-defensins (HNPs) immunolocalize to azurophil granules (blue staining). (**e**,**f**) Constitutive expression of hBD-1 visualized by immunoperoxidase staining of human renal tubules (**e**) and bronchus (**f**). There is lumenal staining of hBD-1-containing mucus.

one or more tissues. For example, hBD-1 expression is upregulated and hBD-2 expression is induced in monocytes exposed to bacteria, LPS or interferon-γ[53,54]. The defensins hBD1–hBD4 are expressed in keratinocytes, and hBD2–hBD4 are induced by stimulation with tumor necrosis factor, interleukin-1β, interferon-γ and phorbol myristate acetate, by exposure to bacteria, and during calcium-induced cellular differentiation of keratinocytes[43,55]. Interleukin-1β markedly stimulates hBD-2 mRNA expression by tracheal epithelial cells[56,57], and infectious and noninfectious respiratory tract inflammation substantially increases peptides[46,47]. In skin, hBD-2 synthesis is strongly induced by interleukin-1 produced by LPS-stimulated monocytes, and this upregulation is dependent on transcription factor NF-κB[58]. Expression of hBD-2 is also stimulated in several cell types by 1,25 dihydroxyvitamin D_3 (ref. 59). Regulation of hBD-3 mRNA expression is induced strongly by interferon-γ in keratinocytes[55], and hBD-4 mRNA expression in respiratory epithelial cells is upregulated by bacterial exposure or phorbol myristate acetate, presumably through protein kinase C activation[45]. Interleukin-22 upregulates hBD-2 and hBD-3 expression in keratinocytes through activation of the transcription factor STAT3 (ref. 60).

TLRs mediate the induction of β-defensin expression in many tissues. Activation of TLR2 by bacterial lipopeptide or lipotechoic acid (TLR2 agonists) on airway epithelial cells induces hBD-2 mRNA and peptide accumulation[61,62]. TLR2 and TLR4 have been linked to inducible hBD-2 expression in intestinal epithelial cells stimulated with peptidoglycan and LPS, respectively[63], and induction is NF-κB dependent in each case[61–63]. TLR3 agonists (poly(I:C) or double-stranded RNA) activate hBD-1 and hBD-2 expression in uterine epithelial cells[64] and hBD-2 and hBD-3 in airway epithelial cells[65]. Bacterial DNA or synthetic CpG oligonucleotides stimulate hBD-2 expression in airway epithelial cells by activation of TLR9 (ref. 66). These studies have demonstrated an important signaling function for TLRs in the inducible

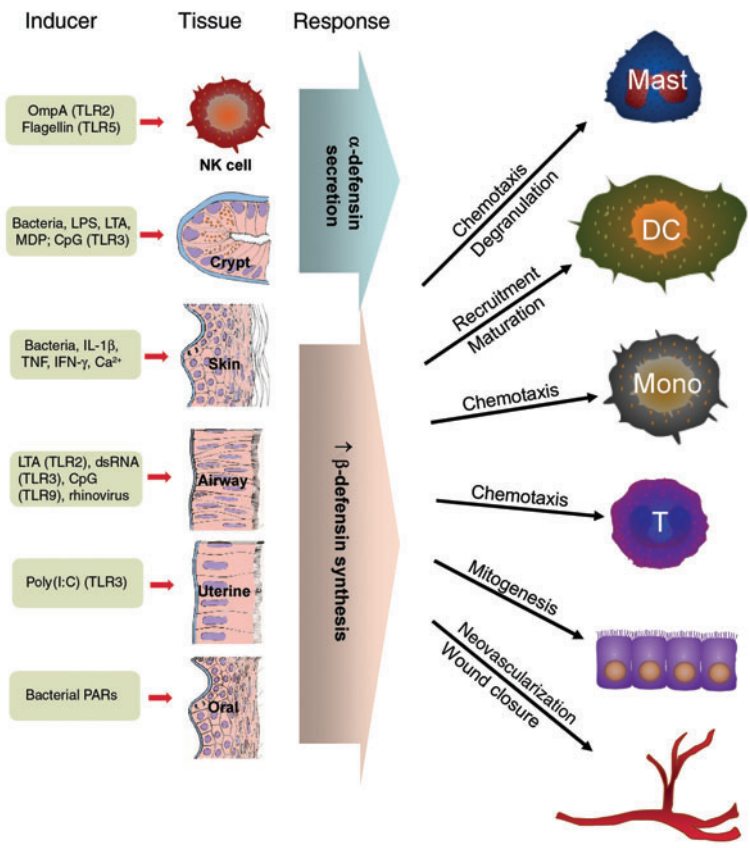

Figure 3 Mobilization, induction and interactions of defensins. Immune functions of defensins may be induced by various physiological stimuli to mobilize pre-formed α-defensins or to upregulate β-defensin expression in various tissues. Released peptides interact with many target cells and tissues to promote secondary responses that may be critical for regulating acute inflammation, the recruitment of adaptive immune cells, angiogenesis and wound healing. NK, natural killer; LTA, lipoteichoic acid; MDP, muramyldipeptide; IL-1β, interleukin-1β; TNF, tumor necrosis factor; IFN-γ, interferon-γ; dsRNA, double-stranded RNA; PAR, protease-activated receptor; Mast, mast cell; DC, dendritic cell; Mono, mononuclear cell; T, T cell.

expression of pathogen-neutralizing defensins in epithelium of many tissues. Additional mechanisms are likely to be operative in eliciting expression of defensins. Notably, microbial proteases activate hBD-2 mRNA expression in oral epithelial cells through the activation of protease-activated receptors[67]. This apparently occurs in a physiological setting (that is, oral mucosa) wherein hBD-2 expression via LPS-TLR4 signaling is inoperative.

Antimicrobial effector functions

Defensins have been suggested as effector molecules in host defense against bacteria, fungi, protozoa and enveloped viruses. Much of the evidence for their antimicrobial function *in vivo* has been inferred from experiments demonstrating that purified defensin preparations kill a wide range of microbes *in vitro*. The α-, β- and θ-defensins are effective microbicides at concentrations in the range of 0.5–5 μM. Conditions used for demonstrating the peptides' antimicrobial properties activity typically include buffers of low ionic strength and neutral pH that allow for optimal killing, as the activity of most α- andβ-defensins is antagonized by 150 mM NaCl, divalent cations and serum components. The θ-defensins differ in this, as they retain most of their bactericidal activity in the presence of physiological concentrations of NaCl[15,16] and of physiological calcium and magnesium as well as serum (D. Tran and M.E.S., unpublished data). The cyclic conformation of θ-defensins seems to be essential for conferring these activities, as the bactericidal activity of decyclized rhesus theta-defensin 1 (RTD-1) is substantially reduced in low ionic conditions and it is nearly inactive in the presence of physiological concentrations of NaCl[15].

The observation that the antimicrobial activities of many defensins are inhibited by physiological components of serum has cast doubt on the idea that defensins have an antibiotic function *in vivo*. Although increasing the salt concentration of assay media antagonizes the microbicidal activity of many defensins *in vitro*, this inhibition is overcome by steeply increasing the peptide to physiological concentrations. For example, the 'candidacidal' concentration (dose killing 90% of the organisms) of a rabbit myeloid α-defensin is a logarithmic function of the NaCl concentration in the incubation mixture, showing that defensin concentrations in the range of 1–10 mg/ml are fungicidal in physiological saline. Defensin concentrations in this range are estimated to exist in the polymorphonuclear leukocyte phagolysosome[68] and in the lumen of the crypts of Lieberkühn[69]. However, little information exists regarding the ionic and macromolecular microenvironments of either of these biological settings or those of most other sites of defensin expression.

Ex vivo studies using isolated intestinal crypts have provided further evidence for the involvement of Paneth cell cryptdins. The secretory responses of Paneth cells are readily assessed by stimulation of isolated small intestinal crypts (**Fig. 2**) with known secretagogs, microorganisms or microbial antigens. Bacteria and bacterial antigens (such as LPS, lipoteichoic acid, lipid A and muramyldipeptide) but not eukaryotic microbes stimulate cryptdin secretion by Paneth cells, and this occurs in an antigen dose–dependent and time-dependent way[69]. Treatment of the Paneth cell secretions with neutralizing antibody to cryptdin eliminates approximately 70% of the released bactericidal activity, indicating that enteric defensins account for a substantial portion of the antimicrobial activity in these preparations. The basis for the selective Paneth cell secretory response to prokaryotes is not understood. Possible involvement of TLR signaling is suggested by the finding that mouse Paneth cells express TLR9 and they are induced to degranulate when mice are challenged intraperitoneally with bacterial (CpG) DNA[52].

Transgenic mouse studies have provided evidence for the physiological function of defensins in antimicrobial host defense. In one study focused on the involvement of enteric α-defensins, the gene encoding MMP-7, the enzyme required for conversion of inactive pro-α-defensins to active cryptdin peptides, was disrupted. MMP-7-null ($Mmp7^{-/-}$) mice lack detectable mature cryptdins and accumulate inactive precursors[23]. Clearance of noninvasive *E. coli* in the small bowel of orally infected mice is considerably diminished in $Mmp7^{-/-}$ compared with parental wild-type mice. Moreover, susceptibility (that is, death) to

oral challenge with *S. typhimurium* is greatly increased in *Mmp7*$^{-/-}$ mice, indicating the importance of cryptdin expression in intestinal immunity to enteric pathogens[23]. The host defense phenotype of mice transgenic for expression of human enteric α-defensin HD-5 has been described in a second, knock-in mouse model[70]. These transgenic HD-5 mice express and process HD-5 properly and exclusively in mouse Paneth cells. In contrast to wild-type mice, these transgenic HD-5 mice are immune to oral challenge with virulent *S. typhimurium*, showing that altering the composition of Paneth cell secretions may have profound effects on enteric host defense[70]. In each of these transgenic models, there was an excellent correlation between the antibacterial activity of the Paneth cell products *in vitro* and their ability to confer immunity *in vivo*.

Similar studies have begun to demonstrate the physiological function of β-defensins. Targeted deletion of the mouse β-defensin-1 gene results in mice deficient in the clearance of *Haemophilus influenzae* from the lung[71] or containing greater numbers of staphylococci in their bladders[72]. Complementary investigations have focused on mechanisms of bacterial resistance to defensins and other antimicrobial peptides. Many of these studies have demonstrated a correlation between antimicrobial peptide resistance *in vitro* with virulence *in vivo*[73]. These investigations have provided strong evidence for the critical physiological function of defensins in antimicrobial host defense.

Mechanism of antimicrobial action

The task of elucidating the molecular mode(s) of defensin action is formidable, particularly as the peptides mediate their antimicrobial effects in a complex milieu that undoubtedly contains synergistic and antagonistic factors. Relatively simple *in vitro* assay systems have been used to characterize defensin-target interactions and to identify factors that modulate their antimicrobial activities. These limitations notwithstanding, the present understanding of defensin antimicrobial mechanisms is that the peptides disable susceptible organisms by disrupting structural elements of the target cell membrane(s). This is supported by the findings that several members of each defensin structural class are effective microbicides against Gram-positive and Gram-negative bacteria, fungi and viruses *in vitro*[7,8]; that the peptides, which are in every case cationic, initially bind electrostatically to the negatively charged microbe surface groups on the target cells in a way that correlates with microbicidal potency[16,74,75]; and that permeabilization of the bacterial or fungal envelope is temporally linked to microbicidal activity[76,77]. Studies of the effects of human neutrophil α-defensins (HNP1–HNP3) on *E. coli* cells have provided insights into the defensin antimicrobial mechanism[76]. In one such study, over a period of 30–60 minutes, the outer and inner membranes of HNP-treated bacteria were permeabilized sequentially, ceased to synthesize DNA, RNA and protein, and became nonviable, as assessed by their ability to produce colony-forming units. Treatment of *Candida albicans* with HNP-1 induced the nonlytic, lethal release of cellular ATP and other low-molecular-weight substances in a way similar to that induced by the salivary peptide histatin 5 (ref. 78). Most of the evidence now available points to membrane depolarization and permeabilization as being the most likely microbicidal mechanism against bacteria and yeast. However, other mechanisms cannot be excluded, such as the possible induction of autolytic enzymes in the target cell or of intracellular targeting after cell permeabilization[79,80].

Other studies have focused on the interactions of defensins of known structure with model membranes. The three-dimensional structures of α- and β-defensins determined so far show an amphiphilic topology, which is postulated to be critical for the interaction with target membranes. It has been proposed, based on crystal or nuclear magnetic resonance structures, that human β-defensin dimers (or oligomers) are the functional structure in solution, but this has yet to be clarified. There is evidence, however, based on the immunoreactivity of human neutrophil α-defensin HNP-1 with monoclonal antibodies, that this peptide exists as a noncovalent dimer in solution, even at very low concentrations[81]. This is consistent with the dimeric structure of crystalline HNP-3, a defensin that differs from HNP-1 by a single N-terminal residue. HNP-1 induces voltage-dependent channels in planar lipid bilayers[82], showing specific interaction with phospholipids. Calorimetric experiments have demonstrated that the specificity in these interactions is determined in part by the inclusion of anionic phospholipids in a membrane mimetic system[83]. HNP-2 (the analog of HNP-1 lacking alanine at position 1) forms stable pores in large unilamellar vesicles, producing a channel estimated to be about 25 Å in diameter. The formation of a peptide-only pore of this size would require assembly of approximately six HNP-2 dimers[84]. Rabbit neutrophil α-defensins and mouse cryptdin-4 are monomeric and interact with model membranes differently than HNPs, as they induce a graded disruption of large unilamellar vesicles without the formation of stable pores. As with HNP-2 (refs. 83,84), disruption of phospholipid vesicles is very much dependent on membrane lipid composition[85].

Compared with α-defensins and β-defensins, the θ-defensin RTD-1 is smaller, lacks chain termini and has almost no amphiphilic character[86]. The cyclic conformation markedly affects the stability of peptide binding to anionic phospholipids[87]. RTD-1 binds to bilayers in a way that induces membrane thinning[88] and can form large cylindrical complexes with 1-palmitoyl-2-oleoyl-phosphatidylcholine–1-palmitoyl-2-oleoyl-phosphatidylglycerol (POPC-POPG) bilayers[89]. Each of those studies demonstrated a mechanism whereby RTD-1 distorts the phospholipid bilayer structure in a way that may explain the membrane-disrupting, antimicrobial properties of θ-defensins noted *in vitro.* These findings must also be considered in the context of experiments showing that θ-defensins have lectin-like activities that seem to mediate the ability of these peptides to protect lymphocytes from infection by T- and M-tropic strains of human immunodeficiency virus 1 (refs. 18,90,91).

Defensins have been linked to the bridging of innate and acquired immune responses. Although it has been known for some time that HNPs are chemotactic for monocytes and T cells[92,93], the finding that naive T cells and immature dendritic cells are mobilized by α-defensins[94] has led to further studies showing involvement of β-defensins as well. Recruitment of memory T cells and immature dendritic cells by hBD-2 is reportedly mediated via human chemokine receptor 6 (CCR6), the receptor for the chemokine MIP3α[95], and hBD-3 and hBD-4 are also monocyte chemotaxins[45,96]. These studies demonstrate a link between the rapid expression of antimicrobial defensins and the recruitment of adaptive immune cells capable of directing a long-lasting cellular and/or humoral response to a potential pathogen[97]. Additional studies have demonstrated that α-defensins and β-defensins are very effective in promoting antigen-specific immune responses[5].

In sites of inflammation, there is probably a concentration gradient of 'free' defensin peptides that have been delivered to affected tissues by infiltrating leukocytes (mostly α- or θ-defensins; β-defensins in birds and ungulates) or that are locally induced and produced by epithelia (β-defensins). Given the evidence for receptor-mediated recruitment of adaptive immune cells by defensins, it is not unexpected that defensins have been suggested as modulators of tissue processes associated with acute inflammation. Several neutrophil α-defensins (human, guinea pig and rabbit) induce secretion of histamine from mast cells that can be inhibited by pertussin toxin[98]. Release is rapid and dose dependent and does not correlate with antibacterial potency. The defensin hBD2 is chemotactic for mast cells and, as with the induction of histamine release, this activity is also pertussis toxin sensitive[99].

Defensins are also associated with the resolution of inflammation, and they stimulate fibroblast and epithelial cell division[100,101], induce neovasculogenesis[102] and enhance wound closure[103]. Defensins may participate in the regulation of acute inflammation by recruiting and activating mast cells and in the resolution phase by stimulating the formation of granulation tissue antecedent to the restoration of parenchyma and connective tissue after an inflammatory insult.

There is abundant evidence that antimicrobial peptides are involved in host defense processes of organisms ranging from plants to mammals[1]. Moreover, studies of mammalian defensin genes have shown the rapid evolution of these peptides. We speculate that in early life forms, defensins and other antimicrobial peptides provided simple but efficient mechanisms for host resistance to microbial colonization. As gene duplication and diversification occurred, organisms retained specific genes when their products conferred additional advantages to the host. For example, the regulatory functions of various defensins, reviewed here, may have emerged by selection of a more 'sophisticated' system of innate immunity. The evolutionary processes that led to the emergence and perpetuation of the cyclic θ-defensins in nonhuman primates and the possible consequences of their subsequent extinction in humans, chimpanzees and gorillas remain topics of interest.

Concluding remarks

Antimicrobial peptides are among the most ancient elements of the immune system, as indicated by the fact that the structural and functional attributes of mammalian defensins are easily recognized in plants and insects. The rapid evolution and diversity of the α- and β-defensin gene families, considered in the context of their varied antimicrobial and immune regulatory activities, indicate myriad functions for defensins in mammalian host defense. Although genetic manipulation of defensin expression in mice, as described here, has provided compelling evidence for the antibacterial effector function of defensins *in vivo*, research is just beginning to unravel other functional aspects of the mammalian 'defensinome'.

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COMPETING INTERESTS STATEMENT

The authors declare competing financial interests (see the *Nature Immunology* website for details).

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1. Hoffmann, J.A., Kafatos, F.C., Janeway, C.A. & Ezekowitz, R.A. Phylogenetic perspectives in innate immunity. *Science* **284**, 1313–1318 (1999).
2. Janeway, C.A., Jr & Medzhitov, R. Innate immune recognition. *Annu. Rev. Immunol.* **20**, 197–216 (2002).
3. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **415**, 389–395 (2002).
4. Levy, O. Antimicrobial proteins and peptides: anti-infective molecules of mammalian leukocytes. *J. Leukoc. Biol.* **76**, 909–925 (2004).
5. Yang, D., Biragyn, A., Hoover, D.M., Lubkowski, J. & Oppenheim, J.J. Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu. Rev. Immunol.* **22**, 181–215 (2004).
6. Zanetti, M. Cathelicidins, multifunctional peptides of the innate immunity. *J. Leukoc. Biol.* **75**, 39–48 (2004).
7. Ganz, T. Defensins: antimicrobial peptides of innate immunity. *Nat. Rev. Immunol.* **3**, 710–720 (2003).
8. Lehrer, R.I. Primate defensins. *Nat. Rev. Microbiol.* **2**, 727–738 (2004).
9. Schutte, B.C. & McCray, P.B., Jr. β-defensins in lung host defense. *Annu. Rev. Physiol.* **64**, 709–748 (2002).
10. Patil, A., Hughes, A.L. & Zhang, G. Rapid evolution and diversification of mammalian alpha-defensins as revealed by comparative analysis of rodent and primate genes. *Physiol. Genomics* **20**, 1–11 (2004).
11. Semple, C.A., Rolfe, M. & Dorin, J.R. Duplication and selection in the evolution of primate beta-defensin genes. *Genome Biol.* **4**, R31 (2003).
12. Taudien, S. *et al.* Polymorphic segmental duplications at 8p23.1 challenge the determination of individual defensin gene repertoires and the assembly of a contiguous human reference sequence. *BMC Genomics* **5**, 92 (2004).
13. Lynn, D.J., Lloyd, A.T., Fares, M.A. & O'Farrelly, C. Evidence of positively selected sites in mammalian α-defensins. *Mol. Biol. Evol.* **21**, 819–827 (2004).
14. Leonova, L. *et al.* Circular minidefensins and posttranslational generation of molecular diversity. *J. Leukoc. Biol.* **70**, 461–464 (2001).
15. Tang, Y.Q. *et al.* A cyclic antimicrobial peptide produced in primate leukocytes by the ligation of two truncated α-defensins. *Science* **286**, 498–502 (1999).
16. Tran, D. *et al.* Homodimeric θ-defensins from rhesus macaque leukocytes: isolation, synthesis, antimicrobial activities, and bacterial binding properties of the cyclic peptides. *J. Biol. Chem.* **277**, 3079–3084 (2002).
17. Nguyen, T.X., Cole, A.M. & Lehrer, R.I. Evolution of primate θ-defensins: a serpentine path to a sweet tooth. *Peptides* **24**, 1647–1654 (2003).
18. Cole, A.M. *et al.* Retrocyclin: a primate peptide that protects cells from infection by T- and M-tropic strains of HIV-1. *Proc. Natl. Acad. Sci. USA* **99**, 1813–1818 (2002).
19. Zeya, H.I. & Spitznagel, J.K. Antimicrobial specificity of leukocyte lysosomal cationic proteins. *Science* **154**, 1049–1051 (1966).
20. Eisenhauer, P.B. & Lehrer, R.I. Mouse neutrophils lack defensins. *Infect. Immun.* **60**, 3446–3447 (1992).
21. Mackewicz, C.E. *et al.* alpha-Defensins can have anti-HIV activity but are not CD8 cell anti-HIV factors. *AIDS* **17**, F23–F32 (2003).
22. Chalifour, A. *et al.* Direct bacterial protein PAMP recognition by human NK cells involves TLRs and triggers α-defensin production. *Blood* **104**, 1778–1783 (2004).
23. Wilson, C.L. *et al.* Regulation of intestinal α-defensin activation by the metalloproteinase matrilysin in innate host defense. *Science* **286**, 113–117 (1999).
24. Ghosh, D. *et al.* Paneth cell trypsin is the processing enzyme for human defensin-5. *Nat. Immunol.* **3**, 583–590 (2002).
25. Wu, E.R., Daniel, R. & Bateman, A. RK-2: a novel rabbit kidney defensin and its implications for renal host defense. *Peptides* **19**, 793–799 (1998).
26. Satchell, D.P. *et al.* Interactions of mouse Paneth cell α-defensins and α-defensin precursors with membranes. Prosegment inhibition of peptide association with biomimetic membranes. *J. Biol. Chem.* **278**, 13838–13846 (2003).
27. Valore, E.V. & Ganz, T. Posttranslational processing of defensins in immature human myeloid cells. *Blood* **79**, 1538–1544 (1992).
28. Wu, Z. *et al.* From pro defensins to defensins: synthesis and characterization of human neutrophil pro α-defensin-1 and its mature domain. *J. Pept. Res.* **62**, 53–62 (2003).
29. Daher, K.A., Selsted, M.E. & Lehrer, R.I. Direct inactivation of viruses by human granulocyte defensins. *J. Virol.* **60**, 1068–1074 (1986).
30. Mandal, M. & Nagaraj, R. Antibacterial activities and conformations of synthetic α-defensin HNP-1 and analogs with one, two and three disulfide bridges. *J. Pept. Res.* **59**, 95–104 (2002).
31. Maemoto, A. *et al.* Functional analysis of the α-defensin disulfide array in mouse cryptdin-4. *J. Biol. Chem.* **279**, 44188–44196 (2004).
32. Wu, Z. *et al.* Engineering disulfide bridges to dissect antimicrobial and chemotactic activities of human β-defensin 3. *Proc. Natl. Acad. Sci. USA* **100**, 8880–8885 (2003).
33. Diamond, G. *et al.* Tracheal antimicrobial peptide, a cysteine-rich peptide from mammalian tracheal mucosa: Peptide isolation and cloning of a cDNA. *Proc. Natl. Acad. Sci. USA* **88**, 3952–3956 (1991).
34. Diamond, G. & Bevins, C.L. Endotoxin upregulates expression of an antimicrobial peptide gene in mammalian airway epithelial cells. *Chest* **105**, 51S–52S (1994).
35. Selsted, M.E. *et al.* Purification, primary structures, and antibacterial activities of β-defensins, a new family of antimicrobial peptides from bovine neutrophils. *J. Biol. Chem.* **268**, 6641–6648 (1993).
36. Schutte, B.C. *et al.* Discovery of five conserved β-defensin gene clusters using a computational search strategy. *Proc. Natl. Acad. Sci. USA* **99**, 2129–2133 (2002).
37. Rodriguez-Jimenez, F.J. *et al.* Distribution of new human β-defensin genes clustered on chromosome 20 in functionally different segments of epididymis. *Genomics* **81**, 175–183 (2003).
38. Zhou, C.X. *et al.* An epididymis-specific β-defensin is important for the initiation of sperm maturation. *Nat. Cell Biol.* **6**, 458–464 (2004).
39. Yudin, A.I. *et al.* ESP13.2, a member of the β-defensin family, is a macaque sperm surface-coating protein involved in the capacitation process. *Biol. Reprod.* **69**, 1118–1128 (2003).
40. Bensch, K.W., Raida, M., Magert, H.J., Schulz-Knappe, P. & Forssmann, W.G. hBD-1: a novel β-defensin from human plasma. *FEBS Lett.* **368**, 331–335 (1995).
41. Valore, E.V. *et al.* Human β-defensin-1: an antimicrobial peptide of urogenital tissues. *J. Clin. Invest.* **101**, 1633–1642 (1998).
42. Harder, J., Bartels, J., Christophers, E. & Schroder, J.M. A peptide antibiotic from human skin. *Nature* **387**, 861 (1997).
43. Harder, J., Bartels, J., Christophers, E. & Schroder, J.M. Isolation and characterization of human β-defensin-3, a novel human inducible peptide antibiotic. *J. Biol. Chem.* **276**, 5707–5713 (2001).
44. Harder, J. & Schroder, J.M. Psoriatic scales: a promising source for the isolation of human skin-derived antimicrobial proteins. *J. Leukoc. Biol.* **77**, 476–486 (2005).
45. Garcia, J.R. *et al.* Human β-defensin 4: a novel inducible peptide with a specific salt-sensitive spectrum of antimicrobial activity. *FASEB J.* **15**, 1819–1821 (2001).
46. Hiratsuka, T. *et al.* Increased concentrations of human β-defensins in plasma and bronchoalveolar lavage fluid of patients with diffuse panbronchiolitis. *Thorax* **58**, 425–430 (2003).
47. Ross, D.J. *et al.* Increased bronchoalveolar lavage human β-defensin type 2 in

bronchiolitis obliterans syndrome after lung transplantation. *Transplantation* **78**, 1222–1224 (2004).
48. Ong, P.Y. *et al.* Endogenous antimicrobial peptides and skin infections in atopic dermatitis. *N. Engl. J. Med.* **347**, 1151–1160 (2002).
49. Nomura, I. *et al.* Cytokine milieu of atopic dermatitis, as compared to psoriasis, skin prevents induction of innate immune response genes. *J. Immunol.* **171**, 3262–3269 (2003).
50. Ganz, T. Extracellular release of antimicrobial defensins by human polymorphonulear leukocytes. *Infect. Immun.* **55**, 568–571 (1987).
51. Ayabe, T. *et al.* Modulation of mouse Paneth cell α-defensin secretion by mIKCa1, a Ca^{2+}-activated, intermediate conductance potassium channel. *J. Biol. Chem.* **277**, 3793–3800 (2002).
52. Rumio, C. *et al.* Degranulation of paneth cells via Toll-like receptor 9. *Am. J. Pathol.* **165**, 373–381 (2004).
53. Duits, L.A., Ravensbergen, B., Rademaker, M., Hiemstra, P.S. & Nibbering, P.H. Expression of β-defensin 1 and 2 mRNA by human monocytes, macrophages and dendritic cells. *Immunology* **106**, 517–525 (2002).
54. Fang, X.M. *et al.* Differential expression of α- and β-defensins in human peripheral blood. *Eur. J. Clin. Invest.* **33**, 82–87 (2003).
55. Harder, J., Meyer-Hoffert, U., Wehkamp, K., Schwichtenberg, L. & Schroder, J.M. Differential gene induction of human β-defensins (hBD-1, -2, -3, and -4) in keratinocytes is inhibited by retinoic acid. *J. Invest. Dermatol.* **123**, 522–529 (2004).
56. Singh, P.K. *et al.* Production of β-defensins by human airway epithelia. *Proc. Natl. Acad. Sci. USA* **95**, 14961–14966 (1998).
57. Tsutsumi-Ishii, Y. & Nagaoka, I. Modulation of human β-defensin-2 transcription in pulmonary epithelial cells by lipopolysaccharide-stimulated mononuclear phagocytes via proinflammatory cytokine production. *J. Immunol.* **170**, 4226–4236 (2003).
58. Liu, L., Roberts, A.A. & Ganz, T. By IL-1 signaling, monocyte-derived cells dramatically enhance the epidermal antimicrobial response to lipopolysaccharide. *J. Immunol.* **170**, 575–580 (2003).
59. Wang, T.T. *et al.* Cutting edge: 1,25-dihydroxyvitamin D_3 is a direct inducer of antimicrobial peptide gene expression. *J. Immunol.* **173**, 2909–2912 (2004).
60. Wolk, K. *et al.* IL-22 increases the innate immunity of tissues. *Immunity* **21**, 241–254 (2004).
61. Hertz, C.J. *et al.* Activation of Toll-like receptor 2 on human tracheobronchial epithelial cells induces the antimicrobial peptide human β defensin-2. *J. Immunol.* **171**, 6820–6826 (2003).
62. Wang, X. *et al.* Airway epithelia regulate expression of human β-defensin 2 through Toll-like receptor 2. *FASEB J.* **17**, 1727–1729 (2003).
63. Vora, P. *et al.* Beta-defensin-2 expression is regulated by TLR signaling in intestinal epithelial cells. *J. Immunol.* **173**, 5398–5405 (2004).
64. Schaefer, T.M., Fahey, J.V., Wright, J.A. & Wira, C.R. Innate immunity in the human female reproductive tract: antiviral response of uterine epithelial cells to the TLR3 agonist poly(I:C). *J. Immunol.* **174**, 992–1002 (2005).
65. Proud, D., Sanders, S.P. & Wiehler, S. Human rhinovirus infection induces airway epithelial cell production of human β-defensin 2 both *in vitro* and *in vivo*. *J. Immunol.* **172**, 4637–4645 (2004).
66. Platz, J. *et al.* Microbial DNA induces a host defense reaction of human respiratory epithelial cells. *J. Immunol.* **173**, 1219–1223 (2004).
67. Chung, W.O., Hansen, S.R., Rao, D. & Dale, B.A. Protease-activated receptor signaling increases epithelial antimicrobial peptide expression. *J. Immunol.* **173**, 5165–5170 (2004).
68. Ganz, T., Selsted, M.E. & Lehrer, R.I. Antimicrobial activity of phagocyte granule proteins. *Sem. Resp. Infect.* **1**, 107–117 (1986).
69. Ayabe, T. *et al.* Secretion of microbicidal α-defensins by intestinal Paneth cells in response to bacteria. *Nat. Immunol.* **1**, 113–118 (2000).
70. Salzman, N.H., Ghosh, D., Huttner, K.M., Paterson, Y. & Bevins, C.L. Protection against enteric salmonellosis in transgenic mice expressing a human intestinal defensin. *Nature* **422**, 522–526 (2003).
71. Moser, C. *et al.* β-Defensin 1 contributes to pulmonary innate immunity in mice. *Infect. Immun.* **70**, 3068–3072 (2002).
72. Morrison, G., Kilanowski, F., Davidson, D. & Dorin, J. Characterization of the mouse β defensin 1, Defb1, mutant mouse model. *Infect. Immun.* **70**, 3053–3060 (2002).
73. Bader, M.W. *et al.* Regulation of *Salmonella typhimurium* virulence gene expression by cationic antimicrobial peptides. *Mol. Microbiol.* **50**, 219–230 (2003).
74. Lehrer, R.I., Szklarek, D., Ganz, T. & Selsted, M.E. Correlation of binding of rabbit granulocyte peptides to *Candida albicans* with candidacidal activity. *Infect. Immun.* **49**, 207–211 (1985).
75. Lehrer, R.I., Ganz, T., Szklarek, D. & Selsted, M.E. Modulation of the in vitro candidacidal activity of human neutrophil defensins by target cell metabolism and divalent cations. *J. Clin. Invest.* **81**, 1829–1835 (1988).
76. Lehrer, R.I. *et al.* Interaction of human defensins with *Escherichia coli*. Mechanism of bactericidal activity. *J. Clin. Invest.* **84**, 553–561 (1989).
77. Boniotto, M. *et al.* A study of host defence peptide β-defensin 3 in primates. *Biochem. J.* **374**, 707–714 (2003).
78. Edgerton, M. *et al.* Salivary histatin 5 and human neutrophil defensin 1 kill *Candida albicans* via shared pathways. *Antimicrob. Agents Chemother.* **44**, 3310–3316 (2000).
79. Helmerhorst, E.J. *et al.* The cellular target of histatin 5 on *Candida albicans* is the energized mitochondrion. *J. Biol. Chem.* **274**, 7286–7291 (1999).
80. Sahl, H.G. *et al.* Mammalian defensins: structures and mechanism of antibiotic activity. *J. Leukoc. Biol.* **77**, 466–475 (2005).
81. Panyutich, A.V., Voitenok, N.N., Lehrer, R.I. & Ganz, T. An enzyme immunoassay for human defensins. *J. Immunol. Methods* **141**, 149–155 (1991).
82. Kagan, B.L., Selsted, M.E., Ganz, T. & Lehrer, R.I. antimicrobial defensin peptides form voltage-dependent ion-permeable channels in planar lipid bilayer membranes. *Proc. Natl. Acad. Sci. USA* **87**, 210–214 (1990).
83. Lohner, K., Latal, A., Lehrer, R.I. & Ganz, T. Differential scanning microcalorimetry indicates that human defensin, HNP-2, interacts specifically with biomembrane mimetic systems. *Biochemistry* **36**, 1525–1531 (1997).
84. Wimley, W.C., Selsted, M.E. & White, S.H. Interactions between human defensins and lipid bilayers: evidence for formation of multimeric pores. *Protein Sci.* **3**, 1362–1373 (1994).
85. Hristova, K., Selsted, M.E. & White, S.H. Critical role of lipid composition in membrane permeabilization by rabbit neutrophil defensins. *J. Biol. Chem.* **272**, 24224–24233 (1997).
86. Trabi, M., Schirra, H.J. & Craik, D.J. Three-dimensional structure of RTD-1, a cyclic antimicrobial defensin from Rhesus macaque leukocytes. *Biochemistry* **40**, 4211–4221 (2001).
87. Abuja, P.M., Zenz, A., Trabi, M., Craik, D.J. & Lohner, K. The cyclic antimicrobial peptide RTD-1 induces stabilized lipid-peptide domains more efficiently than its open-chain analogue. *FEBS Lett.* **566**, 301–306 (2004).
88. Weiss, T.M. *et al.* Two states of cyclic antimicrobial peptide RTD-1 in lipid bilayers. *Biochemistry* **41**, 10070–10076 (2002).
89. Buffy, J.J. *et al.* Solid-state NMR investigation of the selective perturbation of lipid bilayers by the cyclic antimicrobial peptide RTD-1. *Biochemistry* **43**, 9800–9812 (2004).
90. Munk, C. *et al.* The θ-defensin, retrocyclin, inhibits HIV-1 entry. *AIDS Res. Hum. Retroviruses* **19**, 875–881 (2003).
91. Wang, W., Cole, A.M., Hong, T., Waring, A.J. & Lehrer, R.I. Retrocyclin, an antiretroviral θ-defensin, is a lectin. *J. Immunol.* **170**, 4708–4716 (2003).
92. Territo, M.C., Ganz, T., Selsted, M.E. & Lehrer, R.I. Monocyte-chemotactic activity of defensins from human neutrophils. *J. Clin. Invest.* **84**, 2017–2020 (1989).
93. Chertov, O. *et al.* Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J. Biol. Chem.* **271**, 2935–2940 (1996).
94. Yang, D., Chen, Q., Chertov, O. & Oppenheim, J.J. Human neutrophil defensins selectively chemoattract naive T and immature dendritic cells. *J. Leukoc. Biol.* **68**, 9–14 (2000).
95. Yang, D. *et al.* Beta-defensins: linking innate and adaptive immunity through dendritic and T cell CCR6. *Science* **286**, 525–528 (1999).
96. Garcia, J.R. *et al.* Identification of a novel, multifunctional β-defensin (human β-defensin 3) with specific antimicrobial activity. Its interaction with plasma membranes of *Xenopus* oocytes and the induction of macrophage chemoattraction. *Cell Tissue Res.* **306**, 257–264 (2001).
97. Yang, D., Biragyn, A., Kwak, L.W. & Oppenheim, J.J. Mammalian defensins in immunity: more than just microbicidal. *Trends Immunol.* **23**, 291–296 (2002).
98. Befus, A.D. *et al.* Neutrophil defensins induce histamine secretion from mast cells: mechanisms of action. *J. Immunol.* **163**, 947–953 (1999).
99. Niyonsaba, F., Iwabuchi, K., Matsuda, H., Ogawa, H. & Nagaoka, I. Epithelial cell-derived human β-defensin-2 acts as a chemotaxin for mast cells through a pertussis toxin-sensitive and phospholipase C-dependent pathway. *Int. Immunol.* **14**, 421–426 (2002).
100. Murphy, C.J., Foster, B.A., Mannis, M.J., Selsted, M.E. & Reid, T.W. Defensins are mitogenic for epithelial cells and fibroblasts. *J. Cell. Physiol.* **155**, 408–413 (1993).
101. Aarbiou, J. *et al.* Human neutrophil defensins induce lung epithelial cell proliferation in vitro. *J. Leukoc. Biol.* **72**, 167–174 (2002).
102. Chavakis, T. *et al.* Regulation of neovascularization by human neutrophil peptides (α-defensins): a link between inflammation and angiogenesis. *FASEB J.* **18**, 1306–1308 (2004).
103. Aarbiou, J. *et al.* Neutrophil defensins enhance lung epithelial wound closure and mucin gene expression *in vitro*. *Am. J. Respir. Cell Mol. Biol.* **30**, 193–201 (2004).