Defensins in innate antiviral immunity

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Abstract | Defensins are small antimicrobial peptides that are produced by leukocytes and epithelial cells, and that have an important role in innate immunity. Recent advances in understanding the mechanisms of the antiviral action(s) of defensins indicate that they have a dual role in antiviral defence, acting directly on the virion and on the host cell. This Review focuses on the antiviral activities and mechanisms of action of mammalian defensins, and on the clinical relevance of these activities. Understanding the complex function of defensins in innate immunity against viral infection has implications for the prevention and treatment of viral disease.

The innate immune system provides the first line of defence against a wide range of microorganisms before the development of adaptive immune responses. Toll-like receptors (TLRs) are pattern-recognition receptors that have an important role in the innate immune response. They act as initiators of the innate immune response by providing the host with the ability to recognize pathogen-associated molecular patterns (PAMPs)[1]. By contrast, antimicrobial peptides function as important effectors of innate immunity[2]. The roles of these two arms of innate immunity in the control of viral infection have recently been recognized[3,4]. In this Review, we discuss the antiviral activity of antimicrobial peptides. Antimicrobial peptides, such as defensins and cathelicidins (BOX 1), are small molecules that are mainly produced by leukocytes and epithelial cells. These peptides have a broad range of actions against microorganisms, including Gram-positive and Gram-negative bacteria, fungi and viruses[5–8]. Although the antiviral activity of defensins was first reported in 1986 (REF. 9), recent studies have shed light on the multiple and complex mechanisms by which defensins inhibit viral infection. Defensins can block viral infection by directly acting on the virion or by affecting the target cell and thereby indirectly interfering with viral infection. Furthermore, defensin production can be induced by cytokines or TLR activation, and can modulate adaptive immune responses. This Review focuses on the antiviral functions of mammalian defensins, and highlights the recent advances in our understanding of the molecular mechanisms of their antiviral activities and the potential clinical relevance of these functions.

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An overview of mammalian defensins

Classification and structure. Defensins are cysteine-rich, cationic peptides with β-pleated sheet structures that are stabilized by three intramolecular disulphide bonds between the cysteine residues[7,10]. Mammalian defensins are classified into three subfamilies, the α-, β- and θ-defensins, which differ in their distribution of and disulphide links (bonds) between the six conserved cysteine residues. The disulphide linkages of cysteine residues in α-defensins are between the first and the sixth cysteine residues (Cys^1–Cys^6), Cys^2–Cys^4 and Cys^3–Cys^5, whereas in β-defensins, the linkages are Cys^1–Cys^5, Cys^2–Cys^4 and Cys^3–Cys^6. By contrast, θ-defensins have a circular structure with the cysteine residues linked as Cys^1–Cys^6, Cys^2–Cys^5 and Cys^3–Cys^4 (REF. 11).

The α-defensins are synthesized as prepropeptides, which contain an amino-terminal signal sequence, an anionic propiece and a carboxy-terminal mature peptide of approximately 30 amino acids[7]. Human α-defensin-1, -2, -3 and -4 are also designated as human neutrophil peptides (HNP1, HNP2, HNP3 and HNP4) because they are mainly expressed by neutrophils[12]. HNP1, HNP2 and HNP3 are synthesized by promyelocytes, which are neutrophil precursor cells in the bone marrow, and the mature peptides are stored in primary granules of neutrophils[7]. Unlike HNPs, human α-defensin-5 (HD5) is released as a propeptide that is processed extracellularly[13,14]. The θ-defensins are composed of two α-defensin-like precursor peptides of nine amino acids that are connected by a post-translational head-to-tail ligation[11,15,16].

The contribution of defensin structure to defensin function might vary depending on the function. For example, disulphide bonds are not required for the antibacterial functions of HNP1, human β-defensin-3 (HBD3) and the mouse Paneth-cell-derived α-defensin cryptdin-4 (REFS 17–19). However, having the correct disulphide bonding is important for the chemotactic activity that has been attributed to HBD3 (REF. 18). Similarly, the direct effect of the α-defensin HNP1 or θ-defensins on the virion is abolished when disulphide

Box 1 | **Antiviral activity of other antimicrobial peptides**

Similar to defensins, other antimicrobial peptides have broad and diverse activity against both enveloped and non-enveloped viruses. The mechanisms of antimicrobial action are multiple and complex and include direct effects on the virion, as well as effects on the target cell and on innate and adaptive immunity.

Cathelicidins are another important group of mammalian antimicrobial peptides[8]. Human cathelicidin LL37 is highly expressed by neutrophils and by numerous mucosal epithelial cell types. Expression of LL37 is constitutive or induced in response to inflammatory stimuli. Similar to defensins, LL37 has chemotactic activity and other activities that are mediated through alterations in receptor-mediated cell signalling.

LL37 and its mouse homologue, cathelicidin-related antimicrobial peptide (CRAMP), have been shown to inhibit vaccinia virus replication[107]. The activity of LL37 against vaccinia virus is independent of salt concentration. Vaccinia virus treated with LL37 has altered morphology, indicating that LL37 might have a direct effect on the virion. Importantly, the physiological role of cathelicidin has been shown in CRAMP-deficient mice. These mice have enhanced morbidity or mortality following exposure to vaccinia virus compared with control wild-type mice.

Anti-HIV activity of other antimicrobial peptides from other species has been explored. High concentrations of indolicidin, a bovine cathelicidin, have a direct inhibitory effect on HIV *in vitro*[108]. Dermaseptin S4, caerin 1.1, caerin 1.9 and maculatin 1.1, which are antimicrobial peptides isolated from amphibian skin, block HIV infection before or during viral entry and disrupt virions[109,110]. In addition, these amphibian antimicrobial peptides inhibit monocyte-derived dendritic-cell-mediated infection of T cells in *trans*.

bonds are disrupted by treatment with the reducing agents dithiothreitol and iodoacetamide[9,20]. Mutagenic studies of cryptdin-4 show that, in contrast to native cryptdin-4, disulphide-bond variants of cryptdin-4, in which cysteine is substituted with alanine, are susceptible to proteolysis by matrix metalloproteinase 7, indicating that the disulphide bonds might have a role in protection from degradation by proteinases[17]. The importance of the conserved disulphide bonds in the antiviral functions of defensins in target cells remains to be explored.

Cell sources and tissue distribution. Leukocytes and epithelial cells are the main sources of mammalian defensins. So far, six human α-defensins have been identified[8]. HNP1, HNP2 and HNP3, which differ only in the first amino acid, account for 5–7% of total neutrophil proteins[21]. By contrast, HNP4, which has an amino-acid sequence distinct from the HNP1, HNP2 and HNP3 sequences, comprises less than 2% of total defensins in neutrophils[22]. HNP2 is thought to be a proteolytic product of HNP1 and/or HNP3 because no gene that encodes HNP2 has been found[7]. Although the highest level of HNP expression is found in granulocytes, HNPs are also found in other immune cells, at mucosal surfaces and in various tissues[23–26] (TABLE 1). In addition, cells can absorb and internalize HNPs[27–29]; however, it is not clear whether the uptake of defensins is required for defensin functions, including antiviral activity. Although leukocyte defensins are conserved evolutionally and have been isolated from many species, including humans, rabbits, rats, guinea pigs and hamsters, mice lack α-defensin expression by neutrophils[7]. Instead, mice express many enteric α-defensin-like peptides known as cryptdins in intestinal Paneth cells[7,10]. Similarly, HD5 and HD6 are produced mainly by intestinal Paneth cells[7] but are also found in other tissues, such as the salivary glands, the female genital tract and the inflamed large bowel[25,30–32]. In addition, increased concentrations of HD5 have been observed in urethral secretions of men with *Neisseria gonorrhoeae* infection and urethritis associated with *Chlamydia trachomatis* infection[14].

Although 28 human β-defensins[33] have been identified by gene-based searches, six human β-defensins (HBD1, -2, -3, -4, -5 and -6) are expressed mainly by epithelial cells[7,8]. Whereas HBD1 is constitutively expressed by epithelial cells, expression of HBD2 and HBD3 can be induced by viruses, bacteria, microbial products (for example, endotoxin) and pro-inflammatory cytokines, such as tumour-necrosis factor (TNF) and interleukin-1β (IL-1β)[7,34–37]. HBD1, HBD2 and HBD3 have all been detected in various epithelial-cell tissues[25,38,39], although the mechanisms of induction of their expression in

Table 1 | **Distribution and source of defensins**

Defensin	Tissue distribution	Cell source	Synthesis and regulation
HNP1, HNP2 and HNP3	Placenta, intestinal mucosa and cervical mucus plug	Neutrophils*, monocytes, macrophages, natural killer cells, B cells and γδ T cells	Constitutive
HNP4	Not determined	Neutrophils*	Constitutive
HD5 and HD6	Salivary glands, small bowel, inflamed large bowel, stomach, eye, female genital tract (HD5 only), breast milk and inflamed urethral lumen	Intestinal paneth cells* and vaginal epithelial cells (HD5 only)	Constitutive or inducible, such as by sexually transmitted infection
HBD1	Oral and nasal mucosa, lungs, plasma, salivary glands, small and large bowel, stomach, skin, eyes, mammary glands, urogenital tract and kidneys	Epithelial cells*, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes	Constitutive or inducible in response to interferon-γ, lipopolysaccharide and peptidoglycan
HBD2 and HBD3	Oral and nasal mucosa, lungs, plasma, salivary glands, small and large bowel, stomach, skin, eyes, mammary glands, urogenital tract and kidneys	Epithelial cells*, monocytes, macrophages, monocyte-derived dendritic cells and keratinocytes	Inducible in response to viruses, bacteria, lipopolysaccharide, peptidoglycan, lipoproteins, cytokines (IL-1β, TNF) and growth factors
HBD4	Gastric antrum and testes	Epithelial cells*	Constitutive or inducible in response to PMA and bacteria

*Main cellular source. HBD, human β-defensin; HD, human α-defensin; HNP, human neutrophil peptide; IL-1β, interleukin-1β; PMA, phorbol 12-myristate 13-acetate; TNF, tumour-necrosis factor.

response to microbial products have been shown to be distinct from each other[37]. Expression of HBD1 and HBD2 has been detected in monocytes, macrophages and monocyte-derived dendritic cells (DCs)[40], indicating that HBD1 and HBD2 are not exclusively epithelial-cell-associated. Both human α- and β-defensins have been found in breast milk[41,42], indicating a role for defensins in protecting infants from infection. Constitutive expression of HBD4 seems to be restricted to the testes and gastric antrum, although HBD4 expression can be induced in human respiratory epithelial cells after exposure to phorbol 12-myristate 13-acetate (PMA) or bacterial infection *in vitro*[43]. HBD5 and HBD6 are specifically expressed in the human epididymis[44].

Three θ-defensins have been found in leukocytes from rhesus macaques: rhesus θ-defensin-1 (RTD1), RTD2 and RTD3 (REFS 11,15,16). Although RNA transcripts homologous to the rhesus θ-defensin gene (*DEFT*) are found in human bone marrow, these transcripts contain a premature stop codon in the upstream signal sequence, which abolishes subsequent translation[45]. Retrocyclin, an artificially made circular peptide based on the sequence of the mature peptide that would be encoded by the human θ-defensin pseudogene, shows antiviral activity *in vitro*[46].

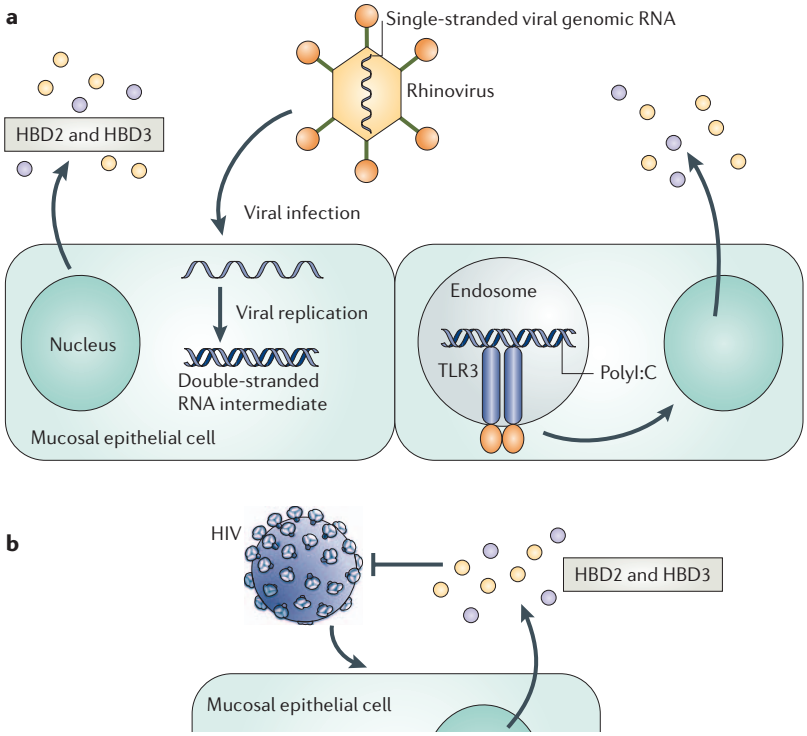

Figure 1 | **Induction of defensin expression in response to viral infection at the mucosal epithelium.** In response to viral infection, the production of human β-defensin-2 (HBD2) and HBD3 is induced by epithelial cells. **a** | In the case of rhinovirus infection, the induction of HBD expression requires active viral replication in the epithelial cell, involving the production of a double-stranded viral RNA intermediate. This RNA intermediate may activate Toll-like receptor 3 (TLR3)-mediated induction of HBD expression, as shown for the synthetic double-stranded RNA mimic polyinosinic–polycytidylic acid (polyI:C). **b** | HIV X4 and R5 viruses induce expression of HBD2 and HBD3 by mucosal epithelial cells, but this induction does not require viral replication. In turn, HBDs directly inhibit both X4 and R5 strains of HIV.

Defensins in response to viral infection

Induction of defensin expression. In response to viral infection, target cells can produce cytokines, chemokines and other antiviral factors to control viral replication. In a similar way to the cytokine induction that occurs as an early innate immune response to viral infection, HIV-1 induces mRNA expression of *HBD2* and *HBD3*, but not *HBD1*, in normal human oral epithelial cells, even in the absence of HIV-1 replication[47] (FIG. 1). These cells lack cell-surface expression of the HIV entry receptors CD4, CC-chemokine receptor 5 (CCR5) and CXC-chemokine receptor 4 (CXCR4), or galactosylceramide, so it is unclear what interactions between the virus and the cell are responsible for this induction of β-defensin expression. Similarly, expression of HBD2 and HBD3, but not HBD1, are induced in bronchial epithelial cells exposed to human rhinovirus[34,35] (FIG. 1).

In contrast to HIV-mediated induction of *HBD* gene expression, active replication of rhinovirus is required for the induction of *HBD* gene expression. Induction of *HBD2* gene expression in response to human rhinovirus infection is mediated by nuclear factor-κB (NF-κB) activation but is independent of IL-1 (REF. 34). Furthermore, a similar profile of *HBD* gene expression is induced in response to polyinosinic–polycytidylic acid (polyI:C), a ligand for TLR3, indicating that the intracellular double-stranded RNA intermediate that is generated during replication of rhinovirus might be involved in the upregulation of HBD2 and HBD3 expression[34,35]. Stimulation of TLR3 has also been shown to induce HBD1 and HBD2 expression by uterine epithelial cells[48]. In addition, stimulation of TLR2 and TLR4 with peptidoglycans and lipopolysaccharides can induce HBD2 expression by keratinocytes and vaginal epithelial cells[49,50]. By contrast, recognition of bacterial proteins, such as outer membrane protein A from *Klebsiella pneumoniae* and flagellin from *Escherichia coli* through TLR2 and TLR5, respectively, can induce the release of HNP1, HNP2 and HNP3 from $CD3^+CD56^+$ natural killer T cells[51].

Defensins as chemotactic agents. Some α- and β-defensins have chemotactic activity for T cells, monocytes and immature DCs, and can induce cytokine production by monocytes and epithelial cells[8]. Therefore, defensins might control viral replication by modulating the immune system, in addition to acting as direct effectors (FIG. 2). Increasing evidence indicates that some activities of defensins are receptor mediated, resulting in activation of downstream signalling events. For example, the chemotatic activity of HBD1, HBD2 and HBD3 for memory T cells and immature DCs is mediated through binding to CCR6, which is the receptor for CC-chemokine ligand 20 (CCL20; also known as MIP3α)[52,53]. In addition, HBD2 has multiple activities on mast cells, including induction of mast-cell migration, degranulation and prostaglandin D_2 production. These activities can be blocked by pertussis toxin and a phospholipase C inhibitor, indicating that $G_{i\alpha}$-protein-coupled receptor(s) and phospholipase C signalling pathways are involved[54].

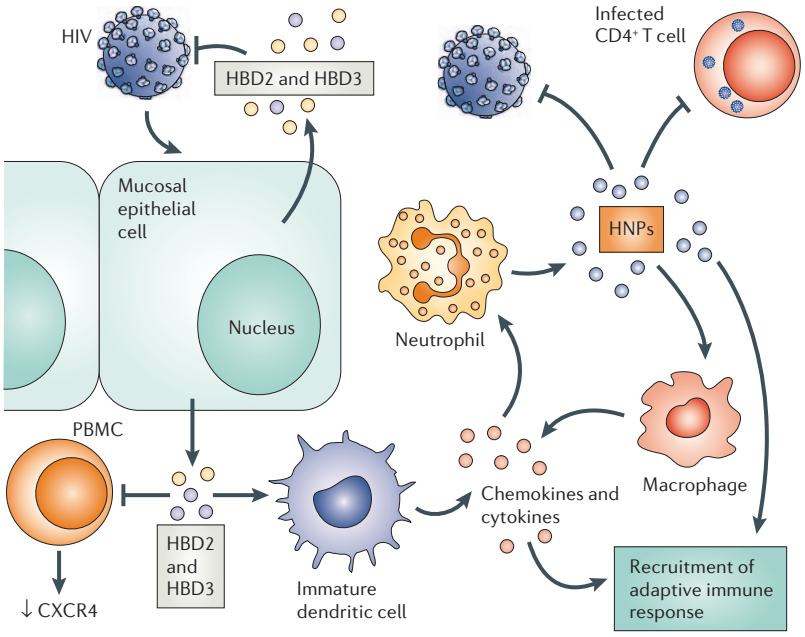

Figure 2 | **Roles of defensins in mucosal immunity against HIV infection.** In the case of HIV, human β-defensins (HBDs) are released from the mucosal epithelium in response to viral infection. HBDs have a direct effect on HIV virons and indirectly can affect HIV infection through downregulation of CXC-chemokine receptor 4 (CXCR4) expression by peripheral-blood mononuclear cells (PBMCs) in the absence of serum. Neutrophils can release neutrophil α-defensins (human neutrophil peptides, HNPs) in response to stimulation by CC-chemokines. HNPs can either directly inactivate HIV virions or suppress HIV replication by altering target cells. HNPs can also upregulate CC-chemokine expression by macrophages. HNPs and HBDs can further recruit T cells, monocytes and immature dendritic cells (DCs) and trigger adaptive immunity to control viral infection.

Murine β-defensin-2 can recruit bone-marrow-derived immature DCs through CCR6 and can induce DC maturation through TLR4 (REF. 55).

Although the specific receptors responsible for the chemotactic activity of HNP1, HNP2 and HNP3 have not been identified, their chemotactic activity can also be blocked by pertussis toxin, indicating the involvement of a $G_{i\alpha}$-protein-coupled receptor(s)[56,57]. Several studies have implicated a role for specific receptors in other biological functions of HNPs[58–61]. For example, HNPs bind to low-density-lipoprotein-receptor-related proteins and interact with protein kinase Cα (PKCα) and PKCβ, leading to decreased smooth-muscle contraction in response to phenylephrine[62]. HNPs also interact with adrenocorticotropic hormone receptors and heparan sulphate proteoglycans (HSPGs) to modulate other biological activities[60,61]. HNP1 has been shown to inhibit the activity of conventional PKC isoforms in a cell-free system[63]. This PKC inhibitory activity seems to be important for the HNP1-mediated inhibition of HIV replication in primary $CD4^+$ T cells[64]. Taken together, these studies indicate that several biological functions of human α- and β-defensins might be mediated through interaction with receptors and subsequent regulation of cell-signalling pathways. However, the role of these receptor interactions and signalling pathways in defensin-mediated antiviral activities remains to be determined.

Specific antiviral effects

Defensins have a dual role in antiviral activity (FIG. 3). One aspect of antiviral activity involves direct interaction with viral envelopes, possibly in a similar way to the antibacterial activity of defensins, and the other involves indirect antiviral activity through interactions with potential target cells. These defensin–cell interactions are complex and possibly mediated by interacting with cell-surface glycoproteins and/or interfering with cell-signalling pathways that are required for viral replication. TABLE 2 summarizes the activities of defensins and other antimicrobial peptides on viral replication.

Direct effect on the virion. HNP1 was originally reported to have a direct effect on several enveloped viruses but not on non-enveloped viruses[9]. Among those enveloped viruses tested, HNP1 has a potent direct inhibitory effect on herpes simplex virus-1 (HSV-1) and HSV-2, a moderate direct effect on vesicular stomatitis virus (VSV) and influenza virus, and little direct effect on cytomegalovirus (CMV)[9]. The differential inhibitory effect of HNP1 against different enveloped viruses might be due to variability in the lipid composition of the viral envelopes of different viruses, as the lipid composition of bacterial membranes has been shown to influence membrane permeabilization by rabbit neutrophil defensins[65]. The exact mechanism of direct inactivation of the virion by defensins is not clear. Current models (FIG. 3a), including viral membrane disruption or binding to viral glycoproteins, need to be further investigated.

Factors such as serum and salt are known to alter the functions of defensins *in vitro*. Therefore, the different antiviral mechanisms of defensins might be operative in mucosal surfaces rather than blood, depending on the salt concentration or the presence of serum. This seems to be the case with the direct antiviral effect. Serum has been shown to diminish the direct effect of defensins on the virion[9,64]. High concentrations of defensins are known to cause cytotoxicity in the absence of serum, and this is associated with changes in cell-membrane permeability in a similar way to the antibacterial activity of the defensins. This cytotoxicity can be abolished by the presence of serum[66,67] and defensin-mediated cytotoxicity might partially account for the antiviral effect[28]. In addition, most defensins have potent direct antibacterial activities in conditions of low salt concentration[68]. However, the required conditions for optimum activity vary depending on the specific function of defensins. For example, neither a low concentration of salt nor the absence of serum are required for the chemotactic effects of defensins[52,57]. Therefore, it is important to define the experimental conditions carefully when examining the antiviral activities of defensins.

HIV. Inhibition of HIV replication by synthetic guinea-pig, rabbit and rat α-defensins was first reported in 1993 (REF. 69), when it was shown that these peptides could inhibit HIV-1 infection *in vitro* following viral entry into transformed $CD4^+$ T cells in the presence of serum[69]. The anti-HIV activity of HNP1, HNP2 and HNP3 has recently

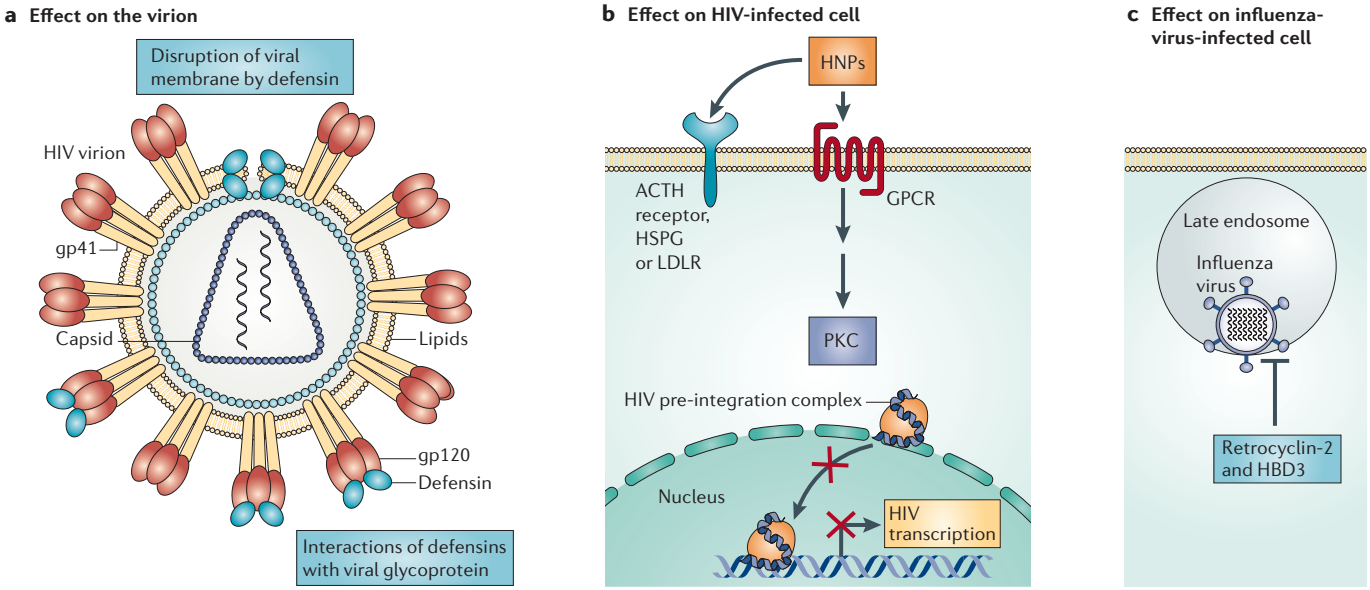

Figure 3 | **Mechanisms of antiviral activity by defensins. a** | In the absence of serum (such as at mucosal surfaces), defensins inactivate enveloped virus particles by disrupting viral envelopes or by interacting with viral glycoproteins, such as HIV gp120. **b** | In the presence of serum, defensins act on target cells, possibly through interaction with G-protein-coupled receptors (GPCRs) and/or other cell-surface receptors, such as adrenocorticotropic hormone (ACTH) receptor, heparan sulphate proteoglycan (HSPG) and low-density-lipoprotein receptor (LDLR), resulting in alterations in downstream signalling, as has been shown for protein kinase C (PKC). These interactions can result in antiviral activity by blocking nuclear import of the pre-integration complex or blocking transcription of viral RNA. **c** | Defensins can block fusion of the viral membrane with the endosome of the host cell by crosslinking viral glycoproteins (influenza virus haemagglutinin, Sindbis virus E1 and baculovirus gp64), thereby preventing viral replication. HBD3, human β-defensin-3; HNPs, human neutrophil peptides.

been investigated[28,70,71]. HNP1, HNP2 and HNP3 all have similar activities against HIV primary isolates[72], in contrast to their differential chemotactic activities on monocytes, which HNP3 does not effect[73]. HNP1, HNP2 and HNP3 have at least two mechanisms of anti-HIV activity. They can inhibit HIV-1 replication by a direct interaction with the virus as well by affecting the target cells[28,64,71,74]. In the absence of serum, HNP1 can directly inactivate the virus before it infects a cell[64]. In the presence of serum and at non-cytotoxic concentrations (low dose), HNP1 acts on infected cells and blocks HIV-1 infection at the steps of nuclear import and transcription. Furthermore, in primary $CD4^+$ T cells, HNP1 interference with PKC signalling is associated with the ability of HNP1 to inhibit infection after HIV enters the cell, although other signalling pathways might also be involved[64]. For example, in macrophages, HNP1 and HNP2 upregulate the expression of CC-chemokines, which could contribute to inhibition of HIV through competition for receptors[75] (FIG. 2). CC-chemokines can also induce the release of HNPs from neutrophils by degranulation[76]. Both effects could have a role *in vivo* in an innate immune response to HIV. At the mucosal surface, HNPs might work to directly inactivate the virions in the absence of serum; however, in the presence of serum, their inhibitory effect would largely be on the infected cell.

HNPs are positively charged, so direct binding to HIV virions through charge interactions might account for some of their direct inhibition of HIV virions, as well as for their sensitivity to serum through competing interactions with serum proteins. HNP1, HNP2 and HNP3 have been reported to function as lectins, by binding to the HIV envelope glycoprotein gp120 and CD4 with high affinity, although their interference with the interaction between HIV gp120 and CD4 has not been well defined[74]. HNP binding to gp120 is strongly attenuated by serum, therefore accounting for the loss of the direct effect on the virion in the presence of serum. Interestingly, in contrast to HNP1, HNP2 and HNP3, HNP4 acts in a lectin-independent manner and does not bind to CD4 or HIV gp120 (REFS 72,74). However, HNP4 inhibits HIV replication more effectively than HNP1, HNP2 and HNP3 (REF. 72), although it is not clear whether the antiviral activity of HNP4 is mediated through a direct effect on virions or on the infected cells.

Other α-defensins, including HD5, mouse cryptdin-3 and cryptdin-4, and rhesus macaque myeloid α-defensin-3 (RMAD3) and RMAD4 have been tested for their ability to block HIV infection[77]. At the high concentrations associated with cytotoxicity, RMAD4 blocks HIV replication, whereas cryptdin-3 enhances viral replication. The other peptides tested do not have anti-HIV activity in the assay systems reported. The mechanism of enhanced HIV replication by cryptdin-3 and the effect of these peptides on HIV replication following viral entry are not clear. Because experiments carried out with these defensins used a transformed cell line, alternative assay systems including primary cells

Table 2 | **Antiviral activities of defensins and other antimicrobial peptides**

Defensins	Viruses	Effect	References
α-Defensins			
HNP1, HNP2 and HNP3	HIV-1, HSV-1, HSV-2, VSV, influenza virus, CMV, adenovirus and papillomavirus	Inhibitory	9,28,64,70–72, 74,75,84,87,88
HNP1	Echovirus, reovirus and vaccinia virus	None	9,107
HNP4	HIV-1	Inhibitory	72
HD5	Papillomavirus	Inhibitory	87
RMAD4	HIV-1	Inhibitory	77
Guinea pig NP1	HIV-1	Inhibitory	69
Rat NP1	HIV-1	Inhibitory	69
Rabbit NP1	HIV-1 and HSV-2	Inhibitory	69,83
Cryptdin-3	HIV-1	Enhanced	77
β-Defensins			
HBD1	HIV-1 and vaccinia virus	None	47,78,107
HBD2	HIV-1 and adenovirus	Inhibitory	47,78,88
	Rhinovirus and vaccinia virus	None	34,107
HBD3	HIV-1 and influenza virus	Inhibitory	47,78,81
HBD6	PIV-3 (*in vivo*)	Enhanced	86
Sheep BD4	PIV-3 (*in vivo*)	Inhibitory	85
θ-Defensins			
Retrocyclin-1 and retrocyclin-2	HIV-1, HSV-2 and influenza virus	Inhibitory	20,46,74, 79,81,84
RTD1, RTD2 and RTD3	HIV-1 and HSV-2	Inhibitory	74,79,84
Other antimicrobial peptides			
LL37	Vaccinia virus	Inhibitory	107
CRAMP	Vaccinia virus (*in vitro*, *in vivo*)	Inhibitory	107
Indolicidin	HIV-1	Inhibitory	108
Dermaseptin S4	HIV-1	Inhibitory	109
Caerin 1.1 and caerin 1.9	HIV-1	Inhibitory	110
Maculatin 1.1	HIV-1	Inhibitory	110

BD4, β-defensin-4; CMV, cytomegalovirus; CRAMP, cathelicidin-related antimicrobial peptide; HBD, human β-defensin; HNP, human neutrophil peptide; HSV, herpes simplex virus; NP1, neutrophil peptide 1; PIV, parainfluenza virus; RMAD, rhesus macaque myeloid α-defensin; RTD, rhesus θ-defensin; VSV, vesicular stomatitis virus.

will help to better define the anti-HIV activity of these defensins. For example, HNP1 causes post-entry inhibition of HIV in primary CD4$^+$ T cells and macrophages but not in several transformed T-cell lines[64,71].

The anti-HIV activities of HBD2 and HBD3 have been shown under different conditions[47,78]. One condition used mimics the oral mucosal environment, with low salt concentrations and the absence of serum[47], and another condition used has high salt concentrations and the presence of serum[78]. Similar to HNP1 (REF. 64), HBD2 and HBD3 have dual anti-HIV activities through direct interactions with the virus and indirectly by altering the target cell. HBD2 and HBD3 have been shown, by electron microscopy, to interact with cellular membranes as well as HIV virions, although membrane disruption is not apparent[47]. HBD2 does not affect cell–cell fusion but instead inhibits the formation of early reverse-transcribed HIV DNA products[78]. There are conflicting reports on the downregulation of expression of HIV co-receptors by β-defensins. In studies reported by Sun *et al.*[78], HBD1 and HBD2 did not modulate cell-surface HIV co-receptor expression by primary CD4$^+$ T cells. By contrast, Quinones-Mateu *et al.*[47] showed HBD2- and HBD3-mediated downregulation of surface CXCR4 but not CCR5 expression by peripheral-blood mononuclear cells (PBMCs) at high salt conditions and in the absence of serum. These conflicting reports might be due to differences in the source of the defensin and/or experimental conditions used (that is, the presence or absence of serum). Interestingly, HBD2 is constitutively expressed in healthy adult oral mucosa but the level seems to be diminished in HIV-infected individuals[78].

Retrocyclins, and RTD1, RTD2 and RTD3, function as lectins and can inhibit HIV entry[20,46,74,79], and they inhibit several HIV-1 X4 and R5 viruses, including primary isolates[20,74,79]. Unlike α- and β-defensins, retrocyclin does not seem to inactivate the HIV virion directly, although it is not clear whether the experiments reported so far were carried out under serum-free conditions[46]. Retrocyclin does, however, bind to HIV gp120 as well as CD4 with high affinity, which is consistent with inhibition of viral entry[46,79]. This high-binding affinity of retrocyclin for glycosylated gp120 and CD4 is mediated through interactions with their *O*-linked and N-linked sugars[80]. Serum strongly reduces the binding of retrocyclin to gp120 (REF. 74). It remains to be determined whether the interactions with HIV glycoproteins are similar to those recently reported with influenza virus glycoproteins[81]. Nevertheless, studies on retrocyclin-1 analogues indicate that modification of this peptide can enhance its potency against HIV *in vitro*[82], indicating the therapeutic potential of such analogues.

HSV. Several defensins, including HNP1, HNP2, HNP3, HNP4, θ-defensins (RTDs and retrocyclin) and a rabbit α-defensin, neutrophil peptide 1 (NP1), have anti-HSV activity[83,84]. HNP1 has a direct effect on HSV virions, which is abolished in the presence of serum[9], although the mechanism of this direct effect is not clear. The anti-HSV activities of HNP1, HNP2, HNP3 and retrocyclin-2 occur by inhibiting viral attachment and entry, but they have no effect following entry of the virus[84]. With the exception of HNP4, α-defensins and θ-defensins interact with the *O*- and N-linked glycans of HSV-2, indicating that defensins might be acting as lectins to prevent HSV-2 gB from interacting with its receptor HSPGs[84].

Compared with HNPs, the rabbit α-defensin NP1 has more positively charged amino-acid residues[68]. It has a direct effect on HSV-1 and HSV-2 virions, and inhibits HSV replication at the steps of fusion and entry[83]. Unlike α- and θ-defensins, which do not inhibit HSV replication after viral entry[9,84], NP1 can suppress HSV-2 infection following viral entry[83].

Influenza virus. The unique mechanism by which retrocyclin-2 inhibits the entry of influenza virus has recently been described[81]. Retrocyclin-2 blocks the step of viral fusion mediated by influenza virus haemagglutinin (HA). In a similar manner, it inhibits fusion mediated by other viral proteins such as baculovirus gp64 and Sindbis virus (alphavirus) E1 proteins. By acting as a lectin, retrocyclin-2 interferes with virus-mediated fusion by crosslinking and immobilizing cell-membrane glycoproteins. Accordingly, pre-treatment of either HA-expressing cells or target cells with retrocyclin-2 inhibits fusion. In a similar manner to retrocyclin-2, HBD3 has an inhibitory effect on HA-mediated fusion and membrane-protein mobility. The study by Leikina *et al.*[81] indicates that a common mechanism might account for a broad range of activity of the innate immune response against viruses that use a common pathway of membrane fusion for entering host cells.

Parainfluenza virus. Respiratory syncytial virus (RSV) and the parainfluenza virus types 1–4 (PIV-1–4), which are members of the Paramyxoviridae family, are major causes of respiratory diseases, particularly in young children. Induction of expression of sheep β-defensin-1 and other antimicrobial proteins, such as surfactant protein A (SP-A) and SP-D, correlates with a decrease in PIV-3 replication in neonatal lambs[85]. However, activity of defensins against paramyxoviruses *in vitro* has not been reported. Adenovirus-mediated HBD6 expression increases neutrophil recruitment and inflammation in the lungs of neonatal lambs[86]. Unexpectedly, PIV-3 infection of neonatal lambs is enhanced during the treatment with adenovirus-mediated gene therapy and expression of HBD6 further exacerbates PIV-3 infection[86]. Nonetheless, it is not clear whether this enhancement of PIV-3 infection results from an HBD6-mediated increase in PIV infection or induction of a deleterious inflammatory response.

Non-enveloped viruses. HNPs do not seem to have a direct effect on the virions of several non-enveloped viruses, including echoviruses and reoviruses[9]. Similarly, HBD2 does not directly inactivate rhinovirus[35]. However, defensins might act on infected cells and suppress non-enveloped viral replication after viral entry. Using pseudoviruses carrying green fluorescent protein, HNP1 and HD5 have been recently shown to inhibit various types of papillomavirus[87]. These defensins do not affect the initial binding of the virion and endocytosis but block virion escape from endosomes. HNP1 has also been shown to inhibit adenovirus infectivity, although the assay system used in this study cannot distinguish between HNP1 effects on the virion or the cell[88]. Nevertheless, further studies are needed to define the mechanism(s) by which defensins suppress non-enveloped viral infection.

Polymorphisms of human defensin genes

Host genetic polymorphisms clearly influence susceptibility to viral infection and disease progression, as has been shown for HIV infection[89–92]. The human α-defensin genes *DEFA1* (encoding HNP1) and *DEFA3* (encoding HNP3) have polymorphisms in both copy number and the location of 19-kilobase (kb) tandem repeats on chromosome 8p23.1 (REFS 93,94). Gene expression of HNP1 and HNP3 at the RNA level in leukocytes correlates with the number of copies of the corresponding gene[94]. Similarly, a β-defensin gene cluster, including *DEFB4* (encoding HBD2), *DEFB103* (encoding HBD3) and *DEFB104* (encoding HBD4), is polymorphic in copy number, with a repeat size of at least 240 kb[95]. The *DEFB104* copy number correlates with the level of transcription. Although correlation between the protein levels of defensins and their gene-copy numbers has not been reported, it is tempting to speculate that variable expression levels of these defensins could lead to differential susceptibility to infection with or progression of infectious diseases.

Polymorphisms in the *DEFB1* gene (encoding HBD1) have been associated with susceptibility to diseases, including chronic obstructive pulmonary disease (COPD) and asthma, and are associated with the severity of cystic-fibrosis-associated pulmonary disease[96–100]. Although viral infections are one of the main triggers of exacerbations of obstructive airway diseases such as asthma and COPD[101], the association of polymorphisms in *DEFB1* with susceptibility to viral respiratory infections is not known. Interestingly, a single-nucleotide polymorphism in the 5′ untranslated region of *DEFB1* has been reported to be associated with perinatal transmission of HIV-1 in a cohort of Italian children[102]. However, the significance of this mutation in the control of HIV-1 infection remains to be explored. Sequence analysis of θ-defensin pseudogenes (*DEFT*) in HIV-1-exposed seronegative female sex-workers from Thailand showed that all subjects had premature stop codons[103]. Therefore, restoration of endogenous θ-defensin production does not account for the resistance to HIV-1 infection in these women.

Clinical implications

The role of HNP1, HNP2 and HNP3 in HIV pathogenesis in humans was first indicated when these peptides were reported to account for the soluble anti-HIV activity of $CD8^+$ T cells isolated from patients who were infected with HIV but remained free of AIDS for a prolonged period (long-term non-progressors, LTNPs)[70]. HNP1, HNP2 and HNP3 were detected in the cell-culture media of stimulated $CD8^+$ T cells from normal healthy controls and LTNPs but not from HIV progressors. Subsequently, studies on the cell source of defensins showed that HNP1, HNP2 and HNP3 were probably produced by co-cultured monocytes and residual granulocytes of allogeneic normal donor irradiated PBMCs that were used as feeder cells and not by the $CD8^+$ T cells themselves[28,29]. It is still unclear why HNP1, HNP2 and HNP3 were found in co-culture systems using $CD8^+$ T cells from normal controls and LTNPs but not when using $CD8^+$ T cells from HIV progessors. In all cases, the $CD8^+$ T cells were co-cultured with irradiated PBMCs from the same source[70]. One possible explanation is that the $CD8^+$ T cells from infected and non-infected individuals vary in their ability to trigger the release of HNPs from the co-cultured

cells and/or vary in their ability to take up and release HNPs. Using similar co-culture systems, concentrations of HNP1, HNP2 and HNP3 were measured in $CD8^+$ T-cell supernatants and cervicovaginal mononuclear cells derived from HIV-exposed seronegative individuals, HIV-infected patients and normal controls[104]. Higher levels of HNP1, HNP2 and HNP3 were found in $CD8^+$ T cells from HIV-exposed seronegative individuals and HIV patients compared with normal controls. Although RNA encoding HNP1, HNP2 and HNP3 was detectable in PBMCs and cervicovaginal biopsies, the specific cell source was not determined from this approach[104]. Although these studies provide some interesting correlations, association between HNPs and HIV infection rate and/or disease progression needs to be carefully evaluated in the context of *in vivo* infection.

The association between HNP1, HNP2 and HNP3 production in breast milk and the transmission of HIV has also been investigated[105]. In a case-control study of HIV-positive women, concentrations of HNP1, HNP2 and HNP3 in breast milk correlated with the amount of HIV RNA in breast milk, which was a strong predictor of transmission. However, after adjusting for the amount of HIV RNA in breast milk, higher concentrations of HNP1, HNP2 and HNP3 in breast milk were associated with a decreased incidence of intrapartum or postnatal HIV transmission. The concentrations of HNP1, HNP2 and HNP3 in the plasma or serum in these HIV-infected women was not analysed, so the role of HNP1, HNP2 and HNP3 in maternal systematic viral control and transmission could not be assessed. There are several other anti-HIV factors in breast milk, including HBD2, lactoferrin, secretory leukocyte inhibitor and chemokines, which could have a role in modifying mother-to-child HIV transmission.

There is a correlation between the abundance of several anti-HIV proteins, including HNP1, HNP2 and HNP3, and cell-associated HIV replication in lymphoid follicles compared with extrafollicular lymphoid tissue[106]. Expression of these antiviral proteins is significantly lower in the follicular region, where HIV replication is concentrated, compared with the extrafollicular regions in lymph nodes from HIV-positive individuals. These regional differences in expression of antiviral proteins have not been described in lymph nodes from HIV-seronegative individuals.

Concluding remarks

Leukocytes and mucosal epithelial cells are the main cell types that produce defensins. In response to viral infection, infected cells produce defensins, chemokines and cytokines to directly control viral infection and to recruit leukocytes including neutrophils to the site of infection (FIG. 2). Release of defensins by local or recruited cells can suppress viral infection by direct inactivation of the virion and by altering the target cell, for example, by interfering with cell-signalling pathways that are required for viral replication. Although there are some common pathways by which defensins can interfere with viral infection, as in the case of retrocylin-2 interference with the membrane-fusion process of several viruses, different defensins also have distinct mechanisms of inhibition that seem to be more virus and target-cell specific.

Increasing evidence indicates that defensins, which have long been recognized as natural antimicrobial peptides, have antiviral activity. However, many questions remain unanswered regarding their role in transmission and disease progression as well as the potential to exploit these activities for the development of new therapeutics and microbicides. Further studies on their mechanism and range of antiviral activity might identify other common pathways of action. The presence of inhibitory levels of defensins at important mucosal sites of viral entry, particularly in the setting of an inflammatory response, might shed light on their role in innate immune responses. In this regard, the crosstalk between TLR activation and defensin production in the control of viral infection requires further delineation, as does the role of defensins in modulating cytokine or chemokine production. The complex diversity of defensins in mammals, as well as apparent differences in mechanisms of defensin action, challenge our understanding of the role of defensins in viral pathogenesis in humans. Further studies focused on the contribution of the structure of defensins to their various antiviral activities, as well as standardization of assays used to assess their biological function, could identify some unifying principles and will contribute to their development as new drugs for the prevention of infection.

Note added in proof

In a recent publication, retrocyclin-1, a synthetic θ-defensin derived from the human pseudogene sequence, has been shown to inhibit HIV-1 by inhibiting envelope-mediated fusion. Retrocyclin-1 binds directly to the C-terminal heptad repeat of HIV envelope protein gp41, preventing formation of the six helix bundle required for fusion. This binding seems to be independent of glycan binding[111].

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Competing interests statement
The authors declare no competing financial interests.

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