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β-Defensins: Multifunctional Modulators of Infection, Inflammation and More?

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Key Words
Antimicrobial peptides · Host defense · Toll-like receptor · Defensin

Abstract
Defensins comprise one of the largest groups of host defence peptides, present throughout evolution, in fungi and flowering plants as well as in invertebrates and vertebrates. These cysteine-rich, cationic peptides have a common ability to kill a broad range of microorganisms including bacteria, yeast and viruses. As such, they are a strong component of the arsenal that is an organism's innate immunity. It is becoming increasingly clear, however, that antimicrobial action is only one of the numerous roles of these multifunctional peptides. In recent years, the functions of defensins in immunomodulation have been widely investigated, and their involvement in other processes (such as fertility) is becoming evident. This review addresses recent advances in the immunomodulatory activity of β-defensins as well as the involvement of β-defensins in fertility, development, wound healing and cancer.

Introduction

In mammals, defensins are a multigene family with many species-specific clades and this duplication allows selection for species-specific function. In the majority of mammals there are different types of defensin that are distinguishable by their genomic organization, cysteine spacing and intramolecular disulfide connectivities. These are β-defensins (which are considered the ancestral gene type), α-defensins (present before placental and marsupial divergence) [1] and θ-defensins (octodecapeptides that arose in primates from an α-defensin gene; for excellent reviews see [2] and [3]). θ-Defensin genes are no longer functional in humans [4] but are in the rhesus macaque *(Macaca mulatta)* and olive baboon *(Papio anubis)*.

In the human genome, many β-defensins occur in a cluster on chromosome 8p23.1 and other β-defensin genes have been identified by computational analysis in clusters at 20p13, 20q11.1 and 6p12 [5]. These β-defensin genes mostly consist of two exons, the first encodes the leucine-rich signal and prosequence, and the second exon encodes the mature peptide. Analysis of the largest murine β-defensin gene cluster on chromosome 8 showed that the DNA and protein sequence of the signal region encoded by exon 1 is highly conserved amongst genes that are physically close. Conversely, the functional mature peptide encoded by exon 2 is not well conserved.

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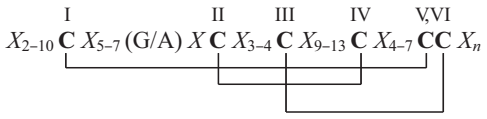
However, β-defensins generally have a 6-cysteine motif and are stabilized by disulfide bridging between cys I–V, cys II–IV and cys III–VI [6] as shown below.



The typical free N-terminus and 3 antiparallel β-sheet structure of a β-defensin are shown in figure 1.

In mammals, the evolution of β-defensins has been complex with both rapid positive and negative selective pressure acting on the gene family [7–9]. The positive selection may be driven by exposure to particular pathogens, and indeed β-defensins demonstrate different profiles of antimicrobial activity and tissue distribution (reviewed by [2, 10]) suggesting that the molecular diversity and rapid evolution of β-defensins have provided the host organism with a range of specific responses to diverse pathogens [11]. It is also possible that this duplication allows specialization to different functions.

An example of the specialization of β-defensins is demonstrated by evolutionary analyses of the β-defensin-like toxin genes present in the platypus *(Ornithorhynchus anatinus)*. Examination of the platypus genome sequence revealed β-defensin-like peptide genes. These encode peptides which are the main components of the substance produced from the venomous spurs on the hind legs of the males [12]. The genes have evolved by duplication and diversification of β-defensins and are chromosomally adjacent to them. Despite low sequence homology, the genomic structure and 6-cysteine motif is retained. The expression pattern of these genes is wider than would be predicted for a specific venomous function, which implies additional unknown functions [13].

The literature has recently exploded with papers dedicated to understanding the in vivo function of defensins and this review seeks to present the expanding function of β-defensins in immunity and beyond. An unequivocal way to investigate function is to link gene mutation to phenotype or disease. This is complicated as there is a block of genes in the major β-defensin locus on human chromosome 8 that is subject to copy number variation [14]. A link between copy number and disease has been reported, where an increase in copy number is associated with an increased risk of psoriasis [15]. A link between copy number and Crohn's disease has also been reported but the copy number associations are confusing and technically challenging, with different populations having a reduced [16] or increased [17] association, or none at all [18]. Polymorphisms in the promoter of *DEFB1* (which is not a copy number variable) have also been associated with Crohn's disease [19]. In addition, tooth decay and oral *Candida* infection in some populations have also been linked to *DEFB1* polymorphisms [20, 21]. Although disease genetics have implicated defensins in inflammatory disease, the role of β-defensins in these diseases remains unclear.

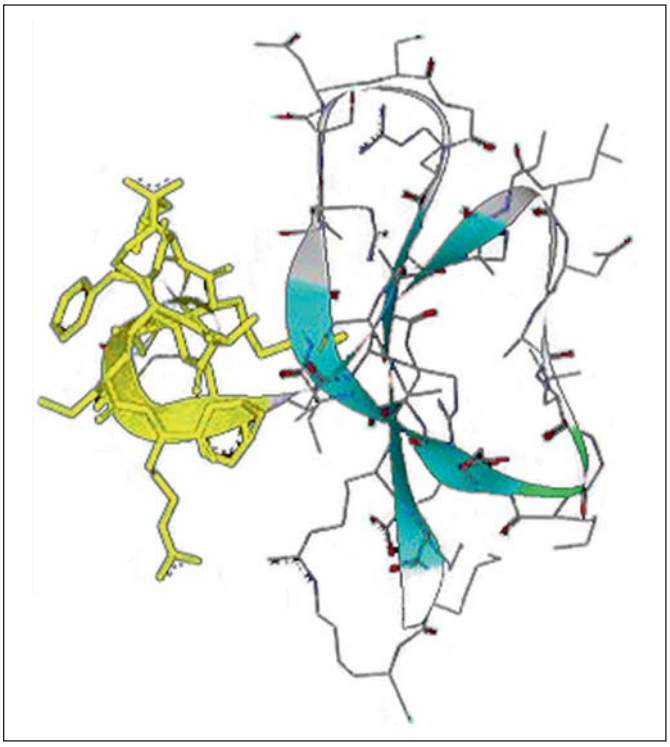


Fig. 1. β-Defensin structure. Homology model of β-defensin Defb14 based on the structure of hBD3, with antimicrobially potent residues 6–17 highlighted in yellow and β-sheets in blue [32].

β-Defensins as Antimicrobial Peptides

A major feature of innate immunity is rapid recognition and response to a foreign pathogen. In the test tube under nonphysiological conditions (low sodium chloride and low serum) defensins effectively and rapidly kill microorganisms including bacteria, viruses and yeast. The antimicrobial mode of action has not been clarified for all β-defensins and pathogens; however, hBD3 has been found to disrupt cell wall biosynthesis by binding lipid-II-rich regions of the cell wall [22]. A similar mechanism has been shown for the fungal defensin, plectasin [23].

The bactericidal activity of defensins in vitro does not necessarily reflect functional activity in vivo. There is, however, evidence in knockout mice that pathogen clearance is at least one aspect of defensin function. Deletion of the gene matrilysin produces animals with incorrectly processed and reduced levels of mature intestinal α-defensins (cryptdins) and these animals display reduced microbial killing in the gut [24]. In addition, expression of the human intestinal α-defensin HD5 makes mice less susceptible to oral challenge with virulent *Salmonella typhimurium* [25]. The antimicrobial effects of β-defensins in vivo have also been shown in mice with reduced levels of murine β-defensin 10. These mice also show defective killing of several major components of the intestinal microbiota [19]. Deletion of the mouse *Defb1* gene results in increased *Staphylococcus* species in the normally sterile urine [26] and an inability to clear *Haemophilus influenza* from the airway after challenge [27]. Recently, antimicrobial activity of human β-defensin 1 (hBD1) was shown to be dependent on structure. This defensin displays weak antimicrobial activity in the oxidized form but was shown to be a potent antimicrobial against commensal bacteria in its reduced form [28]. Such a change in structure might occur during redox reaction in vivo and it was shown that thioredoxin reductase localizes with hBD1 in tissues and in vitro can precipitate disulfide bond reduction.

It is thus clear that β-defensins are involved in antimicrobial clearance; however, it is worth noting that the concentrations of peptide required for antimicrobial activity are high compared to those described in the next section on the immunomodulatory properties of β-defensins.

β-Defensins as Proinflammatory Mediators of the Immune Response

In addition to displaying potent microcidal properties, β-defensins also play a part in other aspects of innate and adaptive immunity. β-Defensin expression is associated with the development of psoriasis, thereby suggesting a proinflammatory effect of these peptides and defensins have indeed been shown to be proinflammatory in a number of studies (discussed in this section). Many of these proinflammatory effects occur via defensin-receptor binding and it appears that β-defensins are 'promiscuous' ligands interacting with a variety of receptors; this may result from electrostatic binding due to their cationic nature. This was recently demonstrated by hBD2 which was shown to electrostatically interact with heparin sulphate (a glycosamineglycan used by many chemokines) [29].

The first remarkable example of β-defensin/receptor interaction was when Yang et al. [30] demonstrated that hBD2, and to a lesser extent hBD1, induced chemoattraction of CD4+ memory T cells and immature dendritic cells, by binding to CCR6. The chemoattraction of CCR6-expressing cells was also demonstrated by hBD3 and interestingly hBD3 and hBD4 have also been shown to attract macrophages [31] which do not express CCR6, suggesting involvement of a different receptor. Functional interaction of hBD3 with CCR6 or macrophages was dependent on both peptide structure and a particular cysteine (cysV) residue [31, 32]. DEFB14, the mouse ortholog of hBD3, also chemoattracts human CCR6-expressing cells and both mouse and human macrophages [32, 33].

It has been shown in vitro that mouse defensin Defb8 and a 5-cysteine allele (Defr1) present in C57Bl/6 mice also attract immature dendritic cells and CD4+ T cells independently of CCR6 [34], and recent work suggests that CCR2 mediates monocyte/macrophage migration in response to hBD3 [35, 36].

In addition to binding CCR6 and CCR2, β-defensins have also been shown to interact with Toll-like receptors (TLRs) on antigen-presenting cells. Murine beta-defensin 2 (mBD2) induced the costimulatory molecules CD40, CD80 and CD86 on immature dendritic cells [37]. This was shown to be mediated by mBD2 interacting with TLR-4. mBD2 also stimulated immature dendritic cells to mature, resulting in T cell stimulation and an adaptive immune response. hBD3 has also been described as an activator of antigen-presenting cells, through binding to TLR1 and TLR2 and subsequent activation of MyD88-dependent signaling [38]. A β-defensin-induced increase in proinflammatory cytokine levels in immune cells has recently been demonstrated in human macrophages [36]. The expression of the *TNFα, IL-1α, IL-6, IL-8* and *CCL18* gene transcripts was significantly increased in these cells after treatment with hBD3; however, the protein levels of these cytokines were not measured. A recent study on the mechanism of hBD3 induction of proinflammatory cytokines demonstrated that hBD3 increases IL-1β, IL-6 and IL-8 (at the protein level) in human monocytes through a TLR1/2 mechanism [39].

Additionally, studies by Niyonsaba et al. [40] have shown that β-defensins have a proinflammatory effect on human keratinocytes. Treatment of primary keratinocytes with hDB2, hBD3 and hBD4 increased the expression of proinflammatory mediators, such as monocyte chemoattractant protein-1, macrophage inflammatory

protein-3α, RANTES, IL-6, IL-10 and IP-10. This induction was shown to be dependent on the interaction of defensins with a G-protein-coupled receptor. hBD2, hBD3 and hBD4 were also shown to enhance the expression of the novel pruritogenic mediator IL-31 in mast cells [41]. In addition to directly promoting an inflammatory response by proinflammatory cytokine induction, β-defensins have also been shown to suppress neutrophil apoptosis [42]. This study showed that hBD3 binds to CCR6 at the neutrophil cell surface, initiating an increase in the antiapoptotic protein Bcl-xL and inhibits caspase 3 activity. The prolonged life span of these neutrophils is an inflammatory event beneficial for the clearing of invading microorganisms. Thus, by inhibiting neutrophil apoptosis and promoting the production of proinflammatory cytokines and chemokines, β-defensins amplify the immune response. Collectively, these studies demonstrate that β-defensins bind to several cell surface receptors and enhance the immune response.

β-Defensins as Proinflammatory Suppressors

A recent and perhaps contradictory function of β-defensins is the discovery that these peptides also demonstrate an ability to attenuate a proinflammatory response.

This phenomenon has previously been described for α-defensins. A study on the matrilysin-deficient mice, without mature α-defensins in the intestine, showed that these mice were more susceptible to dextran-sulfate-sodium-induced colitis than wild-type controls. IL-1β levels in the α-defensin-deficient mice were significantly increased and it was ultimately shown that α-defensins were able to inhibit the production of IL-1β [43]. Miles et al. [44] also demonstrated an immunosuppressive effect of α-defensins released by apoptotic and necrotic neutrophils. It was shown that a proinflammatory stimulus from LPS and a T cell surrogate stimulus (CD40 ligand with IFNγ) were inhibited by the presence of α-defensins. Thus, α-defensins from dying neutrophils inhibit the stimulation of macrophages and may play a part in regulating the resolution of inflammation.

β-Defensins (which are expressed mainly by epithelia) are also capable of inhibiting inflammation. Human β-defensins 1, 2, 3 and 4 are induced on exposure to bacterial infection, proinflammatory stimuli and also endogenous danger signals [45–50]. In addition, basal levels of β-defensins are present in epithelial cells in the absence of an inducing stimulus, and hBD3 has been shown to be expressed in noninflammed tissues in the oral cavity [51, 52]. Low basal levels of this β-defensin may play a role in maintaining a noninflammatory environment before an immune response has been elicited, perhaps by neutralizing the effects of continual low-level exposure to commensal and pathogenic microbial antigens. This idea is consistent with our current studies demonstrating that hBD3 has an immunosuppressive effect in the presence of LPS [53]. The induction of TNFα and IL-6 in LPS-treated mouse and human macrophages was significantly suppressed by the presence of 0.5–1 μM hBD3 (2.5–5 μg/ml). Furthermore, at this concentration, proinflammatory proteins were not induced, and microarray analysis demonstrated a lack of proinflammatory gene expression. The proinflammatory effect of β-defensins observed in the studies by Funderburg et al. [38] and Niyonsaba et al. [41] is at slightly higher concentrations (4–6 μM, 20–30 μg/ml). This is not the first time that opposing effects have been observed for an immunomodulating antimicrobial peptide; the cathelicidin LL-37 has been shown to demonstrate a biphasic effect, being proinflammatory at concentrations above 20 μg/ml but anti-inflammatory at concentrations of 1–5 μg/ml [54]. Indeed, in vitro β-defensins chemoattract immune cells at concentrations in the 1–100 ng/ml range and, at these concentrations, other immunomodulating effects would not be observed. Concentrations of β-defensins in vivo are not well established at areas of inflammation; however, in psoriatic lesions, for example, hBD2 has been shown to range from 2.3 to 157 μM (the equivalent of 10–680 μg/ml) [55]. Further, Jansen et al. [56] found that the serum levels of hBD2 in psoriasis patients were up to 190 ng/ml, which was interpreted to be derived from local production by the keratinocytes. This suggests that the concentrations to which keratinocytes and infiltrated cells in the epidermis are exposed must be several orders of magnitude higher. They went on to use the production of hBD2 in reconstructed skin as a model for human epidermis, and a very strong immunohistochemistry signal was observed after stimulation with IL-1α, TNF-α and IL-6. ELISA for hBD2 found that these 8-mm-diameter skin cultures secreted approximately 66 ± 19 ng hBD2 per 24 h into the tissue culture medium. Their seemingly conservative estimation of the hBD2 local concentration in the compartment of the stimulated epidermis was 1.2 mg/ml (0.3 mM), which is far higher than concentrations required by in vitro studies for biological effect.

It is clear that β-defensins have a variety of different functions that are determined by the level of expression. It is feasible that defensins combine pro- and anti-inflammatory effects depending on disease state and pathogen

exposure. Defensins expressed at lower levels may also be involved in resolution of the immune response. For example, defensins may be expressed at a high level at the site of pathogen entry resulting in a proinflammatory response involving the chemoattraction of macrophages and other immune cells. As the danger is neutralized and defensins and other proinflammatory molecules decrease, defensins may then have a role in resolving inflammation.

It has been shown that the structure of defensins plays a part in the immunomodulatory effect. As discussed above, the importance of structure had previously been demonstrated for hBD3-CCR6 interaction in chemotaxis [31, 32]. We recently demonstrated that while canonically folded hBD3 results in the suppression of an LPS induction of TNFα, a derivative of hBD3 without a disulfide-stabilized structure has no inhibitory effect on LPS-induced TNFα levels, as shown in figure 2 [57]. So the immunomodulatory functions of β-defensins are also dependent on structural integrity.

The mechanism by which β-defensins neutralize a proinflammatory response is not fully known, but a number of potential mechanisms have been investigated. One possible mechanism is the binding of highly positively charged defensins to negatively charged ligands (e.g. LPS) thereby interfering with ligand receptor binding. In addition, defensins may act as antagonists for receptors used by proinflammatory stimuli. Receptors and or cell membranes may be disrupted or altered by defensins, as has been shown for the antimicrobial LL-37 which also has immunosuppressive effects [58, 59]. It is also possible that β-defensins activate a receptor and induce expression of anti-inflammatory mediators. The few studies addressing the immunosuppressive mechanism of β-defensins are discussed below.

DEFB123 was shown to prevent LPS-induced TNFα secretion in murine macrophages [60]. This defensin was shown to bind LPS in an endotoxin-binding assay; hence the suppressive effect was determined as being caused by DEFB123 sequestering LPS. In fact, DEFB123 efficiently bound LPS to the extent that mice given LPS did not die from endotoxic shock. Similarly, Pingel et al. [61] showed that hBD3 attenuated a proinflammatory cytokine response to HagB (a hemagglutinin of *Porphyromonas gingivalis*). HagB was shown to induce 22 cytokines and the presence of hBD3 selectively inhibited IL-6, IL-10, TNFα and GM-CSF. It was demonstrated that hBD3 binds to HagB bacterial antigens resulting in the attenuation of an antigen-induced proinflammatory cytokine response through dendritic cell receptors. However, the proinflammatory mechanism of HagB is not fully known, so it

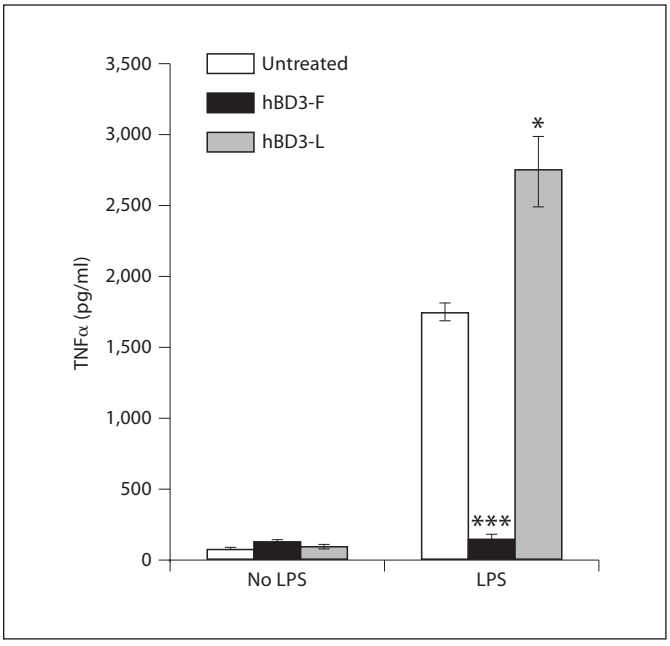


Fig. 2. Structural integrity is required for hBD3 immunosuppressive effect. Treatment of RAW264.7 macrophage cell line with LPS results in an increase in TNFα levels (measured by ELISA). In the presence of LPS, oxidized and canonically folded hBD3 (hBD3-F; black bars) inhibits this induction of TNFα. In contrast, linear hBD3 (hBD3-L) does not inhibit LPS-induced TNFα.

is possible that hBD3 either prevents a specific antigen binding a dendritic cell receptor or hBD3 may disrupt the conformation of HagB. The suppression of only 4 of the 22 cytokines that were investigated suggests that hBD3 is not simply neutralising all HagB activity, otherwise all cytokine induction would be abolished. Therefore, hBD3 is altering the ability of HagB to bind particular receptors. Experiments showing that hBD3 selectively inhibits aspects of MAP kinase signalling pathways validate these findings. Treatment with HagB induces phosphorylation of the stress-activated kinases p38 and JNK, and the growth-related kinases ERK1/2. The presence of hBD3 selectively inhibits ERK phosphorylation, and this presumably allows the selective inhibition of TNFα, IL-6, IL-10 and GM-CSF. Studies on the effects of hBD3 on these pathways will aid in the understanding of hBD3 immunosuppressive function.

hBD3 also binds herpes simplex virus, but hBD1 and hBD2 do not [62]. The presence of hBD3 prevented entry of the virus into cervical epithelial cells and this was shown to be caused by two mechanisms. Firstly, hBD3 bound to glycoprotein B, the surface protein which is required for viral entry into the cells; this effect of hBD3 has

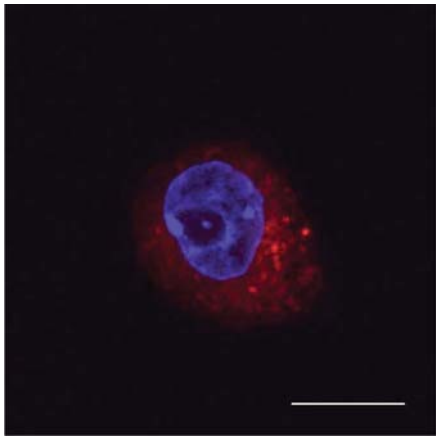


Fig. 3. hBD3 rapidly enters macrophages. The image shows a single cell (from the macrophage-like cell line RAW264.7) 10 min after the addition of hBD3 labeled with a TAMRA fluorochrome (red). Nuclear staining with DAPI (blue) demonstrates that at this time-point, hBD3 accumulates in the cytoplasm. Picture kindly provided by Dr. Heather MacPherson, MRC Human Genetics Unit, Edinburgh, UK.

also been demonstrated with the influenza virus [63]. Secondly, hBD3 also bound heparin sulphate – the receptor for the herpes simplex virus. This binding effectively prevented viral entry. Interestingly, the α-defensins HNP-1 and HD5 blocked viral gene expression, but this was not observed for hBD3. Hazrati et al. [62] further demonstrated that specific defensins intervene at different stages of the viral life-cycle, which suggests specific and diverse actions of defensins on antiviral activity.

Although hBD3 binds viral components, it does not bind the bacterial component LPS. The immunosuppressive effects of hBD3 have been shown to be independent of LPS binding; it was demonstrated that hBD3 did not bind LPS in an endotoxin-binding assay [53, 60]. In addition, macrophages treated with hBD3 for an hour and then washed extensively to remove any unbound material did not exhibit a proinflammatory response when LPS was subsequently added [57]. We have demonstrated that hBD3 rapidly enters macrophages (see fig. 3) and affects Toll-like receptor signalling pathways downstream of TLR4. hBD3 affects signalling through both the MyD88-dependent and independent pathways. In this way, it prevents NFκB activation and the subsequent transcription of proinflammatory genes. Analysis of LPS-induced gene transcription demonstrates that the presence of hBD3 results in suppressed gene transcription. This suggests an additional novel mechanism for β-defensin immunosuppressive activity [57].

The immunoinhibitory effects of β-defensins in vivo are still to be fully explored. To date, an in vivo immunosuppressive effect has been described for hBD3 and mouse β-defensin 1 [46, 53]. Cytokine response was compared in mice injected intraperitoneally with either LPS alone or LPS with hBD3. Mice receiving LPS and hBD3 had significantly lowered levels of circulating TNFα, compared to the LPS controls. The degree of inhibition provided by hBD3 was comparable to the extent of inhibition shown by IL-10 administration, which protects mice from endotoxic shock [64], suggesting that hBD3 may provide similar protection. Recently, β-defensin 1 has been shown to play a role in the prevention of viral inflammation in immune cells. Mice deficient in mBD1 (the mouse ortholog of hBD1) were exposed to the influenza virus and were found to lose weight more rapidly and, ultimately, to die sooner than wild-type controls. It was subsequently shown that mice deficient in mBD1 demonstrate a greater immune cell influx into the lungs [46]. This suggests that mBD1 is involved in early immune responses to inflammation which prevent or clear this influx. It also shows that mBD1 has an immunosuppressive mechanism and does not simply inactivate viral effects on the host by binding the virus and inhibiting viral replication.

Recently, the mouse ortholog of hBD3 (Defb14) was shown to dampen T-cell-driven immune reactions. Treatment with DEFB14 switched CD4+ T cells to regulatory T cells, and DEFB14 injection in vivo suppressed the induction of contact hypersensitivity [65]. Thus DEFB14 may also have an immunosuppressive role by taming T-cell-driven reactions.

These studies clearly demonstrate an immunosuppressive effect of β-defensins on both bacterial and viral components. It is therefore necessary to conclude that these antimicrobial β-defensin peptides have a fascinating ability to both promote and suppress (and/or resolve) an inflammatory response. In addition to these immunomodulatory roles, other functions of β-defensins have emerged. The remainder of this review will discuss some of these exciting directions.

β-Defensin CBD3 and Its Effect on Coat Color

An unexpected and intriguing example of the promiscuity of β-defensins in vivo was revealed by dog coat color genetics [66]. Candille et al. [66] demonstrated that the dominant coat color allele at the dog K locus was a variant of canine β-defensin *103* (CB103, the dog ortholog of the gene that encodes hBD3). In all black dogs, the re-

searchers found at least one allele that contained a 3-base-pair deletion in addition to the loss of a glycine residue from the N-terminal of the mature processed peptide. This allele was more efficiently secreted from cells and has a higher affinity than the wild-type peptide for the dog melanocortin 1 receptor (MC1R). This receptor controls production of eumelanin, the black/brown hair pigment. CBD103 also competes with agouti-signaling protein (ASP) for binding to this receptor. ASP antagonizes MC1R, resulting in the production of pheomelanin, a red/yellow pigment. Expression of either normal or mutant dog *CBD103* in wild-type mice suppressed ASP, resulting in a change in mouse hair color from agouti (hair with a yellow stripe) to black. Presumably, the increased abundance and stronger affinity for the MC1R allows mutant β-defensin to competitively inhibit ASP in melanocytes, enabling the production of eumelanin and black hair. This dominant mutation, that arose in the domestic dog and has presumably been selected as a desirable coat color by breeders, was also found in North American wolves and derives from mating with domestic dogs. The mutation has risen to a high frequency in forested habitats where dark color would be advantageous, demonstrating a molecular signature of positive selection [67]. Binding of a defensin to melanocortin receptors is interesting as these receptors mediate a variety of signalling processes that include not only pigmentation but also weight control. In humans, there is no evidence that defensins interact with MC1R and although melanocortin receptors have been implicated in controlling inflammation through cyclic antimicrobial peptide induction, we found no evidence using *Mc1r* and *Mc3r* knockout mice that the anti-inflammatory effect of hBD3 was mediated through this receptor [53].

Wound Healing

hBD3 is highly expressed in keratinocytes and especially at wound sites in response to growth factors [45]. In addition, it promotes the proliferation and migration of keratinocytes through phosphorylation of epidermal growth factor (EGF) receptor and STAT proteins [40]. Further evidence of its importance in wound healing is the fact that excisional wounds created on the backs of Yorkshire pigs and infected with *Staphylococcus aureus* and transfected with virally introduced hBD3 fared significantly better in the time it took for the wound to close compared to controls (with no hBD3) [68]. The canonical wound healing regulatory pathway, along with calcium mobilization, also regulates CCR6-directed epithelial cell migration in the intestine through hBD2 [69], which has been seen to promote migration, wound healing of endothelial cells and the formation of capillary-like tubes with human umbilical vein endothelial cells [70]. hBD2 is also upregulated in regenerating corneal epithelium [71].

Role of β-Defensins in Fertility

Many β-defensins are expressed in the male reproductive tract. The first defensin-like peptide to be isolated from the epididymis was sperm-associated antigen 11 isoform e, Spag11e (also known as Bin1b, Ep2). *Bin1b* is present in the main cluster of β-defensin genes and the peptide structure was found to correspond to β-defensin structure with a similar positive charge and conserved cysteines [72]. Expression was found primarily in the caput (head) region of the epididymis (proximal to the testes) and was absent from the cauda region (distal). Subsequently, hBD5 and hBD6 (and the mouse orthologs Defb12 and Defb15) were shown to be expressed in the epididymis, particularly in the columnar epithelium lining the caput region [73, 74]. In addition, a smaller cluster of genes (located on chromsome 20 in humans) shows variable expression at different areas of the epididymis [75]. Interestingly, the antimicrobial peptide, cathelicidin (hCAP18; the propeptide of LL-37), has also been identified in the epithelium of the epididymis, but unlike defensins it was shown to be expressed primarily in the cauda region. It was also present in high concentrations on spermatozoa, suggesting that it plays a role in conception [76].

The epididymis is continuous with the urethra and is therefore at risk of exposure to microbes. Bacterial infection of the epididymis is a common cause of acute epididymitis. Epididymally expressed defensins have been shown to be antimicrobially active [72–74]. hBD5 and hBD6 and their mouse othologs (Defb12 and Defb15) were exclusively expressed in the epididymis, with the major site of expression being cells in the columnar epithelium lining the caput region while a smaller cluster of genes (located on chromsome 20 in human) were variably expressed along the epididymis [75]. In contrast to the increase in β-defensin expression observed at mucosal surfaces, it was recently shown that LPS-induced inflammation of the epididymis decreased expression of β-defensins in the caput [77, 78]. Interestingly, this reduction in β-defensins affected sperm motility, and it is becoming increasingly clear that β-defensins have a role in sperm maturation and fertility.

Bin1b levels were shown to be important for sperm motility in rats. Immature, reduced-motility sperm taken from the caput region of rats increased in mobility after the addition of exogenous Bin1b peptide. This is thought to be mediated by the Bin1b-induced uptake of calcium ions and implies a role of Bin1b in sperm maturation [79]. Immunization of rats with Bin1b results in high anti-Bin1b serum levels and subsequent clearance of Bin1b protein and these rats produce sperm with reduced motility [80]. Bin1b has been shown to bind the sperm head, an ability that has also been reported for rodent peptide Defb15 which binds to the glycocalyx of the sperm head. In vivo RNAi silencing of the rat *Defb15* gene down to 50% of normal expression levels resulted in sperm with reduced total and progressive motility and a reduced ability to fertilize eggs. Our own work targeting gene *Defb15* also shows homozygous males with low motility sperm and a reduced fertility phenotype (unpublished data).

A mutation in *DEFB126* has recently been shown to be present in the population at a very high frequency (45% in European and 47% in Chinese cohorts) [81]. Tollner et al. [82, 83] and Yudin et al. [84] had previously shown this peptide to be important for cervical mucus penetration and for binding sperm to the oviductal epithelium. It is hypothesized that DEFB126 is involved in sperm immunorecognition and that sialic acid moieties which bind DEFB126 are responsible for a cloaking effect [85].

Some defensins have an extended tail region beyond the core 6-cysteine motif. DEFB126 has an extra 60 amino acids, including an additional cysteine, and it is also extremely rich in the serine/threonine residues required for O-linked glycosylation. The mouse ortholog of DEFB126 (Defb22) was shown to be a major constituent of the glycocalyx on the sperm head [86]. Interestingly, Defb15 has a 20-amino-acid extension with an extra cysteine and potential O-linked glycosylation sites. The *DEFB126* mutation is a 2-nucleotide deletion resulting in a frame shift and aberrant mRNA. The sperm from men homozygous for these alleles have a less O-linked glycosylated glycocalyx and exhibit reduced penetration of hyaluronic acid gel (a surrogate for human cervical mucus) compared to wild-type or heterozygous sperm. The progressive motility and morphology of these sperm was normal, however. In a prospective cohort study of newly married couples trying to conceive, pregnancy was less likely if the male partner was homozygous for the DEFB126 mutant sequence [81]. The high frequency in the population for a gene allele that leads to infertility in the homozygous state implies either a heterozygous advantage or a balancing mutation phenomenon. Interestingly, and perhaps of relevance here is the fact that flowering plants use various cysteine-rich peptides at different steps of the pollen-pistil interaction. Recently it has been shown that LUREs (members of the cysteine-rich peptide super-gene family) are pollen tube attractants derived from the synergid cell of *Torenia fournieri* [87]. It is presumed that these LUREs have evolved from defence genes. Future investigation of defensins in reproduction will uncover the detailed role of these molecules in fertility.

Roles for β-Defensins in Development

Further diverse functions of defensin-like genes including pollen viability and seed maturation have been demonstrated in tomatoes [88]. The expression of the tomato defensin DEF2 was shown to be differentially regulated during early flower development suggesting a role for defensins in development.

β-Defensin-like genes, *defbl1*, *defbl2* and *defbl3* have also been found in a variety of tissues in zebrafish [89]. These genes showed no significant homology to any mammalian β-defensins, but were found to contain the classic defensin 6-cysteine motif and were predicted to adopt a similar tertiary structure. In addition, two of the genes *(defbl1* and *defbl2)* are flanked by genes that are linked to the main β-defensin cluster in mammals, including *SPAG11*. Subsequent work reported *defbl1* expression in the larval skin and swim bladder, suggesting involvement of defensins in zebrafish development [90]. We find *defbl1* is strongly expressed in the early developing zebrafish (12 h after fertilization) and suppression of this expression results in a developmental delay (unpublished data).

A role in development has been suggested for some murine β-defensin genes. Suppression of epididymis-expressed *Defb15* in rats resulted in decreased fertility; however, offspring derived from the mutant sperm also demonstrated developmental failure [79]. In addition, murine *Defb19* has been implicated in testis development [91] and murine *Defb50* has been detected (by in situ hybridization) in the developing embryonic brain and spinal cord at E14.5 (EMAGE database [92]). It is possible that some of these genes are involved in both development and immunity. *Toll* was first identified in *Drosophila melanogaster* embryos as a determinant of dorsoventral patterning [93], but was later found to have a role in the antifungal response in adult *D. melanogaster* [94]. It is possibly pertinent that the mature form of spätzle, the

ligand for Toll, is a 7-cysteine molecule with three intramolecular disulphide bonds forming a 'cysteine knot structure'.

The Role of β-Defensins in Cancer

It first became apparent in the early 2000s that defensins may play a part in the regulation of carcinogenesis when several studies reported altered expression of β-defensins in cancers. It was shown that many renal cell carcinomas, prostate cancers [95], basal cell carcinomas [96] and oral squamous cell carcinomas (OSCC) [97] either lacked or minimally expressed hBD1 protein. A likely cause of reduced hBD1 protein levels is the genetic rearrangement of chromosome 8p22–23 (the area that encodes hBD1) which is commonly found in these tumor types. However, in contrast to these findings, other groups have found β-defensins to be overexpressed in cancer. hBD1 expression was shown to be increased in renal cell carcinomas [98], both hBD1 and hBD2 were detected at high levels in the serum of patients with lung cancer [99] and hBD3 protein was shown to be overexpressed in OSCC tissue [100].

It has been shown in oral carcinoma that tumor aggressiveness is associated with macrophage infiltration [101]. Kawsar et al. [102] showed that premalignant cells in oral carcinomas overexpressed hBD3; they suggest that this overexpression may function to recruit macrophages to the lesion, enhancing the progression of oral cancer. In addition, hBD3 was shown to be induced by EGF and expression of the EGF receptor and its ligand, EGF, are found in OSCC. Thus, EGF drives the production of hBD3 which recruits macrophages and results in advancement of oral cancer, placing hBD3 at a pivotal position in this disease. In contrast to studies which may simply see the inappropriate expression of β-defensins as the general phenomenon of the dysregulation of gene expression in the cancer cell, hBD3 has also been shown to chemoattract tumor-associated macrophages, which are indicative of progressive tumors. This involves a mechanism whereby hBD3 utilizes the chemokine receptor CCR2, suggesting that it may act as a tumor cell attractant [36].

It has recently been reported that hBD2 and hBD3 may function as proto-oncogenes in OSCC, while hBD1 might function as a tumor suppressor [103]. This study showed that growth of an OSCC cancer cell line decreased after the addition of hBD1, but increased after treatment with hBD2 or hBD3. Similarly, in prostate cancer cells, hBD1 was shown to decrease the growth rate [104]. Expression of hBD1 in late-stage prostate cancer cell lines (PC3 and DU145) caused rapid cell death. In comparison, in a normal prostate epithelial cell line or in an early-stage prostate cancer cell line, it had no effect on viability. Functional studies determined that hBD1 induced cell death via the disruption of cell membrane potential and caspase-mediated apoptosis. It therefore appears that it is involved in the destruction of cancer cells and dysregulation of hBD1 expression allows uncontrolled tumor progression.

Opinion on the role of β-defensins in cancer remains divided. While the role of hBD1 in renal cell carcinoma and prostate cancer has been somewhat resolved, the role of β-defensin in OSCC requires further attention. It has been postulated that the contradictory findings on the role of β-defensins in OSCC may be due to varying levels of inflammation and cytokine production in cancers [97]. Indeed, given the differing effects of β-defensins in the context of immunomodulatory functions, it is not surprising that defensin expression and function in cancers is similarly complex.

Conclusion

It is clear that β-defensins are major components of the armory that is our immune system, but their functional activities extend beyond that to the adaptive immune system, fertility, wound healing and involvement in cancer. They are involved both in the proinflammatory process of immunity and the required resolution. Further work and in vivo study on these interesting molecules are required to fully understand their functional repertoire and to realize their therapeutic potential.

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