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Pro-inflammatory and pro-apoptotic properties of Human Defensin 5

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ABSTRACT

Defensins are cationic antimicrobial peptides that play an important role in innate immunity by primarily acting against microbes. Their antimicrobial properties have been widely studied and are well understood. Defensins contribute to regulation of host immunity also. Their effects on cells of the host however are less well understood. Here, we report on the pro-inflammatory and apoptotic properties of Human Defensin 5, the major antimicrobial peptide of ileal Paneth cells. We find that HD-5 up-regulates expression of genes involved in cell survival and inflammation in a NF-kB-dependent fashion in epithelial cells. Further, we find that HD-5 has pro-apoptotic effects on intestinal epithelial cells as well as primary CD4+ T cells.

1. Introduction

Human defensins are cationic peptides of the innate immune system with the ability to kill a variety of microbial pathogens, including bacteria, fungi and viruses [1,2]. Based on a difference in disulfide connectivity of six conserved cysteine residues, defensins have been divided in two families, termed α and β. Both families are believed to have evolved from a common ancestral β-defensin gene [3,4], and share similar tertiary structures despite low amino acid sequence identity [5–7].

In addition to their antimicrobial activities, defensins play a significant role in adaptive and innate immunity. Defensins have been shown to chemoattract a variety of host cells [8–10] and have shown the ability to modulate host cell cytokine responses [11,12]. Defensins of the α-family are expressed predominantly in neutrophils (termed human neutrophil peptides or HNP) or in specialized intestinal epithelial cells called Paneth cells in the case of Human Defensin 5 and 6 (HD-5 and HD-6) [13,14]. Recently, a specific deficiency of HD-5 was observed in patients with ileal Crohn's disease, a chronic inflammatory disease [15,16]. These findings have led to the notion that HD-5 as an innate immune effector molecule may play an important role in the maintenance of mucosal balance and that deficiency of HD-5, resulting in weakening of mucosal antibacterial capacity may contribute to pathogenesis [17]. Although the antibacterial properties of HD-5 have been described [18,19], little is known about immunomodulatory properties of the peptide on host cells. Here, we describe the immunomodulatory properties of HD-5.

2. Materials and methods

2.1. Solid phase peptide synthesis

Chemical synthesis of HD-5 and HD-5Abu, a linear, unstructured form of HD-5 in which the six cysteine residues are replaced by isosteric α-aminobutyric acid (Abu) was carried out as described [20]. The molecular mass of the peptides was verified by electrospray ionization mass spectrometry (ESI-MS) as described [20].

2.2. Cell culture

Subconfluent monolayers of Caco-2 cells were maintained in RPMI 1640 medium (Gibco), supplemented with 10% FBS (Valley Biomedical, Winchester, VA), 2 mM L-glutamine (Quality Biological, Gaithersburg, MD), 20 mM HEPES, 1× nonessential amino acids, 1 mM sodium pyruvate and 5% Penicillin/Streptomycin (Sigma) in a humidified incubator at 37 °C with 5% CO_2. Caco-2 cells were used between passages 18–35 and were plated at a density of 4×10^5 cells/cm^2.

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR) and gene expression analysis

Caco-2 cells were cultured in 12-well plates in the presence of serum. Cells were gently washed twice with serum-free medium and incubated for a further 6 h in serum-free medium containing the peptide5 at a final concentration of 50 μg/ml. Total RNA was extracted and transcribed into cDNA using the FastLane Cell cDNA

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kit (Qiagen) according to the manufacturer's instructions. Gene expression analysis was subsequently carried out using Signal Transduction Pathway Finder PCR arrays from SABiosciences according to manufacturer's protocol. Data were evaluated using the ΔΔCt method and gene expression fold upregulation was plotted according to instructions on the company website.

2.4. Evaluation of cytokine secretion

Caco-2 cells were cultured in 96-well plates in the presence of serum. Cells were gently washed twice with serum-free medium and incubated for a further 24 h in serum-free medium containing the peptides at indicated concentrations. To study the role NF-kB in signal transduction, cells were pre-incubated for 2 h in the presence of 50 μg/ml M-132 (Sigma). CD4+ T cells were purified from PHA-activated peripheral blood monocytes obtained from healthy volunteer donors of the New York Blood Bank. Following activation, CD-4+ T cells were isolated by negative selection according to the manufacturer's instructions (Mylteni Biotec), washed in serum-free medium and exposed to peptide at 25 μg/ml for 4 h. Culture supernatants were collected for measurement of IL-8 using the Luminex-100 system (Bio-Rad).

2.5. Cell viability

The effect of defensin peptides on Caco-2 cell viability was assessed by measuring the mitochondrial activity using MTS assays according to the manufacturer's instructions (Cell Titer 96 proliferation assay, Promega). The cells were gently washed twice with serum-free medium and incubated for a further 24 h in serum-free medium containing the peptides at a final concentration of 20, 50 or 100 μg/ml. The number of viable cells was determined using a standard curve of serially diluted untreated cells in each experiment.

2.6. Apoptotic assays

Caspase-3/-7 activity in Caco-2 cell lysates was measured using the fluorogenic Ac-DEVD-AMC peptide substrate. Cells were gently washed twice with serum-free medium and exposed to the peptides at a final concentration of 20, 50 or 100 μg/ml. The general caspase inhibitor zVAD was incubated for 2 h before exposure of the cells to HD-5 at 50 μM. Cells were lysed in 200 μl of lysis buffer (20 mM HEPES, 50 mM NaCl, pH 7.2, 10 mM DTT containing 1% CHAPS, 1 mM EDTA, 2 mM PMSF, leupeptin (10 μg/ml; Sigma) and pepstatin A (10 μg/ml; Sigma) for 30 min on ice. After centrifugation (7000g, 10 min), protein concentration was determined using the BCA Protein assay reagent (Bio-Rad). Subsequently, 20 μg of each sample was diluted to a final volume of 200 ml in assay buffer (20 mM HEPES, 50 mM NaCl, pH 7.2, 10 mM DTT, 0.1% CHAPS containing 100 mM Ac-DEVD-AMC) in a 96-well plate. Fluorescence was determined (excitation 360 nm, emission 460 nm) with a Cary Eclipse fluorescence spectrophotometer (Varian, Palo Alto, CA). Background fluorescence was determined using the assay buffer only.

Apoptosis in purified CD4+ T cells was determined using the Annexin-FITC apoptosis kit from Calbiochem according to manufacturer protocol. Purified CD4+ T cells (2×10^5 cells/ml) were exposed to peptide in serum-free medium and subsequently analyzed by flow cytometry (FACSCalibur, BD Biosciences).

3. Results

3.1. Interaction of HD-5 with cells of the host

To study the functional effects of the HD-5 peptide on host cells, Caco-2 intestinal epithelial cells were exposed to the peptide at a concentration of 50 μg/ml for 6 or 16 h and the differential gene expression was compared to untreated control cells (Fig. 1). HD-5 induced the expression by more than 15-fold after 6 h or 8-fold after 16 h of three major groups of genes in Caco-2 cells: (1) regulation of cell survival (BCL2A1, BIRC2, BIRC3, FAS, TANK, TNF); (2) pro-inflammatory (IL-2, IL-8, CCL-20, TNFα); (3) cell adhesion (MMP7, MMP10, SELE, VCAM1). Notably, transcription of 50% of the genes induced by HD-5 is NF-κB-dependent. Pre-incubation of Caco-2 cells with the broad NF-κB neutralizing agent M-132

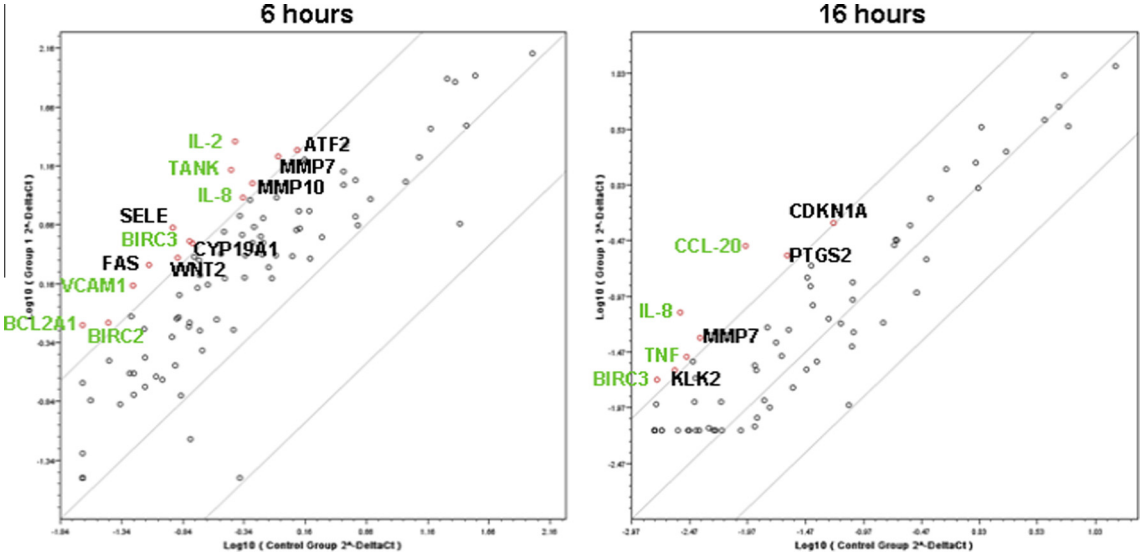

Fig. 1. Upregulation of gene expression by Caco-2 cells treated with HD-5 (50 μg/ml) for 6 h or 16 h versus control untreated cells. Expression was determined by RT^2 Profile Human Signal Transduction Pathway Finder PCR array (SABiosciences) and data were analyzed using the ΔΔCt method. Only genes with a more than 15-fold upregulation compared to control after 6 h or more than 8-fold upregulation for 16 h are listed. Transcription of genes marked in green is NF-kB-dependent. *Abbreviations:* ATF2: Activating Transcription Factor2; BCL2A1: B-cell Lymphoma2-related protein A1; BIRC2/BIRC3: Baculoviral IAP Repeat Containing; CCL20: Chemokine (C-C motif) Ligand 20; CDKN1A: Cyclin-dependent kinase inhibitor A1; CYP19A1: Cytochrome P450; FAS:TNF receptor superfamily member 6; IL: InterLeukin; KLK2: Kallikrein-related peptidase 2; MMP: MatrixMetalloProtease; PTGS2: Prostaglandin-endoperoxidase synthase 2; SELE: Selectin E; TANK:Traf family member associated NF-kB Activator; VCAM1: Vascular Cell Adhesion Molecule-1; WNT2: Wingless-type MMTV integration site family member 2.

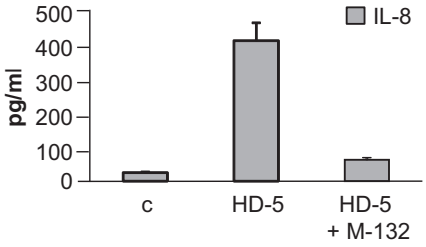

Fig. 2. Induction of IL-8 secretion by HD-5 is NF-$_\kappa$B-dependent. Caco-2 cells were preincubated with the NF-$_\kappa$B inhibitor M-132. Subsequently, Caco-2 cells were exposed to HD-5 peptides at 50 μg/ml for 24 h in serum-free medium. Protein secretion was determined by ELISA; means ± SEM from three independent experiments for HD-5 are shown.

negated any observed secretion of IL-8, confirming the results from RT-PCR experiments (Fig. 2).

We previously observed that IL-8 secretion diminished at higher HD-5 concentrations and at longer exposure times. Given that HD-5 affects expression of cell survival genes after 6 h, we tested for cytotoxic effects of the HD-5 peptides by measuring metabolic activity using the MTS assay (Fig. 3A). Incubation of the cells with HD-5 for 24 h at a concentration of 50 μg/ml resulted in a 20% decreased mitochondrial activity and by more than 60% at a concentration of 100 μg/ml. No decreased activity was observed for a linear HD-5 Abu peptide, in which the six cysteine residues have been replaced by butyric acid. Incubation of the cells with peptide for 6 h did not noticeably alter cell viability. Since Caco-2 cell viability was affected by HD-5, we examined the possibility that HD-5 induces programmed cell death. Apoptotic cell death is characterized by activation of caspases, including caspase-3/-7. Therefore, caspase-3/-7 activity in cellular lysates was determined after exposure to the peptides at a concentration of 50 μg/ml for 6 h or 24 h. Activity was determined by measuring fluorescence of AMC after cleavage of the DEVD peptide substrate (Fig. 3B). After 6 h, no significant caspase-3/-7 activity was detected for any of the peptides as compared to untreated cells. After 24 h, caspase activity was significantly increased in cells treated with HD-5. The broad-spectrum caspase inhibitor zVAD was able to abrogate this HD-5 induced caspase activity. We determined the effect of HD-5 on cytoskeletal reorganization as a second measure for apoptosis since we found that HD-5 induces expression of cell adhesion genes as well as activates caspase enzymes. Compared to control cells, HD-5 induces a dramatic disruption of the actin cytoskeleton. Concentration-dependent secretion of IL-8 as well as disruption of the actin cytoskeleton and decreased cell viability was observed also upon exposure of HD-5 to Jurkat cells (not shown). Taken together, these data suggest that HD-5 can mediate NF-$_\kappa$B-dependent signaling at low concentrations and negatively affects host cell viability through caspase activation at higher concentrations or prolonged exposure.

We used purified CD4 T cells to examine the effects of HD-5 on primary cells, rather than cell lines. Purified CD4+ T cells were exposed to increasing concentrations of HD-5 in serum-free medium and assayed for cytokine release by ELISA and apoptosis by flow cytometry. As expected, HD-5 induced secretion of IL-8 and IL-2 by these cells, in accordance with our RT-PCR results (Fig. 4A). Additionally, exposure to HD-5 induced secretion of interferon-gamma (Fig. 4A). No secretion of IL-4, IL-1β or TNFα could be observed by these cells following exposure to the peptide. Prolonged exposure of the peptide induced predominantly apoptotic events even at concentrations of HD-5 as low as 5 μg/ml. At this concentration, approximately 50% of the cell population was undergoing early or late stage apoptotic events (Fig. 4B). At increased peptide concentrations, the percentage of cells undergoing late stage apoptotic events or necrotic events increased 8- to 10-fold compared to

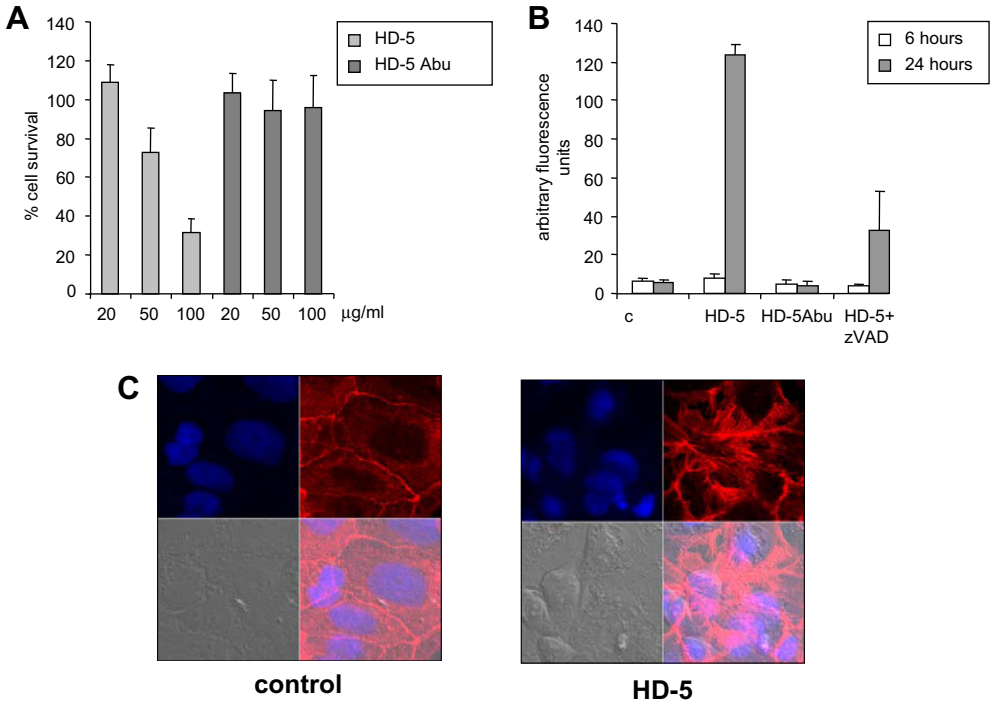

Fig. 3. Pro-apoptotic effects of HD-5. (A) Caco-2 cells were exposed to the peptides at concentrations of 20, 50 or 100 μg/ml for 24 h in serum-free medium. Mitochondrial activity was measured by MTS colorimetric measurement and plotted as percentage of untreated control cells. (B) Cells were exposed to the peptides at a concentration of 50 μg/ml for 6 or 24 h in serum-free medium. Caspase-3/-7 activity in cellular substrates was determined using the Ac-DEVD-AMC peptide substrate. Caspase activity of HD-5 was inhibited by pre-incubation of the cells with the broad caspase inhibitor zVAD (50 μM) for 2 h. Data represent three individual experiments. (C) Cytoskeletal reorganization induced by HD-5. Caco-2 cells were exposed to HD-5 at a concentration of 50 μg/ml for 24 h in serum-free medium. Reorganization of the actin cytoskeleton was visualized by phalloidin staining using Hoechst counterstaining to visualize cellular nuclei.

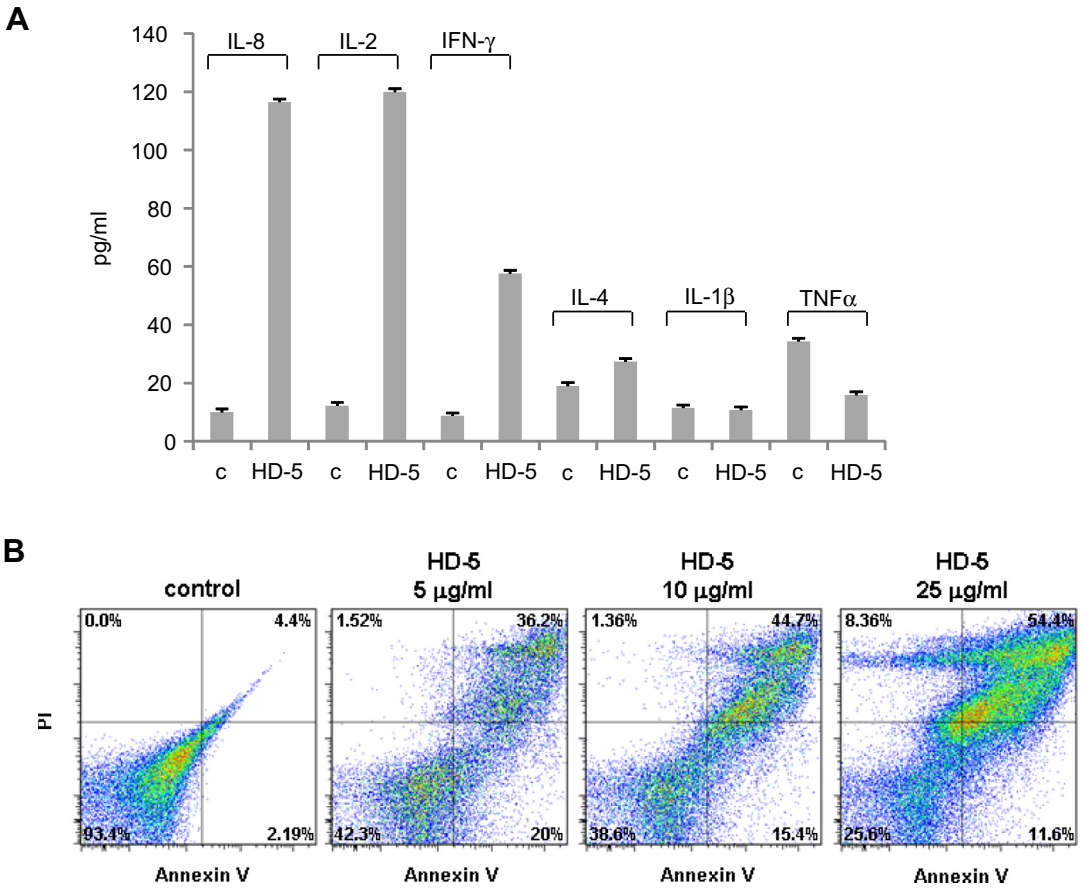

Fig. 4. HD-5 induces interleukin secretion and apoptosis on CD4+ T cells. (A) Purified CD4+ T cells derived from PHA-activated peripheral blood monocytes were exposed to HD-5 at 25 μg/ml for 4 h. Interleukins in the supernatant were detected by ELISA using the Luminex assay from Invitrogen. Means ± SEM for one of two independent experiments carried out in triplicate is shown. (B) HD-5 was exposed to purified CD4+ T cells derived from PHA-activated peripheral blood monocytes at the indicated concentrations for 6 h and assayed for apoptotic events by flow cytometry. One out of three representative experiments is shown.

control, indicating that HD-5 dramatically effects the survival of primary CD4+ T cells. Linearized HD-5 was unable to induce either cytokine release or apoptosis.

4. Discussion

Several recent studies have shown abnormally low expression levels of HD-5 patients suffering from Crohn's disease. Diminished HD-5 levels have been linked to two independent underlying signaling pathways. First, mutations in NOD2/CARD15 gene encoding an intracellular receptor for the bacterial peptidoglycan component muramyl dipeptide have been linked with susceptibility to Crohn's disease [21,22]. Second, defensin deficiency has been linked to the Wnt/β-catenin/Tcf-4 pathway, independent from the NOD2/CARD15 genotype [16]. These observations have led to the suggestion that decreased levels of α-defensin secreted by Paneth cells located at the base of small intestinal crypts weaken the antimicrobial defense of the ileal mucosa [23]. Indeed, HD-5 deficiency in man alters the bacterial composition of the gut [15]. Transgenic overexpression of HD-5 in mice protects against oral challenge with Salmonella [24] and alters the gut microbiome also [25]. Interestingly, HD-5 transgenic mice show a striking loss of segmented filamentous bacteria and have fewer IL-17-producing lamina propria T cells [25]. Loss of IL-17-producing T cells however was also observed in wild-type mice with functional defensins, in the specific absence of this class of bacteria. Our studies suggest a concentration-dependent effect of defensins. In the healthy intestine, HD-5 is continuously secreted at low levels. The concentration of HD-5 in ileal fluid has been quantified to range between 6–30 μg/ml [26]. Concentrations of pro-HD-5 stored in granules of Paneth cells has been estimated between 90 and 450 μg/cm^2 of ileal surface area, translating roughly into 50–250 μg/ml upon complete degranulation and processing [18]. At these concentrations, we predict that the antibacterial activity of defensins would be minimal, due to presence of salts and cations in intestinal fluid, known to inhibit its antibacterial activity [27]. We find here that at low or steady-state concentrations, HD-5 has pro-inflammatory properties. In healthy individuals, these properties are likely beneficial in maintaining epithelial integrity and regulating controlled infiltration of immune cells into underlying tissues. In a recent study describing allograft rejection of intestinal tissue, which closely resembles Crohn's disease, it was noted that decrease in epithelial antimicrobial peptides preceded visible epithelial injury [28]. One of the earliest visible signs of epithelial damage in this study was increased apoptosis of the epithelium. Furthermore, reduced alpha-defensin expression was recently found to be associated with inflammation but not with NOD2 mutations [29]. The observed inflammatory changes resulted in loss of epithelium, suggesting that lower expression of alpha-defensins is a consequence of ileal inflammation in Crohn's disease.

Barrier integrity of the mucosa is generally believed to be regulated by signaling of key regulator cytokines to cells of the adaptive immune system. A contrasting view is offered in several studies with epithelial-specific deletions of crucial regulators of signaling pathways. For example, specific deletion of NEMO, a master regu-

lator of NF-$_\kappa$B signaling in intestinal epithelial cells, causes spontaneous colitis in mice [30]. Impaired NF-$_\kappa$B led to epithelial apoptosis, translocation of bacteria into the mucosa and reduced expression of defensins. Epithelial-specific deletion of RelA, a subunit of NF-$_\kappa$B causes spontaneous intestinal disease and death [31]. Deletion of RelA elevated basal rates of epithelial apoptosis and proliferation and was accompanied by diminished expression of anti-apoptotic genes and defensins. More recently, intestinal epithelial-specific deletion of MyD88, a key regulator of Toll-like receptor mediated signaling [32], was found to cause spontaneous colitis in aged mice. Disease was characterized by increased bacterial translocation and decreased defensin expression [33]. Surprisingly, epithelial apoptosis or permeability was not found to be increased. Although not confirmed in man, degranulation of murine Paneth cells from isolated crypts by LPS or other stimuli results in estimated defensin concentrations as high as 20 mg/ml in the crypt lumen [34]. Our studies indicate that at these concentrations, defensins not only kill micro-organisms instantly, but induce strong pro-inflammatory and pro-apoptotic effects as well. Based on the above and on our findings, we suggest that defensin deficiency affects both the gut microbiome and host immunity and may contribute to disease development and progression, likely however only in combination with genetic or environmental factors. Treatment or prevention of disease with HD-5 as a therapeutic agent may be beneficial, however may accelerate the development or be harmful in cases of active disease.

Various studies have indicated specific interactions between defensins and host cell receptors. Both α- and β-defensins have been shown to act chemotactic to a variety of cells, including naïve and mature T cells, mast cells and macrophages [9], immature dendritic cells [35] and monocytes [36]. Members of the β-defensin family have been shown to interact with a variety of cellular receptors, including the chemokine receptor CCR6 [8,37], Toll-Like Receptor 4 [38] and more recently the melanocortin 1 receptor [39], causing black coat color in domestic dogs. Defensins share striking common characteristics with chemokines, such as size, cationic charge, disulfide bonding and biological activities [40,41]. Predominantly based on the ability of defensins to act as chemoattractants, it was concluded that α-defensins may share a common receptor, distinct from β-defensins [9,10]. The only member of the α-defensin family with reported receptor interactions is Human Neutrophil Peptide-1 or HNP-1. Interaction of HNP-1 with the purinergic receptor PY2 on airway epithelial cells has been reported [42]. More recently however, the activation of macrophages by HNP-1 was shown to be insensitive to pertussis toxin, as well as independent of purinergic receptors, Toll-like Receptor and CD18 signaling [43]. In agreement with the above mentioned studies, we showed that signaling in Caco-2 cells is relatively insensitive to pertussis toxin treatment [27]. Further, pre-incubation with the PY2-receptor specific antagonist suramin did not alter the IL-8 secretion induced by HD-5 in Caco-2 cells, suggesting that this pathway is not involved (unpublished observation). Previously, it was suggested that α-defensins chemoattract cells via a unique receptor, predominantly involving p38 and ERK pathways [9]. Our previous studies and this work indicate that HD-5 signals with similar pathway specificity [27,44] and activates NF-$_\kappa$B (this work). To date, no specific receptors for HD-5 have been identified.

Acknowledgments

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