

β -defensin expression in the canine nasal cavity

by

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Abstract

Defensins are a family of endogenous antibiotics that are important in mucosal innate immunity, but little is currently known about defensin expression in the nasal cavity. Herein expression of canine β -defensin (cBD)1, cBD103, cBD108 and cBD123 RNA in the respiratory epithelium (RE), cBD1 and 108 RNA in the olfactory epithelium (OE), and cBD1, cBD108, cBD119 and cBD123 RNA in the olfactory bulb (OB) is reported. cBD1 and 103 were also expressed in the canine nares and tongue. cBD102, cBD120, and cBD122 RNA expression was undetectable in the tissues examined. cBD103 transcript abundance in canine nares showed a 90 fold range of inter-individual variation. Murine β -defensin 14 expression mirrors that of cBD103 in the dog, with high expression in the nares and tongue, but has little to no expression in the RE, OE or OB. High expression of cBD103 in the nares may provide indirect protection to the OE by eliminating pathogens in the rostral portion of the nasal cavity, whereas cBD1 and 108 may provide direct protection to the RE and OE.

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List of Abbreviations

A431	human epithelial cell line, squamous cell carcinoma derived
AD	atopic dermatitis
cBD	canine β -defensin
CCR	CC chemokine receptor
CNS	central nervous system
CNV	copy number variable/variation
Cys	cysteine
DEFA	α -defensin gene, human
DEFB	β -defensin gene, human
Defb14	murine β -defensin 14
DNA	deoxyribonucleic acid
HaCaT	human keratinocyte cell line, melanoma derived
hBD	β -defensin peptide, human
HD	α -defensin peptide, human Paneth cells
HIV	human immunodeficiency virus
HNP	α -defensin peptide, human neutrophils
IFN- γ	interferon gamma
IL	interleukin
LPS	lipopolysaccharides
MCR	melanocortin receptor
mg	milligrams
MIC	minimal inhibitory concentration(s)

NF-kB	nuclear factor kB
ng	nanograms
nM	nanomolar
OE	olfactory epithelium
OMP	olfactory marker protein
ORN	olfactory receptor neurons
OSCC	oral squamous cell carcinoma
RE	respiratory epithelium
rHagB	recombinant hemagglutinin B
RNA	ribonucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction, semi-quantitative
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
SNP	single nucleotide polymorphism(s)
TLR	Toll-like receptor(s)
TNF- α	tumor necrosis factor alpha
ug	micrograms
ul	microliter

uM micromolar

VNO vomeronasal organ

Chapter 1: The Innate Immunity

In multicellular organisms the innate immune system is the first line of defense against pathogenic insult, but for many years it was viewed as an inert, rather unexciting part of the immune system. The innate immunity is now understood to be a complex and dynamic arrangement of leukocytes, as well as physical and biochemical barriers that work together to respond to environmental stimuli quickly and efficiently. The leukocytes of the innate immunity include neutrophils, eosinophils, macrophages and natural killer cells. The physical barriers consist of cellular structures such as tight junctions, keratinization of epithelial surfaces, and the mechanical properties of cellular secretions. The biochemical barrier is a milieu of cellular products that neutralize toxins and serve as endogenous antibiotics. Part of the biochemical barrier is composed of defensins, a multifunctional peptide family that exhibit antibiotic activity and modulate the adaptive immunity.

Section 1: Defensins

Defensins have a wide range of antibacterial, antiviral, antifungal and antiprotozoal activity. While especially numerous in species that do not have adaptive immunities, such as plants and insects, defensins are also prominent in the innate immunity of vertebrates (Carvalho Ade and Gomes, 2009). Humans have ~40 defensins clustered on several chromosomes, with similar numbers found in murine, canine and other primate genomes (Patil et al., 2005).

Defensin genes encode peptides that are small, cationic and amphiphilic, with diverse primary amino acid sequences. However, all defensins have six conserved cysteine residues. The pairing of the cysteine residues to form three disulfide bridges is the basis for differentiating the defensin subfamilies (Figure 1): α (cys¹-cys⁶, cys²-cys⁴, cys³-cys⁵), β (cys¹-cys⁵, cys²-cys⁴, cys³-cys⁶) and θ (cys¹-cys¹, cys²-cys³, cys²-cys³ dimer) (Drakopoulou et al., 1998; Tang et al., 1999; Cederlund et al., 2011).

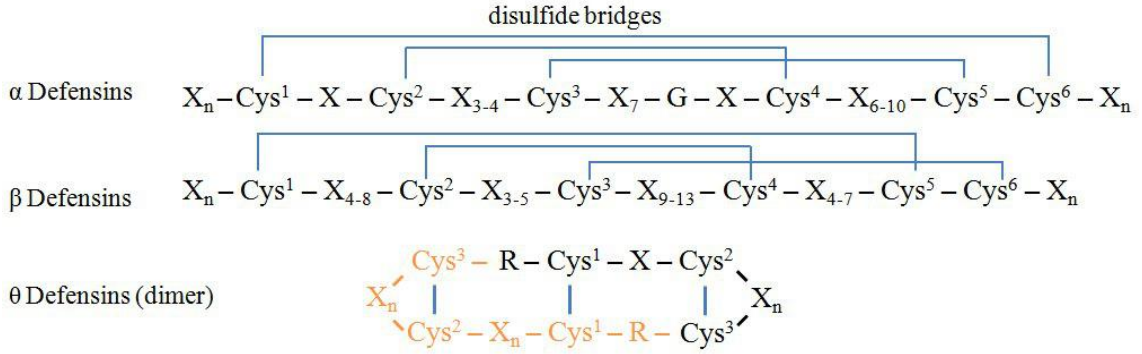


Figure 1: General sequence structure and cysteine pairs of the α -, β - and θ - defensin subfamilies. Conserved cysteine (Cys), glycine (G), arginine (R), variable amino acid (X) (Ganz, 2003; Garcia et al., 2008; Lehrer and Lu, 2012).

Section 2: The α -defensins

The α -defensins are thought to have arisen from a duplication of the β -defensin gene cluster and are only present in marsupial and some placental mammals (Patil et al., 2004). Humans have five α -defensin genes which encode six peptides (Table 1). In addition to the distinct cysteine pairing, α -defensins have a conserved glycine residue between cysteines 3 and 4 that is important for proper folding and antibacterial function (Ganz, 2003; Zhao et al., 2012).

Location	Gene	Protein	Peptide Sequence
Neutrophils	DEFA1	HNP-1	A CYCRIPACIAGERRYGT C IIYQGRLWAFCC
	DEFA1 or 3	HNP-2	- C YCRIPACIAGERRYGT C IIYQGRLWAFCC
	DEFA3	HNP-3	D CYCRIPACIAGERRYGT C IIYQGRLWAFCC
	DEFA4	HNP-4	VCS CRLVFCRRTLRVGNCLIGGVSFTYCCTRV
Paneth Cells	DEFA5	HD-5	ATCYCRTGRCATRESLSGVCEISGRLYRLCCR
	DEFA6	HD-6	AFTCHCRRSCYSTEYSYGTCTVMGINHRFCCL

Table 1: Primary cellular source, genomic and protein nomenclature, and amino acid sequence of human α -defensins. Human neutrophil peptides, HNP-1, HNP-2, and HNP-3 sequences (bold) differ by a single N-terminal amino acid (highlighted red). HNP-2 is derived from proteolytic cleavage of DEFA1 or DEFA3 gene products. This slight change in sequence influences antibiotic and chemotactic activity. Alanine (A, non-polar), aspartic acid (D, negatively charged), cysteine (C, polar). Human defensins, HD-5 and HD-6 are expressed primarily in the gut.

Human α -defensins, known as human neutrophil peptides (HNP-1 through 4) are synthesized during the promyelocyte stage of neutrophil development and are stored as an inactive pro-peptides (Ganz, 2003). HNPs comprise 50% of the protein content within the azurophilic granules of neutrophils and are found to a lesser extent in other leukocytes (Quinn et al., 2008). Human defensins (HD-5 and -6) are found in the granules of the Paneth cells in the small intestines. Low levels of HD-5 have also been reported in the respiratory mucosa (Frye et al., 2000).

Stored pro- α -defensins are neutral, with the arginine rich (cationic) mature α -defensin sequence balanced by a negative, N-terminal pro-sequence. Upon stimulation the pro-piece is enzymatically cleaved from the mature peptide sequence. Matrix metalloproteinase 7 (MMP-7) cleaves α -defensins in murines. However mature α -defensins are found in the lower intestinal tract of MMP-7 knock-out mice, indicating other host or microbial-derived proteases can effect murine α -defensin maturation (Wilson et al., 1999; Mastroianni et al., 2012). In humans, α -defensins are proteolytically cleaved to the mature form by trypsin, chymotrypsin, neutrophil elastase and proteinase 3 (Ghosh et al., 2002; Tongaonkar et al., 2012).

Relatively high local concentrations (in the range of micrograms per milliliter) of α -defensins are necessary for antibiotic activity. To minimize dilution and optimize the environmental factors such as pH and salt concentrations, α -defensins are secreted into relatively contained environments, such as the crypts of Lieberkühn. The azurophilic granules containing high concentrations of α -defensins can also merge with phagolysosomes within the neutrophils. α -defensins are present in various bodily fluids and are especially prominent in the gut where they are thought to help manage the large numbers of commensal biota (Cunliffe, 2003; Lehrer and Lu, 2012). Insufficient defensin expression is associated with decreased tolerance to normal gut microflora, contributing to chronic inflammatory disorders such as Crohn's disease and ulcerative colitis (Wehkamp et al., 2006; Gersemann et al., 2011). α -defensin secretion is variable and stimulus dependant. In neutrophils isolated from allergic individuals, a greater proportion of the α -defensin content of neutrophils is secreted in response to their allergen, compared to other stimuli (Kaiser and Diamond, 2000).

Section 3: The β -defensins

The β conformation is the most ancient and abundant form of defensin with ~30 β -defensin genes clustered on chromosomes 8p23, 6p12, 20q11.1 and 20p13 in humans, with corresponding syntenic clusters in other mammalian genomes (Patil et al., 2005). Human β -defensins have official and historic nomenclatures which are both commonly used in the literature. Official nomenclature follows DEFB#, with the # representing the number assigned to that gene and is, in general, in the order of discovery. The resultant protein is referred to as hBD#, except for the first three, which are still almost exclusively referred to by their common name (Table 2). When required for clarity, both the genomic and historic names are used in this paper. It is worth noting that hBD2 is encoded by the DEFB4 gene, leading to confusion with hBD4 which is encoded by DEFB104.

Gene	Historic Name (protein)	Official Name (protein)
β-defensins		
DEFB1	hBD1	β -defensin-1
DEFB4	hBD2	β -defensin-4
DEFB103	hBD3	β -defensin-103
DEFB104	hBD4	β -defensin-104
DEFB105	hBD5	β -defensin-105
DEFB106	hBD6	β -defensin-106
DEFB107	hBD7	β -defensin-107
DEFB108	hBD8	β -defensin-108
hBD#	hBD-#	β -defensin-#

Table 2: Defensin genomic and protein nomenclature according to Human Genome Organization – Gene Nomenclature Committee (HGNC). In current literature the first three to eight β -defensins are commonly referred to by their historic name. Note that hBD2 is encoded by the DEFB4 gene, and hBD4 is encoded by the DEFB104 gene.

In β -defensins, the net positive charge is generated by a high arginine and lysine content. The pro-sequence, which neutralizes α -defensins during storage, is small or completely absent in β -defensins. The mechanisms that neutralize β -defensins within the secreting cell is unknown (Ganz, 2003). β -defensins are proteolytically processed to the mature form by signal peptidase and secreted (Beckloff and Diamond, 2008).

β -defensins are widely expressed throughout the body and are primarily produced by epithelial cells. The regulation and expression of β -defensins is extremely complex. Some β -defensins are constitutively expressed, while others are only expressed upon exposure to stimuli (Liu et al., 2002). The ability of a cell to upregulate defensin expression is defensin-specific and stimuli-specific. Human gingival epithelial cells can upregulate DEFB1 expression by 2-4 fold, whereas DEFB4A and DEFB103 (encoding hBD2 and hBD3) expression is increased by 150 and 20 fold respectively, upon incubation with various stimuli (Sørensen et al., 2005).

Section 4: The θ -defensins

The θ -defensins, also called retrocyclins, are derived from a duplication event of the α -defensin gene cluster and have potent anti-viral activity (Patil et al., 2004). In humans and closely related primates, θ -defensins are transcribed in several tissues, but a premature stop codon truncates the RNA, leading to degradation. No detectable protein product is found in humans, but θ -defensins are expressed in the leukocytes of macaques (*Macacca mulatta*) and orangutans (*Pongo pygmaeus abelii*) (Venkataraman et al., 2009). These oligo peptides are 9 amino acids long, forming cyclic dimers that are post-translationally ligated. The three canonical defensin disulfide bridges are formed by the pairing of two intramolecular cysteines and one intermolecular pair (Figure 1) (Lehrer and Lu, 2012). Ongoing research is searching for ways to induce viable θ -defensin expression in human cells for anti-HIV and other antiviral applications (Venkataraman et al., 2009).

Section 5: Defensin diversity

The complexity of the defensin repertoire is based in the 40+ defensin genes, with subsequent layers of diversity added through variations at the genetic, RNA and protein level (O'Neil et al., 1999). Defensins are single nucleotide polymorphic (SNP), with DEFB1, DEFB104A and DEFB103 genes containing ~395, 39, and 3 annotated SNPs respectively, in the Genecards database (Safran et al., 2002; Genecards, 2013). Some of these SNPs encode non-synonymous amino acid substitutions which may influence defensin function and expression. While DEFB1 is fixed at two copies per diploid

genome, the other α - and β -defensins clustered on chromosome 8 are copy number variation (CNV), ranging from 5 to 12 and 2 to 8 copies per diploid genome, respectively (Linzmeier and Ganz, 2005; Nuytten et al., 2009; Groth et al., 2010). CNV effects defensin transcription and translation, and is associated with propensities towards inflammation, infection and some forms of cancer (Linzmeier and Ganz, 2005; Huse et al., 2008; Jansen et al., 2009; Milanese et al., 2009; Janssens et al., 2010). The levels of SNP and CNV of β -defensins on chromosomes 6 and 20 have not yet been investigated. Similarly, little is known about epigenetic influences, but a recent report showed histone deacetylases regulate constitutive DEFB1 expression in epithelial cell line A549 (Kallsen et al., 2012).

Alternative splicing generates several distinct defensin transcripts. Human DEFB119 and DEFB120 are the product of a single gene and share N-terminal sequence encoded by a common 5' exon, but have distinct C-termini which are encoded by different exons (Radhakrishnan et al., 2005). Similarly, the canine defensins originally reported as cBD1, cBD2 and cBD3 have identical N-terminal sequences but vary at the C-termini, and were later renamed cBD122 (Patil et al., 2005). At the protein level, several isoforms of DEFB1 can be isolated from human urine and show varying degrees of antibiotic activity (Zucht et al., 1998). These isoforms are thought to be generated by different proteolytic cleavage at the N-terminus.

The effects of altering or replacing amino acids can have mercurial effects on antibiotic activity. For example, HNP-1, HNP-2 and HNP-3 (Table 1) vary by a single N-terminal amino acid but have different antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes* and antifungal activity against *Candida albicans* (Raj et al., 2000; Ericksen et al., 2005). By contrast, hBD3 remains potent against *E.coli* and *S.aureus* even when the first 10 amino acids are deleted (Hoover et al., 2003). Thus, predicting the functional effects of SNPs, different isoforms and alternative cleavage for any defensin is difficult.

Section 6: Defensin regulation

Multiple receptors, major intracellular signaling pathways, epigenetic factors, negative and positive feedback loops, and micro-environmental factors all play a role in cell-specific and stimuli-specific defensin expression. Pathogen-associated molecular patterns (PAMPs), whole microorganisms, infectious agents and cytokines have been shown to differentially induce defensin expression (Table 3) (Harder et al., 2004; Premratanachai et al., 2004; Sørensen et al., 2005; Kimball et al., 2006; Ji et al., 2007).

Defensin	Constitutive	TNF- α	IFN- γ	<i>P.aerg</i>	<i>P.ging</i>	<i>S.gord</i>	<i>F.nucl</i>	<i>C.alb</i>
DEFB1	Yes	nc	nc	nc	nc	nc	nc	
DEFB4 (hBD2)	No	+	nc	+	+	+	+	
DEFB103	No	+	+	+	nc	+	+	
DEFB104	Yes							
DEFB105	No							
DEFB106	No							
DEFB107	Yes							
DEFB108	No	+	+					
DEFB109	Yes	+	+					+
DEFB110	No							-
DEFB111	Yes	+	+					
DEFB112	No	+	+					
DEFB113	No							
DEFB114	No	+	+					+

Table 3: Differential regulation of defensin RNA expression by cytokines, pathogenic and commensal microorganisms in gingival and epidermal keratinocytes. Several defensins are constitutively expressed while others are expressed in response to tumor necrosis factor alpha (TNF- α), interleukin-1 β (IL-1 β), interferon gamma (IFN- γ), pathogenic bacteria *Pseudomonas aeruginosa* (*P.aerg*), *Porphyromonas gingivali* (*P.ging*), pathogenic fungi *Candida albicans* (*C.alb*) or commensal bacteria *S. gordonii* (*S.gord*) and *F. nucleatum* (*F.nucl*). No change in expression (nc), increased expression (+), decreased expression (-), not assayed (). (Harder et al., 2004; Premratanachai et al., 2004; Kimball et al., 2006; Ji et al., 2007)

Receptors: Toll-like receptors (TLRs) are evolutionarily conserved pattern recognition receptors and can regulate defensins expression. *E.coli* lipopolysaccharides (LPS) can induce hBD2 expression in intestinal epithelial cell line SW480 (Vora et al., 2004). Similarly HaCaT, a spontaneously transformed keratinocytes cell line (melanoma), upregulates hBD2 expression after LPS stimulation (Seo et al., 2001). In

primary culture, *E. coli* LPS produces a 5 fold increase in hBD2 in human gingival epithelial cells co-cultured with murine fibroblast (Mathews et al., 1999).

However, epithelial cell lines A431, T84 and Caco-2 are insensitive to LPS (Seo et al., 2001; Vora et al., 2004; Sparatore et al., 2005). In primary cultures of human gingival epithelial cells, hBD2 transcription is reportedly unaltered by LPS from *E. coli*, *Aggregatibacter actinomycetemcomitans* or *Fusobacterium nucleatum* (Krisanaprakornkit et al., 2002; Laube et al., 2008).

This apparent discrepancy of hBD2 induction in epithelial cells via LPS stimulation *in vitro* could be due to the variation of TLR and co-factor expression in epithelia cell populations. TLR4 is responsible for detecting LPS, and TLR4 is known to be low or absent on the T84 and Caco-2 cell lines. Therefore it is not unreasonable that these cell lines would be unresponsive to LPS (Baker et al., 2003; Lebre et al., 2006). However A431 cell lines express TLR4, and other pro-inflammatory molecules are expressed in response to LPS (Evdonin et al., 2010). So why hBD2 is not upregulated in A431 cells remains unclear but may be linked to a TLR accessory molecule (MD-2), which has been shown to be critical for hBD2 expression (Vora et al., 2004). To date no one has reported the presence of MD-2 in A431 cells. Primary epithelial cell sensitive to LPS stimulation were co-cultured with mitomycin-treated murine fibroblasts, whereas the LPS insensitive epithelial cells were in relatively pure cultures (Mathews et al., 1999; Krisanaprakornkit et al., 2000). The fibroblasts may have been secreting cytokines that influenced defensin expression (Liu et al., 2003). Cytokines can transactivate defensin expression in keratinocytes that are LPS insensitive. This has been demonstrated by incubating epithelial cells with the supernatant of LPS stimulated lymphocytes or monocytes (Sørensen et al., 2005). Different secreted factors from the leukocytes induce hBD1, hBD2, and hBD3 RNA expression in epidermal cultures. hBD2 upregulation was dependant on IL-1, whereas hBD3 was influenced by epidermal growth factor receptor and IL-6. Blocking these factors did not influence hBD1 expression, indicating different cytokines and regulatory factors are responsible for the transactivation of all three defensins. The ability of lymphocyte or mononuclear cell supernatants to induce hBD

expression was dependent upon both the leukocyte and the stimuli (Sørensen et al., 2005).

A single study shows murine β -defensin 2 induces dendritic cell maturation in a TLR4 dependant mechanism (Biragyn et al., 2002), indicating the TLR-defensin relationship is potentially cyclic, with TLRs regulating defensins expression, and defensins serving as ligands for TLRs. Further research will be necessary to fully explore this relationship. However, it is clear that TLRs are not the only receptors involved since epithelial cells insensitive to *A. actinomycetemcomitans* and *F. nucleatum* LPS, will strongly produce hBD2 and hBD3 in response to the cell wall extracts and whole bacterium (Krisanaprakornkit et al., 2000; Laube et al., 2008). Protease-activated receptors (PARs) also regulate defensin expression. PARs are expressed in human epithelial cells and are activated in the presence of gingipains (proteases secreted by *P. gingivalis*) and have been shown to mediate hBD2 upregulation (Dommisch et al., 2007).

Intracellular signaling: DEFB1 and DEFB4 genes contain NF- κ B binding sites, indicating NF- κ B can regulate defensin expression in response to stimuli. Indeed, pathogenic bacterium, *P. gingivalis*, induces hBD2 transcription via the IKK α /TRAF3 arm of the NF- κ B pathway in gingival epithelial cells (Chung and Dale, 2008). However, the induction of hBD2 by commensal bacterium, *F. nucleatum*, is NF- κ B independent in oral epithelial cells (Krisanaprakornkit et al., 2002), indicating multiple intracellular pathways upregulate hBD2 expression and is dependent upon the stimulus.

Defensins may also play a role in regulating their own transcription. hBD1, hBD2, and hBD3 are all found in the cytosol and nucleus of cells. When exogenous added to oral squamous cell carcinoma cultures, hBD1 can differentially enhance or suppress the expression of hBD1, hBD2 and hBD3 through unknown mechanisms (Winter et al., 2011).

While hBD1 through 3 are the most widely studied defensins, human gingival keratinocytes constitutively express DEFB1, 103, 104, 107, 109, 111, 112, while DEFB102, 108, 109 and 114 are differentially regulated by proinflammatory cytokines and microorganisms (Premratanachai et al., 2004). This abundance of defensin

expression indicates more work is necessary to establish an accurate profile of defensin expression in various types of epithelium.

Chapter 2: Defensin Activities

Section 1: Antibacterial activity

While all defensins tested show some degree of antibiotic activity, few defensins have been extensively characterized. hBD1, hBD2, and hBD3 show pathogen-specific antimicrobial activity which is influenced by both the physical properties of the defensin, as well as the bacteria. For example hBD3 demonstrates minimal inhibitory concentrations (MICs) ranging from 3 ug/ml for *Escherichia coli* DH5 α to over 250 ug/ml for *P. gingivalis* strain W50 (Joly et al., 2004). Since defensins have such high primary amino acid sequence variability it is difficult to extrapolate or predict antimicrobial activity, so currently each defensin must be individually characterized.

Bacterial component: The ability of defensins to discriminate between bacterial and mammalian cells is thought to arise from the different composition of the lipid bilayers. When incubated with bacterial or mammalian membrane mimetics, defensins actively integrate in the bacterial membranes. While defensins are not excluded from the mammalian cells (Winter et al., 2011), the electrostatic attraction between the positively charged defensins and the anionic bacterial lipids is thought to increase defensin concentration around the bacterial membranes (White et al., 1995; Jing et al., 2005). The presence of physiological levels of salt greatly diminishes defensins antimicrobial activity by interfering with these charge interactions, as well as defensin dimerization.

The hydrophobic portion of the defensin integrates into the bacterial lipid bilayer, forming pores that disrupt ion gradients and asymmetry of the inner and outer membrane leaflets. These defensin pores are responsible for the rapid killing kinetics of a substantial portion of microbes (Sahl et al., 2005) and may also enhance defensin access to the intracellular compartment. Secondary antibiotic effects are thought to arise from

defensin mediated alterations in RNA transcription and inhibition of protein and peptidoglycan synthesis (Sharma and Khuller, 2001; Zasloff, 2002; Cole et al., 2004; Sahl et al., 2005). However, the exact mechanisms by which defensins exert these intracellular changes have not yet been elucidated.

In contrast to the anionic bacterial phospholipids, neutral cholesterol and zwitterion lipids in mammalian membranes do not actively attract defensins. Cholesterol also adds rigidity to the mammalian lipid bilayer, inhibiting pore formation and may be responsible for the ability of defensins to enter but not lyse mammalian cells (Glukhov et al., 2005; Brender et al., 2012).

Defensin component: The overall net charge of the defensin is thought to play the largest role in defensin antibiotic potency (Reynolds et al., 2010). hBD1, hBD2 and hBD3 have net charges of +5, +7, and +11 respectively, with general antibiotic activity scaling with increasing positive charge. Specifically, hBD1, hBD2, hBD3 have MICs of 100, 75 and 25 ug/ml respectively against *P. aeruginosa*; and MICs of >50, 10 and 5 ug/ml respectively against *S. aureus* (reviewed in Diamond et al., 2008).

However, overall net charge is not the only determining factor in antibacterial activity. hBD1 is four fold more effective against *A. actinomycetemcomitans* and *P. gingivalis* than hBD-3 (Ouhara et al., 2005). Also, C-terminal fragments of hBD1, hBD2 and hBD3, with charges of +5, +5 and +7 respectively, have about the same antibiotic activity against *E.coli* and *S. aureus* (Krishnakumari et al., 2006). The spatial distribution of the charged/neutral and hydrophilic/hydrophilic residues within the defensin are also important for protein conformation, dimerization, and antibacterial activity (White et al., 1995; Wu et al., 2003; Pazgier et al., 2007; Reynolds et al., 2010).

While most defensins have a high net positive charge, there are exceptions. Murine β -defensin 2 (Defb2), with a charge of +2, has antibiotic activity and stimulates dendritic cell maturation (Biragyn et al., 2002). The β -defensin derivative mellitin, from bee venom, is a neutral antibiotic peptide but is also highly cytotoxic to mammalian cells (Yamaguchi and Ouchi, 2012). In humans DEFB108 has a net charge of +1 at neutral pH, and is widely expressed throughout the respiratory and digestive tracts, skin and male

reproductive tract (Uhlen M, 2010). The functional activities of DEFB108 have not yet been assessed, but might yield insight into the mechanisms and importance of the cationic charge for defensin function.

It is also of note that the highly conserved disulfide bridges are not essential to antibacterial activity. In some cases, directed mutagenesis of the cysteines or linearization of the defensin peptide leads to enhanced antimicrobial activity against specific microorganisms (Varkey and Nagaraj, 2005; Taylor et al., 2008).

Mixtures of defensins: *In vitro*, defensins have been characterized as isolated peptides against a single pathogen. *In vivo*, multiple defensins are expressed together, creating admixtures of defensins determined by cell type and environment. While the importance of defensin combinations is not understood in terms of combating infection *in vivo*, a study by Chen et al. (2005) shows the potential for two or more defensins to enhance *in vitro* antibacterial activity. Concentrations of hBD1, hBD2 and hBD3 that are sub-lethal when used alone, worked synergistically against gram positive *S.aureus* and additively against gram negative *E.coli*. Different combinations of these three defensins showed different levels of synergy that was pathogen specific. This study indicates *in vitro* estimations of defensin concentrations necessary for *in vivo* efficacy may be grossly overestimated. The ubiquitous expression of some defensins ensures that defensins expressed in response to stimuli will be joining the defensin(s) already being constitutively expressed (e.g. DEFB1). It is unknown if the 30+ remaining human β -defensins show similar coactivity, but the potential for a vast array of synergistic or additive activity against diverse bacterial populations is clear.

Section 2: Antiviral activity

Like defensin antibacterial properties, antiviral activity of α -, β - and θ - defensins are defensin-specific and virus-specific. *In vitro*, HNP-1, hBD2 and hBD3 demonstrate anti-HIV activity, whereas hBD1 does not (Quiñones-Mateu et al., 2003; Wang et al., 2004; Chang et al., 2005). HNP-1 also exhibits antiviral activity against cytomegalovirus, vesicular stomatitis virus, and papillomavirus, but is ineffective against vaccinia virus and echovirus (Table 4) (Klotman and Chang, 2006).

Virus	Type	Family	HNP-1
HIV	enveloped retrovirus	precursor to AIDS	active
Cytomegalovirus	enveloped dsDNA virus	herpes family	active
Vesicular stomatitis	enveloped dsDNA virus	rabies family	active
Papilloma virus	non-enveloped	warts	active
Vaccina virus	enveloped dsDNA virus	small pox family	inactive
Echovirus	non-enveloped RNA virus	gastrointestinal	inactive

Table 4: HNP-1 activity/inactivity against a variety of viruses is representative of the variability of defensin antiviral activities.

Defensins also show a variety of pre- and post-infection effects including direct binding of the viral particles, binding target cell receptors or co-factors, inhibiting viral integration, endosomal exit, nuclear import, transcription and replication (Chang et al., 2005; Hazrati et al., 2006; Furci et al., 2007; Smith and Nemerow, 2008). The α -defensins also exert antiviral activity against Influenza A Virus (IAV) by inducing aggregation and enhancing neutrophil phagocytosis (Doss et al., 2009). Unlike antibacterial activity, some antiviral activity is dependent on the defensin disulfide bridges and protein conformation (Smith et al., 2010).

Section 3: Association with disease

DEFB1 SNP -44 G to C is associated with a higher rate of persistent nasal carriage of *S.aureus*, a known risk factor for nosocomial infection (Nurjadi et al., 2013). DEFB1 SNPs are also associated with different rates of mother-to-child transmission of HIV. HIV exposed but seronegative individuals are also more likely to have certain DEFB1 SNPs (Braida et al., 2004; Zapata et al., 2008; Ricci et al., 2009). Still other mutations in DEFB1 are associated with asthma, allergies, irritable bowel disease, lepromatous leprosy, chronic obstructive pulmonary disease, cystic fibrosis, lupus erythromyosis, and mortality due to sepsis (Chen et al., 2007; Prado-Montes de Oca, 2010; Sandrin-Garcia et al., 2012).

It is unknown if these associations are directly caused by hBD1. hBD1 which is ubiquitously expressed in most tissues, but shows the lowest antibacterial activity against *S.aureus* and does not inhibit HIV infection of oral keratinocytes *in vitro* (Quiñones-

Mateu et al., 2003; Diamond et al., 2008) Synergistic activity of hBD1 with other endogenous antibacterial and antiviral components of the innate immunity might also enhance functional importance of this defensin *in vivo* (Chen et al., 2005). This association of DEFB1 SNPs with various inflammatory or infectious diseases could also be the product of downstream effects. The -44 G to C SNP of DEFB1 is associated with lower translation and induceability of both DEFB1 and DEFB103. hBD1 can also upregulate hBD3 transcription (Kalus et al., 2009; Winter et al., 2011; Nurjadi et al., 2013), so lower DEFB1 might influence hBD3 expression at several levels. This is important because hBD3 is a broad spectrum and potent antibiotic, and lower levels of this defensin has been linked to increased pathogen load at various epithelial surfaces (Zanger et al., 2011).

Chapter 3: Alternative Functions

In addition to their role as endogenous antibiotics, defensins are promiscuous ligands which interact with several receptors involved in cell migration, glucocorticoid production, hair pigmentation, and lipid catabolism (Quinn et al., 2008). The strict conservation of the six cysteines and disulfide bridges that characterize the defensin peptides may be required for some, but not all of these receptor mediated effects.

Section 1: Chemotaxis

Defensins coordinate the innate and adaptive arms of the immune system by recruiting specific cells to the site of inflammation or infection. Select α - and β -defensins induce the migration of adaptive immune cells via the CC chemokine receptors (CCR) on memory T cells and immature dendritic cells (CCR6), as well as on monocytes and possibly mast cells (CCR2) (Yang et al., 1999; García et al., 2001; Niyonsaba et al., 2002; Röhrle et al., 2010). These chemotactic properties are functional at concentrations lower than necessary for *in vitro* antibiotic activity, forming a bell curve, with maximal response observed at 10 ng/ml for HNPs, hBD2 and hBD3 and 100 ng/ml for hBD1 (Yang et al., 2000; Pazgier et al., 2007; Taylor et al., 2008; Röhrle et al., 2010). While individual defensins have been tested, to date, the effects of different mixtures of β -

defensins on chemoattraction of adaptive immune cells have not been investigated. If the chemotactic properties of defensins are synergistic or antagonistic, the type and amount of defensins expressed would influence the recruitment of adaptive immune cells.

Microenvironment can alter the effectiveness of defensin chemotactic function. *In vitro*, dendritic cells that have been exposed to TNF- α no longer migrate in response to HNPs (Yang et al., 2000). Conversely TNF- α stimulated neutrophils will become chemotactically responsive to high concentrations (5ug/ml) of hBD2 (Niyonsaba et al., 2004). Concentration and cytokine dependency may provide temporal and spatial separation of defensin chemotactic and antibiotic function.

Defensin induced chemotaxis may also play a role in normal wound healing and tumor angiogenesis. *In vitro*, hBD2 induces the migration of endothelial cells via the $\alpha v \beta 3$ integrin (Baroni et al., 2009). Subsequent proliferation and structural organization of the endothelia is also enhanced in the presence of hBD2. While not much is known about hBD2/ $\alpha v \beta 3$ interactions, several factors influence the ability of defensins to interact with CCR2 and CCR6 expressing cells. Overall defensin conformation and the distribution of the amino acids within three dimensional space is essential for receptor interaction. HNP-1, HNP-2, and HNP-3 differ by a single N-terminal amino acid (Table 1) but only HNP-1 and HNP-2 chemoattract monocytes, whereas HNP-3 is inactive (Territo et al., 1989). A different study showed even when the cysteine residues, disulfide bridges and overall tertiary structure is retained, chemotactic activity is disrupted by the mutation of N-terminal residues on hBD1 and mutating the second amino acid position in murine Defb14 from iso/leucine to glycine or lysine (Pazgier et al., 2007).

The exact role of the disulfide bridges in chemotactic activity is unclear. Site directed mutagenesis of the cysteines to alanines of murine Defb14 demonstrates only the fifth cysteine, not the disulfide bridges themselves, must be retained for chemotactic activity (Taylor et al., 2008). However, Wu et al. (2003) showed the pairings of the cysteine residues of hBD3 greatly changed the strength of chemoattraction of CCR6 expressing human embryonic kidney cells (HEK) and monocytes. Of interest, the alteration of hBD3 disulfide bridges to the α -defensin conformation (cys¹-cys⁶, cys²-cys⁴,

cys³-cys⁵) decreased the chemoattraction of CCR6 expressing cells by 100 fold and totally abolished the migration of CCR2 expressing cells. The alteration of the disulfide bridges may lock hBD3 into conformations that are less effective in binding the CC chemokine receptors.

The effect of linearizing defensin peptides has similar conflicting reports in the literature. Substitution of hBD3 cysteines with cysteine analog (α -aminobutyric acid, Aba) creates a linear peptide with no chemotactic activity (Wu et al., 2003). However, iodoacetamide (IAM) capping of hBD3 cysteines inhibits disulfide bridging, but IAM-hBD3 can still induce migration of CCR6 expressing cells (Taylor et al., 2008). The apparent discrepancy may be explained by the ability of the modified peptide to attain and retain wild-type peptide conformation in the absence of the disulfide bridges. Possibly the thermodynamic properties that inhibit the disulfide bridges in Aba-hBD3 also inhibit normal folding, whereas this may not be true for the IAM-hBD3. However, it seems clear that the disulfide bridges are neither absolutely necessary nor sufficient for binding CCR6 and CCR2. Taken together it is clear that the N-terminal portion of the defensin peptide is important for chemotaxis (Tyrrell et al., 2010).

Section 2: Immunomodulation, pro-inflammatory effects

In addition to inducing the migration of some adaptive immune cells, defensins elicit both pro- and anti-inflammatory responses from stationary and migratory cells that are both defensin-specific and target cell-specific. Toll-like receptors (TLR), environmental factors and the exact sub-population of target cells are some of the factors that determine if defensins illicit pro- or anti-inflammatory effects.

Murine β -defensin 2 (mBD-2) induces the production of pro-inflammatory cytokines (IL-12, IL-1 α , and IL-1 β) and maturation of dendritic cells via TLR4 (Biragyn et al., 2002). The mature mBD-2 sequence and disulfide bridges are important for the interaction with TLR4, given that pro-mBD-2, mBD-2 with scrambled disulfide bridges and mBD-3 are unable to elicit the same response from dendritic cells. In humans, hBD3 activates monocytes in a TLR 1/2 dependant mechanism (Funderburg et al., 2007). This

demonstrates defensins are both regulated by multiple TLR and are potentially ligands for multiple TLRs.

Section 3: Immunomodulation, anti-inflammatory effects

What determines if defensins will exert pro- or anti-inflammatory effects is not known, but may be due to environmental factors, target cell receptor expression, and the leukocytes present (Harvey et al., 2013). Dying neutrophils can exert both pro- or anti-inflammatory effects on local macrophages depending on if they were undergoing necrosis or apoptosis (Miles et al., 2009). Necrotic neutrophils enhance antigen presentation on macrophages thus enhancing the potential for inflammation, whereas apoptotic neutrophils have the opposite effect (Barker et al., 1999). The reason for this has not been conclusively demonstrated, but a case may be made for defensin involvement.

In other studies human α -defensins and hBD3 inhibit production of several pro-inflammatory cytokines (e.g. IL-1 β , TNF- α , IL-6) in LPS stimulated mononuclear cells and hBD3 suppresses IFN- γ mediated macrophage stimulation (Shi et al., 2006; Funderburg et al., 2007; Semple et al., 2010; Semple and Dorin, 2012).

Section 4: Neutralization of pro-inflammatory molecules

Pathogen components called endotoxins can elicit a potent inflammatory response, even if the pathogen is no longer viable. An important function of the innate immunity is to neutralize endotoxins. Lipopolysaccharides (LPS) are major cell wall components of gram negative bacteria and potentiate toxic shock. LPS binding protein (LBP) binds LPS and enhances the interaction with CD14 on monocytes and macrophages, leading to increased TNF- α , IL-6 and other pro-inflammatory cytokines (Figure 2). HNP-1, hBD2, DEFB123 and other cationic peptides directly bind LPS and inhibit the interactions between LPS and LBP, thus attenuating the pro-inflammatory cytokine production (Scott et al., 2000; Motzkus et al., 2006). When administered therapeutically, DEFB123 decreased the mortality of LPS induced toxic shock in the murine model.

While direct binding of LPS may account for some of the protective effects of defensins, hBD3 does not bind LPS, nor block the MAP kinase signaling cascade, but has been reported to decrease pro-inflammatory cytokine production in macrophages after the addition of LPS (Motzkus et al., 2006; Semple et al., 2010). Similar effects are seen with α -defensins. Even after human peripheral blood mononuclear cells (PBMC) have been stimulated with LPS and ATP, HNP-1 dramatically decreases IL-1 β production (Shi et al., 2006). This indicates there are at least two mechanisms by which defensins are able to ameliorate toxic shock.

LPS is not the only toxic compound that can be bound by various defensins. Defensins also bind the recombinant form of pathogenic *P. gingivalis* adhesion molecules, hemagglutinin B (rHagB) and fimbriin A (rFimA), *in vitro*. Binding affinity of HNPs and hBDs for rHagB or rFimA is defensin-specific. HNP-1, HNP-2 and hBD3 all bound with greater affinity than hBD1 and hBD2 (Dietrich et al., 2008). Binding of these bacterial proteins may target endotoxins for uptake by phagocytes and inhibit their interaction with cell surface molecules which induce pro-inflammatory cytokine production in macrophages, thus neutralizing the toxicity of the endotoxins.

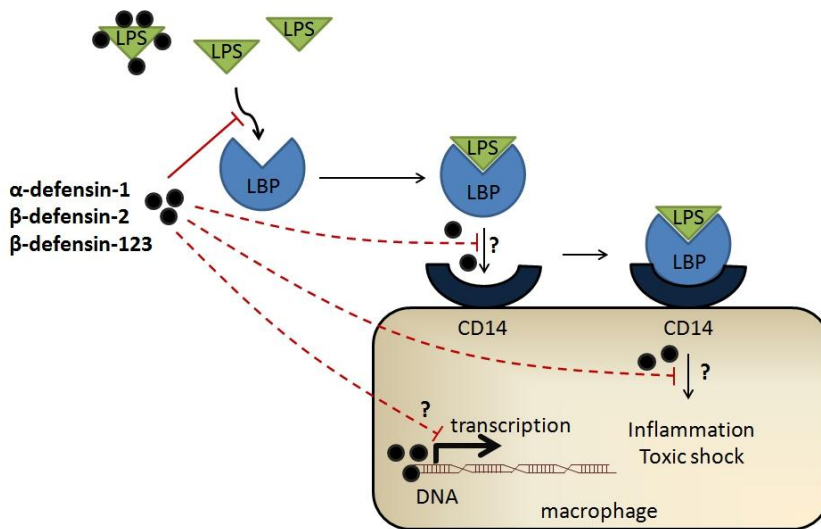


Figure 2: HNP-1, β -defensin 2, and β -defensin 123 can neutralize toxic bacterial components (e.g. LPS) through direct binding, inhibition of LPS - binding protein (LBP) interaction, and target cell interactions. While this function is common to many cationic peptides, some defensins (β -defensin-3) cannot bind LPS (Scott et al., 2000; Motzkus et al., 2006).

Section 5: Defensins as adjuvants

Defensins are attractive as potential adjuvants due to their immunomodulatory activity. By chemoattracting specific populations of helper T cells defensins may help skew the adaptive immune response towards long lasting immunity (Biragyn et al., 2002; Biragyn, 2005).

An *in vivo* mouse study showed intranasal administration of rHagB, with selected α - or β -defensins enhanced IgG antibody production and decreased inflammatory markers in nasal secretions (Kohlgraf et al., 2010). *In vitro*, covalent linking of defensins to weakly immunogenic molecules or tumor antigens enhanced the adaptive immune response (Biragyn, 2005). The efficacy of defensins as adjuvants is defensins-specific, as demonstrated by the differential immune response elicited by mBD2 and mBD3 when linked to the same antigen. The mBD3-antigen molecule induces a strong antibody response but failed to protect mice from subsequent tumor challenge, whereas the mBD2-antigen molecule induced only a moderate antibody response but protected 50% of the mice from tumor challenge (Oppenheim et al., 2003). This difference between mBD2 and mBD3 was IFN- γ dependant and opens up the potential for different defensin admixture to induce different humoral and cellular responses to vaccines.

Section 6: Defensins and the hypothalamic-pituitary-adrenal axis

The hypothalamic-pituitary-adrenal (HPA) axis is an important systemic regulator of metabolic homeostasis, stress, immunity, and reproduction in vertebrates. The HPA axis incredibly complex and composed of multiple receptor and ligand interactions, but our interest lies in the melanocortin receptors (MCRs), POMC derivatives (ACTH, α -MSH) and defensins.

In the 1980's it was discovered that a peptide isolated from rabbit lung inhibited the release of corticosterone from ACTH stimulated rat adrenal cells. This peptide called corticostatin-1 (CS-1), could reversibly bind the ACTH receptor, competitively inhibiting the effects of ACTH (Qlnzhang et al., 1987; Zhu et al., 1989). Immunohistochemistry of HPA axis tissues showed CS-1 present in the pituitary and adrenal cortex, but little to no

expression in the hypothalamus. However, CS-1 expression was induced in the adrenal gland and hypothalamus in rabbits with induced peritonitis, focal abscesses, or given Freund's adjuvant, thus co-localizing CS-1 to all the tissues involved in the HPA axis (Tominaga et al., 1992). Corticostatin-1 was later identified as an α -defensin and the ACTH receptor, the melanocortin 2 receptor (MC2R). Human HNP-4 also has weak corticostatic properties (Singh et al., 1988), but after this initial flurry of research, very little attention was paid to this link between defensins and the melanocortin receptors.

Defensin-MCR interaction came back to the forefront of defensin research in 2007, when Candille *et al.* showed that a common mutation in canine β -defensin 103, called cBD103 Δ G23, found in Labrador Retrievers as well as Great Danes, Basenjis, Poodles and Golden Retrievers was responsible for the dominant black coat color in dogs (Candille et al., 2007; Leonard et al., 2012). The deletion removes a single glycine residue from N-terminal sequence of the mature peptide, enhancing the binding affinity of cBD103 for the melanocortin 1 receptor (mc1r). cBD103 Δ G23 competitively displaces the agouti protein, leading to the production of eumelanin (black/brown pigment) instead of pheomelanin (yellow pigment). The cBD103 Δ G23 peptide retains the antimicrobial activity of wild-type cBD103 peptide (Leonard et al., 2012), and thus is not deleterious to the innate immunity.

Transgenic mice expressing cBD103 Δ G23 not only had dark coats but also weighed significantly less than their non-transgenic littermates, suggesting that this defensin can also bind the melanocortin 4 receptor (mc4r), which regulates energy homeostasis. The caveat to this finding was that mice with the wild type cBD103 also had dark coat colors and weighed less, indicating that both forms of the canine defensin can bind the mouse melanocortin 1 and 4 receptors *in vivo*. *In vitro*, cBD103 and the Δ G23 mutant and human hBD3 had different affinities for the human melanocortin 4 receptor. The localization of melanocortin receptors and defensins in many of the same tissues opens up the possibility that defensins may serve as a link between the immune system and processes regulated by the MCRs. However, other than altering the coat color in the dog, no other phenotype has yet been definitively attributed to the

cBD103ΔG23 mutation. Of the 40+ human defensins, few have been tested for binding capacity to the melanocortin receptors.

A single study indicates that, *in vivo*, local steroidogenesis may be influenced by the genes that can determine coat color. In seeking to validate the utility of cortisol measurement from hair samples in dogs, Bennett and Hayssen reported that measurable cortisol levels were decreased in canines with black coat color. When hair samples were collected from the same dog, and separated by color, the black hair had measurably less cortisol than the other samples. The authors hypothesized that the influences driving the hair color were altering the sequestration of cortisol in the hair shaft (Bennett and Hayssen, 2010). However, it may also be possible that the protein(s) responsible for inducing eumelanin production via the mc1r, is also influencing steroidogenesis controlled by mc2r. While the binding affinity of cBD103ΔG23 has not been established for mc2r, it has been demonstrated that many ligands (e.g. ACTH, α-, β-, and γ-MSH) can bind multiple melanocortin receptors (Ducrest et al., 2008). So it is possible cBD103ΔG23 or the peptide involved in the recessive black hair phenotype may be interacting with multiple melanocortin receptors to influence both coat color and local steroidogenesis.

Section 7: Defensins and reproduction

Almost all β-defensins are expressed to some degree in the epididymis and/or testis in rats, dogs, and man (Patil et al., 2005; Pazgier et al., 2006; Leonard et al., 2012). However, no link between defensins and reproductive fitness was reported until 2011 when Tollner *et al.* showed that in humans, a frame-shift mutation in DEFB126 leads to perm with altered glyocalyx composition and a decreased ability to penetrate cervical mucus analogs (Tollner et al., 2011). Couples were also more likely to have greater difficulty in achieving pregnancy when the male was homozygous for the DEFB126 mutation.

Considering the extensive level of defensin expression in the male reproductive tract, it would not be surprising if future research uncovered a much larger role for defensins in fertility and reproduction. The reproductive tract is another tissue in which

both MCRs (Schiöth and Watanobe, 2002) and defensins are co-expressed. While co-expression does not prove interaction it does suggest a potential link between defensins, the innate immunity, the HPA-gonadal axis and reproductive fitness.

Section 8: Defensins and cancer

Altered defensin expression is associated with several types of cancer (Droin et al., 2009). In over 80% of all primary cell cultures isolated from prostate cancers and renal clear cell carcinomas, hBD1 is significantly downregulated at the RNA and protein level (Donald et al., 2003). Decreased expression was due to promoter mutations and chromosomal instability around the DEFB1 locus. Restoration of DEFB1 expression in a renal cancer cell line enhances apoptosis (Sun et al., 2006), indicating DEFB1 may facilitate apoptosis in cells exhibiting pre-cancerous alterations in metabolism, thus acting as a tumor suppressor gene.

Exogenous hBD1 suppresses DEFB1 transcription after 48 hours in oral squamous cell carcinomas, indicating hBD1 exposure exerts negative feedback on its own expression (Winter et al., 2011). In normal cells, hBD1 is found throughout the cytoplasm and nucleus, by contrast, hBD1 accumulates almost exclusively in the nucleus of malignant salivary gland tumors (Wenghoefer et al., 2008). If hBD1 acts as a transcription factor to suppress defensin expression, translocation or trapping of hBD1 in the nucleus may cause perpetual under expression of DEFB1. This would mimic the observed decrease in DEFB1 in prostate and renal clear cell cancers due to genetic abnormalities.

Not all defensins are tumor suppressors. hBD2 and hBD3 may be proto-oncogenes. One study showed 50 nM exogenous hBD2 and hBD3 enhances proliferation of OSCC (Winter et al., 2011). However in a different study hBD2 concentrations of <1 nM enhanced proliferation, whereas 10 nM to 1 uM of hBD2 inhibited proliferation of malignant epithelial cell lines (A549, adenocarcinoma and A431, epidermoid carcinoma) (Zhuravel et al., 2011). This inconsistency could be due to the different origins of the cell lines, or be cancer-related alterations in normal cellular function. However, if lower hBD2 concentration enhances proliferation, this could potentially explain why hBD2

expression is downregulated in human papilloma associated cervical cancer (Coffelt and Scandurro, 2008).

The involvement of defensins in cell cycle regulation, angiogenesis and recruitment of tumor enhancing adaptive immune cells (e.g. myeloid-derived dendritic cells) (Röhrl et al., 2012) indicates further study is needed to define the role of defensins in cancer.

Chapter 4: Animal Modeling

Section 1: The canine model

There are several human and canine afflictions that share common symptoms and etiologies, therefore show similar responses to therapy (Van Damme et al., 2009; Parker et al., 2010). Companion animals are often exposed to the same environmental factors that can affect disease development and progression, as well as incur traumas that mirror the range of human injuries not seen in murine models (Seok et al., 2013). The demand for high quality health care for companion animals is second only to what is expected for humans, and canine genetic diversity, along with breed-specific diseases or phenotypes, help uncover underlying genetic mechanisms that are difficult to identify in the human population. In recent years the dog has emerged as a potential bridge between mice and men in the study of disease, especially that of infection and inflammation.

The potential use of dogs as models of human disease is well established for cancer (Rowell et al., 2011), but could also be important for inflammatory processes which are not well modeled in murines. An allergic skin condition associated with recurrent infection called atopic dermatitis (AD), is the most common human pediatric chronic inflammatory skin condition worldwide. AD affects 10% of dogs, 17% of children and 0.9% of adults (Williams, 2006; Horii et al., 2007; Marsella and Girolomoni, 2009; Van Damme et al., 2009). In AD, common environmental allergens trigger an inappropriate inflammatory response. Alterations in the expression several innate immune genes, including defensins, have been reported. In particular, hBD3 is

downregulated in AD skin, which might contribute to the high rate of infection in AD lesions (Kisich et al., 2008). The canine paralog of hBD3, cBD103, has been reported to be downregulated in the skin of dogs with AD (Howell et al., 2006; Van Damme et al., 2009); however a single study reported that cBD103 levels were unaffected by AD (Leonard et al., 2012). It has been shown that mobilization of the human paralog, hBD3, is critical for keratinocytes mediated killing of *S. aureus*, which could indicate that even if cBD103 levels were unaltered in some cases of AD, bacterial activity may still be hindered.

Recent attention has turned towards the connection between chronic inflammation and various psychiatric and neurodegenerative diseases, as well as traumatic brain injury. As modulators of the innate and adaptive immunities, and as promiscuous ligands, defensins are well suited to play a role in the development and/or progression in central nervous system pathologies. hBD1 and hBD2 can be expressed in neuronal tissues but very little is known about the expression of the other 30+ defensins in the central nervous system (Hao et al., 2001; Su et al., 2010). Already increased α -defensin expression has been put forth as a potential marker for identifying individuals more likely to develop schizophrenia in at risk populations (Craddock et al., 2008). Of particular interest to this study is the discovery that olfactory neuronal stem cells demonstrate the same altered physiology observed in CNS neurons in cases of schizophrenia, Alzheimer's and Parkinson's (Ghanbari et al., 2004; McCurdy et al., 2006; Mackay-Sim, 2012). The abundance of the canine olfactory mucosa represents an ideal source for studying disease related neuronal changes. These samples would allow direct comparison from the same individual during disease progression or before and after therapy. Thus, studying the innate immunity of the olfactory receptor neurons has the potential to greatly advance the field of neuroscience and psychiatry.

Section 2: Insufflated medicine

Understanding the innate immunity of the nasal cavity may also help in therapeutic drug delivery. Proximity, neural connections, shared vasculature and lymphatics cement the intimate connection between the nasal mucosa and the central nervous system (CNS). While this close association is exploited by several pathogens, it

also offers an attractive route to deliver therapeutic agents to the CNS. Intranasal administration circumvents many of the challenges that can hinder systemic delivery: dilution, hepatic and renal elimination, sequestration and the blood brain barrier (BBB). The BBB presents the largest challenge to neurologic and psychiatric medication, excluding 98% of small and ~100% of large molecules.

Nasal insufflations deliver high local concentrations to the nasal epithelia. Transport of molecules to the CNS is possible via olfactory neurons (Thorne et al., 2004), trigeminal nerves, olfactory ensheathing cells, perivascular channels, and lymphatic vessels (Dhuria et al., 2009). Depending on the route, some molecules are quickly absorbed and transported to the brain within 30 minutes of delivery, while others can take days (Thorne et al., 2004; Dhuria et al., 2009). CNS delivery is also dependent on several factors including dose, tissue integrity, and the properties of the molecule itself (e.g. size, charge, lipophilicity). Different routes deliver the drugs to different area of the brain, with the olfactory nerves associated with high anterior concentrations (olfactory bulb) and trigeminal nerves associated with both the anterior (accessory olfactory bulb) and posterior deposition (brain stem) (Dhuria et al., 2009).

Small lipophilic molecules (cocaine, 0.3kDa), larger molecules (leptin, 16kDa and nerve growth factor, 28kDa) and even stem cells have been shown to enter the CNS via the nasal mucosa (Danielyan et al., 2009; Dhuria et al., 2009). This opens up the exciting idea of autologous harvesting and administration of stem cells using the nasal mucosa to treat neurologic diseases. However, the innate immune system is largely responsible for inefficient drug delivery of insufflated medicines, with 1% or less of the administered drug reaching the CNS (Thorne and Frey, 2001; Illum, 2004). Thus, study of the innate immunity of the nasal cavity may shed light on methods to enhance the delivery and efficacy of CNS targeted therapies.

Chapter 5: The Nasal Cavity

Section 1: Gross anatomy and microanatomy

Besides the difference in size, the general organization of the nasal cavity is well conserved in mammals and is composed of four main regions distinguished by function and cellular makeup. For thorough comparative anatomic analyses of the nasal cavity of mice and men, and the anatomy of the dog nose please see reviews by Harkema et al. and Craven et al. (Harkema et al., 2006; Craven et al., 2007).

The nasal cavity is divided laterally by the nasal septum into two symmetric chambers. The externally visible structure called the nares is responsible for directing airflow into the nasal cavity. The nares are covered with keratinized or non-keratinized stratified squamous epithelia and are confluent with the epidermis. In man, caudal to the nares, respiratory epithelium lines the floor, inferior and middle turbinates of the nasal cavity. Respiratory epithelium (RE) is composed of pseudostratified ciliated columnar cells and numerous goblet cells. Underlying the epithelium are seromucous glands and a dense vasculature. Blood vessels in the RE dilate or constrict in response to the environment, providing the heat and humidity necessary to condition the inspired air.

The boundaries between the respiratory and olfactory epithelium are not fixed, with interspersed pockets of one in the other (Morrison and Costanzo, 1990). In general human olfactory epithelium (OE) is located on the superior turbinate and ceiling of the nasal cavity and comprises only a small proportion of the nasal mucosa. Microvillar cells, basal cells, pseudostratified ciliated columnar cells, and olfactory receptor neurons (ORNs) comprise the OE. Two features make olfactory receptor neurons unique: 1. ORNs are the only neurons directly exposed to the external environment and 2. ORNs are one of the few neuronal populations that are involved in adult neurogenesis, turning over about every 90 days (Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 2006). The turnover of ORNs is thought to eliminate cells that are potentially infected or otherwise damaged. Division of basal cells give rise to multipotent progenitors that can differentiate into neurons or other cell types in the olfactory epithelium. Olfactory ensheathing cells create a protective tube around the axon of the ORN, this tube remains

in place during neuronal regeneration and is active in phagocytizing cellular debris associated with neuron degeneration (Li et al., 2005; Dhuria et al., 2009).

While important for pheromone detection in other mammals, the existence of the vomeronasal organ (VNO) in man is subject to debate (Meredith, 2001). In dogs and rats the VNO is located in the rostral portion of the nasal cavity and consists of a small blind pocket lined with both respiratory and sensory epithelia. The sensory epithelium containing chemosensory neurons distinct from those found in the olfactory epithelium.

The orientation and the surface area of the turbinates are the two most striking anatomical differences between the nasal cavities of man and the nasal cavities of mice, rats and dogs. In man, the orientation follows a general inferior-superior layout, whereas the orientation is rostral-caudal in other animals. The turbinates in humans are simple, but animals that rely heavily on olfaction, have amazingly complex turbinate structures that increase the surface area by several orders of magnitude.

Section 2: Defensin in the human nasal cavity

The nasal cavity presents a unique challenge to the immune system and has evolved to balance populations of commensal bacteria, while confining opportunistic bacteria and eliminating a wide array of potential pathogens. Viruses, such as herpes simplex, Borna disease, rabies, as well as prions and meningitis-causing pathogens such as *Neisseria meningitidis* bacteria and *Naegleria fowleri* amoeba can also exploit the intimate connection between the nasal mucosa and the brain, using the nasal cavity as a point of entry into the central nervous system (Zanusso et al., 2003; Mori et al., 2005; Sjölander and Jonsson, 2010; Yoder et al., 2012). However, the low incidence of infection due to nasal pathogens indicates a robust and effective innate immunity.

The mucociliary blanket is the most prominent feature of the innate immunity in the nasal cavity. This thick mucus layer that coats the nasal mucosa is secreted by epithelial cells, goblet cells, submucosal seromucous glands and Bowman's glands. The mucous traps airborne particulate matter while the ciliated epithelia beat in a coordinated manner to create a current that draws the mucous towards the nasopharynx, where it is swallowed. The composition and mechanical properties of the mucous varies by location within the nasal cavity (Harkema et al., 2006). RNA transcriptional analysis shows

innate immune genes are differentially expressed in the respiratory epithelia and olfactory epithelia, indicating antibiotic properties in the mucous could also vary by location (Roberts et al., 2007).

The few studies that have looked at human defensin expression in the nasal mucosa and nasal secretions have focused on the lower turbinates, representing respiratory epithelium (Cole et al., 1999; Laudien et al., 2011). No one has described defensin expression specifically in the olfactory epithelium.

The composition and antibacterial properties of nasal secretions varies by individual. DEFB1 is constitutively transcribed in the inferior turbinates and hBD1 is found consistently in the nasal secretions (Lee et al., 2002). Whereas DEFB4 and DEFB103 (encoding hBD2 and hBD3) are not consistently expressed in the inferior turbinates and nasal secretions of healthy individuals (Lee et al., 2002; Laudien et al., 2011). All three β -defensins are upregulated in cases of chronic sinusitis, nasal polyps, and upon exposure to bacteria (Cole et al., 1999; Dauletbaev et al., 2002; Lee et al., 2002; Chen and Fang, 2004; Quinn and Cole, 2007; Laudien et al., 2011).

hBD3 is thought to be one of the most potent β -defensin, showing activity against a wide variety of pathogens, and works synergistically with defensins, lactoferrin and lysozyme that are also expressed in nasal secretions (Mathews et al., 1999; Lee et al., 2002; Chen et al., 2005). While the importance of hBD3 has not been established in the nasal mucosa, hBD3 is critical for oral keratinocyte mediated killing of *S. aureus*, which is a common opportunistic pathogen in both the oral and nasal cavities (Maisetta et al., 2003; Kisich et al., 2007). Decreased hBD3 is also associated with higher rates of nasal carriage of *S.aureus*, a well documented risk factor for nosocomial infection (Huang and Platt, 2003; Zanger et al., 2011; Nurjadi et al., 2013). From this, we can deduce that when the expression of hBD3 is impaired or naturally low in some individuals, *S.aureus* can persistently colonize the nasal cavity, serving as a reservoir for infection and subsequent re-infection. In healthy individuals, lower hBD3 levels can also be correlated to the severity of skin infections (Zanger et al., 2010)

Altered defensin expression in response to stimuli is often manifest in diseases such as HIV, cystic fibrosis and Wegner's granulomatosis (WG) (Dauletbaev et al., 2002; Alp et al., 2005; Luisa-Thienhaus et al., 2011). WG is a disease of the blood vessels

which is characterized by chronic sinus infection. WG patients show decreased hBD3 in nasal secretions, as well as an inability to upregulate hBD3 expression to the same degree as the healthy controls upon stimulation (Luisa-Thienhaus et al., 2011).

Section 3: Canine β -defensins

There have been two highly influential papers written on the general subject of canine β -defensins. The first was a 2005 study conducted by Patil *et al.* comparing β -defensin genes in man, chimp, dog, mouse and rat. All five species had several dozen β -defensins, located on syntenic chromosomal regions within their respective genomes. Between these species, general defensin clusters are largely conserved as a whole, but duplication events and mutations have created defensins repertoires that are species specific. Exclusive sets of defensin genes are common to the genomes of primates and murines (human β -defensin 2, and murine β -defensin 3 through 8), primates and canines (β -defensin 104, 108, 144, 120, 133 and 134) and murines and canines (β -defensins 138 through 140) (Patil et al., 2005).

The second formative paper on basic canine defensin research was published in 2012 by Leonard et al., who systematically analyzed the transcription of all known canine β -defensins in a variety of tissues. Canine β -defensins have tissue-specific expression and, like other mammalian defensins, almost all are expressed in the male reproductive tract. cBD1, cBD103, cBD108, cBD119, cBD120, cBD122, cBD123, cBD124 are expressed in two tissues from the respiratory tract, the tongue and lungs as well as in the epidermis (Table 7). Leonard's paper focused on cBD103 and cBD103 Δ G23, which can interact with various melanocortin receptors. This deletion of the N-terminal glycine residue from the mature peptide had similar antibacterial activity as the wild-type cBD103 against clinically relevant canine pathogens. They showed Labrador Retrievers and Golden Retrievers were more likely to have between 2 to 5 copies of cBD103 (Leonard et al., 2012), whereas Doberman Pinschers, German Shepherds, and Rottweilers showed no CNV.

Erles and Brownlie showed that cBD1, cBD103, and cBD108 are constitutively expressed in canine tracheal epithelial cells. These defensins can also be downregulated in response to LPS and canine viral pathogens (Erles and Brownlie, 2010). The ability of some viruses to depress defensins expression is also seen in young pigs challenged by porcine reproductive and respiratory syndrome virus, and may be partly due to viral suppression of pro-inflammatory cytokine production by surrounding cells.

In the case of canine atopic dermatitis (AD), alterations in defensins have been reported skin samples in both humans and dogs. cBD1 RNA levels are elevated in dogs with AD compared to unaffected individuals. In AD dogs, lesion skin also had increased cBD1 expression compared to non-lesional skin. By contrast, cBD103 expression is downregulated in atopic dermatitis lesions, similar to what is reported in humans (Kisich et al., 2008; Van Damme et al., 2009; Santoro et al., 2011). However, others have reported cBD103 expression was not significantly different in AD lesions compared to skin without lesions (Leonard et al., 2012). It is unclear if different primer sets, interindividual defensin expression, breed differences or some other factor is responsible for the discrepancy. Canine defensin expression in the literature is also variable (Table 5).

Skin cBDs	Leonard et al.	van Damme et al.	Wingate et al.	Consensus
1	+	+	+	Yes
102	-	-	+/-	Variable
103	+	+	+	Yes
104	-	-		No
105	-	+/-		Variable
106	-			No
107	-	+		Variable
108	+	+/-		Variable
109	-			No
110	-			No
111	-			No
112	-			No
113	-			No
114	-			No
116	-			No
117	-			No
118	-			No
119	+			Yes
120	-			No
122	+	+/-	+/-	Variable
123	+	-		Variable
124	+	-	+/-	Variable
125	-			No
126	-			No
127	-		+/-	Variable
128	-			No
129	-			No
130	-			No
131	-			No
132	-			No
134	-	-		No
135	-	-		No
136	-	-		No
137	-			No
138	-	+/-		Variable
139	-	+/-		Variable
140	-	-		No
141	-			No
142	-			No

Table 5: Canine epidermal defensins expression shows variable expression, possibly due to breed or individual variation. Leonard et al. from beagle and unidentified breed, van Damme et al. from beagles and mixed breed dogs, Wingate et al. from hounds and beagle. Detected (+), not detected (-), inconsistent detection (+/-), not assayed (empty).

Chapter 6: Hypotheses

Little is currently known about defensin expression in the nasal cavity, which is anatomically and functional distinct from the oral cavity and lungs. It is the aim of this work to characterize the defensin RNA profile in multiple locations within the oronasal cavity and olfactory bulb of the canine. Three main hypotheses were formulated:

1. Canine β -defensins are expressed throughout the nasal cavity in tissue specific patterns.

Rationale: Since β -defensins show tissue specific expression patterns in the epidermis, oral mucosa and lungs, basal defensins expression in the nasal epithelia must be established empirically. Using published gene sequences or previously reported primers, canine defensin RNA expression will be determined for several tissues in the oronasal cavity and olfactory bulb.

2. Canine β -defensin 103 (cBD103) is involved in protecting the olfactory epithelium, therefore will be co-expressed with olfactory marker protein (OMP).

Rationale: Human DEFB103 is potent broad spectrum antibacterial peptide and is important in both epidermal and oral health in humans. The canine paralog, cBD103 has shown to be active against a number of canine pathogen, therefore may be important in protecting the olfactory epithelia in dogs. Olfactory marker protein is exclusively expressed by olfactory receptor neurons, therefore will indicate which tissue samples contain olfactory epithelium.

3. cBD103 RNA expression will show interindividual variation in the canine population.

Rationale: Within the human population there is a range of inter-individual variation of DEFB103 RNA expression, with low expression and low induceability correlated to an increased risk of *S. aureus* colonization in the nares.

cBD103 expression may show similar variability within the canine population and serve as a potential marker for selecting dogs with robust innate immunities.

Chapter 7: Materials and Methods

Section 1: Canine and murine primers for reverse transcriptase polymerase chain reaction

Canine and rat β -defensin and reference gene primers whose sequences were identified in previous studies were used (Table 6) (Zhang et al., 2004; Patil et al., 2005; Sang et al., 2005; Etschmann et al., 2006; Van Damme et al., 2009; Leonard et al., 2012). Primers for cBD103, olfactory marker protein (OMP) and reference gene, GAPDH, identified by accession numbers, were designed using OligoPerfect primer design software (Invitrogen). Amplicon sequencing verified primer specificity.

Primers				
RT-PCR	Forward Primer	Reverse Primer	Amplicon Size	Source
cBD1	TGAGGCCTCTCTACTTGTCTGCTGC	TCCTGGCACAGATGTACTGGTCAGA	117	Leonard <i>et al.</i>
cBD102 (DEFB4A)	GGAGAAACAAGATATCACCCCTA	CAC TGCTCCCAATACAAAGAT	250	NM_001113715.1
cBD103	GTACCTAIGGCTGTGTGTC	AGTTTATAGAAAGAAAACCGAATT	373	NM_001129980
cBD103 12s/117a	TTACCTTCTCCTCCTGTGGCCCTTG	CCGACCGCTCCTTATTCTGCAAT	105	Leonard <i>et al.</i>
cBD108	TCGCCCTGCTCTCTTTCTGAG	CGTTCCAGGCAAAACTCCTG	104	Leonard <i>et al.</i>
cBD119	CCTCTTTTTGTCAATCCTTCTGGC	GGTAGGGCTGTTCAAGTCTTTCTGC	122	Leonard <i>et al.</i> ,
cBD120	CCTCTTTTTGTCAATCCTTCTGGC	AGCACCGTTTACGATTTTCGC	131	Leonard <i>et al.</i> ,
cBD122	CTTTTTGCTGACTTTGGCTGC	TTGGCTGGAACCTTGGGCTTC	174	Leonard <i>et al.</i> ,
cBD122-ISO1	CCTGAAGACATGAAGGCTTT	CCTGTCCCAAGTCTGATCTC	285	Patil <i>et al.</i> , (cBD1)
cBD122-ISO2	CCTGAAAGACATGAAGGCTTT	TTTAGTGGGCTATCTTCACCCAGAAGA	429	Patil <i>et al.</i> , (cBD2)
cBD122-ISO3	CCTGAAGACATGAAGGCTTT	GCCTACACCTA TATGGTCAACT	254	Patil <i>et al.</i> , (cBD3)
cBD123	ATGAAGCTCCTTTGGCTGACTG	TTTTCTCTCGGCTGGA ACTTGGGC	188	Leonard <i>et al.</i>
OMP	GACCTGACCAAGCAGATG	AGGTGATGAGGAAGT ACATGA	376	XM_844636
GAPDH	TGGCATCGTGAAAGGCCTCAT	GTGGGTGTCACTGTTGAAAGTC	368	XM_003434387
RPS5	GCTCTTTGGAAATGGAGCAC	GCATCATCATTGAGTTGGTCAGG	182	Leonard <i>et al.</i>
Defb14	TCATCTTGTCTTGGTGCCCTG	CTGCAGCATTTTGTACCTCTGT	160	Patil <i>et al.</i>
rGAPDH	AGGGCTGCCTTCTCTTGTGAC	TGGGTAGAAATCATACTGGAACATGTA	100	Zhang <i>et al.</i>
RT-qPCR	Forward Primer	Reverse Primer	Amplicon Size	
cBD103	GCCGCTGCTTACTTGTACCT	CCTCATGACCAACAGGCTTC	112	van Damme <i>et al.</i>
HPRT	TGCTCGAGATGTGATGAAGG	TCCCCTGTTGACTGGTCATT	191	Etschmann <i>et al.</i>

Table 6: RT-PCR and RT-qPCR primer sets for canine and murine defensins, olfactory marker protein (OMP) and reference genes (GAPDH, HPRT, and RPS5).

Section 2: Sample collection

All dogs were euthanized for reasons unrelated to this study and a full complement of samples from each dog included 10 tissues (Figure 3) nares, alar fold, rostral and caudal maxillary turbinates, ethmoid turbinate, olfactory bulb, vomeronasal organ, control tongue (filiform papillae) and taste buds (circumvallate papillae). Two beagles had lyme disease, and a third female beagle had renal failure. Despite the disease state of these three dogs none of the oronasal or brain tissue collected had any overt signs of infection or inflammation.

Tissues were stored in RNAlater (Invitrogen) at 4°C for short term and -20°C for long term. 100 milligrams (mg) tissue including the epithelium and underlying connective tissue was weighed out and total RNA was extracted in Trizol (Invitrogen). Nasal tissues from three normal and one diabetic Sprague-Dawley rat were collected and processed in the same manner.

Sample	Location	Epithelia / Tissue
A	nares	stratified squamous (SS)
B	alar fold	SS + respiratory
C	rostral concha	respiratory
D	caudal concha	respiratory
E	ethmoid labyrinth	olfactory
F	olfactory bulb	brain
VNO	vomeronasal organ	respiratory + chemosensory
CT	control tongue	SS
CP	circumvalate papillae	SS + sensory

Figure 3: Location of tissue samples taken from the canine oronasal cavity and brain and types of epithelia present.

Section 3: Semi-quantitative reverse transcriptase polymerase chain reaction

RT-PCR reactions for cBD102, cBD103, cBD122-ISO1, cBD122-ISO2, cBD122-ISO3, OMP and GAPDH (Table 6) were assembled on ice as follows: 2x Master Mix (AccessQuick, Promega), 200 nM forward primer, 200 nM reverse primer, 100 ng RNA, +/- 2.5 units AMV RT, water up to final volume of 25ul. Reaction conditions were as follows: reverse transcription carried out at 46°C for 45 minutes, followed by a denaturing step at 95°C for 2 minutes. Amplification was carried out during 22-37 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. Final extension was carried out at 70°C for 10 minutes, followed by a 4°C hold. Negative controls without reverse transcriptase were used with GAPDH primers to monitor DNA contamination. RT-PCR products were run on 2% agarose gels in TAE and images were captured using a digital camera. Optimal cycle number for cBD103, OMP and GAPDH (37, 28, and 22 cycles respectively) was empirically determined to be the point at which a range of band intensities was evident, e.g. tissues with high expression showed strong bands, tissues with lower expression had weak bands and other tissues had no bands.

RT-PCR reactions for cBD1, cBD103 (12s/117a), cBD108, cBD119, cBD120 and cBD123 or reference gene, RPS5 (Table 6) were assembled on ice as follows: 2x Master Mix (One-Step RT-PCR with SYBR green, Biorad), 300 nM forward primer, 300 nM reverse primer, 100 ng RNA, +/-0.5 ul iSCRIPT MMLV RT, water to final volume of 30 ul. Reaction conditions are as follows: reverse transcription was carried out at 50°C for 10 minutes, followed by a denaturing step at 95°C for 10 minutes. Amplification was carried out for 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. Final extension was carried out at 70°C for 10 minutes. An 80 cycle melt curve started at 55°C and increased by 0.5°C every cycle, the temperature was held for 10 seconds every cycle. A 80 cycle re-annealing curve started at 95°C and decreased by 0.5°C every cycle, temperature was held for 2 seconds. All samples were held at 4°C until removed from MyiQ (Biorad). cBD103 (12s/117a) samples were run for 30 cycles due to background issues. RTPCR products were run on 2% agarose gels in TAE and images were captured using a digital camera.

Section 4: Quantitative reverse transcriptase polymerase chain reaction for cBD103

RNA for RT-qPCR was extracted as above, then treated with DNase (DNAfree, Ambion) as per manufacturer's instructions before RNA was diluted to 25 ng/ul. RT-qPCR reactions for cBD103 and reference gene, HPRT (Table 6) were assembled on ice as follows: SYBR green mix, 300 nM forward primer, 300 nM reverse primer, 50 ng RNA, +/-0.5ul iSCRIPT RT, and water to final volume of 12.5ul. All reactions were run in duplicate. Cycling conditions are as follows: Reverse transcription carried out at 50°C for 10 minutes, followed by denaturation step at 95°C for 10 minutes. Amplification with 30 to 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds. Final extension at 70°C for 10 minutes. An 80 cycle melt curve started at 55°C and increased by 0.5°C every cycle, the temperature was held for 10 seconds every cycle.

Standards for the RT-qPCR were established by cloning the cBD103 and HPRT amplicons into pGEMT plasmids. Plasmid concentrations were quantified using a sensitive fluorescent dye (Quant-iT™ PicoGreen dsDNA Kit, Invitrogen). Using the plasmid size and insert size, the number of amplicon copies could be determined for each ug of plasmid isolated. cBD103 and HPRT containing plasmids were diluted to 10⁸ gene copies per ul. Serial dilutions of 10⁵ to 10² copies/reaction were used to create the standard curve and calculate sample copy numbers. Amplification efficiency was at least 95% for all PCR reactions.

Complete reverse transcription was assumed for all reactions. It must be noted that cBD103 negative controls with no reverse transcriptase had positive signals that arose at ~cycle 35 for 4 different primer sets (not listed). The melting profile is the same as the amplicon, so primer dimer is an unlikely explanation. All of these primer sets spanned the intron so genomic DNA contamination would have yielded a product that would have a considerably higher melting temperature and would be 1144 base pairs longer than the cBD103 cDNA sequence. Even when all new solutions, pipettors, tips, plates and a different room were used this false positive remained. Therefore, all data points used for the quantitative analysis of cBD103 RNA were those that cross the threshold several cycles before the negative controls.

Chapter 8: Results

Section 1: β -defensin expressed in canine oronasal cavity and brain

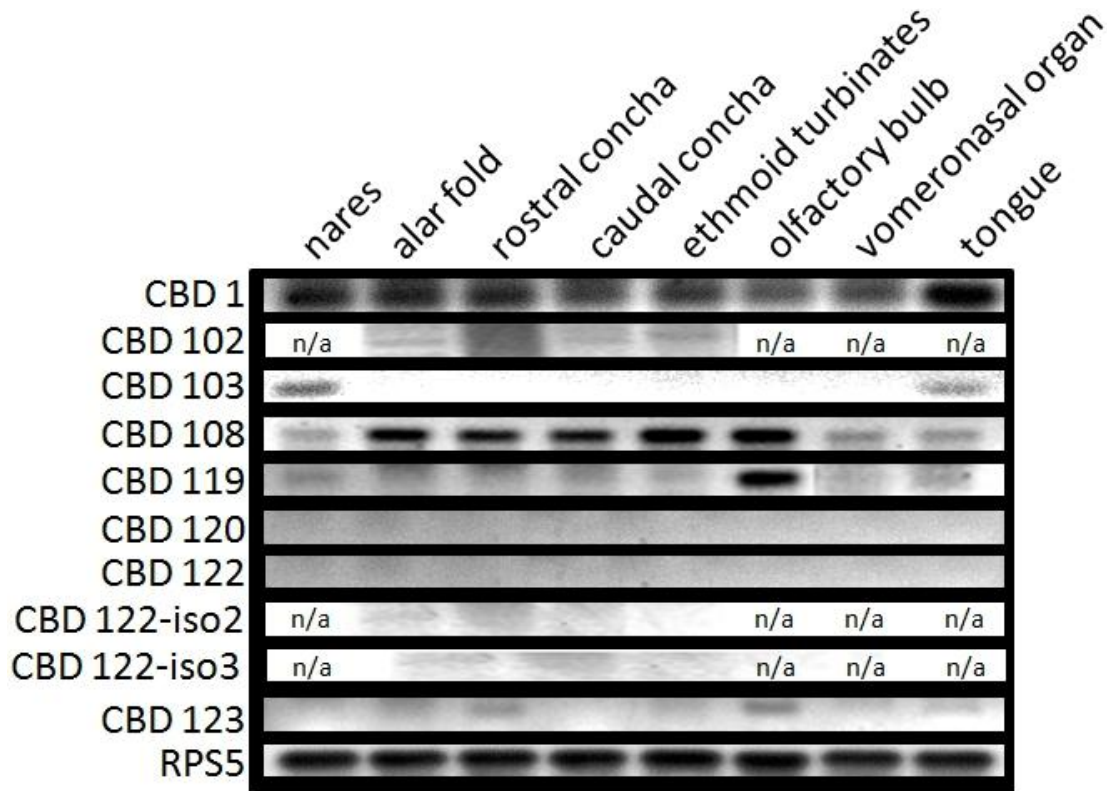


Figure 4: cBD expression in canine oronasal tissue and brain. cBD1 is ubiquitously expressed. cBD108 is highly expressed in all respiratory and olfactory epithelia and the olfactory bulb. cBD119 is highly expressed in the olfactory bulb. cBD123 is weakly expressed in respiratory epithelia and olfactory bulb. RPS5 is a reference gene. Samples not collected (n/a). RT-PCR products from various tissues from a single dog, run on a 2% agarose gel.

In the canine nasal cavity some defensins are expressed ubiquitously, while others are tissue specific and are segregated, in part, by the type of epithelial layer present (Figure 4). The nares and tongue express cBD1, cBD103, 108 and 119 RNA. The rostral and caudal concha, ethmoid turbinates and olfactory bulb express high levels of cBD1 and 108. The nares, tongue and VNO express lower levels of cBD1 and 108. cBD123 appears to be slightly expressed in the rostral concha and olfactory bulb. cBD1, 108, 119 are highly expressed in the olfactory bulb. cBD123 is slightly expressed in the

olfactory bulb. Whereas cBD102, 120, and the three isoforms of cBD122 were not detectable in any of the oronasal tissues sampled.

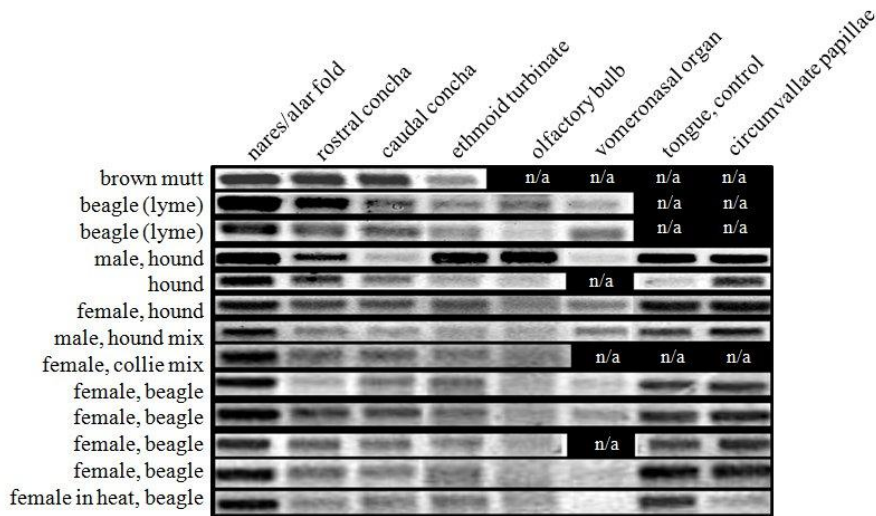


Figure 5: cBD103 RNA expression in oronasal and brain tissues of several dogs. cBD103 is highly expressed in the nares/alar fold and tongue of all dogs. Little to no expression of cBD103 is seen in most samples of olfactory epithelia, olfactory bulb or VNO. Different sexes and breeds are represented and labeled. Samples not collected (n/a). RT-PCR products run on 2% agarose gel.

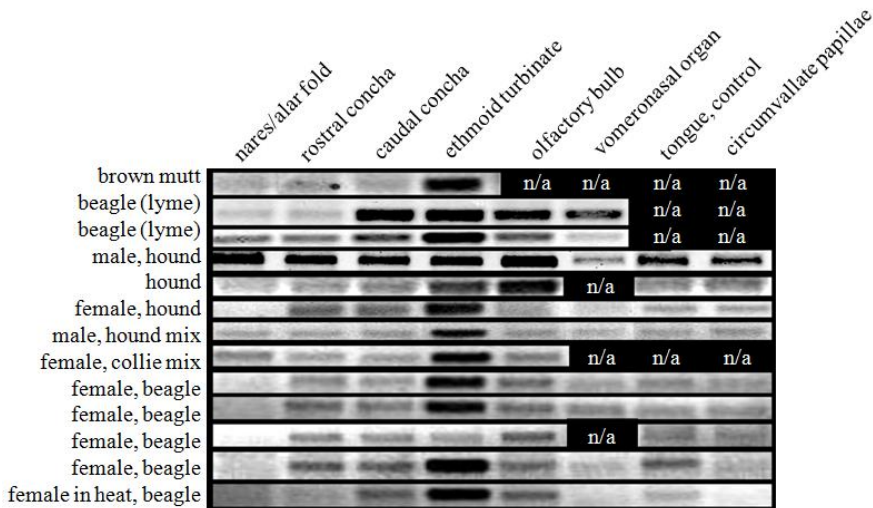


Figure 6: OMP RNA expression in oronasal and brain tissues of several dogs. OMP expression generally peaks in the ethmoid turbinates and olfactory bulb as expected. Different sexes and breeds are represented and labeled. Samples not collected (n/a). RT-PCR products run on 2% agarose gel.

cBD103 is highly expressed in the nares/alar fold samples but, in general, expression tapers off in the more caudal samples (Figure 5). In contrast, olfactory marker protein (OMP) expression is low in the nares/alar fold and peaks in the ethmoid turbinates indicating the presence of olfactory receptor neurons (Figure 6). Thus, cBD103 is not co-expressed with OMP.

Section 2: Variation of cBD103 RNA Expression

cBD103 was detectable in the nares and tongue samples, showing a 10 fold difference in expression between the two tissues (Figure 7).

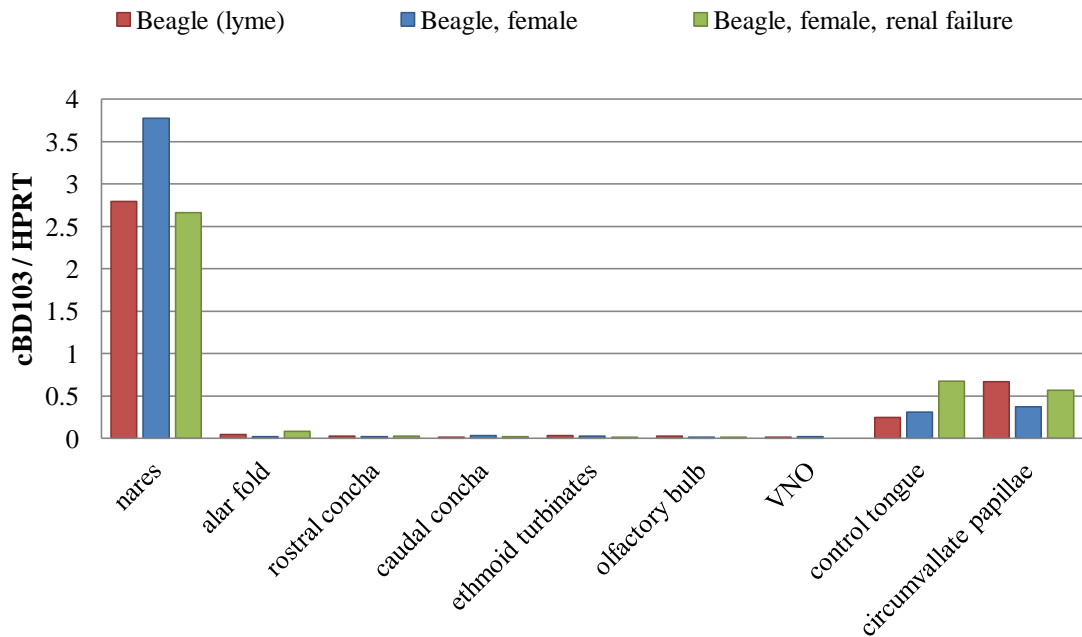


Figure 7: cBD103 RNA was found in the nares and tongue samples. There is a ~10 fold difference in cBD103 RNA levels between the two tissues. cBD103 expression in the oronasal cavity of dogs with lyme disease and renal failure did alter cBD103 expression outside the normal range of expression established in Figure 8. cBD103 RT-qPCR data normalized to reference gene HPRT.

In the nares, cBD103 shows a 90 fold level of interindividual variation (Figure 8). That dog 8 had normal HPRT RNA levels indicates the low cBD103 numbers are not due to degradation or loss of RNA during processing, but rather reflects the inter-individual variation demonstrated by other canine defensins (Van Damme et al., 2009). The average number of cBD103 RNA copies found in 50 ug of RNA was $\sim 1 \times 10^4$ copies. cBD103 expression was not notably different in the beagles with lyme disease or renal failure (Figure 7).

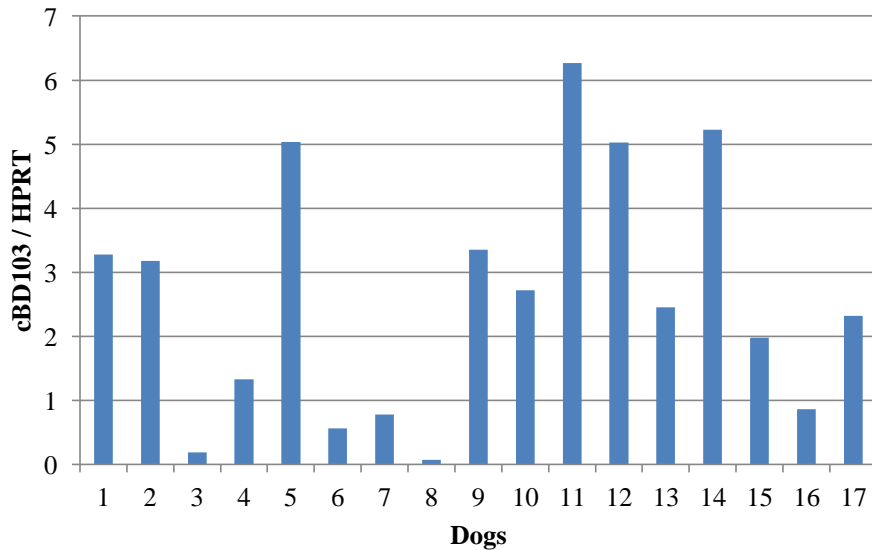


Figure 8: cBD103 RNA expression in the alar fold shows a ~ 90 fold range of variation between several dogs. cBD103 RT-qPCR data normalized to reference gene HPRT.

Section 3: Comparative expression of hBD3 paralogs in rat and dog

Human DEFB103 paralogs, rat Defb14 and canine cBD103, showed a conserved pattern of expression in the tissues sampled (Figure 9). Samples from three normal Sprague-Dawley rats showed Defb14 RNA transcription in the nares/alar fold and tongue samples, but none in the VNO, OB, respiratory or olfactory epithelium. DefB14 expression was not noticeably altered in the diabetic Sprague-Dawley rat.

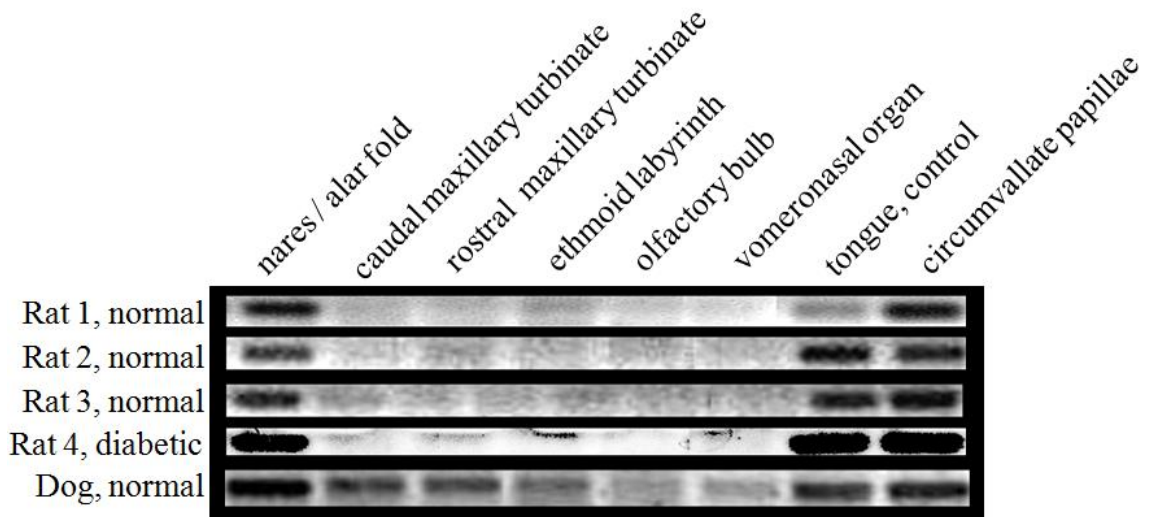


Figure 9: Comparative expression of DEFB103 paralogs in murine and canine oronasal tissues and olfactory bulb. The pattern of Defb14 expression is not altered in normal and diabetic Sprague-Dawley rats in the tissues tested. Representative cBD103 RNA expression pattern included comparison. RT-PCR products run on 2% agarose gel.

Chapter 9: Discussion

Section 1: Defensins in the canine oronasal cavity

β -defensin expression in the canine oronasal cavity and olfactory bulb is summarized on Table 7.

	nares	alar fold	rostral concha	caudal concha	ethmoid turbinates	olfactory bulb	VNO	tongue, CP control	CP
cBD1	++	++	++	++	++	++	++	++	++
cBD103	++	+	-	-	-	-	-	++	++
cBD108	+	++	++	++	++	++	+	+	+
cBD119	+/-	-	-	-	-	+	-	-	-
cBD120	-	-	-	-	-	-	-	-	-
cBD122	-	-	-	-	-	-	-	-	-
cBD123	-	-	+/-	-	-	+/-	-	-	-

Table 7: Summary of cBD expression in the canine oronasal cavity and olfactory bulb. High expression (++), low expression (+), low or no expression (+/-) or (). Vomeronasal organ (VNO), circumvallate papillae (CP).

cBD1 shares the highest sequence homology and similar ubiquitous expression pattern with DEFB1 (Valore et al., 1998; Zucht et al., 1998; Pazgier et al., 2006; Prado-Montes de Oca, 2010). The presence of cBD1 throughout oronasal cavity indicates potential importance in the innate immunity of the respiratory system. In humans, SNPs in hBD1 are associated with increased risk of respiratory issues such as asthma and chronic pulmonary obstruction disease (reviewed in Prado-Montes de Oca, 2010). If cBD1 polymorphisms are linked to similar predispositions in dogs, the canine could serve as a model for understanding the role of defensins in respiratory infection and inflammation.

cBD103 is not uniformly expressed in the respiratory system, showing strong expression in the nares and tongue, but little to no expression in the respiratory, olfactory epithelia, or the olfactory bulb. cBD103 transcription in the alar fold shows high levels of interindividual variation (Figure 8). Human DEFB103 is also sporadically expressed

in nasal tissue and nasal secretions (Nishimura et al., 2003; Harder et al., 2004; Laudien et al., 2011).

The expression pattern of cBD103 and homology with hBD3 indicates it might be important in regulating commensal and pathogenic bacteria in the nares (Laudien et al., 2011). Since cBD103 is not co-expressed with OMP, and expression is limited to rostral tissues, it is unlikely cBD103 provides protection specifically for olfactory epithelia. However, by eliminating potential pathogens in the rostral portion of the nose cBD103 may be conferring indirect protection to the olfactory epithelia. Other defensins, such as cBD1 and cBD108, may be directly involved in guarding the olfactory receptor neurons from infection.

The ~10 fold difference in cBD103 transcript abundance between the nares and tongue is similar to human DEFB103 expression in these two tissues (Harder et al., 2001). However, Leonard *et al.* (2012) reported cBD103 expression was almost the same in both skin and tongue. This apparent disparity might be due to the location of the skin samples. Leonard *et al.* used abdominal skin, while nares tissue was used for this research. Immunohistochemistry of DEFB103 and cBD103 indicates the presence of hair follicles, as well as the number of squamous layers, would impact apparent defensin RNA levels in the skin from different locations.

Compared to healthy dogs, the two dogs infected with Lyme disease, *Borrelia burgdorferi*, did not have abnormal cBD103 RNA expression levels in the oronasal cavity and olfactory bulb. This could be due to the stage of the infection, which may have been early. Renal failure also did not radically alter cBD103 expression in the oronasal tissues or olfactory bulb of the single canine case investigated. Diabetes in a single Sprague-Dawley rat also did not apparently influence the DEFB103 paralog, Defb14, expression in the nasal cavity. Larger sample sizes would be necessary to verify these observations are generally applicable.

cBD108. Both human and canine β -defensin 108 are expressed throughout the respiratory tract and several other tissues (Uhlen M, 2010; Leonard et al., 2012). Comparison of cBD108 expression in the nasal cavity reveals that histologically similar

tissues, such as the respiratory and olfactory epithelia and the epithelia in the VNO, can have different patterns of defensin expression. LPS, viruses, cytokines and fungi can regulate the expression of β -defensin 108 in humans and canines (Premratanachai et al., 2004; Erles and Brownlie, 2010).

β -defensin 108 is one of the defensins that is present in the human and canine genome, but absent in the murine. If β -defensin 108 regulation and expression is conserved in human and canines, the dog may prove to be a valuable model for the studying the role of this defensin in chronic inflammatory and infectious processes. DEFB108 is also an unusual defensin since, at neutral pH, the overall net charge is only +1, which may influence the antibiotic activity and immunomodulatory functions. There are also three potentially functional copies of DEFB108 on human chromosomes 4, 8 and 11 and two pseudogenes (Semple et al., 2006). Further work is necessary to determine if cBD108 would be a good model for studying hBD-108.

cBD119 & cBD123. In humans, both DEFB119 and DEFB123 are not part of the prototypic defensin cluster on chromosome 8, but reside on chromosome 20q11.21 which also includes DEFB115 through DEFB124 (Pazgier et al., 2006). These defensins were at first categorized as “testis-specific,” however the canine paralogs are expressed outside of the male reproductive tract (Leonard et al., 2012). SNP, CNV, regulation and function of these defensins remain to be characterized.

Little is known about cBD119, other than it is expressed in the canine skin, lung and now, in the olfactory bulb. cBD123 RNA was expressed at very low levels in the rostral concha and olfactory bulb (Figure 4), but is also expressed in the skin, lungs, bone marrow and male reproductive tract (Leonard et al., 2012). Human DEFB123 is active against a range of gram positive and negative bacteria, supporting the general assumption that the defensin family as a whole can be regarded as antimicrobial peptides. DEFB123 also binds LPS and decreases pro-inflammatory cytokine production in monocytic cells, which is not common to all defensins (Motzkus et al., 2006). Further study will be necessary to determine if cBD123 shows conserved antimicrobial activity and LPS binding capacity.

cBD102, cBD120 and cBD122. Neither cBD102, cBD120, nor cBD122 were detectable in the canine nasal cavity. cBD102 is unique to the canine genome and thought to be a recent duplication of cBD103 (Patil et al., 2005). However, it is not expressed in the same pattern as cBD103 and, so far, has shown to be expressed only in canine testes. cBD120 RNA expression has been reported in the canine lung, bone marrow, testes and epididymis (Leonard et al., 2012). cBD122 is an unusual defensin, having an additional exon, compared to the two-exon structure of most β -defensins. cBD122 was originally reported as cBD1, cBD2 and cBD3, but later renamed cBD122 (Patil et al., 2005). It is thought all three RNAs are due to the alternative splicing during the transcription of a single gene.

Section 2: Defensins in the olfactory bulb

The expression of various defensins in the canine olfactory bulb indicates the dog might be a useful model for studying defensins in the context of the innate immunity of the central nervous system. cBD1, 108 and 119 are expressed in the anterior portion of the brain called the olfactory bulb, however their exact cellular source has yet to be described. All these defensins are expressed in tissues outside of the CNS (Leonard et al., 2012). Expression of cBD119 in the olfactory bulb, but not the olfactory mucosa (ethmoid turbinate sample) indicates that olfactory receptor neurons are not expressing cBD119. Future studies will be necessary to identify the cells which express cBD1, 108 and 119 in the olfactory bulb.

The local expression of defensins in the brain and the promiscuity of defensin-receptor interactions (e.g. multiple MCRs, CCRs, and $\alpha\beta3$) strongly suggests the possibility that defensins can modulate neural function, which would provide a nice symmetry to the recent discovery that several neuropeptides are structurally similar to defensins and also exert antimicrobial activity (Schluesener et al., 2012). The cross-over activities of neuropeptides and defensins may provide a clearer picture of how the innate immunity affects neural function in health and disease.

To date, few papers have been published that have investigated the expression of defensins in neuronal tissue, even though chronic inflammation and dysregulation of the

immune system is associated with traumatic brain injury, neurodegenerative disease such as Alzheimer's, Huntington's, obesity and normal changes in the aged brain (Godbout et al., 2005; Williams et al., 2012). As changes in diet, a sedentary lifestyle and increased life span has produced a corresponding increase in the number of people affected by obesity, Alzheimer's, and other types of dementia, there is both a scientific and societal benefit to increasing our knowledge of the potential regulators of inflammation and the innate immunity in the central nervous system.

Human astrocytes, microglia and meningeal fibroblasts constitutively express DEFB1, whereas hBD2 expression can be induced in astrocytes by LPS and cytokines (Hao et al., 2001; Su et al., 2010). Several murine defensins are expressed in the neonatal and adult brain, however the cellular source was not identified (Maxwell et al., 2003).

While, immunohistochemistry data on Human Protein Atlas (Uhlen M, 2010) is circumstantial, several defensins appear to be expressed by specific neurons and associated cells within the human CNS (Table 8), and provide a good starting point for future defensin research in the human brain.

It has also been recently reported that the activity of neutrophil elastase (NE), which processes pro- α -defensins to their mature form, effects the onset and propensity towards obesity, insulin resistance, and fatty liver disease. NE levels are elevated in obese individuals, whereas NE knock-out rats fed high fat diets are resistant to becoming obese, retain better insulin sensitivity and have less liver damage (Sun and Yang, 2004). As a mediator of inflammation and a substrate of NE, α -defensins could play a role in the downstream effects of increased NE activity. Future research may reveal both CNS expressed and systemic α - and β -defensins link inflammatory processes, energy homeostasis and several disease processes.

	Neurons in cerebral cortex	Endothelial cells in cerebral	Glia cells in cerebral cortex	Neurons in hippocampus	Glia cells in hippocampus	Neurons in lateral ventricle	Glia cells in lateral ventricle	Purkinje cells in cerebellum	Granular layer of cerebellum	Molecular layer of cerebellum
DEFB104		+								
DEFB107								+		
DEFB108		+							+	
DEFB113	++			+		++		++		
DEFB114	++			+				+		
DEFB118		++								
DEFB119	++					++		++		
DEFB121	++		+	+	+	++	+	++	++	
DEFB125	+									
DEFB129	+						+	+		
DEFB131	+	+	+	+		+		++	++	+
DEFB132			++		++		++		++	
DEFB133	++	+		++		++	+	++	++	+

Table 8: Immunohistochemistry for several defensin antibodies in various central nervous system cell populations, as reported by Human Protein Atlas (Uhlen M, 2010). Strong or moderate staining (+ +), weak staining (+), no staining ().

Chapter 10: Conclusions and Future Prospects

The mammalian defensins consist of several dozen small, cationic peptides, containing 6 conserved cysteine residues. Functionally defensins serve as endogenous antibiotics and chemokines for select adaptive immune cells. The alteration of normal defensin expression may contribute to tissue specific pathogenesis. Here we show several

defensins are expressed in the nasal oronasal cavity and olfactory bulb, and may play a critical role in these tissues.

cBD1 and cBD 108 RNAs are ubiquitously expressed in the canine oronasal cavity and olfactory bulb. cBD119 RNA is localized to the olfactory epithelia and olfactory bulb, while cBD123 RNA is expressed in respiratory epithelia and the olfactory bulb. cBD103 RNA was expressed in the nares, alar fold and tongue. Since interindividual variation is common for defensin expression, further study is necessary to determine if the expression pattern of cBD1, cBD103, cBD108, cBD119, and cBD123 nasal expression is consistent in the larger canine population and across multiple breeds.

cBD103 RNA is 10 fold more abundant in the nares than in the tongue, which is consistent with what has been reported in human literature for hBD3. cBD103 RNA is expressed in the alar fold of all dogs sampled and shows a 90 fold range of interindividual variation. cBD103 RNA does not co-localize with OMP, a marker for olfactory receptor neurons. This makes it unlikely cBD103 it is directly involved in protecting the olfactory epithelia. However, cBD103 in the nares and respiratory epithelia may confer indirect protection to the olfactory epithelia by killing and neutralizing potential pathogens in the rostral portion of the nasal cavity. Further study is necessary to determine if cBD103 and other defensins are upregulated in nasal mucosa upon pathogenic challenge.

The expression of cBD1, cBD108, cBD119 and cBD123 in the olfactory bulb indicates that defensins play a role in the innate immunity of the central nervous system. As promiscuous ligands, defensins are also ideally situated to modulate neuronal activity via the melanocortin receptors or other receptors.

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