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Transcription of canine toll-like receptor 2, β -defensin 1 and β -defensin 103 in infected atopic skin, non-infected atopic skin, healthy skin and the CPEK cell line

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ABSTRACT

Dogs and humans with atopic dermatitis (AD) have a high prevalence of recurrent staphylococcal pyoderma. β -Defensins (BDs) and toll-like receptors (TLRs) are important in innate immunity against bacterial skin infections, and decreased BD and TLR2 expression has been associated with human AD. However, findings from recent studies of BD expression in human and canine AD have been variable and contradictory. The aim of this study was to further our understanding of the role of antimicrobial proteins in canine AD by quantifying mRNA for canine (c) BD1, cBD103 and TLR2 in healthy skin ($n = 17$ dogs), matched samples of atopic skin with and without active infection by *Staphylococcus pseudintermedius* ($n = 13$ dogs), and the canine keratinocyte cell line CPEK cultured with 5 ng/ml tumour necrosis factor alpha (TNF α) and 10 μ g/ml lipopolysaccharide (LPS). mRNA for cBD1, CB103 and TLR2 were detected in all samples. TNF α significantly increased transcription of cBD1, cBD103 and TLR2 in the CPEK cells. mRNA for cBD103 was also significantly increased after stimulation with LPS. There were no significant differences in mRNA levels for cBD1, cBD103 or TLR2 in healthy, non-infected atopic or infected atopic skin. Canine AD did not appear to be associated with altered expression of cBD1, cBD103 and TLR2 in these dogs. Other studies have reported both increased and decreased expression of these antimicrobial peptides in canine AD and pyoderma, and therefore further investigation of the clinical significance of these mediators is required.

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1. Introduction

Human and canine atopic dermatitis (AD) patients frequently suffer from recurrent staphylococcal colonisation and infection (Bieber, 2010; Fazakerley et al., 2010). This susceptibility has been associated with changes in innate immunity, including altered expression

of β -defensins (BDs) and toll-like receptors (TLRs). It has been reported that BD levels in atopic skin are lower than in the skin of psoriasis patients (Nomura et al., 2003; Ong et al., 2002), and TLR2 and human (h) BD1 polymorphisms have been associated with severe phenotypes of AD (Ahmad-Nejad et al., 2004; Prado-Montes de Oca et al., 2007). However, more recent studies reported that hBD1, hBD2 and hBD3 were elevated in lesional atopic and psoriatic skin compared to non-lesional and healthy skin, and that their expression did not correlate with the severity of AD or *Staphylococcus aureus* colonisation (Gambichler et al., 2008; Harder et al., 2010).

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Findings from studies on BDs in canine AD have been variable and contradictory. Altered BD mRNA expression in atopic compared to healthy skin, including increased canine (c) BD1 and cBD2 expression but decreased cBD3 and cBD103 expression, has been reported (Santoro et al., 2011; van Damme et al., 2009). Another study, in contrast, found that cBD103 transcription was comparable in non-lesional atopic, lesional atopic and healthy skin (Leonard et al., 2012b). More recent findings, however, have been different again with increased cBD103 and decreased cBD1 transcription in atopic compared to healthy skin (Santoro et al., 2012). The role of TLRs in canine AD has not been studied, and it is therefore difficult to determine the role of altered BD and TLR expression in the pathogenesis of canine AD and staphylococcal skin infections.

The aim of our study was to further assess the role of BDs and TLRs in canine AD by quantifying mRNA for cBD1, cBD103 and TLR2 in lesional atopic skin infected with *Staphylococcus pseudintermedius*, non-lesional atopic skin without infection, healthy skin, and the canine keratinocyte cell line CPEK.

2. Materials and methods

2.1. Subjects

Samples were taken from 13 dogs with AD. These consisted of seven females (all neutered) and six males (one neutered) with a mean age of 2.4 years (range 1.5–6.8). The breeds included were West Highland white terrier (3), boxer (2), Labrador retriever (2), crossbred (3), and golden retriever, German shepherd dog and Shih Tzu (1 each). None of the subjects had received topical or systemic antimicrobial or anti-inflammatory treatment for at least three weeks prior to sampling. AD was diagnosed according to currently accepted criteria and by exclusion of other pruritic diseases (DeBoer and Hillier, 2001). Briefly, hair plucks, coat brushings, skin scrapes, *Sarcoptes* specific IgG serology, cytology and trial therapy were used to eliminate ectoparasites and *Malassezia*. All dogs underwent a trial with a novel home-cooked or commercial single protein diet, or a commercial hydrolysed hypoallergenic diet for at least six weeks to eliminate adverse food reactions. The healthy dogs consisted of 12 males (two neutered) and five females (two neutered) with a mean age of 4.4 years (range 2.0–10.8) presented for euthanasia. The breeds included were crossbred (5), Staffordshire bull terrier (5), German shepherd dog (3), and cairn terrier, pug, border collie and golden retriever (1 each). The dogs had no history of skin disease or other conditions likely to affect the skin or immune function. The study was approved by The University of Liverpool Ethics Committee. Informed consent was obtained from the owners.

2.2. Sample collection

Skin biopsies were collected from the ventral body. In the atopic dogs, 0.5 ml of 2% lidocaine was infiltrated into the sub-cutis underlying the biopsy site and samples were collected using a 6 mm biopsy punch. Skin biopsies from the healthy dogs were collected in a similar fashion within

30 min of euthanasia. One sample was used from each healthy dog. Two matched samples from each atopic dog were used: one from lesional skin with clinical signs and cytology consistent with staphylococcal infection, and another from a nearby area of non-lesional skin without bacterial colonisation. The presence or absence of *S. pseudintermedius* in the atopic and control dogs was confirmed using stained adhesive tape strips and cultures, as previously described (Fazakerley et al., 2009). Infected skin was characterised by the presence of neutrophils and both intracellular and extracellular cocci, whereas only sparse extracellular bacteria were seen on non-lesional and healthy skin. Cytology was performed before the skin biopsies, which were taken from skin immediately adjacent to the cytology and culture sites to avoid any artefacts induced by these procedures. Biopsies for the mRNA studies were immediately placed into RNAlater® (Qiagen Ltd., Crawley, UK) and stored at –20 °C. Biopsies for histopathology were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sagittal 4 µm sections were cut parallel to the hair follicles and stained with haematoxylin and eosin. Five images (400× magnification) of the superficial interfollicular dermis were captured for quantitative analysis using Object-Image (Norbert Vischer, The University of Amsterdam, The Netherlands; <http://simon.bio.uva.nl/Object-Image/object-image.html>; last accessed 03.09.2012). The image software was used to compute the proportion of each image area that consisted of cell nuclei compared to cytoplasm and extracellular matrix, which yields a reliable estimate of cell numbers (Nuttall et al., 2004).

2.3. CPEK cells

A canine progenitor epidermal keratinocyte cell line (CPEK; CELLnTEC Advanced cell Systems, Bern, Switzerland) was cultured in complete CnT-09 medium (CELLnTEC). Near confluent CPEK cells were harvested from 75 cm² flasks using trypsin/EDTA (TE; 0.25%/0.1%; Life Technologies Ltd., Paisley, UK). The suspensions were spun down (160 g for 5 min), re-suspended in complete CnT-09 and adjusted to 3×10^6 cells/ml. An equal volume of freezing media (CELLnTEC) was added and the suspensions were stored immediately at –80 °C in 1 ml aliquots. After 24 h the cryotubes were transferred to liquid nitrogen until required. The 1 ml cryotubes were defrosted at 37 °C and then diluted in 10 ml CnT-09 before centrifugation at $160 \times g$ for 5 min. The cell pellet was re-suspended in 10 ml CnT-09 and centrifuged a second time before being re-suspended in 5 ml CnT-09 and seeded into a 25 cm² flask. The flasks were incubated for at least 48 h and passaged once the cells reached confluence. Following optimisation studies (data not shown) passage 2–6 CPEK cells were seeded into 12 well plates at a density of 1×10^5 cells/well in 1 ml CnT-09 and incubated at 37 °C for 48 h until near confluence. 1 ml of 20 µg/ml LPS (Sigma, Poole, UK), 1 ml of 10 ng/ml human TNFα in CnT-09 or 1 ml of CnT-09 alone was added to appropriate wells and the plates incubated for a further 12 (TNFα) or 24 h (LPS) at 37 °C before harvesting (optimisation data not shown). All the wells were in triplicate.

2.4. mRNA extraction and cDNA synthesis

Total RNA extraction from the CPEK cultures and skin biopsies was carried out using Trizol[®] (Life Technologies). Briefly, the CPEK cultures were washed with sterile PBS and then agitated with 1 ml of Trizol[®] for 2 min at room temperature. The skin biopsies were removed from the RNAlater[®], snap frozen in liquid nitrogen, mechanically pulverised in a liquid nitrogen cooled chamber and then mixed with 1 ml Trizol[®]. The RNA was purified using a standard chloroform/isopropanol and RNasin[®]/DNase[®] protocol (Promega, Southampton, UK). The RNA was cleaned up using RNeasy[®] columns (Qiagen, Crawley, UK) according to the manufacturer's instructions. The quantity and quality of RNA was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA) according to the manufacturer's instructions. All the RNA was stored at -80°C until required. cDNA was prepared from total RNA with an integrity number ≥ 6 using oligo (dT) primers and SuperScript[™] II[®] reverse transcriptase according to the manufacturer's instructions (Life Technologies). All cDNA was kept at -20°C until required. Control non-reverse transcriptase samples were produced using 1 μl of molecular grade water instead of reverse transcriptase.

2.5. Primer design

Primers (Table 1) were derived from published sequences (NCBI: www.ncbi.nlm.nih.gov; canine genome: www.ensembl.org/index.html) using the Roche Universal Probe Library Assay Design Centre (www.roche-applied-science.com/servlet/). The specificity was checked by performing Basic Local Alignment Search Tool (BLAST) searches. Preliminary studies established that the most appropriate reference genes were RPL13A and CG14980 for the skin samples (Wood et al., 2008), and HIRAP and SDHA for the CPEK samples (Fazakerley, 2009).

2.6. Quantitative real time polymerase chain reaction (qPCR)

All qPCR assays were performed in triplicate on 384 well PCR plates using a TaqMan[™] ABI PRISM 7900 SDS

(Applied Biosystems, CA, USA). Each well contained 10 μl of reaction mix consisting of 5 μl Taqman[™] 2 $\times$ PCR master mix (Universal Mastermix, Applied Biosystems), 0.1 μl 20 μM forward primer, 0.1 μl 20 μM reverse primer, 0.1 μl 10 μM probe (Exiquon[®]; Roche Diagnostics Ltd.), 0.1 μl molecular grade water and 4.6 μl of template cDNA or water for the negative controls. Amplifications were performed using the manufacturer's recommended protocol of 50°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min.

The specificity of the PCR products was checked by DNA sequencing (Lark Inc., Essex, UK), alignment (JustBio Sequence Aligner; www.justbio.com/aligner/index.php) and BLAST searches. The primer efficiencies were determined by running standard dissociation curves using tenfold serial dilutions of cDNA (10^{-1} – 10^{-6}), with amplification efficiencies of 93 to 107% with an R^2 value >0.98 considered acceptable. The data was analysed using Sequence Detection System (SDS[®]) software (v 2.2.1; Applied Biosystems) with the detection threshold of each assay set manually at 0.02. mRNA expression was compared to the reference genes using the formula $2^{-(\Delta\text{CT}_{\text{test}} - \Delta\text{CT}_{\text{control}})}$, where ΔCT (cycle threshold) is the difference between the target and reference gene expression (Wood et al., 2008).

2.7. Statistical analysis

Transcription levels in the lesional atopic, non-lesional atopic and healthy skin samples were expressed as the mean $\pm$ SD for each group. The transcription data from the skin biopsies were not normally distributed (Kolmogorov–Smirnov tests; Graphpad Prism 5[®]; Graphpad Inc., San Diego, CA, USA) and therefore Kruskal–Wallis tests with Dunn's post-tests were used to compare mRNA levels in the three groups. Transcription levels in the CPEK cells were expressed as the mean $\pm$ SEM for the triplicate wells. The data from the stimulated CPEK cells were normalised to non-stimulated control cells and expressed as a fold change (non-stimulated cells = 1.0). The data were tested using unpaired *T*-tests. One-way ANOVA with Dunn's post-tests was used to compare the proportion of the histopathological images from the skin biopsies that consisted of cell nuclei. *P* values <0.05 were considered significant.

Table 1
Gene primer sequences.

Gene	Name	Primers
CBD1	Canine beta-defensin 1 (CBD1; ortholog of human BD1)	F: tgtcctcttcttcttcttcttcttctt R: ggggagaagttgacagtg
CBD103	Canine beta-defensin 103 (cBD103; ortholog of human BD3)	F: tgttcttgatgcctgttcca R: ggaagaacagcggcctatc
TLR2	Toll-like receptor 2 (TLR2)	F: gttcaggcccttcttcttcttctt R: aagaggtgtttcccaagtg
CG14980	Protein coding gene; unknown function	F: gcagggaaggattctccag R: ggggtccagtaagaaatcttccataa
RPL13A	Ribosomal protein L13a	F: ctgccccacaagaccaag R: gggatcccatcaaacact
HIRAP	HIRA interacting protein 5 isoform 2	F: aattcagaacatgctgcaatttta R: tgattcatcatcataacctgttc
SDHA	Succinate dehydrogenase subunit A	F: ggtggcacttctacgacacc R: atgtagtggatggcgtcctg

3. Results

3.1. Histopathology

Healthy skin was characterised by a thin epidermis with basket-weave stratum corneum, normal hair follicles and associated structures, and a loose array of collagen fibres with a sparse infiltrate of fibrocytes, mast cells and mononuclear cells in the dermis. There were few differences between non-lesional atopic and healthy skin. These included occasional mild hyperkeratosis and acanthosis, sparse lymphocyte exocytosis, and a mild superficial, perivascular mononuclear cell infiltrate.

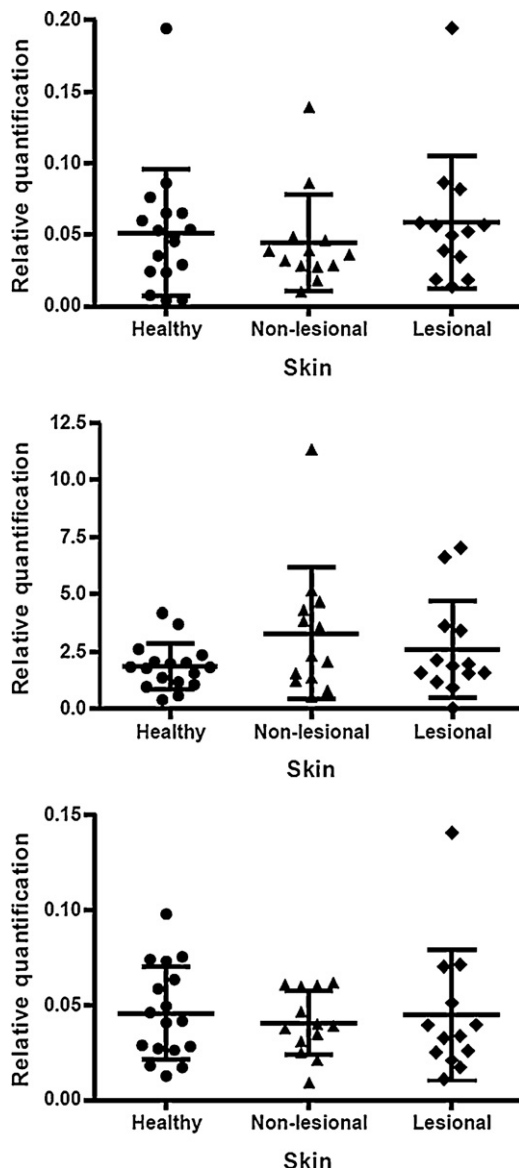


Fig. 1. mRNA levels of (a) cBD1, (b) cBD103 and (c) TLR2 in healthy skin ($n = 17$), lesional atopic skin infected by *S. pseudintermedius* ($n = 13$) and non-lesional atopic skin without infection ($n = 13$). The expression levels were normalised to the reference genes RPL13A and CG14980. The graphs show the individual data points. Bars represent the mean and standard deviation.

Lesional atopic skin was characterised by basket-weave to lamellar hyperkeratosis, acanthosis and spongiosis, lymphocyte exocytosis, and a superficial perivascular to interstitial infiltrate of mononuclear cells, mast cells, eosinophils and neutrophils, with occasional microabscesses. The mean area occupied by cell nuclei was significantly greater in the dermis of lesional atopic skin (0.69% [SD 0.18]) than in either non-lesional atopic (0.27% [SD 0.06]) or healthy skin (0.23% [SD 0.04]) ($p < 0.0001$).

3.2. cBD1, cBD103 and TLR2 mRNA expression in canine skin

mRNA for cBD1 (Fig. 1a), cBD103 (Fig. 1b) and TLR2 (Fig. 1c) was detected in all the samples of infected atopic, non-infected atopic and healthy skin. There were, however, no significant differences in the level of transcription of cBD1, cBD103 or TLR2 between infected atopic, non-infected atopic and healthy skin.

3.3. cBD1, cBD103 and TLR2 mRNA expression in CPEK cells

mRNA for cBD1, cBD103 and TLR2 was constitutively expressed in the CPEK cell line. The changes in mRNA levels following treatment with LPS and TNF α are illustrated in Fig. 2. Treatment with LPS resulted in a significant increase in cBD103 transcription ($p < 0.05$). Levels of mRNA for cBD1 and TLR2 also increased but the changes were not significant. Treatment with TNF α , however, significantly increased transcription of cBD1 ($p < 0.01$), cBD103 ($p < 0.05$) and TLR2 ($p < 0.01$).

4. Discussion

We did not find any differences in mRNA expression for cBD1, cBD103 and TLR2 in lesional atopic skin infected with *S. pseudintermedius*, non-lesional skin without infection and healthy skin. This corroborates a recent study showing similar expression of cBD103 in lesional atopic, non-lesional atopic and healthy canine skin (Leonard et al., 2012b). However, an earlier study reported a two-fold reduction in expression of cBD103 mRNA in lesional and non-lesional atopic compared to healthy skin, and it has been hypothesised that this predisposes atopic dogs to staphylococcal colonisation and infection (Leonard et al., 2012a; van Damme et al., 2009). In contrast, a 1.9-fold increase in cBD103 expression in atopic compared to healthy skin was recently described (Santoro et al., 2012). However, the physiological significance of such small changes is uncertain, and therefore these findings taken together could suggest that altered expression of cBD103 is not associated with canine AD.

Microbial colonisation could have affected the results. We specifically included lesional atopic skin from sites of infection with *S. pseudintermedius*, but in other studies of BD expression in canine AD the dogs were treated with systemic or topical antimicrobials prior to sampling and/or microbial colonisation and infection was not reported (Leonard et al., 2012b; Santoro et al., 2011; van Damme et al., 2009). However, the only other study to specifically include samples from infected atopic skin reported marked increases in cBD103 expression compared to non-infected

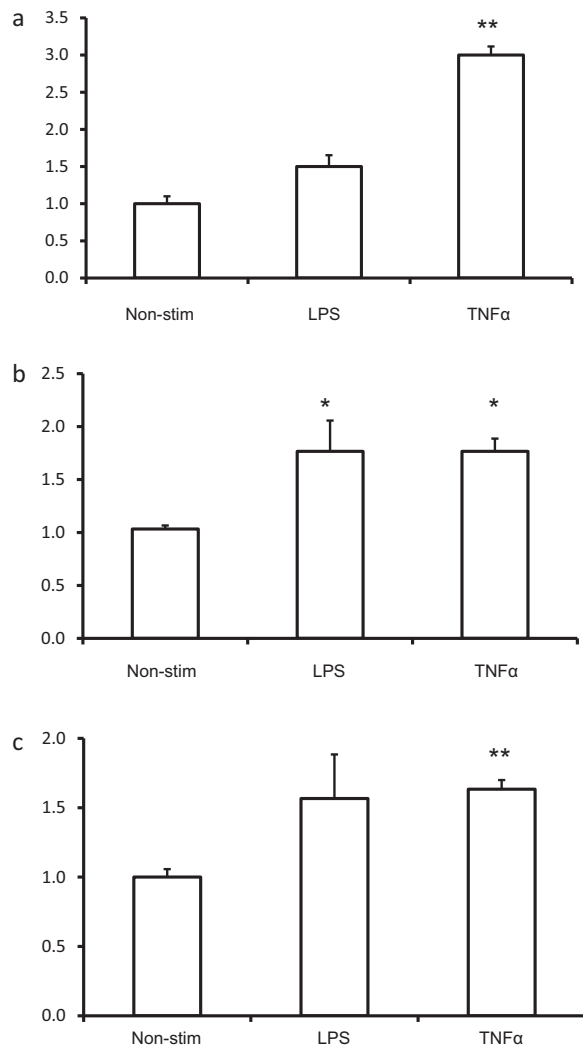


Fig. 2. mRNA levels for (a) cBD1, (b) cBD103 and (c) TLR2 in the CPEK cell line. mRNA expression levels were normalised to the reference genes HIRAP and SDHA. The data are expressed as the fold change in gene expression relative to the non-stimulated control cells, where expression in the control cells = 1. Non-stim – CPEK cells cultured in complete CnT-09 medium only. LPS – CPEK cells cultured in complete CnT-09 medium with 10 µg/ml LPS. TNFα – CPEK cells cultured in complete CnT-09 medium with 5 ng/ml TNFα. The columns represent the mean plus SEM of triplicate wells. * $p < 0.05$, ** $p < 0.01$.

atopic (3.8 times) and healthy (4.4 times) skin (Santoro et al., 2012). The reasons for this discrepancy are unknown. cBD103 is the canine homologue of hBD3, which is expressed in skin and human keratinocyte cell lines following stimulation with T-helper (TH) 1 cytokines, bacteria and bacterial products (Dinulos et al., 2003; Menzies and Kenoyer, 2005; Seo et al., 2001). Lesional infected skin in our study was clearly different from the non-lesional and healthy skin, with significant inflammatory changes and infiltration of mononuclear cells, neutrophils and eosinophils. Chronic lesional atopic skin in dogs is dominated by TH1 cytokines, including IFNγ and TNFα (Nuttall et al., 2002), which up-regulate hBD3

expression in human keratinocytes (Howell et al., 2006; Nomura et al., 2003). However, this can be suppressed by TH2 cytokines, particularly IL-4 and IL-13 (Howell et al., 2006; Kisich et al., 2008; Nomura et al., 2003), which dominate acute lesions in canine AD (Nuttall et al., 2002). The precise cytokine milieu may therefore be important in determining expression and function of cBD103, and studies using defined time points in experimental patch test models and *in vitro* cultures are required.

We did not find any differences in cBD1 expression between infected atopic skin, non-infected atopic skin and healthy skin. In a similar study in atopic dogs, expression of cBD1 was decreased (0.48×) in infected skin compared to non-infected skin, although there was no difference with healthy skin (Santoro et al., 2012). The significance of the small change in infected skin is unclear, and therefore these findings may corroborate our results. In an earlier study, however, there was a marked increase in cBD1 expression in lesional (12-fold) and non-lesional (5-fold) atopic skin compared to healthy skin in dogs (van Damme et al., 2009). A further study using an experimental beagle model also reported increased cBD1 transcription in atopic skin, which was enhanced following allergen challenge (Santoro et al., 2011). The reasons for the differing results are unclear, although the staphylococcal colonisation and infection status of the atopic skin in the latter two studies are unknown.

Expression of TLR2 in atopic canine skin has not been reported but findings in human AD have been contradictory. Macrophages and CD4+ T cells isolated from atopic humans show reduced TLR2 expression compared to those from healthy individuals (Niebuhr et al., 2009). In addition, expression could be up-regulated by TLR2-ligands in healthy but not atopic macrophages. In contrast, similar levels of TLR2 expression have been reported in healthy and atopic human keratinocytes, and in the skin of patients with atopic dermatitis and psoriasis (McGirt and Beck, 2006; Nomura et al., 2003). A further study found that TLR2 was up-regulated in atopic skin compared to healthy skin (de Jongh et al., 2005).

This is the first study of both BD and TLR2 expression in CPEK cells, which are used as *in vitro* keratinocyte models to study canine AD and innate immunity (Hendricks et al., 2012; Shibata et al., 2008). The cells expressed mRNA for cBD1, cBD103 and TLR2, which could be up-regulated following culture with the pro-inflammatory mediators LPS and TNFα. TNFα in particular is associated with chronic lesions in canine AD, where its expression correlates with mononuclear cell infiltration and clinical severity (Nuttall et al., 2004). LPS also induces BDs and TLR2 expression in human keratinocytes, although it is typically a Gram-negative associated TLR4 agonist (Holand et al., 2009; Seo et al., 2001). It would be interesting to study the effect of killed whole cell and staphylococcal protein extracts, but in our preliminary studies these resulted in poor CPEK cell viability. In another study, FSL-1, Pam3CSK4 and staphylococcal peptidoglycan did not up-regulate TLR2, TLR4, IL-8 and TNFα in CPEK cells (Hendricks et al., 2012). Our results suggest that CPEK cells may be a useful *in vitro* model to study innate immunity in canine skin. However, these are a derived cell

line and may not be representative of healthy or atopic keratinocytes.

Over 60 AMPs are now recognised in animals and humans. We selected cBD1 and cBD103 as these are consistently expressed in canine skin, are the orthologues of hBD1 and hBD3, and there was evidence of their involvement in AD (Leonard et al., 2012a). We could not reliably amplify mRNA for cBD122 and cBD102, which appear to be predominantly found in the lung and testis rather than skin (Leonard et al., 2012a; van Damme et al., 2009). At the time of the study we could not reliably amplify and/or specific sequences were not available for cBD2, cBD3 and cBD127. Further studies of a wider repertoire of AMPs in canine skin are therefore warranted.

It is difficult to draw firm conclusions from this and other studies. Some authors have concluded that canine AD is associated with decreased expression of certain BDs or that some patients are unable to up-regulate expression sufficient to prevent infections (Leonard et al., 2012a; van Damme et al., 2009). Others have concluded that expression is increased in inflamed and infected atopic skin (Santoro et al., 2011, 2012). It is likely that cBDs have a role in defence against micro-organisms, and this may affect the susceptibility of some dogs with AD to secondary infection. However, BDs have many other functions and interactions (including regulating the commensal microflora, cell signalling, chemotaxis, coat colour and reproduction), which could complicate the expression pattern in an individual (Leonard et al., 2012a). In addition, breed specific polymorphisms in the cBD103 gene have been described in golden and Labrador retrievers (Leonard et al., 2012b). These did not impair activity against meticillin-susceptible and meticillin-resistant *S. pseudintermedius*, but it is possible that these and as yet unknown BD variants could affect expression patterns and function in the skin. Further studies are therefore required to understand the role of innate immunity in canine AD.

Conflict of interest statement

Tim Nuttall and Neil McEwan have received lecture and consultancy fees, and research funding from Virbac Animal Health. These were unrelated to this study, and the authors do not have any conflicts of interest to report.

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