

# A canine keratinocyte cell line expresses antimicrobial peptide and cytokine genes upon stimulation with bacteria, microbial ligands and recombinant cytokines

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## ABSTRACT

Keratinocytes (KC) are the main cellular components of the stratum corneum that constitutes a solid physical skin barrier representing the first line of defense against pathogens. Moreover, KC are potent producers of inflammatory mediators and antimicrobial peptides (AMP) when activated through their pattern recognition receptors. In atopic dermatitis (AD) the protective skin barrier may be compromised due to barrier disruption, secondary infection and accelerated secretion of inflammatory cytokines which may also affect AMP expression in the skin. In the present study, we addressed the responses of a canine KC cell line upon exposure to *Staphylococcus pseudintermedius*, typically found on canine atopic skin during secondary infections, and stimulation by individual AD-associated ligands and cytokines. All stimuli induced a significant increase in expression of the pro-inflammatory cytokine genes tumor necrosis factor (TNF)-α and interleukin (IL)-8, but with different kinetics. Limited effects were observed on AMP gene expression except for K9CATH which was significantly upregulated upon bacterial infection but with none of the individual AD-associated ligands. Interestingly, K9CATH possessed antimicrobial activity towards *Staphylococcus pseudintermedius*, indicating that K9CATH expression is a specific defense reaction towards bacterial infection and not part of a general pro-inflammatory profile of KC.

## 1. Introduction

In the stratum corneum of the skin, the major constituents, keratinocytes (KC) embedded in their lipid matrix, establish a solid physical barrier representing the first line of defense against pathogens (Santoro et al., 2015b, Chermprapai et al., 2018). Moreover KC are potent producers of inflammatory mediators and antimicrobial peptides (AMP) when activated through their pattern recognition receptors (PRRs) as observed in both human (Bitschar et al., 2017) and canine (Leonard et al., 2012) studies, thereby contributing to local immune homeostasis. Canine skin homeostasis may be disturbed by changes in the

microbiome as well as the micro-environment that contribute to alterations of innate and adaptive immune responsiveness (Santoro et al., 2015b). One of the most common skin disorders in dog is atopic dermatitis (AD). Of the multitude of microbiota colonizing the canine skin, those most frequently involved in exacerbation of AD due to secondary infection are the Gram-positive *Staphylococcus pseudintermedius* and the yeast *Malassezia pachydermatis*; however, Gram-negative bacteria may contribute as well (Miller et al., 2013).

In acute disease AD is characterized by a Th2-type cytokine response, whereby IL-4 and IL-13 dominate the microenvironment of keratinocytes (Jassies-van der Lee et al., 2014). In contrast, in the

**Abbreviations:** AMP, antimicrobial peptide; KC, keratinocyte; PRR, pattern recognition receptor; AD, atopic dermatitis; cBD, canine β-defensins; K9CATH, canine cathelicidin; MSCEK, the canine keratinocyte cell line

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chronic phase of AD the Th1-type cytokine IFN- $\gamma$  is most prominent (Schlotter et al., 2011). In addition to release of typical Th1 and Th2-related cytokines, the expression of IL-17 is increased in acute lesions of human AD skin (Eyerich et al., 2009). Alterations in Th1, Th2 and Th17-type cytokine responses may have differential impact on both expression of AMP and pro-inflammatory cytokines which may render the skin more susceptible to infection (Eyerich et al., 2009; Gittler et al., 2012).

Two families of AMP, cathelicidins and  $\beta$ -defensins, are important in the first line of defense against pathogens (Gallo and Hooper, 2012). A broad antimicrobial spectrum (Gallo and Hooper, 2012), as well as immunomodulatory activity has been ascribed to these peptides (Jenssen et al., 2006), that are produced by both immune cells (often neutrophils) and epithelial tissues (Sang et al., 2007; Leonard et al., 2012). The  $\beta$ -defensins, cBD1 and cBD103, and the only known cathelicidin K9CATH are found in canine atopic skin and their expression is higher relative to that in healthy skin (Santoro et al., 2011, 2013), which is comparable to what is reported for their homologues in human AD (Harder et al., 2010). In contrast, gene expression levels of cBD102, cBD107 and cBD108 were found to be low in atopic dog skin (van Damme et al., 2009). In view of the putative clinical impact of bactericidal and immune-modulatory properties of AMP (Leonard et al., 2012), better understanding of AMP as well as cytokine expression by, and their impact on canine KC may contribute to the identification of therapeutic targets for modulation of defense mechanisms in the canine skin.

The aim of this study was to determine the effects of viable *S. pseudintermedius*, synthetic microbial ligands, and AD-related cytokines on expression of AMP and pro-inflammatory cytokine genes of a canine keratinocyte cell line, thus, mimicking interactions occurring in the skin of dogs with (allergic) dermatitis. In addition, the antimicrobial activity of K9CATH to viable *S. pseudintermedius* was assessed.

## 2. Materials and methods

### 2.1. MSCEK cell line

The canine keratinocyte cell line (MSCEK) was cordially provided by Koji Nishifuji, Tokyo University, Japan (Ide et al., 2010). The cell line is derived from a skin biopsy of a healthy dog and passaged over 90 times in culture. Cells were grown in William's E Medium (Life Technologies, Carlsbad, USA) supplemented with 10% of Nu-Serum IV Growth medium supplement (Corning, New York, USA); 5 ng/ml of Epidermal Growth Factor (EGF; Sigma, New Jersey, USA); 100 pM of Cholera Toxin (Sigma, New Jersey, USA); Penicillin-streptomycin (100 units/mL of penicillin and 100  $\mu$ g/ml of streptomycin; Life Technologies, Carlsbad, USA). MSCEK were seeded in 12 well-plates, (coated with type 1 collagen; Corning, New York, USA)  $1 \times 10^5$  cells/well in a volume of 1 ml, grown at 37 °C in a humidified atmosphere at 5% CO<sub>2</sub>. The culture medium was refreshed every 48 h until cells were confluent, prior to use.

### 2.2. *Staphylococcus pseudintermedius* preparation

An overnight culture of methicillin-susceptible *S. pseudintermedius*, a clinical isolate from dog skin with bacterial infection (provided by the Veterinary Microbiological Diagnostic Center, VMDC, Faculty of Veterinary Medicine, Utrecht University, The Netherlands), was prepared by picking a colony from the selective agar and dispersing it into Miller Hilton broth (MHB) for selection of a mid-logarithmic phase bacteria. OD measurement at 620 nm was performed to determine the bacterial density using the formula: OD value  $0.1 \approx 1 \times 10^8$  CFU/ml and subsequently diluted to the desired density for each experiment.

### 2.3. Co-culture of *S. Pseudintermedius* and MSCEK

Freshly-prepared  $10^4$ ,  $10^6$  and  $10^8$  CFU of *S. pseudintermedius* were added to a > 90% confluent MSCEK cell monolayer, in 12 well-plates. To optimize the incubation time, bacteria and cells were co-cultured for 1 and 4 h at 37 °C After incubation wells were washed with PBS to remove bacteria not involved with keratinocytes. To assess the total number of MSCEK associated bacteria (adhesion + invasion), cells were lysed with 1% Triton-X100 in PBS for 5 min to release all associated bacteria. The resultant bacterial suspension was diluted 10–10000 fold and plated out on Tryptone Soy Agar (TSA; Oxoid LTD, Hampshire, UK).

To assess invasion, in separate wells, after co-culture and washing as described above, cells were incubated with gentamicin (25  $\mu$ g/ml) for 1 h at 37 °C to kill extracellular bacteria. Cells were washed with PBS, 1% Triton-x100 in PBS was added, and lysate was plated to determine the number of invaded bacteria.

### 2.4. Stimulation of MSCEK cells with *S. Pseudintermedius*

Confluent MSCEK cultures were incubated with  $10^8$  CFU/ml *S. pseudintermedius* for 4 h at 37 °C or culture medium alone as negative control. After killing extracellular bacteria with gentamicin (25  $\mu$ g/ml) and washing, cells were lysed at time points 0, 2, 4, 6, 24 and 48 h for qPCR analysis of AMP and pro-inflammatory cytokine gene activity. All stimulation conditions were repeated in three independent experiments.

### 2.5. Stimulation of MSCEK cells with selected synthetic microbial ligands and recombinant cytokines

MSECKS were stimulated with the synthetic microbial ligands Pam3CSK4 (10  $\mu$ g/ml), LTA (10  $\mu$ g/ml), TBD (20  $\mu$ g/ml) (Invivogen, Carlsbad, CA) and LPS (50  $\mu$ g/ml) (Sigma, New Jersey, USA). Alternatively MSECKS were stimulated with the recombinant canine cytokines TNF- $\alpha$ , IFN- $\gamma$  and IL-17 (20 ng/ml) and IL-4 and IL-13 at 50 ng/ml (R&D systems, Minnesota, USA). Cell cultures without stimulants (medium only) were used as a negative control. After 7, 24 and 48 h incubation cells were harvested for qPCR analysis of AMP and pro-inflammatory cytokine gene expression.

### 2.6. Expression of AMP and pro-inflammatory cytokine genes

RNA was isolated using the FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit (FAVORGEN-Europe, Vienna, Austria) according to the manufacturer's recommendation. Five hundred nanogram of RNA per sample was used for cDNA synthesis by the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories Inc., Hercules, USA) according to the manufacturer's instruction. RT-qPCR was performed with 1  $\mu$ l cDNA (diluted 1:10) as a template using IQ™ SYBR® Green (Bio-Rad Laboratories Inc. licensed by Molecular Probes, Eugene, USA) in a MyiQ™ single-color real-time PCR detection system (Bio-Rad laboratories, Richmond, USA). Total reaction volume was 25  $\mu$ l with primer concentration of 300 nM for the AMP (cBD1, cBD103 and K9CATH) and 400 nM for the reference gene RPS19, and the pro-inflammatory cytokine genes IL-8 and TNF- $\alpha$ . The primer pairs were used as either previously described or custom designed by the program of NCBI using Primer3 and BLAST (Table S1). The PCR conditions consisted of 5 min of initial denaturation at 95 °C, followed by 40 cycles of 20 s at 95 °C, 30 s of T<sub>m</sub> at the specific temperatures for each primer pair and 30 s elongation at 72 °C (in case the T<sub>m</sub>  $\geq$  58 °C, the elongation step was skipped) (Brinkhof et al., 2006). Relative mRNA expression levels were determined by the MyiQ software and calculated by the Pfaffl method normalized to the reference gene RPS19 (Schlotter et al., 2009).

## 2.7. Antimicrobial activity of K9CATH

The K9CATH was synthesized using Fmoc solid-phase synthesis as described previously (Bikker et al., 2006). The peptide was purified to > 95% by reverse phase high-performance liquid chromatography prior to biological testing. Peptide was dissolved in water.

Different concentrations of K9CATH (0–40  $\mu\text{M}$ ) were added to  $2 \times 10^6$  CFU/ml of *S. pseudintermedius* in a polypropylene 96-well plate and incubated for 3 h at 37 °C in 50% MHB medium. Subsequently, bacteria were diluted 10–1000 fold and 100  $\mu\text{l}$  from each dilution was plated out on TSA plates. After 24-hour incubation at 37 °C, colonies on the plates were counted.

## 2.8. Statistical analyses

To compare the differences in relative expression of mRNA cBD1, cBD103, K9CATH, TNF- $\alpha$  and IL-8 by the MSCEK cells stimulated by *S. pseudintermedius*  $10^8$  CFU/ml at indicated time-points to the baseline (0 h) (Fig. 2), a general linear model was used and corrected by Dunnett's Post Hoc test at the significance level  $P < 0.05$ . Non-parametric Kruskal-Wallis corrected by Dunn's multiple comparisons testing was performed for the comparison between any contrasts of multiple groups (Figs. 3 and 4). Statistical analysis was performed by GraphPad Prism 8.0 and SPSS.

## 3. Results and discussion

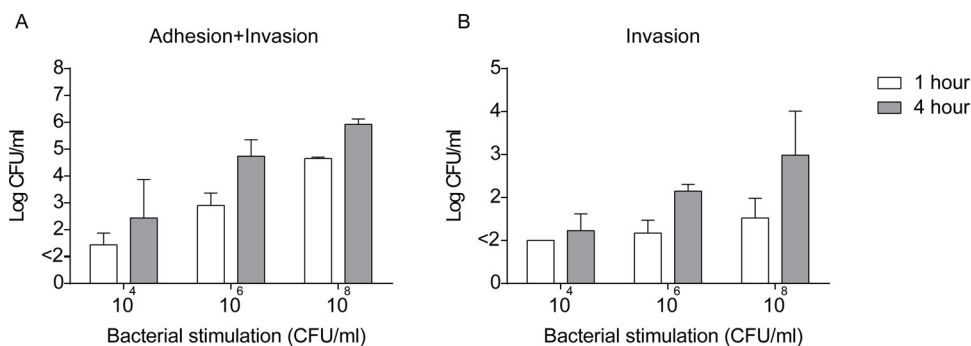
As a first step towards understanding the response of KC towards *S. pseudintermedius*, regularly involved in secondary infection in AD, interaction of these bacteria with MSCEK cells was investigated. Adhesion and invasion of *S. pseudintermedius* were assessed at three different bacterial densities during 1 or 4 h of incubation. Both adhesion and invasion of bacteria were time- and dose-dependent as shown by increasing bacterial counts (Fig. 1A and B). Nonetheless, the number of invaded (intracellular) bacteria after incubation of MSCEK with *S. pseudintermedius* for 1 h was low, i.e. under or just above the detection limit of 2 Log CFU, at all bacterial densities. Longer incubation of MSCEK cells with *S. pseudintermedius* enhanced the bacterial invasion at  $10^8$  CFU/ml, which was still less than 1% of the total number of associated bacteria (adhesion + invasion), indicating that MSCEK cells are relatively well equipped to prevent invasion.

Next, the response of MSCEK cells upon interaction with *S. pseudintermedius* was studied. Stimulation with  $10^8$  CFU/ml for 4 h resulted in significantly increased expression of the gene of the pro-inflammatory cytokine IL-8 at 4 and 6 h after exposure (Fig. 2A). Gene expression of TNF- $\alpha$  was not affected at these early time points (Fig. 2B). The pro-inflammatory cytokine (TNF- $\alpha$  and IL-8) responses to *S. pseudintermedius* occurred in a time-dependent fashion (Fig. 2A and B) in line with previous studies using human KC in co-culture with live or killed *S. aureus* (Sasaki et al., 2003; Wiegand et al., 2009; Secor et al., 2011). In AD, increased expression of TNF- $\alpha$  and IL-8 in the skin may induce maturation of antigen presenting cells and neutrophil migration

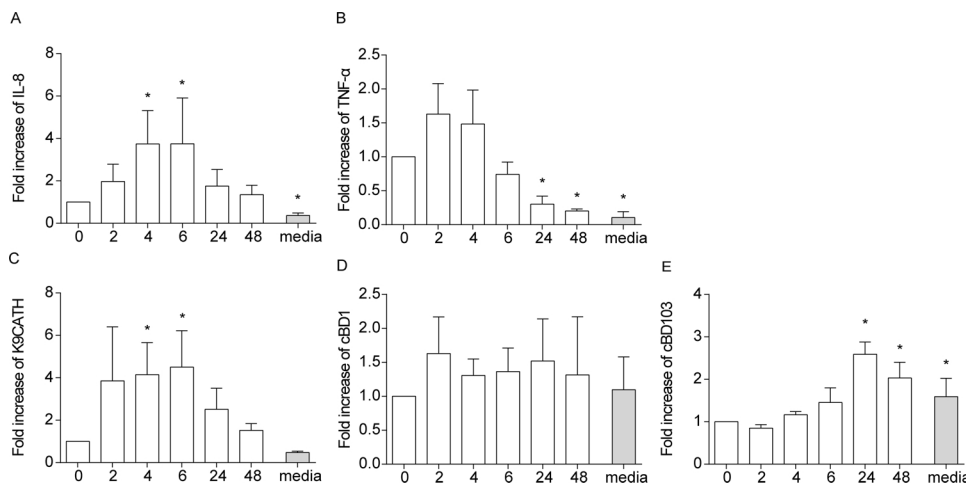
to sites of infection *in vivo* (Koga et al., 2008). Interestingly expression of the K9CATH gene followed the same pattern of induction, peaking at 4 and 6 h after infection, with higher than 4 fold increase (Fig. 2C) (of note: some samples of 0 h were below the detection limit, indicating that the shown fold increase is an underestimation). On the other hand the defensins cBD1 and cBD103 did not increase significantly (Fig. 2D and E), showing that expression of AMP is differentially regulated for each (family of) AMP, as has been noted before, in humans (Midorikawa et al., 2003) and dogs (Santoro and Maddox, 2014). At later time-points cBD103 was significantly expressed as compared to the expression at 0 h, but this was partially an effect of culturing as cells incubated with control medium also showed increased expression. Differences in constitutive *versus* induced expression level were observed between AMP (Leonard et al., 2012). For example, hBD-1 is constitutively expressed in epithelial cells but not upregulated during inflammation due to lacking of NF- $\kappa\text{B}$  transcription factor binding sites in the promotor region. Whereas, the promotor region of hBD-2 and hBD-3 (the orthologue of cBD103) consist of functional NF- $\kappa\text{B}$  binding sites exhibiting inducible expression in human skin upon inflammation or infection (Leonard et al., 2012). The constitutive expression may be important for an innate defense mechanism and the induced expression may help limit the severity of clinical symptoms. Although the observed effects on gene expression of AMP and cytokines in this study are relatively mild, exposure to *S. pseudintermedius* caused a clear pro-inflammatory state of MSCEKs.

Since bacteria consist of a complex mixture of potential stimulatory compounds, the effects observed with viable bacteria were related to MSCEK stimulation with single ligands, hence *via* individual PRP in order to obtain an insight in the complexity of bacterial immune stimulation. Incubation of MSCEK cells with LPS, Pam3CSK4 and TBD (ligands for TLR4, TLR2/1 and MINCLE, respectively) had significant effects on pro-inflammatory cytokines, especially TNF- $\alpha$  showing more than a 20 fold increase at 7 h (Fig. 3A). IL-8 mRNA levels were upregulated up to 5 fold compared to the control, although mainly after long incubation times (48 h, Fig. 3B). On the contrary, LTA, a TLR2 agonist and major component of the Gram positive bacterial cell wall, had no effect on pro-inflammatory cytokine production (Fig. 3A and B). With respect to AMP, small increases in cBD1 expression were observed after long incubation times with LTA (Fig. 3C), but there was no effect on cBD103 (Fig. 3D) or K9CATH where gene expression often remained under the detection limit of the assay for all stimulations (data not shown). The observed induction of pro-inflammatory cytokines by *in vitro* stimulated KC is comparable to that described earlier in human (Sasaki et al., 2003; Niebuhr et al., 2011) and canine studies (Hendricks et al., 2012). Clearly, the kinetics and extents of induction using individual ligands were different, mostly less intense, from that after whole bacterial stimulation, indicating that a multi factor based stimulation happens in the latter situation that cannot be simply reproduced by single ligands.

Besides the stimulatory impact of bacterial ligands, the inflammatory state of the skin in AD itself is likely to have an effect on cytokine and AMP expression of keratinocytes. Therefore, we also



**Fig. 1.** Adhesion and invasion assay of *S. pseudintermedius* to the MSCEK cell line. Three different densities ( $10^4$ ,  $10^6$  and  $10^8$  CFU/ml) of freshly-prepared *S. pseudintermedius* were added to the MSCEK cells and incubated for 1 and 4 h at 37 °C. A) To assess all bacteria associated with MSCEK (adhesion + invasion). B) To assess bacterial invasion.



**Fig. 2.** MSCEK stimulated by live *S. pseudintermedius*. Relative mRNA expression of IL-8 (A), TNF- $\alpha$  (B), K9CATH (C), cBD1 (D) and cBD103 (E) by MSCEK cells unstimulated (media) or following 0, 2, 4, 6, 24 and 48 h after an initial stimulation of 4-hours with *S. pseudintermedius*,  $10^8$  CFU/ml, at indicated hours assessed by qRT-PCR normalized to the reference gene RPS19. Fold increase calculated by the relative gene expression at the indicated time points compared to the relative expression at 0 h. Data are expressed as mean  $\pm$  SEM fold increase of two to three independent experiments in triplicate. Significance level at \*  $P < 0.05$ .

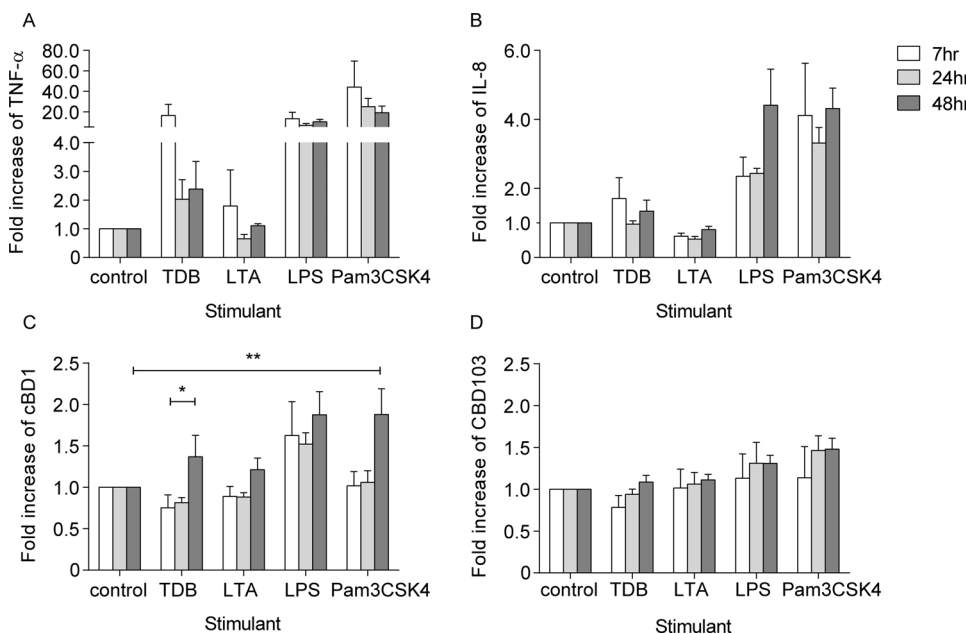
analyzed the effect of AD-related cytokines involved in this inflammatory state on cBD1, cBD103, TNF- $\alpha$  and IL-8 gene expression.

Incubation of MSCEK with recombinant cytokines did not lead to statistically significant difference for any of the tested AMP genes (Fig. 4A and B). Again gene expression of K9CATH was below the detection level for most samples (data not shown). Contrary to AMP gene expression after stimulation with AD related cytokines, TNF- $\alpha$  was able to induce a 15-fold higher expression of IL-8, as compared to the control at 48 h (Fig. 4C). The presence of IFN- $\gamma$  significantly decreased the expression of IL-8 at 24 h (Fig. 4C). The presence of Th-2 associated cytokines IL-4, IL-13, or pro-inflammatory cytokine IL-17 had no significant effect on either AMP or pro-inflammatory cytokine mRNA levels.

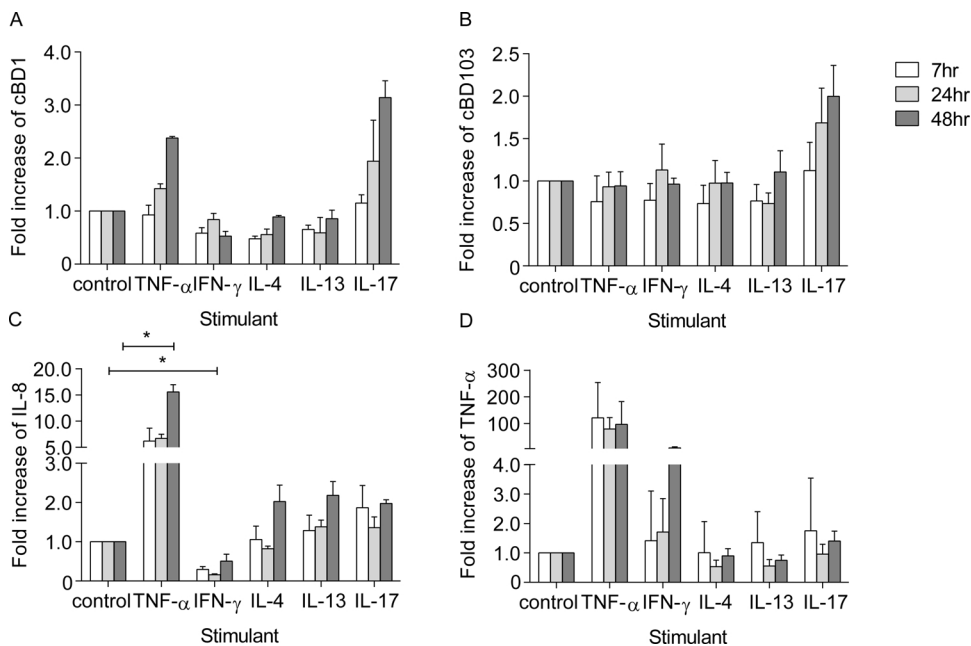
Despite the differences in kinetics and extent of induction, the main difference between stimulation with ligands/cytokines and viable *S. pseudintermedius* was the induction of the K9CATH that was significantly increased upon stimulation with the whole viable bacteria. This prompted us to also test the antimicrobial activity of this peptide against the bacterium. The minimum bactericidal concentration (MBC) of K9CATH was 30  $\mu$ M but significant killing activity was also observed in the 10–20  $\mu$ M range ( $> 2$ log reduction in number of viable bacteria, data not shown). In another study a minimal inhibitory concentration (MIC) of  $> 30$   $\mu$ M of K9CATH against *S. aureus* was reported (Sang

et al., 2007). Other AMP such as of human (LL-37), mouse (CRAMP) and pig (PR-39) (Gallo et al., 1997; Sang et al., 2007; Veldhuizen et al., 2014) have similarly high MBC or MIC, which could indicate that *Staphylococci* are relatively resistant to (some) AMP compared to other bacteria. These results contrast, lower MIC values previously determined for K9CATH against other Gram positive and Gram negative bacteria (Sang et al., 2007). Overall, it is an interesting observation that *S. pseudintermedius* induces an antimicrobial compound (K9CATH) that is not directly induced by individual PRR ligands or components of the pro-inflammatory immune response *in vitro*, thought to be important on and individual basis.

The alterations in the AMP and inflammatory cytokine expressions upon stimulation of the MSCEK cell with microbial components and analogs are comparable to those of the widely used canine keratinocyte progenitor cell line (CPEK) (Hendricks et al., 2012; Mullin et al., 2013), or canine primary KC from healthy donors (Santoro et al., 2015a). Moreover, the increased expression of pro-inflammatory cytokines and AMP by the MSCEK in co-culture with *S. pseudintermedius* are in agreement with the finding in human KC co-cultured with *S. aureus* (Sasaki et al., 2003; Menzies and Kenoyer, 2005; Wiegand et al., 2009; Secor et al., 2011; Wanke et al., 2011). Even though one should be careful extrapolating the research done in cell lines to the *in vivo* situation, these first results with the MSCEK cell line indicate that it can



**Fig. 3.** MSCEK stimulated by selected synthetic microbial ligands. Relative TNF- $\alpha$  (A), IL-8 (B), cBD1 (C) and cBD103 (D) mRNA expression by MSCEK cells stimulated for 7, 24 and 48 h with selected PRR ligands: synthetic TDB (20  $\mu$ g/ml), LTA (10  $\mu$ g/ml), LPS (50  $\mu$ g/ml) and Pam3CSK4 (10  $\mu$ g/ml) as assessed by qRT-PCR normalized to the reference gene RPS19. Fold increase was calculated by the relative gene expression of stimulated cells compared to non-stimulated cells (control) at the same time-points. Data are expressed as mean fold increase  $\pm$  SEM of at least three independent experiments. Significant differences between groups ( $P < 0.05$ ,  $P < 0.01$  denoted by \*, \*\*, respectively) were tested by non-parametric Kruskal-Wallis and Dunn's multiple comparison.



**Fig. 4.** MSCEK stimulated by selected recombinant (canine) cytokines. Relative cBD1 (A), cBD103 (B), IL-8 (C) and TNF- $\alpha$  (D) mRNA expression by MSCEK cells stimulated for 7, 24 and 48 h with selected recombinant canine cytokines: TNF- $\alpha$  (20 ng/ml), IFN- $\gamma$  (20 ng/ml), IL-4 (50 ng/ml), IL-13 (50 ng/ml) and IL-17 (20 ng/ml) as assessed by qRT-PCR normalized to the reference gene RPS19. Fold increase was calculated by the relative gene expression of stimulated cells compared to non-stimulated cells (control) at the same time-points. Data are expressed as mean fold increase  $\pm$  SEM of at least three independent experiments. Significant differences between groups ( $P < 0.05$  denoted by \*) were tested by non-parametric Kruskal-Wallis and Dunn's multiple comparison.

be an easy and useful new tool to study the KC role in immune reactivity in the canine skin. Since a good understanding of the complex immune response in atopic dermatitis is currently lacking, results of the present study potentially extended with those of similar studies with canine keratinocytes may unveil new therapeutic targets for AD and other (chronic) inflammatory disorders.

#### Declarations of interest

None.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.vetimm.2018.11.009>.

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