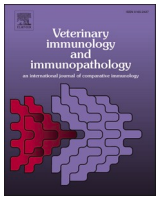
Veterinary Immunology and Immunopathology 238 (2021) 110291

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm



Research paper

The immune response in bovine primary dermal fibroblasts is influenced by Interleukin 8 promoter haplotype and vitamin D

Megan B. O'Brien [a,b], Charlotte L. Beynon [a], Rachel M. McLoughlin [b], Kieran G. Meade [a,c,*]

[a] Animal & Bioscience Research Department, Animal & Grassland Research and Innovation Centre, Teagasc, Grange, Co Meath, Ireland
[b] Host-Pathogen Interactions Group, School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin, Dublin 2, Ireland
[c] School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland

ARTICLE INFO

Keywords:
Interleukin-8
Vitamin D
Fibroblast
Haplotype
Holstein-Friesian

ABSTRACT

Interleukin-8 (IL-8) is a potent inflammatory chemokine, and two gene promoter haplotypes have been previously reported to segregate in cattle populations. Our earlier work showed how these divergent IL8 genotypes influence IL-8 expression and other immune response parameters at a systemic level. Here we extend that work to characterise the influence of haplotype on the local immune response – in primary bovine dermal fibroblasts. Furthermore, we also investigated how this response is modulated by 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). Significant induction of *IL8* expression was observed in cells from both haplotypes at 3 and 24 h post-stimulation with the TLR1/2 ligand, Pam3CSK4 and with the TLR4 ligand, LPS. *IL8* expression was elevated in response to both LPS and Pam3CSK4 in fibroblasts carrying the IL8-h1 haplotype and this result was supported by significantly enhanced IL-8 protein secretion. Gene expression profiles for other known fibroblast immune mediators (*SAA3* and *CCL20*) did not show significant differences between haplotypes but *NOS2* gene expression was significantly elevated in response to vitamin D, even above the level detected in response to both TLR ligands. In conclusion, this work has demonstrated that the IL-8 response of dermal fibroblasts is dependent on IL8 haplotype and that the immune response profile in these cells is significantly differentially regulated by 1,25 $(OH)_2D_3$. Fibroblasts have important immune response capacity and their function in driving inflammatory responses (including iNOS) is underappreciated. Understanding the relationship between cattle genotype and immune function is critically important for uncovering sustainable solutions for animal disease.

1. Introduction

Interleukin-8 is a critical and potent chemokine that regulates neutrophil influx to the site of infection. Initially produced by a number of cell types including epithelial cells, IL-8 signals through the ligation of two receptors - known as CXCR1 and CXCR2, expressed on polymorphonuclear cells including neutrophils (Bosch et al., 2002; Richardson et al., 2003). Different protein isoforms have been identified in humans which are responsible for alternative chemotactic recruitment of neutrophils and also neutrophil function (Galligan and Coomber, 2000; Maheshwari et al., 2009). As a result, IL-8 has a broad role in the activation and regulation of inflammation and therefore a critical influence on pathology and the outcome of infection.

At a genetic level, single nucleotide polymorphisms (SNPs) in *IL8* have been identified in humans, and associated with multiple inflammatory diseases including periodontitis, bronchiolitis and tuberculosis (Hacking et al., 2004; Ma et al., 2003; Pigossi et al., 2019). Genetic changes have been identified in both coding and non-coding regions of the human gene but relevantly to this study, SNPs in the promoter region of the *IL8* gene confer differential expression levels of *IL8* and thereby influence disease susceptibility (Hull et al., 2000; Ma et al., 2003). Inflammation is a core component of diseases in all species, but these relationships have not been as extensively characterised in non-model organisms.

In cattle, our early work identified a panel of SNPs in the promoter region of the bovine *IL8* gene, which are inherited as distinct haplotypes (Meade et al., 2012). These haplotypes (IL8-h1 and IL8-h2), are retained in most cattle breeds and our efforts have focused on understanding the implications of these haplotypes for the bovine immune response. Initial characterisation of these haplotypes was performed *in vitro* using serial deletion analysis of promoter constructs transfected into a mammary epithelial cell line. This work demonstrated that IL8-h2 was functionally

* Corresponding author at: School of Agriculture and Food Science, University College Dublin, Belfield, Dublin 4, Ireland.
E-mail address: kieran.meade@ucd.ie (K.G. Meade).

https://doi.org/10.1016/j.vetimm.2021.110291
Received 11 January 2021; Received in revised form 2 July 2021; Accepted 4 July 2021
Available online 8 July 2021

more responsive to LPS and TNFa stimulation (Meade et al., 2012). *In vivo* functional analysis of the role of the haplotypes on the immune response was conducted at a systemic level, where calves of each IL8 haplotype were stimulated with LPS *in vivo*. Clear dominance of IL8-h2 was detected in terms of driving the *IL8* response (Stojkovic et al., 2016). The practical relevance of this haplotype was illustrated by the detection of a genetic association between IL8-h2 and somatic cell count (SCC) - a marker of mastitis (Stojkovic et al., 2017). However, the effect of each haplotype on the endogenous expression levels of IL-8 has not been previously assessed but could have important implications for the activation and regulation of the local immune response.

In terms of local immunity, previous work developed a primary fibroblast cell model in cattle and this model showed that these cells were capable of producing IL-8 in response to the bacterial ligand, LPS (Green et al., 2011). Furthermore, the same group identified that fibroblast profiles were both predictive of and correlated with the response to experimental infection with a Gram-negative *E. coli* bacteria in the mammary gland (Korkmaz et al., 2018). We therefore selected this cell model to investigate the local immune response profile in calves homozygous for either the IL8-h1 or the IL8-h2.

Evidence for regulation of *IL8* expression by the hormone-like vitamin D is also evident in the literature but has not been investigated previously in cattle. The active form of vitamin D -1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) has been documented to reduce IL-8 production in bacterially–challenged monocyte derived macrophages in human studies (Dauletbaev et al., 2015). The mechanism of action by which $1,25(OH)_2D_3$ reduces IL-8 protein production is thought to involve the vitamin D receptor (VDR) interfering with pro-inflammatory transcription factors such as NF-κB or *via* its direct action as a transcription factor in order to regulate gene expression (Harant et al., 1997; Ryynanen and Carlberg, 2013). Similarly $1,25(OH)_2D_3$ decreases IL-8 protein levels in human fibroblasts in response to LPS and also Gram-negative bacteria (Rostkowska-Nadolska et al., 2010; Tang et al., 2013). In contrast, the active form of vitamin D has been shown to increase *IL8* expression in human monocyte cell lines in the absence of stimulation (Ryynanen and Carlberg, 2013). Collectively, these findings suggest a cell-type and stimulus-dependent mechanism of $1,25(OH)_2D_3$ effect on *IL8* expression, and this could have important relevance for disease-susceptibility in cattle.

2. Materials and methods

2.1. Blood sampling, DNA extraction and genotyping

All procedures described were conducted under ethical approval from the Teagasc Animal Ethics Committee and experimental license from the Irish Health Products Regulatory Authority in accordance with the Cruelty to Animals Act 1876 and the European Communities (Amendments of the Cruelty to Animals Act 1876) Regulations, 1994. Blood (8 mL) was collected *via* the jugular vein into sodium heparin vacutainers. DNA was extracted using the Maxwell® DNA extraction instrument according to manufacturer's instructions. The quantity and purity of extracted DNA was measured using the Nano-Drop ND-1000 (Thermo Scientific). A TaqMan assay was carried out on the Applied Biosystems 7500 Fast Real-Time PCR System according to manufacturer instructions (Applied Biosystems). Primers and probes were designed to amplify the region within which the tag IL8 promoter SNP is located: chr6:91739266-91739367. This variant is within the same haplotypic block as all other IL8 promoter SNPs as previously demonstrated (Meade et al., 2012), and it was used to determine the haplotype of each animal. TaqMan probe reporter sequences used were VIC–ATGGTTGCATATTGTG, FAM–ATGGTTGCGTATTGTG.

2.2. Animal selection, tissue collection and cell culture

Six one-year old Holstein-Friesian heifers from each haplotype group (IL8-h1 and IL8-h2) were selected for the study based on genotyping results. A small 5 mm punch was collected from the ear of each heifer using an ear punch and transferred to a 1.5 mL microfuge tube before being transported to the lab on ice. The tissue sample was first washed in HBSS containing 1% penicillin/streptomycin and 1% amphotericin B before being macerated using a scalpel. The macerated tissue was transferred to a 15 mL tube containing 10 mL RPMI with 1% collagenase I (Thermo Scientific). The tubes were put in a shaking incubator at 37 °C rotating at 150 rpm for four hours. After incubation, the cell suspension was passed through a 70 μM mesh filter into a 50 mL tube containing 15 mL HBSS with 10 % FBS and centrifuged at 200×*g* for 10 min. The cells were then resuspended in RPMI with 1% penicillin/streptomycin, 1% amphotericin B, 1% insulin-transferrin-selenium and 10 % FBS, counted using a haemocytometer, then transferred to T25 flasks and grown to confluency at 37 °C and 5% CO_2. For stimulation experiments, cells at passage 3/4 were seeded at a density of 1×10^5 cells/mL in six-well plates and allowed to adhere overnight. Cells were then stimulated with 1 μg/mL LPS (*E. coli,* O55:B5) (Sigma) or 1 μg/mL Pam3CSK4 (InvivoGen) with or without a two-hour pre-treatment of 10 nM $1,25(OH)_2D_3$ (Sigma) for 3 h or 24 h at 37 °C and 5% CO_2. After stimulation, cell supernatant was removed and frozen at −20 °C while cells were transferred to Trizol (Thermo Fisher Scientific).

2.3. Immunocytochemistry

Cells at passage 3/4 were seeded on four-well glass slides at a density of 1×10^5 cells/mL and grown overnight at 37 °C, 5% CO_2. Medium was removed and cells were fixed for 10 min in a 1:1 solution of acetone and methanol. The solution was then removed and a 3% solution of hydrogen peroxide in methanol was added for 20 min. to stop endogenous peroxide activity. A wash step was performed by adding 1% PBS-tween for 5 min. twice. The cells were then blocked for non-specific binding with 10 % goat serum in PBS for 1 h at room temperature. The primary antibodies were then added (mouse anti-human cytokeratin antibody, mouse anti-human vimentin antibody or IgG1 isotype control) at 1:100 dilution overnight at 4 °C. A wash step was performed before the secondary antibody (anti-mouse) was added for 30 min. at room temperature. Another wash step was performed before addition of the chromogen diaminobenzidine (DAB) for 1 min. Cells underwent a further wash step before addition of haematoxylin stain for 1 min. Cells were washed a final time, mounted with VectaMount™, cover slips applied and examined using an Olympus BX41 upright microscope.

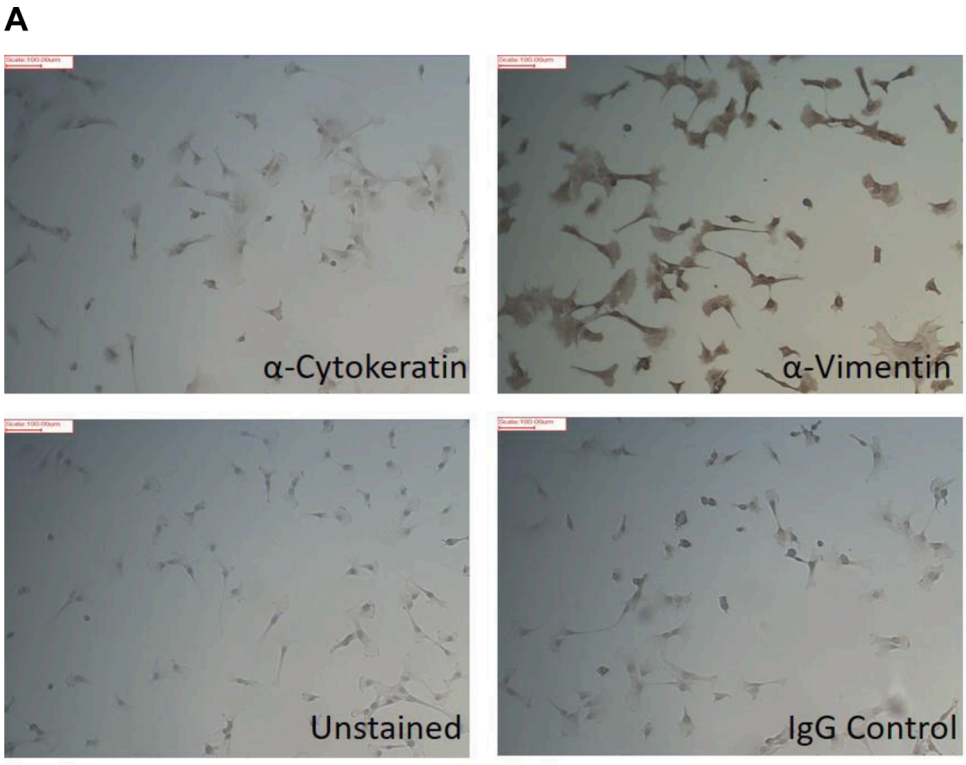
2.4. RNA extraction, cDNA synthesis and qPCR

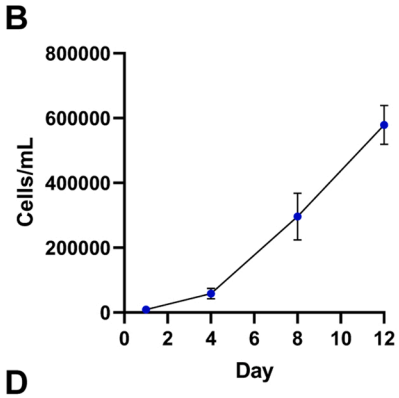
A combination method of Trizol and the RNeasy Plus Mini Kit (Qiagen) was used to extract RNA. Chloroform was added to the cell pellet in Trizol and shaken vigorously. The solution was then centrifuged for 15 min, 12,000×*g* at 4 °C. The RNA containing aqueous layer was transferred to a clean microfuge tube. An equal amount of 70 % ethanol was then added and mixed vigorously. This solution was then transferred to a RNeasy kit column. The manufacturer's instructions were then followed from this point. RNA quantity and purity were measured using the Nano-Drop ND-1000 and quality was assessed using the RNA 6000 Nano Kit on the 2100 Bioanalyser (Agilent Technologies) according to manufacturer's instructions. The RNA samples were diluted down to the sample with the lowest concentration. The RNA was then converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Scientific) according to manufacturers' instructions. cDNA samples were prepared on a MicroAmp™ Fast Optical 96-Well Reaction Plate (Applied Biosystems) with primers using Fast SYBR® Green Master Mix (Applied Biosystems) according to the manufacturer's instructions. The following primers were designed using NCBI Primer-BLAST:

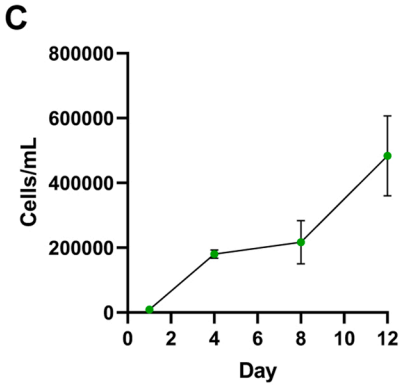
KRT18 forward–ATTTCAGTCTTGGCGACGCT, *reverse*-GCCTCAGTGCCTCAGAACTT; *VIM forward*-TGCGCTCAAAGGGACTAACG, *reverse*-TCGAGCGCCATCTTGACATT; *RPS15 forward*-GCGACATGATCATTCTA

CCCG, *reverse*-GGTAGTGGCCGATCATCTCA; *IL8 forward*- CATTCCACACCTTTCCACCC, *reverse*- CCTTCTGCACCCACTTTTCC; *SAA3 forward*-CTCAAGGAAGCTGGTCAAGG, *reverse*- CTTCGAATCCTCCCGTACCT; *NOS2 forward*- GATCCAGTGGTCGAACCTGC, *reverse*- CAGTGATGGCCGACCTGATG; *CCL20 forward*- CAGCAAGTCAGAAGCAAGCA, *reverse*-TTTGGATCTGCACACACAGC; *VDR forward*- GAGGGGAACCGTCCTTTGAG, *reverse*- GAGAAGCTGGTTGGCTCCAT.

No template and no reverse transcriptase controls were also included. The samples were run on a 7500 Fast Real-Time PCR System (Applied Biosystems). The following parameters were used: 95 °C for 20







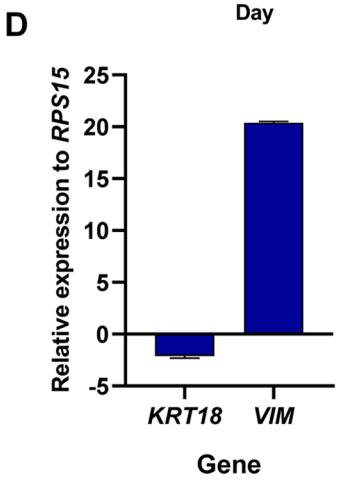


Fig. 1. Distinct characteristics of bovine dermal fibroblasts isolated from the ear. Cells were isolated from a 5 mm section of tissue from the ear by collagenase digestion. The isolated cells were then grown in 12 well plates before being passaged. **(A)** Immunohistochemical staining for cytoskeletal proteins α-cytokeratin and α-vimentin. Scale bar indicates 100 μM. **(B)** Growth curve indicates increase in cell number over twelve days of culture after first passage and **(C)** after cryopreservation. Error bars indicate SEM of n = 3. **(D)** Relative *KRT18* (keratin-18) and *VIM* (vimentin) gene expression to reference gene *RPS15*. Error bars indicate SEM of n = 12.

s, 40 cycles of 95 °C for 3 s and 60 °C for 30 s. From a panel of five reference genes, *RPS15* was selected to use for normalisation based on a low M-value calculated by the geNorm tool in the GenEx software v.5.2.7.44. Cq values were adjusted for primer efficiency and replicates averaged. The values were then normalised to the reference gene and gene expression was calculated relative to the unstimulated control sample for each animal (Schmittgen and Livak, 2008).

2.5. ELISA

The IL-8 ELISA used to measure concentration in cell supernatants was carried out as previously described (Cronin et al., 2015).

2.6. Statistical analysis

Statistical analysis was carried out using GraphPad Prism 8 software. Cell stimulation data was assessed for statistical significance using a two-way repeated measures ANOVA (or mixed models) and a P-value of <0.05 was considered as significant.

3. Results

3.1. Morphological and ontological characterisation of cells isolated from ear punch identifies dermal fibroblasts

Following cell isolation by collagenase digestion, immunohistochemistry confirmed the strong presence of α-vimentin and lack of α-cytokeratin indicating the cells to be fibroblasts (Fig. 1A). Investigation of cell growth revealed cells were actively growing after first passage, increasing cell number over the course of twelve days (Fig. 1B). This was also the case after cryopreservation but at a slightly reduced rate (Fig. 1C). After these investigations, cells were then used for PAMP stimulation experiments between passages three and four. Fibroblast cell type was further confirmed using qPCR analysis which showed increased expression of *VIM* over *KRT18* (Fig. 1D).

3.2. Induction of IL8, CCL20, SAA3, NOS2 and VDR in response to LPS

IL8 expression was significantly induced by LPS at both timepoints in both IL8-h1 and IL8-h2 (Fig. 2A&B). At 24 h, a significant difference in induction was apparent between haplotypes with elevated expression in cells from IL8-h1 (Fig. 2B). *CCL20* expression was significantly induced by LPS in IL8-h1 animals at both timepoints (Fig. 2C&D) with maximal expression at 3 h post-stimulation. A significant induction of *SAA3* was also detected in cells carrying both haplotypes in response to LPS at 3 h (Fig. 2E) but maximal expression was apparent at 24 h (Fig. 2F). Expression of *NOS2* was significantly induced by LPS in IL8-h1 and IL8-h2 animals at both timepoints (Fig. 2G&H). Although *VDR* expression is also induced in response to LPS, levels are comparatively lower and only reached statistical significance after 24 h (Fig. 2I&J). Generally, high inter-animal variation in gene expression is more prevalent in IL8-h1 compared to IL8-h2.

3.3. $1,25(OH)_2D_3$ modulates gene expression in response to LPS depending on IL8 haplotype

No major modulation of $1,25(OH)_2D_3$ on the LPS-mediated response of *IL8, CCL20* or *SAA3* expression was apparent after 3 or 24 h stimulation (Fig. 2A–F). However, induction of *NOS2* was significantly increased by the addition of $1,25(OH)_2D_3$ in both haplotypes at both timepoints (Fig. 2G&H). Although a trend of increased *VDR* expression was observed by the addition of $1,25(OH)_2D_3$ in combination with LPS, these differences did not reach statistical significance (Fig. 2I&J).

3.4. Induction of IL8, CCL20, SAA3, NOS2 and VDR in response to Pam3CSK4

Expression of *IL8* was significantly induced by Pam3CSK4 alone in both haplotypes at 3 and 24 h (Fig. 3A&B) with higher levels at the later time point. *CCL20* was also significantly induced by Pam3CSK4 alone at 3 and 24 h in both IL8-h1 and IL8-h2 animals (Fig. 3C&D). *SAA3* expression was induced by Pam3CSK4 but levels were low at 3 h (Fig. 3E). At 24 h, *SAA3* was significantly induced by both haplotypes (Fig. 3F). Again, *NOS2* expression was significantly induced by Pam3CSK4 at both timepoints in both IL8-h1 and IL8-h2 (Fig. 3G&H) with levels considerably higher at 24 h. *VDR* expression was only significantly induced by Pam3CSK4 in cells from IL8-h2 animals at 24 h (Fig. 3I&J).

3.5. $1,25(OH)_2D_3$ modulates gene expression in response to Pam3CSK4 depending on IL8 haplotype

$1,25(OH)_2D_3$ had no effect in modulating *IL8* expression in response to Pam3CSK4 at 3 h (Fig. 3A). However, after 24 h, there was a significant reduction in *IL8* expression for IL8-h1 but not IL8-h2 animals (Fig. 3B). There was no significant modulation of *CCL20* or *SAA3* expression in response to Pam3CSK4 by $1,25(OH)_2D_3$ (Fig. 3C–F). As detected in response to LPS, the addition of $1,25(OH)_2D_3$ significantly increased *NOS2* expression in response to Pam3CSK4 for IL8-h1 and IL8-h2 at both timepoints (Fig. 3G&H). Expression of *VDR* in response to Pam3CSK4 was only detected for IL8-h1 cells with the addition of $1,25(OH)_2D_3$ for both haplotypes at 3 h (Fig. 3I&J).

3.6. IL-8 protein production differs between haplotypes in response to LPS and Pam3CSK4

At 3 h post-stimulation, IL-8 protein induction in response to LPS was not significantly altered between haplotypes (Fig. 4A) but at 24 h a significant difference was detected (Fig. 4B). The induction of IL8 was significantly higher in IL8-h1 cells relative to IL8-h2. The addition of $1,25(OH)_2D_3$ did not significantly reduce IL-8 protein levels at either time point.

In response to Pam3CSK4 simulation, induction levels of IL-8 in both haplotypes were similar at the 3 h time point (Fig. 4C). At 24 h, stimulation with Pam3CSK4 significantly induced IL-8 in cells carrying both IL8-h1 and IL8-h2 (Fig. 4D). IL-8 protein expression levels were also significantly higher for IL8-h1 relative to IL8-h1 (Fig. 4D). The addition of $1,25(OH)_2D_3$ with Pam3CSK4 did significantly change IL-8 expression levels in cells of either haplotype at 3 or 24 h.

4. Discussion

Multiple previous studies have examined systemic bovine immune responses in whole blood or in peripheral leukocytes and mononuclear cells isolated from blood (Bai et al., 2019). However, such analyses preclude the detection of responses from resident cells in the local microenvironment, where infection is usually initiated. Early local innate responses are critical to the recruitment of circulating immune cells and thereby to the trajectory of the subsequent immune response. Although not traditionally viewed as canonical immune cells, fibroblasts have important roles in both the activation and regulation of the inflammatory response (Jordana et al., 1994). Disruption to the epithelium at mucosal surfaces is thought to expose the underlying stromal fibroblasts and their ability to secrete chemokines and promote polymorphonuclear (PMN) cell influx is thought to endow them with important ability to control the switch to chronic inflammation (Buckley et al., 2001; Van Linthout et al., 2014). Empirical understanding of fibroblast function in cattle is limited, although previous work has developed a primary cell culture model. This model was used to identify cattle with *in vitro* response profiles which mirrored the response to

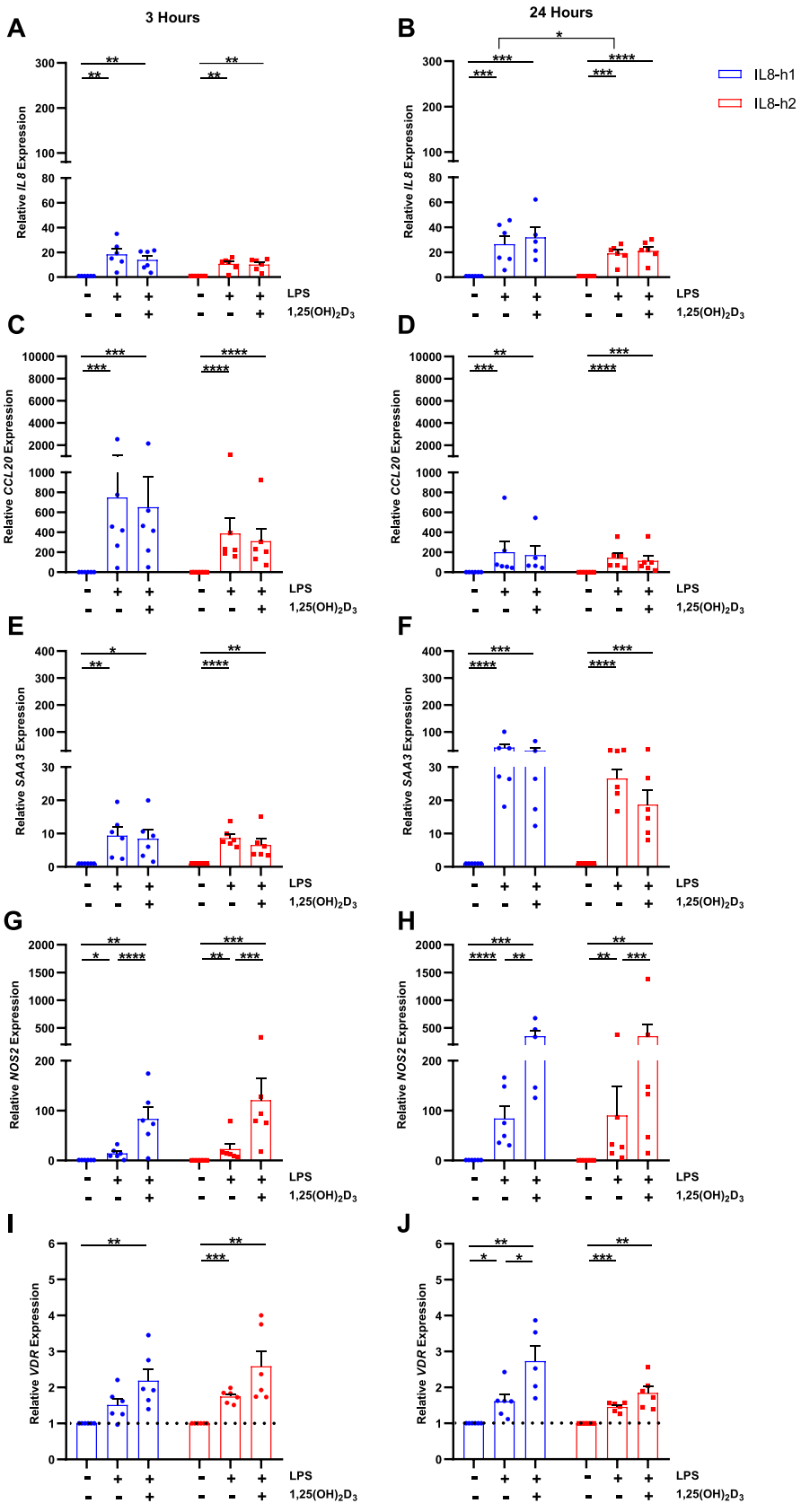


Fig. 2. Gene expression of *IL8, CCL20, SAA3, NOS2* and *VDR* following 3 and 24 h stimulation of LPS +/- $1,25(OH)_2D_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 μg/mL LPS +/- 10 nM $1,25(OH)_2D_3$ for 3 and 24 h plus an unstimulated control at 37 °C, 5% CO_2. Gene expression is shown for IL8-h1 in blue and for IL8-h2 in red and presented as relative to the unstimulated control. Induction is shown of (A) *IL8* (C) *CCL20* (E) *SAA3* (G) *NOS2* and (I) *VDR* after 3 h and (B) *IL8* (D) *CCL20* (F) *SAA3* (H) *NOS2* and (J) *VDR* after 24 h in response to LPS +/- $1,25(OH)_2D_3$. Error bars represent the SEM of n = 5/6. Significance is shown between stimuli (straight lines) or between haplotypes (branched lines) where $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ and **** $P<0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

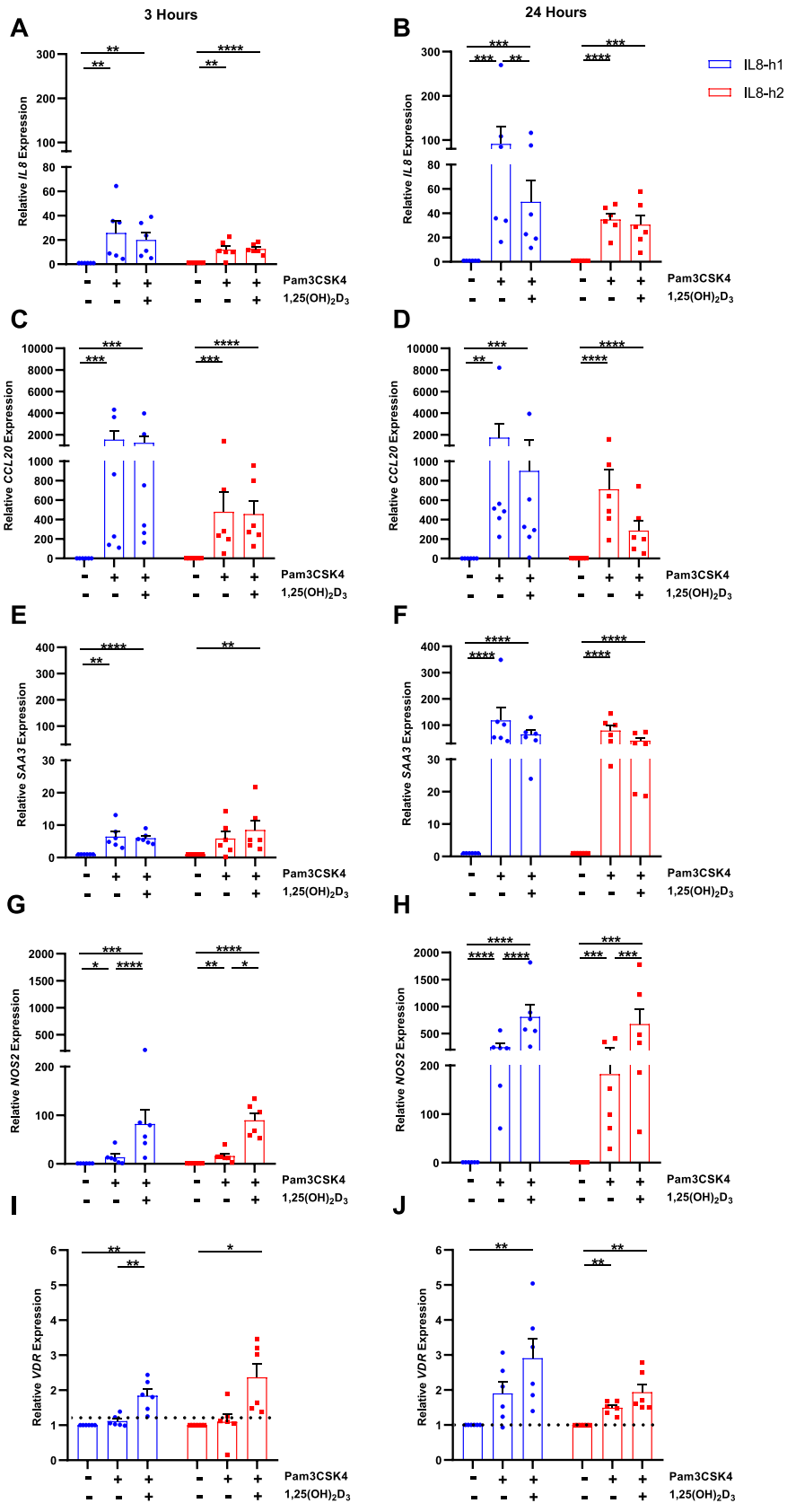


Fig. 3. Gene expression of *IL8, CCL20, SAA3, NOS2* and *VDR* following 3 and 24 h stimulation of Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 μg/mL Pam3CSK4 +/- 10 nM 1,25(OH)$_2$D$_3$ for 3 and 24 h plus an unstimulated control at 37 °C, 5% CO_2. Gene expression is shown for IL8-h1 in blue and for IL8-h2 in red and presented as relative to the unstimulated control. Induction is shown of (A) *IL8* (C) *CCL20* (E) *SAA3* (G) *NOS2* and (I) *VDR* after 3 h and (B) *IL8* (D) *CCL20* (F) *SAA3* (H) *NOS2* and (J) *VDR* after 24 h in response to LPS +/- 1,25(OH)$_2$D$_3$. Error bars represent the SEM of n = 5/6. Significance is between stimuli (straight lines) where *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and **** $P<0.0001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

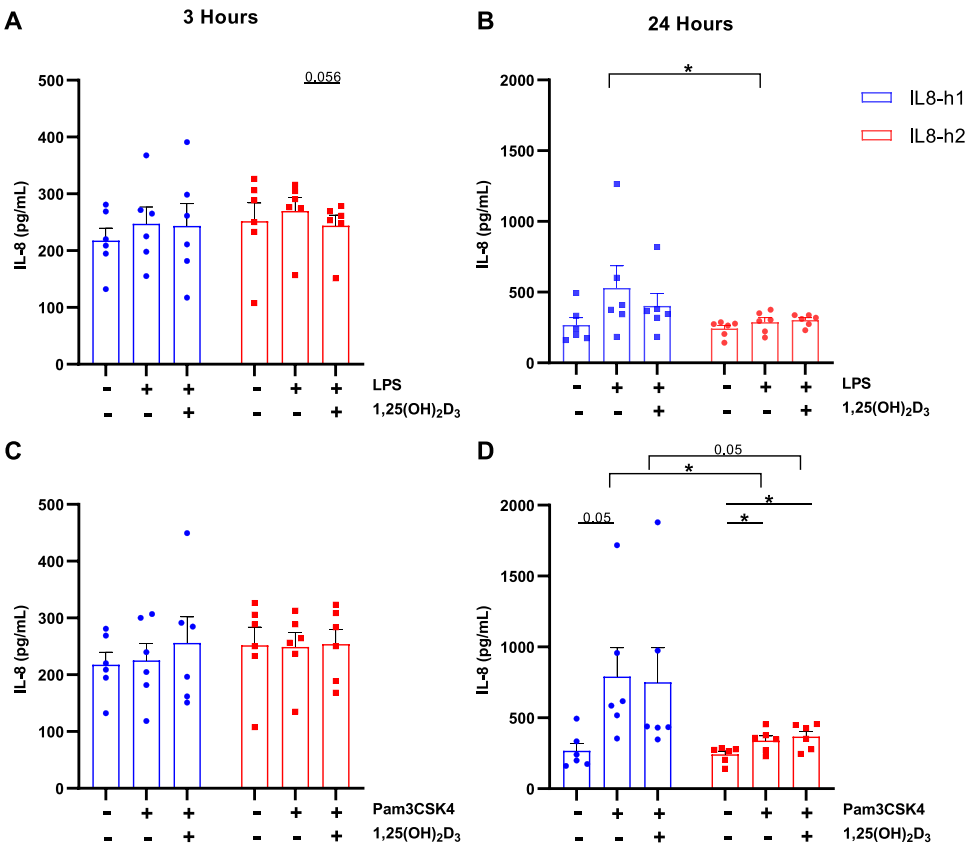


Fig. 4. IL-8 protein expression following 3 h and 24 h stimulation of LPS +/- 1,25(OH)$_2$D$_3$ or Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in primary dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 μg/mL LPS +/- 10 nM 1,25 (OH)$_2$D$_3$ (2 h pre-treatment) for (A) 3 and (B) 24 h or 1 μg/mL Pam3CSK4 +/- 10 nM 1,25 (OH)$_2$D$_3$ (2 h pre-treatment) for (C) 3 h and (D) 24 h plus an unstimulated control at 37 °C, 5% CO_2. IL-8 levels in IL8-h1 and IL8-h2 cell stimulation supernatants was assessed *via* ELISA and results are shown for IL8-h1 in blue and for IL8-h2 in red. Error bars represent the SEM of n = 6. Significance is shown between stimuli (straight lines) or between haplotypes (branched lines) where *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experimental infection in the same animals *in vivo* (Kandasamy et al., 2011). High and low IL-8 responder cattle were identified, and their response profile may be directly relevant to their interleukin 8 haplotype, although that was not investigated.

We previously identified two major IL8 genetic haplotypes in cattle and relevantly both are carried at approximately equal frequencies in the Holstein-Friesian breed of cattle (Meade et al., 2012). Characterisation of the functional role of these haplotypes showed that they are responsible for significantly divergent systemic *IL8* expression profiles in calves stimulated with bacterial endotoxin, LPS, *in vivo* (Stojkovic et al., 2016). *In vitro* analysis of the effect of haplotype on endogenous IL8 has not previously been performed and therefore in this current study, we continued our functional characterisation of these haplotypes in the fibroblast cells, representing a local immune response model.

Primary fibroblasts cultured from cattle with distinct IL8 haplotypes showed significantly different *IL8* response profiles in response to bacterial PAMPs – the TLR ligand, LPS and the TLR1/2 ligand, Pam3CSK4. *IL8* was significantly induced in response to both PAMPs, as expected, at 3 and 24 h. Of note, expression level of this chemokine was consistently higher at the 24 h time point. In addition, a significant difference between cells homozygous for IL8-h1 and IL8-h2 was detected for *IL8* gene expression in response to LPS as well as for IL8 protein expression levels in response to both LPS and Pam3CSK4 showing a clear effect of haplotype on IL8 production in fibroblasts. This finding supports an important association between IL8 genetic haplotype and IL-8 chemokine function that could have important relevance for the ability of cattle carrying this haplotype to resist and respond to infection. Interestingly, in contrast to previously published results, the IL8 dominant haplotype in this study (IL8-h1) is the haplotype that displayed reduced IL8 expression in our earlier analysis of systemic IL8 responses (Stojkovic et al., 2016). Results therefore suggest that functional analysis performed at a systemic level in peripheral blood does not directly transfer to the analysis of resident cells, a finding that has important consequences for measuring immune responses. The reason underlying the change in the responsiveness of cells carrying either haplotype could be explained by a possible divergent regulatory context (*e.g.* transcription factors) in circulating immune *versus* resident cell types.

A secondary objective of the work performed here was to assess the role of vitamin D on the innate immune gene expression. The interrelationship between inflammatory and potential regulatory molecules would be expected to have important significance for the activation of an early and effective immune response. The association between IL8 and vitamin D has been the subject of multiple previous studies and in general, findings have shown that vitamin D down regulates *IL8* expression in stimulated cells but can also induce expression under normal conditions (Rostkowska-Nadolska et al., 2010; Ryynanen and Carlberg, 2013). We did not find evidence of a regulatory effect of vitamin D on IL8 expression in fibroblasts in this study. However, results did show a strong induction of *NOS2* gene expression in response. The NOS2 gene encodes inducible nitric oxide synthase (iNOS) which has important roles in immune signalling and has antimicrobial properties (Xue et al., 2018). Previous studies in cattle have shown vitamin D to modulate expression of *NOS2* in monocytes and fibroblasts by significantly enhancing its response to LPS (Boylan et al., 2020; Nelson et al., 2010). That enhancement is conserved in the fibroblast response assessed here which showed significant elevation of *NOS2* expression above what was detected in response to both LPS and Pam3CSK4, and gene expression levels were notably higher at 24 h post-stimulation.

Our data also shows that fibroblasts express high levels of CCL20 at both time points. Although not regulated in response to vitamin D, the very high PAMP-mediated expression of the *CCL20* gene indicates that secretion of this chemokine is a core component of fibroblast immune function in cattle, as has been shown in other species (Hosokawa et al., 2005), as well as in previous bovine studies (Green et al., 2015). SAA3 expression was also elevated at 24 h and encodes a major acute phase protein. Expression of *SAA3* has been documented in many bovine

epithelial tissues including from the mammary gland, intestine and in bovine dermal fibroblasts (Green et al., 2015; Murata et al., 2020).

In conclusion, IL8 haplotype determines the *IL8* response profile in primary fibroblast cells. These primary fibroblasts are not only responsive to the bacterial ligands LPS and Pam3CSK4 but also to vitamin D. The significant upregulation of *NOS2* expression in response to vitamin D could provide a mechanism to enhance the protective effects of antimicrobial inducible nitric oxide synthase (iNOS).

Authors contributions

Conceived the study: KGM. Performed experiments and interpreted data: MOB, CLB, KGM and RMcL. Wrote the manuscript: MOB, CLB, KGM and RMcL. All authors read and approved the final manuscript.

Funding

This work was funded by a Teagasc Walsh Scholarship to MOB (RMIS no. 0005GE).

Ethics statement

All procedures described were conducted under ethical approval and experimental license from the Irish Health Products Regulatory Authority in accordance with the Cruelty to Animals Act 1876 and in agreement with the European Union (Protection of Animals Used for Scientific Purposes) regulations 2012 (S.I. No. 543 of 2012).

Consent for publication

Not applicable.

Availability of data and material

The datasets analysed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors report no declarations of interest.

Acknowledgements

The authors would like to acknowledge the technical support provided by Susana Flores Villalva, Margaret Murray and Eddie Mulligan.

References

Bai, H., Shabur, T.M.A., Kunii, H., Itoh, T., Kawahara, M., Takahashi, M., 2019. Evaluation of the immune status of peripheral blood monocytes from dairy cows during the periparturition period. J. Reprod. Dev. 65, 313–318.

Bosch, I., Xhaja, K., Estevez, L., Raines, G., Melichar, H., Warke, R.V., Fournier, M.V., Ennis, F.A., Rothman, A.L., 2002. Increased production of interleukin-8 in primary human monocytes and in human epithelial and endothelial cell lines after dengue virus challenge. J. Virol. 76, 5588–5597.

Boylan, M., O'Brien, M.B., Beynon, C., Meade, K.G., 2020. 1,25(OH)D vitamin D promotes NOS2 expression in response to bacterial and viral PAMPs in primary bovine salivary gland fibroblasts. Vet. Res. Commun. 44, 83–88.

Buckley, C.D., Pilling, D., Lord, J.M., Akbar, A.N., Scheel-Toellner, D., Salmon, M., 2001. Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. Trends Immunol. 22, 199–204.

Cronin, J.G., Hodges, R., Pedersen, S., Sheldon, I.M., 2015. Enzyme linked immunosorbent assay for quantification of bovine interleukin-8 to study infection and immunity in the female genital tract. Am. J. Reprod. Immunol. 73, 372–382.

Dauletbaev, N., Herscovitch, K., Das, M., Chen, H., Bernier, J., Matouk, E., Berube, J., Rousseau, S., Lands, L.C., 2015. Down-regulation of IL-8 by high-dose vitamin D is specific to hyperinflammatory macrophages and involves mechanisms beyond up-regulation of DUSP1. Br. J. Pharmacol. 172, 4757–4771.

Galligan, C.L., Coomber, B.L., 2000. Effects of human IL-8 isoforms on bovine neutrophil function in vitro. Vet. Immunol. Immunopathol. 74, 71–85.

Green, B.B., Kandasamy, S., Elsasser, T.H., Kerr, D.E., 2011. The use of dermal fibroblasts as a predictive tool of the toll-like receptor 4 response pathway and its development in Holstein heifers. J. Dairy Sci. 94, 5502–5514.

Green, B.B., McKay, S.D., Kerr, D.E., 2015. Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome. BMC Genomics 16, 30.

Hacking, D., Knight, J.C., Rockett, K., Brown, H., Frampton, J., Kwiatkowski, D.P., Hull, J., Udalova, I.A., 2004. Increased in vivo transcription of an IL-8 haplotype associated with respiratory syncytial virus disease-susceptibility. Genes Immun. 5, 274–282.

Harant, H., Andrew, P.J., Reddy, G.S., Foglar, E., Lindley, I.J., 1997. 1alpha,25-dihydroxyvitamin D3 and a variety of its natural metabolites transcriptionally repress nuclear-factor-kappaB-mediated interleukin-8 gene expression. Eur. J. Biochem. 250, 63–71.

Hosokawa, Y., Hosokawa, I., Ozaki, K., Nakae, H., Matsuo, T., 2005. Increase of CCL20 expression by human gingival fibroblasts upon stimulation with cytokines and bacterial endotoxin. Clin. Exp. Immunol. 142, 285–291.

Hull, J., Thomson, A., Kwiatkowski, D., 2000. Association of respiratory syncytial virus bronchiolitis with the interleukin 8 gene region in UK families. Thorax 55, 1023–1027.

Jordana, M., Sarnstrand, B., Sime, P.J., Ramis, I., 1994. Immune-inflammatory functions of fibroblasts. Eur. Respir. J. 7, 2212–2222.

Kandasamy, S., Green, B.B., Benjamin, A.L., Kerr, D.E., 2011. Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during Escherichia coli mastitis. J. Dairy Sci. 94, 5963–5975.

Korkmaz, F.T., Elsasser, T.H., Kerr, D.E., 2018. Variation in fibroblast expression of toll-like receptor 4 and lipopolysaccharide-induced cytokine production between animals predicts control of bacterial growth but not severity of Escherichia coli mastitis. J. Dairy Sci. 101, 10098–10115.

Ma, X., Reich, R.A., Wright, J.A., Tooker, H.R., Teeter, L.D., Musser, J.M., Graviss, E.A., 2003. Association between Interleukin-8 gene alleles and human susceptibility to tuberculosis disease. J. Infect. Dis. 188, 349–355.

Maheshwari, A., Voitenok, N.N., Akalovich, S., Shaik, S.S., Randolph, D.A., Sims, B., Patel, R.P., Killingsworth, C.R., Fallon, M.B., Ohls, R.K., 2009. Developmental changes in circulating IL-8/CXCL8 isoforms in neonates. Cytokine 46, 12–16.

Meade, K.G., O'Gorman, G.M., Narciandi, F., Machugh, D.E., O'Farrelly, C., 2012. Functional characterisation of bovine interleukin 8 promoter haplotypes in vitro. Mol. Immunol. 50, 108–116.

Murata, E., Kozaki, S., Murakami, T., Shimizu, K., Okada, A., Ishiguro, N., Inoshima, Y., 2020. Differential expression of serum amyloid A1 and A3 in bovine epithelia. J. Vet. Med. Sci. 82, 764–770.

Nelson, C.D., Reinhardt, T.A., Thacker, T.C., Beitz, D.C., Lippolis, J.D., 2010. Modulation of the bovine innate immune response by production of 1alpha,25-dihydroxyvitamin D(3) in bovine monocytes. J. Dairy Sci. 93, 1041–1049.

Pigossi, S.C., Anovazzi, G., Finoti, L.S., de Medeiros, M.C., Mayer, M.P.A., Rossa Junior, C., Scarel-Caminaga, R.M., 2019. Functionality of the Interleukin 8 haplotypes in lymphocytes and macrophages in response to gram-negative periodontopathogens. Gene 689, 152–160.

Richardson, R.M., Marjoram, R.J., Barak, L.S., Snyderman, R., 2003. Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation. J. Immunol. 170, 2904–2911.

Rostkowska-Nadolska, B., Sliupkas-Dyrda, E., Potyka, J., Kusmierz, D., Fraczek, M., Krecicki, T., Kubik, P., Zatonski, M., Latocha, M., 2010. Vitamin D derivatives: calcitriol and tacalcitol inhibits interleukin-6 and interleukin-8 expression in human nasal polyp fibroblast cultures. Adv. Med. Sci. 55, 86–92.

Ryynanen, J., Carlberg, C., 2013. Primary 1,25-dihydroxyvitamin D3 response of the interleukin 8 gene cluster in human monocyte- and macrophage-like cells. PLoS One 8, e78170.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative CT method. Nat. Protoc. 3, 1101–1108.

Stojkovic, B., McLoughlin, R.M., Meade, K.G., 2016. In vivo relevance of polymorphic Interleukin 8 promoter haplotype for the systemic immune response to LPS in Holstein-Friesian calves. Vet. Immunol. Immunopathol. 182, 1–10.

Stojkovic, B., Mullen, M.P., Donofrio, G., McLoughlin, R.M., Meade, K.G., 2017. Interleukin 8 haplotypes drive divergent responses in uterine endometrial cells and are associated with somatic cell score in Holstein-Friesian cattle. Vet. Immunol. Immunopathol. 184, 18–28.

Tang, X., Pan, Y., Zhao, Y., 2013. Vitamin D inhibits the expression of interleukin-8 in human periodontal ligament cells stimulated with Porphyromonas gingivalis. Arch. Oral Biol. 58, 397–407.

Van Linthout, S., Miteva, K., Tschope, C., 2014. Crosstalk between fibroblasts and inflammatory cells. Cardiovasc. Res. 102, 258–269.

Xue, Q., Yan, Y., Zhang, R., Xiong, H., 2018. Regulation of iNOS on immune cells and its role in diseases. Int. J. Mol. Sci. 19.