Functional Relevance of Interleukin 8 Haplotype for the Innate Immune System in Holstein-Friesian Cattle

Megan O'Brien MSc, BSc

This thesis is submitted for the degree of Doctor of Philosophy (PhD)

March 2021

School of Biochemistry and Immunology

Trinity College Dublin

Supervisors: Prof Rachel M McLoughlin and Dr Kieran G Meade

Declaration

I declare that this thesis has not been submitted as an exercise for a degree at this or any other university and it is entirely my own work, except where otherwise stated.

I agree to deposit this thesis in the University's open access institutional repository or allow the Library to do so on my behalf, subject to Irish Copyright Legislation and Trinity College Library conditions of use and acknowledgement.

I consent to the examiner retaining a copy of the thesis beyond the examining period, should they so wish.

_____

Megan O'Brien

Publications

Publications arising from this thesis:

O'Brien MB, McLoughlin RM and Meade KG. Application of the TruCulture® whole blood stimulation system for immune response profiling in cattle. *Vet Immunol Immunopathol.* 221, 110025 (2020).

O'Brien MB, Beynon CL, McLoughlin RM, and Meade KG. The immune response in bovine primary dermal fibroblasts is influenced by Interleukin 8 promoter haplotype and vitamin D. *Vet Immunol Immunopathol.* 238, 110291 (2021).

O'Brien MB, McLoughlin RM, Nelson CD and Meade KG. Seasonal profile in circulating levels of interleukin 8 and 25(OH) vitamin D is IL-8 haplotype dependent in Holstein-Friesian calves. *Vet Immunol Immunopathol.* 238, 110287 (2021).

Additional publications:

Boylan M, **O'Brien MB**, Beynon C and Meade KG. 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 (2020).

De Matteisa G, Scatàa MC, Grandonia F, Crisàa A, **O'Brien MB**, Meade KG and Catillo G. Effect of Interleukin 8 haplotype on cellular and molecular immune traits in periparturient dairy cows. *Vet Immunol Immunopathol.* 238, 110288 (2021).

Donnellan EM, **O'Brien MB**, Meade KG and Fair S. Comparison of the uterine inflammatory response to frozen-thawed sperm from high and low fertility bulls. *Theriogenology*. 176, 26-34 (2021).

Acknowledgments

I must start by thanking my ever supportive and patient supervisors, Kieran and Rachel. Thank you so much for taking me on as a student in the first place and giving me the most amazing opportunity I could ever ask for. Kieran, you have been a brilliantly supportive and enthusiastic mentor to me for the past few years. You have given me the space to grow and develop as a researcher while also providing me with excellent advice and motivation especially in the times where I could never see the positives! Rachel, you have given me so much guidance and brilliant input on my project while being the most inspiring role model for a young woman in science. Thank you for everything you have done for me.

A huge special thanks go to all the amazing technicians at Teagasc Grange who provided me with so much help and support over the years. Eddie, I wouldn't have gotten half as far without all your help with collecting blood and tissue samples. Thank you for not getting tired of my endless requests and for always being available to help (and for not holding it against me being a dub!). Margaret and Joe, you never got tired of answering my endless questions, you were always there to help process samples and most importantly you were always available for a chat!

I also need to give a special thanks to Gina in Tennessee for kindly allowing me to go to Knoxville to work with her and her group. I learned so much and had such a wonderful experience there. A big thanks to Leszek, Tory, Emily, Clay, Tiffany and Melea for looking after me so well and helping with my experiments.

Thank you to all the past and present members of the Immunobiology group at Teagasc. It has been a wild ride and I never expected to leave with so many lifelong friends. Amy, my former work wife and fellow complainer about life, you were such a wonderful and supportive friend to me and never said no when I needed help or advice. Nick, never failed in making me laugh and never failed in giving me a fright, your positivity kept me going. Up Tipp! Charlotte, I owe so much to you in educating me in the finer skills of cell culture. It really is true to say that I wouldn't have gotten half as far without you. Susana, where to begin! You've done so much for me this past year from driving me to the factory, helping me collect samples, feeding me cake, buying me tequila... I could go on! Thank you so much for being such a lovely friend. There are so many others to thank: Cian, Eimear, Laura, Sajad, Sarah, Paul, Ronan and Bojan. Also,

to all the friends I've made at Teagasc over the years who have made it such a great experience: Dayle, Dagmara, Shauna, Javi, Paul, Irene, Gabi, Gaelle, Niamh and Cathy.

A special thanks as well to past and present members of the Host-Pathogen Interaction Group at Trinity: Alannah, Keenan, Tracey, Vivienne, Eoin, Stephen, Emilio and especially to Jenny and Andrew for putting up with all my requests for help and advice.

My friends have been such a wonderful support to me over the years from listening to all my complaining, my anxieties to picking me up and helping stay positive. I don't know where I would be without you guys, you mean the world to me. Thank you so so much: Martha, Ciara, Claire, Amy, Anne-Marie, Hannah, Drew and Carol. I also want to give a special thanks to Eleanor and Michael for looking after me these last few weeks. It hasn't been easy!

The biggest thanks of all goes to my family. Mam and Dad, you've never stopped believing in me. You've been such a rock in my life. You trusted me completely when I knew this is what I wanted to do and have never wavered in your support since. My sisters, Gemma and Louise, you always listened to my rants and kept my head screwed on. Always there to give me lifts and available for coffee and chats. My brother in laws, Bernard and Terry, you're essentially my big brothers now and always look out for me. And lastly, the mischief makers in my life who always make me smile and remind me it's good to be a big child sometimes: Lilly-Rose, Harry, Jamie and Ben. Thank you from your Auntie, the scientist.

Table of Contents

List of Figures

List of Tables

Abbreviations

ACD	Acid citrate dextrose
ACTB	β actin
ACTG1	γ actin
AFU	Average fluorescence unit
AP-1	Activator protein-1
BSA	Bovine serum albumin
BTV	Blue tongue virus
CARD	Caspase repeat domain
CCL20	Chemokine ligand 20
CCR7	Chemokine receptor 7
CD3	Cluster of differentiation 3
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD14	Cluster of differentiation 14
CHOP	C/EBP homologous protein
CXCL1	CXC motif chemokine ligand 1
CXCL5	CXC motif chemokine ligand 5
CXCL6	CXC motif chemokine ligand 6
CXCR1	Chemokine (CXC motif) receptor 1
CYP24A1	Cytochrome P450 Family 24 Subfamily A Member 1
CYP27A1	Cytochrome P450 family 27 subfamily A member 1
CYP27B1	Cytochrome P450 family 27 subfamily B member 1
CYP2R1	Cytochrome P450 family 2 subfamily R member 1
CXCR1	Chemokine (CXC motif) receptor 1
CXCR2	Chemokine (CXC motif) receptor 2
ELISA	Enzyme-linked immunosorbent assay

FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde 3-phosphate
GC	Group-specific component
GUSB	Glucuronidase β
HIF1A	Hypoxia inducible factor 1 α
HSP90AB1	Heat shock protein 90 alpha family class B member
IFN-α	Interferon α
IFN-β	Interferon β
IFN-γ	Interferon γ
IL-1β	Interleukin 1 β
IL-8	Interleukin 8
IL-4	Interleukin 4
IκB	Inhibitor of nuclear factor kappa B
iNOS	Inducible nitric oxide synthase
LGP2	Laboratory of genetics and physiology
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
MACS	Magnetic activated cell sorting
MAPK	Mitogen-activated protein kinase
MDA5	Melanoma differentiation associated factor 5
MHCII	Major Histocompatibility Complex II
MYD88	Myeloid differentiation factor 88
NET	Neutrophil extracellular trap
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NOD	Nucleotide-binding oligomerization domain
NLR	NOD –like receptor
NRF	NF-κB repressing factor

NLRP	NOD-like receptor family pyrin domains
PAMP	Pathogen associated molecular pattern
PBS	Phosphate buffered saline
RPS15	Ribosomal protein S15
RSV	Respiratory syncytial virus
SAA3	Serum Amyloid A 3
VDBP	Vitamin D binding protein
VDR	Vitamin D Receptor

Abstract

Calves rely heavily on their innate immune response in the post-birth period before their adaptive immune system develops. Interleukin 8 (IL-8) is a major chemoattractant predominantly associated with neutrophils response to infection. The genetic variation at this gene promoter is likely to have functional consequences for both inflammation and infectious disease susceptibility. Previous work by our group identified two distinct haplotypes (IL8-h1 and IL8-h2) in the IL-8 gene promoter region carried at equal frequencies (~ 50%) in the Holstein-Friesian breed. Calves carrying IL8-h2 produce significantly higher levels of IL-8 after *in vivo* systemic stimulation with lipopolysaccharide (LPS). However, little is understood about IL8 haplotype effect on the IL-8 response and the wider innate immune system within the first six months of life. It also remains unknown if levels of IL-8 secreted by local cell types differ between haplotypes. Additionally, vitamin D has been described to modulate the IL-8 response but its role in calf immunity and the specific effects of IL8 haplotype has not been previously investigated. The aims of this project were I. To investigate immune cell populations and the systemic innate immune response in calves with different IL8 haplotypes to bacterial, viral and fungal PAMPs over the first year of life; II. To establish the relationship between IL8 and circulating vitamin D levels in peripheral blood and expression of vitamin D pathway genes; and III. To establish a local primary cell model to examine the effect of IL8 haplotype on innate immune gene expression and the response to vitamin D.

Haematological profiling of genotyped Holstein-Friesian calves showed neutrophils, monocytes, eosinophils and basophils were increased at specific time points in IL8-h2 calves across the first ten months of life in comparison to IL8-h1 calves. Using whole blood culture systems, peripheral blood from these calves was exposed to a bacterial (LPS), viral (poly (I:C)) and fungal (zymosan) PAMPs. No significant differences in IL-8 were identified in response to any PAMP which may be due to the absence of a cell type in peripheral blood which produces IL-8. Examination of basal IL-8 levels in serum along with vitamin D showed that IL8-h2 calves produced significantly higher levels of IL-8, concurrently with lower circulating levels of vitamin D. The opposite relationship was apparent for IL8-h1 calves which produce relatively lower levels of IL-8 but higher levels of vitamin D. Further analysis *in vitro* showed vitamin D to significantly reduce IL-8 from monocytes at a basal level. Finally, primary dermal

fibroblasts isolated from genotyped cattle were stimulated with LPS and Pam3CSK4. IL8-h1 cells showed significantly increased levels of IL-8 in response to Pam3CSK4 when compared with IL8-h2 fibroblasts. This indicates a cell specific and PAMP specific IL-8 response which is dependent on IL8 haplotype. To conclude, the results here support a significant effect of IL8 haplotype on both systemic and local innate immune response in Holstein-Friesian cattle and further demonstrates functional interaction between IL8 haplotype and vitamin D levels in peripheral blood. Therefore, IL8 haplotype is likely to have important relevance for immune competence and *in vivo* susceptibility of cattle to multiple diseases.

Chapter 1. General Introduction

1.1 Interleukin-8 and regulation of expression

1.1.1 Introduction to interleukin-8 expression, regulation and function,

Interleukin 8 (IL-8), also known as C-X-C chemokine ligand 8 (CXCL8), is a member of the chemokine subfamily with a CXC motif which is characterised by an amino acid separation between the first two cysteines. Its amino acid sequence in the bovine species is approximately 101 amino acids in length. In humans, it is 99 amino acids in length with several active isoforms [1]. Gene activation is under control of several transcription factors including nuclear factor kappa B (NF-κB) and activator protein-1 (AP-1) [2]. It has also been shown to be negatively regulated by micro ribonucleic acids (miRNAs) and other mechanisms [3]. Additionally, *IL8* gene expression patterns also depend on the presence of SNPs (single nucleotide polymorphisms) in the gene, some of which are inherited together as a haplotype. Humans and cattle with a particular IL8 haplotype have been shown to demonstrate different IL-8 responses to pathogen-associated molecular patterns (PAMPs) to *in vitro* and *in vivo* infections, respectively [4, 5]. In its action as a chemoattractant for leukocytes, and predominantly neutrophils, IL-8 binds to C-X-C motif chemokine receptor 1 (CXCR1) or C-X-C motif chemokine receptor 2 (CXCR2) [6]. Divergent expression responses have also been detected in other genes in the IL-8 axis where animals with different CXCR1 and CXCR2 receptor haplotypes respond differently to infection [7]. Gene regulation therefore plays a critical role in how *IL8* expression is initiated and regulated.

1.1.2 Signalling pathways involved in interleukin-8 expression

There are several receptors involved in initiating expression of *IL8* as one of many effector molecules of the innate immune response, and which individually recognise specific ligands to create an appropriate signalling response to the pathogen. These receptors include Toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like receptors (NLRs) or NOD-like receptors and also retinoic acid-inducible gene I-like receptors (RIG-I) or RIG-I-like receptors.

TLRs are significant in the early activation of the IL8 by recognising PAMPs. TLRs are denoted according to the PAMP they recognise [8]. Genes encoding ten different bovine TLRs (numbered TLR1-TLR10) have been annotated to date in the bovine genome [9]. The PAMPs which each TLR recognises is summarised in Table 1.1.

Depending on the TLR activated a different signalling pathway is usually activated. The most commonly studied of these receptors TLR4, operates through the adaptor molecules myeloid differentiation factor 88 (MYD88) and toll-interleukin 1 receptor domain containing adaptor protein (TIRAP). Once this signalling pathway is activated via kinases the transcription factor NF-κB is freed from its cytosolic IκBα protein complex to initiate expression of important innate immune response pro-inflammatory cytokines in the nucleus, such as *TNFA*, *IL1B* and *IL8* [10, 11].

Unlike TLR4 which is expressed on the surface of cells, some TLRs are expressed internally on endosomes where they detect viruses which may have infiltrated the cell. These include TLRs 3, 7 and 8. TLR9 is also expressed internally on endosomes and detects a bacterial DNA known as CpG DNA [9]. The signalling pathway activated through TLR3 which recognised dsRNA involves the use of the adaptor molecule known as TIR-domain-containing adapter-inducing interferon-β (TRIF) in order to initiate expression of anti-viral molecules known as type-1 interferons (IFNs) [12]. In cattle, the IL-8 protein was shown to be significantly secreted in response to activation of TLR3 by the agonist poly (I:C), which is a synthetic version of dsRNA [13].

The NLR family includes members such as NOD1 and NOD2 which recognise bacterial peptidoglycans within the cell cytosol. Their structure includes a caspase repeat domain (CARD) and leucine-rich repeat (LRR) domain. Upon activation they signal through mitogen-activated protein kinase (MAPK) and TNF receptor-associated factor 3 (TRAF3) in order to activate NF-κB [14]. The importance of these receptors in the innate immune response has been demonstrated in periparturient cows that are generally immunologically compromised. These cows show a down regulation of NOD1 expression on neutrophils compared with cows in mid lactation, and neutrophil function was reportedly defective with a reduction in phagocytosis as well as reduced ROS generation [15].

Other members of this family include the NOD-like receptor family pyrin domains (NLRPs). These receptors are mostly involved in formation of the inflammasome - NLRP1 and NLRP3, in particular. The inflammasome is essentially a collection of proteins which combine to form a system capable of promoting the activation of key pro-inflammatory cytokines such as IL-1β. The inflammasome is essential for protection against pathogen invasion [14]. In 2012, Thacker *et al*. described

a small peptide that activates the NLRP3 inflammasome and was successful in significantly reducing the somatic cell count (SCC) of dairy cows with mastitis when compared with the standard antibiotic treatment. This would suggest that targeting an innate immune response such as the inflammasome complex may be more successful than direct targeting the bacteria itself [16]. This receptor pathway has been demonstrated in cattle to also initiate expression of *IL8,* in turbinate cells isolated from the nasal cavity, in response to intracellular bacteria *C. abortus* [17].

This RIG like receptor family includes RIG-I and two less well described members; melanoma differentiation associated factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). Their primary function is to recognise viral signatures and initiate an IFN response. Both RIG-I and MDA5 recognise a variety of viruses that include ssRNA, dsRNA and DNA viruses. RIG-I itself contains two CARD domains and what is known as an Asp-Glu-Ala-Asp or DEAD helicase. The activation of the RIG-I pathway results in an ‘’opening‘’ of the RIG-I structure which allows it to interact with additional adaptor molecule known as IFN-β promoter stimulator-1 (IPS-1). The expression of IFN genes occurs and initiates the antiviral response [18].

This receptor family has been reported involvement in the bovine response to bluetongue virus (BTV). The authors observed a decrease in expression of IFNβ following knockdown of RIG-I and family member MDA5. Decreased expression was not observed in the case of TLR3 which would lead us to believe that the RIG-I pathway is important for control of this particular virus [19]. While this receptor pathway is primarily responsible for initiating IFN responses, it has also been shown to be involved in IL-8 production in cattle as shown in endometrial stromal cells where the short interfering RNA (siRNA) was used to interfere with a component of the RIG-I pathway knowns as IFN regulatory factor 3 (IRF3) resulted in decreased IL-8 when induced by poly(I:C) [13].

Table 1.1 Bovine TLRs and their recognised PAMPs. Specific TLRs recognise their own unique ligand in order to initiate an appropriate response to the pathogen (adapted from: [12]).

TLR	PAMP recognised
TLR1	Triacylated lipoproteins
TLR2	Triacylated/diacylated lipoproteins
TLR3	Double stranded RNA (dsRNA)
TLR4	Lipopolysaccharide (LPS)
TLR5	Bacterial flagellin
TLR6	Diacylated lipoproteins
TLR7	Single stranded RNA (ssRNA)
TLR8	ssRNA
TLR9	CpG DNA
TLR10	Unknown (possibly a lipoprotein)

1.1.3 Transcriptional regulation of interleukin-8 gene expression

NF-κB is a well characterised transcription factor regulating the expression of many inflammatory genes, including IL-8. While under the control of NF-κB, other transcription factors also have a significant role in IL-8 activation such as AP-1 and C/EBP homologous protein (CHOP) [2]. NF-κB itself is a hetero-dimeric structure composed from several different subunits which include p65 or RelA, RelB, c-Rel, p50 and p52. It is found in a protein complex in the cytosol before relocating to the nucleus [20]. This occurs when the NF-κB inhibitor, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (IκB), is released and allows NF-κB to travel and bind to *IL8* promoter to activate gene transcription [20]. The pro-inflammatory pathway which leads to activation of IL-8 gene expression is mediated by subunits p50 and p65 interacting with the promoter region of the gene. While it has also been reported that IL-8 gene activation is repressed through a pathway where the RelB and p50 subunits are involved. This is as a result of the neutrophil elastase (NE) enzyme inducing activation of this alternative pathway in an effort to regulate inflammation by inhibiting neutrophil recruitment [21].

AP-1 is another key transcription factor involved in activation of *IL8* expression [22]. It also composed of a number of subunits which include Jun-B, Jun-D and also c-Jun. AP-1 is consistently found bound to the *IL8* promoter region in addition to its other target genes and becomes activated by multiple mitogen-activated protein kinase (MAPK) pathways by mechanisms including phosphorylation [23]. Binding of PAMPs to multiple TLRs and also tumour necrosis factor-α (TNF-α) binding to its receptor TNF receptor-1 (TNFR1) can initiate *IL8* expression through this transcription factor and has been shown in using *P. aeruginosa* stimulated human bronchial epithelial cells [2].

Two other transcription factors which also regulate *IL8* expression are C/EBP homologous protein (CHOP) and nuclear factor interleukin 6 (NF-IL-6) [22]. CHOP becomes activated through the MAPK p38 pathway [24], while NF-IL-6 works with NF-κB to transcribe *IL8* [25].

One element involved in inhibiting the expression of *IL8* is the repressor protein NF-κB repressing factor (NRF). It is a key regulator of the transcription factor NF-κB and ensures transcription of *IL8* is controlled [22]. The area NRF binds to on the *IL8* promoter is a region overlapping with the response element for NF-κB thus ensuring

repression of *IL8* expression [22]. Mutations in this regulatory element resulted in a reduction of *IL8* expression which was induced by IL-1α, highlighting the importance of considering SNPs or mutations in the promoter region [26]. NRF has also been reported to be induced by NE in order to control *IL8* expression [27].

IL8 expression can also be controlled by epigenetic regulation. Histone deacetylase acting on the *IL8* promoter region inhibited *IL8* expression in human airway epithelium which was induced by TNF-α [28]. Also, methylation of particular areas of the *IL8* promoter region was found to inhibit expression of *IL8* in human chrondocytes in addition to repressing the effects of transcription factors including NF-κB and AP-1 [29].

Additionally, a number of miRNAs have also been implicated in repressing *IL8* expression and also controlling IL-8 protein production. miRNA-93 was shown to be involved in controlling translation of IL-8 mRNA by the ability to bind to the 3'untranslated region (UTR) in human bronchial epithelial cells infected with *P. aeruginosa* [30]. The binding results in a repressive effect which interferes with the mRNA being translated. This effect was also confirmed by high expression of miRNA-93 by the cell under normal conditions [30]. Another miRNA, known as miRNA-181, also binds to the 3' UTR of IL-8 mRNA and produces a similar effect as was shown in human fibroblasts when stimulated with LPS [31]. Again, the study showed that high levels of miRNA-181 expression resulted in reduced levels of IL-8 [31]. Much of the regulation of *IL8* in the bovine remains relatively unknown however the role of polymorphisms in the gene its effects on regulation have been somewhat characterised.

1.1.4 Role of genetic variation in IL8 signalling

The presence of single nucleotide polymorphisms (SNPs) in the interleukin-8 gene are an important component which impact on both the mRNA expression level and IL-8 isoform. SNPs in the promoter region can alter how and which transcription factors can bind and regulate gene expression. SNPs can be inherited together from one parent in a haplotype block [32].

IL8 haplotypes have been identified in humans as well as cattle and also impact on *IL8* expression in addition to functional effects on neutrophils and other cell types [4]. SNPs present in the *IL8* promoter region in humans also result in two separate haplotypes and have been investigated in the context of periodontitis in the gum. Using Gram-

negative bacteria associated with periodontitis, monocytes and lymphocytes from individuals carrying each of the haplotypes (Hap-1 and Hap-2) were infected and Hap-1 monocytes showed increased expression of *IL8* and *TNFA* and this was also observed in Hap-1 lymphocytes [4]. In addition, Hap-1 monocyte derived macrophages (MDMs) showed decreased ability to phagocytose bacteria but increased ability to generate reactive oxygen species (ROS) against them [4]. These results suggest that *IL8* haplotype has the potential to impact the functional ability of cells in addition to impacting expression of *IL8* itself and also the expression of other cytokines. Another study examined neutrophils from individuals possessing these haplotypes and showed a significant difference in *IL8* gene expression with Hap-1 neutrophils showing higher expression levels [33]. Results also showed neutrophils from Hap-1 individuals had increased chemotactic capability when compared with Hap-2 [33]. This highlights the downstream effects that *IL8* haplotype plays in influencing how neutrophils themselves can respond to stimuli. Another study also supports a major role for *IL8* haplotype on respiratory disease susceptibility in humans [34]. Airway epithelial cells from individuals possessing Hap-2 showed increased expression of *IL8* in response to TNF-α which wasn't observed in lymphocytes isolated from these individuals, indicating a cell-type specific response [34]. It was discovered that the SNPs in haplotype 2 affected transcription factor binding, which thus affected *IL8* transcription resulting in the conclusion that this haplotype could increase susceptibility to RSV (respiratory syncytial virus) disease as high expression of *IL8* could contribute to tissue damage [34].

1.1.5 Cattle IL8 promoter haplotypes

While work in cattle has been more limited, previous work by our group has identified the SNPs present in the haplotypes examined in this thesis as being critical for the amount of IL-8 produced following an *in vivo* challenge with LPS in Holstein-Friesian calves [5]. The two haplotypes, IL8-h1 and IL8-h2, displayed very different IL-8 secretion profiles which differed most distinctly twelve hours post challenge where IL8-h2 had significantly elevated levels IL-8 in the serum when compared with IL8-h1 [5]. These haplotypes were initially identified in a population of sixty cattle. Thirty of which were Holstein-Friesian and thirty Norwegian Red. The twenty-nine polymorphic sites present in the IL8 promoter region (see Figure 1.1) segregate into two haplotypes. The ratio of each haplotype was interesting in the Holstein-Friesian population as it was near 50:50 for the two haplotypes (IL8-h1 – 48%, IL8-h2 – 52%) indicating a possible

advantage of each haplotype depending on the context of infection i.e. bacterial versus viral through a process of 'balancing' selection.

The polymorphic sites include two insertion/deletion sites which are predicted *in silico* to impact binding of transcription factors thus potentially regulating gene expression levels. This was shown in a bovine mammary epithelial cell line that were transfected with luciferase reporter plasmids representing each haplotype and cells simulated with TNF-α. Again, IL8-h2 cells showed significantly more activation when compared with IL8-h1 [35]. In the case of these haplotypes, the type of stimulant used may also impact expression levels. When a bovine endometrial cell line which was transfected with plasmids representing each haplotype, in addition to another plasmid expressing a BoHV4 (bovine herpes virus 4) viral product, IL8-h1 cells showed higher *IL8* promoter activity when compared with IL8-h2 cells [36]. This may indicate that virally activated transcription factors may more effectively activate *IL8* expression in IL8-h1 animals.

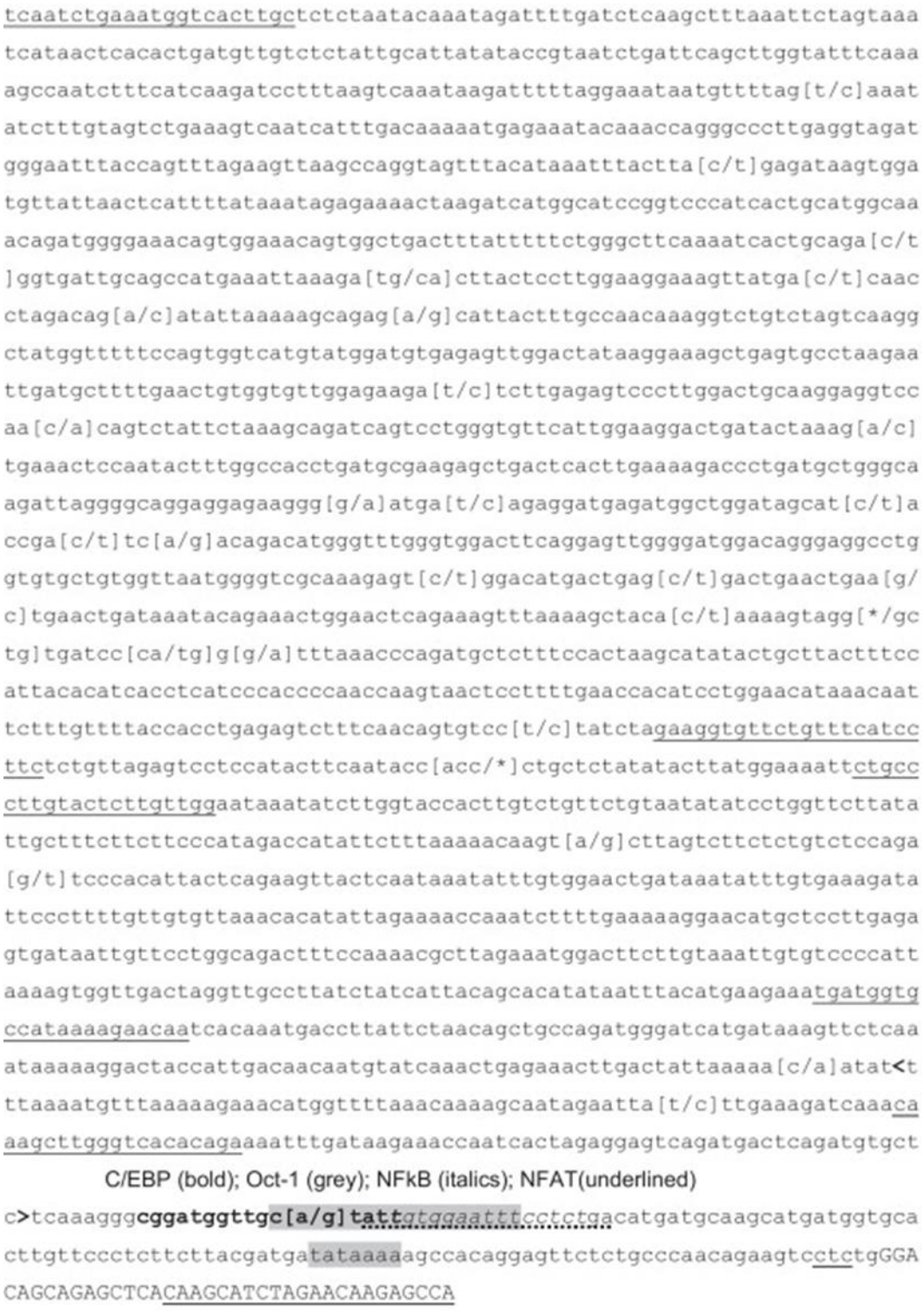

Figure 1.1 Cattle IL8 promoter region showing polymorphic sites associated with IL8 promoter haplotypes. Twenty-nine polymorphic which diverge between the haplotypes are distinguished by square brackets where the first letter indicates presence in IL8-h1 and the second in IL8-h2. An asterisk (*) indicates a deletion in that haplotype. The transcription start sites are underlined. Specific binding sites for each transcription factor are indicated in the image. Taken from Meade *et al.* 2012 [35].

1.1.6 Cattle IL8 receptor haplotypes

Haplotypes have also been investigated across other genes in the IL8 axis including receptors CXCR1 and CXCR2. These receptors are members of the G-coupled protein receptor family and become phosphorylated and internalised upon ligand binding. However the speed with which this occurs differs between the two receptors with it being quicker for CXCR2 which may indicate activation of different downstream signalling pathways [37]. CXCR1 is the primary receptor which binds IL-8 in order to initiate a number if immune function by neutrophils including chemotactic migration and respiratory burst. CXCR2 also binds IL-8 in addition to other chemokines including CXCL1 and CXCL5 [38]. While both receptors are primarily expressed by neutrophils, they have also been reported to be expressed by a number of other traditional immune cells such as monocytes, natural killer (NK) cells, basophils and T-cells [39]. Additionally, CXCR1 and CXCR2 expression by human gut epithelial cells has been shown basally and to be increased during inflammation [40]. An investigation into whether epithelial cells which express IL8 receptors are facilitating migration of neutrophils across mucosal sites using human primary broncho-epithelial cells revealed that this did not seem to be the case as blocking CXCR1 and CXCR2 did not reduce neutrophil trans-epithelial migration [41]. This indicates possibly another role for IL8 receptor expression at mucosal sites. In the case of cattle, CXCR1 expression has been identified during mastitis in the mammary gland however, this expression may be the result of neutrophil infiltration into the area [42]. It is not entirely inconceivable that CXCR1 could be expressed by mammary epithelial cells and play a role during infection in the bovine species.

CXCR1 haplotypes were investigated using an *in vivo* infection model where mammary glands of cattle of five different CXCR1 haplotypes were infected with *S. uberis*. Cattle with a specific haplotype which changed the resultant amino acid sequence (VWHRR) displayed a significantly different response when compared with the other haplotypes. Cattle of this haplotype all required antibiotic intervention in addition to demonstrating increased bacterial counts in the mammary gland, increased inflammation (assessed by severity scores of the udder) and also high somatic cell scores [7]. Studies on *CXCR2* SNPs showed that cattle which were CC homozygous for a SNP in *CXCR2* or heterozygous neutrophils showed significantly reduced chemotaxis in response to IL-8 when compared with neutrophils from cattle who were GG [43]. When survival and ROS

production by neutrophils from cattle with these *CXCR2* SNPs were assessed *in vitro* in response to phorbol myristate acetate (PMA), neutrophils from CC homozygous individuals showed significantly increased ability to survive when exposed to IL-8 [44]. Additionally, an impaired ability by neutrophils from cattle of this genotype to produce ROS in response to PMA was also observed [44]. These studies show the importance of an animal's underlying genetic background in determining their subsequent response to infection.

Overall, it is increasingly clear that genetic variation in the *IL8* gene has important consequences for immune cell functionality and protein secretion. This also can result in impactful downstream effects on disease susceptibility and outcome following infection. This genetic variation is also present across the IL8/CXCR axis and SNPs present in the receptor cause comparable differential effects on the immune response to those reported for the chemokine gene. It hasn't been previously explored in the literature whether possession of a particular IL8 haplotype along with a particular CXCR1 haplotype would be detrimental or advantageous for the overall immune response to infection.

1.2 Interleukin-8 and the innate immune response

1.2.1 Introduction to interleukin-8 and the innate immune response

The systemic innate immune response occurs when chemokines such as IL-8, form a chemoattractant gradient from a local site, to recruit neutrophils from peripheral blood during infection of the tissue such as the mammary gland during mastitis. Immediately after birth, calves are exposed to multiple antigens that activates their innate immune response. However, at this stage their adaptive immune system which provides T and B-cell responses, is still naïve so they rely heavily on their innate immune response [45]. Even though the immune system is naïve, it still is at risk of providing an excessive response to an antigen therefore neonates are programmed to produce a minimal amount of pro-inflammatory mediators in order to limit any damage caused by inflammation. Pathology caused by excessive inflammation could be detrimental to a calf at this young age and could result long term effects on vital organs or death [46].

The majority of infectious diseases which cattle develop begin in local tissue sites including the skin, lung, mammary gland and endometrium [47-49]. The swift initiation of a robust innate immune response is the responsibility of local immune cells such as tissue resident macrophages but also epithelial cells and fibroblasts which can secrete IL-8 to recruit neutrophils and clear the infection before requiring an adaptive immune response. As mentioned previously, genetic variation in the *IL8* gene can result in gene expression changes which could hinder the early innate immune response and result in increased disease susceptibility [34]. Therefore, the early innate immune response is critical for efficient clearing of infection and whether it progresses to systemic chronic infection.

1.2.2 Factors influencing the innate immune response in young calves

The innate immune response is heavily relied upon at birth as is the calf's passive immunity. This is provided to the calf via maternal antibodies which are maintained after birth by the consumption of nutrient rich colostrum. Over time, passive immunity gradually wanes as the calf moves from a milk diet to a grass/grain diet. Also, at this time the calf is beginning to produce its own antibodies through the process of active immunity. The calf is responding to antigens entering the body and producing antibodies against them while also gaining immune memory [50]. Not only are calves vulnerable to pathogens in early life they also are subject to stressors which may have a negative effect

on their immune system. These stressors include transportation, dehorning, castration and weaning. Stressors can cause inflammatory responses which will challenge the immune system of these calves when they are already in weakened immune state particularly during the period of what is known as the window of susceptibility [51]. This is the period where maternal antibodies are being cleared from circulation and also the calf's own antibodies are low as illustrated in Figure 1.1. This window in particular is where the innate immune response is heavily relied upon to provide protection against pathogens [50].

Colostrum has a large effect on the new-born calf's' immune status. It is recommend a calf receives colostrum within two hours of birth and no more than 4 hours after as their ability to absorb IgGs (immunoglobulin Gs) can subsequently diminish [52]. The quality of colostrum can also influence the effectiveness of the calf's immune response. The most important content is IgGs which enables passive immunity for the calves. Antibodies are transported from peripheral blood to the mammary gland via the neonatal Fc receptor which is found on mammary epithelial cells [53]. The presence of an adequate amount of growth promoting factors is particularly important for immediate weight gain over the first week of life which is crucial calves energy needs in mounting an effective immune response [54].

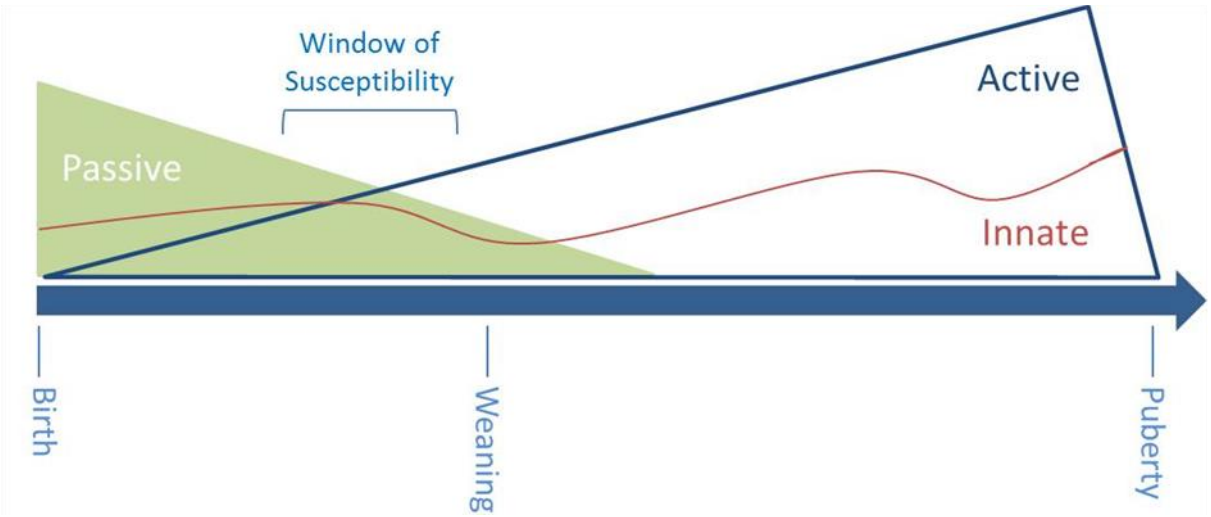

Figure 1.2 The immune system of the calf in early life

The first six months of a calf's life contain a number of stressors which can results in large variation in the capabilities of the immune systems. The gradual turnover of maternal antibodies presents a window of susceptibility where the calf's endogenously produced antibodies are only beginning to be made (adapted from: [50]).

1.2.3 Cellular components of the systemic innate immune response

Some of the cell types involved in the innate immune response and are key responders and producers of IL-8 include granulocytes (neutrophils, eosinophils and basophils), macrophages and monocytes. The neutrophil is the most abundant leukocyte in peripheral blood [55]. It is seen as an important early cell defence against bacteria, however more recent evidence suggest previously underappreciated roles in viral defence [56]. These will be discussed in further detail.

Eosinophils are a less abundant granulocyte when compared to the neutrophil. They also contain granules important for pathogen killing. Granules are contained in vesicles which are released upon activation and eosinophils perform many similar functions to neutrophils such as phagocytosis and also releasing extracellular DNA traps. However, they also play an additional role in maintaining tissue homeostasis and repair. They do this by releasing cytokines such as transforming growth factor β (TGF-β) and interleukin 13 (IL-13), which play strong roles in promoting fibrosis [57]. They have also been reported to be efficient in parasite killing and have been observed proliferating in response to helminth infection [58]. Eosinophils have also been described in faciolosis infection in beef cattle [59]. Basophils, another type of granulocyte, have been known to be involved in allergic responses in humans. However, they are now being considered to have more of modulatory role in directing the Th2 arm of adaptive immunity by expressing interleukin 4 (IL-4) [60]. They are also considered to be involved in the bovine immune response to parasites such as *A. americanum* [61].

Macrophages are found within tissues and act as a monitor continuously surveying the environment for potential pathogens. They can start phagocytosis early during infection and begin expressing pro-inflammatory cytokines and chemokines to initiate the innate immune response [45]. Bovine macrophages have been particularly well characterised in respiratory disease such as tuberculosis (TB). Macrophages resident to alveolar sites are essential in the response to *M. bovis.* As these are the first cell type to come into contact with the pathogen it is no surprise that they upregulate important chemokines such as IL-8 to recruit additional immune cells to the sight of infection [62]. Most tissue-resident macrophages are established from monocytes with their primary role to replenish populations. Current knowledge is that bovine monocytes are present in three distinct populations based on CD14 and CD16 expression. They have been given the title classical (c), intermediate (int) and non-classical (nc). cMonocytes are defined as carrying

out traditional functions such as phagocytosis whereas intMonocytes are shown to be more inflammatory, producing high levels of reactive oxygen species (ROS) and IL-1β. The role of bovine ncMonocytes is still unclear but they could carry out a similar role to their human/mouse counterpart in viral defence [63]. Monocyte function can also vary in young calves within the first months of life. Monocytes (CD14+) from calves sampled over the first eight months showed a variation in the ability to phagocytose *S. aureus* and *E. coli* as well as in the production of ROS. Phagocytic function was increased in the first month compared to the other months. This was also true for ROS production at month two compared to other seven months. It is thought that this follows the pattern of when colostrum effects are waning. After five months, the calf's immune system is beginning to stabilise [64].

Interestingly, the bovine species has a high circulating level of γδ T-cells and young calves in particular have the highest (50-60%) [65]. Similar to its adaptive T-cell counterpart (which possesses a αβ receptor), the γδ T-cell has a γ chain and δ chain as its receptor and works as part of the IIR. In mice and humans, γδ T-cells are found at very low concentrations in circulation (sometimes as low as 1%) compared to their bovine counterparts. However, they can be found in higher numbers within tissues particularly at mucosal surfaces [66]. Bovine γδ T-cells have distinct subsets based on their expression of surface receptor known as workshop cluster 1 (WC1). This a type of scavenger receptor which is primarily expressed on γδ T-cells found in peripheral blood. There is further subdivision based on expression of certain WC1 genes such as WC1.1 and WC1.2. Each is seen to react to specific pathogens. WC1 is considered to act as a co receptor for the γδ T-cell receptor (γδ TCR) and was found to also act as a pathogen recognition receptor (PRR) for the *Leptospira* species. There is also evidence of alternative splicing that results in secretion of WC1 by removal of the transmembrane domain suggesting it may function as an antimicrobial peptide [67] .

1.2.4 Interleukin-8 and the local innate immune response

The local immune response is an essential component of innate immunity that ensures a rapid reaction to infection. This early response is key to the resolution of infection and if inadequate could result in a longer and chronic infection. Some of the cell types involved in local immunity include epithelial, stromal or fibroblasts, endothelial and additionally immune cells such as tissue resident macrophages [68].

A number of infections in cattle begin in a local site. Mastitis, a common infection of the mammary gland, begins when bacteria such as *E. coli* or *S. aureus* infiltrate the tissue in teat of cows. Endometritis, an infection of the endometrium post-partum, begins when bacteria pervade the endometrial tissue following epithelial barrier disruption during birth. These infections, if not managed correctly, can results in more chronic infections than can be detected by systemic markers present in peripheral blood [47, 48].

Epithelial cells form a fundamental barrier through tight junctions on top of the stromal cells which protects from the infiltration of bacteria while also being exposed to an environment of microbes [69]. Disruption of this barrier can result in bacteria penetrating the stromal layer resulting in the underlying fibroblasts secreting inflammatory cytokine and chemokines such as IL-8 in order to request cells including monocytes and neutrophils provide support in clearing the infection [68, 69]. Fibroblasts from humans have demonstrated the ability to produce high levels of IL-8 and recruit neutrophils via chemotaxis [70]. In the human lung, IL-8 secretion by fibroblasts has been described to be induced by resident macrophages through the production of TNF-α. This action would result in recruitment of a large number of neutrophils to the lung during infection [71]. Human epithelial cells have similarly been shown to produce IL-8 in recruitment of neutrophils but interestingly, this effect was enhanced by the addition of endothelial cells in a bi-culture showing that cellular crosstalk is a substantial feature in the early local immune response [72].

Epithelial and endothelial cells have been shown to be excellent producers of IL-8 in order to attract neutrophils into the area during an infection of the mammary gland in cattle. Using an *in vivo E. coli* infection model, it was shown that epithelial cells primarily produce IL-8 in the first eight-twelve hours post infection after which it gradually decreases by twenty-four hours post infection [73]. At this time, there is increase in IL-8 secretion by endothelial cells, which had previously remained lower producers when compared to epithelial cells at the earlier timepoints, which may be due to a more sustained response by the endothelial cells [73]. Viral infections also result in sustained IL-8 response from bovine epithelial cells as demonstrated using an *in vitro* model of BoHV-4 in bovine endometrial epithelial cells. IL-8 levels remained increased over the first six days of infection when compared with the uninfected control levels [74]. There is a spike in IL-8 at day 7 which is when cell death is at its greatest and the infection

is persisting. This is where chronic sustained infection begins and where IL-8 secretion can become negative due to excessive inflammation and infiltration of immune cells [74].

The underlying stromal cell population becomes important when the epithelial cell barrier is breached. In the bovine species, dermal fibroblasts and IL-8 have been used as tool to predict systemic immune responses. Primary dermal fibroblasts were treated with LPS and the amount of IL-8 secreted was used to divide animals in high and low responder groups for a further evaluation by an *in vivo* LPS challenge. It was found that systemic IL-8 levels in serum corresponded with the high and low responders i.e. high responders produced significantly higher levels of IL-8 in serum [75]. This work was followed up with a similar study involving an *in vivo* infection of the mammary gland with *E. coli*. Again, IL-8 production by primary dermal fibroblasts was used to divide animals into high and low responders in addition to using IL-6 production [76]. Interestingly, IL-8 and IL-6 levels were significantly correlated. It was also found that high responder animals were quicker to clear the infection when compared with low responder animals. However, the author points out that these animals may also be vulnerable to extensive tissue damage due to excessive inflammation [76]. There is also a possibility that these animals have an underlying genotype which is causing high and low production of IL-8 such as the IL8 haplotypes. Infection by the virus, BoHV-4, was shown to also upregulate expression of *IL8* and subsequent protein production in bovine endometrial stromal cells by examining promoter activity of the *IL8* gene [77]. It was discovered that a transcription factor known as ORF50 (open reading frame 50) was upregulated in response to BoHV-4 infection and this transcription factor had the ability to bind to a region of the *IL8* promoter to initiate gene expression [77].

This highlights the importance of SNPs present in the promoter region of *IL8* and how they can result in selective expression of *IL8* depending on the ability of the transcription factor to effectively bind and can occur in multiple cell types. This could also be of critical importance for the early innate immune response during a local infection if animals of a certain haplotype are slower or unable to respond to certain transcription factors.

1.3 IL-8 and the neutrophil

As discussed previously, the innate immune response is the first line of defence against the entry of pathogens into the body. One of the earliest cytokines to be produced upon a cell's interaction with a pathogen is IL-8. Its primary role is to attract neutrophils to the site of infection by chemoattractant gradient. IL-8 is particular type of cytokine known as a chemokine. Chemokines are broken up into subgroups based on amino acid sequence. The majority fall into the categories of CXC or CC the former of which provides IL-8 with its other term CXCL8. A number of cell types can produce IL-8 because of it's in importance during early infection. These cell types include epithelial and endothelial cells, fibroblasts, monocytes and macrophages [78].

1.3.1 IL-8 Function in neutrophil recruitment and activation

Rapid induction of IL-8 occurs in response to the presence of stimuli such as LPS via signalling through PRRs (pathogen recognition receptors). The secretion of IL-8 outwards from the infection site forms a gradient. When IL-8 encounters a neutrophil, it binds to CXCR1 or CXCR2 which begins the process of chemotaxis. Neutrophils also become primed to respond to pathogens by increasing ROS production. The neutrophils of cattle possessing polymorphisms in the CXCR2 genes are defective in migrating to infections sites [43]. IL-8 acting alone on neutrophils produces a different transcriptional profile than a bacterial product such as LPS. Many genes upregulated by IL-8 are not upregulated by LPS. These genes include *ACTG1* (gamma –actin) and *TPM4* (tropomyosin 4) which are both involved in cytoskeleton. LPS had a more profound effect in activating gene expression of typical pro-inflammatory cytokines such as IL-1β. This would indicate that IL-8 has more of an effect on mobility of the neutrophil and less of an inflammatory effect. Interestingly, it was shown that a combination of LPS and IL-8 can in fact cause higher expression of IL-1β than LPS alone. This would suggest when a neutrophil reaches its destination IL-8 still plays a pro-inflammatory role rather than just acting as a chemoattractant [79].

The neutrophil will begin to follow the gradient of IL-8 which becomes more concentrated closer to the point of origin. Neutrophil activation occurs which results in it being able to initiate a number of different functions which includes phagocytosis, release of ROS along with other granules and also the formation of NETs (neutrophil extracellular traps) [80].

Phagocytosis

Phagocytosis is a key mechanism used by neutrophils to engulf and kill bacteria. The process involves a large and rapid change in gene expression. Interestingly, this expression profile can differ depending on the stimulus of activation. This is particularly true in relation to cell death post phagocytosis. Two ways in which phagocytosis can be initiated include antibody mediated and complement mediated, representing adaptive and innate immunity respectively [81]. Once a pathogen is phagocytosed other neutrophil functions are employed to carry out killing such ROS and granules as seen in Figure 1.2 [80].

IL-8 alone does not encourage phagocytosis; however, its presence at the site infection has an enhancement effect on the neutrophils ability to engulf bacteria. Concentrations of between 40 ng/mL and 400ng/mL significantly increase phagocytosis of *S. aureus* by neutrophils from 2-4 month old calves [82]. Phagocytic ability has been reported to be affected by age in both human and bovine studies. Neonatal calf neutrophils have a reduced ability to phagocytose *E. coli* compared to older calves (3 weeks and above). This was only shown were reduced amounts of bacteria were used along with a shorter incubation. When bacterial numbers were increased with time neonatal and older claves had similar phagocytic ability [83]. Possibly neonatal calves may be slower in responding to bacterial burden as a study examining phagocytosis is young calves over six months showed that the number of cells phagocytosing *E. coli* remained fairly constant across the first six months of life [84]. Stressors in early life such as weaning can also decrease phagocytosis ability in calves and this can take up to one week to recover [85].

Oxidative burst and granule release

Also known as respiratory burst, this important function involves the release of ROS and oxygen free radicals (O_2^-) along with derivatives such as hydrogen peroxide or H_2O_2 [86]. As is this case with phagocytosis, oxidative burst is increased in an environment of bacteria and IL-8 [82]. Production of ROS occurs when a pathogen in present inside the phagosome. The enzyme NADPH (reduced nicotinamide adenine dinucleotide phosphate) oxidase generates ROS from free radicals (Figure 1.3). These molecules are highly effective at killing pathogens and are a key mechanism in host defence [80]. Neonatal calves (1 hr after birth) have been observed to show increased

production of ROS by their neutrophils when compared with calves that are between one and two months of age. It is thereby an important mechanism employed by calves at this age when their immune system is still naïve [83].

Bovine neutrophil granules contain a number of different components which include antimicrobial peptides, elastase, lysozyme and collagenase. These all function to damage and kill pathogen invaders. Interestingly, bovine neutrophils from other species including humans by containing larger granules but contain less of some components such elastase and lysozyme [87]. A study carried out comparing the activity of these enzymes between blood neutrophils and milk neutrophils during an experimental mastitis infection in cows confirmed that neutrophils present in milk actually show less activity than their counterparts in blood. This may suggest that neutrophils recruited from blood may have contain more of these enzymes in their granules [88].

Neutrophil extracellular traps

NETs are a relatively more recently discovered function of neutrophils that are associated with a form of cell death known as NETosis. These NETs are composed of DNA, histones, elastase, myeloperoxidase, lactoferrin and gelatinase amongst other granular peptides. The purpose of these NETs is to trap and kill microbes to prevent the spread of infection. While trapped within the NET bacteria effects are neutralised by proteases which target the toxins produced. This action has been observed against *S. flexneri* and *S. aureus* [89]. The largely accepted view was that neutrophils die in the process of producing NETs in a process that is activated by high levels of ROS within the cell [90]. This was considered a unique type of cell death along with others such as apoptosis and necrosis and was named NETosis. There has been evidence to suggest that neutrophils can survive the process and release NETs in a more controlled fashion which would limit any immunopathology. This would involve the release of mtDNA (mitochondrial DNA) only and not nuclear DNA [91]. There are cases where nuclear DNA is released and it has been suggested that high concentrations of stimulants and longs incubations may play a role [92]. In the case of neonates, it has been reported that human new-borns (premature and term) production of NETs is significantly lower when compared to adults. This coincided with reduced levels of elastase and extracellular bacterial killing. Whether this is a mechanism by which damage is limited to prevent pathology or just a deficit in the innate immune response is still unknown [93]. There

appears to be systems in place to prevent excessive NET presentation in the case of lactoferrin coating the NETs. Lactoferrin, as mentioned previously, is present on NETs but before NET release it is found to congregate at the cellular membrane. Okubo *et al.* has established that lactoferrin inhibits NET production in humans and its addition in neutrophil stimulations showed also localising to the cell membrane. This would suggest it has some role in regulating NET formation or their release from the cell [94]. Bovine neutrophils also produce NETs against a host of pathogens including parasites and bacteria. NETs were discovered to effective in limiting the action of the parasite *N. caninum* in the early stage of the innate immune response [95]. They are also protective in the mammary innate immune response during infection with *E. coli* where they are extremely effective in killing of the pathogen [96]. Surprisingly, NETs are not induced by *Mycoplasma bovis* with even ROS production being low in response to the infection. There was is also evidence to suggest that when NETs are stimulated via a different stimulant *M. bovis* could evade NET capture by releasing nucleases to cause degradation [97]. The initial non-expression of NETs against *Mycoplasma bovis* could be explained by its size compared to other pathogens. There is a mechanism in place to release NETs in response to larger sized organisms such as fungi as they cannot be phagocytised. Branzk *et al.* suggests that the ability or inability to phagocytose a pathogen is the driver in whether NETs are formed or not. This would explain why viruses have been reported to stimulate NET formation even though they are small in size. Certain pathogens are better NET stimulants than others [98].

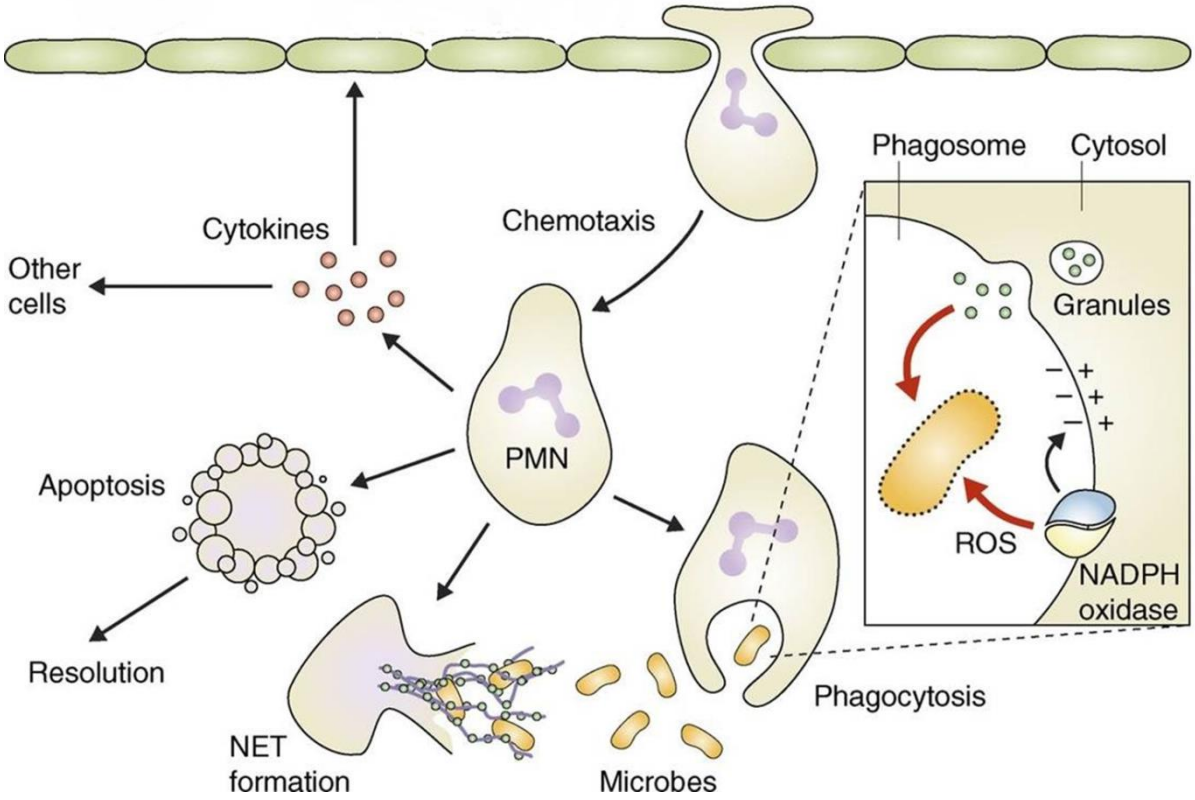

Figure 1.3 An overview of neutrophil function during infection

Neutrophils respond to pathogen infection by carrying out a number of different functions which include chemotaxis, phagocytosis, ROS generation and NET formation (adapted from [80]).

1.3.2 Neutrophil subsets

For many years, neutrophils had been described as short lived as once they home to the infection they carry out their function they die however they have been shown to have the ability to re-enter circulation after crossing the endothelial barrier into tissue during chemotaxis [99]. In more recent years, there is an expanding knowledge of neutrophil heterogeneity, longevity and how different neutrophils subsets can develop in circulation. During different states of human disease and inflammation, neutrophils which have been classified based on different cell surface marker expression and also phenotype have been described. They include what are commonly referred to as low density neutrophils or LDNs which are named due to their residence in the low-density fraction during neutrophil separation. They are known to be present in a large array of human diseases including rheumatoid arthritis (RA), asthma and also cancer [100]. This subset is thought to be an early stage of the neutrophil which shows less complex granularity. It remains to fully elucidated whether this subset develops in response to disease or whether they could be responsible for disease [101]. Another subset that has been described in mice are known as tumour associated neutrophils or TANs. Interestingly, this particular can display two phenotypes known as N1 and N2 similar to M1/M2 macrophages. The two phenotypes develop in response to different cytokines and display different in response to tumours. N1 is anti-tumour and develops in response to IFN-β while N2 is pro-tumour and develops in response to TGF-β [102]. Myeloid derived suppressor cells (MDSCs) have been extensively described in the literature in mice and are closely associated with monocytes. However, granulocyte MDSCs are considered a subset of neutrophils and display functions that help the tumour in its progression [100]. It is important to mention that more recently there is debate about considering these as exact subsets of neutrophils rather than just the plasticity of neutrophils that change phenotype and surface marker expression in response to their environment [100].

Recent work in the bovine species has described regulatory neutrophils which can influence the adaptive immune response via suppressing T-cell activity. These regulatory neutrophils express MHC-II and are functionally different than classical neutrophils where observed ROS production was much greater for the regulatory neutrophils and they also displayed a vastly differently transcriptomic profile compared with their

classical counterparts. The most interesting observation was that the addition of regulatory neutrophils to activated T-cells completely suppressed T-cell proliferation in a majority of the animals [103]. This exciting work presents new avenues to be explored in bovine neutrophil function beyond their killing capacity via phagocytosis and NET formation.

1.4 Vitamin D

Vitamin D was originally considered to be only important for the health and development of the skeletal system but in recent years it has been discovered to have immunomodulatory properties. There is now emerging evidence that vitamin D has crucial effects on overall immune system function [104]. Sources of vitamin D are primarily UVB (ultraviolet B) rays and also diet. Vitamin D can be found naturally in two forms D_2 and D_3. In the case of cattle, vitamin D_2 (also known as ergocalciferol) can be sourced from grass or grass products and fungi, whereas vitamin D_3 (also referred to as cholecalciferol) is synthesised in the skin via UVB or can come from milk and concentrates. Before vitamin D can utilised by the immune system, it undergoes a number of enzymatic steps to reach its active form [105].

The traditional route by which cattle produce vitamin D_3 begins with exposure to UVB rays via sunlight. The chemical 7-dehydroxycholesterol or pro-vitamin D_3 is present in the skin and UVB rays can convert it to pre-vitamin D_3. This process occurs very quickly upon exposure to UVB and reaches what is considered a maximum level of pre-vitamin D_3 within hours. Two other related products known as lumisterol and tachysterol are also formed in this process. Lumisterol continues to be formed even after pre-vitamin D_3 has reached its maximum level. However, when pre-vitamin D_3 levels fall, lumisterol can be converted to pre-vitamin D_3. This mechanism ensures that even when there is only a very short exposure time to UVB rays, there is a slow conversion thermally of pre-vitamin D_3 via isomerisation to produce vitamin D_3 and also conversion of lumisterol to pre-vitamin D_3 [106]. In humans, melanin in the skin acts as an absorber of UVB rays and reduces the ability of the skin to convert pre-vitamin D_3 to vitamin D_3. This is a reason why darker skinned individuals in areas close to the equator are considered to have lower vitamin D levels [107]. Cattle show a large variety of skin melanin patterns depending on their breeding. Holstein-Friesians for example have patches of light and dark skin that vary in size depending on the individual animal. This in area that is undergoing more studies to understand how cattle breed may impact on skin conversion of vitamin D_3.

Vitamin D_2/D_3 can also enter the body from dietary via intestinal absorption [105]. Cattle consume grass and grass products such as silage which is their primary source of vitamin D_2. Vitamin D_2 is also known as ergocalciferol and is converted to this form from ergosterol through UVB radiation in plants and fungi (ref). However, it is

present at low concentrations and is considered to be less efficient compared to its D_3 which can be accessed much easier [108]. Vitamin D_3 can be found or fortified in milk replacer and concentrate feed and can also be provided by supplements. D_2/D_3 is transported around the circulatory system by VDBP (vitamin D binding protein). VDBP is a transport protein responsible for shuttling vitamin D in its many forms throughout the bloodstream. It is encoded for by the *GC* (group-specific component) gene. It is important as it has a major influence over bioavailability of vitamin D metabolites [109].

The liver is where D_2/D_3 first undergoes enzymatic conversion to its in-active form of 25(OH)D by the process of 25-hydroxylation by CYP2R1 (also known as vitamin D 25-hydroxylase) carried out by hepatocytes. This can also be performed by CYP27A1 (also known as sterol 27-hydroxylase) [110]. This in-active form of vitamin D can itself be locally converted to active vitamin D by certain immune cells and epithelial cells which will be discussed later. 25(OH)D is transferred by VDBP to the kidneys where it is converted to $1,25(OH)_2D$ or active vitamin D by CYP27B1 (or 1α-hydroxylase). Active vitamin D is then circulated by VDBP to different tissues in order to carry out functions in calcium homeostasis and immune modulation. The active and in-active can also be degraded in the kidneys by the action of cytochrome P450 family 24 subfamily A member 1 or 24-hydroxylase (CYP24A1) which performs hydroxylation to form $1,24,25(OH)_2D$ and 24,25(OH)D which are removed by excretion [111]. A summary of the main components involved in the vitamin D pathway can be observed in Figure 1.4.

The vitamin D status of an individual is usually established by measuring serum levels of 25(OH)D. This is currently the preferred method due to the in-active form being more present in serum as a reservoir. Active vitamin D tends to carry out functions or be in-activated and excreted via the kidneys. Although there are now suggestions that ratios of different vitamin D metabolites may be a more accurate method [112]. The recommended serum levels of 25(OH)D for humans in the UK are above 25 nmol/L or 10 ng/mL [113]. This is in stark contrast to the US were recommended levels are above 30 ng/mL [114]. Currently, cattle in the US are also subject to this recommended level however, it is now believed that levels for dairy cows may re need to be increased. There is also a need for supplementation where cattle are housed indoors [115].

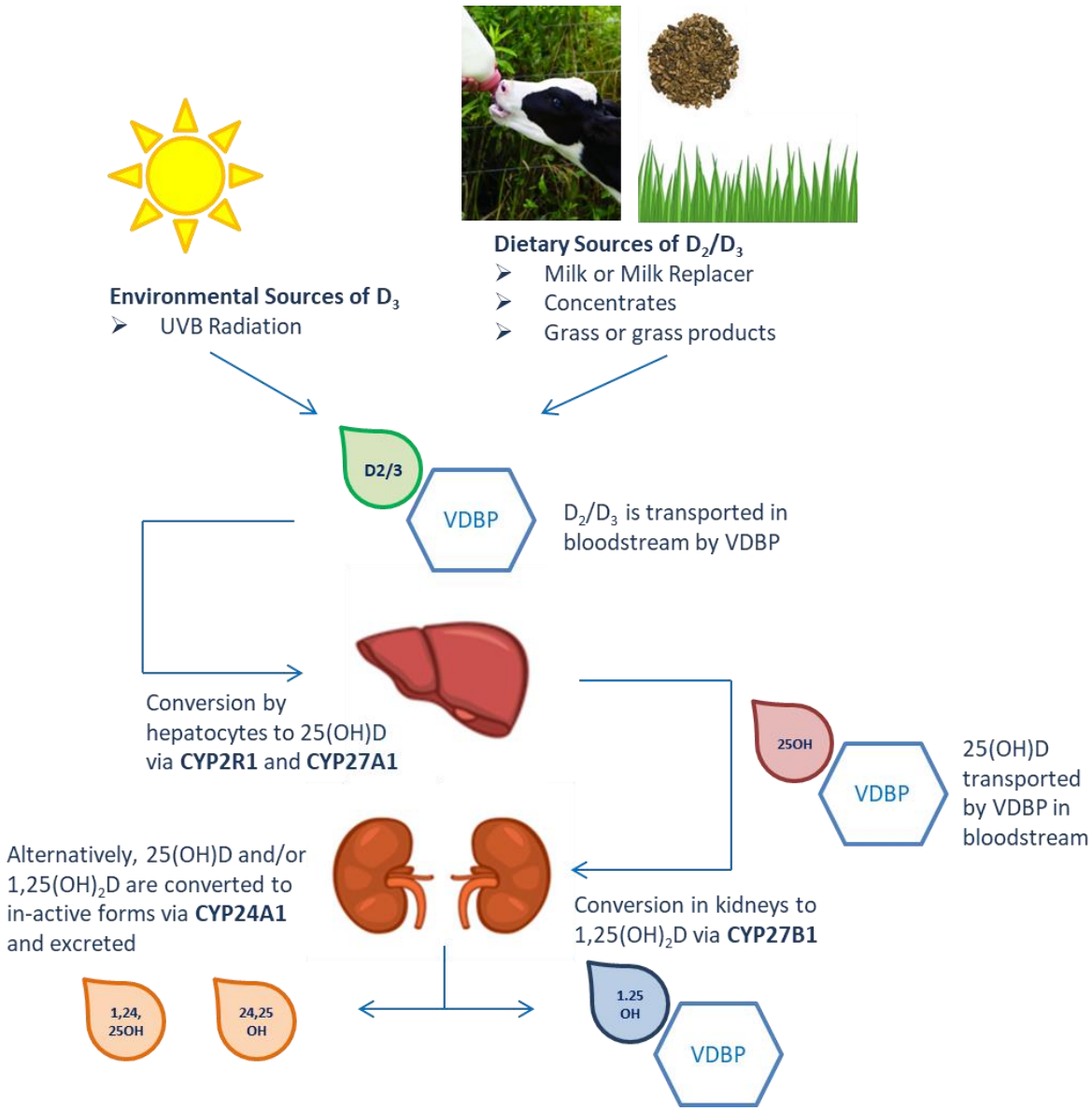

Figure 1.4 The process of vitamin D absorption and utilisation in cattle

Vitamin D is attained through two primary sources: food and sunlight. Before it can be utilised it is processed through enzymatic pathways in the liver and kidneys (adapted from [105], [116] and [117]).

1.4.1 The role of vitamin D in the innate immune response

Vitamin D has wide effects across the immune system including both the innate and adaptive branches. It can carry out immunomodulatory effects in its active and in-active forms. However, 25(OH)D must be locally converted to $1,25(OH)_2D$. In order for this to happen the immune cell in question must express CYP27B1. Bovine monocytes have been shown to express CYP27B1 in response to LPS stimulation and convert $25(OH)D_3$ to $1,25(OH)_2D_3$ indicating that monocytes respond to bacterial infection by insuring increased amounts of active vitamin D_3 are available. A feedback loop also exists when there is an over availability of $1,25(OH)_2D_3$. CYP24A1 is highly expressed when in the presence of $1,25(OH)_2D_3$ only in order for it to be degraded. Innate immune response related genes such as iNOS (inducible nitric oxide synthase), RANTES (regulated on activation, normal T cell expressed and secreted) and IL-1β were seen to be increased in response to LPS and $1,25(OH)_2D_3$ rather than just LPS alone [118]. A separate study carried out on human macrophages also found increased IL-1β in response to LPS and $1,25(OH)_2D_3$. The macrophages showed a reduced phagocytic capacity as well as a significant reduction in the amount of *E. coli* phagocytosed in the presence of LPS and $1,25(OH)_2D_3$. This may indicate that vitamin D can alter macrophage phenotypes [119]. DBP also plays a role in influencing monocyte responses to vitamin D_3. When serum is depleted of DBP, production of cathelicidins is further increased in response to $1,25(OH)_2D_3$ or $25(OH)D_3$ compared with serum containing DBP. Bioavailability of vitamin D_3 is therefore controlled by DBP [109].

Although not strictly an immune cell, epithelial cells are not just a barrier cell but play an important role in the innate immune response by alerting immune cells to the presence of infection. They can also convert in-active vitamin D_3 to its active form but this seems to be tissue specific [120]. In a viral infection with RSV, human bronchial epithelial cells responded to $1,25(OH)_2D_3$ by increasing expression of CYP27B1. In the case of rhinovirus, the presence of $1,25(OH)_2D_3$ resulted in the modulation of the innate immune response by reducing expression of IL-6 and IL-8 at a mRNA and protein level. However, there was in increase in important ISGs for the anti-viral response and also cathelicidins which have previously been identified as showing increased expression in response to vitamin D [121]. Cathelicidins are small peptides with antimicrobial properties that are released by a number of cell types in including epithelial cells. Endocervical epithelial cells (human) produced higher amounts of cathelicidin mRNA

when stimulated with $1,25(OH)_2D_3$ compared to LPS. A combination of both produced even higher levels than the individual stimulations. This again points towards vitamin Ds immunomodulatory role in the innate immune response of epithelial cells to limit inflammation and promote antimicrobial peptide expression [122].

The overall impact of vitamin D supplementation on preventing or reducing severity of infection in humans is still widely discussed in the literature. There is particular scrutiny on how supplementation trials are conducted in humans and whether they are actually capturing a direct effect of vitamin D. These discussions have come to the forefront most recently during the COVID-19 pandemic. There have been a large number of observational studies recording that supplementation with vitamin D can reduce the likelihood of developing severe COVID-19. However, these studies contain confounding factors such as the fact that many of the precipitants which display low levels of vitamin D such as older individuals in long term care (which spend large amounts of time indoors) or individuals from ethnic or minority groups (largely darker skin) are all described as being disproportionately affected by the pandemic [123, 124]. Better designed intervention trials are required in the future in order to investigate the link between vitamin D and major respiratory diseases such as COVID-19 [125].

1.4.2 Vitamin D and IL-8

There a several publications in the literature which show a definite relationship between IL-8 expression and vitamin D metabolites. $1,25(OH)_2D_3$ binds to the VDR expressed on the target cell whereby then it may bind VDR binding sites on specific genes. The *IL8* gene cluster has been shown to be responsive to $1,25(OH)_2D_3$ stimulation. It has been established that the cluster, which contains other chemokine genes such as *CXCL1, CXCL5* and *CXCL6,* contains a region of high rate open chromatin. Within this region there is a VDR binding site just downstream from the *IL8* transcription start site which is most likely the where $1,25(OH)_2D_3$ bound to VDR can modulate gene expression in the cluster. Stimulation of a monocyte cell line with $1,25(OH)_2D_3$ of a course of eight hours resulted in time dependent significant increase in *IL8* expression as well as other chemokines in the cluster. However, the biggest increases were seen in *IL8.* There same result occurred in the case of differentiation into macrophages but the expression levels of *IL8* were lower overall. This would suggest that differentiated cells may be less susceptible to modulation by vitamin D [126]. The effect is not just limited to inducing expression of IL-8 but also downregulation depending on the mechanism of activation.

In the case of a monocyte derived macrophage (MDM) in a hyper inflammatory state, $1,25(OH)_2D_3$ has an anti-inflammatory effect on IL-8 production (during bacterial stimulation) reducing levels in a dose dependent manner. However, the effect is the opposite in the case of what are considered as 'non-responder' MDMs (low levels of IL-8 upon bacterial stimulation). $1,25(OH)_2D_3$ increases IL-8 expression in a dose dependent manner and in both cases the higher doses of $1,25(OH)_2D_3$ has a statistically significant effect. The in-active form $25(OH)D_3$ has the same effect as its active form [127].

The effects of vitamin D are likely to depend upon whether the cell in question is in pro-inflammatory state via activation by a PAMP or if it was in a 'resting' state. Vitamin D can stimulate a cell to produce IL-8 to attract neutrophils via chemotaxis most likely in early infection [126]. This not only seems to be the case in monocytes/macrophages but also in neutrophils. Neutrophils significantly increase their IL-8 production when pre-treated with vitamin D and stimulated with LPS in an effort to attract more neutrophils. Interestingly, in this study it was found that macrophages didn't respond differently to vitamin D pre-treatment compared to the vehicle control [119]. This may be because of the differentiated state of the macrophage being less IL-8 responsive to $1,25(OH)_2D_3$ as described by Ryynamen *et al.* It was also suggested that after vitamin D binds to VDR it then interacts with the *IL8* gene cluster to initiate expression. It may also be suppressing components of activated pro-inflammatory pathways such as NF-κB, which would explain its dual immunomodulatory effect being both pro and anti-inflammatory [126, 128]. The NF-κB pathway inducing *IL8* expression has been observed to be influenced by $1,25(OH)_2D_3$ in a melanoma cell line. TNF-α induces *IL8* expression via NF-κB signalling. When a biologically relevant dose (20 nM) of $1,25(OH)_2D_3$ is applied, it reduces IL-8 levels by approximately 50%. The mechanism by which this occurred was through $1,25(OH)_2D_3$ interfering with transcription factors preventing them from binding to the promoter region of the *IL8* gene. The area in question is where NF-κB is known to bind, thus confirming that $1,25(OH)_2D_3$ bound to its VDR prevents NF-κB from binding to the *IL8* promoter [129]. Another study further investigated how the VDR interferes with NF-κB binding and established this was through interference with the IKKβ (IκB kinase β) protein which is involved in degradation of the IκB protein. While IκB remains bound to NF-κB it cannot enter the nucleus and initiate *IL8* expression. VDR can directly bind IKKβ to prevent it from phosphorylating IκB and thus not allowing for its degradation and the ability for NF-κB

to enter the nucleus [128]. This result was further supported when a previous study had identified that primary fibroblasts isolated from VDR knockout mice had markedly increased expression of pro-inflammatory cytokines such as IL-1β and IL-6 which resulted from increased activity of NF-κB [130].

Vitamin D status clearly has ramifications overall innate immune function however, it has notable immunomodulatory properties in the case of *IL8* expression. The VDR has been shown to have the ability to directly bind the *IL8* promoter region and initiate *IL8* expression while also being able modulate expression by interfering with the ability of the transcription factor NF-κB to bind to its target gene promoter [126, 128]. This investigative work has almost been exclusively carried out in humans and mice, so it is unknown whether these effects of vitamin D on *IL8* are true for the bovine species. Also, the presence of SNPs in the promoter region of *IL8* could have negative impact in the ability of VDR to bind to the region thus affecting the immunomodulatory effects of vitamin D on *IL8.*

1.5 Hypothesis and Aims

We hypothesise that IL8 promoter haplotype (IL8-h1 and IL8-h2) directly influences IL-8 responses both systemically and locally in primary dermal fibroblasts. Additionally, these divergent haplotypes influence cattle vitamin D status.

The specific aims of the project were:

1. Investigate the peripheral blood IL-8 response of calves with different IL8 haplotypes to bacterial, viral and fungal PAMPs.
2. Establish the influence of IL8 haplotype on vitamin D levels in peripheral blood and on the IL-8 response to vitamin D.
3. Examine the effect of IL8 haplotype on the IL-8 response of primary dermal fibroblasts and investigate whether vitamin D modulates these responses differently between both haplotypes.

Chapter 2. Materials and Methods

Table 2.1 List of general reagents

Reagent	Company
Cell isolation, culture and assays	
1,25(OH)$_2$D$_3$, vitamin D	Sigma-Aldrich
Accutase cell detachment solution	Biolegend
Anti-bovine granulocyte monoclonal antibody	Kingfisher Biotech
Amphotericin B solution	Sigma-Aldrich
Anti-Mouse IgG MicroBeads	Miltenyi Biotec
Bovine serum albumin (BSA)	Sigma-Aldrich
Collagenase II	Sigma-Aldrich
CD14 MicroBeads, human	Miltenyi Biotec
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich
Distilled, sterile, nuclease-free water	Gibco (Thermo Fisher Scientific)
EDTA	Sigma-Aldrich
Ethanol	Sigma-Aldrich
Fetal bovine serum (FBS)	Gibco (Thermo Fisher Scientific)
Fixative-Free Lysing Solution, High-Yield Lyse	Invitrogen (Thermo Fisher Scientific)
Hank's balanced saline solution	Gibco (Thermo Fisher Scientific)
LS Column (MACS)	Miltenyi Biotech
IL-8, bovine, recombinant	Kingfisher Biotech
Insulin-Transferrin-Selenium-Ethanolamine (ITS-X) solution	Gibco (Thermo Fisher Scientific)
LPS from *E. coli*	Sigma-Aldrich
Micrococcal nuclease	New England Biolabs
Pam3CSK4	InvivoGen
Penicillin-Streptomycin	Invitrogen (Thermo Fisher Scientific)
Phosphate buffered saline (PBS)	Gibco (Thermo Fisher Scientific)
PicoGreen dsDNA stain	Thermo Fisher Scientific
Poly(I:C) (HMW)	InvivoGen
Recovery™ Cell Culture Freezing Medium	Gibco (Thermo Fisher Scientific)
RPMI 1640 + GlutaMAX	Gibco (Thermo Fisher Scientific)
Trypan blue	Sigma-Aldrich
Zymosan	InvivoGen
Molecular biology	
Chloroform	Sigma-Aldrich
Custom SNP Genotyping Assays	Applied Biosystems (Thermo Fisher Scientific)
Ethanol, molecular grade	Sigma-Aldrich
Fast SYBR Green PCR Master Mix	Applied Biosystems (Thermo Fisher Scientific)
High-Capacity cDNA Reverse Transcription Kit	Applied Biosystems (Thermo Fisher Scientific)
H_2O, molecular grade	Sigma-Aldrich
Fast SYBR Green Master Mix	Applied Biosystems (Thermo Fisher Scientific)
Maxwell® 16 DNA Purification Kit	Promega
Primers, qPCR	Sigma-Aldrich
RNA 6000 Nano Kit, bioanalyser	Agilent Technologies

RNeasy Mini Kit	Qiagen
TaqMan Universal PCR Master Mix	Applied Biosystems (Thermo Fisher Scientific)
TE buffer	Sigma-Aldrich
TRIzol	Ambion (Thermo Fisher Scientific)
TruCulture tubes (LPS, poly (I:C), zymosan)	Myriad RBM
ELISA	
0.5M Sulfuric acid	Sigma-Aldrich
25(OH)D ELISA Kit	Eagle Biosciences
Bovine IL-1β ELISA Kit	Thermo Fisher Scientific
BupH™ Carbonate-Bicarbonate Buffer	Thermo Fisher Scientific
DPBS, no calcium, no magnesium	Gibco (Thermo Fisher Scientific)
Fish skin gelatin	Sigma-Aldrich
Mouse anti-sheep IL-8 monoclonal antibody	Bio-Rad
Polyclonal goat anti-rabbit horseradish peroxidase	Agilent Technologies
Rabbit anti-sheep IL-8 polyclonal antibody	Bio-Rad
Recombinant Bovine IL-8	Kingfisher Biotech
SureBlue reserve TMB microwell peroxidase substrate	KPL
Tween 20	Sigma-Adrich
Immunocytochemistry	
DAB Substrate Kit, Peroxidase (HRP)	Vector Laboratories
Goat serum	Gibco (Thermo Fisher Scientific)
Haemotoxylin	Dako
Hydrogen peroxide	Sigma-Aldrich
Methanol	Sigma-Aldrich
VectaMount	Vector Laboratories
Flow cytometry	
AbC™ Anti-Mouse Bead Kit	Invitrogen (Thermo Fisher Scientific)
APEX™ Alexa Fluor® 488 Antibody Labelling Kit	Invitrogen (Thermo Fisher Scientific)
APEX™ Pacific Blue™ Antibody Labelling Kit	Invitrogen (Thermo Fisher Scientific)
Attune™ Focusing Fluid 1X	Invitrogen (Thermo Fisher Scientific)
Propidium Iodide	Invitrogen (Thermo Fisher Scientific)

Table 2.2 List of general materials

Reagent	Company
Cell isolation, culture and assays	
3.5 mL transfer pipette, sterile	Starstedt
10/25 mL plastic pipettes	Starstedt
15/50 mL tubes	Starstedt
6-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)
12-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)
24-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)
96-well flat bottom tissue culture plates	Cruinn Diagnostics (Cellstar)
96-well flat bottom plates, black	Cruinn Diagnostics (Cellstar)
70µm filters	Fisher Scientific
Cryotubes	Thermo Fisher Scientific
Forceps with curved teeth	Lennox
QuadroMACS™ Separator	Miltenyi Biotec
Petri dishes	Greiner Bio-One
Scalpel, disposable, sterile	Swann-Morton
T175 Tissue culture flask, vented	Starstedt
T75 Tissue culture flask vented	Starstedt
Vacutainers, acid citrate dextrose	Greiner Bio-One
Vacutainers, EDTA	Greiner Bio-One
Vacutainers, sodium heparin	Greiner Bio-One
Molecular biology	
MicroAmp™ Optical 96-well reaction plates	Applied Biosystems (Thermo Fisher Scientific)
MicroAmp™ Optical adhesive film	Applied Biosystems (Thermo Fisher Scientific)
0.5/1.5/2.0 ml nuclease-free, sterile microcentrifuge tubes	Sigma-Aldrich (Eppendorf)
ELISA	
96 well ELISA plates	Greiner Bio-One
Immunocytochemistry	
EZ SLIDE 4 well glass culture slides	Merck Millipore
Flow cytometry	
3mL tubes	Starstedt

Table 2.3 List of antibodies used

Antibody	Supplier	Species
Anti-bovine CD3	Washington State University	Mouse
Anti-bovine CD4, FITC	Bio-Rad	Mouse
Anti-bovine CD8	Washington State University	Mouse
Anti-bovine CD11c	Washington State University	Mouse
Anti-bovine Granulocyte (G1)	Washington State University	Mouse
Anti-bovine sIgM (B-cells)	Washington State University	Mouse
Anti-bovine TCR γδ	Washington State University	Mouse
Anti-bovine WC1	Washington State University	Mouse
Anti-mouse IgG, FITC	Southern Biotech	Goat
IgG1 Negative control	Bio-Rad Antibodies	Mouse
IgG2a Negative Control	Bio-Rad Antibodies	Mouse
Vimentin	Sigma-Aldrich	Mouse
Cytokeratin	Dako	Mouse
Anti-Mouse HRP	Sigma-Aldrich	Rabbit

Table 2.4 List of equipment used

Reagent	Company
7500 Fast Real-Time PCR System	Applied Biosystems (Thermo Fisher Scientific)
ADVIA 2120	Bayer Healthcare
Attune Flow Cytometer	Applied Biosystems (Thermo Fisher Scientific)
Bioanalyser 2100	Agilent Technologies
GloMax Multi+ Detection System	Promega
Graphpad Prism 8	Graphpad Software Inc.
Mastercycler gradient	Eppendorf
Maxwell 16 Automated nucleic acid extraction system	Promega
NanoDrop ND-1000 Spectrophotometer	NanoDrop Technologies
Olympus BX41 upright microscope	Leica Biosystems

2.1 Ethics statement

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.

2.2 Animal details

For the systemic immunity experimental work, ten male Holstein-Friesian calves were purchased from a commercial dairy farm and moved the Teagasc Animal and Biosciences Research Centre, Grange, Co Meath 3 weeks after birth. Calves were immunised on arrival with Bovipast® RSP. They were kept outdoors on pasture throughout the experimental period which began one week after arrival. The calves were bucket fed 4 L of milk replacer twice daily for one month after arrival. After this period, it was reduced to 2 L once a day for a further two weeks before weaning. They were also provided with calf pellets and fresh water *ad libitum.*

For the vitamin D supplementation trial, sixteen male Holstein-Friesian calves were purchased from a commercial dairy farm. They were removed from the dam and fed 6 L of colostrum within 4 h of birth and were transported to the research farm within 24-48 h. All calves were group housed and bucket fed with milk replacer. Calves were fed with 3 L of milk replacer from 0 to 14 days, 6 L from 15 to 60 days of age, and then 3 L from 60 to 70 days. They were also provided with calf pellets and fresh water *ad libitum*. Weaning was done at 70 days of average age, then a commercial pellet was offered once a day. The vitamin D supplemented group were moved outdoors on pasture after weaning. The control group were kept in confinement during the duration of the trial and were provided with alfalfa hay and silage *ad libitum*.

For the local immunity experimental work, ten one –year old Holstein-Friesian heifers were used. At the time of sampling, the heifers were outdoors on pasture at Teagasc Oakpark, Co Carlow where they were provided with fresh water *ad libitum.*

2.3 DNA extraction, IL8 genotyping and vitamin D pathway gene SNP search

Blood was collected in 9 mL EDTA coated vacutainers via jugular venepuncture. Whole blood was used for DNA extraction via the Maxwell® 16 DNA Purification Kit

for blood. The extraction was carried out using the Maxwell® 16 Instrument via the manufacturer's instructions. The extracted DNA was stored in 1.5 mL microfuge tubes at -20°C.

A TaqMan genotyping assay (Applied Biosystems) was designed to amplify the region containing a promoter SNP (chr6: 91739266-91739367). The assay was carried out in a 96 well reaction plate with each well containing 10 ng of DNA in 11.25 µL of molecular grade water, 1.25 µL of 20X SNP genotyping assay and 12.5 µL of 2X TaqMan Universal PCR Master Mix. This gave each well a final volume of 25 µL. The cycling parameters used are shown in table 2.4.

Table 2.5 Cycling parameters for TaqMan genotyping assay.

	Step 1	Step 2	Step 3	Step 4	Step 5
Temp °C	60	95	92	60	60
Time	30 sec	10 min	15 sec	1 min	30 sec

40 Cycles

TaqMan Genotyping assays were also designed to amplify regions containing SNPs: rs134037344, rs132651447, rs43315175 and rs209571234. The same reaction volumes and cycling parameters were used as stated above. The Applied Biosystems 7500 Fast Real-Time PCR System software presents the data as an allelic discrimination plot from which the animals' haplotype can be discerned based on its position on the plot.

2.4 TruCulture and whole blood culture

TruCulture (Myriad RBM) tubes which were pre-filled upon purchase with LPS, poly (I:C), zymosan along with a control containing just the proprietary medium, were left to thaw at room temperature for 1 hour prior to use. Blood was drawn directly into each tube via jugular venepuncture. The tubes were transferred within 30 min to a heat block and incubated at 37°C for 24 hours. After 24 hours, the tubes were centrifuged at 500 x g for 10 min. The tubes were opened and the Valve filter Serplas was inserted via a plunger to separate the cells and supernatant. The supernatant was then transferred to a 1.5 mL microfuge tubes and the cells were transferred to Trizol for RNA extraction.

For whole blood culture, blood was collected in 9 mL heparin coated vacutainers. 1 mL of blood was then transferred to each well of a 6 well plate containing 2 mL of RPMI 1640 with 1% penicillin/streptomycin, within 30 min of being collected. Cells were then stimulated with one of the following LPS (100 ng/mL) (Sigma), Poly (I:C) (30 µg/mL) (InVivogen), zymosan (50 µg/mL) (Sigma) or a negative control (no stimulant). The plates were then incubated at 37°C 5% CO_2 for 24 hours. After 24 hours, the well contents were transferred to microfuge tubes and centrifuged at 500 x g for 10 min. The supernatant was transferred to 1.5 mL microfuge tubes and cells were transferred to Trizol for RNA extraction.

2.5 Flow cytometry

1 mL of blood collected in a 9 mL heparin coated vacutainer was lysed in 10 mL of high yield lysis buffer (Life Technologies) for 10 min. The solution was then centrifuged at 300 x g for 10 min. The supernatant was discarded, and the pellet was resuspended in 2mL of PBS with 1% BSA. 200 µL per well was transferred to a 96 well plate.

Primary antibodies WC1, γδTCR, CD3 and CD8 (Washington State University) were conjugated to the Pacific Blue and AlexaFlur490 fluorophores using the Apex antibody labelling kits (Thermo Fisher Scientific) according to manufacturer's instructions. The CD4 antibody was purchased conjugated to FITC (Bio-Rad). 0.5 µL of each of these was added per well and the cells incubated for 30 min at 4°C in the dark. Afterwards, the plate was centrifuged at 300 x g for 5 min. The supernatant was discarded and 200 µL of PBS 1% BSA was added to each well to resuspend the cells. This wash step was carried out a further 2 times.

The sIgM antibody was unconjugated, therefore it had a secondary FITC antibody added after 1 wash step. This plate was incubated at 4°C in the dark for 30 min and wash step was carried before resuspension in 200 µL of PBS 1% BSA.

30,000 events were acquired using the Attune flow cytometer (Applied Biosystems) from a leukocyte gate based on FSC SSC profile. The results were analysed using the Attune flow cytometry software. Isotype controls were also run on the system.

2.6 Haematological analysis

Haematological profiles were established from blood collected in 6 mL EDTA coated vacutainers and processed using the ADVIA 2120 haematology system to acquire total lymphocyte, monocyte, neutrophil, basophil and eosinophil cell numbers. The system uses a light scatter, cytochemical staining and nuclear density to measure total leukocyte counts.

2.7 Neutrophil separation via magnetic activated cell sorting (MACS)

Blood was collected using acid citrate dextrose (ACD) vacutainers by jugular venepuncture. Two filled vacutainers per animal were transferred to a 50 mL tube. This was then filled up to the 50 mL mark with high-yield lysis buffer and incubated at room temperature for 10 min. Occasional inversion was performed to facilitate lysis. The lysed blood was then centrifuged for 10 min at 300 x g. The cells were then washed by resuspending the pellet in 5 mL of MACS buffer (D-PBS, 2mM EDTA, 0.5% BSA) and centrifuging at 300 x g for 10 min. The cell pellet was then resuspended in 1 mL of MACS buffer. An aliquot was taken from the cellular suspension and cells were counted using the Attune flow cytometer based on events/µL from a leukocyte gate formed on FSC SSC profile. A granulocyte-specific mouse anti-bovine G1 primary antibody (Washington State University) is added to the cell suspension (1:500 dilution) and incubated at 4°C for 15 min. A wash step was carried out and the pellet was resuspended in MACS buffer (80 µL per 10^7 total cells). Anti-mouse microbeads were added (20 µL per 10^7 total cells) and the suspension was incubated at 4°C for 15 min. A final wash step was carried out and the pellet resuspended in 500 µL. The MACS columns were placed into the magnetic separator and rinsed with 3 mL of MACS buffer. The 500 µL cell suspension was added to the column and allowed to flow by gravity. The flow-through was collected for flow cytometry analysis. The column was then washed with 3 mL of MACS buffer three times. The column was then removed and placed in a 50 mL tube where 5 mL of MACS buffer was added and pushed through the column using the plunger. An aliquot of both the positively and negatively selected cells were then analysed using FSC and SSC on the flow cytometer. The positively selected cell suspension was then centrifuged and resuspended in RPMI.

2.8 NET assay

Isolated neutrophils (1 x 10^6 cells) were added to each well of a 24 well plate containing 500 µL of RPMI 1640 with 1% penicillin/streptomycin. Cells were stimulated with LPS (200 µg/mL) along with a negative control. Zymosan (1 mg/mL) acted as a positive control. Cells were then incubated for 1 hour at 37°C 5% CO_2. 50 µL of nuclease buffer was then added to each well. This buffer contained 0.1 U/µL of micrococcal nuclease (New England Biolabs) to break up DNA strands. The plates were then centrifuged at 300 x g for 5 min. Supernatants were transferred to a black 96 well plate, 100 µL/ well in duplicate. PicoGreen (Thermo Scientific) was diluted 1:200 in TE buffer and 100 uL was added per well. The plate was then incubated at room temperature for 4 min in the dark. Fluorescence was measured using the GloMax Multi+ Detection System (Promega) using the blue light setting for excitation at 490 nm and emission between 510-570 nm. Output was expressed as average fluorescence unit (AFU).

2.9 Monocyte separation via MACs

Blood was collected using acid citrate dextrose (ACD) vacutainers by jugular venepuncture. Two filled vacutainers per animal was transferred to a 50 mL tube. 50 mL of high-yield lysis buffer was added, and cells incubated at room temperature for 10 min with occasional inversion to facilitate lysis. The lysed blood was then centrifuged for 10 min at 300 x g. Following a wash step, the pellet was resuspended in 5 mL of MACS buffer and centrifuged at 300 x g for 10 min. The cell pellet was then resuspended in 1 mL of MACS buffer. An aliquot was taken from the cellular suspension and cells were counted using a flow cytometer. The cells were then washed again, and the pellet resuspended in MACS buffer (80 µL per 10^7 total cells). CD14 microbeads were added (20 µL per 10^7 total cells) and the suspension was incubated at 4°C for 15 min. A final wash step was carried out and the pellet resuspended in 500 µL. The MACS columns were placed into the magnetic separator and rinsed with 3 mL of MACS buffer. The 500 µL cell suspension was added to the column and allowed to flow by gravity. The flow-through was collected for flow cytometry analysis. The column was then washed with 3 mL of MACS buffer three times. The column was then removed and placed in a 50 mL tube where 5 mL of MACS buffer was added and pushed through the column using the plunger. An aliquot of both the positively and negatively selected cells were then analysed using FSC and SSC on the flow cytometer. The positively selected cell suspension was then centrifuged and resuspended in RPMI.

2.10 Monocyte stimulations

Monocytes were isolated from whole blood via MACS as described above. 1 x 10^6 cells were added per well to a 12-well plate in 1 mL of RPMI 1640 with 1% penicillin/streptomycin, 1% ampicillin B and 1% FBS.

For *in vitro* vitamin D experiments, monocytes were stimulated with either 0.5 - 20 ng/mL recombinant bovine IL-8 (plus a 100 ng/mL LPS positive control) or 10-200 nM $1,25(OH)_2D_3$ (plus an 8 uL sterile ethanol vehicle control) over the course of 1 – 18 hours. For the vitamin D supplementation experiments, monocytes were stimulated with 100 ng/mL LPS for 6 hours.

After each stimulation, plates were spun at 300 x g for 5 mins, supernatant was collected and stored at -20℃ and cells were transferred to Trizol.

2.11 Vitamin D supplementation model*

Blood was taken via jugular venepuncture from six-month old male Holstein-Friesian calves undergoing a vitamin D supplementation trail for whole blood culture and monocyte isolation via MACS as described previously. The trial was a randomized complete block design with a two-by-two factorial arrangement of treatments, calves were randomly assigned to one of two treatments. Treatments are summarised in Table 2.6.

The diet of the control calves consisted of commercial pellets which contained the industry standard concentration of vitamin D. For the supplemented diet, the milk replacer and pellets were supplemented with vitamin D_3 (Rovimix D3 500, DSM Nutritional Products). The vitamin D_3 was prepared from dry powder concentrate (500,000 IU/g) to the desired concentration by dissolving in distilled water. The supplements were prepared fresh weekly and stored at 4°C. Supplements were added once daily to the milk replacer, and top dressed on the pellets. The pellets were offered to calves *ab libitum* at approximately 0800 h in the day to ensure consumption.

*Study conducted as part of another students PhD experimental work

Table 2.6 Summary of vitamin D treatments received by calves undergoing a vitamin D supplementation trial

	Control	**Supplemented**
Injection (Birth)	Vehicle	50,000 IU
Milk Replacer (Week 1-12)	6000 IU/Kg	10,000 IU/Kg
Pellets (Week 10-24)	2000 IU/Kg	4000 IU/Kg
Environment	Indoor	Outdoor

2.12 Primary dermal fibroblast 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 1 mL RPMI with 1% collagenase I (Thermo Scientific). The tubes were put in a shaking incubator at 37^{o}C rotating at 150 rpm for 4 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 x g for 10 mins. 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, 5% CO_2.

For stimulation experiments, cells at passage 3/4 were seeded at a density of $1x10^5$ 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 three hours or twenty-four hours at 37°C, 5% CO_2. After stimulation, cell supernatant was removed and stored at -20^{o}C while cells were transferred to Trizol (Thermo Fisher Scientific).

2.11 Immunocytochemistry

Cells at passage 3/4 were seeded on four-well glass slides at a density of $1x10^5$ cells/mL and grown overnight at 37^{o}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 mins to stop endogenous peroxide activity. A wash step was performed by adding 1% PBS-tween for 5 mins twice. The cells were then blocked for non-specific binding with 10% goat serum in PBS for 1 hour 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^{o}C. A wash step was performed before the secondary antibody (anti-mouse) at 1:1000 dilution was added for 30 mins 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.

2.13 RNA extraction, cDNA synthesis and qPCR

2.13.1 RNA extraction

A combination method of Trizol and the RNeasy Plus Mini Kit (Qiagen) was used to extract RNA from whole blood collected via the TruCulture tubes/ heparin vacutainers for whole blood culture assays. Chloroform was added to cells plus Trizol (200 µL/mL of Trizol) in a 1.5 mL microfuge and shaken vigorously. The solution was then centrifuged for 15 min, 12,000 x g at 4°C. The RNA containing aqueous layer was taken and moved 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 was measured using the NanoDrop and then quality was assessed using the RNA 6000 Nano Kit (Agilent Technologies) on the Bioanalyser according to manufactures instructions. Samples were then stored at -80°C prior to further processing.

2.13.2 cDNA synthesis

The RNA samples were diluted down to approximately 50 ng/µL. The RNA was then converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to manufactures' instructions. The conditions were used for the conversion are shown in table 2.1.

Table 2.7 Conditions used for cDNA conversion

	Step 1	Step 2	Step 3	Step 4
Temp (ºC)	25	37	85	4
Time (min)	10	120	5	∞

2.13.3 Primer design and qPCR

Gene DNA sequences were taken from GenBank® and the primers were designed using the Primer-BLAST program. All primers were intron spanning (were possible) to eliminate the risk of genomic DNA being amplified. The primers were then produced by Sigma. Primer details are available in Table 2.8.

2.13.4 qPCR

cDNA samples were prepared with primers using SYBR green according to the manufacturer's instructions. The samples were run on a 96-well PCR plate with negative and no reverse transcriptase controls in the Applied Biosystems 7500 Fast Real-Time PCR System. The cycling parameters used are shown in Table 2.7.

Table 2.8 Cycling parameters for qPCR analysis

	Step 1	Step 2	Step 3	Step 4	Step 5	Step 6	Step 7
Temp (°C)	95	95	60	95	60	95	60
Time (sec)	20	3	30	15	60	15	15

40 Cycles Melt Curve

Primer efficiencies were calculated using a series of dilutions of cDNA pooled from all samples. Standard curves were prepared using a semi-log regression line plot where Ct value was plotted against the log of the cDNA concentration. The formula used to calculate efficiency was $E = (10^{-1/Slope}-1)$. Primers with efficiencies of between 0.8 and 1.2 were only used.

Results from qPCR were analysed using the GenEx software package (version 5.2.1.3) where the raw Ct values were used as input. Results were corrected for primer efficiency. The GeNorm tool was used the access the potential of five candidate housekeeping genes: *ACTB, GAPDH, GUSB, RPS15* and *HSP90AB1*. There stability of expression was calculated using an M value where the lowest value was considered the most stable.

Table 2.9 Primer sequences for genes used in qPCR

Gene	Forward Primer	Reverse Primer
ACTB	AGATGACCCAGATCATGTTCGA	TGACCCCGTCACCGGAGTCCATCACGAT
CCL20	CAGCAAGTCAGAAGCAAGCA	TTTGGATCTGCACACACAGC
CYP24A1	TGAGAATCAGTTGCCACGGG	TTTGCGGACAATTCCTTTGGG
CYP27A1	GGGCAGCTACGCCTCTTATT	ATCTGAGGCCCTACTCGGTT
CYP27B1	TGGGACCAGATGTTTGCATTCGC	TTCTCAGACTGGTTCCTCATGGCT
eDEFB4A	AGGCTCCATCACCTGCTCCTC	ACAGGTGCCAATCTGTCTCATGC
GAPDH	CTCCCAACGTGTCTGTTGTG	TGAGCTTGACAAAGTGGTCG
GUSB	ACCATCGCCATCAACAACAC	TCCCGCGTAGTTGAAGAAGT
HIF1A	CAGAAGAACTTTTGGGCCGC	TACAATGCACTGGGGCTGAG
HSP90AB1	GCATGAAGGAGACGCAGAAG	TCCTTGAGCTGCTGTACACA
IL8	CATTCCACACCTTTCCACCC	CCTTCTGCACCCACTTTTCC
KRT18	ATTTCAGTCTTGGCGACGCT	GCCTCAGTGCCTCAGAACTT
NOS2	GATCCAGTGGTCGAACCTGC	CAGTGATGGCCGACCTGATG
RPS15	GCGACATGATCATTCTACCCG	GGTAGTGGCCGATCATCTCA
SAA3	CTCAAGGAAGCTGGTCAAGG	CTTCGAATCCTCCCGTACCT
TLR4	CTTGCGTACAGGTTGTTCCTAA	CTGGGAAGCTGGAGAAGTTATG
VDR	GAGGGGAACCGTCCTTTGAG	GAGAAGCTGGTTGGCTCCAT
VIM	TGCGCTCAAAGGGACTAACG	TCGAGCGCCATCTTGACATT

2.14 ELISA

The bovine IL-8 ELISA used to measure concentration in cell supernatants and serum was carried out as instructed by Cronin *et al.* [131]. The bovine IL-1β ELISA (Thermo Fisher Scientific) and bovine 25(OH)D ELISA (Eagle Biosciences) were carried out as per the manufacturer's instructions using bovine standards, prepared as previously described by Nelson *et al.* [132]. VDBP ELISA was carried out by Heartland Assays (Iowa State University Research Park, IA, USA).

2.15 Statistical analysis

Haematological, IL-8 and 25(OH)D serum data were all analysed using SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Each variable was assessed for normality by generating and plotting residuals. If the residuals of a variable were found to have an abnormal distribution, a log transformation was used to normalise the data. An outlier in the IL-8 dataset was identified and once this was omitted, data could only then be normalised. Differences in haplotype groups were calculated by month using PROC MIXED which was then used to compute the least means squares of fixed effects. A P-value of ≤ 0.05 was considered statistically significant.

All other data was assessed for normality by generating and plotting residuals and were analysed using two-way repeated measures ANOVA (or mixed models) protocol on GraphPad Prism 8. A P-value of <0.05 was considered significant. We acknowledge the low numbers of animals present in many experiments which may impact on results.

Chapter 3. IL8 haplotypes confer distinct systemic innate immune responses

3.1 Introduction

The systemic innate immune response plays an important role early during infection in setting up a network by which cells such as neutrophils, monocytes and macrophages can be recruited to the site of infection. An important player in this network is IL-8, a key orchestrator of the neutrophil response. SNPs have been previously identified in the promoter region of the *IL8* gene of cattle which segregate in two distinct haplotypes in the Holstein-Friesian breed [35]. Previous work on the systemic effects of IL8 haplotype, have shown cattle with different IL8 haplotypes produce differing levels of IL-8 in peripheral blood in response to an LPS challenge. IL8-h2 produced significantly higher serum levels of IL-8 at 12 hr post-challenge [5]. Considering the measurable effect of haplotype on systemic IL-8 levels shown in cattle it may also be true that haplotype effects may have wider reaching effects/impacts on innate immunity. Interestingly, in humans SNPs identified in the promoter region of the *IL8* gene, which also segregate into two haplotypes, have been shown to also effect the amount of IL-8 secreted in response to gram-negative bacterial infection in addition other pro-inflammatory cytokines such as TNF-α and IL-1β. Additionally, the IL8 haplotype of these individuals also affected the chemotactic capability of their neutrophils [4]. In the case of viral ligands, an association was shown between an IL8 haplotype in humans and susceptibility to RCV disease. This particular haplotype (6 SNPs) showed an increase in *IL8* gene expression using a reporter gene assay [34]. More recently, human IL8 haplotypes have been functionally characterised using CRISPR/Cas9 genome editing where the ATC/TCC haplotype was linked to increased IL-8 levels and chemotaxis of neutrophils following poly (I:C) stimulation of HEK293T carrying the haplotype when compared with normal HEK293T cells [133]. The response to poly (I:C) nor fungal ligands has not been investigated in cattle IL8 haplotypes.

In addition to IL8 haplotype influencing levels of IL-8 secreted in calves, age related changes may also play a role in the systemic production of IL-8. The previous study examining the effect of IL-8 haplotype during an LPS challenge was carried out on one-month old calves. Changes in circulating immune cell populations have been documented to occur in calves in the first six months of life. Most notably in the case of CD3+ cells, CD21+ cells (B-cells) and γδ T-cells [84, 134, 135]. Interestingly, IL-8 production by B-cells and subsequent lymphocyte recruitment has been documented in humans. B-cells isolated from human tonsil samples have been shown to produce IL-8

and this was proposed as an avenue by which T-cells that express the IL-8 receptor CXCR1 are recruited to the lymph node [136]. Subtle changes in the numbers of these immune cell populations due to age in combination with differing levels of IL-8 secretion may therefore contribute to what was previously seen in cattle where lymphocyte levels were shown to be altered with differing IL-8 haplotypes over the course of an *in vivo* LPS challenge. IL8-h2 having been shown to have increased lymphocyte numbers compared to IL8-h1 [5].

The primary cellular source of IL-8 in the systemic circulation in Holstein-Friesian cattle has not yet been characterised. IL-8 is known to be produced by a number of cell types most notably monocytes and macrophages [137]. Considering IL-8 is the primary chemokine involved in the early neutrophil response, it is generally tissue resident macrophages, epithelial and stromal cells which would be major neutrophil recruiters during tissue damage or infection [138]. During *P. haemolytica* infection in cattle, the alveolar macrophage is the primary source of IL-8 in a biphasic manner [139]. Therefore, it is likely monocytes are a key producer of IL-8 in the blood as it is a precursor to the macrophage [140]. However, human neutrophils themselves have been shown to produce IL-8 after 24 hours of stimulation with LPS *ex vivo* [141]. This presumably occurs when the first neutrophils arrive at the infection site and begin to express IL-8 to recruit additional neutrophils. Lymphocytes such as CD4+ T-cells have also been shown to produce IL-8, spontaneously and in response to IL-8 stimulation, in humans [142]. As have human γδ T-cells which have the ability to recruit neutrophils via IL-8 chemotaxis following stimulation with phosphoantigen [143]. It remains to be investigated whether neutrophils, monocyte or lymphocytes are the key IL-8 producing cell type in bovine peripheral blood.

Given the importance of IL-8 in recruiting neutrophils, previous work has also examined whether IL8 haplotypes affect neutrophil function. Chemotaxis assays carried out using serum from genotyped calves challenged with LPS revealed no significant differences between haplotypes on neutrophil migration. There was also no observable difference in *S. aureus* killing in the presence of serum from calves with two different haplotypes [5]. A relatively new discovered function of neutrophils known as NETs has not yet been investigated in calves with these haplotypes. Cattle are now known to produce NETs against a variety of pathogens including the parasite *N. caninum* and also

M. bovis [95, 97]. In the case of the latter, the microbe has evolved ways to overcome capture by NETs using nucleases to digest DNA [97]. The resulting differences in IL-8 secretion in bovine peripheral blood due to IL8 haplotype may have important downstream effects for neutrophil NET function considering IL-8 itself has been reported as a stimulus for NET formation in human studies [89]. A study performed on macrophages isolated from humans with different IL8 promoter haplotypes showed differences in their ability to perform effector functions such as phagocytosis and generation of ROS [4].This may be also true for neutrophils isolated from individuals with distinct IL8 haplotypes and has not been previously investigated in cattle or humans.

The importance of IL-8 in early innate immune response for neutrophil recruitment and function has been extensively demonstrated in the literature. Our group has previously shown that differing IL8 haplotype in cattle results in alternative levels of IL-8 secreted by peripheral blood cells with IL8-h2 showing higher levels [5]. This has only been shown *in vivo* in one-month old calves. It is unknown whether age, particularly at the beginning of life, would impact on IL-8 production from calves with each haplotype. Additionally, IL-8 has now also been shown to be involved in recruitment of lymphocytes such as T helper cells as well as been produced by them in the case of γδ T-cells in humans [136, 143]. Lymphocytes were previously shown to be elevated in cattle with the IL8-h2 haplotype during an *in vivo* LPS challenge and it is remains to be seen does this occur under basal conditions and across the early life of the calf [5]. It was therefore aimed to assess immune cell populations in peripheral blood from calves with different IL8 haplotypes across early life and discover the origin of IL-8 in bovine peripheral blood.

Also, IL-8 has been shown to be secreted in response to viral ligands and differentially secreted depending on IL8 haplotype as demonstrated in humans [34, 133]. It remains to be investigated what effect viral, and even fungal ligands, have on IL-8 secretion by peripheral blood cells from calves with different IL8 haplotype. The aim, therefore, was to assess the peripheral blood response to LPS, poly (I:C) and zymosan in calves of different IL8 haplotypes across early life using whole blood culture systems in addition to the numbers of immune cell populations by flow cytometry and haematological analysis.

Finally, the relatively recent discovery of the NET formation by neutrophils has not been previously examined in cattle with these haplotypes and considering IL-8 is a promoter of NET formation [89], the fact that these haplotypes result in alternative levels of IL-8 secretion may be significant in their ability to form NETs. As such, it was aimed to investigate NET formation in calves of different IL8 haplotypes during early life.

3.3 Results

3.3.1 Immune cell populations in calves with distinct IL8 haplotypes

The Holstein-Friesian breed was chosen for this study due to the approximately 50:50 ratio of each IL8 haplotype within this population [35]. Blood was taken from thirty-two one-month old calves and genotyping was carried out using a custom designed TaqMan genotyping assay which detects a haplotype-linked SNP within the IL8 promoter region. The allelic discrimination plot (Figure 3.1) allowed us to choose six calves from each haplotype for functional analyses.

At months 1-10, blood samples were collected, and absolute numbers of individual cell populations established using the ADVIA 2120 haematology analysis system. Significant haplotype differences in specific cell populations were detected across months two, four and eight in addition to differences approaching significance at months three and six (Figure 3.2). Neutrophil numbers were significantly different between the haplotype groups at month four where IL8-h2 showed increased numbers compared to IL8-h1 (Figure 3.2A). Generally, IL8-h2 displayed higher variation in neutrophil numbers when compared with IL8-h1, most notably in the first six months. Increases in monocyte numbers in IL8-h2 compared to IL8-h1 approached significance (P=0.059) at month six and were significantly raised in IL8-h2 at month nine (Figure 3.2B). Eosinophil numbers differed at month three (P=0.052) and significantly so at month four where IL8-h2 again had increased numbers when compared to IL8-h1 (Figure 3.2C). Basophil numbers also significantly differed at month two with IL8-h2 displaying higher amounts compared to IL8-h1 (Figure 3.2D) In terms of adaptive immune cells, interestingly there were no differences in lymphocyte numbers between haplotypes were detected at any age (Figure 3.2E).

Generally, there was a large drop in lymphocytes and basophils from month nine to ten in both haplotypes whereas there was a large increase in neutrophil numbers. Where significant differences in cell populations i.e. neutrophils, monocytes, eosinophils and basophils are observed, IL8-h2 always had the increased numbers. Across all timepoints, IL8-h2 appears to show greater variation in neutrophil numbers compared to IL8-h1. These increased IL8-h2 cell numbers are mostly seen in early months such as months two; three and four, where stability (less variation) in the neutrophil numbers is more present for IL8-h1. The results here suggest that IL8 haplotype impacts immune cell

numbers at specific months in a calf's early life, but this impact does not appear to extend to overall lymphocyte numbers.

In order to assess whether specific lymphocyte populations may differ between the two haplotypes, flow cytometry was carried out with cells markers for B-cells (sIgM), CD4 T-cells (CD3+CD4+), CD8 T-cells (CD3+CD8+) and γδ T-cells (γδTCR+ and WC1+). A difference was previously seen in lymphocyte numbers following *in vivo* LPS challenge where IL8-h2 showed increased numbers at 12hr post challenge. It was therefore important to establish if this was also true at a basal level across the calf's early life. Flow cytometry analysis was carried using the gating strategy shown in Figure 3.3 and results are presented as percentage of cells which stained positive for the marker in that gate (Figure 3.4). No significant differences in the percentages of CD4 T-cells, CD8 T-cells or γδ T-cells at any age were detected between haplotypes (Figure 3.4A, C&D). A significant difference in B-cell percentages at month two was observed where IL8-h1 showed a higher percentage of cells expressing sIgM (Figure 3.4B). This could possibly be a result of a haplotype specific response to a booster vaccination around this period. In general, however, these data imply that IL8 haplotype does not affect circulating lymphocyte populations.

Before assessing differences in IL-8 production between the two haplotypes, it was important to establish the primary source of IL-8 in whole blood from cattle. Major blood cell populations: neutrophils, lymphocytes and monocytes were separated from 4/5 calves via MACS and stimulated with LPS for 24 hours *in vitro* before assessing IL-8 production by ELISA. IL-8 is significantly and primarily produced by monocytes in response to LPS (Figure 3.5). There is also a significant induction of IL-8 in response to LPS by lymphocytes although much lower levels when compared with monocytes (Figure 3.5).

Overall, IL8 haplotype does appear to have some effect on levels of circulating innate immune cells such as granulocytes and monocytes but does not significantly impact lymphocytes. IL8-h2 appeared to have the increased numbers of these cell types when compared with IL-8h1. The significant haplotype effect in B-cells at month two could be result in IL8-h1 responding better to a booster vaccination during this period. Finally, monocytes can be considered the primary producer of IL-8 in bovine systemic circulation in addition to smaller quantity produced by lymphocytes.

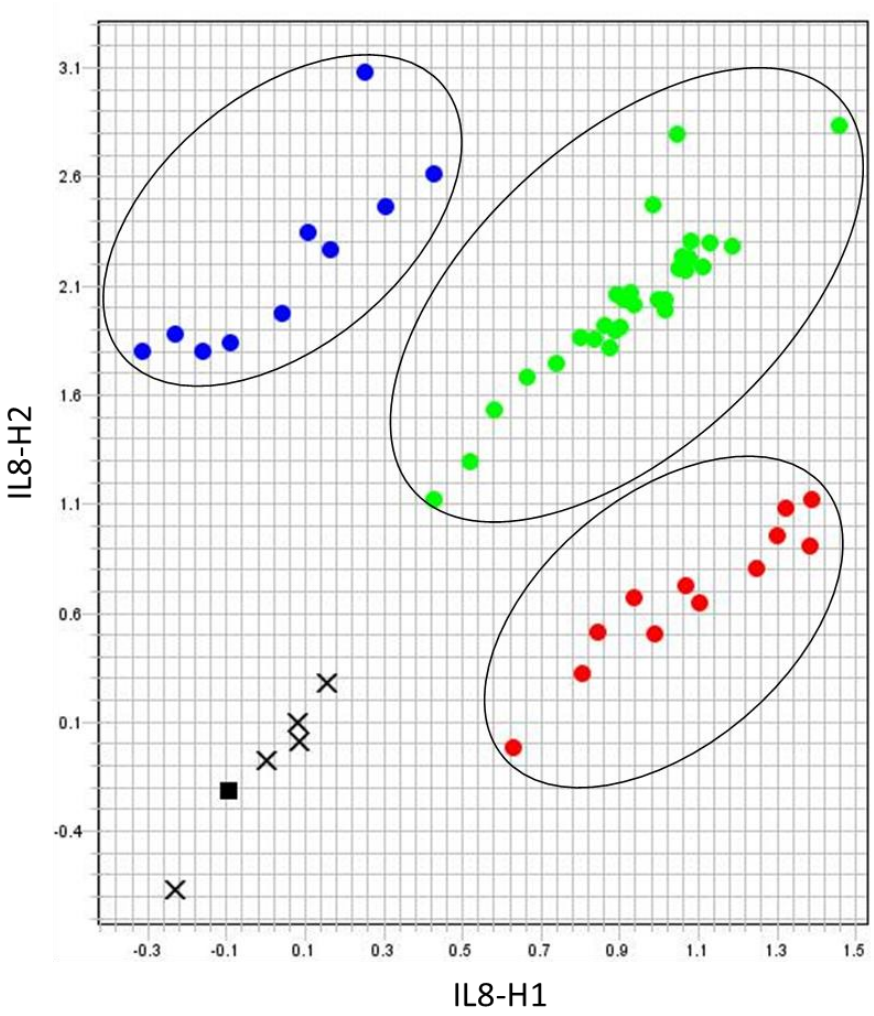

Figure 3.1 Allelic discrimination plot of calf IL8 haplotypes. Thirty-two one-month old Holstein-Friesian calves were genotyped in duplicate using a custom TaqMan genotyping assay for a SNP within the promoter region of the *IL8* gene in order ascertain *IL8* haplotype. Fluorescent signals distinct for each haplotype allowed us the discriminate between homozygote IL8-h1 (blue), homozygote IL8-h2 (red) and heterozygote (green). Undetermined animals where marked with an X. Negative controls (■) and positive controls were also included.

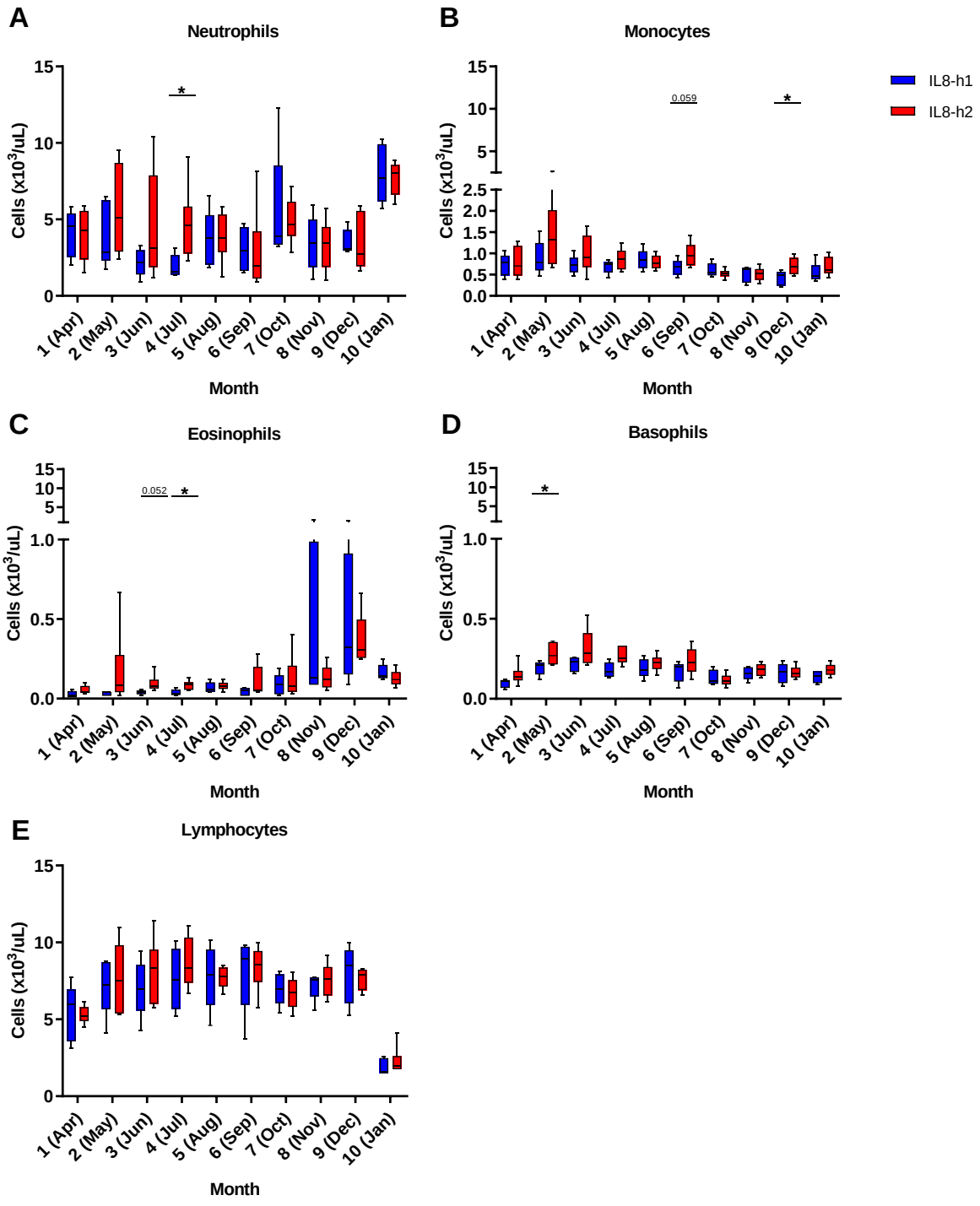

Figure 3.2 Haematological profiles of calves with different *IL8* haplotypes. Blood from calves was taken via neck venepuncture monthly and processed by the ADVIA 2120 system. The results for each month are presented as cells/ mL for (A) neutrophils, (B) monocytes, (C) eosinophils, (D) basophils and (E) lymphocytes. Error bars represent the min/max of n=5/6. Significance is between haplotypes where *P<0.05.

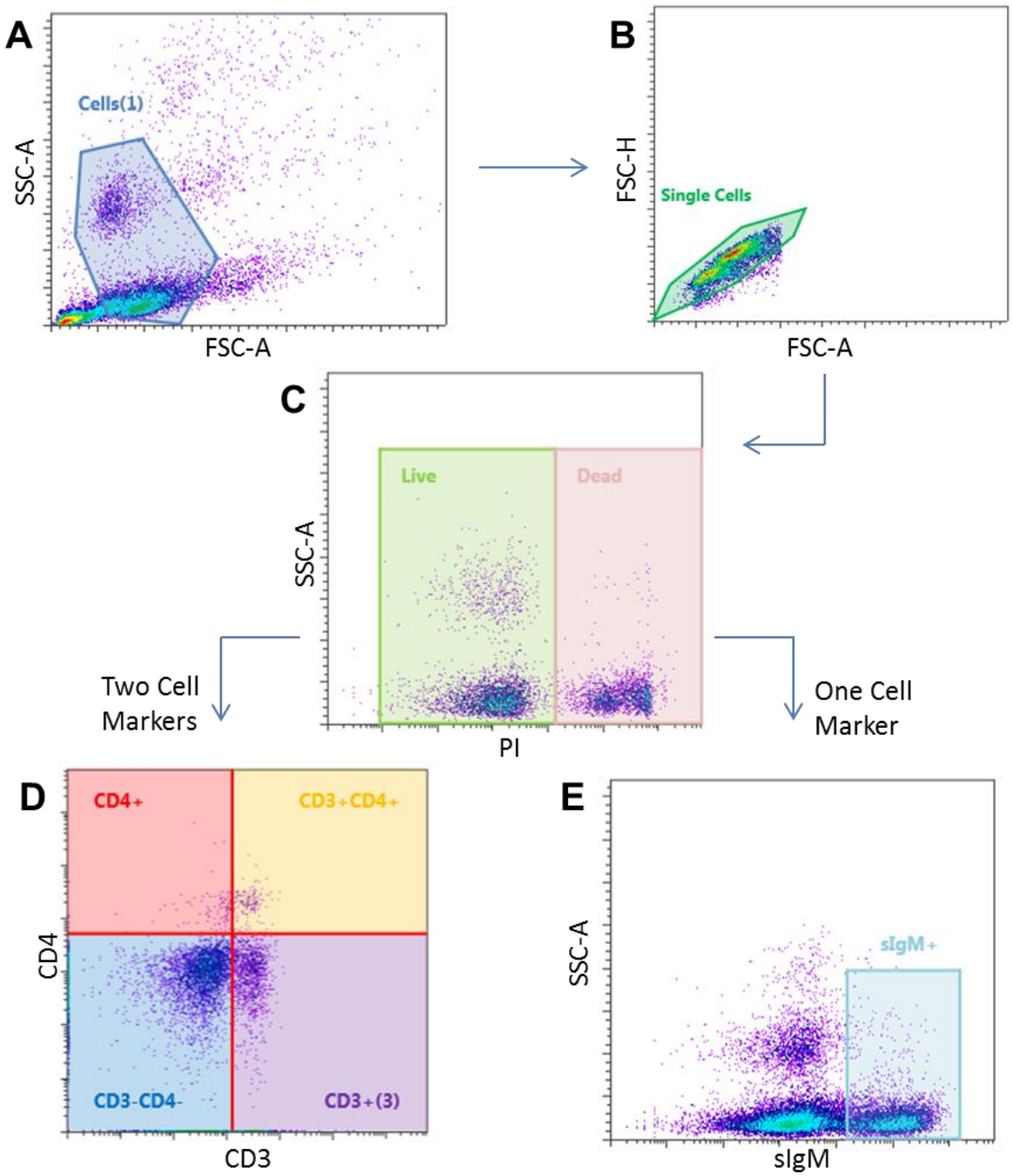

Figure 3.3 Gating strategy for flow cytometry analysis. Blood was taken from calves in sodium heparin vacutainers and RBCs were lysed. Cells were then stained with antibodies against CD3, CD4, CD8, sIgM, γδTCR and WC1 in addition to a PI viability stain and analysed by flow cytometry. The first gate was created for (A) granulocytes and mononuclear cells, these cells were then gated for (B) single cells only and then cells were gated for (C) live cells only. Cells were then identified based on surface expression of (E) single marker (sIgM+ = B-cells) or a (D) double marker (CD3+CD4+ = CD4 T-cells, CD3+CD8+ = CD8+ T-cells, γδTCRWC1+ = WC1+γδT-cells).

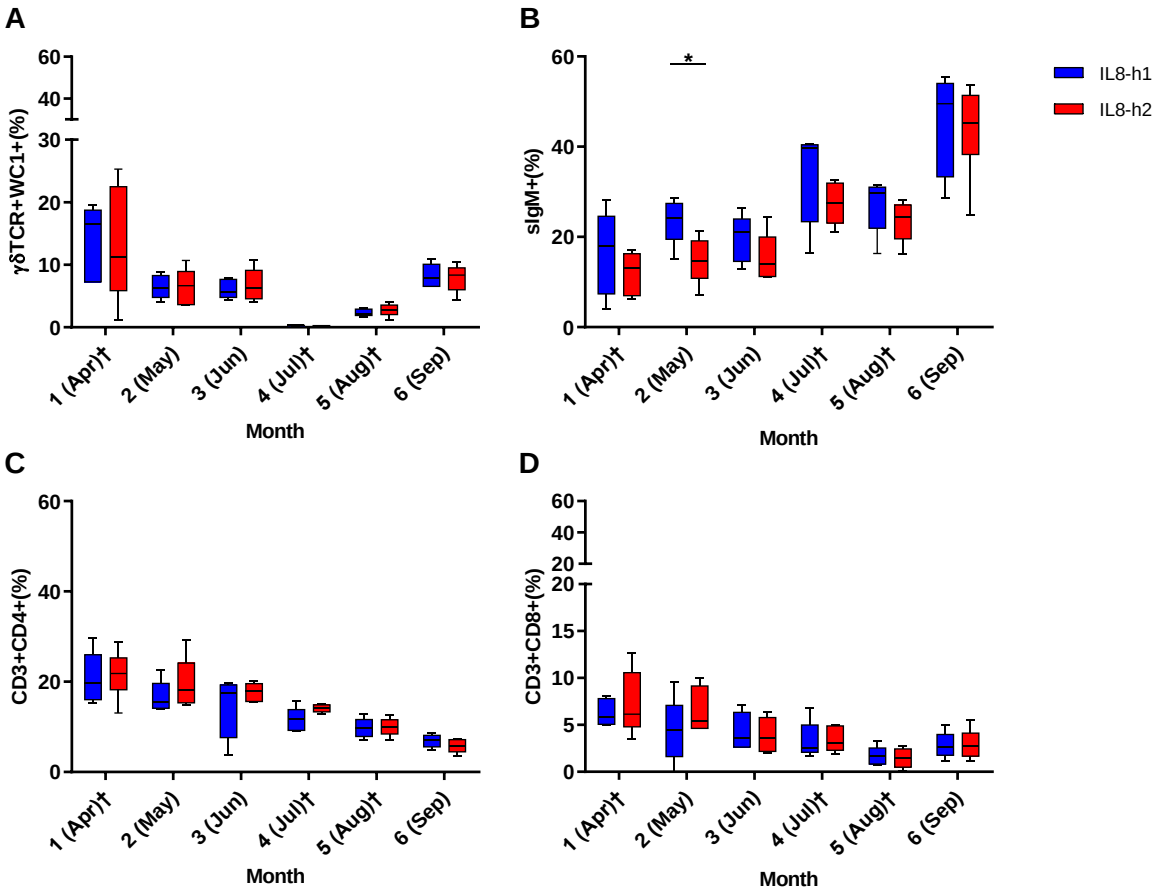

Figure 3.4 Percentage of cell populations stained positive for corresponding cell markers. The distribution of cells positive for (A) γδTCR and WC1 (B) sIgM, (C) CD3 and CD4 and (D) CD3 and CD8 in whole blood obtained from calves' homozygote for each IL8 haplotypes. Error bars represent the min/max values of n=5/6. Significance is between haplotypes where *P<0.05. † Cells were only gated as single cells, no live/dead staining performed.

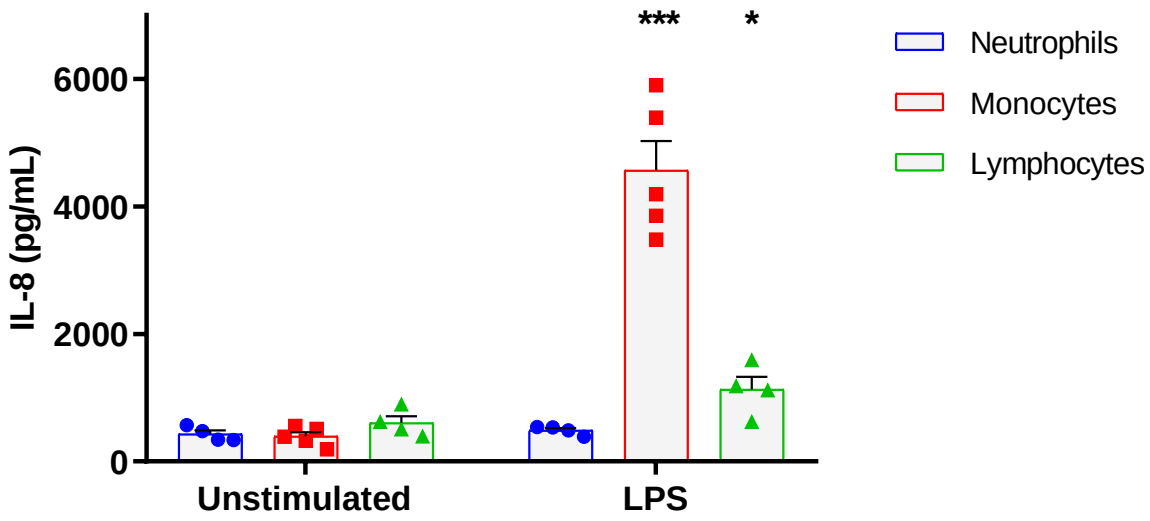

Figure 3.5 IL-8 production by neutrophils, monocytes and lymphocytes in response to LPS. Neutrophils, monocytes and lymphocytes were isolated from the peripheral blood of 5/6 month old calves by MACS. The cells were plated at $1x10^6$ cells/well and stimulated with 100 ng/mL of LPS for 24 hr at 37°C, 5% CO_2. An unstimulated negative control was also included. IL-8 levels in supernatant were measured by ELISA. Error bars represents SEM of n=4/5 animals. Significance is between unstimulated control and LPS where *P<0.05 and ***P<0.001.

3.3.2 Functional characterisation of *IL8* haplotypes innate immune response against LPS, poly (I:C) and zymosan using whole blood culture (WBC) systems

IL8-h2 calves demonstrated significantly higher levels of circulating monocytes and granulocytes early in life at months two, three, four and six. Considering this result, it was also important to assess whether IL8-h2 also showed different induced responses to bacterial, viral and fungal PAMPS when compared with IL8-h1 calves. Both a commercial whole blood culture system known as TruCulture® and a conventional whole blood culture system using tissue culture plates was used to assess variance in response to the two systems. The TruCulture® system employs a prefilled tube containing 2 mL of medium and the stimulant which 1 mL of blood is bled directly into from the vein. The tubes are then incubated on a heating block at 37°C for twenty-four hours. The conventional blood culture system was designed based on the TruCulture® tubes where 1 mL of blood was transferred from a heparin vacutainer to 2 mL of RPMI 1640 , containing the stimulant, in a six well plated and incubated at 37°C, 5% CO_2 for twenty-four hours.

Levels of IL-1β were first examined in both haplotypes in response to LPS, poly (I:C) and zymosan using the TruCulture® system (Figure 3.6). There was no significant difference between haplotypes although there was a trend which saw increased levels of IL-1β production in response to LPS for IL8-h2 across all months (Figure 3.6A). This trend was slightly less in response to zymosan (Figure 3.6C). When using the conventional whole blood culture system to look IL-1β, the only significant difference between haplotypes could be seen at month six for zymosan stimulation where IL8-h2 produced much higher levels (Figure 3.7C). There was no significant difference between haplotype responses to LPS although there appears to be a trend at months four and six where IL8-h2 has a slightly higher response (Figure 3.7A). The major difference between the two systems in IL-1β response is in the levels produced in response to zymosan (Figure 3.6C and Figure 3.7C).

In the case of the IL-8 response using the TruCulture® system (Figure 3.8), the only significant differences between the haplotypes was seen with the poly (I:C) stimulus (Figure 3.8B). This, however, seems to be a result of a significant control level difference. There appears to be no real induction of IL-8 by poly (I:C) from Figure 3.8B as the control levels follow nearly the same pattern as the induced levels. There is a large difference in the magnitude of the response at month one in the case of LPS and zymosan (Figure 3.8A

and 3.8C) compared to the other months. This may be explained by the young age of the calves. Using the conventional whole blood culture system to observe the IL-8 response (Figure 3.9), a similar response was seen to poly (I:C) (Figure 3.9B) in that the only significant difference (at month six) between the haplotypes is due to a basal level difference. The were no evident differences in IL-8 levels between the haplotypes in response to LPS or zymosan (Figure 3.9A and 3.9C).

Having observed differences in cytokine responses between the haplotypes in both systems, it was necessary to establish whether there were also observable differences between the conventional and commercial whole blood culture assays themselves. To do this, the assays were directly compared at month three of the study, again looking at IL-1β and IL-8 responses to LPS, poly (I:C) and zymosan (Figure 3.10). Both systems induced a response when compared with control samples. However, in the case of IL-1β responses, there was a significant difference between the two assays in response to zymosan where the where the whole blood culture elicited much higher levels than the TruCulture system (Figure 3.10A). For IL-8, there was a highly significant difference between the assays for both LPS and zymosan where again there were much higher levels in the whole blood culture than TruCulture (Figure 3.10B). The results observed here show that discrepancies between assays in some part may be due to the origin of LPS and zymosan used by the company and by us or possibly due to the ability of the cells to respond in each specific environment.

Due to the closed nature of the TruCulture® system, oxygen is absent for the cells during the twenty-four hour incubation period. Therefore, it was important to assess whether increased cell death or increased hypoxia occurred in this system, which could affect the results obtained. The assays were carried out using the same parameters as previously mentioned. At the end of the incubation period half of the cells were stained using PI and the other half stored in trizol for RNA extraction. Generally, cell death appeared to be greater in the conventional whole blood culture system (Figure 3.11). This was significant for the control, LPS and zymosan stimulation. To establish whether hypoxia may be increased in the TruCulture system, RNA was extracted from the whole blood after both assays had been run. This was converted to cDNA and qPCR was performed. The suitability of five reference genes for qPCR was evaluated: *GAPDH, ACTB, GUSB, RSP15* and *HSP90AB*. This was carried out using the geNorm tool available on the GenEx qPCR analysis software. The tool applies an M value to each

gene as a measure of its expression stability across control and treated samples. The lowest M value is deemed the most stable gene. In the case of this experiment, *RSP15* and *HSP90AB* (Figure 3.12A) were given the same lowest M value and therefore both were used as reference genes by averaging their values. *HIF1A* was measured gene expression as an indication of whether the cells may have been experiencing hypoxia. The gene expression was made relative to the lowest expressing sample. As shown in Figure 3.12B, there were no significant differences in gene expression between the two culture systems which would indicate that the TruCulture® system does not create hypoxic conditions. This was also true in the case of non-stimulated versus stimulated.

Taken together these results would indicate that any variance between the two systems is most likely caused by the origin of the stimulants used. A possible explanation for the increased cell deaths seen in the whole blood culture system may be due to a longer processing time for the samples as the blood needs to be transferred to the culture plates unlike the TruCulture® system where the blood is bled directly into the tubes and incubated immediately.

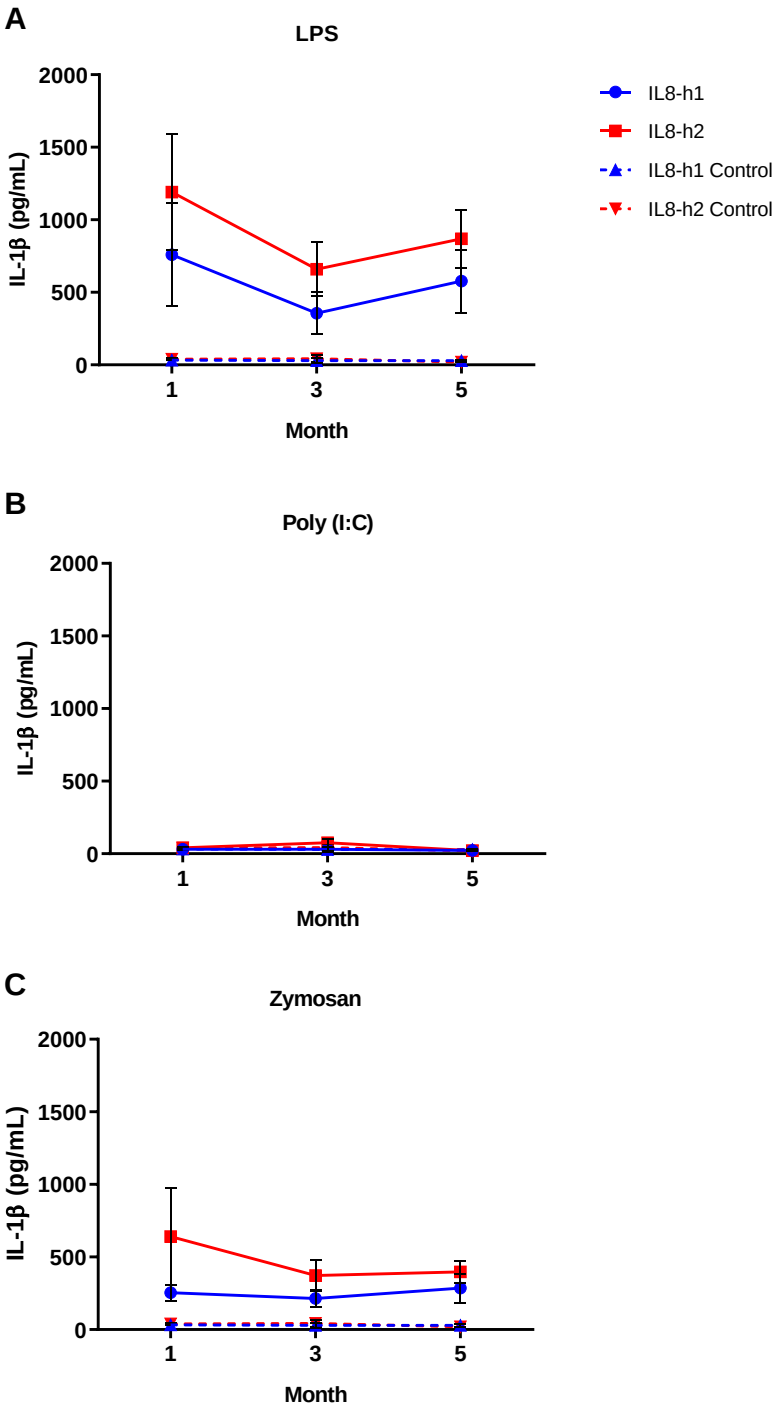

Figure 3.6 IL-1β expression in whole blood of *IL8* haplotypes in response to LPS, poly (I:C) and zymosan using TruCulture®. Blood was taken via jugular venepuncture on months 1,3 and 5 and stimulated for 24 hours at 37°C with (A) LPS (100 ng/mL) (B)Poly (I:C) (20 µg/mL) and (C)zymosan (50 µg/mL) using TruCulture® . IL-1β concentration was measured in the supernatant by ELISA and represented in pg/mL. Error bars represent the SEM of n=5.

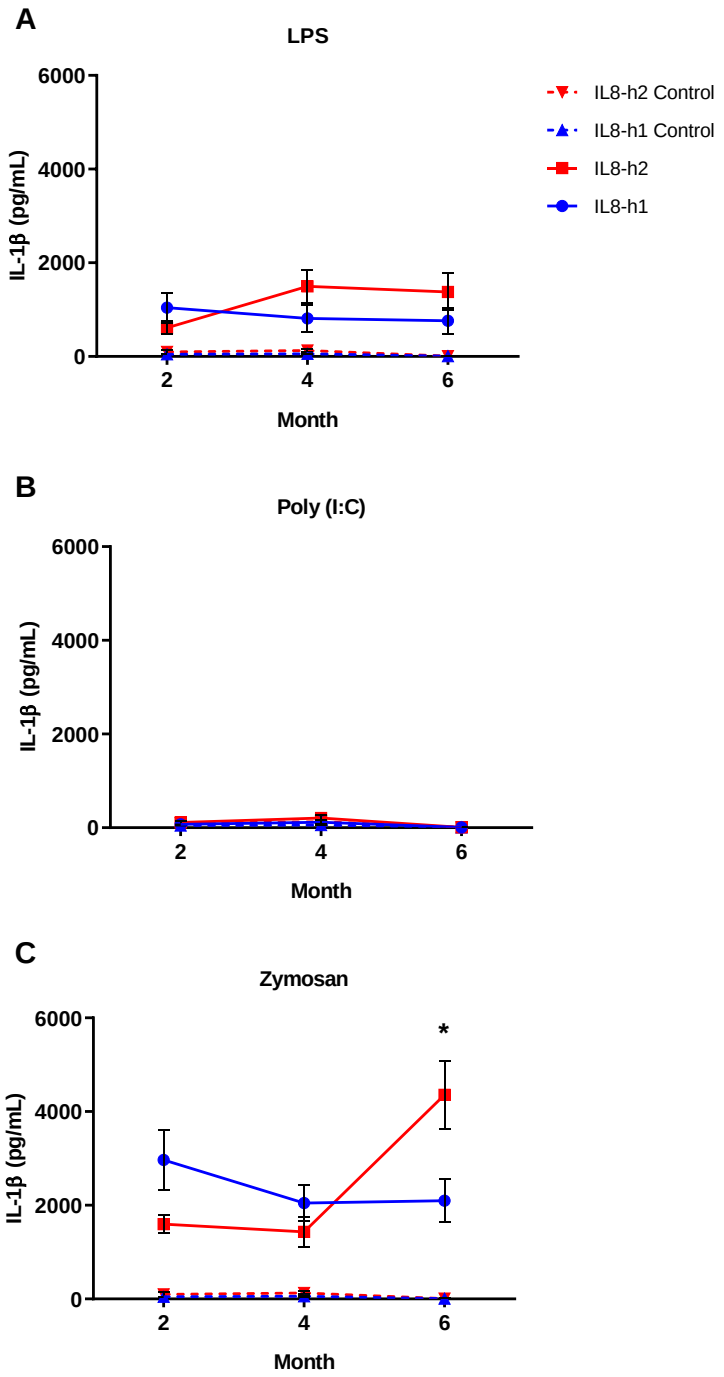

Figure 3.7 IL-1β protein expression of *IL8* haplotypes in response to LPS, poly (I:C) and zymosan using a conventional whole blood culture system. Blood was taken via jugular venepuncture and stimulated for 24 hours at 37°C with (A) LPS (100 ng/mL) (B)Poly (I:C) (20 µg/mL) and (C)zymosan (50 µg/mL) using a conventional whole blood culture system. IL-1β concentration was measured from the supernatant by ELISA and represented in pg/mL. Error bars represent the SEM of n=5. Significance is between haplotypes where *P<0.05.

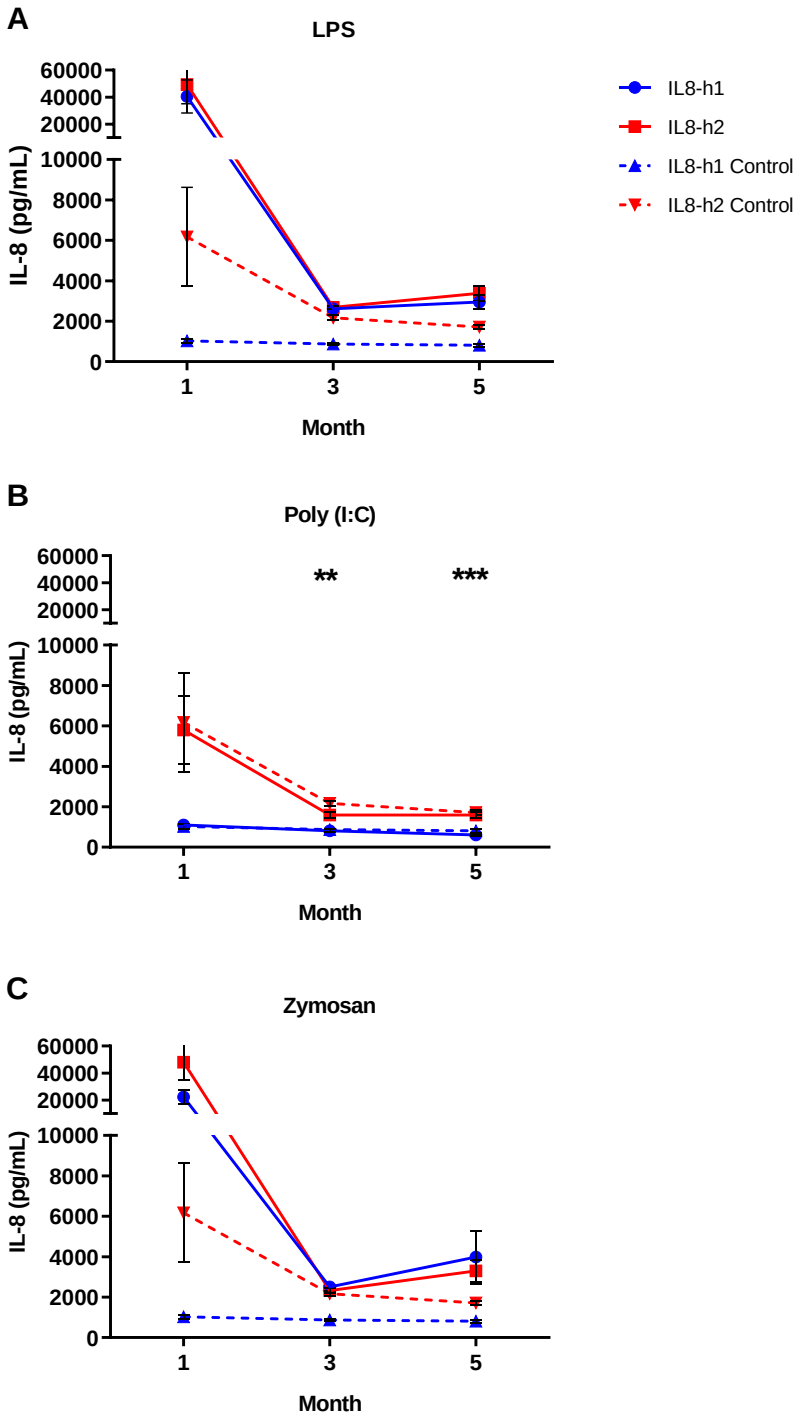

Figure 3.8 IL-8 protein expression of *IL8* haplotypes in response to LPS, poly (I:C) and zymosan using TruCulture®. Blood was taken via jugular venepuncture and stimulated for 24 hours at 37°C with (A) LPS (100 ng/mL) (B)Poly (I:C) (20 µg/mL) and (C)zymosan (50 µg/mL) using TruCulture® (months 1, 3 and 5). IL-8 concentration was measured from the supernatant by ELISA and represented in pg/mL. Error bars represent the SEM of n=5. Significance is between haplotypes where **P<0.01 and ***P<0.001.

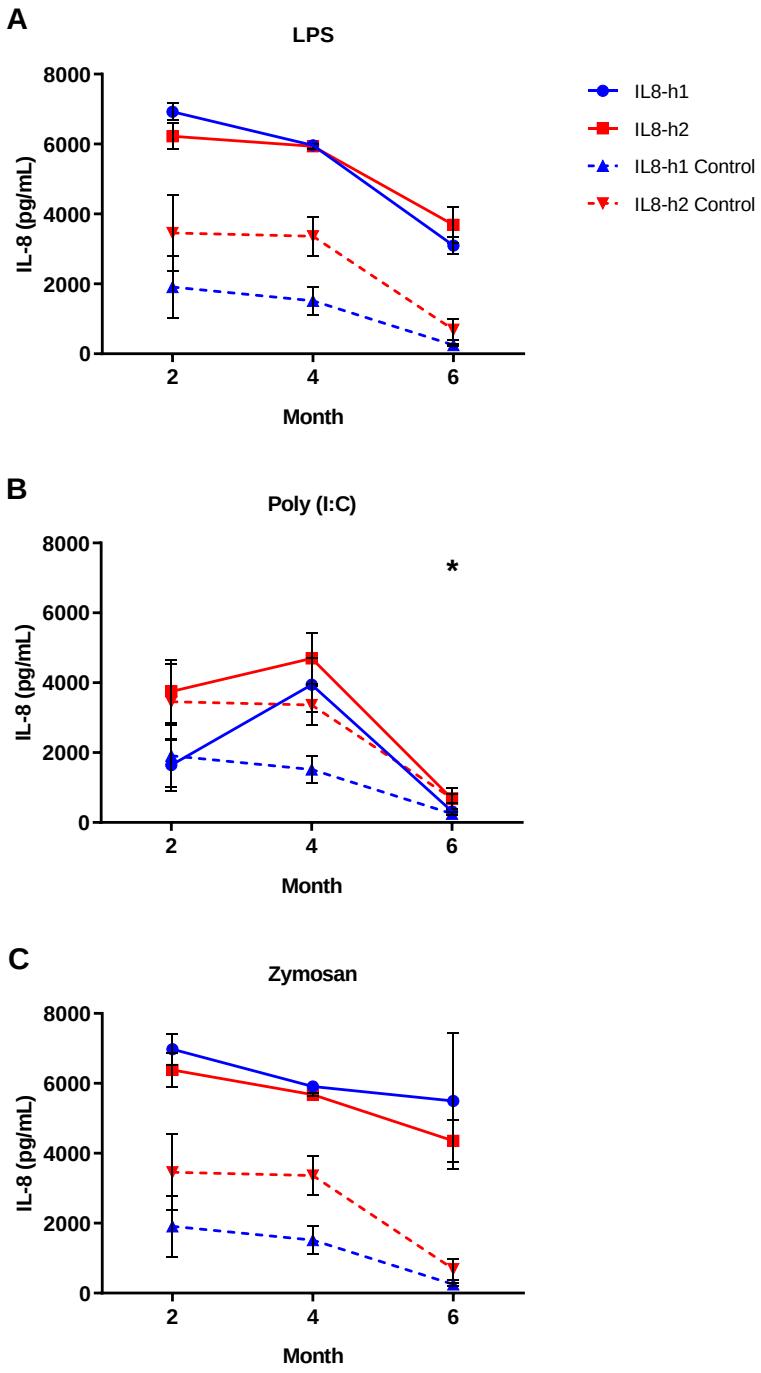

Figure 3.9 IL-8 protein expression of *IL8* haplotypes in response to LPS and control using a conventional whole blood culture system. Blood was taken via jugular venepuncture and stimulated for 24 hours at 37°C with (A) LPS (100 ng/mL) (B)Poly (I:C) (20 µg/mL) and (C)zymosan (50 µg/mL) using a conventional whole blood culture system (months 2, 4 and 6). IL-8 concentration was measured from the supernatant by ELISA and represented in pg/mL. Error bars represent the SEM of n=5. Significance is between haplotypes where *P<0.05.

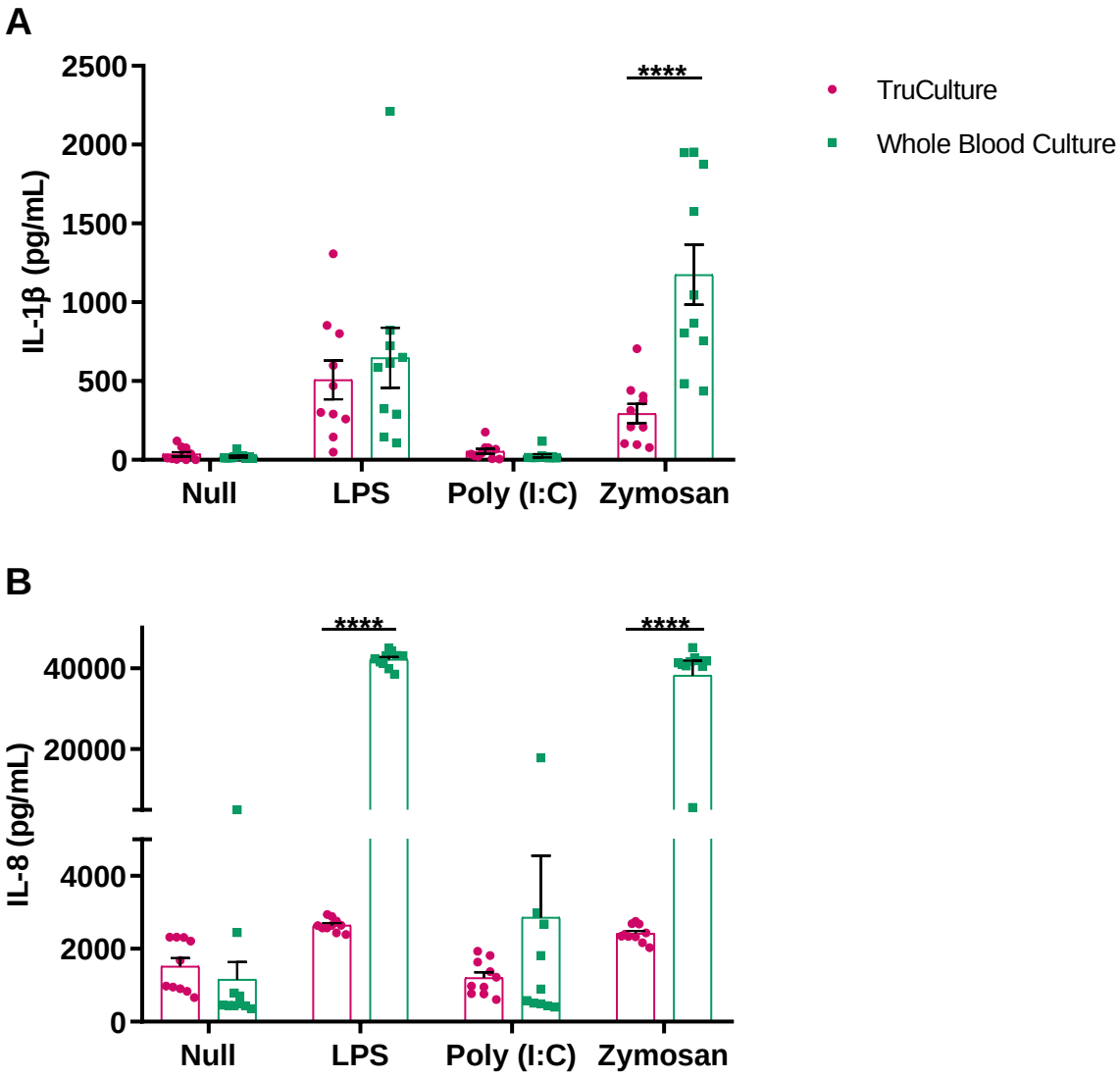

Figure 3.10 Comparison of IL1β and IL-8 levels in stimulated blood from three-month-old Holstein-Friesian calves using TruCulture® and a conventional whole blood culture system. Blood was taken via jugular venepuncture and stimulated for 24 hours at 37°C with LPS (100 ng/mL), poly (I:C) (20 µg/mL) and zymosan (50 µg/mL) using each system. (A) IL-1β and (B) IL-8 concentration was measured from the supernatant by ELISA and represented in pg/mL. Error bars represent the SEM of n=10. Significance is between systems where ***P<0.001.

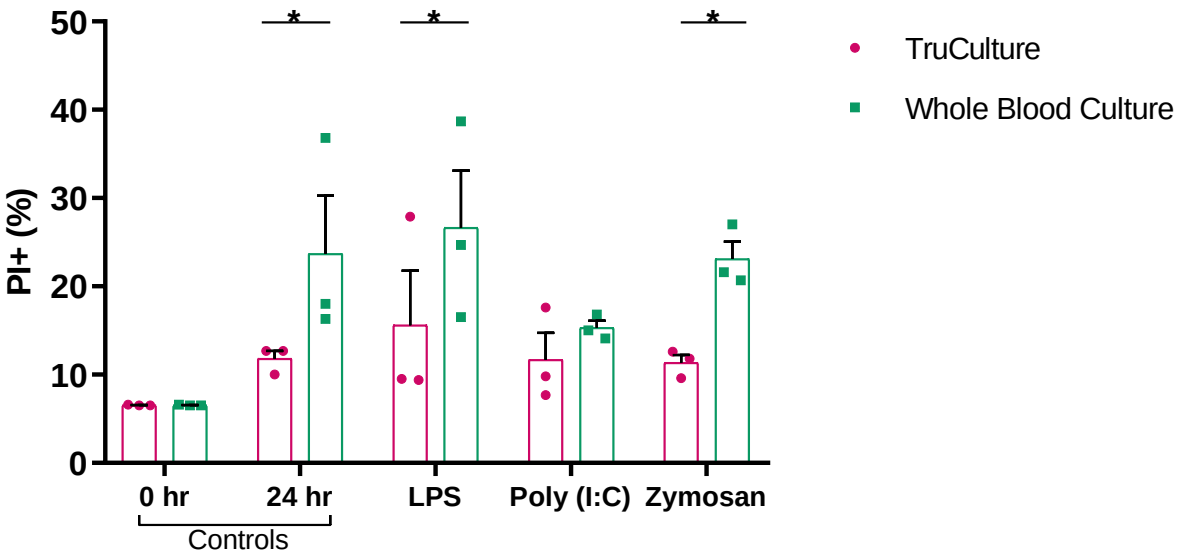

Figure 3.11 Cell viability comparison of TruCulture® and a conventional whole blood culture system. Blood was taken via jugular venepuncture from three-month-old calves. 0 hr control was immediately stained with PI and fluorescence measured by flow cytometry. Other samples were then stimulated at 37°C for 24 hr using the two culture systems with LPS (100 ng/mL), poly (I:C) (20 µg/mL) and zymosan (50 µg/mL). A negative control of unstimulated blood (24 hr control) was also included. The RBCs were lysed and then the cells were stained with PI and fluorescence was measured by flow cytometry. Bars represent the min/max of n=3. Significance is between systems where **P<0.01.

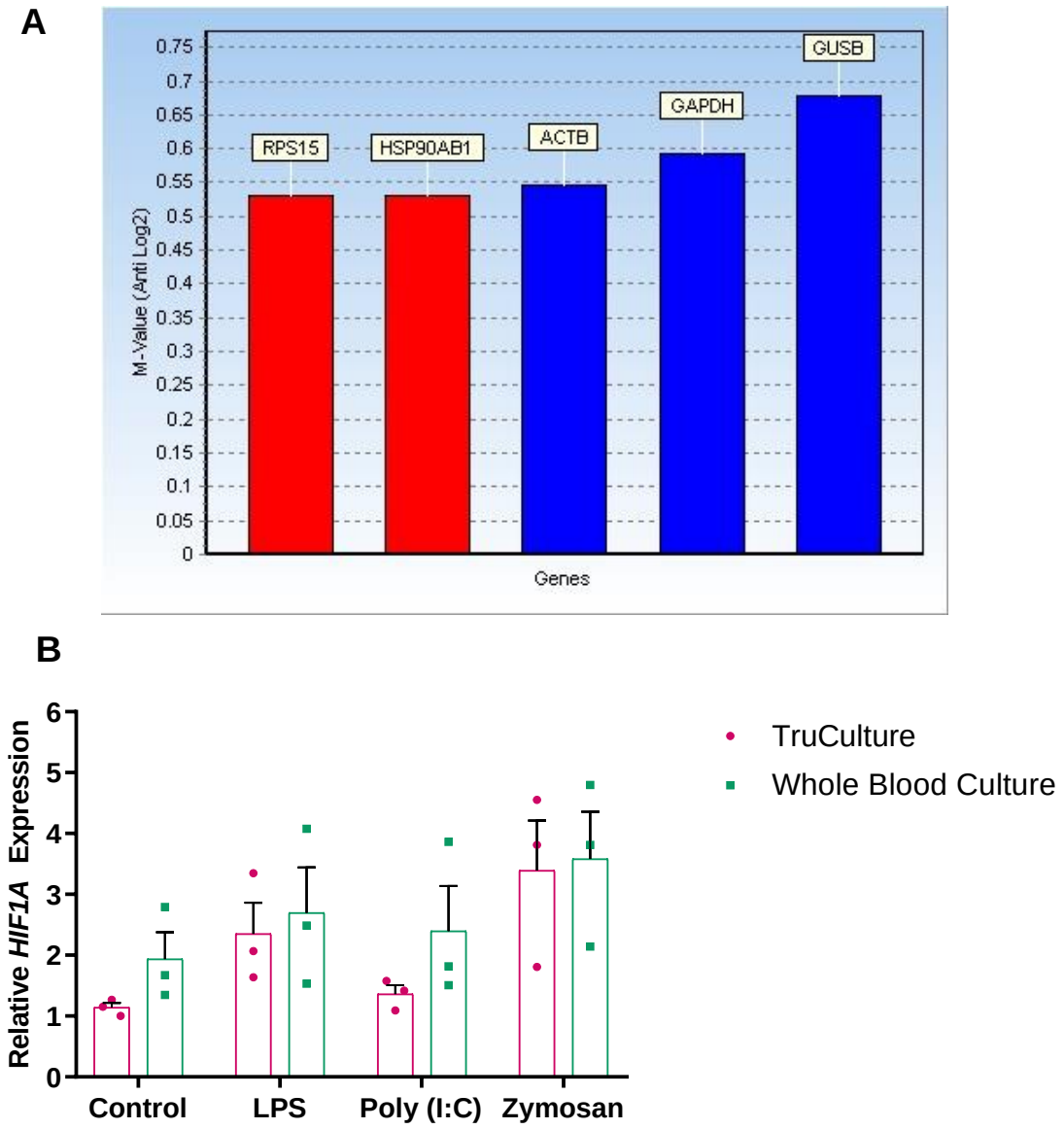

Figure 3.12 *HIF1A* gene expression and reference gene stability in whole blood stimulated using TruCulture® and a conventional whole blood culture system. RNA was extracted from blood stimulated by using the two culture systems and cDNA converted for qPCR analysis. Five reference genes were assessed using geNorm where (A) M value was plotted to show recommended reference genes (red). (B) HIF1A expression was assessed in blood stimulated at 37°C for 24 hr using the two systems with LPS (100 ng/mL), poly (I:C) (20 µg/mL) and zymosan (50 µg/mL). A negative control of unstimulated blood was also included. Data is presented as relative gene expression compared to lowest expressed sample. Error bars represent the SEM of n=3.

3.3.3 *IL8* haplotype NET function in response to PAMPs

After determining differences in the ability of peripheral blood leukocytes from calves with different IL8 haplotypes to produce IL-8 particular at a basal level, an important neutrophil effector function of which IL-8 can induce directly was evaluated [89]. Neutrophils isolated via MACS from calves with different IL8 haplotypes are potentially exposed to different circulating levels of IL-8 in peripheral blood resulting in different abilities to form NETs. An assay was set up based on Carretta *et al.* to measure NET function by staining for extracellular DNA after neutrophil stimulation [113]. Neutrophils were isolated from whole blood from which Holstein-Friesian calves with different IL8 haplotypes by MACS using an antibody specific for bovine granulocytes. The purity of the granulocyte population was always between 80-95% with the majority of the granulocyte's being neutrophils (Figure 3.13B). Cell viability was assessed using PI staining (Figure 3.13C) and cell death was confirmed to always be below 3%.

To optimise the assay, neutrophils were isolated from Holstein-Friesian calves and incubated with increasing concentrations of LPS for thirty minutes or one hour. The incubation times of thirty minutes or one hour were used based on previous published studies [144]. There was no major difference in the amount of extracellular DNA measured between incubation times, as can be seen in Figure 3.14, therefore one hour was chosen. A range of LPS concentrations (10-200 µg/mL) were then evaluated to choose the best at inducing NETS. A high concentration (1 mg/mL) of zymosan was used as a positive control as it has been shown to induce the maximum amount of extracellular DNA [113]. A concentration of 200 µg/mL LPS (Figure 3.15) induced the optimum amount of extracellular DNA therefore this LPS concentration was used for further studies.

Blood was isolated from IL8 genotyped Holstein-Friesian calves at months four, five and six of age and stimulated with LPS to induce NET formation and assessed by measurement of extracellular DNA. There was no significant difference in NET activation between haplotypes at month four (Figure 3.16A). At month five, there was a significant difference between haplotypes where IL8-h1 produced a larger amount of extracellular DNA indicative of NET formation in the presence of the positive control, zymosan (Figure 3.16B). At month six, IL8-h1 again produced significantly more extracellular DNA this time in the case of LPS stimulation (Figure 3.16C). These data suggest that neutrophils from IL8-h1 is more functionally capable of producing NETs.

There is much debate in the literature about neutrophil death after NET formation [92]. Neutrophils were assessed at month four of this study by staining with PI after the assay was performed to measure cell death. The FSC/SSC profiles of the neutrophils changed drastically after the one-hour stimulation even in the case of the unstimulated control (Figure 3.17). Cell death also increased. PI staining for the neutrophils at month four can be seen in Figure 3.18. Although there were no significant differences between the haplotypes in terms of cell death, there were significant differences between the stimulations. The positive control zymosan appeared to induce significantly lower cell death than the unstimulated negative control. A possible explanation for this may be due to a higher amount of DNA being expelled from the zymosan stimulated neutrophils thus PI had less to bind to in those cells. The cell death for the LPS stimulated neutrophils was significantly higher than the negative control which suggests that cell death during NET formation differs depending on the stimulus. Due to technical problems in removing cells from the plates, the staining at months five and six could not be performed. The results presented here indicate that the ability to form NETs differs between the haplotypes and NET formation was induced more effectively by IL8-h1 as indicated by increased extracellular DNA.

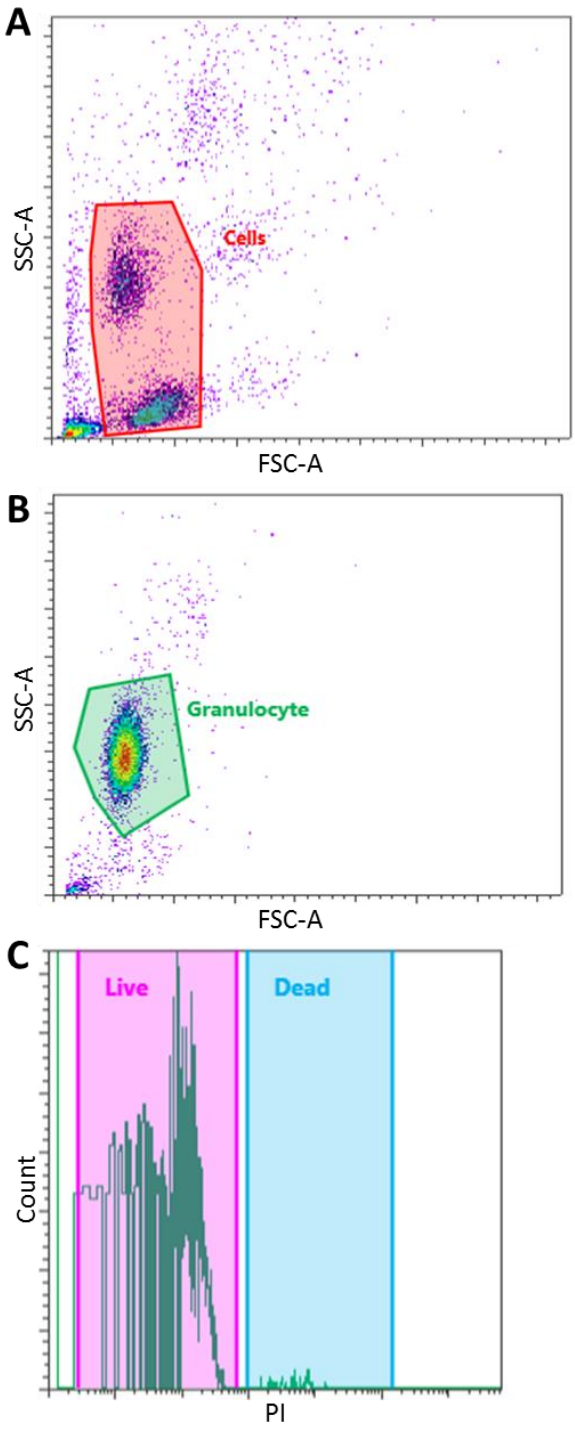

Figure 3.13 Neutrophil purity assessed via flow cytometry following MACS separation. Whole blood was collected from Holstein-Friesian calves in ACD vacutainers and placed in lysis buffer to remove RBCs. The peripheral bloods cells were then run through the flow cytometer (A) to assess cell populations and numbers for MACS. Granulocytes were then isolated via MACS and (B) the purity of the granulocyte population was assessed by forming a gate based on FSC and SSC along with (C) cell viability via PI staining.

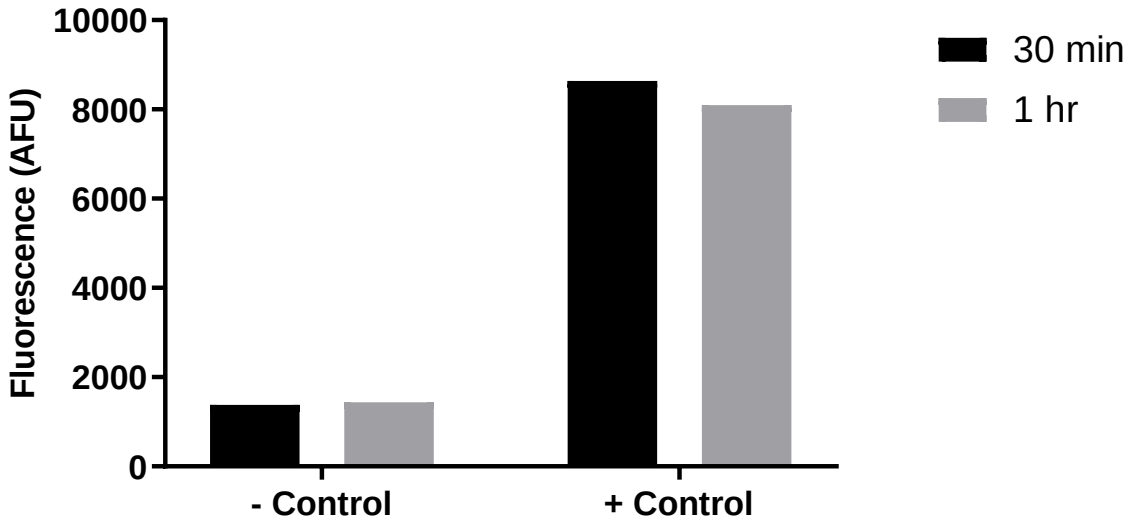

Figure 3.14 Comparison of incubation time required for NET formation in neutrophils. Neutrophils were isolated from peripheral blood via MACS and plated at $1x10^6$ cells/well. They were stimulated for 30 min and 1 hr at 37°C, 5% CO_2 with either medium (- control) or 1 mg/mL zymosan (+ control). Supernatants were stained with PicoGreen dye to assess NET formation and fluorescence was measured, n=1.

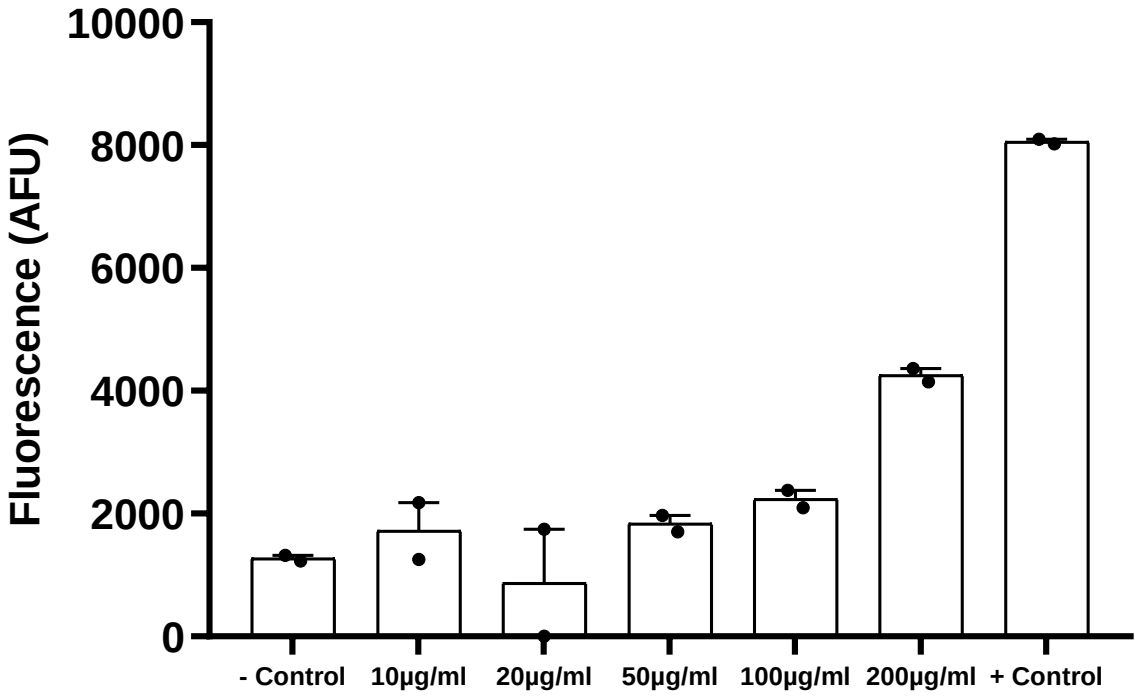

Figure 3.15 LPS dose response assessment for NET formation in neutrophils. Neutrophils were isolated from peripheral blood via MACS and plated at $1x10^6$ cells/well. They were stimulated for 1 hr at 37°C, 5% CO_2 with medium (- control) ,1 mg/mL zymosan (+ control) and increasing concentrations of LPS (10, 20, 50, 100 and 200 µg/mL). Supernatants were stained with PicoGreen dye to assess NET formation and fluorescence was measured. Error bars represent the SEM of n=2.

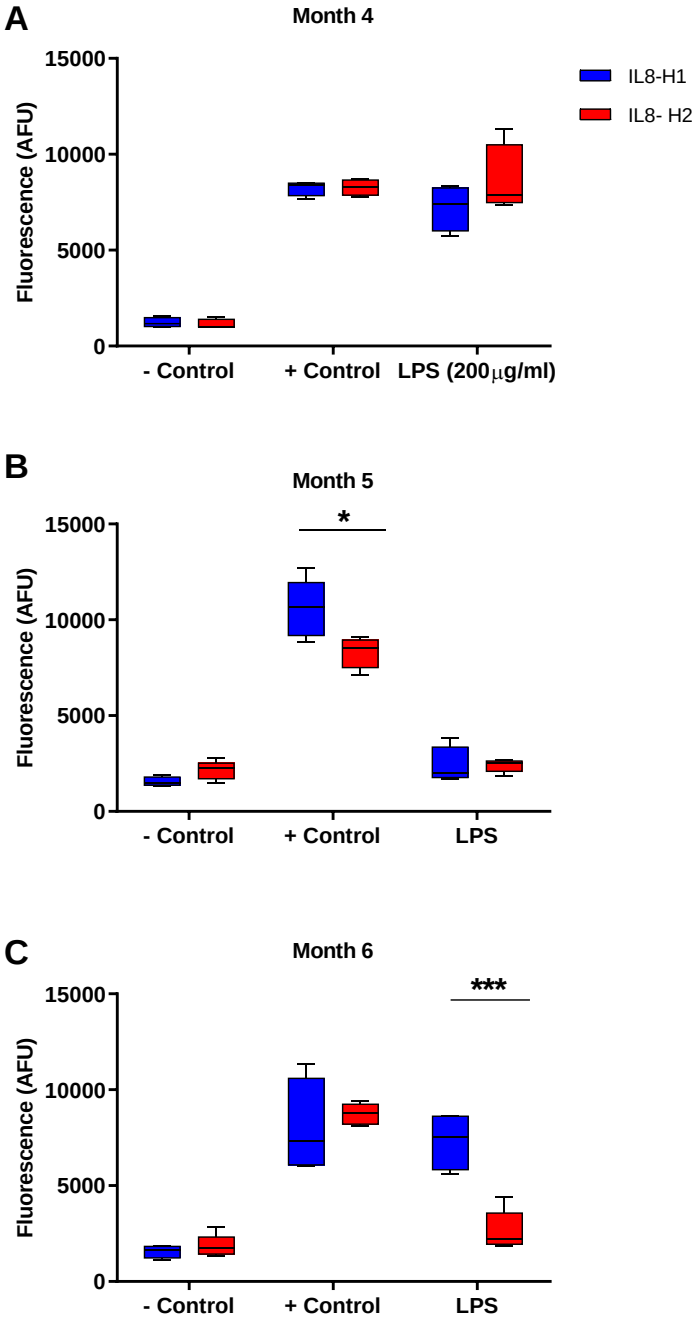

Figure 3.16 NET production in *IL8* haplotypes stimulated using LPS. Neutrophils were isolated via MACS from *IL8* genotyped animals at month 4 (A), month 5 (B) and month 6 (C) of age. They were plated at $1x10^6$ cells/well and stimulated for 1 hr at 37°C, 5% CO_2 with either medium (- control), 1 mg/mL zymosan (+ control) or 200 µg/mL LPS. Supernatants were stained with PicoGreen dye to assess NET formation and fluorescence was measured. Each bar represents ± SEM, n=5/6 animals, *P<0.05 and ***P<0.001 between haplotype.

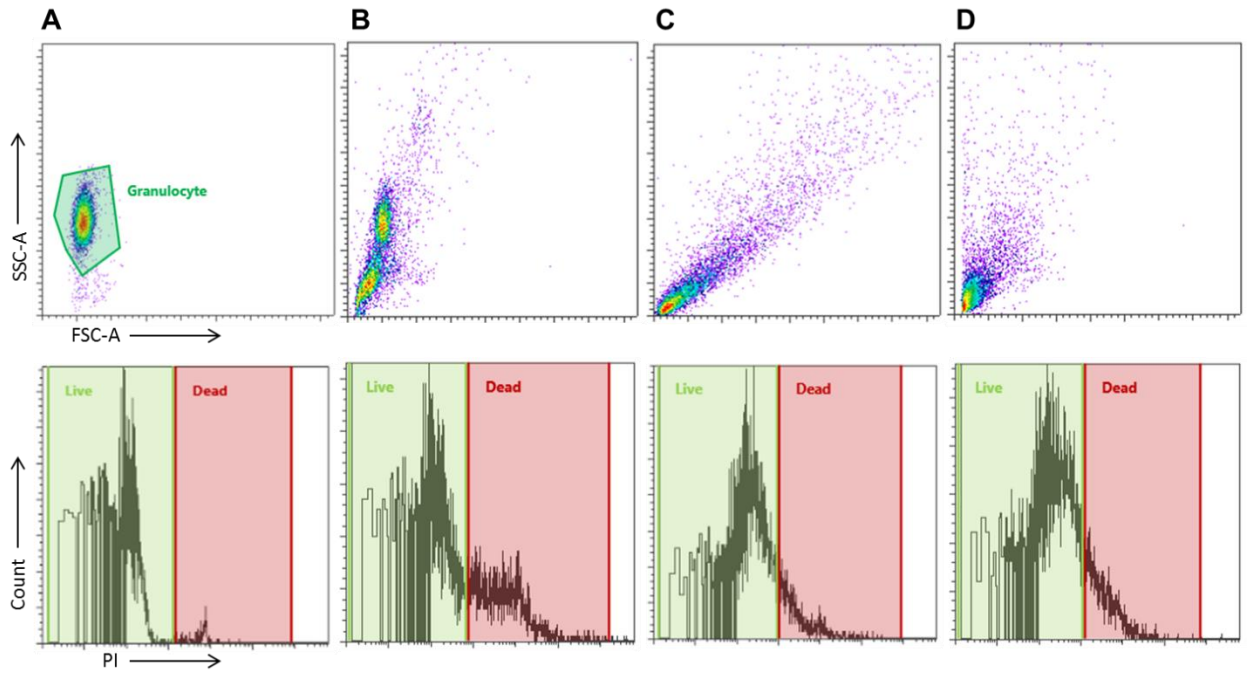

Figure 3.17 Neutrophil death before and after stimulation of NET formation. Neutrophils were isolated via MACS from *IL8* genotyped animals at month 4. The cell viability was assessed by PI staining and visual readouts (A) before stimulation and then after stimulation for 1 hr at 37°C, 5% CO_2 with either (B) medium (- control), (C)1 mg/mL zymosan (+ control) or (D)200 µg/mL LPS.

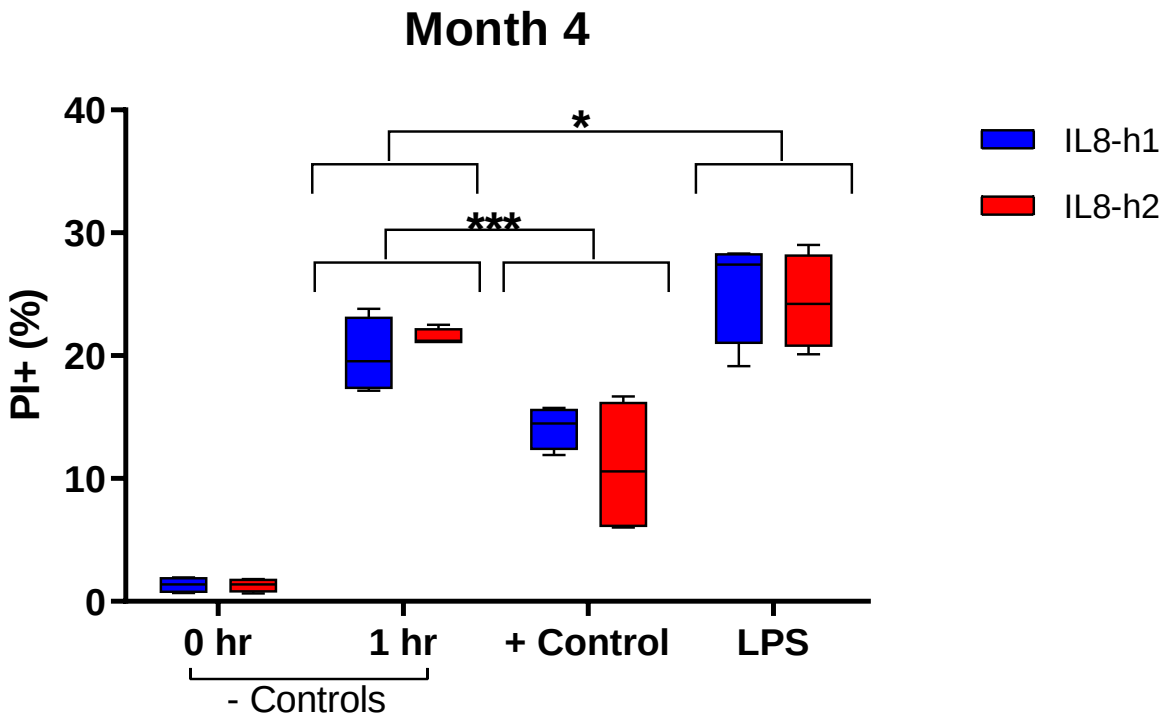

Figure 3.18 Live/Dead cell staining of neutrophils at month four before and after NET assay was performed. Neutrophils were isolated via MACS from *IL8* genotyped animals at month four. They were plated at $1x10^6$ cells/well and stimulated for 1 hr at 37°C, 5% CO_2 with either medium (- control), 1 mg/mL zymosan (+ control) or 200 µg/mL LPS. The cell viability was assessed by PI staining**.** Each bar represents ± SEM, n=5/6 animals**,** *P<0.05 and ***P<0.001 between treatments.

3.3 Discussion

IL8 haplotype has previously been demonstrated to impact systemic immunity by causing a difference in IL-8 levels produced by peripheral blood leukocytes in response to an *in vivo* LPS challenge in one-month old Holstein-Friesian calves. Additionally, it was also shown that IL8 haplotype affects levels of circulating lymphocytes during the LPS *in vivo* challenge [5]. In humans, IL8 haplotypes was shown to effect expression levels of *IL8* peripheral blood as well as impacting effector functions of macrophages such as phagocytosis an ROS generation [4]. It was therefore aimed to examine how IL8 haplotype effects peripheral immune cell population changes across a calf's early life. Also, how IL8 haplotype effects IL-8 and IL-1β secretion in response to viral and fungal PAMPs as well as bacterial. Finally, it was aimed to examine how IL8 haplotype influenced the neutrophil effector function of NET formation which had not previously been examined in either humans or cattle.

Our work has shown that IL8 haplotype affects circulating numbers of neutrophils, monocytes, eosinophils and basophils but not lymphocytes. IL8-h2 calves display increased number of circulating neutrophils, monocytes, eosinophils and basophils across multiple timepoints. The significant difference observed in absolute neutrophil numbers at month four where IL8-h2 had higher numbers could be explained by haplotype differences in levels of IL-8 at multiple timepoints. IL8-h2 has been previously shown to produce significantly higher levels of IL-8 in response compared to IL8-h1 [5]. This would indicate the presence of a basal level variation in IL-8 in these animals. In the case of monocyte differences where IL8-h2 showed higher monocytes levels compared to IL8-h1 at month six and nine, it has been shown that monocytes express CXCR1 and CXCR2 which are the receptors for IL-8. They can also respond to IL-8 in the same manner as neutrophils [145]. It is therefore likely that the significant haplotype differences in monocytes at months nine and close to significance at month six are due to elevated IL-8 levels. Again, high monocyte numbers in IL8-h2 most likely correspond with high IL-8 levels in IL8-h2 control samples. Eosinophils were also observed to be increased in IL8-h2 calves at months four and five. Basophils were increased at month two. Eosinophils and basophils also respond to IL-8 in a similar manner to neutrophils [146, 147]. Therefore, an increased level of IL-8 in circulation could also explain the increased numbers of these cell types in peripheral blood. No significant haplotype difference was seen in lymphocyte numbers at any time which was

notable as Stojkovic *et al*. reported a significant difference in lymphocyte numbers at twelve hours post LPS challenge. However, the difference was observable at every timepoint during the LPS challenge, from 0 hr up to 216 hr. Therefore, it is reasonable to assume the difference in lymphocyte numbers may be as a result of the treatment i.e. the LPS challenge [5]. This would suggest that during a systemic LPS challenge *in vivo* there is a haplotype difference lymphocyte numbers which would not be observable with the *ex vivo* assay used in this study.

It also investigated whether specific lymphocyte population may be proportionally different between the haplotypes. In particular, γδ T-cells are present at quite a high proportion in calves compared to other species so they may possibly play a strong role in the innate immune response [135]. However, no haplotype difference was seen for this cell type or other lymphocytes such as CD4 T cells and CD8 T-cells. There was a difference in the proportion of cells expressing sIgM between the haplotypes at month two indicating that an increase in B cells was significantly higher for IL8-h1 compared to IL8-h2. A possible explanation for this may be that calves received a booster vaccine between one and two months of age. This may have resulted in a B-cell expansion the remnants of which was observed as IL8-h1 calves may respond better to vaccination with an increased B-cell population. This may indicate that IL8 haplotype may affect adaptive immune responses. While previous studies have not identified IL8 haplotype as specifically affecting B-cell numbers or function, IL8 haplotype has been shown in humans to affect adaptive immune response by selectively polarising T-cells to either Th1 or Treg phenotypes depending on the haplotype of the individual [4]. Whether this impact of IL8 haplotype on adaptive immune responses is true for cattle also would require further experimental investigation.

There also appears to be age related change in the proportion of different sub-populations within the lymphocyte population independent of haplotype. There is gradual increase in sIgM positive cells across the six months while the proportion of CD4+, CD8+ and γδ+ T-cells generally decrease. Overall lymphocyte numbers do not change across time, but the proportions of these sub-populations do. These results have been seen previously in the case of γδ T-cells decreasing in numbers as calves age and B-cells gradually increasing [84, 148].

The blood cell population responsible for IL-8 production was confirmed to be monocytes. After observing monocytes as the primary producer of IL-8 in whole blood, lymphocytes were also seen to produce some IL-8. This has been documented in humans also where T-cells were seen to produce IL-8 [149]. The decrease in CD4+ T-cells may explain more muted IL-8 response from the calves as they grow older.

Having detected significant differences in neutrophil, monocyte, eosinophil and basophil numbers at multiple timepoints, the IL-1β and IL-8 responses were examined from whole blood in response to bacterial, viral and fungal PAMPs in calves of these haplotypes in order to assess whether haplotype differences occur across early life. Additionally, it was investigated whether haplotype differences occur in cytokines other than IL-8 such as IL-1β and in response to viral and fungal antigens as well as bacterial. The impact of using different assay systems was also established as the this was the first time the TruCulture system had been used in cattle so it was essential to ensure it worked effectively for the bovine species by comparing to a conventional whole blood culture assay. When measuring the IL-1β response to PAMPs using the conventional whole blood culture system, a significant difference between haplotypes was only seen at month six with zymosan simulation where IL8-h2 produced higher levels of IL-1β. The TruCulture® system showed no significant differences between haplotypes but there was a trend showing increased IL-1β in IL8-h2 calves for LPS and zymosan at months one, three and five. Zymosan is known to be a strong driver of pro-inflammatory cytokines and is regularly used as an inducer of arthritis in mouse models in part due to its ability to drive a high IL-1β response [150]. Zymosan generally produced higher levels of IL-1β compared with LPS and poly (I:C). Zymosan used in the whole blood culture system tended to show a significantly higher IL-1β response and increased cell death compared to TruCulture®. The variation could be down to origin of zymosan used in each culture system as the same concentration was used.

There was a lack of induction of IL-8 in response to poly (I:C) and the difference in IL-8 levels observed was due to a basal level difference between the haplotypes. The concentration of poly (I:C) used was most likely too low as the TruCulture® system (20 μg/mL) is designed for human assays so possibly cattle studies may require a higher concentration [151]. There was an inability to purchase TruCulture® tubes containing a higher concentration of poly (I:C) therefore the same concentration was used for the

conventional whole blood culture in order to make the assays comparable. Studies using poly (I:C) in cattle are uncommon as generally viral products or transfection are used [36]. TLR3 activation can initiate IL-8 expression so a dose response curve should provide us with the appropriate concentration to use [152]. It is interesting to note that no significant haplotype differences in the IL-8 response was seen to LPS in either culture system when a previous *in vivo* LPS challenge described IL8-h2 haplotype as producing significantly higher IL-8 [5]. While it was shown that monocytes are key drivers of IL-8 levels in bovine peripheral blood, the discrepancy seen between the *ex vivo* LPS stimulation here and the previously described *in vivo* LPS challenge, could be explained by the exclusion of cell types such as endothelial, epithelial and stromal from the *ex vivo* whole blood culture system. This would suggest that the key drivers of the IL-8 level differences between haplotypes in an *in vivo* setting are local endothelial, epithelial and stromal cells rather than circulating immune cells. Endometrial epithelial cells with the IL8-h2 promoter have been shown to have more activity in response to TNFα and LPS which would suggest they may express more IL-8 [36].

In this study two different whole blood stimulation systems were used to validate the TruCulture® system for use in cattle. Upon comparing the two systems directly at month three, there were significant differences in the response to stimulants most notably in the IL-8 response to LPS and zymosan. The most likely explanation for this is the origin of the LPS and zymosan used in both assays. Different LPS serotypes have been shown to elicit largely variable responses, most notably in the case of IL8 expression [153]. Cell death was increased in the conventional whole blood culture system compared to the TruCulture® system in the case of the control, LPS and zymosan. No difference in gene expression of *HIF1A* was seen therefore hypoxia is not a factor in any differences seen between the two assays. It is probable that the increased cell death and the higher levels of IL-8 are linked. The zymosan A from *S. cerevisiae* used in this system seemed to induce high pro-inflammatory response as evidenced by IL-1β levels. There is a possibility of the TruCulture® system using a zymosan from a different origin which may explain the differences seen between the two systems.

Finally, it was hypothesised that IL8 haplotype would alter the ability of neutrophils to form NETs due to different levels of IL-8 in the peripheral blood of Holstein-Friesian calves with distinct IL8 haplotypes. Also, a previous human study

showed IL8 haplotype to affect the ability of macrophages to carry out other effector functions including phagocytosis and ROS generation [4]. It was shown that IL8 haplotype affects neutrophil ability to form NETs. NET formation was significantly decreased for IL8-h2 calves compared to IL8-h1 calves at months five and six. The initial study which identified NET function in neutrophils reported IL-8 to be an inducer of NETs [89]. Considering these haplotypes display a difference in IL-8 levels during *in vivo* LPS stimulation [5], and now additionally a basal level difference *ex vivo* has been identified, this may be contributing to the significant haplotype differences at month five in the positive control and at month six in LPS treated neutrophils. The assay used in this case stains extracellular DNA however an additional visual method would be useful in validating NET formation. Extracellular DNA can be realised in the case of other forms of cell death such as apoptosis and necrosis [154]. Overall IL8-h1 produced more NETs than IL8-h2. This is notable as this haplotype was shown to produce lower levels if IL-8 in response to LPS during an *in vivo* challenge [5]. However, in humans, phagocytosis was shown to be increased in macrophages from individuals with an IL8 haplotype that showed reduced expression of *IL8* in peripheral blood cells. Interestingly, no differences in cell death were observed between the haplotypes. There is much debate in the literature about whether neutrophils die after NET formation. Some studies have reported cells still being viable after NET stimulation in response to *S. aureus* [144]. Significant differences were seen in cell viability depending on the stimulation but not between haplotypes. Less cell death was seen in the case of the positive control compared to the negative control even though more NETs would have been produced. The use of PI as a cell viability may not be ideal for this type of assay as it binds to dsDNA within a cell where the membrane has been compromised. It may have been the case that there wasn't enough DNA available for binding after the NET assay. However, LPS had the highest amount of cell death at that month and also a high level of NET production. Another explanation for this discrepancy could be different types of NETosis being employed depending on the stimulus. So called ''suicidal'' NETosis involves complete release of cell contents while ''vital'' NETosis occurs quicker and the membrane does not disintegrate. The neutrophil can still carry out functions afterwards [155].

In conclusion, *IL8* haplotype differences were identified in circulating cell populations including neutrophils, monocytes, eosinophils and basophils in a calf's early months of life while also confirming the major source of IL-8 in blood as monocytes.

Additionally, it was shown that IL8 haplotype influences the calf's systemic response to bacterial and fungal PAMPs at different stages of their early life while validating the use of a standardised whole blood culture system in cattle. Lastly, it was established that IL8 haplotypes affects the ability of neutrophils to for NETs. There were clear systemic differences in the levels of IL-8 protein between the IL8 haplotypes which are particularly evident at basal levels where IL8-h2 shows higher levels. It was interesting to note also the differences seen here in response to LPS using this *ex vivo* whole blood stimulation system where there was no haplotype effect, and the previous study using an *in vivo* LPS challenge where IL8-h2 showed increased IL-8 levels [5]. Further investigation in chapter 4 will establish serum level IL-8 differences between IL8 haplotypes. Additionally, the differences seen between the results for the *ex vivo* whole blood culture in this chapter and the previous results for the *in vivo* LPS challenge which are hypothesised to be due to exclusion of local cell types in the *ex vivo* model will be further investigated using a local cell culture model in chapter 5.

Chapter 4. IL8 haplotype influences key components of the vitamin D pathway

4.1 Introduction

It is now understood that calves with different IL-8 haplotypes vary significantly in their peripheral blood IL-8 response to PAMPs such as LPS and zymosan and that responses change over the course of their early life as shown in chapter three. The negative controls (unstimulated peripheral blood) used in those experiments also showed significantly different IL-8 levels between haplotypes. Therefore, it was hypothesised that there are underlying differences in IL-8 levels in serum between the haplotypes. Additionally, vitamin D is now understood to be an important modulator of the innate immune response as well as its traditionally understood function in calcium homeostasis. It also has a particularly unique relationship with IL-8 which appears to be the result of its ability to regulate IL-8 gene expression via interference with NF-κB and also by its receptor VDR binding to the *IL8* gene [128, 129]. In humans, an inverse relationship has been noted between IL-8 and vitamin D levels in serum [156]. It is therefore hypothesised that this relationship between IL-8 and vitamin D is also present in cattle and differs based on IL8 haplotype.

It has been shown that different forms of vitamin D can regulate *IL8* expression *in vitro*. Monocytes that were treated with the active form of vitamin D; $1,25(OH)_2D_3$, showed increased expression of IL-8 over eight hours. This result was also seen with the inactive form; 25(OH)D. It is believed this was because of $1,25(OH)_2D_3$ binding to the VDR which in turn acts as a transcription factor, acting on the promoter region to initiate *IL8* gene expression [126]. Other studies have reported that vitamin D reduces IL-8 secretion by macrophages but only in the situation where the cell is already in an inflammatory or activated state [127]. The suggestion is that vitamin D bound to the VDR is interfering with NF-κB signalling in a certain capacity [128]. The effects of vitamin D appear to also vary depending on cell type. $1,25(OH)_2D_3$ was shown to significantly increase IL-8 levels in human neutrophils but not macrophages [119]. No study has investigated the role that high levels of IL-8 may have on the vitamin D pathway. Considering that calves possess two different IL8 promoter haplotypes it is hypothesised that this could affect the expression of genes in the vitamin D pathway.

Specific SNPs in vitamin D pathway genes have also been associated immune function as well as disease susceptibility and outcomes in cattle and humans. Genetic variation in the *GC* gene, which encodes the vitamin D binding protein, was shown to be associated with susceptibility to clinical mastitis as well as an increased SCC [157]. This

would suggest that polymorphisms in this gene of the vitamin D pathway may affect how individuals respond to infection. Interestingly, this gene is located just upstream from the IL8 gene. SNPs in other vitamin D pathway genes have not been investigated on whether they are associated with immune function.

It also remains to be established whether vitamin D supplementation would have a significant effect on the response to infection. A number of recent studies have linked vitamin D supplementation to improved survival and reduced severity of infection with COVID-19 [158, 159]. Also, a study conducted in mice showed how supplementation can improve immune function. Using an immunosuppressed mouse model, vitamin D supplementation was shown to significantly improve thymus and spleen indices as well as CD4 and CD8 T-cell ratios in blood [160]. Vitamin D has also been shown to improve the innate immune response by upregulating key receptors including TLR2 and NOD2 in an *in vitro S. pneumoniae* infection of monocyte derived dendritic cells [161]. With evidence pointing towards vitamin D possibly having significant effects in modulating pro-inflammatory cytokine and chemokine production in addition to increasing receptor expression during the innate immune response, it was important for us to know the vitamin D status of the calves in this study as it may influence how they respond to PAMP stimulation.

The inactive form of vitamin D; 25(OH)D is generally used by healthcare professionals to assess the vitamin D status of an individual. There is no official recommendation of a specific level of vitamin D that should be present in circulation of cattle although the human recommendation of >30 ng/mL is generally used as a benchmark [115]. However, age and energy requirements of the animal should also be considered for example, weaning, pregnancy, post-partum etc. Young calves in the US were shown not to have sufficient vitamin D levels (~30 ng/mL) until at least four months of age. This was true for herds in both the north and the south of the country indicating that the amount of sunlight the calves received had no effect at this age. The author recommended increased supplementation for calves at a young age [132]. Other studies which have examined the impacts of vitamin D deficiency have reported significant outcomes in relation to disease susceptibility. In a mouse model of rhinosinusitis, deficiency of vitamin D resulted in increased PMN and lymphocyte infiltration into the nasal passage in addition to increased IFN-γ and IL-6 in nasal lavage [162]. Vitamin D deficiency was also linked to significant decreased staining of 1α-hydroxylase (encoded

by *CYP27B1* gene) in the nasal epithelial cells [162]. Researchers in Finland were able to link vitamin D deficiency in military personnel to increased incidence rate of respiratory infection [163]. It remains to be investigated how vitamin D deficiency directly impacts IL-8 production, particularly in cattle, however the in-active form of vitamin D; 25(OH)D (routinely measured in serum) and IL-8 have been presented as potential biomarkers for melanoma indicating a potential dynamic relationship between the two molecules [156].

With increasing evidence showing vitamin D can modulate expression of *IL8* in humans, it remains unclear whether this is also true for cattle. Additionally, it is also unknown if this immunomodulatory action of vitamin D is affected by IL8 haplotype and if this is the case, is the mechanism through IL-8 effecting the expression of genes in the vitamin D pathway. It was therefore aimed to assess basal levels of 25(OH)D and IL-8 in the serum of calves with both Il-8 haplotypes. Following this it was also aimed to establish the nature of the relationship between IL-8 and vitamin D using an *in vitro* system of bovine monocytes. Finally, the role of vitamin D supplementation was investigated in modulating *IL8* expression and protein production in monocytes and whole blood from Holstein-Friesian calves.

4.2 Results

4.2.1 IL-8, 25(OH)D and VDBP levels in serum of calves with different IL8 haplotypes

As previously shown in chapter three, IL-8 levels significantly differed in the whole blood of IL8-h1 and IL8-h2 in the negative control (null) samples. As these samples were incubated for twenty-four hours it was necessary to confirm the levels were as a result of an *in vivo* haplotype difference and not because of a random effect occurring in the culture system. This was confirmed by measuring serum IL-8 levels in the genotyped calves. Serum samples were taken at monthly intervals and IL-8 levels were measured by ELISA. Highly significant differences between haplotypes were identified at months two, three, six, seven and eight (Figure 4.1). IL8-h2 showed much higher levels than IL8-h1. The levels of IL-8 for both haplotypes also increased to October before decreasing back down in January. The end of October was when the calves were moved in indoors for the winter season before being turned out in January.

After observing changes in IL-8 levels across months, it was then examined whether there were changes in 25(OH)D levels across months. The 25(OH)D levels for both haplotypes increase from one month old to seven months old at which point the levels begin to drop (Figure 4.2). After seven months, the calves were housed indoors for the winter season. Interestingly, there is a significant haplotype difference in levels of 25(OH)D at month five, six and seven where levels in IL8-h1 calves were greater. This trend was also seen earlier at months three and four were levels began to increase above the recommended level of 30 ng/mL.

In order to investigate whether differences in VDBP between haplotypes was influencing the serum 25(OH)D levels, VDBP levels were assessed using ELISA. A subset of calves' serum of each haplotype from months five, six and seven was used for testing. There were no significant differences between the haplotypes at any of the months although there was a slight increase in IL8-h2 at month six (Figure 4.3).

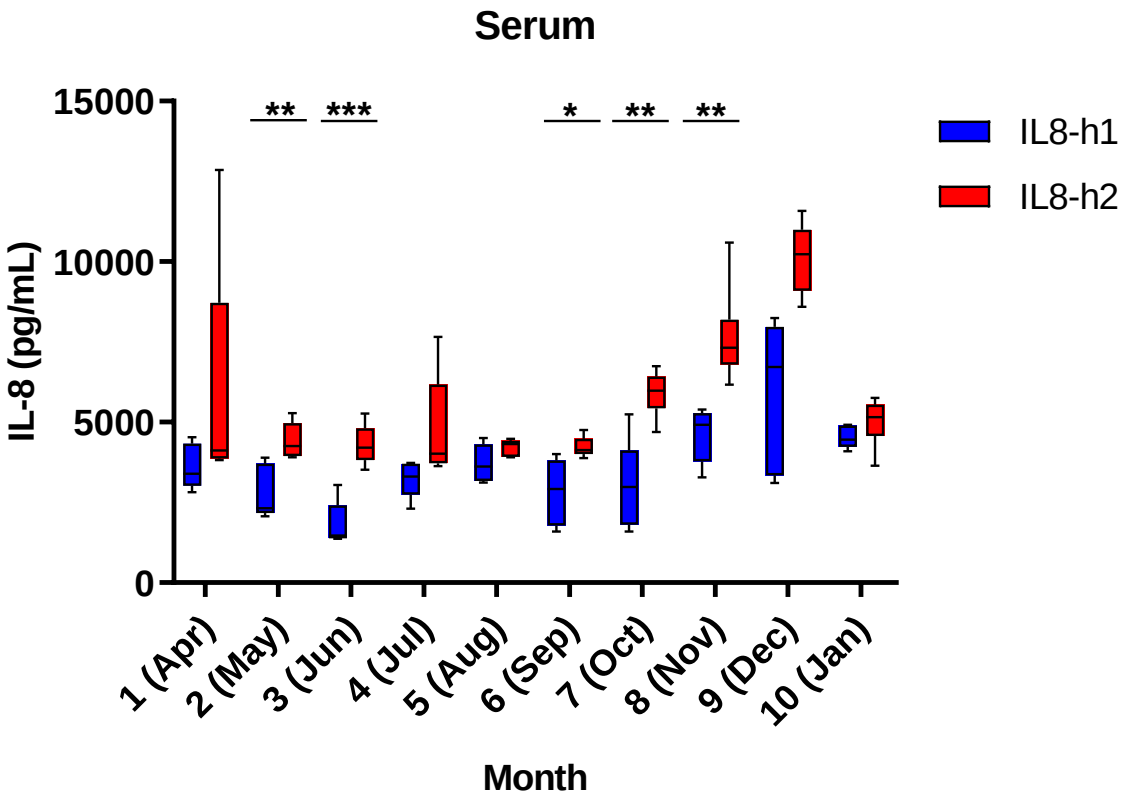

Figure 4.1 Basal expression of IL-8 protein in serum of calves with different *IL8* haplotypes. Serum samples from genotyped calves were taken at monthly intervals and IL-8 levels were assessed by ELISA. Concentrations are presented as pg/mL and bars represent the min/max values of n=5/6. Significance is between haplotypes where *P<0.05, **P<0.01 and ***P<0.001.

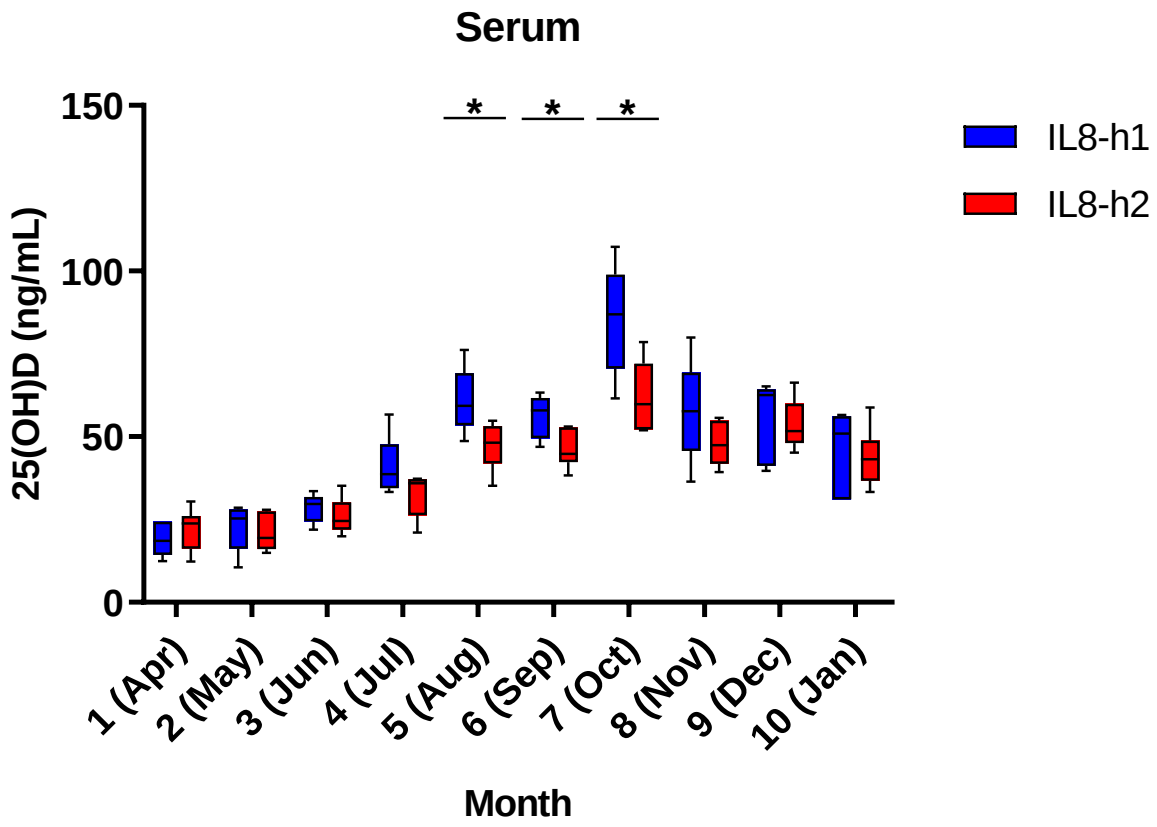

Figure 4.2 Levels of 25(OH)D in the serum of calves with different IL8 haplotypes. Serum samples from genotyped calves were taken at monthly intervals and 25(OH)D levels were assessed by ELISA. Concentrations are presented as ng/mL and bars represent the min/max values of n=5/6. Significance is between haplotypes where *P<0.05.

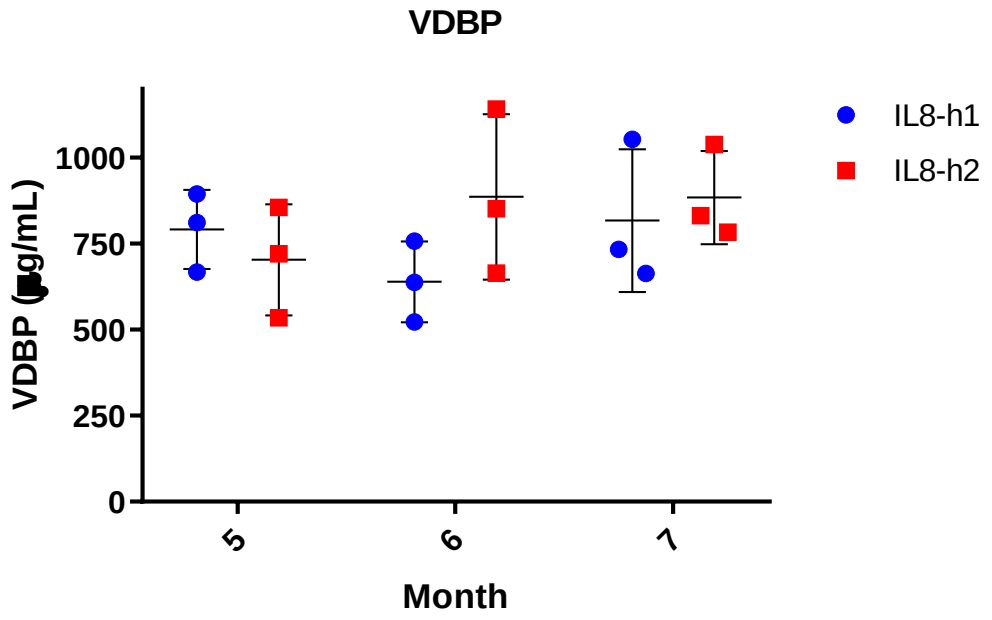

Figure 4.3 Levels of vitamin D binding protein (VDBP) in the serum of calves with different IL8 haplotypes. Serum samples from genotyped calves were taken at five, six and seven months of age and VDBP levels were assessed by ELISA. Concentration is presented as µg/mL and error bars represent the SEM of n=3.

4.2.2 Vitamin D pathway associated gene expression in whole blood from calves with two different IL8 haplotypes

With evidence now indicating a connection between serum IL-8 and vitamin D levels, gene expression of vitamin D synthesis genes was examined in both haplotypes. We hypothesised that gene expression of enzymes involved in vitamin D conversion between its active and inactive form (*CYP27A1, CYP27B1 and VDR)* are differentially expressed depending on IL8 haplotype. RNA extracted from whole blood culture stimulations carried out previously (Chapter three) were used to analyse gene expression. The RNA from the control and LPS stimulated whole blood samples of the six-month-old calves were used as this was the age that saw the most significant haplotype differences in IL-8 and 25(OH)D levels. It was necessary to include LPS stimulated samples as a previous study on bovine monocytes identified the gene *CYP27B1* only to be expressed during LPS stimulation [118]. Genes known to be modulated by vitamin D were also included such as defensins (*eDEFB4A*) and *NOS2* which encodes iNOS [118, 164]. *IL8* gene expression was also measured and compared to protein levels at month six. In addition, *TLR4 (*as the whole blood was stimulated with LPS) was included to investigate whether the genotyped calves were responding to LPS in the same manner.

RNA was extracted from the whole blood samples and its quality was assessed using the bioanalyser before conversion to cDNA. Gene expression of *IL8* was not significantly different between haplotypes however, levels in IL8-h1 were higher than IL8-h2 in response to LPS (Figure 4.3A). *TLR4* expression approached significantly (P=0.077) increased expression in IL8-h1 calves as compared to IL8-h2 calves in response to LPS (Figure 4.3B). A significant difference between haplotypes was observed for the *eDEFB4A* in the control samples. This primer is specific for eight different β defensin genes in cattle which are difficult to design individual primers for due to highly similar sequences. *eDEFB4A* gene expression was significantly higher in IL-h1 control compared to IL8-h2 control (Figure 4.3C). No significant difference in *NOS2* expression was detected between the two haplotypes (Figure 4.3D). *CYP27A1,* which converts inactive vitamin D to active, expression was not significantly different between the two haplotypes however, expression levels were higher in the IL8-h1 control (Figure 4.3E). *CYP27B1*, which is also involved in local conversion to active vitamin D, also showed increased expression in IL8-h1 control when compared with IL8-h2 (Figure 4.3F). There was no difference in *VDR* expression between the haplotypes (Figure 4.3G).

There is evidence of a basal haplotype difference resulting in increased expression *CYP27B1* and *eDEFB4A* for the IL8-h1 haplotype that could possibly be the result of elevated levels of 25(OH)D in IL8-h1 animals described in the previous section.

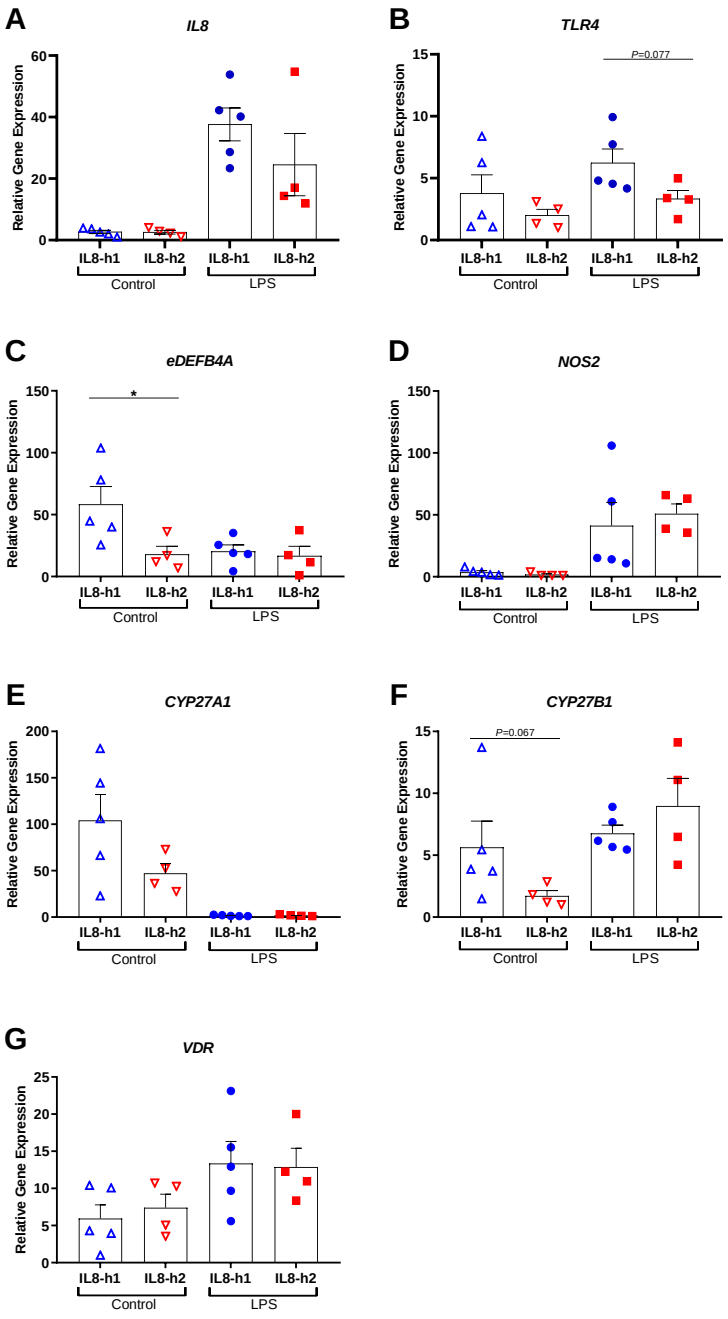

Figure 4.4 Gene expression of *IL8, TLR4, eDEFB4A, NOS2, CYP27A1, CYP27B1* and *VDR* from whole blood of six month old genotyped calves stimulated with LPS via a conventional whole blood culture system. Whole blood of IL8 genotyped animals was stimulated with 100ng/mL of LPS for 24 hr at 37°C, 5% CO_2. (A) *IL8* (B) *TLR4* (C) *eDEFB4A* (D) *NOS2* (E) *CYP27A1*, (F) *CYP27B1* and (G) *VDR* expression was assessed and data is presented as relative gene expression compared to the lowest expressed sample. Error bars represent the SEM of n=4/5. Significance is between haplotypes where *P<0.05.

4.2.3 Analysis of SNPs present in vitamin D pathway genes of calves with different IL8 haplotypes

Having observed significant haplotype differences in IL-8 and 25(OH)D at a protein level in serum and also vitamin D pathway gene expression in whole blood, we began to investigate whether these differences could be explained by polymorphisms in the genes themselves. We decided to target two genes; *GC* (encodes vitamin D binding protein or VDBP) and *CYP27A1* for a number of reasons. *GC* is located ~1 Mb upstream from the *IL8* gene (Figure 4.4A) presenting a possibility the two may be in linkage disequilibrium where SNPs in this gene are being inherited along with a certain IL8 haplotype. *CYP27A1* is located ~1.5 Mb downstream from the IL-8 receptor genes *CXCR1* and *CXCR2* (Figure 4.4B) which have also been shown to have unique haplotypes in cattle [7, 43]. *CYP27A1* also showed differential gene expression between the IL8 haplotypes. A previous whole exome sequencing study identified a number of SNPs in the *GC* and *CYP27A1* [165]. We chose two of these SNPs from each gene which were labelled GC SNP1, GC SNP2, CYP27A1 SNP1 and CYP27A1 SNP2. They were chosen based on their locations where three are synonymous variants and one being an upstream variant (Figure 4.5).

Custom SNP genotyping assays were designed for each SNP and used to calculate allele frequencies for each SNP. The allele frequencies for GC SNP1 and GC SNP2 were the same for both haplotypes with no particular allele segregating with a specific haplotype (Figure 4.6). For CYP27A1 SNP1 and CYP27A1 SNP2 the results were similar (Figure 4.7) where no particular SNP seeming to co-segregate along with an IL8 haplotype. Overall, there appears to be no evidence of these particular SNPs playing a role in the IL8 haplotype differences seen in serum 25(OH)D levels.

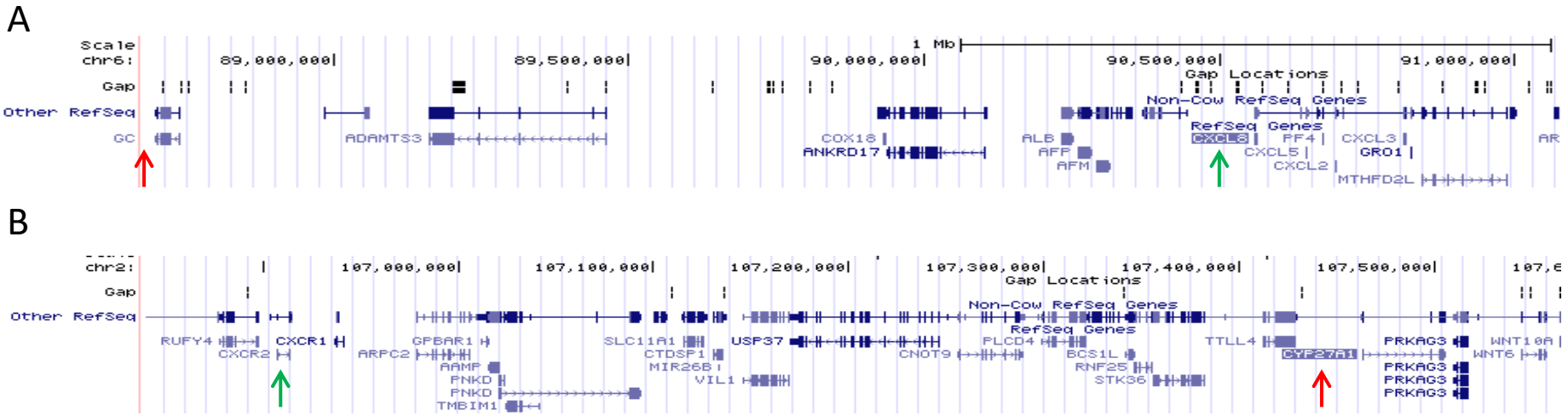

Figure 4.5 GC and CYP27A1 locations on their respective chromosomes in the bovine genome. Images taken from USCS Genome Browser show the location of (A) *GC* gene (red arrow) in respect to *CXCL8/IL8* (green arrow) on chromosome six and (B) *CYP27A1* (red arrow) in respect to *CXCR1/CXCR2* (green arrow) on chromosome two in the bovine genome.

Figure 4.6 GC SNP1, GC SNP2, CYP27A1 SNP1 AND CYP27A1 SNP2 locations on their respective genes. Images taken from GeneView in dbSNP show the locations of (A) GC SNP1 and (B) GC SNP2 in the *GC* gene, and (C) CYP27A1 SNP1 and (D) CYP27A1 SNP2 in the *CYP27A1* gene.

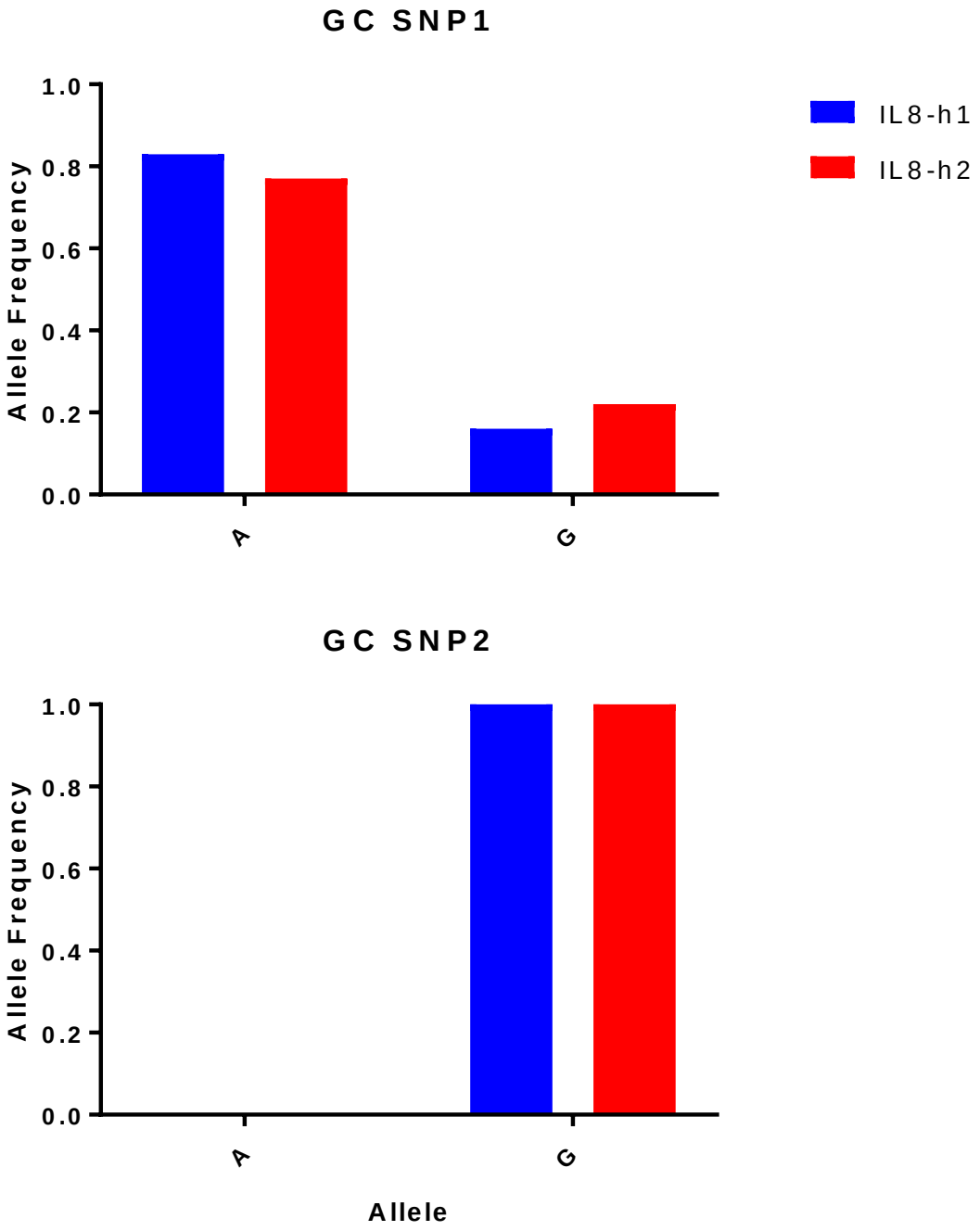

Figure 4.7 Allele frequencies of GC SNP1 and GC SNP2 in the *GC* gene of calves with different IL8 haplotypes. Genomic DNA was extracted from the blood of each calf and a custom SNP genotyping assay was performed. Allele frequencies for each SNP were calculated by assessing the number of animals for each IL8 haplotype (n=9) which were heterozygote and homozygote for each allele.

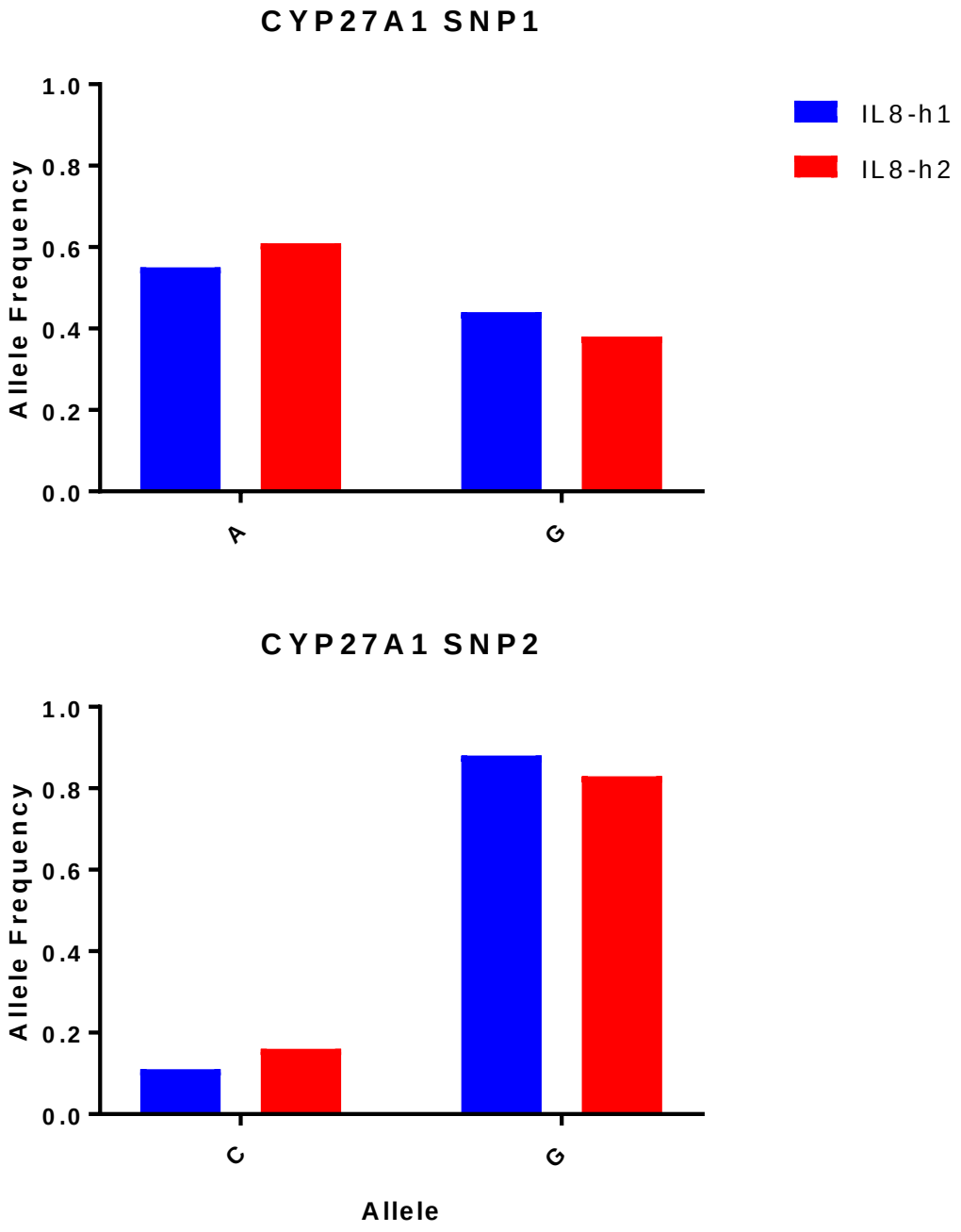

Figure 4.8 Allele frequencies of CYP27A1 SNP1 and CYP27A1 SNP2 in the *CYP27A1* gene of calves with different IL8 haplotypes. Genomic DNA was extracted from the blood of each calf and a custom SNP genotyping assay performed. Allele frequencies for each SNP were calculated by assessing the number of animals in each IL8 haplotype (n=9) which were heterozygote and homozygote for each allele.

4.2.4 *In vitro* analysis of vitamin D and IL-8 effects on monocytes

In order to further establish the relationship between IL-8 and vitamin D, monocytes isolated from Holstein-Friesian calves were stimulated with IL-8 and vitamin D and the expression of vitamin D pathway genes examined. The optimum stimulation time was first established. Monocytes were isolated using MACS and stimulated with 1 ng/mL recombinant bovine IL-8 for one, three, six, twelve and eighteen hours. Expression of vitamin D pathway genes *VDR, CYP27A1, CYP27B1* and *CYP24A1* was analysed. There were no significant differences between timepoints for any of the genes (Figure 4.9 A-D). Therefore, further IL-8 stimulations where conducted over a three-hour time period in order to assess early responses to IL-8.

A range of IL-8 concentrations (0.5-20 ng/mL) were used to stimulate monocytes in order to assess its ability to activate expression of vitamin D pathway genes. There was no significant induction of *VDR* in response to any concentration of IL-8 (Figure 4.10A) in contrast the positive control LPS did induce expression of *VDR.* A similar result was observed for *CYP27A1* where again there was no significant induction by IL-8, but induction was observed for LPS (Figure 4.10B). *CYP27B1* expression is slightly increased with upwards of 5 ng/mL IL-8 but this seems to be just one animal (Figure 4.10C). The positive control LPS does increase expression of *CYP27B1.* There is a slight increase of *CYP24A1* expression in response to IL-8, but this tends to be just one animal (Figure 4.10D). Again, there is high expression in response to LPS.

Gene expression in response to $1,25(OH)_2D_3$ after three hours was also examined in monocytes. A low 10 nM concentration of $1,25(OH)_2D_3$ significantly decreased expression of *VDR* while the 100 and 200 nM concentrations kept expression stable (Figure 4.11A). The opposite was true for *CYP27A1* where the 200 nM concentration significantly decreased expression while expression remained stable for the other two concentrations (Figure 4.11B). Much like *VDR,* expression of *CYP27B1* was also significantly decreased in response to 10 nM $1,25(OH)_2D_3$ and expression stayed stable for the 100 and 200 nM concentration stimulations (Figure 4.11C). *CYP24A1* expression was significantly increased in response to 100 and 200 nM of $1,25(OH)_2D_3$ and close to significant for 10nM (P=0.069) (Figure 4.11D). *IL8* expression was significantly decreased in response to 10 nM $1,25(OH)_2D_3$ and there was also small decrease in response to the 200 nM concentration (Figure 4.11E).

A six-hour stimulation of monocytes with $1,25(OH)_2D_3$ was also carried out and gene expression analysed. There was no significant difference in gene expression of *VDR* in response to $1,25(OH)_2D_3$ unlike what was seen at three hours where expression was significantly reduced by 10 nM $1,25(OH)_2D_3$ (Figure 4.12A). The response of *CYP27A1* to $1,25(OH)_2D_3$ was similar to what was seen at three hours where a high concentration (200 nM) of $1,25(OH)_2D_3$ significantly reduced gene expression (Figure 4.12B). Expression of *CYP27B1* remained significantly reduced by 10 nM $1,25(OH)_2D_3$ after six hours, in addition there is also a close to significant reduction by the 100 nM concentration (P=0.055) (Figure 4.12C). Expression of *CYP24A1* remained very high in response to all concentrations of $1,25(OH)_2D_3$ after six hours much like what was seen after three hours (Figure 4.12D). There is a small increase in *IL8* expression which wasn't observed at three hours especially in response to 200 nM $1,25(OH)_2D_3$ however this was driven by inter-animal variation (Figure 4.12E).

IL-8 protein production was measured in cell supernatant after three, six and also twenty-four hours stimulation with $1,25(OH)_2D_3$. There was no significant induction of IL-8 protein in response to any concentration of $1,25(OH)_2D_3$ after three hours stimulation (Figure 4.13A) or six hours stimulation, however one animal showed much higher levels of IL-8 at six hours, for both the controls and all $1,25(OH)_2D_3$ concentrations ,when compared with the others (Figure 4.13B). There was again no significant induction of IL-8 protein in response to $1,25(OH)_2D_3$ after twenty-hours although one animal showed increased production in response to each concentration of $1,25(OH)_2D_3$ (Figure 4.13C).

IL-8 protein itself appears to have no effect on expression of vitamin D pathway enzymes in monocytes therefore it is most likely not affecting serum 25(OH)D levels through modulating expressing of these genes. Active vitamin D or $1,25(OH)_2D_3$ can reduce IL-8 levels in monocytes at a low dose and early at a basal level but does not increase gene expression or protein secretion of IL-8 in monocytes. It also appears to reduce expression of *VDR* and *CYP27B1* at a basal level.

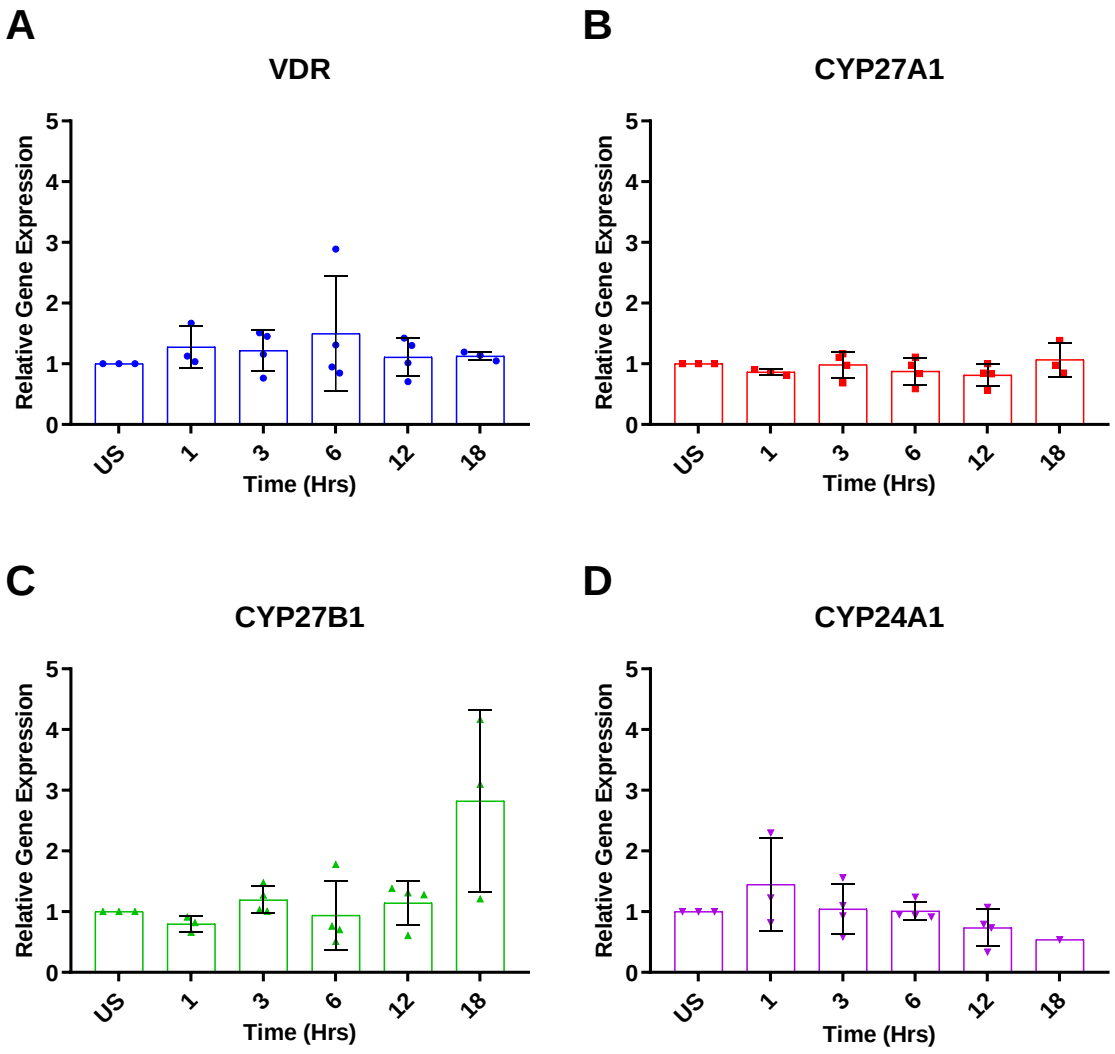

Figure 4.9 Gene expression of *VDR*, *CYP27A1*, *CYP27B1* and *CYP24A1* following one, three, six, twelve- and eighteen-hours stimulation of 1 ng/mL of recombinant bovine IL-8 in bovine monocytes isolated from whole blood via MACS. Monocytes were stimulated with 1 ng/mL IL-8 for one, three, six, twelve- and eighteen-hours plus an unstimulated control at 37°C, 5% CO_2. Gene expression was assessed and presented as relative to the unstimulated control. Induction is shown of (A) *VDR* (B) *CYP27A1* (C) *CYP27B1* and (D) *CYP24A1*. Error bars represent the SEM of n=3/4 animals.

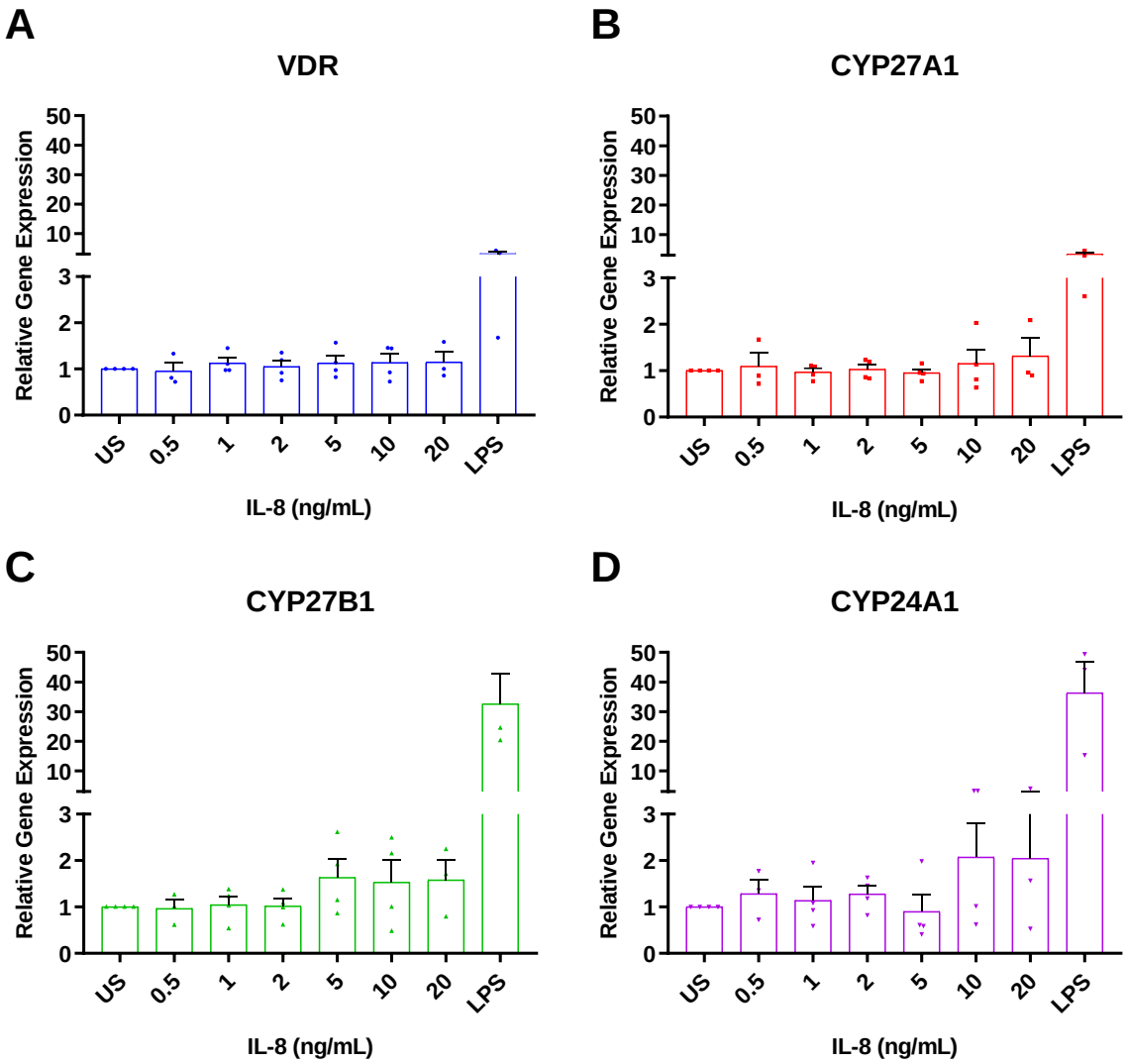

Figure 4.10 Gene expression of *VDR, CYP27A1, CYP27B1* and *CYP24A1* following three hours stimulation of 0.5 - 20 ng/mL of recombinant bovine IL-8 in bovine monocytes isolated from whole blood via MACS. Monocytes were stimulated with 0.5 - 20 ng/mL IL-8 for three hours plus an unstimulated control (US) and a LPS positive control (100 ng/mL) at 37°C, 5% CO_2. Gene expression was assessed and presented as relative to the unstimulated control. Induction is shown of (A) *VDR* (B) *CYP27A1* (C) *CYP27B1* and (D) *CYP24A1*. Error bars represent the SEM of n=3/4 animals.

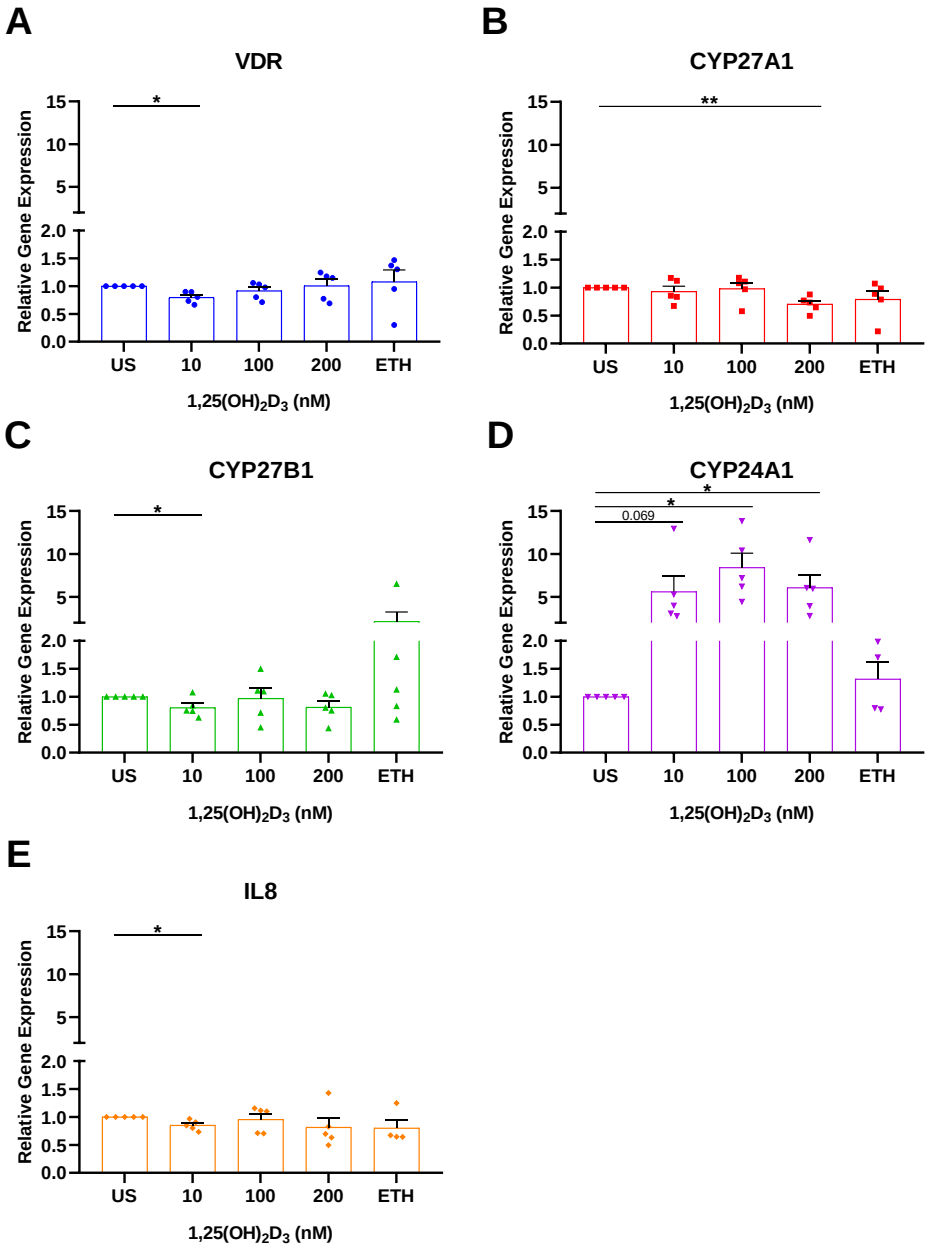

Figure 4.11 Gene expression of *VDR*, *CYP27A1*, *CYP27B1*, *CYP24A1* and *IL8* following three hours stimulation of 10 - 200 nM $1,25(OH)_2D_3$ in bovine monocytes isolated from whole blood via MACS. Monocytes were stimulated with 10 - 200 nM $1,25(OH)_2D_3$ for three hours plus an unstimulated control (US) and an ethanol vehicle control (ETH) at 37°C, 5% CO_2. Gene expression was assessed and presented as relative to the unstimulated control. Induction is shown of (A) *VDR* (B) *CYP27A1* (C) *CYP27B1* (D) *CYP24A1* and (E) *IL8*. Error bars represent the SEM of n=4/5 animals. Significance is between control and stimuli where *P<0.05 and **P<0.01.

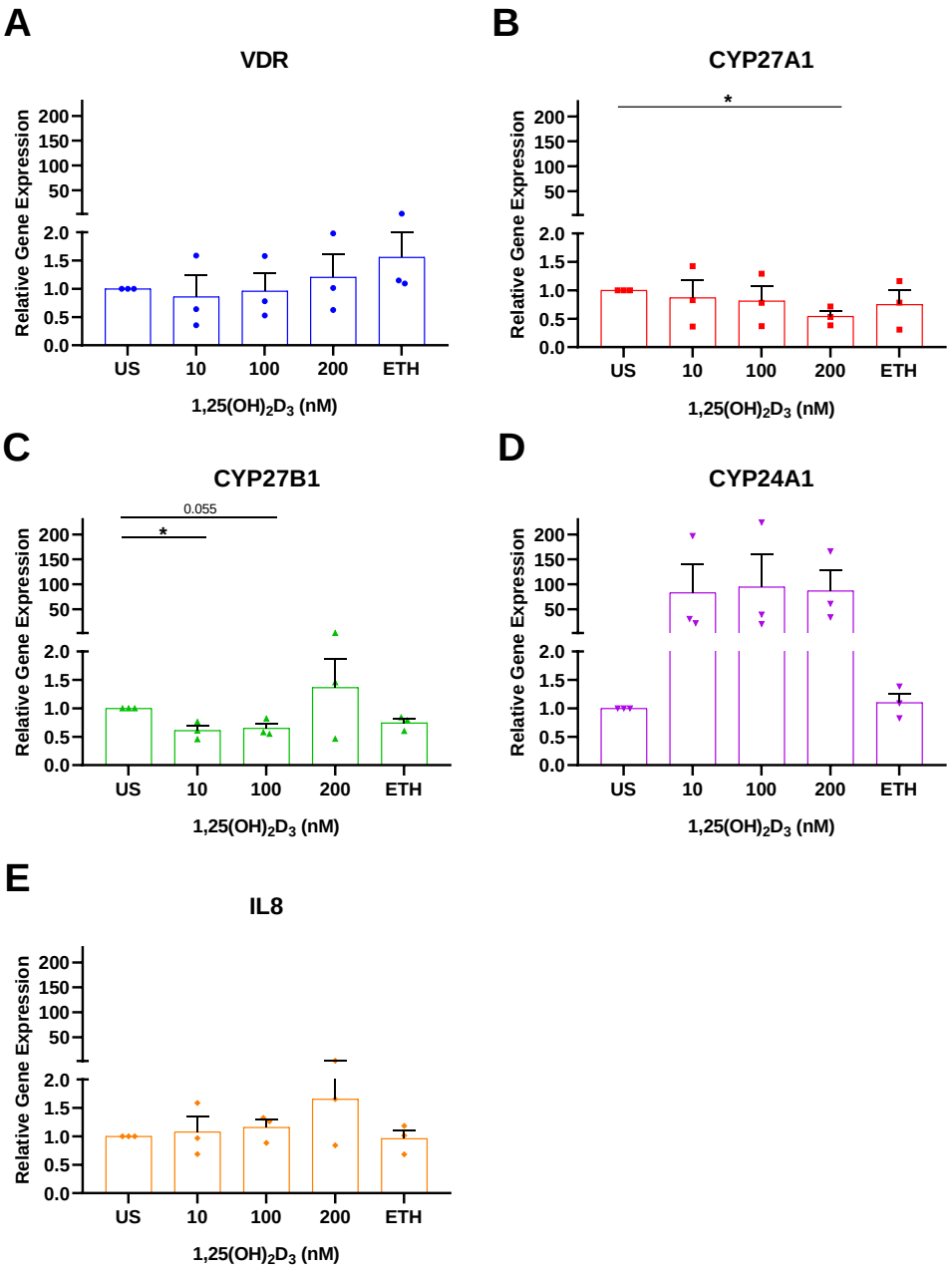

Figure 4.12 Gene expression of *VDR*, *CYP27A1*, *CYP27B1*, *CYP24A1* and *IL8* following six hours stimulation of 10 - 200 nM 1,25(OH)$_2$D$_3$ in bovine monocytes isolated from whole blood via MACS. Monocytes were stimulated with 10 - 200 nM 1,25(OH)$_2$D$_3$ for six hours plus an unstimulated control (US) and an ethanol vehicle control (ETH) at 37^{o}C, 5% CO_2. Gene expression was assessed and presented as relative to the unstimulated control. Induction is shown of (A) *VDR* (B) *CYP27A1* (C) *CYP27B1* (D) *CYP24A1* and (E) *IL8*. Error bars represent the SEM of n=3 animals. Significance is between control and stimuli where *P<0.05.

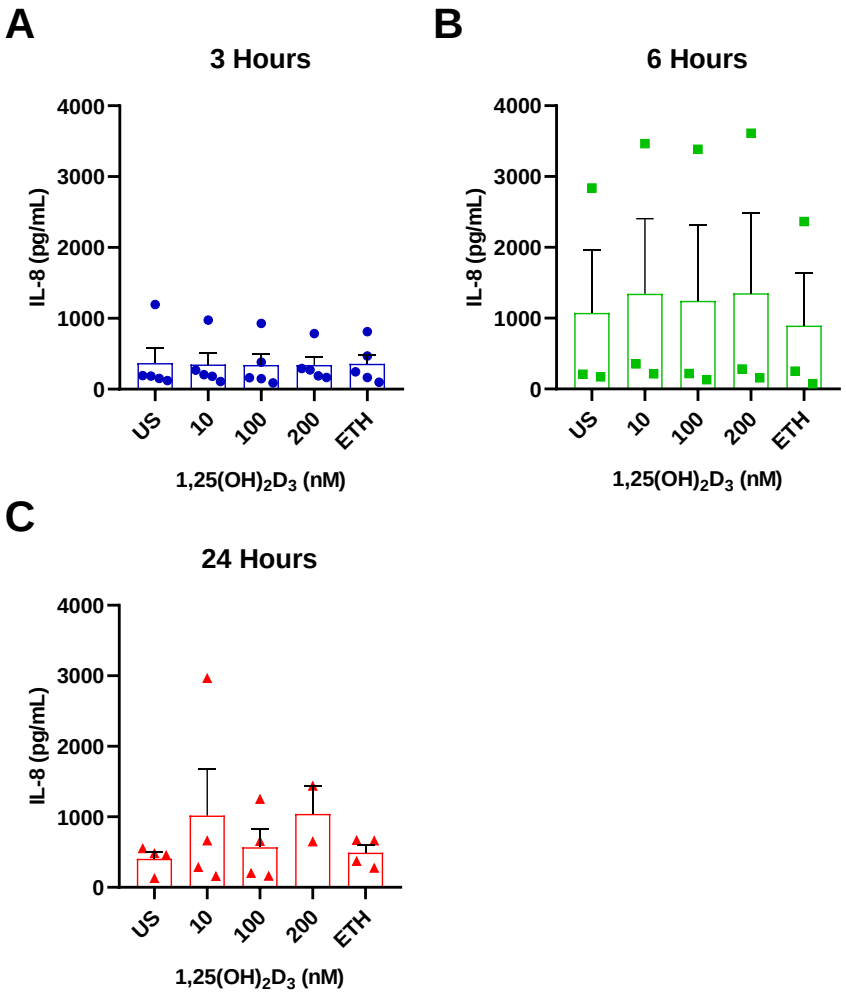

Figure 4.13 Production of IL-8 protein following three, six and twenty-four hours stimulation of 10 - 200 nM $1,25(OH)_2D_3$ in bovine monocytes isolated from whole blood via MACS. Monocytes were stimulated with 10 - 200 nM $1,25(OH)_2D_3$ for 3, 6 and 24 hours plus an unstimulated control (US) and an ethanol vehicle control (ETH) at 37°C, 5% CO_2. Production of IL-8 was assessed by ELISA from the cell stimulation supernatant. Induction is shown at (A) three (B) six and (C) twenty-four hours. Error bars represent the SEM of n=3-5 animals.

4.2.5 The effect of vitamin D supplementation on *IL8* expression in calves

Having previously seen vitamin D to reduce *IL8* expression at a basal level in monocytes *in vitro*, it was important to further confirm if this was the case *in vivo*. To do this, new-born calves were supplemented with vitamin D from birth, while a control group was included in the study where no additional supplementation to feed was provided. After six months, blood samples were taken from both groups of calves, serum was collected, and monocytes were isolated and stimulated in vitro with LPS for 6 hours. In addition, whole blood was also subjected to same stimulation conditions for six hours in order to investigate whether the of vitamin D on whole blood differed to monocytes alone.

IL-8 and 25(OH)D levels in the sera were assessed by ELISA. Levels of IL-8 protein were not significantly different between the supplemented and control calves though mean levels from the supplemented calves were lower than in the control calves (Figure 4.14A). Inter-animal variation was high. 25(OH)D levels between the supplemented and control calves were close to significance (P=0.099) where levels were higher for supplemented calves (Figure 4.14B). Again, inter-animal variation was high.

There was a significant induction of *IL8* in response to LPS in monocytes from both supplemented and control calves (Figure 4.15A). In whole blood, the was again a significant induction of *IL8* expression in response to LPS for both sets of calves (Figure 4.15B). There were no significant differences between supplemented and control groups in *IL8* gene expression. Generally, however, mean expression levels were much more elevated in the control calves due to higher inter-animal variation.

There was a significant reduction of *CYP27A1* in monocytes upon stimulation with LPS from supplemented and control calves when compared with the zero-hour control (Figure 4.16A. Interestingly, LPS significantly increased *CYP27A1* expression in monocytes from control calves when compared with the six-hour control (Figure 4.16A). The situation was quite different in whole blood where some calves showed very high induction of *CYP27A1* while a number showed a reduction for LPS. This case was the same for both the supplemented and control calves (Figure 4.16B). There was a significant difference in *CYP27A1* expression levels in monocytes between the supplemented and control groups with elevated levels in the supplemented calves (Figure 4.16A).

There was a significant induction of *CYP27B1* by LPS in monocytes from both sets of calves (Figure 4.17A). The situation was different in whole blood where a significant induction of *CYP27B1* was seen for control calves in response to LPS but not for supplemented calves (Figure 4.17B). A significant difference in *CYP27B1* expression levels between supplemented and control groups was seen in LPS stimulated whole blood (Figure 4.17B). Generally, expression levels were much more elevated in monocytes compared to whole blood.

The only significant induction of *CYP24A1* was seen in LPS stimulated whole blood from supplemented calves and not control calves (Figure 4.18B). There was no induction of expression in monocytes from either sets of calves upon stimulation with LPS (Figure 4.18A). There was a close to significant difference (P=0.059) in *CYP24A1* expression levels between supplemented and control groups in LPS stimulated whole blood (Figure 4.18B).

There was a significant induction of *NOS2* in monocytes from both supplemented and control calves in response to LPS (Figure 4.19A). The situation in whole blood was slightly different. There was significant induction of *NOS2* for the control calves in response to LPS but no significant induction for the supplemented calves (Figure 4.19B). However, there was no significant difference in expression levels between the supplemented and control groups of calves for either monocytes or whole blood.

There was a significant induction of *VDR* by LPS in monocytes from both supplemented and control calves (Figure 4.20A). In LPS stimulated whole blood, there was a significant induction of *VDR* in control calves but not supplemented calves due to a subset of calves in this group not inducing expression of *VDR* in response to LPS (Figure 4.20B). There was a significant difference in *VDR* expression levels between the supplemented and control groups in LPS stimulated monocytes (Figure 4.20A).

IL-8 protein levels in the cell supernatant of LPS treated monocytes and whole blood were assessed by ELISA. There was significant induction of IL-8 protein in response to LPS in monocytes for both supplemented and control calves (Figure 4.21A). For whole blood, there was also a significant induction of IL-8 protein for both supplemented and control calves (Figure 4.21B). There was no significant difference in IL-8 levels between the supplemented and control groups from either monocytes or whole blood.

In order to assess the possible cause of some animals lack of gene induction or large inter-animal variation for *CYP27A1, CYP27B1, CYP24A1* and *VDR* in whole blood, each group of calves were genotyped to assess IL8 haplotype and then the individual calves were plotted on trendline graph for each gene. In each group, three animals were heterozygote (IL8-het), three were IL8-h1 and two were IL8-h2. For *CYP27A1* expression, one animal with IL8-h2 in the control group showed much higher expression levels than the others (Figure 4.22A). In the supplemented group, two IL8-het and two IL8-h2 animals showed much higher expression levels of *CYP27A1* (Figure 4.22B). Interestingly, all IL8-h1 animals in each group showed down regulation of *CYP27A1* in response to LPS (Figure 4.22A&B). For the control group, there was little variation between in *CYP27B1* expression (Figure 4.22C). However. In the supplemented group IL8-h1 animals appeared to show higher expression levels of *CYP27B1* in response to LPS when compared with IL8-h2 animals (Figure 4.22D). In the case of *CYP24A1* expression, again control calves showed little variation in expression levels (Figure 4.22E). However, when you look at supplemented calves, IL8-h2 animals showed much higher expression levels of *CYP24A1* in response to LPS when compared with IL8-h1 animals (Figure 4.22F). There was little variation in *VDR* gene expression for the control calves bar one IL8-h2 animal down regulating expression (Figure 4.22G). In supplemented calves, *VDR* expression levels were higher for IL8-h1 animals while IL8-h2 animals down-regulated *VDR* expression (Figure 4.22H).

While no significant difference was seen between the supplemented and control groups in *IL8* expression levels, generally levels did seem more elevated in the control animals however there was large amount of inter-animal variation. The reduction of *CYP27A1* and *CYP27B1* in supplemented calves do correspond with the *in vitro* observations in monocytes where expression of these genes was reduced with the addition of $1,25(OH)_2D_3$. IL8 haplotype does also appear to play a role in responsiveness to vitamin D supplementation as demonstrated by how expression of *CYP27A1, CYP27B1, CYP24A1* and *VDR* differs in whole blood from supplemented calves.

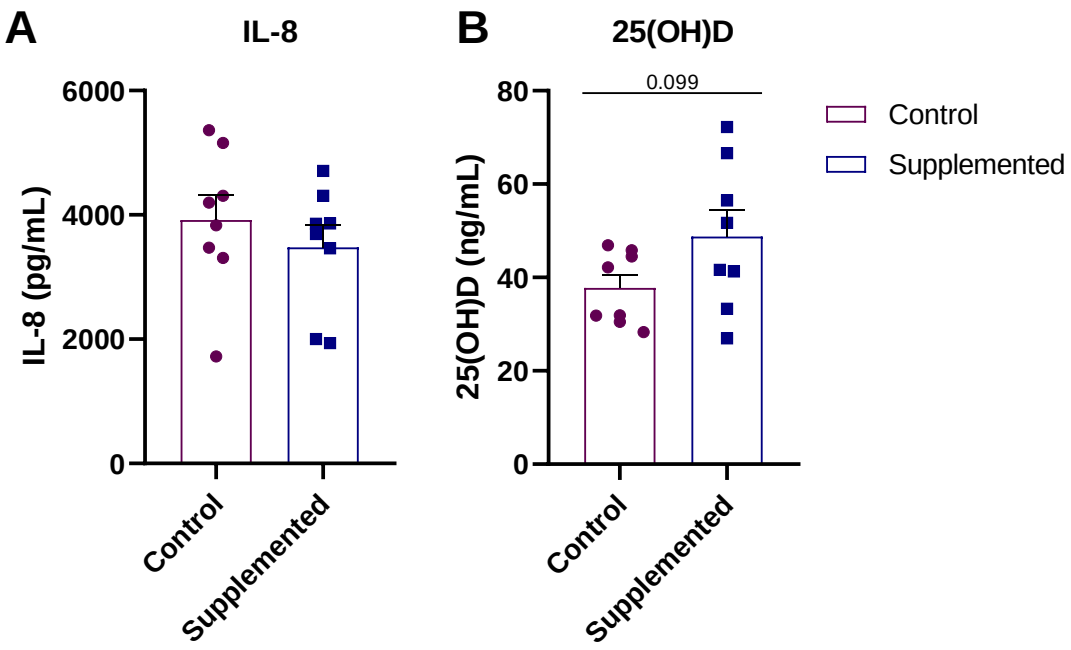

Figure 4.14 Levels of IL-8 and 25(OH)D in serum from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of male Holstein-Friesian calves were also included. (A) IL-8 and (B) 25(OH)D levels were assessed in isolated serum via ELISA. Error bars represent the SEM of n=8 animals.

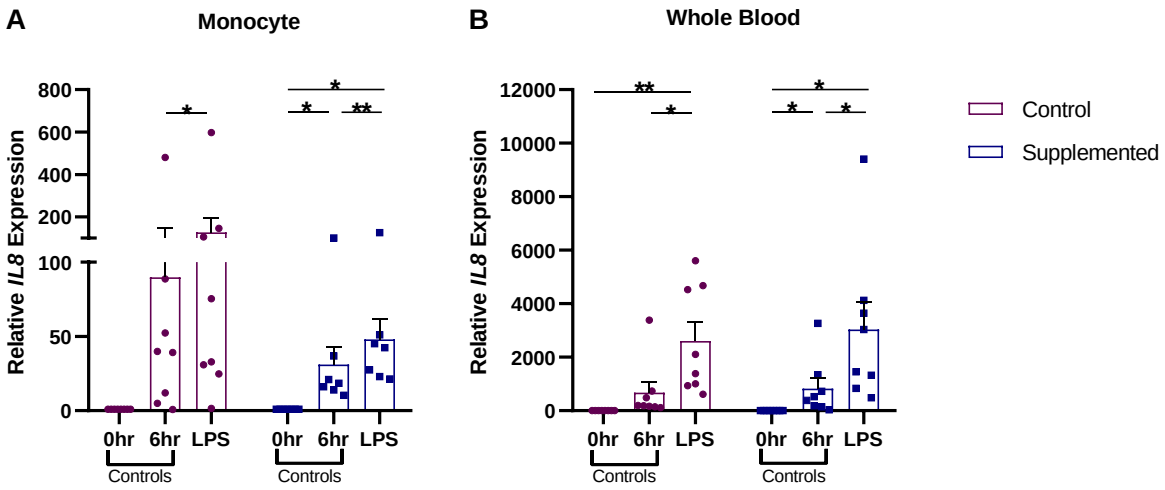

Figure 4.15 Gene expression of *IL8* in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and *IL8* expression was analysed and normalised to the zero-hour control. *IL8* gene expression is shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) where *P<0.05 and **P<0.01.

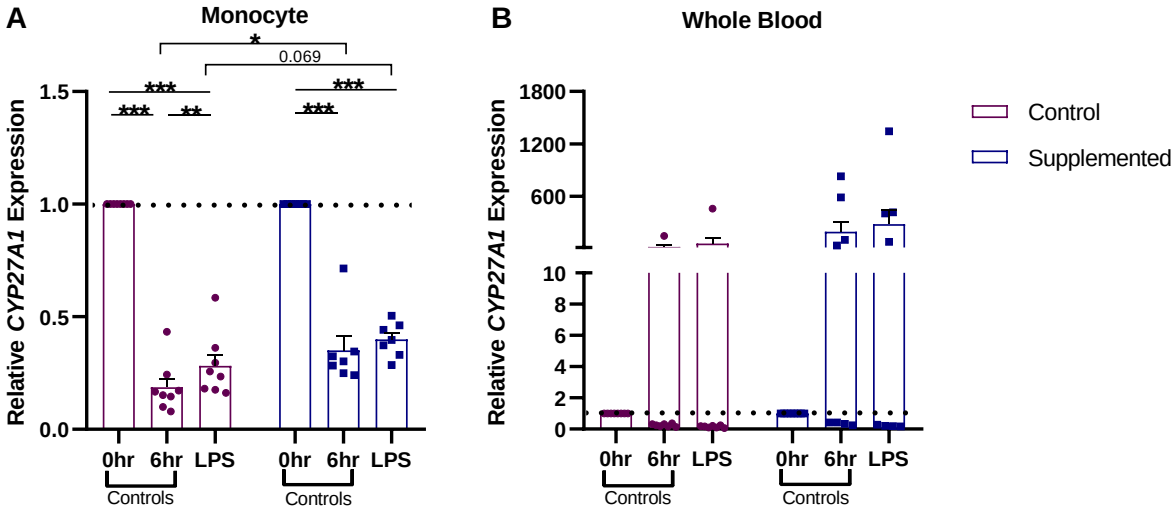

Figure 4.16 Gene expression of *CYP27A1* in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and *CYP27A1* expression was analysed and normalised to the zero-hour control. *CYP27A1* gene expression is shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) or between treatment groups (branched lines) where *P<0.05, **P<0.01 and ***P<0.001.

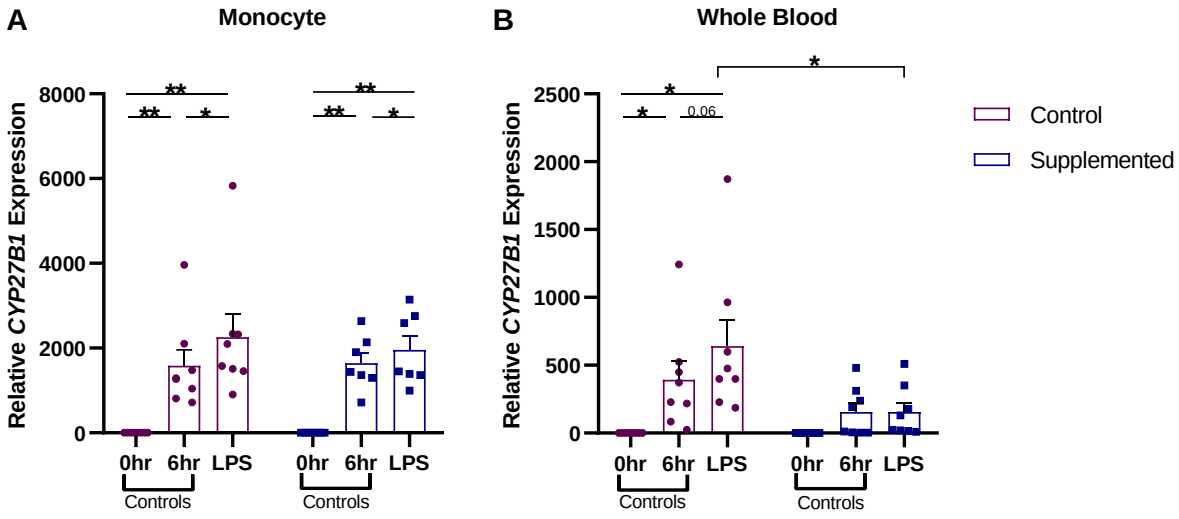

Figure 4.17 Gene expression of *CYP27B1* in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and *CYP27B1* expression was analysed and normalised to the zero-hour control. *CYP27B1* gene expression is shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) or between treatment groups (branched lines) where *P<0.05 and **P<0.01.

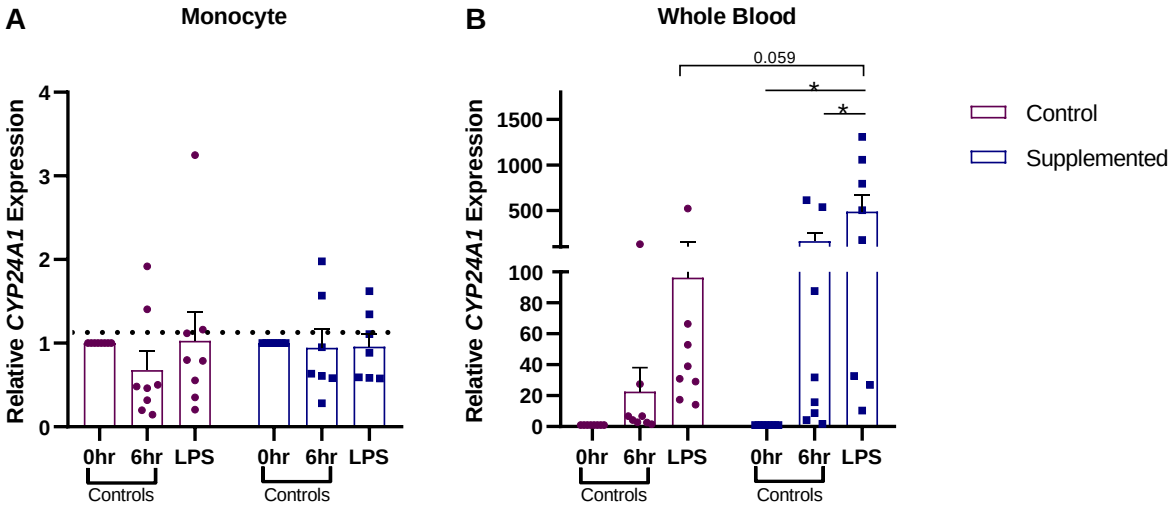

Figure 4.18 Gene expression of *CYP24A1* in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and *CYP24A1* expression was analysed and normalised to the zero-hour control. *CYP24A1* gene expression is shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) or between treatment groups (branched lines) where *P<0.05.

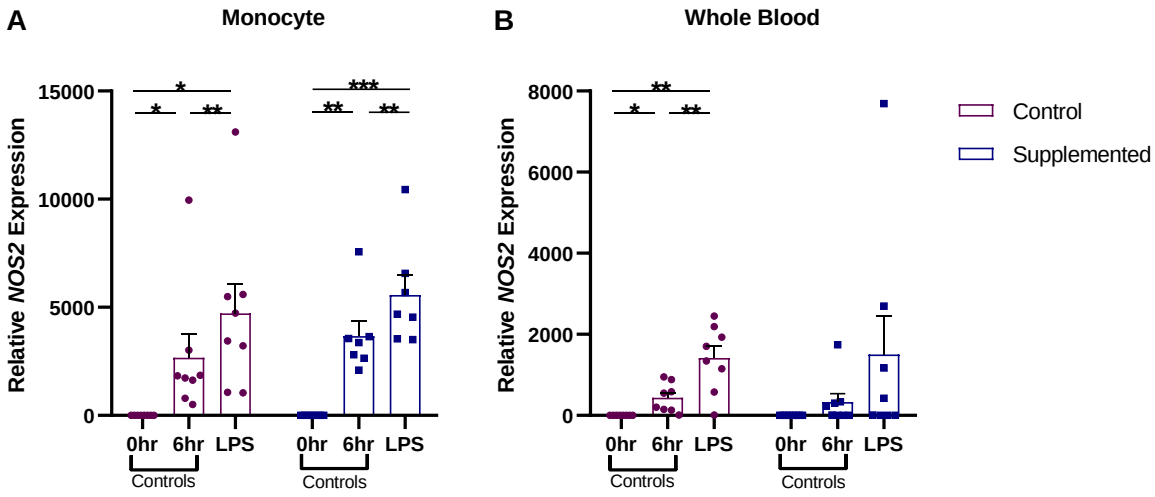

Figure 4.19 Gene expression of *NOS2* in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and *NOS2* expression was analysed and normalised to the zero-hour control. *NOS2* gene expression is shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) or between treatment groups (branched lines) where *P<0.05, **P<0.01 and ***P<0.001.

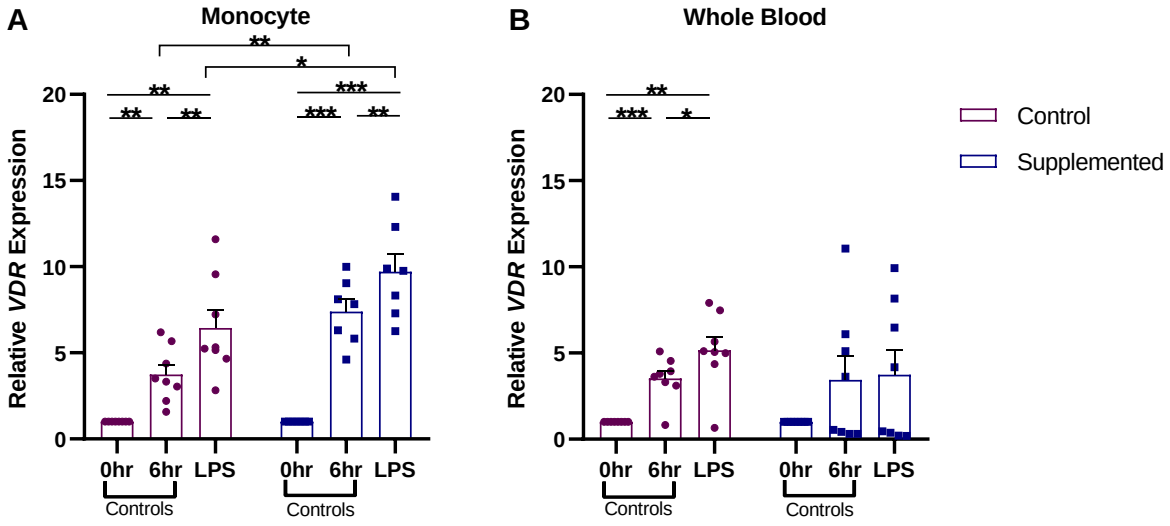

Figure 4.20 Gene expression of *VDR* in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and *VDR* expression was analysed and normalised to the zero-hour control. *VDR* gene expression is shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) or between treatment groups (branched lines) where *P<0.05, **P<0.01 and ***P<0.001.

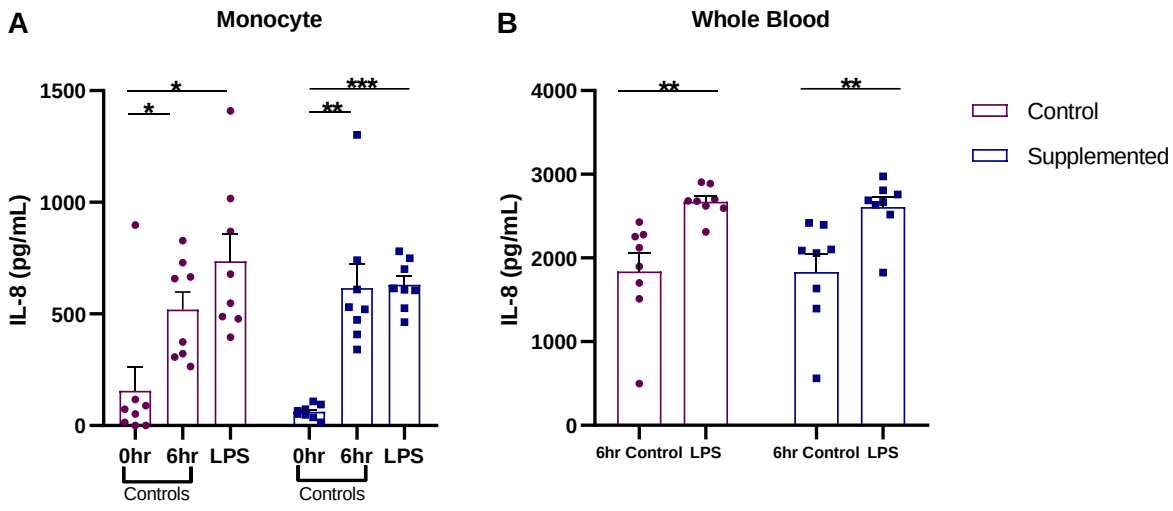

Figure 4.21 Production of IL-8 protein in LPS stimulated monocytes and whole blood isolated from calves supplemented with vitamin D and control calves. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. A control group of calves received no additional supplementation. Monocytes were isolated via MACS and were stimulated along with whole blood with 100 ng/mL LPS for six hours. A six-hour unstimulated control was also included. For monocytes, a zero-hour control was additionally included. IL-8 was measured via ELISA in the cell supernatant. IL-8 protein levels are shown in (A) monocytes and (B) whole blood. Error bars represent the SEM of n=8. Significance is between controls and LPS (straight lines) where *P<0.05, **P<0.01 and ***P<0.001.

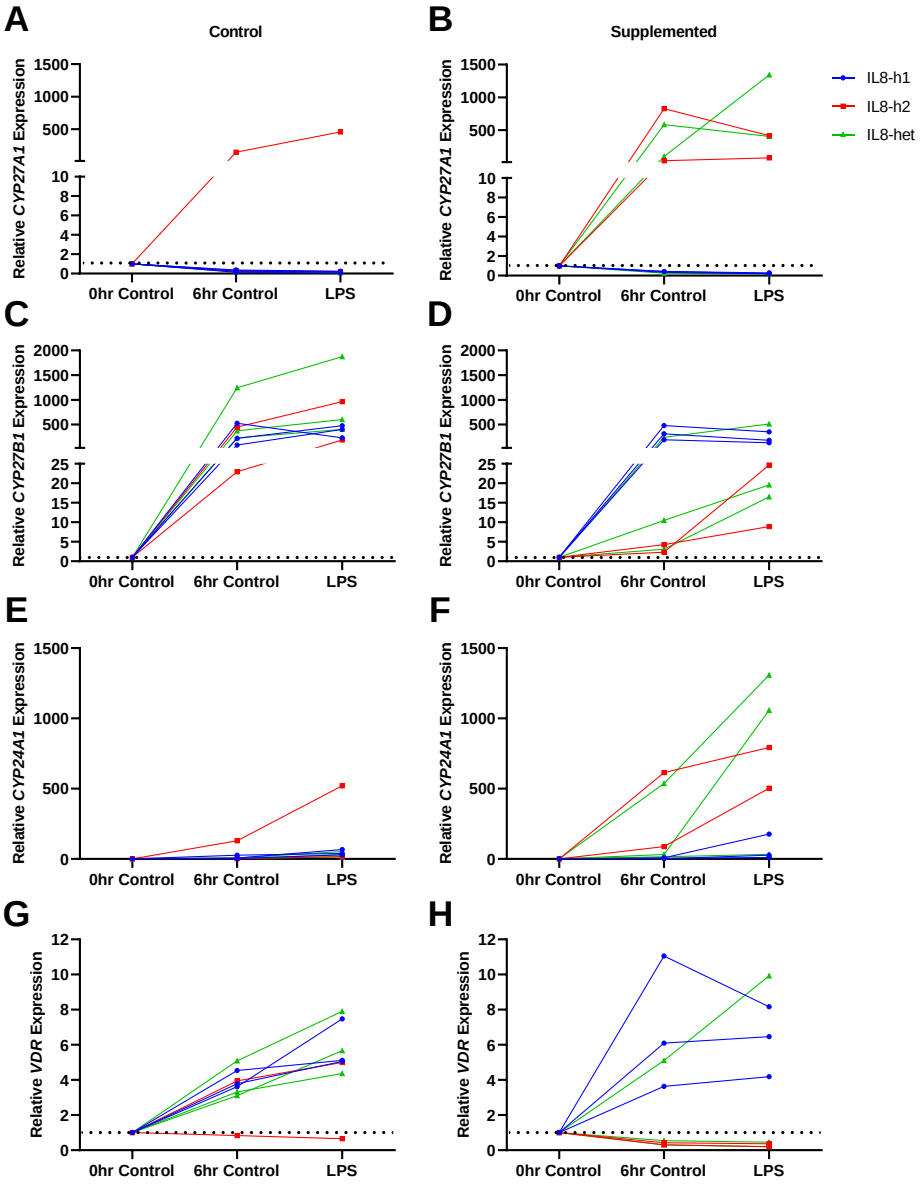

Figure 4.22 Gene expression of *CYP27A1*, *CYP27B1*, *CYP24A1* and *VDR* in LPS stimulated whole blood from calves supplemented with vitamin D and control calves and subsequentially genotyped for IL8 haplotype. Male Holstein-Friesian calves were supplemented with additional vitamin D in their diet from birth and blood samples were taken at six months of age. They were then genotyped for IL8 haplotype. Whole blood was stimulated with 100 ng/mL LPS for six hours. A zero hour and six-hour unstimulated control was also included. RNA was extracted and gene expression was analysed and made relative to the zero-hour control. Induction of (A&B) *CYP27A1*, (C&D) *CYP27B1*, (E&F) *CYP24A1* and (G&H) *VDR* expression is shown in monocytes from control and supplemented calves. Each point with connecting line represents an individual animal with IL8-h1 (blue), IL8-h2 (red) or IL8-hetereozygote (green) haplotype, n=2/3.

4.3 Discussion

It has been established there are serum level differences in IL-8 and 25(OH)D between haplotypes which appear to show an inverse relationship with each other at months six, seven and eight of age. Haplotype differences have also been observed in gene expression between the two haplotypes. An examination was also carried out to establish whether polymorphisms in vitamin D pathway genes were co-segregating with IL8 haplotypes. It has been shown that SNPs in two genes; *GC* and *CYP27A1* were polymorphic in our populations but not co-segregating or in linkage disequilibrium with the IL8 haplotypes. It was confirmed that IL-8 doesn't have a direct effect on the expression of genes which encode elements of the vitamin D pathway, but $1,25(OH)_2D_3$ alone does reduce *IL8* expression in monocytes. Finally, the impact of vitamin D supplementation of six-month old calves was investigated and only a slight reduction in *IL8* expression was detected in monocytes from supplemented calves in addition to divergent expression profiles of vitamin D pathway genes which depended on IL8 haplotype.

Considering IL8-h2 calves have significantly higher IL-8 levels in months six to eight and significantly lower 25(OH)D levels at months five, six and seven, there is most likely a relationship between the two. The most likely explanation for the haplotype divergence is IL-8 influencing serum 25(OH)D levels, due to SNPs present in the IL8 promoter region affecting expression levels, and this is mostly visible in late summer when 25(OH)D levels peak. A seasonal effect of sunlight exposure is most definitely playing a role in regulating circulating vitamin D concentrations. The results seen here correspond with a study carried out on melanoma patients where they were shown to have low 25(OH)D levels compared to healthy controls but much higher IL-8 levels [156]. In that particular study, subjects were in a disease state, but it shows the presence of an inverse relationship between IL-8 and 25(OH)D. The calves in our study were born with two different IL8 promoter haplotypes which are most likely influencing their serum 25(OH)D levels depending on the season.

How IL-8 might be affecting vitamin D status was established by looking the expression of pathway genes in whole blood from the genotyped calves. LPS stimulated blood was also used as previous studies had reported *CYP27B1* to be only expressed when LPS or another stimulus was present [118]. The significant differences observed in *eDEFB4A* expression is interesting as recent studies in cattle have described the active

form of vitamin D, 1,25$(OH)_2$D, increasing β-defensin expression in monocytes [166]. IL8-h1 calves showed higher expression of *eDEFB4A* which would correspond with them having higher 25(OH)D levels. Another gene which showed differential expression was *CYP27B1* which is involved in the local conversion of the vitamin from in-active to active form. Again, this corresponds with the high levels of 25(OH)D in IL8-h1. Gene expression of *TLR4* showed some differential expression between haplotypes upon simulation with LPS indicating there could possibly be difference in how each haplotype respond to LPS stimulation. TLR4 is a major receptor for LPS. When *TLR4* is knocked out in mice they struggle to mount an innate immune response to respiratory syncytial virus that is characterised by a weakened monocyte recruitment and an inability to clear the virus [167]. In relation to *IL8* gene expression, no significant differences were seen in control samples which would provide further evidence that the IL-8 protein levels seen in the supernatant from these samples is coming from an additional source other than peripheral blood cells present in the assay. Also, the IL8-h1 samples appear to show a higher expression of IL8 in response to LPS which may support the idea that the magnitude of an IL8 response shown by IL8-h1 calves is greater than that of IL8-h2 calves as these calves already have a high basal level of IL-8 in serum. This is a noteworthy result considering IL8-h1 was shown to have increased levels of 25(OH)D.

The investigation of vitamin D pathway gene SNPs was to confirm whether any of the alleles co segregated with IL8 haplotype. It was hypothesised that linkage disequilibrium may result in the non-random recombination of haplotype blocks containing both the IL8 promoter SNPs and the GC SNPs. None of the SNPs appeared to be segregating with the IL8 haplotypes which suggest there is no linkage disequilibrium present and that these specific SNPs do not play a role in the haplotype differences observed in vitamin D and IL-8 levels. However, CYP27A1 SNP1 appears to be polymorphic in the population, as observed by the allele frequencies. A larger number of subjects would be useful in validating these results. The possibility must also be accepted that unknown SNPs may be present in these genes and having functional effects. The close proximity of the GC gene to the IL8 gene is still noteworthy considering the role of vitamin D binding protein in transporting both forms of vitamin D in the serum. Studies have already identified vitamin D binding protein as potentially impacting the levels of 25(OH)D in circulation. SNPs in the gene itself are being investigated as to whether they affect binding to 25(OH)D [168]. *GC* is not expressed in peripheral blood so VDBP levels

were investigated in the blood. When VDBP levels were examined in a subset of calves from months five, six and seven, no significant difference in levels were observed. Ideally a larger subset of calves could be used to further investigate this in the future. As VDBP binds much of active and inactive vitamin D in the bloodstream, it is suggested to control the amounts of available or free 25(OH)D thus influencing levels in the serum [169]. Additionally, it also is involved in facilitating interactions of the active and inactive vitamin D forms with monocytes [109]. The fact that no differences in serum VDBP were seen between the two haplotypes most likely means there is another factor controlling 25(OH)D levels.

Assessing whether IL-8 itself can influence genes in the vitamin D pathway had not been previously investigated in the literature. The monocyte was chosen as the model cell due to its importance in vitamin D metabolism [170] and was previously shown in chapter three to be a key producer of IL-8 in peripheral blood from cattle. IL-8 showed no effects on expression of vitamin D pathway genes even at high concentrations even though monocytes have been shown to express *CXCR1* [171]. However, it was confirmed that low levels of $1,25(OH)_2D_3$ alone could downregulate *IL8* expression in monocytes isolated from cattle basally, which is different to what has been shown in humans. $1,25(OH)_2D_3$ alone has been shown to upregulate *IL8* expression in a human monocyte cell line over the course of eight hours [126]. This is not the case in bovine monocytes or possibly also human peripheral blood derived monocytes. It is also possible that presence of an additional stimulus such as LPS may alter how $1,25(OH)_2D_3$ regulates *IL8* expression. With the addition of LPS, $1,25(OH)_2D_3$ was shown to increase IL-8 production by neutrophils but not macrophages [119]. This also shows a cell specific response to $1,25(OH)_2D_3$.

In order to get a clearer picture on how vitamin D systemically affects the innate immune response in addition to *IL8* expression and subsequent protein production, a vitamin D supplementation study was designed. Holstein-Friesian calves were supplemented from birth with the maximum amount of dietary vitamin D allowed under EU regulations and raised outdoors from three months old. Another control group of calves received no additional dietary vitamin D and were raised indoors. The lack of divergence in IL-8 serum levels was unexpected considering what was seen previously in chapter three where six-month-old genotyped calves IL-8 and 25(OH)D profiles significantly differed between haplotypes. Levels of 25(OH)D do differ between the

supplemented and control calves therefore, based on previous results in this chapter, IL-8 levels would be expected to differ too. A previous study on melanoma patients also identified a correlation of high IL-8 levels and vitamin D deficiency in patients with melanoma which didn't exist in healthy controls [156]. This suggests a control mechanism of vitamin D on IL-8 which can become disbalanced under certain circumstances such as cancer. It may be possible vitamin D supplementation has a smaller effect on circulating IL-8 in otherwise healthy subjects as control calves 25(OH)D hovered around ~30 ng/mL at this stage of the study.

An interesting result noted from the supplementation study was how supplementation effects differed in monocytes versus whole blood. Genes such as *CYP27A1, CYP27B1, CYP24A1* and *VDR* displayed very different expression profiles in monocytes or whole blood. In addition, those expression profiles become differentially altered in response to supplementation, again depending on the cellular source. While bovine monocytes have been previously shown to express the conversion enzyme *CYP27B1* in response to LPS, only a slight non-significant reduction in expression was recorded with the addition of vitamin D *in vitro* [118]. This is similar to what was seen in monocytes in this study, however whole blood showed a significant decrease in expression in response to LPS. This indicates other key cell types in blood which also play a large role in vitamin D conversion. Interestingly, IL8 haplotype may also have a role here as IL8-h1 animals display a different expression profile of *CYP27B1* to IL8-h2 animals in whole blood. Supplementation decreases the expression in IL8-h2 animals but not IL8-h1 animals. This may be a small size, but the results do correspond with the immunomodulatory effect of vitamin D on *IL8* where levels are high [127]. *CYP24A1* was also previously shown to be highly expressed in bovine monocytes *in vitro* especially in response to vitamin D alone [118]. However, here only very little expression in monocytes is shown from both supplemented and control animals but very high expression in whole blood from supplemented animals. Again, differing expression profiles are seen depending on IL8 haplotype with IL8-h2 animals showing much higher expression. *VDR* expression also showed a haplotype specific profile in whole blood from supplemented calves where much higher expression was seen with IL8-h1. Considering has been previously hypothesised in the literature to act as a transcription factor for IL8 [126], most likely through interfering with NF-κB signalling [128], this could potentially be a mechanism of how IL-8 levels are reduced in this haplotype. These results also

underline distinct differences between *in vitro* and *in vivo* studies of vitamin D and immunity as *in vitro* a reduction in *VDR* expression was described in monocytes in response to $1,25(OH)_2D_3$, while *in vivo* monocytes from supplemented calves show a significant increase in *VDR* expression. In addition, it also confirms the cell specific response to immunomodulation by vitamin D [119] as there is a clear difference shown here between gene expression profiles of whole blood and monocytes from supplemented calves.

To conclude, IL8 haplotype impacts significantly on vitamin D concentrations in Holstein-Friesian calves, This primarily appears to occur through a mechanism whereby expression levels of vitamin D conversion enzymes are altered depending on haplotype, resulting in differing levels of 25(OH)D in circulating serum. While the SNPs investigated in *GC* and *CYP27A1* do not co-segregate with the SNP used to identify IL8 haplotypes, there is still a divergence of the *CYP27A1* SNPs in the Holstein-Friesians population which could potentially affect activation of expression or expression levels. It also remains to be investigated whether there are other unidentified SNPs present that do possibly co-segregate with IL8 haplotype SNPs and which could possibly impact vitamin D levels, which numerous studies have previously shown [172, 173]. IL-8 alone does not affect vitamin D pathway gene expression; however, vitamin D alone can reduce *IL8* gene expression in monocytes possibly through VDR acting as a transcription factor. This was further confirmed upon supplementation of calves with vitamin D where a slight reduction in *IL8* gene expression in monocytes was seen in addition to a significant increase in *VDR* expression. The mechanism by which IL8-h1 animals show increased 25(OH)D in circulating serum is not particularly clear, however it may be linked to altered *VDR* expression seen in whole blood from IL8-h1 supplemented calves and also the fact that the IL8 promoter region of these animals may be more open to binding by VDR. Further work is required to explore the presence of VDREs in the bovine IL8 haplotype promoter regions and whether there are differences in how VDR affects initiation of expression between the two. Overall, however, this work has demonstrated that the implications of divergent IL8 haplotypes goes beyond just IL-8 levels and may have wider ranging effects for the immune system.

Chapter 5. IL8 haplotype and vitamin D influences the local innate immune response in primary dermal fibroblasts

5.1 Introduction

Following confirmation of a role for IL8 haplotype in influencing the systemic innate immune response via peripheral blood cell numbers and basal IL-8 levels, it was next sought to investigate whether IL8 haplotype influences the local innate immune response. As IL-8 is secreted to attract neutrophils toward the infection site, and local epithelial and stromal cells are key producers of this chemokine [70], it is therefore logical to hypothesise that IL8 haplotype would affect the activation of the local immune response. The early innate immune response is critical to the recruitment of circulating immune cells and also to the trajectory of the subsequent adaptive immune response.

IL8 promoter haplotypes have been previously associated with somatic cell count in Holstein-Friesian cows where high cellular influx into the mammary gland is indicative of subclinical mastitis. These infiltrating cells are predominantly neutrophils responding to the infection, likely as a result of the chemotactic gradient established via inflammatory signalling by the mammary epithelium. IL8-h1 is associated with lower somatic cell scores compared to IL8-h2 and therefore, this haplotype could be considered a predictor of how a cow will respond to mammary gland infection [36]. These haplotypes have also been shown to influence cell-specific responses, with endometrial epithelial cells showing significantly more *IL8* promoter activity in the IL8-h2 cells in response to TNFα stimulation when compared with IL8-h1 cells. However, when these cells were transfected with a BoHV-4 (bovine herpes virus 4) protein expressing plasmid, IL8-h1 cells showed more *IL8* promoter activity when compared with IL8-h2 cells [36]. This suggests that the haplotype-specific activity may depend on the infectious agent in question.

Epithelial cells have a demonstrated role in recruitment of neutrophils via IL-8 however [174], stromal cells such as fibroblasts also play an important role particularly when the epithelial barrier is damaged prior to or during infection [68]. Fibroblasts have been previously isolated from cattle and have been shown to produce IL-8 *in vitro* in response to LPS [75]. In addition, fibroblasts of a dermal origin have been shown to predict how an animal will respond to mammary gland infection. Cattle with elevated fibroblast IL-8 responses to LPS *in vitro* subsequentially showed high levels of inflammation and neutrophil infiltration in the mammary gland when experimentally infected with *E. coli* [76]. This study showed that fibroblast function is therefore not only important in order to understand local sentinel immune responses but also can be

predictive of clinical outcomes and recovery after infection, as shown by a quicker reduction in somatic cell count by cows who showed low IL-8 production by fibroblasts [76]. The identified high and low IL-8 responders in the study were identified by measuring IL-8 secretion from LPS stimulated primary dermal fibroblasts but the role of IL8 haplotype on *IL8* gene expression and subsequent protein production in this cell type was not investigated. Also, it remains to be seen whether the pattern, previously established, of IL8-h2 animals inducing more IL-8 production systemically is replicated in a local *ex vivo* model such as the primary dermal fibroblast [5].

Although not regarded as classical immune cells, fibroblasts are now described being important regulators of the immune response within specific tissues. They can act as pro-inflammatory while also switching to a suppressive phenotype when required [175]. They are therefore a useful model for investigating the impact of IL8 haplotype on the local innate immune response. Bovine dermal fibroblasts have been reported previously to produce significant expression of innate immune genes such as *SAA3* (serum amyloid A), *CCL20* (chemokine ligand 20), *IL6* (interleukin-6) and *TNFA* after stimulation with LPS [176]. *SAA3* and *CCL20* have also been reported to be induced in bovine and human fibroblasts isolated from different tissue sources [177, 178]. Expression of *SAA3* has been documented in many bovine epithelial tissues including from the mammary gland and intestine [177]. Human gingival fibroblasts produced significant *CCL20* expression in response to cytokine stimulation with IL-1β and TNF-α and also with LPS [178]. The viral ligand poly (I:C) with the addition of IL-1β has also been shown to induce *CCL20* in this cell type. In this case, *CCL20* is theorised to attract Th17 cells into the infection site [179]. CCL20 has also been shown to play the same role in human cervical fibroblasts where expression is induced by IL-6 [180]. As some tissue-specific fibroblasts have been shown to express the IL-8 receptor CXCR1 [181], alternate expression levels of IL-8 may result in downstream effects on induction of other inflammatory proteins and chemokines such as *SAA3* and *CCL20.*

As described in chapter four, vitamin D levels in serum differ between calves depending on IL8 haplotype. The relationship between vitamin D and IL-8 has not been previously investigated in local type cells from cattle with these haplotypes. The role for vitamin D in attenuating the *IL8* response has been studied previously in humans and has been shown to have the ability to upregulate and downregulate *IL8* expression which will be described in more detail subsequently. However, its role in regulation of IL8 in the

bovine species has not yet been investigated. The active form of vitamin D, known as $1,25(OH)_2D_3$, has been shown previously to down-regulate the production of IL-8 in human monocyte derived macrophages which had been pre-exposed to bacterial culture media [127]. In addition, this form of vitamin D has been shown to activate expression of *IL8* in a monocyte cell line without any other stimulus [126] suggesting that its action on *IL8* expression depends on whether the cell is activated by an additional stimulus. The mechanism behind how it carries out its regulatory role involves the VDR interfering with pro-inflammatory transcription factors such as NF-κB following the binding of $1,25(OH)_2D_3$ [128, 130]. VDR itself can also act as a transcription factor and bind to gene promoter regions to regulate expression and this is potentially one method of how it initiates *IL8* expression [126]. Vitamin D has also been shown to act on human fibroblasts to regulate *IL8* gene expression. In fibroblasts isolated from the nasal cavity, $1,25(OH)_2D_3$ reduced expression of IL-8 and IL-6 when cells were stimulated with LPS [182]. Gingival fibroblasts were also shown to reduce IL-8 expression levels in response to $1,25(OH)_2D_3$ after bacterial stimulation [183].

Considering IL8 haplotypes do play a role in *IL8* expression and subsequent protein levels systemically in peripheral blood cells, they may also play an important role in the activation and maintenance of the local immune response. IL-8 receptor haplotypes have been previously shown to alter the outcome in local site infections such as the mammary gland and which fibroblasts play a role in [7]. Additionally, vitamin D has also been shown to attenuate *IL8* expression in human peripheral blood cells as well as human fibroblasts, therefore, it may also modulate the IL-8 response of bovine fibroblast by similar mechanisms. Consequently, it is therefore important for us to establish whether IL8 haplotype will result in distinct differences in primary dermal fibroblast *IL8* gene expression and subsequent protein production in response to PAMP stimulation and whether this response may be modulated by vitamin D. To do this, primary dermal fibroblasts were isolated from cattle of each IL8 haplotype, and the expression innate immune genes and Il-8 assessed in response to PAMP stimulation, with and without the addition of vitamin D.

5.2 Results

5.2.1 Characterisation and optimisation of primary dermal fibroblasts isolated from IL8 genotyped animals.

A panel of fifty-nine one-year old Holstein-Friesian heifers were used for the study. Blood samples were taken via the jugular vein and DNA extracted for the custom genotyping assay to determine IL8 haplotype of each animal. Thirteen animals were determined as IL8-h1, fourteen as IL8-h2 and thirty as heterozygotes (Figure 5.1). Two animals could not be determined by the assay. Ten animals with IL8-h1 and ten animals with IL8-h2 were selected and ear punches collected in order to isolate primary dermal fibroblasts.

Upon cell isolation from a small 5 mM tissue sample, cells were grown to confluency for immunocytochemical and qPCR analysis. Cells expressed high levels of vimentin (Figure 5.2A) confirming a fibroblast phenotype. RNA was also extracted from the cells and expression of *VIM* (vimentin) and *KRT18* (keratin-18) measured. Cells were found to exclusively express *VIM* with no *KRT18* detected which again strongly supported the cells being fibroblasts (Figure 5.2B). The growth rate of the cells was also examined to ensure active proliferation. Cells were seeded at 9 $x10^3$ cells/mL on day 0 and counted every four days over the course of twelve days. Cells recovered well after passage and cell counts continued to increase consistently over the course of the twelve days confirming active proliferation (Figure 5.3A). Cell growth was also examined following cryopreservation of the cells. The cells were again seeded at a density of 9 $x10^3$ cells/mL on day 0 following thawing after cryopreservation and counted every four days over the course of twelve days. Cell counts were slightly slower to increase following cryopreservation but nonetheless cell numbers reached were similar to the numbers seen for cells that hadn't been cryopreserved (Figure 5.3B).

Having confirmed an actively proliferating fibroblast phenotype, it was confirmed these cells could respond to a range of PAMPs through the expression of *IL8*. Cells at a passage of 3/4 were stimulated with LPS, Pam3CSK4 and R848 to represent gram-negative bacteria (TLR4 agonist), gram-positive bacteria (TLR2 agonist) and ssRNA virus (TLR7/8 agonist), respectively. $1,25(OH)_2D_3$ was also included to determine its ability to activate gene expression alone. At specific timepoints RNA was extracted for qPCR analysis. LPS induced significant *IL8* expression at three hours for 0.1 μg/mL, at

six hours for all concentrations and twelve hours for 1 and 2 μg/mL. There also appears a biphasic nature to the initiation of expression as a strong activation at three hours (Figure 5.4A), is followed by a lag period at six hours (Figure 5.4B). Expression becomes strong again after twelve hours (Figure 5.4C) and continues at twenty-four hours (Figure 5.4D). Although *IL8* expression was induced at twenty-four hours, it wasn't significant due to a large amount of inter-animal variation. Pam3CSK4 induces similar responses at all time points but expression appeared to peak at twelve hours (Figure 5.4). R848 is a poor inducer of *IL8* expression at all timepoints (Figure 5.4A-D) except for a single animal at twelve hours (Figure 5.4C). The addition of $1,25(OH)_2D_3$ alone does not drive *IL8* expression (Figure 5.4A-D).

The expression of *CCL20* was also examined at each timepoint as this gene was previously identified as a key inflammatory marker in bovine dermal fibroblasts [176]. LPS at 1 μg/mL drives expression most effectively at three (Figure 5.5A) and six hours (Figure 5.5B). At later timepoints, the expression of CCL20 decreased (Figure 5.5C&D). Expression in response to Pam3CSK4 drove the greatest level of CCL20 expression at six hours in particular in response to the 1 μg/mL concentration (Figure 5.5B) after which time expression levels decreased but remained significant at twelve hours for 1 μg/mL (Figure 5.5C). Again, no *CCL20* expression was recorded in response to the viral ligand R848 at any timepoint (Figure 5.5A-D). $1,25(OH)_2D_3$ alone also did not induce *CCL20* expression (Figure 5.5A-D).

IL-8 protein levels in cell supernatants were also quantified. Significant induction of IL-8 in response to LPS was seen only at the twenty-four timepoint in response to both 1 μg/mL 2 μg/mL concentration (Figure 5.6D). The IL-8 response to Pam 3CSK4 was similar to LPS in that significant induction is only seen at the twenty-four hour timepoint, again in response to both the 1 μg/mL and 2 μg/mL concentration (Figure 5.6D). There was no significant induction of IL-8 protein in response to R848 at any timepoint which corresponds with the gene expression results (Figure 5.6A-D). Similarly, $1,25(OH)_2D_3$ alone did not induce IL-8 production at any timepoint (Figure 5.6A-D).

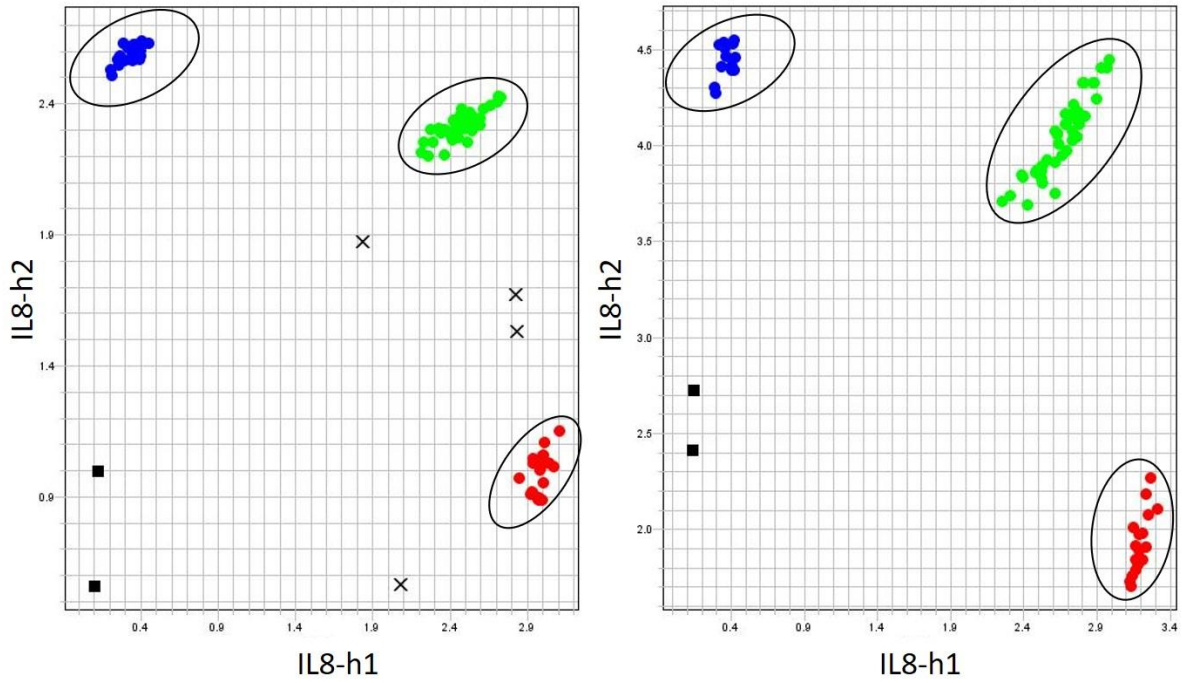

Figure 5.1 Allelic discrimination plots of heifer IL8 haplotypes from which primary dermal fibroblasts were isolated. Fifty-eight one-year old Holstein-Friesian heifers were genotyped in duplicate using a custom TaqMan genotyping assay for a SNP within the promoter region of the *IL8* gene in order ascertain *IL8* haplotype. Fluorescent signals distinct for each haplotype allowed us the discriminate between homozygote IL8-h1 (blue), homozygote IL8-h2 (red) and heterozygote (green). Undetermined animals where marked with an X. Negative controls (■) and positive controls were also included.

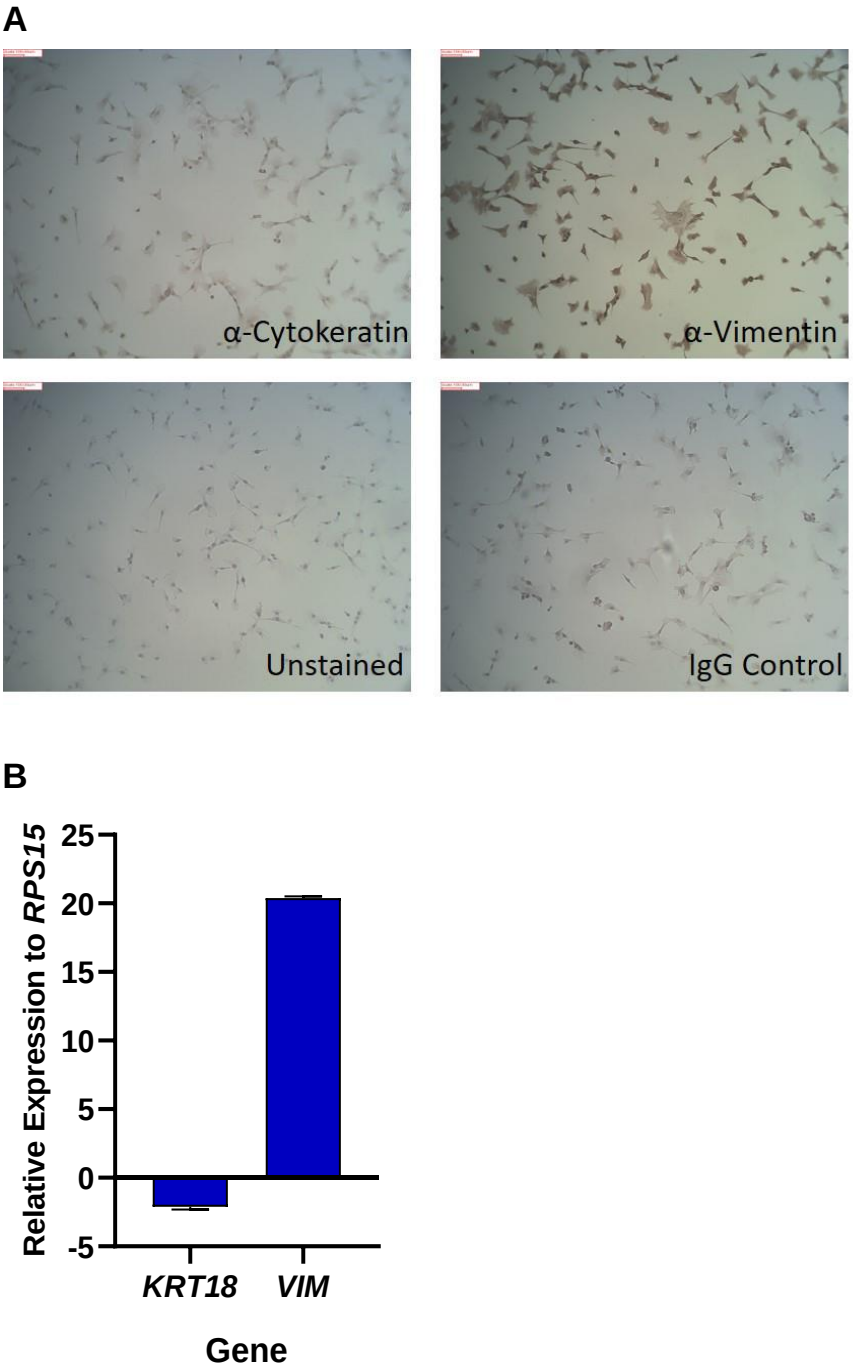

Figure 5.2 Bovine dermal fibroblasts isolated from the ear display high vimentin expression. Cells were isolated from a 5 mM section of tissue from the ear by collagenase digestion. The isolated cells were then grown in 12 well plates before being passaged. (A) Immunohistochemical staining for cytoskeletal proteins α-cytokeratin and α-vimentin. Scale bar indicates 100µM. (B) Relative *KRT18* (keratin-18) and *VIM* (vimentin) gene expression to reference gene *RPS15*. Error bars indicate SEM of n=12.

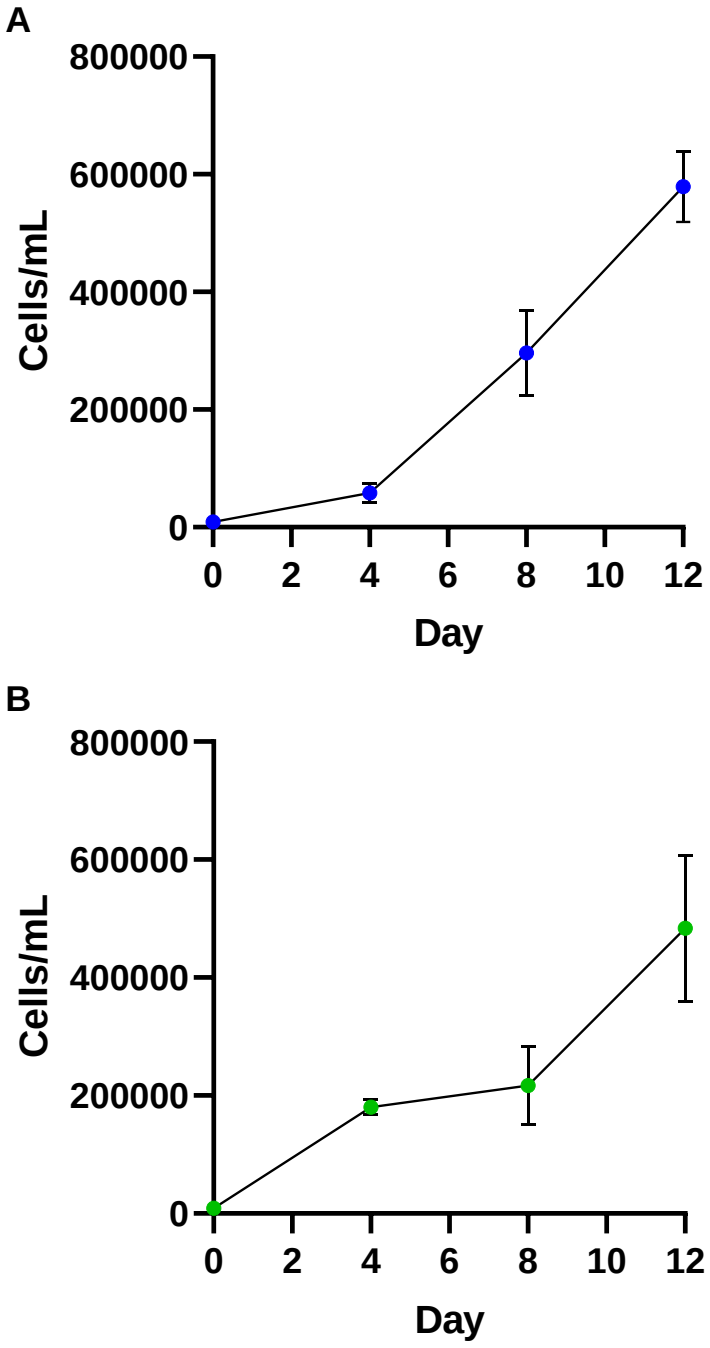

Figure 5.3 Growth characteristics of bovine dermal fibroblasts isolated from the ear. Cells were isolated from a 5 mM section of tissue from the ear by collagenase digestion. The isolated cells were seeded at a density of 9 x 10^3cells/mL in 12 well plates on day 0. A single well of cells was detached by Accutase® and counted using a haemocytometer on days 4, 8 and 12. (A) Growth curve indicates increase in cell number over twelve days of culture after first passage and (B) and after cryopreservation. Error bars indicate SEM of n=3.

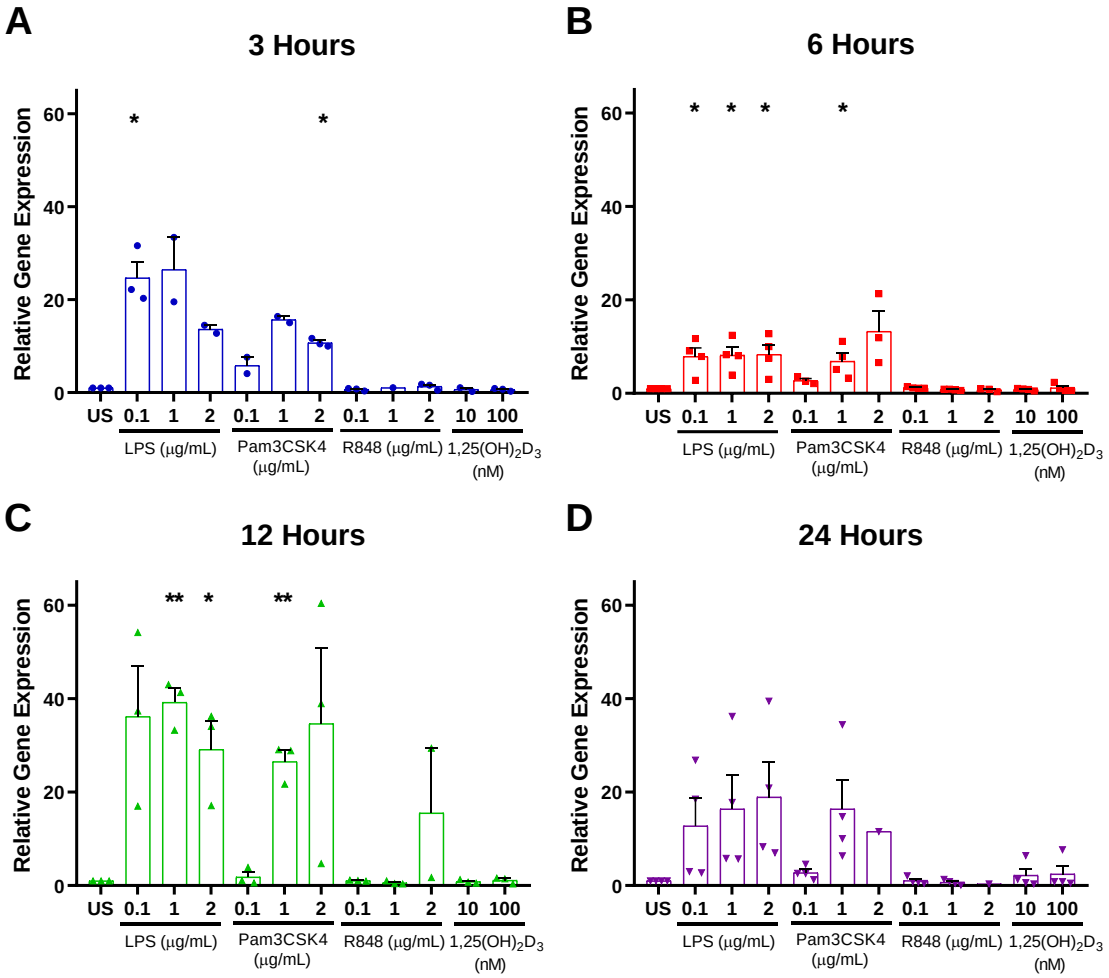

Figure 5.4 *IL8* gene expression in primary dermal fibroblasts in response to LPS, Pam3CSK4, R848 and 1,25(OH)$_2$D$_3$ at 3, 6, 12 and 24 hours. Primary dermal fibroblasts were isolated from one-year old animals via collagenase digestion, grown to confluency and stimulated at passage 3/4 with LPS, Pam3CSK4, R848 (0.1-2 μg/mL) and 1,25(OH)$_2$D$_3$ (10-100 nM) for (A) 3, (B) 6, (C) 12 and (D) 24 hours. At each time point RNA was extracted and gene expression analysed normalised to an unstimulated control (US). Error bars represent the SEM of n=2-4 biological replicates. Significance is between unstimulated (US) and stimuli where *P<0.05 and **P<0.01.

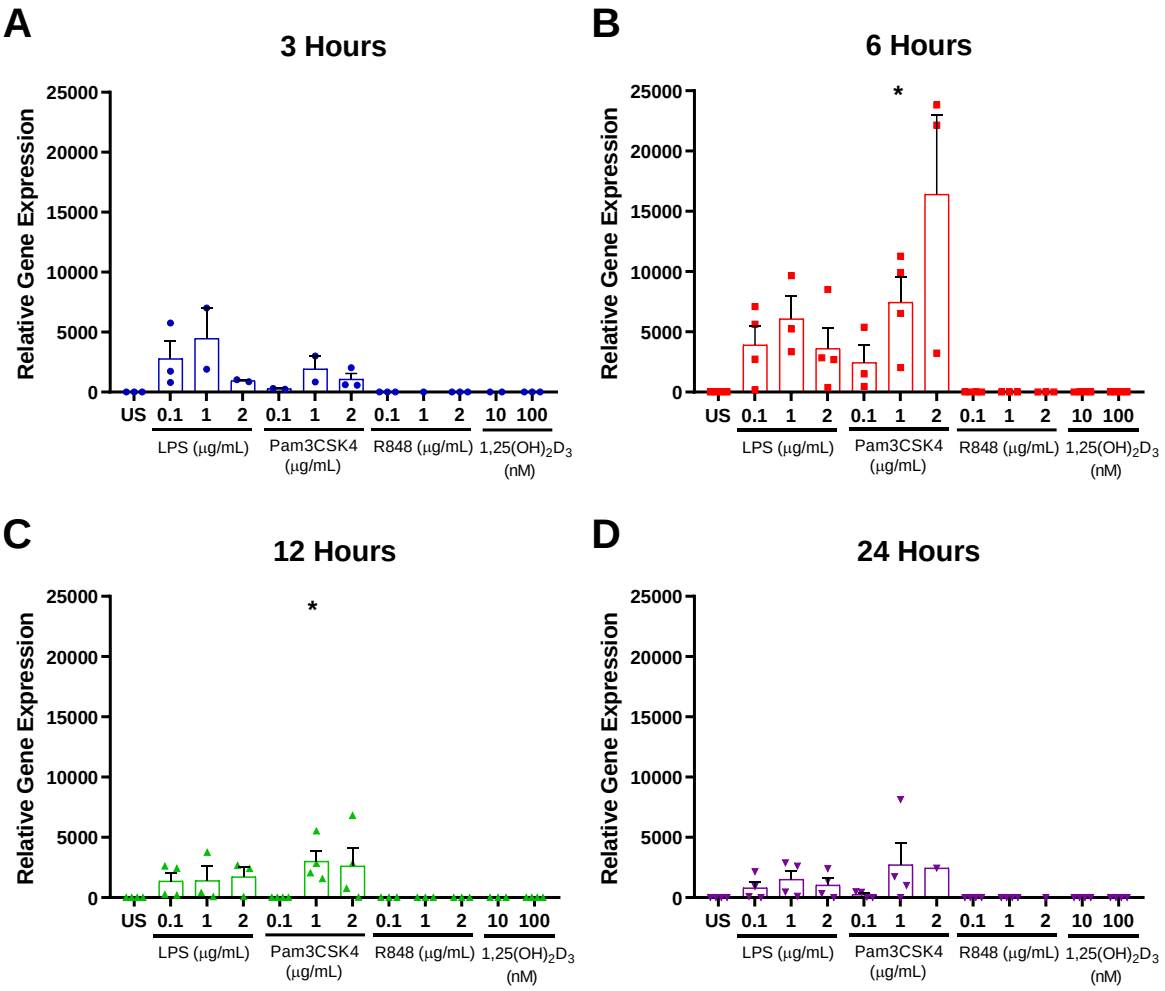

Figure 5.5 *CCL20* gene expression in primary dermal fibroblasts in response to LPS, Pam3CSK4, R848 and 1,25(OH)$_2$D$_3$ at 3, 6, 12 and 24 hours. Primary dermal fibroblasts were isolated from one-year old animals via collagenase digestion, grown to confluency and stimulated at passage 3/4 with LPS, Pam3CSK4, R848 (0.1-2 µg/mL) and 1,25(OH)$_2$D$_3$ (10-100 nM) for (A) 3, (B) 6, (C) 12 and (D) 24 hours. RNA was extracted and gene expression analysed normalised to an unstimulated control (US). Error bars represent the SEM of n=2-4 biological replicates. Significance is between unstimulated (US) and stimuli where *P<0.05.

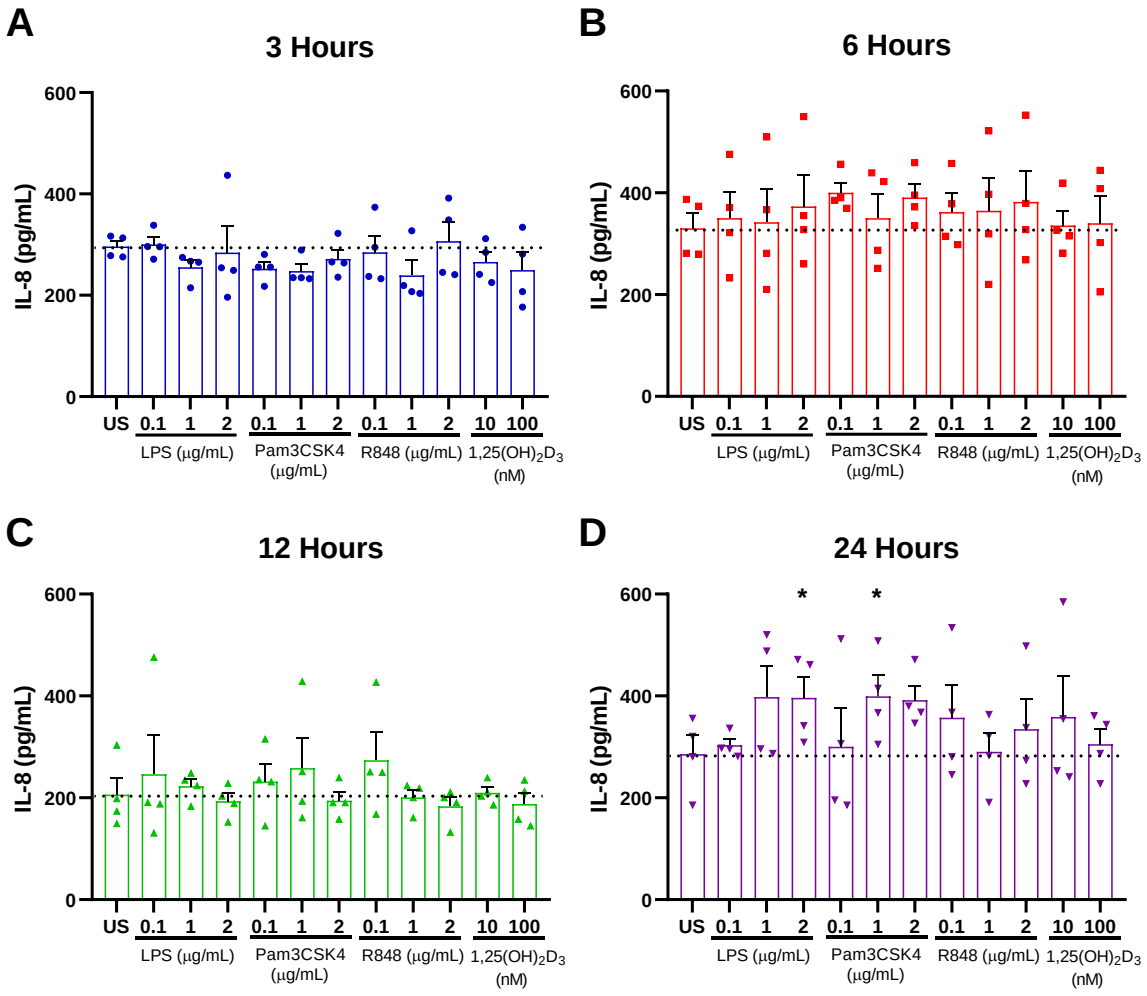

Figure 5.6 IL-8 protein production in primary dermal fibroblasts in response to LPS, Pam3CSK4, R848 and 1,25(OH)$_2$D$_3$ at 3, 6, 12 and 24 hours. Primary dermal fibroblasts were isolated from one-year old animals via collagenase digestion, grown to confluency and stimulated at passage 3/4 with LPS, Pam3CSK4, R848 (0.1-2 µg/mL) and 1,25(OH)$_2$D$_3$ (10-100 nM) for (A) 3, (B) 6, (C) 12 and (D) 24 hours. IL-8 (pg/mL) concentration was measured in supernatant by ELISA. Dotted line represents mean IL-8 level from unstimulated supernatants. Error bars represent the SEM of n=2-4. Significance is between unstimulated (US) and stimuli where *P<0.05.

5.2.2 Local innate immune gene expression of dermal fibroblasts of two distinct IL8 haplotypes.

Having investigated the optimum concentrations of stimuli required and the optimum time point for examining *IL8* expression and subsequent protein production, it was decided to use the 1 μg/mL concentration of both LPS and Pam3CSK4 due to a consistent induction across multiple timepoints while R848 was removed as a PAMP due to lack of induction. An early (three hours) and late (twenty-four hours) timepoint was used in order to observe the initial phase of *IL8* mRNA production and the later phase of IL-8 protein production. Therefore, dermal fibroblasts were isolated from animals of the two distinct IL-8 haplotypes and stimulated with 1 μg/mL of the TLR4 agonist LPS or TLR2 agonist Pam3CSK4 for three and twenty-four hours.

IL8 expression was significantly induced by LPS at both timepoints in both IL8-h1 and IL8-h2 (Figure 5.7A&B). Expression was also significantly induced by Pam3CSK4 in both haplotypes at three and twenty-four hours (Figure 5.7C&D) however, this was only close to significance for IL8-h1 animals (P=0.063) in response to Pam3CSK4 at twenty-four due to a high amount of inter-animal variation (Figure 5.7D). Pam3CSK4 as a stimulant also appears to induce higher levels of *IL8* expression when compared with LPS at the twenty-four timepoint. Generally, high inter-animal is much more prevalent in IL8-h1 compared to IL8-h2 for *IL8* gene expression.

CCL20 expression was not significantly induced by LPS in IL8-h1 animals at either timepoints (Figure 5.8A&B) due to a large amount of inter-animal variation in expression. This was not the case for IL8-h2 which showed close to significant induction (P=0.053) in response to LPS at three hours and a significant induction at twenty-four hours (Figure 5.8A&B). There was no significant induction of *CCL20* in IL8-h1 animals in response to Pam3CSK4 at both timepoints again due to high levels of inter-animal variation (Figure 5.8C&D). There was a significant induction for IL8-h2 animals but only at the twenty-four timepoint and not the three timepoint (Figure 5.8C&D). Again generally, high inter-animal is much more prevalent in IL8-h1 compared to IL8-h2 for *CCL20* gene expression, similar to what was seen for *IL8* expression.

SAA3 expression was also looked at as it had previously been established as a key inflammatory gene stimulated by LPS in bovine dermal fibroblasts [176]. The was a relatively small but significant induction of *SAA3* seen by both haplotypes in response to

LPS at three hours (Figure 5.9A). After twenty-four hours, the significant induction to LPS is maintained in both haplotypes and expression levels become more increased for both (Figure 5.9B). There is also a significant modest induction of *SAA3* in response to Pam3CSK4 by IL8-h1 at the three-hour timepoint and close to significance for IL8-h2 (P=0.066) (Figure 5.9C). After twenty-four hours, induction of expression in response to Pam3CSK4 becomes increased for IL8-h1 animals and is close to significant (P=0.057) (Figure 5.9D). For IL8-h2 animals, there is a significant induction of *SAA3* in response to Pam3CSK4 at twenty-four with increased levels compared to the three-hour timepoint (Figure 5.9D)

NOS2 encodes for inducible nitric oxide synthase or iNOS which has been shown to be highly responsive to vitamin D in both bovine monocytes and fibroblasts and was included in this study so as to examine any changes in response to vitamin D [118, 164]. Expression of *NOS2* was significantly induced by LPS alone in IL8-h1 animals at both timepoints but this wasn't the case for IL8-h2 animals (Figure 5.10A&B). In response to Pam3CSK4 alone, *NOS2* was only significantly induced in IL8-h1 animals at twenty-four hours and not three hours (Figure 5.10C&D). For IL8-h2 animals, there was a significant induction in response to Pam3CSK4 at both timepoints with higher levels at twenty-four hours when compared with three hours (Figure 5.10C&D).

VDR encodes for the vitamin D receptor and was also included in the study to record any changes in response to vitamin D. There was a significant induction in response to LPS alone at both timepoints for each haplotype (Figure 5.11A&B). There was no significant induction of *VDR* in response to Pam3CSK4 alone at the three-hour timepoint (Figure 5.11C). However, after twenty-four hours, a significant induction of *VDR* is seen for both haplotypes (Figure 5.11D). Generally, again, IL8-h1 animals appear to show much more inter-animal variation when compared with IL8-h2.

To determine whether vitamin D modulates expression of *IL8, SAA3, CCL20, NOS2* in and *VDR,* in addition to examining differences in response to vitamin D between haplotype, some cells were pre-treated for two hours with 10 nM $1,25(OH)_2D_3$ prior to stimulation with LPS and Pam3CSK4. While $1,25(OH)_2D_3$ showed no evidence of inducing *IL8* alone previously in bovine monocytes or here in bovine dermal fibroblasts, there is clear evidence in the literature that the addition of vitamin D with a PAMP can alter expression of a number of inflammatory genes including *IL8* [119, 127].

Interestingly, the addition of 1,25$(OH)_2D_3$ showed no significant effect in modulating expression of *IL8* for either haplotype in response to LPS at three and twenty-four hours (Figure 5.7A&B). The same result was also seen in response to Pam3CSK4 at three and twenty-four hours (Figure 5.7C&D).

For *CCL20* expression, 1,25$(OH)_2D_3$ had a close to significant effect in reducing expression in response to LPS for IL8-h2 at both three and twenty-four hours which was not seen for IL8-h1 (Figure 5.8A&B). There was no effect of 1,25$(OH)_2D_3$ on *CCL20* expression in response to Pam3CSK4 for either haplotype at three or twenty-four hours (Figure 5.8C&D).

For *SAA3* expression, 1,25$(OH)_2D_3$ significantly reduced expression for IL8-h1 in response to LPS at twenty-four hours but not at three hours (Figure 5.9A&B). In the case of IL8-h2 the reduction of expression by 1,25$(OH)_2D_3$ was close to significance at twenty-four hours (Figure 5.9B). There was no significant modulation of *SAA3* expression in response to Pam3CSK4 in response to pre-treatment with 1,25$(OH)_2D_3$ by either haplotype at three or twenty-four hours (Figure 5.9C&D).

For *NOS2* expression, the was significant increase in expression in response to LPS with pre-treatment of 1,25$(OH)_2D_3$ for both haplotypes at three hours and for IL8-h1 only at twenty-four hours (Figure 5.10A&B). There was also a significant increase in expression with pre-treatment of 1,25$(OH)_2D_3$ in response to Pam3CSK4 for both haplotypes at three hours and for IL8-h1 at twenty-four hours (Figure 5.10C&D). The effect of 1,25$(OH)_2D_3$ on IL8-h2 *NOS2* expression at twenty-four hours was close to significance (Figure 5.10D).

Finally, for *VDR* expression, 1,25$(OH)_2D_3$ was able to significantly increase expression of *VDR* for IL8-h1 in response to LPS at both three and twenty-four hours while this only occurred at twenty-four hours for IL8-h2 (Figure 5.11A&B). Interestingly, a close to significant difference (P=0.068) between haplotypes in expression levels of *VDR* in response to LPS with the addition of 1,25$(OH)_2D_3$ was observe at twenty-four hours (Figure 5.11B). This haplotype difference in expression levels was only seen for *VDR*. For Pam3CSK4, 1,25$(OH)_2D_3$ significantly boosted *VDR* expression at three hours as Pam3CSK4 alone did significantly induce expression (Figure 5.11C). At twenty-four hours, there was a close to significant effect (P=0.051) of

1,25$(OH)_2D_3$ increasing *VDR* expression in response to Pam3CSK4 for IL8-h1 but not for IL8-h2 (Figure 5.11D).

Generally, there appears to be no significant differences between the haplotypes in expression of *CCL20*, *SAA3* and *NOS2*. There does appear to be higher expression for *IL8* in IL8-h1 animals in response to Pam3CSK4 at twenty-four hours but not significantly so. The addition of 1,25$(OH)_2D_3$ and LPS resulted in a close to significant haplotype difference (P=0.068) in *VDR* expression where IL8-h1 showed higher expression levels after twenty-four hours.

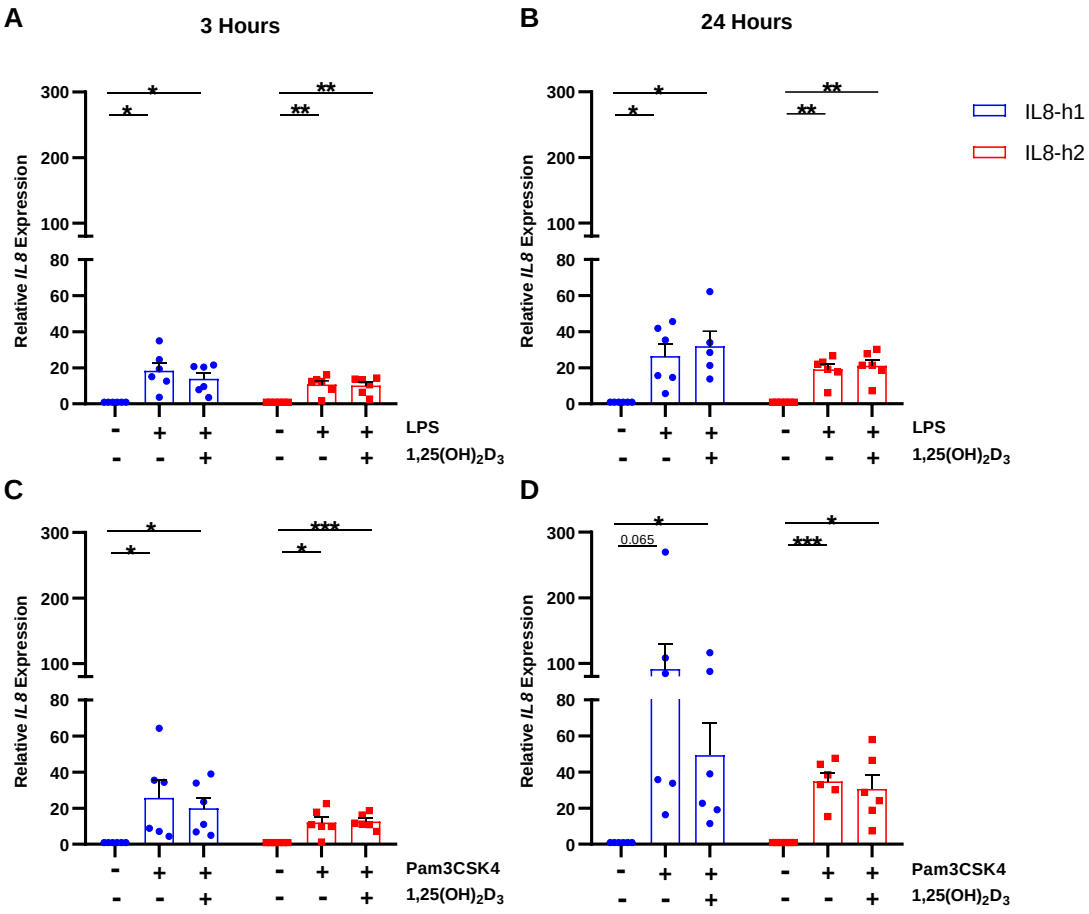

Figure 5.7 Gene expression of *IL8* following 3 and 24 hour stimulation of LPS +/- 1,25(OH)$_2$D$_3$ or Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 µg/mL LPS +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (A) 3 and (B) 24 hours or 1 µg/mL Pam3CSK4 +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (C) 3 hours and (D) 24 hours plus an unstimulated control at 37^{o}C, 5% CO_2. Gene expression of *IL8* was assessed and presented as normalised to the unstimulated control. Error bars represent the SEM of n=6. Significance is between stimuli (straight line) where *P<0.05, **P<0.01 and ***P<0.001

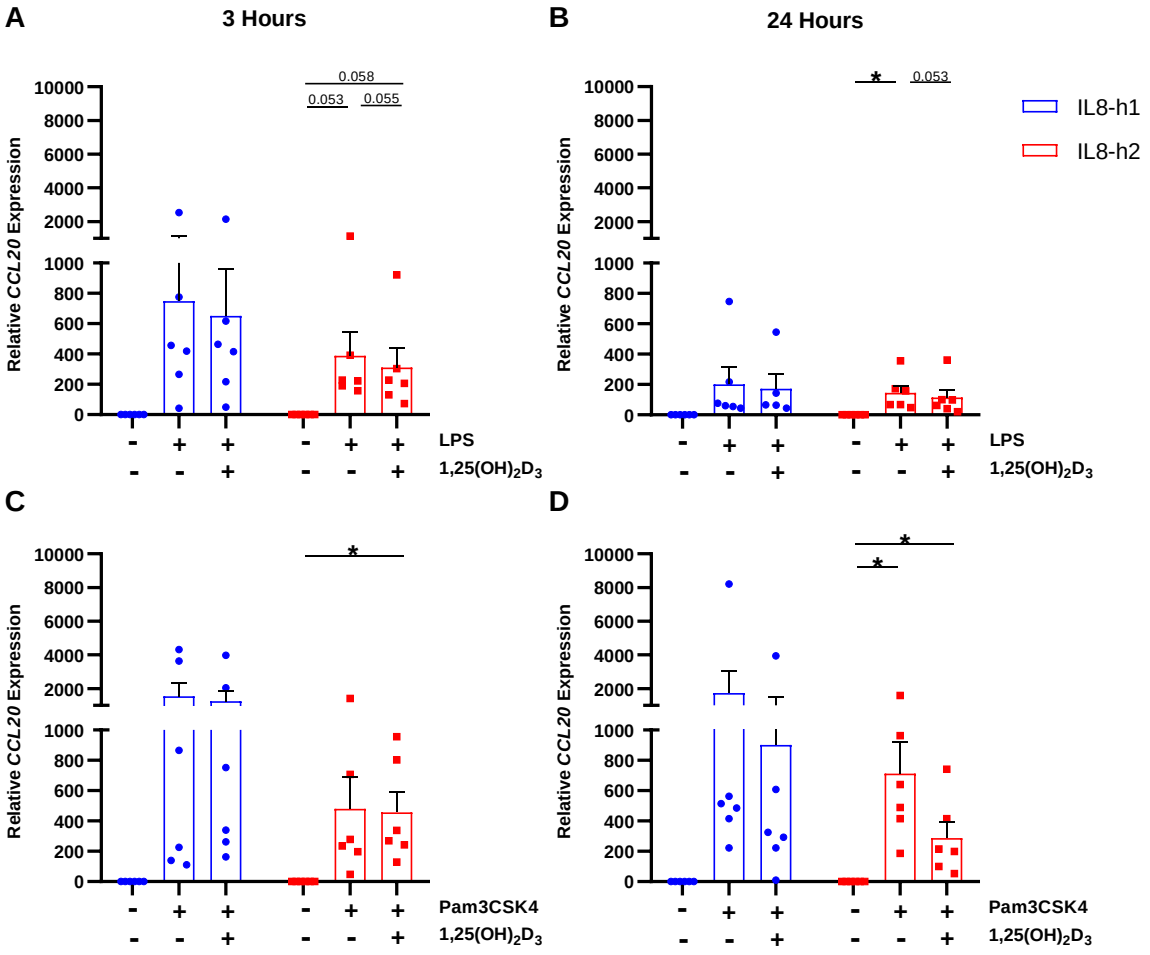

Figure 5.8 Gene expression of *CCL20* following 3 and 24 hour stimulation of LPS +/- 1,25(OH)$_2$D$_3$ or Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 µg/mL LPS +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (A) 3 and (B) 24 hours or 1 µg/mL Pam3CSK4 +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (C) 3 hours and (D) 24 hours plus an unstimulated control at 37^{o}C, 5% CO_2. Gene expression of *CCL20* was assessed and presented as normalised to the unstimulated control. Error bars represent the SEM of n=6. Significance is between stimuli (straight line) where *P<0.05, **P<0.01 and ***P<0.001.

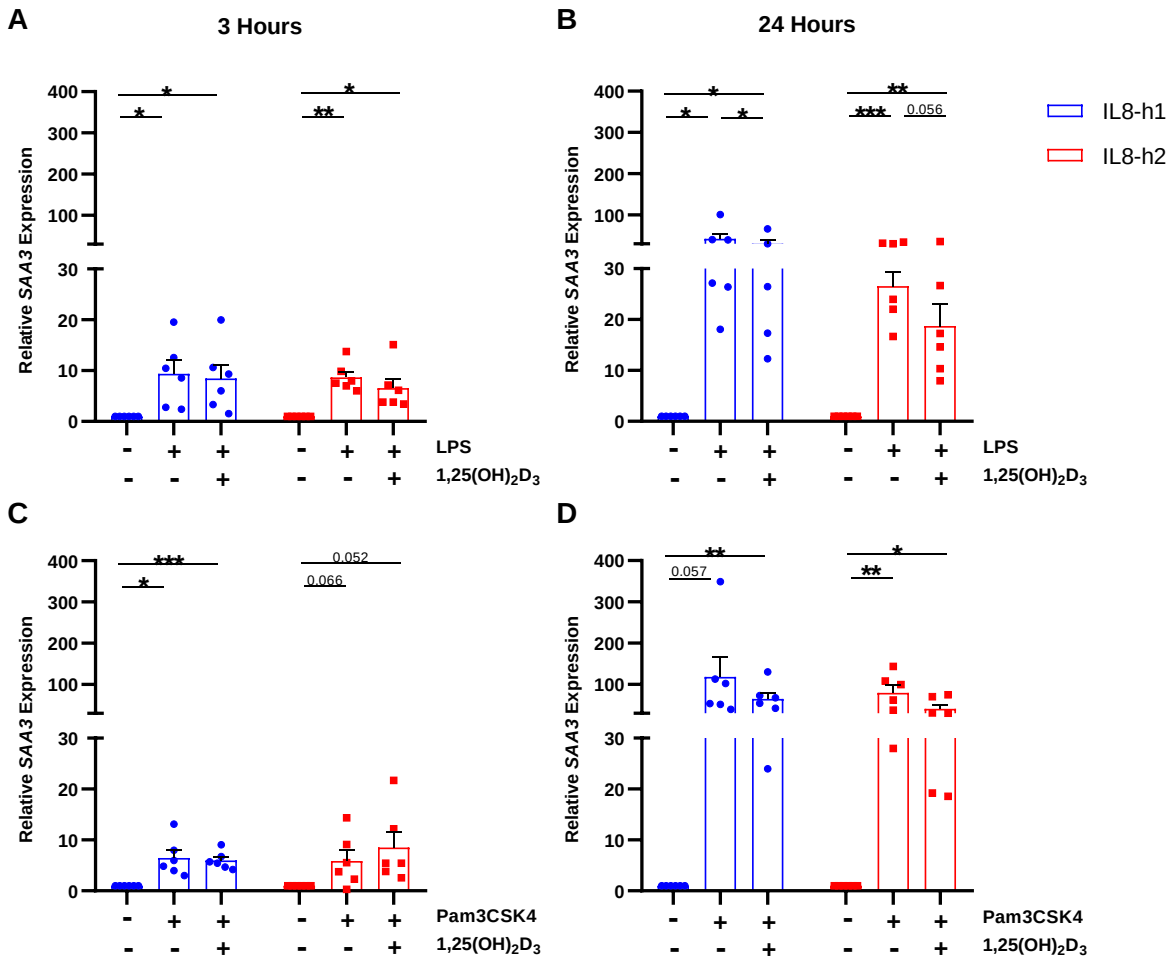

Figure 5.9 Gene expression of *SAA3* following 3 and 24 hour stimulation of LPS +/- $1,25(OH)_2D_3$ or Pam3CSK4 +/- $1,25(OH)_2D_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 µg/mL LPS +/- 10 nM $1,25(OH)_2D_3$ (2 hr pre-treatment) for (A) 3 and (B) 24 hours or 1 µg/mL Pam3CSK4 +/- 10 nM $1,25(OH)_2D_3$ (2 hr pre-treatment) for (C) 3 hours and (D) 24 hours plus an unstimulated control at 37°C, 5% CO_2. Gene expression of *SAA3* was assessed and presented as normalised to the unstimulated control. Error bars represent the SEM of n=6. Significance is between stimuli (straight line) where *P<0.05, **P<0.01 and ***P<0.001.

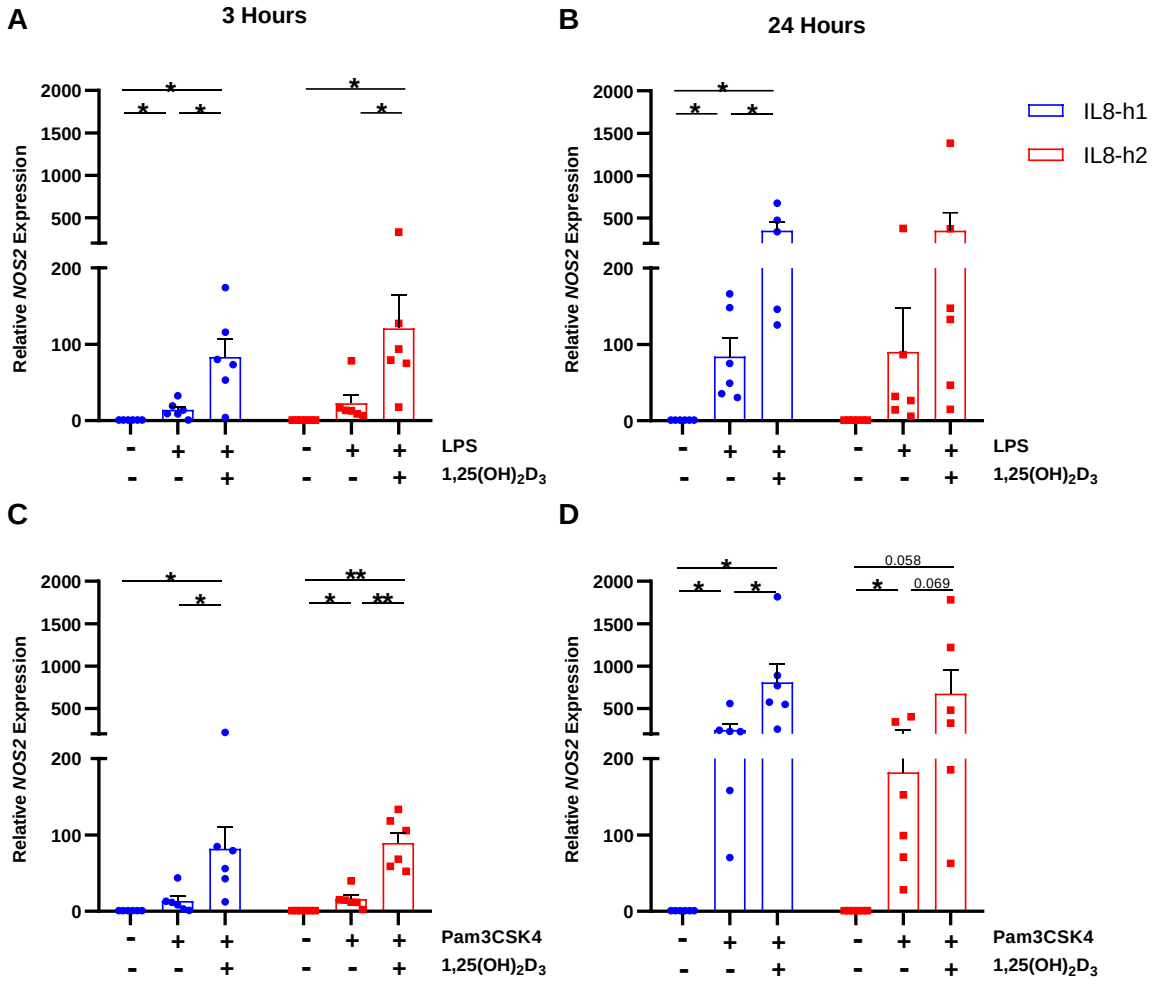

Figure 5.10 Gene expression of *NOS2* following 3 and 24 hour stimulation of LPS +/- 1,25(OH)$_2$D$_3$ or Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 µg/mL LPS +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (A) 3 and (B) 24 hours or 1 µg/mL Pam3CSK4 +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (C) 3 hours and (D) 24 hours plus an unstimulated control at 37°C, 5% CO_2. Gene expression of *NOS2* was assessed and presented as normalised to the unstimulated control. Error bars represent the SEM of n=6. Significance is between stimuli (straight line) where *P<0.05, **P<0.01 and ***P<0.001.

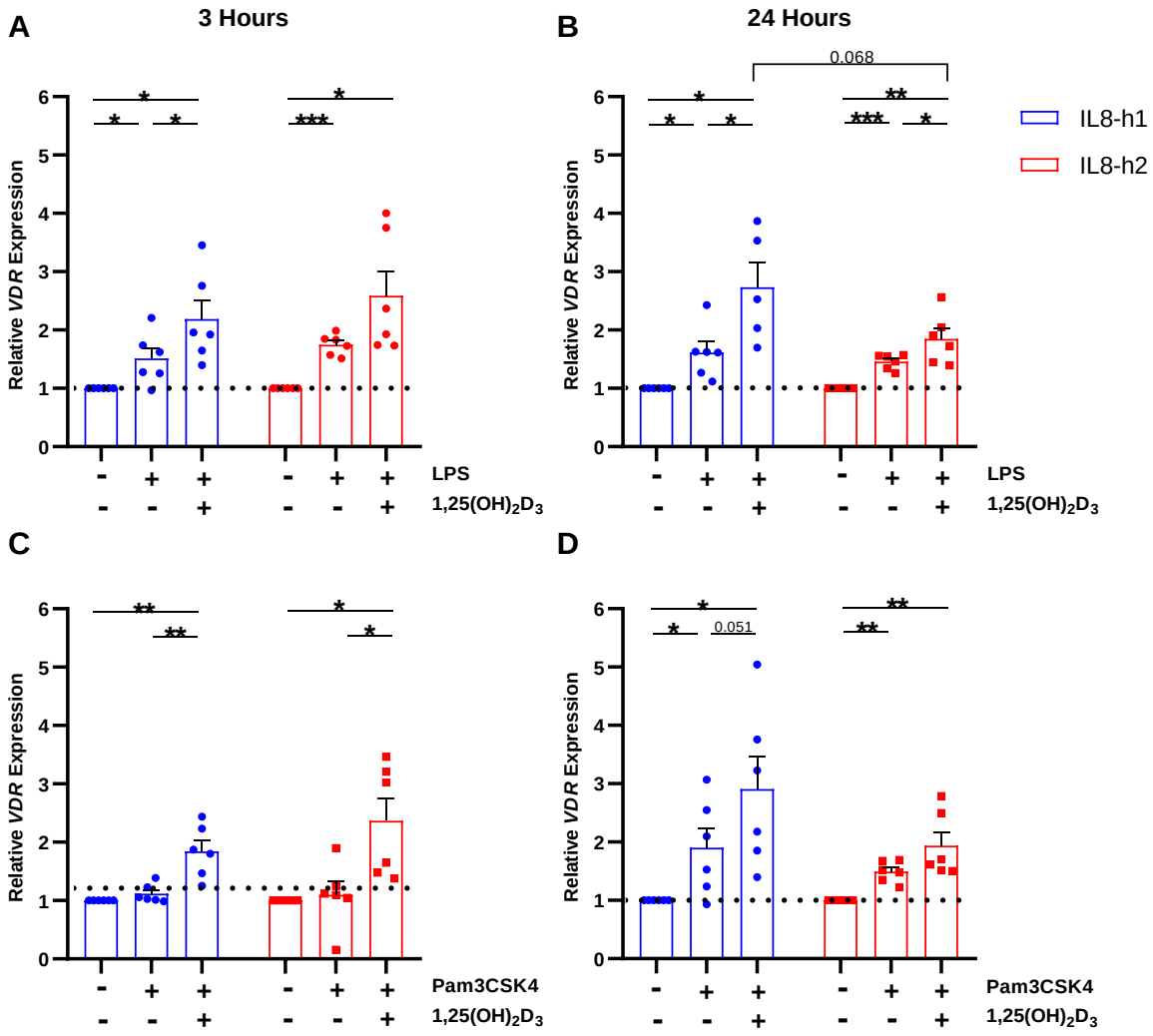

Figure 5.11 Gene expression of *VDR* following 3 and 24 hour stimulation of LPS +/- 1,25(OH)$_2$D$_3$ or Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in bovine dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 µg/mL LPS +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (A) 3 and (B) 24 hours or 1 µg/mL Pam3CSK4 +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (C) 3 hours and (D) 24 hours plus an unstimulated control at 37^{o}C, 5% CO_2. Gene expression of *VDR* was assessed and presented as normalised to the unstimulated control. Error bars represent the SEM of n=6. Significance is between stimuli (straight line) or between haplotype (branched line) where *P<0.05, **P<0.01 and ***P<0.001.

5.2.3 Local IL-8 production of dermal fibroblasts of two distinct IL8 haplotypes.

To determine if whether IL-8 protein levels differed between haplotypes and also whether vitamin D modulate IL-8 levels, IL-8 secretion in the supernatants from the previously describe PAMP stimulations of bovine dermal fibroblast from each IL8 haplotype were measured by ELISA.

Surprisingly, no significant induction of IL-8 protein was seen in response to LPS at three or twenty-four hours for either haplotype (Figure 5.12A&B). However, the addition of $1,25(OH)_2D_3$ significantly reduced IL-8 levels in response to LPS for IL8-h2 at the earlier three-hour timepoint (Figure 5.12A). This effect was not seen for IL8-h1 and was lost at the later twenty-four timepoint for IL8-h2 (Figure 5.12B).

Also, no significant induction of IL-8 protein was seen in response to Pam3CSK4 at three-hours for either haplotype (Figure 5.12C) however, there was a significant induction at twenty-four for both haplotypes (Figure 5.12D). An interesting close to significant (P=0.051) difference between haplotypes was observed in IL-8 levels produced in response to Pam3CSK4 alone at twenty-four hours where IL8-h1 levels are higher (Figure 5.12D). There appears to be no effect of $1,25(OH)_2D_3$ on the IL-8 response to Pam3CSK4 in either haplotype at both timepoints (Figure 5.12C&D).

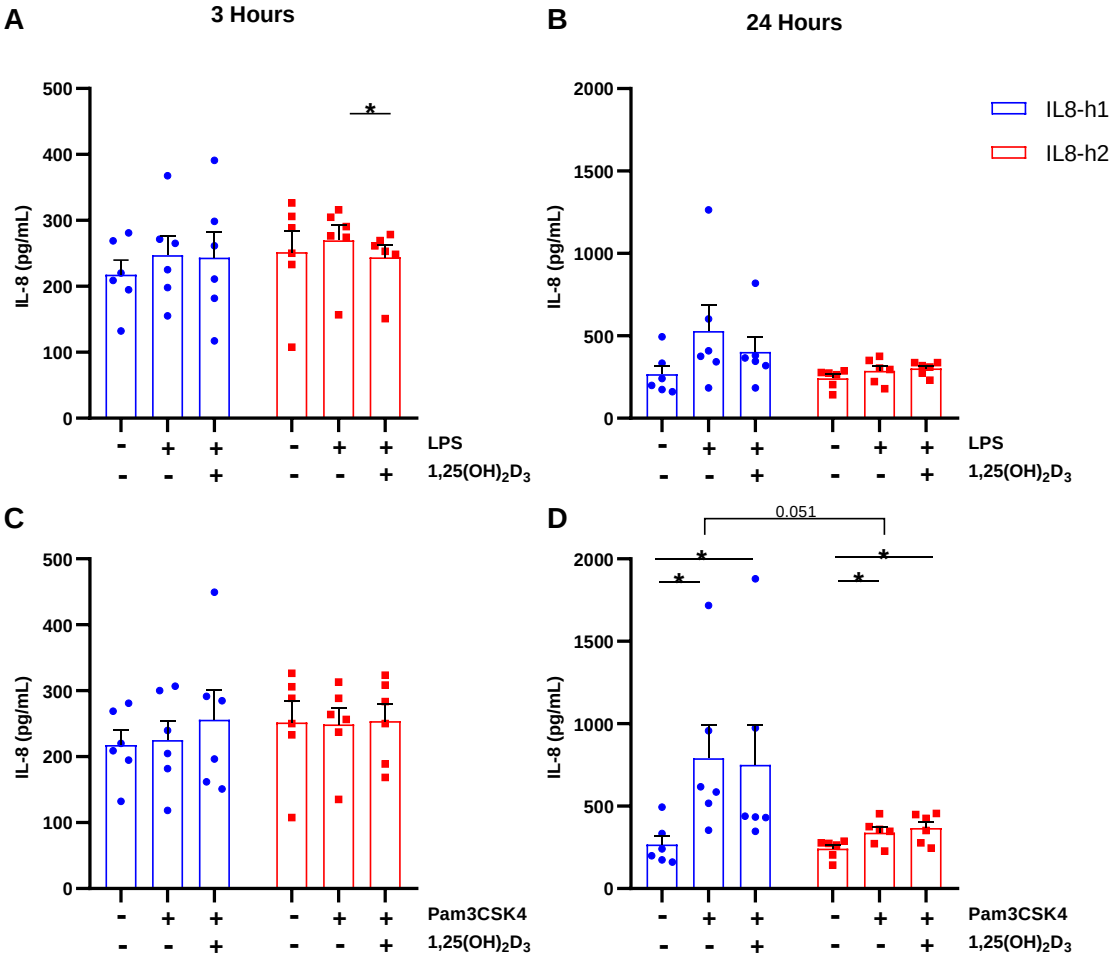

Figure 5.12 IL-8 production following 3 hours and 24 hours stimulation of LPS +/- 1,25(OH)$_2$D$_3$ or Pam3CSK4 +/- 1,25(OH)$_2$D$_3$ in primary dermal fibroblasts from two distinct IL8 haplotypes. Dermal fibroblasts were stimulated with 1 µg/mL LPS +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (A) 3 and (B) 24 hours or 1 µg/mL Pam3CSK4 +/- 10 nM 1,25(OH)$_2$D$_3$ (2 hr pre-treatment) for (C) 3 hours and (D) 24 hours plus an unstimulated control at 37^{o}C, 5% CO_2. IL-8 levels in IL8-h1 and IL8-h2 cell stimulation supernatants was assessed via ELISA. Error bars represent the SEM of n=6. Significance is between stimuli (straight lines) or between haplotypes (branched lines) where *P<0.05.

5.3 Discussion

Analysis of peripheral blood immune cell responses are useful in investigating the systemic implications of infection. However, such approaches don't include the local environment where the infection may originate. Early local innate responses are key determinants of the level of inflammation that controls immune cell infiltrate as well as the degree of resulting tissue damage. It has already been established a role for IL8 haplotype in influencing the systemic innate immune response by generating divergent neutrophil and monocyte numbers and serum IL-8 levels basally in Holstein-Friesian calves in chapters three and four, respectively. The role for a local type cell such as the fibroblast in contributing to overall systemic IL-8 levels in cattle with these divergent haplotypes had not been previously investigated. As this cell type would be heavily involved in the early activation of the innate immune response locally within tissues such as the skin, mammary gland and endometrium, it represents an opportunity to explore the effects of IL8 haplotype on local immunity which may have important consequences for the subsequent trajectory of infection.

Although the fibroblast is not traditionally seen to be a cell of the immune system, it still plays a unique role in activation of immune response by recruitment of monocytes, macrophages and polymorphonuclear cells [70]. Damage to the epithelial layer is a common feature in livestock systems and therefore the response of the underlying stroma (including fibroblasts) has important consequences for the activation of inflammation [47]. Furthermore, dysregulated local inflammatory responses are an important risk factor for chronic disease and tissue pathology [184]. Although knowledge on bovine fibroblast function is not extensive, IL-8 production by primary bovine dermal fibroblasts stimulated with LPS *in vitro* have been shown to correlate with clinical outcomes of *in vivo* infection with *E. coli* in the mammary gland [76]. The high and low IL-8 responders identified in that study may be directly related to their IL8 haplotype. By using the bovine primary dermal fibroblast for this work, it has begun to unravel the role IL8 haplotype plays in influencing IL-8 production in these cells.

After selecting suitable animals from the cohort genotyped using the custom SNP genotyping assay, dermal fibroblasts were successfully isolated from animals of each haplotype and subsequentially characterized. Strong staining for vimentin and corresponding gene expression confirmed the cells were fibroblast in nature as was

previously established [69]. Cell stimulations with a range of PAMPs (LPS, Pam3CSK4 and R8484) and 1,25$(OH)_2D_3$ showed that *IL8* expression was activated with varying concentrations of LPS and Pam3CSK4. LPS has been previously demonstrated to induce *IL8* expression in bovine dermal fibroblasts indicating the cells isolated from the same tissue here are similarly responsive [75]. Pam3CSK4 has similarly been shown to activate *IL8* expression and subsequent protein production in fibroblasts isolated from both humans and cattle. The lack of cellular response to R848 could be explained by an inefficiency of it to activate TLR7/8 in bovine dermal fibroblasts. Other TLR7/8 agonists including Imiquimod and ssRNA40 have be shown to induce IL-8 production in human skin fibroblasts albeit at very low levels, however this may not be the case for the bovine species [185]. The addition of 1,25$(OH)_2D_3$ alone did not result in a significant induction of *IL8*. The literature had previously identified 1,25$(OH)_2D_3$ as a significant driver of *IL8* expression but in a monocyte cell line, and the area of the genome where the *IL8* gene is located, to be particularly sensitive to its action [126]. Therefore, it is not entirely surprising that no evidence of it alone being able to induce *IL8* in these fibroblasts was found.

The expression of *CCL20* was also investigated as marker of innate immune response activation in bovine dermal fibroblasts and was previously shown to be highly expressed in response to LPS [176]. The cells were found to highly express *CCL20* in response to LPS but more so in response to Pam3CSK4 which had not been previously reported. Although other related cell types such as endothelial cells have been shown to express high levels of *CCL20* in response to Pam3CSK4 [186]. The TLR7/8 agonist R848 and 1,25$(OH)_2D_3$ did not induce high *CCL20* expression in this cell type. This has also been shown in human epithelial cells where R848 was unable to significantly induce CCL20 protein [187]. The subsequent IL-8 protein levels in the cell stimulation supernatant was also examined. Significant protein levels were seen only after 24 hours stimulation in response to high concentrations of LPS and Pam3CSK4. This again corresponds with the IL8 mRNA levels seen at three and twenty-four hours indicating the successful translation to protein.

After characterising the cells and ensuring they were responsive to PAMP stimulation, cells isolated from each animals of each haplotype (IL8-h1 and IL8-h2) were stimulated LPS and Pam3CSK4 for 3 hours and 24 hours. This was to successfully examine the early and late response to PAMP stimulation and to pick up late IL-8 protein

production. IL-8 protein secretion in response to Pam3CSK4 was significantly induced by both haplotypes but there was a very close to significant haplotype difference which showed higher levels for IL8-h1. The *IL8* gene expression results observed for Pam3CSK4 at twenty-four hours showed expression was much more elevated in IL8-h1 compared to IL8-h2 although not significantly so. This is an interesting result considering previous work identified IL8-h1 as producing lower IL-8 levels systemically [5]. This previous analysis was carried out using LPS during the *in vivo* challenge so there is a possibility of specific haplotype response depending on the PAMP. The IL-8 gene and protein response to LPS at twenty-four hours is slightly more elevated in IL8-h1 compared to IL8-h2 but not as clearly evident as the response to Pam3CSK4. The analysis performed using an *in vivo* challenge may also not directly translate to a primary local cell model such as the fibroblast due to alternative *IL8* regulation because of the two distinct haplotypes. A previous study linked fibroblast IL-8 production to clinical outcome during mammary gland infection but the same may not be said comparing systemic IL-8 levels and fibroblast IL-8 production in the context of IL8 haplotype [76]. IL8 haplotype did not appear to significantly affect the other fibroblast inflammatory genes investigated, *SAA3* and *CCL20*. IL8 haplotype has been noted to influence genes other than *IL8* in human peripheral monocytes and lymphocytes where *TNFA* was elevated in one haplotype compared to the other but this may be cell specific and the polymorphisms which make up the haplotypes are not exactly the same between the species [4].

Cell were also pre-treated with $1,25(OH)_2D_3$ to investigate its effect on gene expression on the IL8 haplotypes in addition to examining its role in attenuating the innate immune response. *NOS2* expression in response to LPS and Pam3CSK4 was significantly increased with the addition of $1,25(OH)_2D_3$ in the case of both haplotypes but this effect appeared more evident in IL8-h1 compared to IL8-h2. Vitamin Ds role in enhancing *NOS2* expression may be due to VDR possibly acting as a transcription factor on the *NOS2* gene as has been shown in the case of *IL8* in humans [126]. In salivary gland fibroblasts isolated from cattle, the gene encoding inducible nitric oxide (iNOS), *NOS2*, was shown to be significantly enhanced following stimulation by LPS with the addition of $1,25(OH)_2D_3$ [164]. This action of $1,25(OH)_2D_3$ has also been identified in bovine monocytes [118]. *VDR* expression was significantly increased for both haplotypes in response to both LPS and Pam3CSK4 particularly after 24 hours but the addition of

$1,25(OH)_2D_3$ significantly increases it further. However, this effect of $1,25(OH)_2D_3$ increasing *VDR* expression was much more muted in IL8-h2 than in IL8-h1 and close to significantly so. This indicates fibroblasts are vitamin D responsive and possibly more so in the case of IL8-h1 animals. The IL8-h1 haplotype could result in an enhanced responsiveness to vitamin D which in turn results in increased *VDR* expression and the ability for it to act on the *IL8* promoter region. Many studies point towards $1,25(OH)_2D_3$ downregulating *IL8* expression and protein secretion in stimulated cells [127, 182].

The primary dermal fibroblast represents an opportunity for further study of how vitamin D attenuates the innate immune response in determining whether VDR acts as a transcription factor for *IL8* expression in cattle as this area has only been briefly covered by studies on the human species. Here, the dermal fibroblast was used to demonstrate an effect of IL8 haplotype on the production of IL-8 in response to Pam3CSK4 showing that IL8-h1 produced higher levels. This was a novel result as previously IL8-h1 had been shown to produce lower levels of IL-8 systemically in response to an *in vivo* LPS challenge [5]. An effect of IL8 haplotype was also shown in responsiveness to vitamin D in the case of *VDR* expression where IL-h1 expressed higher levels in response to LPS. These results could potentially be explained by a greater responsiveness by IL8-h1 animals to vitamin D, resulting in increased *VDR* expression and in turn, increased expression on secretion of IL-8 by VDR acting on the *IL8* gene. This would represent a novel mechanism in IL8 haplotype influencing the ability of the cell to respond to and use vitamin D for regulating IL-8 secretion during a local infection. Further experimental work would be required to confirm this hypothesis. This work has shown that vitamin D has some key effects of elements of the immune response particularly the ability to upregulate gene expression of *NOS2* and demonstrates the importance of adequate vitamin D levels to maintain an effective innate immune response during a local infection. It also presents a useful model by which further understanding around how VDR may act as a transcription factor for *IL8* expression could be elicited. In particular, the how this transcriptional regulation of vitamin D may differ between IL8 haplotypes.

Chapter 6. General Discussion

Genetic variation can have very significant effects on the immune response of an individual. There are broad estimates that in the region of 20-40% of immune system variation in humans can be attributed to variation in the genome [188]. IL-8 is an important chemokine in the innate immune system responsible for forming chemotactic gradients which recruit immune cells, primarily the neutrophil, to infection sites [189]. Previously identified SNPs in the promoter region of the *IL8* gene, which segregate into two haplotypes (IL8-h1 and IL8-h2) in Holstein-Friesian cattle, were shown to impact the systemic innate immune response, resulting in divergent levels of IL-8 produced during an *in vivo* LPS challenge [5, 35]. These haplotypes were also separately shown to be linked to SCC in Holstein-Friesian cows [36]. In both studies, IL8-h2 was linked to higher IL-8 levels and also SCC showing specific disease relevance [5, 36]. The goal of this current project was to examine the mechanisms by which IL8 haplotype impacts upon both systemic and local innate immune and how these responses may be modulated by vitamin D. Specifically, I examined systemic changes in immune cell populations, chemokine and cytokine responses in peripheral blood and functional responses by neutrophils. I additionally looked at how IL8 haplotype impacts the response to vitamin D in cattle and whether it affects circulating levels of vitamin D in blood. Finally, the role IL8 haplotype plays in the local innate immune response was examined using a primary bovine dermal fibroblast model.

When examining systemic immunity in cattle of both IL8 haplotypes (results summarised in figure 6.1), differences were identified between haplotypes in circulating immune cell populations. Neutrophils, monocytes, eosinophils and basophils were increased at specific timepoints in IL8-h2 calves across the first ten months of their life when compared with IL8-h1 calves. Also, I confirmed monocytes as the major source of IL-8 secretion in peripheral blood. IL8 haplotype was also shown to impact IL-1β responses to fungal PAMPs in whole blood at six months of age but no effect was observed on the IL-8 response to any PAMP. This was in contrast to a previous study which identified IL8-h2 calves being significantly higher secretors of IL-8 in response to an *in vivo* LPS challenge [5]. Here using an *ex vivo* model, no significant differences were recorded in the peripheral blood IL-8 response to LPS between haplotypes. This suggests that an additional cell type, which is a key producer of IL-8, is either being excluded, or is not functioning/responding appropriately in the *ex vivo* whole blood culture model. Local cell types such as epithelial, endothelial and stromal cells have been shown to

produce significant levels of IL-8 in bovine [74, 76]. These cell types have not been previously isolated from cattle genotyped for their IL8 haplotype so, it was hypothesised that cells of this nature could differ significantly in their ability to produce IL-8 in response to PAMPs depending on IL8 haplotype.

While no haplotype differences were observed in LPS induced IL-8 responses in whole blood culture, there was observable difference in IL-8 levels in control samples in the absence of stimulation with IL8-h2 calves generally showing higher levels when compared with IL8-h1 calves suggesting a basal level haplotype difference between these calves. IL-8 levels in the serum of IL8-h2 calves was significantly higher when compared with sera from IL8-h1 across several months of the first ten months of life suggesting that calves with IL8-h2 are already predisposed to increased IL-8 levels when they encounter pathogen during infection. This could result in a higher infiltration of neutrophils from these animals and increased levels of inflammation.

IL8-h1 calves were shown to produce significantly more NETs, based in higher amounts of extracellular DNA measured in the supernatant from stimulated neutrophils, than IL8-h2 calves at months five and six which is an interesting result considering IL-8 levels in serum are lower for this haplotype. However, work in humans demonstrated that, in the case of a key macrophage effector function, an IL8 haplotype which showed decreased *IL8* expression in peripheral blood showed increased ability of macrophages to phagocytose bacteria [4]. Also, lymphocytes from this haplotype were associated with a more a regulatory adaptive phenotype when stimulated with PMA and ionomycin suggesting a lesser inflammatory response [4]. There is a possibility that there may be similar situation occurring with these IL8 haplotypes in cattle where IL8-h1 may be display a more inflammatory profile in relation to neutrophil function but less neutrophil infiltration due to lower levels of IL-8. This would require further work to fully elucidate what the trajectory of an *in vivo* infection would be for calves of each haplotype.

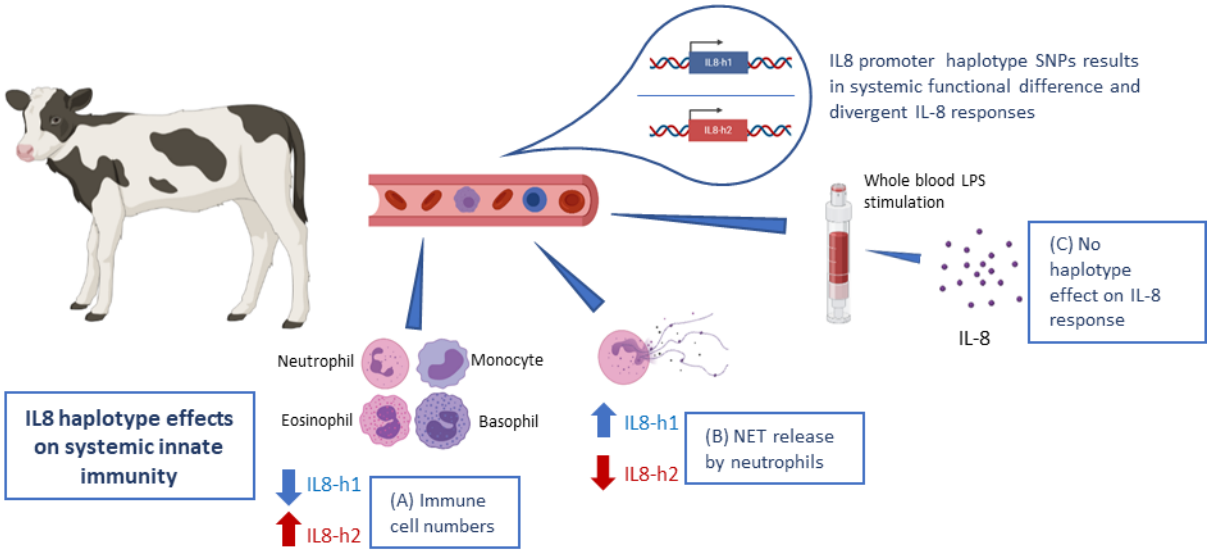

Figure 6.1 The key effects of IL8 promoter haplotype on components of systemic innate immunity in Holstein—Friesian calves. IL8 haplotype impacts neutrophil, monocytes, eosinophil and basophil numbers with increased numbers displayed in IL8-h2 across a number of months (A). NET release from neutrophils also differs depending on IL8 haplotype (B). No haplotype effect on the IL-8 response to LPS using an *ex vivo* whole blood stimulation assay (C). Blue (IL8-h1) and red (IL8-h2) arrows indicate increase or decrease depending on each haplotype.

At this early stage in life for calves there can be huge variation in immune responses. Our group has recently demonstrated (unpublished data) that vitamin D levels are very low in spring born Irish Holstein-Friesian calves and do not increase significantly until at least four months of age which could have implications for the innate immune response. In this study a similar trend is observed where levels remain low in early life but, interestingly 25(OH)D vitamin D levels were significantly increased in the serum from IL-8h1 calves as compared to IL-8-h2 calves across months five, six and seven. This was a very interesting result due to IL8-h1 previously having shown lower IL-8 levels in serum. This therefore demonstrates a novel inverse relationship between 25(OH)D and IL-8 concentration in serum in cattle with these haplotypes.

In attempts to further elucidate this relationship, qPCR analysis was performed on control and LPS stimulated whole blood from month six (where there were significant haplotype differences in both 25(OH)D and IL-8) and showed IL8-h1 calves to have significantly increased expression of e*DEFB4A*, which amplifies a set of defensin genes. Defensins were previously shown to be upregulated by vitamin D in bovine monocytes [118]. Additionally, there was an increase in *CYP27B1* expression which was approaching significance. *CYP27B1* encodes 1-alpha-hydroxylase, which converts inactive vitamin D to active vitamin D suggesting active conversion of the 25(OH)D in serum to $1,25(OH)_2D_3$ for use by cells. I postulated that SNPs in key genes involved in the vitamin D pathway were inherited along with the IL8 haplotypes which could be contributing to the relationship between IL-8 and vitamin D in the serum of these calves. Previously identified SNPs in the gene encoding VDBP, *GC*, and *CYP27A1*, which encodes another conversion known as sterol 27-hydroxylase, were investigated but no co-segregation with IL8 haplotypes was observed. This however does not rule out that other uncharacterised SNPs in vitamin D pathway related genes are being inherited with the IL8 haplotypes as SNPs in the *GC* gene in humans have been previously associated with vitamin D deficiency [190].

Considering that calves are born with these haplotypes which effect secretion levels of IL-8, it was also investigated whether IL-8 itself was playing a role in the vitamin D pathway by attenuating gene expression of key enzymes and receptors including *CYP27A1*, *CYP27B1*, *CYP24A1* and *VDR*. Using the monocyte, which has been previously shown to express these enzymes [118], we observed no significant effect of IL-8 on any of these genes. However, I also wanted to confirm that vitamin D does

attenuate *IL8* expression as had been reported in humans [127], as this had not been previously investigated in cattle. $1,25(OH)_2D_3$ was shown to significantly reduce *IL8* expression at a basal level in monocytes which would correspond with what was detected in the serum of calves with the low IL-8 producing IL8-h1 haplotype.

Having shown an effect of $1,25(OH)_2D_3$ in reducing *IL8* expression in monocytes and also the inverse relationship between IL-8 and 25(OH)D basally in serum, I investigated the possibility that vitamin D supplementation would actually modulate *IL8* gene expression and IL-8 protein secretion in vivo. Calves which were supplemented with additional vitamin D in the diet from birth and raised outdoors were used for *ex vivo* whole blood culture and monocyte stimulation with LPS at six months of age. A control group which received no additional vitamin D and was raised indoors were also included. No effect on IL-8 levels by supplementation was seen in the serum of these calves suggesting that the inverse relationship between IL-8 and 25(OH)D previously seen may be unique to cattle with these IL8 haplotypes. There were three IL8-h1 calves and two IL8-h2 each in the control and supplemented groups, haplotype differences in vitamin D pathway genes including *CYP27A1, CYP27B1, CYP24A1* and *VDR* were observed. IL8-h1 calves showed much higher expression levels of *CYP27B1* and *VDR* in whole which were blood when compared with IL8-h2 calves. This was not seen in monocytes indicting another peripheral blood cell is most likely involved in this difference. Interestingly, *CYP27A1* and *CYP24A1* expression was much higher in IL8-h2 calves when compared with IL8-h1 calves in whole blood. The enzyme which *CYP24A1* encodes is responsible for converting active vitamin D to another inactive form to clear it from the body. This is quite interesting considering IL8-h2 calves were shown to have lower levels of vitamin D in serum. Again, this haplotype effect was seen in whole blood only and not monocytes alone suggesting the involvement or synergistic effect of another peripheral blood cell.

There is evidence here that IL8 haplotype has effects on serum vitamin D status as well as gene expression of vitamin D pathway related genes. Since these haplotypes differ by SNPs in the promoter region of *IL8* [35], and the VDR interferes with *IL8* gene expression [127, 128], VDR action potentially differs in each haplotype due to this. VDR has been shown to act as a transcription factor and bind directly to *IL8* gene in humans to initiate gene expression and also interfere with NF-κB acting as a transcription factor, again in humans [126, 128]. Much is still unknown in cattle about IL-8 and vitamin D however, and this mechanism may differ based on *IL8* haplotype. VDREs have been

previously used to predict areas were VDR binds in the human genome but little is known about their presence in bovine genes [191]. Further work is required to fully elucidate whether there is a VDRE present in the promoter region of the bovine *IL8* gene and whether it differs between haplotypes which could possibly be one explanation for different serum IL-8 levels between the haplotypes. The results summary of the examination of the relationship of IL8 haplotype and vitamin D are present in figure 6.2.

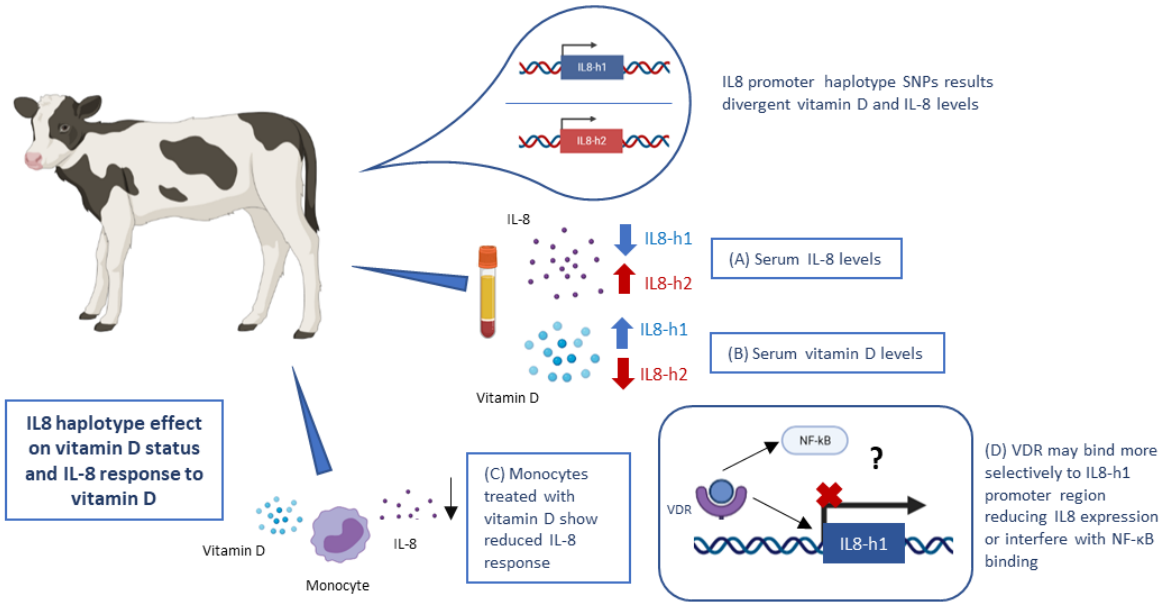

Figure 6.2 The key effects of IL8 promoter haplotype on vitamin D status and IL-8 response to vitamin D in Holstein—Friesian calves. IL8 haplotype impacts serum levels of IL-8 (A) in addition to effecting circulating levels of vitamin D (B). Vitamin D alone reduced IL8 expression in monocytes isolated from Holstein-Friesian calves (C). The mechanism by which circulating IL-8 levels are reduced and vitamin D increased in IL8-h1 calves may be due to more effective binding of VDR to the IL8-h1 promoter or interference with NF-κB binding (D). Blue (IL8-h1) and red (IL8-h2) arrows indicate increase or decrease depending on each haplotype.

Having shown basal level haplotype differences in IL-8 and also shown no LPS effect on whole blood I used a local cell model to investigate whether local tissue cells isolated from cattle with these IL8 haplotypes also differ in their secretion of IL-8 could that be contributing to IL-8 levels in serum (results summarised in figure 6.3). I established that primary dermal fibroblasts produced IL-8 in response to two bacterial PAMPs; LPS and Pam3CSK4. Importantly, there appeared to be haplotype differences with IL8-h1 producing higher levels of IL-8 when compared with IL8-h2 in response to Pam3CK4. This wasn't observed in the the response to LPS stimulation. This a very interesting novel result considering the previous *in vivo* LPS challenge showed IL8-h2 to produce significantly higher levels of IL-8 [5]. There may possibly be a selective response depending on the interaction between PAMP and the IL8 haplotype. In humans, IL8 haplotype was shown to influence the ROS response by macrophages to two different types of bacteria. Macrophages from one haplotype showed much higher generation of ROS compared to the other with a type of bacteria known as *P. gingivalis* while both haplotypes responded similarly to the bacteria *A. actinomycetemcomitans* [4]. This indicates IL8 haplotype can influence innate response differently depending on the pathogen. Additionally the cell type involved in the response can also respond differently depending on IL8 haplotype.

IL8 haplotype also played a role in how the cells responded to vitamin D. This was particular evident in *VDR* expression where $1,25(OH)_2D_3$ enhanced expression in response to LPS. Additionally, $1,25(OH)_2D_3$ also had a major effect on increasing expression of *NOS2,* the gene encoding iNOS, which was detected for both haplotypes. This effect has been previously described in bovine salivary gland fibroblasts [164]. Local dermal fibroblasts in IL8-h1 cattle may potentially be more responsive to vitamin D based on the significantly increased expression of *VDR* which in turn could be acting on the *IL8* gene promoter region as has been a previously described action of VDR in human monocytes [126]. However, this effect may be PAMP specific as haplotype difference in *VDR* expression was only detected in response to LPS and not Pam3CSK4. Nevertheless, the bovine dermal fibroblast does present itself as a useful model for examining the effects of vitamin D on *IL8* gene expression in a local model of infection. This model also highlights that *IL8* haplotype effects on *IL8* expression and protein production are not the same in both a systemic and local setting. In order more fully

understand the role IL8 haplotype plays on the immune response to an infection, an *in vivo* infection model would be required in genotyped cattle.

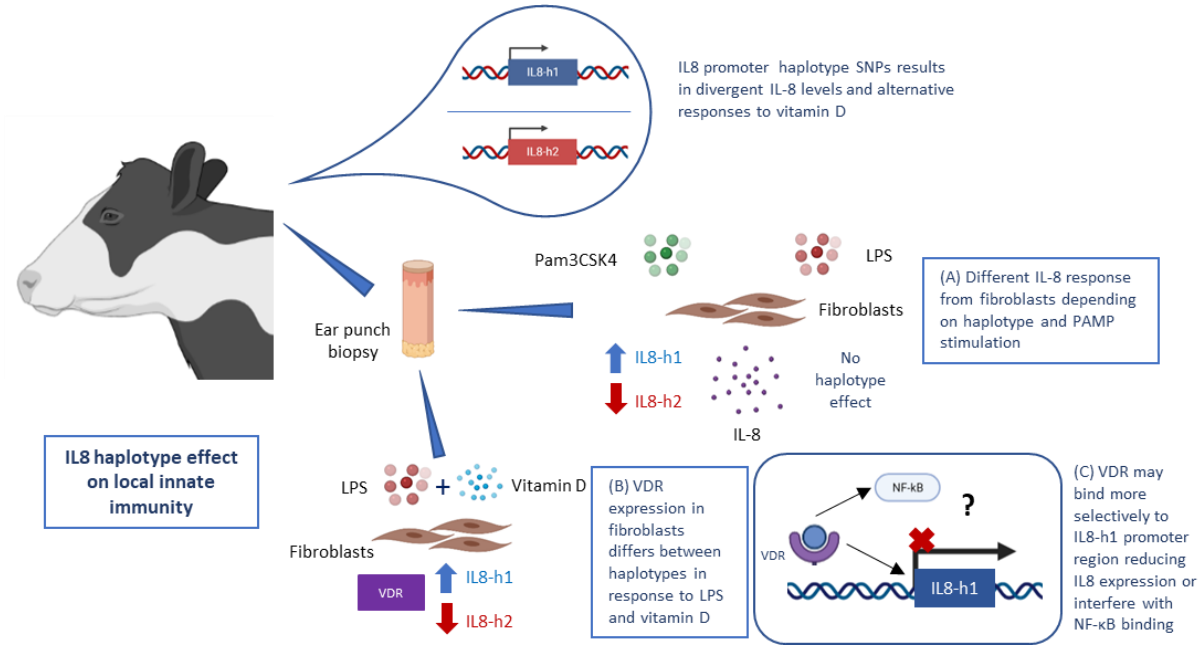

Figure 6.3 The key effects of IL8 promoter haplotype on components on local innate immunity in Holstein—Friesian calves. IL8 haplotype effects the IL-8 response of dermal fibroblasts depending on PAMP stimulation (A) The combination of vitamin D and LPS results in haplotype specific effects on expression of *VDR* in dermal fibroblasts (B). This effect may be enhanced in IL8-h1 cattle due to enhanced binding to the IL8-h1 promoter. Blue (IL8-h1) and red (IL8-h2) arrows indicate increase or decrease depending on each haplotype.

Overall, the results presented in this thesis emphasise the importance of considering an animals' underlying genotype to fully understand how it will respond to infection. These functionally characterised IL8 haplotypes in cattle show differences in circulating immune cell populations, NET formation, serum levels of IL-8 and 25(OH)D and fibroblast IL-8 responses (Figure 6.1) which could have important implications for the animal as a calf with also as cow during common cattle associated diseases such as mastitis and endometritis.

Cows possessing different haplotypes of the IL-8 receptor CXCR1 showed different outcomes during an experimental bacterial infection of the mammary gland where cattle possessing the so called 11 haplotype showed high levels of inflammation, high bacterial count and increased requirement for antibiotics [7]. I had to opportunity to investigate whether a combination of a certain IL8 haplotype with either of two CXCR1 haplotype known as 44 and 14, which presented a moderate inflammatory response to the experimental infection, would have any effect on expression of *IL8* and *CXCR1* (Appendix I). Using a whole blood culture model stimulated with LPS in blood from Holstein-Friesian cows possessing either IL8-h1, IL8-h2 or IL8-het (heterozygote) along with the CXCR1 haplotype 44 or the CXCR1 haplotype 14, I analysed expression of *IL8* and *CXCR1*. IL8-h1 showed significantly less expression of *CXCR1* in both the control and LPS stimulated compared to IL8-h2 and IL8-het in combination with the CXCR1 44 haplotype (Appendix I: Figure 1). Whereas no significant differences in expression for any gene from cows with any IL8 haplotype and the CXCR1 14 haplotype was detected (Appendix I: Figure 2). These results underline that an animal which showed moderate inflammation in an experimental infection could potentially show a different phenotype if it also possesses the IL8-h1 haplotype. This finding highlights once again the importance of gaining further understanding of the role IL8 haplotypes play during infection.

The original hypothesis for this thesis stated that IL8 promoter haplotypes (IL8-h1 and IL8-h2) directly influences IL-8 responses both systemically and locally in primary dermal fibroblasts. Additionally, these divergent haplotypes influence cattle vitamin D status. The work presented here shows that genetic variation in the IL8 promoter region of Holstein-Friesian cattle results in systemic differences in circulating IL-8 and also vitamin D levels. However, using an *ex vivo* whole blood LPS stimulation with LPS, no differences were detected in the IL-8 response between haplotypes. The

exclusion of local cell types such as fibroblasts, epithelial and endothelial cells was possibly why this result didn't correlate with previous work describing a haplotype difference in IL-8 response during an *in vivo* LPS challenge [5]. Local IL-8 responses investigated using a dermal fibroblast model showed that IL8-h1 secreted more IL-8 in response to Pam3CSK4 compared to IL8-h2, however no haplotype difference was detected in response to LPS. The addition of vitamin D during the LPS stimulation of fibroblasts resulted in a haplotype difference in *VDR* expression where IL8-h1 showed higher expression compared to IL8-h2. This links to the systemic results showing higher circulating levels of vitamin D in IL8-h1 and lower levels of IL-8. In figures 6.2 and 6.3, the suspected mechanism by which this may be occurring is presented. The SNPs present in the IL8-h1 promoter region may allow VDR to bind to it more successfully as a transcription factor to reduce IL8 expression or it could also be interfering with other transcription factors such as NF-κB acting on the promoter region. This presents an interesting avenue for the relationship between vitamin D and IL8 haplotype to be investigated in the future.

Future studies could utilise an *in vivo* bacterial infection model such as *S. aureus* or *E. coli* in the mammary gland to monitor infection outcomes in cattle with distinct IL8 haplotypes. This would allow us to fully contextualise the impact of IL8 haplotype on cows with each haplotype by assessing systemic levels of IL-8 in serum in response to the infection. Additionally, local secretion of IL-8 could be assessed in milk along with numbers of infiltrating neutrophils. The use of scoring system could also assess inflammation in the udder and whether these scores would differ between cows with different IL8 haplotypes. This model would give us a major insight into how genetic variation in the IL8 promoter region could have significant downstream effects for infection susceptibility. Fundamentally, the work here has demonstrated that IL8 haplotype plays an important role in the expression of *IL8* and subsequent protein production at both systemic and local levels, in addition to potentially regulating the vitamin D status of cattle. Given the high frequency of these haplotypes, particularly in the Holstein-Friesian population, these insights are ultimately valuable to improve the future selection of cattle for improved disease resistance.

Appendix I

IL8 haplotype and CXCR1 haplotype impacts expression of *CXCR1* in genotyped Holstein-Frisian cows

As part of funding received during the course of my PhD studies, I had the opportunity to travel to the University of Tennessee to work with Dr Gina Pighetti who has published work on CXCR1 haplotypes in Holstein-Friesian. Her group have characterised these haplotypes using an experimental *in vivo* infection model of mastitis [7]. While there I designed a study to investigate the consequences for *IL8* and *CXCR1* gene expression when cow has both a specific CXCR1 haplotype and specific IL8 haplotype. The study design was as follows:

28 Holstein Friesian cows from the University of Tennessee dairy research herd were genotyped for their IL8 haplotype (IL8-h1, IL8-h2 and IL8-het) and CXCR1 haplotype (14 and 44) before blood samples were taken via tail venepuncture. All cows were age and parity matched where possible. They were all between 90-300 days in milk and had SCC less than 250,000.

4 mL of blood was stimulated with 5 μg/mL in a 37℃ shaking water bath for four hours after which blood was transferred to Tempus tubes for RNA extraction. RNA was extracted and converted to cDNA for gene expression analysis. Expression of *IL8* and *CXCR1* was analysed and Ct values were normalised to the reference gene *RPS15*.

A significant difference was observed in *CXCR1* expression for cows with CXCR1 haplotype 44 and IL8 haplotype IL8-h1 where expression was significantly lower for these cows in comparison to cows with CXCR1 haplotype 44 and IL8-h2 or IL8-het (Figure 7.1C&D). No significant differences were seen for *IL8* expression (Figure 7.1A&B).

However, for cows with CXCR1 haplotype 14 and any IL8 haplotype (IL8-h1, IL8-h2 and IL8-het) no significant differences were recorded for either *IL8* or *CXCR1* gene expression (Figure 7.2).

These results demonstrate the significance for *CXCR1* expression if a cow possess both the IL8 haplotype: IL8-h1 and the CXCR1 haplotype: 44. This CXCR1 haplotype was characterised during the *in vivo* infection previously described as showing moderate level of inflammation and bacterial burden [7]. However, if this cow also possessed the IL8-h1 it could possibly result in a different outcome potentially a less or more severe

infection. This study underscores the importance of knowing an animal's genotype in order to fully understand phenotypes seen during infection.

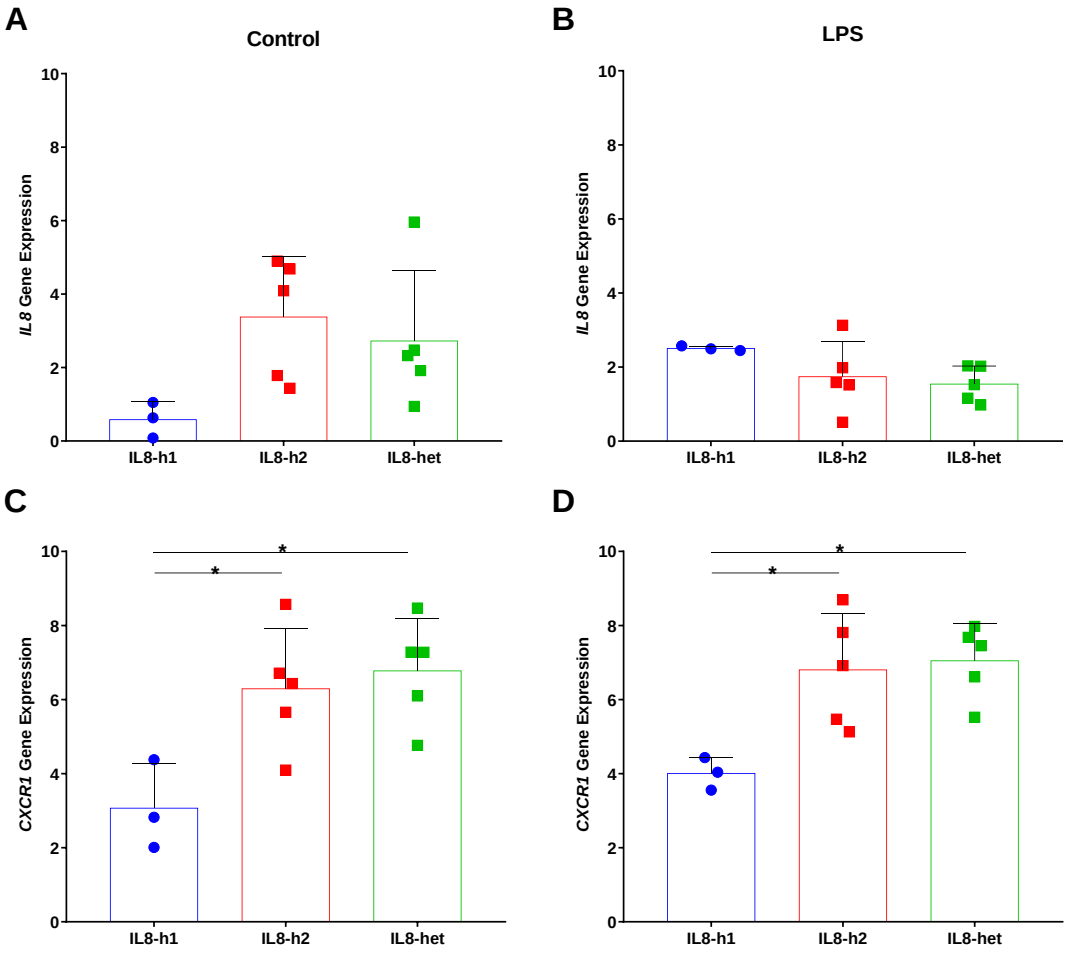

Figure 1 Gene expression of *IL8* and *CXCR1* from whole blood of Holstein-Friesian cows with CXCR haplotype 44 and either IL8-h1, IL8-h2 or IL8-het stimulated with LPS. Whole blood taken from IL8 and CXCR1 genotyped cows was stimulated with 5 µg/mL of LPS for 4 hr in a 37°C shaking water bath. Expression of *IL8* (A&B) and *CXCR1* (C&D) for unstimulated control and LPS was assessed and data is presented as normalised to the unstimulated control. Error bars represent the SEM of n=3-5. Significance is between haplotypes where *P<0.05.

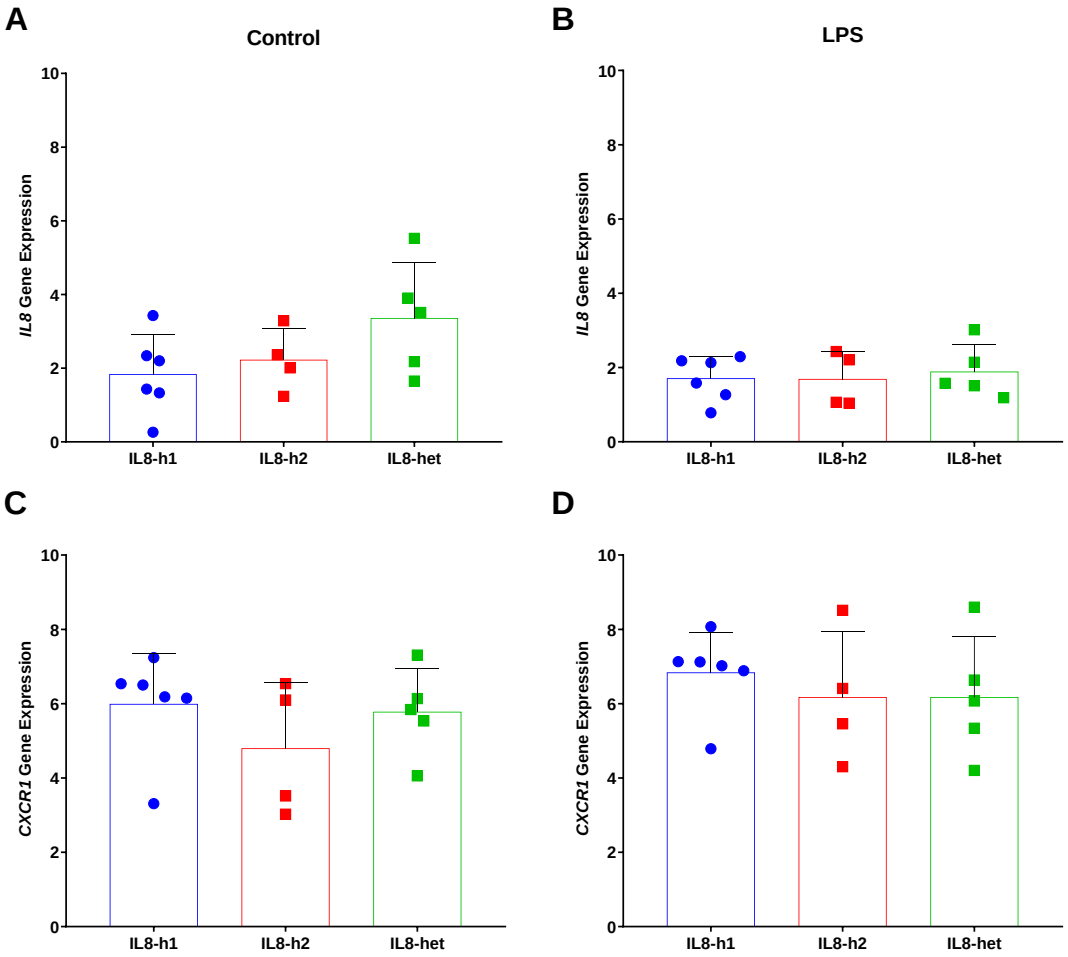

Figure 2 Gene expression of *IL8* and *CXCR1* from whole blood of Holstein-Friesian cows with CXCR haplotype 14 and either IL8-h1, IL8-h2 or IL8-het stimulated with LPS. Whole blood taken from IL8 and CXCR1 genotyped cows was stimulated with 5 µg/mL of LPS for 4 hr in a 37°C shaking water bath. Expression of *IL8* (A&B) and *CXCR1* (C&D) for unstimulated control and LPS was assessed and data is presented as normalised to the unstimulated control. Error bars represent the SEM of n=4-6.

References

1. Miller, M.C. and K.H. Mayo, *Chemokines from a Structural Perspective.* Int J Mol Sci, 2017. **18**(10).
2. Bezzerri, V., et al., *Mapping the transcriptional machinery of the IL-8 gene in human bronchial epithelial cells.* J Immunol, 2011. **187**(11): p. 6069-81.
3. Bhaumik, D., et al., *MicroRNAs miR-146a/b negatively modulate the senescence-associated inflammatory mediators IL-6 and IL-8.* Aging, 2009. **1**(4): p. 402-411.
4. Pigossi, S.C., et al., *Functionality of the Interleukin 8 haplotypes in lymphocytes and macrophages in response to gram-negative periodontopathogens.* Gene, 2019. **689**: p. 152-160.
5. Stojkovic, B., R.M. McLoughlin, and K.G. Meade, *In vivo relevance of polymorphic Interleukin 8 promoter haplotype for the systemic immune response to LPS in Holstein-Friesian calves.* Vet Immunol Immunopathol, 2016. **182**: p. 1-10.
6. Moepps, B., *CXCR1 and CXCR2 and Ligands.* 2015: p. 1-10.
7. Siebert, L., et al., *Genetic variation in CXCR1 haplotypes linked to severity of Streptococcus uberis infection in an experimental challenge model.* Vet Immunol Immunopathol, 2017. **190**: p. 45-52.
8. Beutler, B., *Innate immunity: an overview.* Molecular Immunology, 2004. **40**(12): p. 845-859.
9. Werling, D., J. Piercy, and T.J. Coffey, *Expression of TOLL-like receptors (TLR) by bovine antigen-presenting cells—Potential role in pathogen discrimination?* Veterinary Immunology and Immunopathology, 2006. **112**(1): p. 2-11.
10. Cronin, J.G., et al., *Toll-Like Receptor 4 and MYD88-Dependent Signaling Mechanisms of the Innate Immune System Are Essential for the Response to Lipopolysaccharide by Epithelial and Stromal Cells of the Bovine Endometrium.* Biology of Reproduction, 2011. **86**(2): p. Article 51, 1-9.
11. A. Cates, E., et al., *Functional characterization of bovine TIRAP and MyD88 in mediating bacterial lipopolysaccharide-induced endothelial NF-κB activation and apoptosis*. Vol. 32. 2009. 477-490.
12. Jungi, T.W., et al., *Toll-like receptors in domestic animals.* Cell and Tissue Research, 2011. **343**(1): p. 107-120.
13. Carneiro, L.C., et al., *Coordinated Role of Toll-Like Receptor-3 and Retinoic Acid-Inducible Gene-I in the Innate Response of Bovine Endometrial Cells to Virus.* Front Immunol, 2017. **8**: p. 996.
14. Van Gorp, H., et al., *NOD-like receptors interfacing the immune and reproductive systems.* FEBS J, 2014. **281**(20): p. 4568-82.
15. Tan, X., et al., *Down-regulation of NOD1 in neutrophils of periparturient dairy cows.* Vet Immunol Immunopathol, 2012. **150**(1-2): p. 133-9.
16. Thacker, J.D., et al., *NLRP3 inflammasome is a target for development of broad-spectrum anti-infective drugs.* Antimicrob Agents Chemother, 2012. **56**(4): p. 1921-30.
17. Doull, L., et al., *Late production of CXCL8 in ruminant oro-nasal turbinate cells in response to Chlamydia abortus infection.* Vet Immunol Immunopathol, 2015. **168**(1-2): p. 97-102.
18. Loo, Y.-M. and M. Gale, *Immune signaling by RIG-I-like receptors.* Immunity, 2011. **34**(5): p. 680-692.
19. Chauveau, E., et al., *Sensing and Control of Bluetongue Virus Infection in Epithelial Cells via RIG-I and MDA5 Helicases.* Journal of Virology, 2012. **86**(21): p. 11789.

20. Lawrence, T., *The nuclear factor NF-kappaB pathway in inflammation.* Cold Spring Harb Perspect Biol, 2009. **1**(6): p. a001651.
21. Ho, S.C., et al., *Noncanonical NF-kappaB mediates the Suppressive Effect of Neutrophil Elastase on IL-8/CXCL8 by Inducing NKRF in Human Airway Smooth Muscle.* Sci Rep, 2017. **7**: p. 44930.
22. Hoffmann, E., et al., *Multiple control of interleukin-8 gene expression.* J Leukoc Biol, 2002. **72**(5): p. 847-55.
23. Whitmarsh, A.J. and R.J. Davis, *Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways.* J Mol Med (Berl), 1996. **74**(10): p. 589-607.
24. Winzen, R., et al., *The p38 MAP kinase pathway signals for cytokine-induced mRNA stabilization via MAP kinase-activated protein kinase 2 and an AU-rich region-targeted mechanism.* EMBO J, 1999. **18**(18): p. 4969-80.
25. Matsusaka, T., et al., *Transcription factors NF-IL6 and NF-kappa B synergistically activate transcription of the inflammatory cytokines, interleukin 6 and interleukin 8.* Proc Natl Acad Sci U S A, 1993. **90**(21): p. 10193-7.
26. Nourbakhsh, M., et al., *The NF-kappa b repressing factor is involved in basal repression and interleukin (IL)-1-induced activation of IL-8 transcription by binding to a conserved NF-kappa b-flanking sequence element.* J Biol Chem, 2001. **276**(6): p. 4501-8.
27. Ho, S.C., et al., *Neutrophil elastase represses IL-8/CXCL8 synthesis in human airway smooth muscle cells through induction of NF-kappa B repressing factor.* J Immunol, 2009. **183**(1): p. 411-20.
28. Kim, S.H., et al., *Repression of TNF-alpha-induced IL-8 expression by the glucocorticoid receptor-beta involves inhibition of histone H4 acetylation.* Exp Mol Med, 2009. **41**(5): p. 297-306.
29. Takahashi, A., et al., *Epigenetic regulation of interleukin-8, an inflammatory chemokine, in osteoarthritis.* Osteoarthritis Cartilage, 2015. **23**(11): p. 1946-54.
30. Fabbri, E., et al., *Expression of microRNA-93 and Interleukin-8 during Pseudomonas aeruginosa-mediated induction of proinflammatory responses.* Am J Respir Cell Mol Biol, 2014. **50**(6): p. 1144-55.
31. Galicia, J.C., et al., *MiRNA-181a regulates Toll-like receptor agonist-induced inflammatory response in human fibroblasts.* Genes Immun, 2014. **15**(5): p. 333-7.
32. Gabriel, S.B., et al., *The Structure of Haplotype Blocks in the Human Genome.* Science, 2002. **296**(5576): p. 2225.
33. Pigossi, S.C., et al., *The ATC/TTC haplotype in the Interleukin 8 gene in response to Gram-negative bacteria: A pilot study.* Arch Oral Biol, 2019. **107**: p. 104508.
34. Hacking, D., et al., *Increased in vivo transcription of an IL-8 haplotype associated with respiratory syncytial virus disease-susceptibility.* Genes Immun, 2004. **5**(4): p. 274-82.
35. Meade, K.G., et al., *Functional characterisation of bovine interleukin 8 promoter haplotypes in vitro.* Mol Immunol, 2012. **50**(1-2): p. 108-16.
36. Stojkovic, B., et al., *Interleukin 8 haplotypes drive divergent responses in uterine endometrial cells and are associated with somatic cell score in Holstein-Friesian cattle.* Vet Immunol Immunopathol, 2017. **184**: p. 18-28.
37. Stillie, R., et al., *The functional significance behind expressing two IL-8 receptor types on PMN.* J Leukoc Biol, 2009. **86**(3): p. 529-43.

38. Richardson, R.M., et al., *Role of the cytoplasmic tails of CXCR1 and CXCR2 in mediating leukocyte migration, activation, and regulation.* J Immunol, 2003. **170**(6): p. 2904-11.
39. Bachelerie, F., et al., *International Union of Basic and Clinical Pharmacology. [corrected]. LXXXIX. Update on the extended family of chemokine receptors and introducing a new nomenclature for atypical chemokine receptors.* Pharmacol Rev, 2014. **66**(1): p. 1-79.
40. Kulkarni, N., M. Pathak, and G. Lal, *Role of chemokine receptors and intestinal epithelial cells in the mucosal inflammation and tolerance.* J Leukoc Biol, 2017. **101**(2): p. 377-394.
41. Schulz, C., et al., *Bronchoepithelial expression of CXCR1 and CXCR2 does not facilitate transepithelial migration of neutrophils.* Respiration, 2012. **84**(2): p. 108-16.
42. Mao, Y.J., et al., *Methylation analysis of CXCR1 in mammary gland tissue of cows with mastitis induced by Staphylococcus aureus.* Genet Mol Res, 2015. **14**(4): p. 12606-15.
43. Rambeaud, M. and G.M. Pighetti, *Impaired neutrophil migration associated with specific bovine CXCR2 genotypes.* Infect Immun, 2005. **73**(8): p. 4955-9.
44. Rambeaud, M., R. Clift, and G.M. Pighetti, *Association of a bovine CXCR2 gene polymorphism with neutrophil survival and killing ability.* Vet Immunol Immunopathol, 2006. **111**(3-4): p. 231-8.
45. Barrington, G.M. and S.M. Parish, *Bovine Neonatal Immunology.* Veterinary Clinics of North America: Food Animal Practice, 2001. **17**(3): p. 463-476.
46. Levy, O., *Innate immunity of the newborn: basic mechanisms and clinical correlates.* Nat Rev Immunol, 2007. **7**(5): p. 379-90.
47. Brewer, A., et al., *Qualitative and quantitative differences in endometrial inflammatory gene expression precede the development of bovine uterine disease.* Sci Rep, 2020. **10**(1): p. 18275.
48. Bannerman, D.D., et al., *The bovine innate immune response during experimentally-induced Pseudomonas aeruginosa mastitis.* Veterinary Immunology and Immunopathology, 2005. **107**(3): p. 201-215.
49. Ellis, J.A., et al., *Duration of immunity to experimental infection with bovine respiratory syncytial virus following intranasal vaccination of young passively immune calves.* J Am Vet Med Assoc, 2013. **243**(11): p. 1602-8.
50. Chase, C.C., D.J. Hurley, and A.J. Reber, *Neonatal immune development in the calf and its impact on vaccine response.* Vet Clin North Am Food Anim Pract, 2008. **24**(1): p. 87-104.
51. Hulbert, L.E. and S.J. Moisa, *Stress, immunity, and the management of calves.* J Dairy Sci, 2016. **99**(4): p. 3199-3216.
52. Lorenz, I., et al., *Calf health from birth to weaning. I. General aspects of disease prevention.* Irish Veterinary Journal, 2011. **64**(1): p. 10.
53. Mayer, B., et al., *Expression of the neonatal Fc receptor (FcRn) in the bovine mammary gland*. Vol. 72 Spec No. 2005. 107-12.
54. Stelwagen, K., et al., *Immune components of bovine colostrum and milk.* J Anim Sci, 2009. **87**(13 Suppl): p. 3-9.
55. Knowles, T.G., et al., *Changes in the blood biochemical and haematological profile of neonatal calves with age.* Veterinary Record, 2000. **147**(21): p. 593-598.
56. Gasteiger, G., et al., *Cellular Innate Immunity: An Old Game with New Players.* Journal of Innate Immunity, 2017. **9**(2): p. 111-125.

57. Shamri, R., J.J. Xenakis, and L.A. Spencer, *Eosinophils in innate immunity: an evolving story.* Cell and tissue research, 2011. **343**(1): p. 57-83.
58. Basten, A., M.H. Boyer, and P.B. Beeson, *MECHANISM OF EOSINOPHILIA : I. FACTORS AFFECTING THE EOSINOPHIL RESPONSE OF RATS TO TRICHINELLA SPIRALIS.* The Journal of Experimental Medicine, 1970. **131**(6): p. 1271-1287.
59. Tanimoto, T., et al., *Eosinophilic Proliferative Pylephlebitis in the Liver of Japanese Beef Cattle with Fascioliasis.* Journal of Veterinary Medical Science, 1998. **60**(10): p. 1073-1080.
60. Min, B., G.L. Gros, and W.E. Paul, *Basophils: A Potential Liaison between Innate and Adaptive Immunity.* Allergology International, 2006. **55**(2): p. 99-104.
61. Brown, S.J., R.W. Barker, and P.W. Askenase, *Bovine resistance to Amblyomma americanum ticks: an acquired immune response characterized by cutaneous basophil infiltrates.* Vet Parasitol, 1984. **16**(1-2): p. 147-65.
62. Widdison, S., M. Watson, and T.J. Coffey, *Early response of bovine alveolar macrophages to infection with live and heat-killed Mycobacterium bovis.* Developmental & Comparative Immunology, 2011. **35**(5): p. 580-591.
63. Hussen, J., et al., *Phenotypic and Functional Heterogeneity of Bovine Blood Monocytes.* PLoS ONE, 2013. **8**(8): p. e71502.
64. Batista, C.F., et al., *Evolution of phagocytic function in monocytes and neutrophils blood cells of healthy calves.* J Dairy Sci, 2015. **98**(12): p. 8882-8.
65. Davis, W.C., et al., *Analysis of monoclonal antibodies specific for the γδ TcR.* Veterinary Immunology and Immunopathology, 1996. **52**(4): p. 275-283.
66. Pang, D.J., et al., *Understanding the complexity of γδ T-cell subsets in mouse and human.* Immunology, 2012. **136**(3): p. 283-290.
67. Hsu, H., et al., *WC1 is a hybrid gammadelta TCR coreceptor and pattern recognition receptor for pathogenic bacteria.* J Immunol, 2015. **194**(5): p. 2280-8.
68. Nowarski, R., R. Jackson, and R.A. Flavell, *The Stromal Intervention: Regulation of Immunity and Inflammation at the Epithelial-Mesenchymal Barrier.* Cell, 2017. **168**(3): p. 362-375.
69. Kelly, P., et al., *Improved filtration method to isolate pure populations of primary bovine endometrial epithelial and stromal cells for immunological studies.* Vet Res Commun, 2020. **44**(1): p. 29-39.
70. Brouty-Boyé, D., et al., *Chemokines and CD40 expression in human fibroblasts.* Eur J Immunol, 2000. **30**(3): p. 914-9.
71. Rolfe, M.W., et al., *Pulmonary fibroblast expression of interleukin-8: a model for alveolar macrophage-derived cytokine networking.* Am J Respir Cell Mol Biol, 1991. **5**(5): p. 493-501.
72. Mul, F.P., et al., *Sequential migration of neutrophils across monolayers of endothelial and epithelial cells.* J Leukoc Biol, 2000. **68**(4): p. 529-37.
73. McClenahan, D., et al., *Interleukin-8 expression by mammary gland endothelial and epithelial cells following experimental mastitis infection with E. coli.* Comp Immunol Microbiol Infect Dis, 2006. **29**(2-3): p. 127-37.
74. Chanrot, M., et al., *Bovine herpes virus type 4 alters TNF-alpha and IL-8 profiles and impairs the survival of bovine endometrial epithelial cells.* Reprod Biol, 2017. **17**(3): p. 225-232.
75. Green, B.B., et al., *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, 2011. **94**(11): p. 5502-14.

76. Kandasamy, S., et al., *Between-cow variation in dermal fibroblast response to lipopolysaccharide reflected in resolution of inflammation during Escherichia coli mastitis.* J Dairy Sci, 2011. **94**(12): p. 5963-75.
77. Donofrio, G., et al., *The chemokine IL8 is up-regulated in bovine endometrial stromal cells by the BoHV-4 IE2 gene product, ORF50/Rta: a step ahead toward a mechanism for BoHV-4 induced endometritis.* Biol Reprod, 2010. **83**(6): p. 919-28.
78. Murphy, K.T., P and Walport, M., *Janeway's Immunobiology*. 7th ed. 2008, New York and London: Garland Science.
79. Martinez, F.O., et al., *IL-8 induces a specific transcriptional profile in human neutrophils: synergism with LPS for IL-1 production.* Eur J Immunol, 2004. **34**(8): p. 2286-92.
80. Mócsai, A., *Diverse novel functions of neutrophils in immunity, inflammation, and beyond.* The Journal of Experimental Medicine, 2013. **210**(7): p. 1283.
81. Kobayashi, S.D., et al., *Global changes in gene expression by human polymorphonuclear leukocytes during receptor-mediated phagocytosis: Cell fate is regulated at the level of gene expression.* Proceedings of the National Academy of Sciences, 2002. **99**(10): p. 6901.
82. Mitchell, G.B., B.N. Albright, and J.L. Caswell, *Effect of interleukin-8 and granulocyte colony-stimulating factor on priming and activation of bovine neutrophils.* Infect Immun, 2003. **71**(4): p. 1643-9.
83. Menge, C., et al., *Compensation of preliminary blood phagocyte immaturity in the newborn calf.* Veterinary Immunology and Immunopathology, 1998. **62**(4): p. 309-321.
84. Kampen, A.H., et al., *Lymphocyte subpopulations and neutrophil function in calves during the first 6 months of life.* Vet Immunol Immunopathol, 2006. **113**(1-2): p. 53-63.
85. Hulbert, L.E., et al., *The effects of early weaning on innate immune responses of Holstein calves1.* Journal of Dairy Science, 2011. **94**(5): p. 2545-2556.
86. Chen, Y. and W.G. Junger, *Measurement of oxidative burst in neutrophils.* Methods Mol Biol, 2012. **844**: p. 115-24.
87. Bassel, L.L. and J.L. Caswell, *Bovine neutrophils in health and disease.* Cell and Tissue Research, 2018. **371**(3): p. 617-637.
88. Prin-Mathieu, C., et al., *Enzymatic Activities of Bovine Peripheral Blood Leukocytes and Milk Polymorphonuclear Neutrophils during Intramammary Inflammation Caused by Lipopolysaccharide.* Clinical and Diagnostic Laboratory Immunology, 2002. **9**(4): p. 812.
89. Brinkmann, V., et al., *Neutrophil Extracellular Traps Kill Bacteria.* Science, 2004. **303**(5663): p. 1532.
90. Fuchs, T.A., et al., *Novel cell death program leads to neutrophil extracellular traps.* J Cell Biol, 2007. **176**(2): p. 231-41.
91. Yousefi, S., et al., *Viable neutrophils release mitochondrial DNA to form neutrophil extracellular traps.* Cell Death Differ, 2009. **16**(11): p. 1438-44.
92. Yousefi, S. and H.U. Simon, *NETosis - Does It Really Represent Nature's "Suicide Bomber"?* Front Immunol, 2016. **7**: p. 328.
93. Yost, C.C., et al., *Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates.* Blood, 2009. **113**(25): p. 6419-27.
94. Okubo, K., et al., *Lactoferrin Suppresses Neutrophil Extracellular Traps Release in Inflammation.* EBioMedicine, 2016. **10**: p. 204-215.

95. Villagra-Blanco, R., et al., *Bovine Polymorphonuclear Neutrophils Cast Neutrophil Extracellular Traps against the Abortive Parasite Neospora caninum.* Front Immunol, 2017. **8**: p. 606.
96. Grinberg, N., et al., *β-Hydroxybutyrate Abrogates Formation of Bovine Neutrophil Extracellular Traps and Bactericidal Activity against Mammary Pathogenic Escherichia coli.* Infection and Immunity, 2008. **76**(6): p. 2802-2807.
97. Gondaira, S., et al., *Mycoplasma bovis escapes bovine neutrophil extracellular traps.* Veterinary Microbiology, 2017. **199**: p. 68-73.
98. Branzk, N., et al., *Neutrophils sense microbe size and selectively release neutrophil extracellular traps in response to large pathogens.* Nature Immunology, 2014. **15**: p. 1017.
99. Deniset, J.F. and P. Kubes, *Neutrophil heterogeneity: Bona fide subsets or polarization states?* J Leukoc Biol, 2018. **103**(5): p. 829-838.
100. McKenna, E., et al., *Neutrophils: Need for Standardized Nomenclature.* Front Immunol, 2021. **12**: p. 602963.
101. Bongers, S.H., et al., *Kinetics of Neutrophil Subsets in Acute, Subacute, and Chronic Inflammation.* Front Immunol, 2021. **12**: p. 674079.
102. Burn, G.L., et al., *The Neutrophil.* Immunity, 2021. **54**(7): p. 1377-1391.
103. Rambault, M., et al., *Neutrophils Encompass a Regulatory Subset Suppressing T Cells in Apparently Healthy Cattle and Mice.* Front Immunol, 2021. **12**: p. 625244.
104. Clancy, N., et al., *Vitamin D and neonatal immune function.* J Matern Fetal Neonatal Med, 2013. **26**(7): p. 639-46.
105. Gois, P.H.F., et al., *Vitamin D and Infectious Diseases: Simple Bystander or Contributing Factor?* Nutrients, 2017. **9**(7).
106. Webb, A.R., B.R. DeCosta, and M.F. Holick, *Sunlight regulates the cutaneous production of vitamin D3 by causing its photodegradation.* J Clin Endocrinol Metab, 1989. **68**(5): p. 882-7.
107. Nair, R. and A. Maseeh, *Vitamin D: The "sunshine" vitamin.* Journal of pharmacology & pharmacotherapeutics, 2012. **3**(2): p. 118-126.
108. Houghton, L.A. and R. Vieth, *The case against ergocalciferol (vitamin D2) as a vitamin supplement.* The American Journal of Clinical Nutrition, 2006. **84**(4): p. 694-697.
109. Chun, R.F., et al., *Vitamin D-binding protein directs monocyte responses to 25-hydroxy- and 1,25-dihydroxyvitamin D.* J Clin Endocrinol Metab, 2010. **95**(7): p. 3368-76.
110. Zhu, J.G., et al., *CYP2R1 is a major, but not exclusive, contributor to 25-hydroxyvitamin D production in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 2013. **110**(39): p. 15650-15655.
111. White, J.H., *Vitamin D metabolism and signaling in the immune system.* Rev Endocr Metab Disord, 2012. **13**(1): p. 21-9.
112. Herrmann, M., et al., *Assessment of Vitamin D status - A changing landscape*. Vol. 55. 2016.
113. Carretta, M.D., et al., *Butyric acid stimulates bovine neutrophil functions and potentiates the effect of platelet activating factor.* Vet Immunol Immunopathol, 2016. **176**: p. 18-27.
114. Schleicher, R.L., et al., *The vitamin D status of the US population from 1988 to 2010 using standardized serum concentrations of 25-hydroxyvitamin D shows recent modest increases*. The American Journal of Clinical Nutrition, 2016. **104**(2): p. 454-461.

115. Nelson, C.D., et al., *Vitamin D status of dairy cattle: Outcomes of current practices in the dairy industry.* J Dairy Sci, 2016. **99**(12): p. 10150-10160.
116. Nelson, C.D., et al., *Vitamin D signaling in the bovine immune system: a model for understanding human vitamin D requirements.* Nutrients, 2012. **4**(3): p. 181-96.
117. Jakobsen, J. and E. Saxholt, *Vitamin D metabolites in bovine milk and butter.* Journal of Food Composition and Analysis, 2009. **22**(5): p. 472-478.
118. Nelson, C.D., et al., *Modulation of the bovine innate immune response by production of 1alpha,25-dihydroxyvitamin D(3) in bovine monocytes.* J Dairy Sci, 2010. **93**(3): p. 1041-9.
119. Chen, L., M.S. Eapen, and G.R. Zosky, *Vitamin D both facilitates and attenuates the cellular response to lipopolysaccharide.* Sci Rep, 2017. **7**: p. 45172.
120. Hewison, M., *An update on vitamin D and human immunity.* Clin Endocrinol (Oxf), 2012. **76**(3): p. 315-25.
121. Telcian, A.G., et al., *Vitamin D increases the antiviral activity of bronchial epithelial cells in vitro.* Antiviral Res, 2017. **137**: p. 93-101.
122. Chin-Smith, E.C., N.L. Hezelgrave, and R.M. Tribe, *Host Defense Peptide Expression in Human Cervical Cells and Regulation by 1,25-Dihydroxyvitamin D3 in the Presence of Cytokines and Bacterial Endotoxin.* Reprod Sci, 2017: p. 1933719117737847.
123. Vimaleswaran, K.S., N.G. Forouhi, and K. Khunti, *Vitamin D and covid-19.* BMJ, 2021. **372**: p. n544.
124. The Lancet, D. and Endocrinology, *Vitamin D and COVID-19: why the controversy?* The Lancet Diabetes & Endocrinology, 2021. **9**(2).
125. Giustina, A., et al., *Controversies in Vitamin D: A Statement From the Third International Conference.* JBMR Plus, 2020. **4**(12): p. e10417.
126. Ryynanen, J. and C. Carlberg, *Primary 1,25-dihydroxyvitamin D3 response of the interleukin 8 gene cluster in human monocyte- and macrophage-like cells.* PLoS One, 2013. **8**(10): p. e78170.
127. Dauletbaev, N., et al., *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, 2015. **172**(19): p. 4757-71.
128. Chen, Y., et al., *Vitamin D receptor inhibits nuclear factor kappaB activation by interacting with IkappaB kinase beta protein.* J Biol Chem, 2013. **288**(27): p. 19450-8.
129. Harant, H., et al., *1alpha,25-dihydroxyvitamin D3 and a variety of its natural metabolites transcriptionally repress nuclear-factor-kappaB-mediated interleukin-8 gene expression.* Eur J Biochem, 1997. **250**(1): p. 63-71.
130. Sun, J., et al., *Increased NF-kappaB activity in fibroblasts lacking the vitamin D receptor.* Am J Physiol Endocrinol Metab, 2006. **291**(2): p. E315-22.
131. Cronin, J.G., et al., *Enzyme linked immunosorbent assay for quantification of bovine interleukin-8 to study infection and immunity in the female genital tract.* Am J Reprod Immunol, 2015. **73**(4): p. 372-82.
132. Nelson, C.D., et al., *Assessment of serum 25-hydroxyvitamin D concentrations of beef cows and calves across seasons and geographical locations1.* Journal of Animal Science, 2016. **94**(9): p. 3958-3965.
133. Benakanakere, M.R., et al., *Investigation of the functional role of human Interleukin-8 gene haplotypes by CRISPR/Cas9 mediated genome editing.* Sci Rep, 2016. **6**: p. 31180.

134. Baccili, C.C., et al., *Hematological and immunological development from birth to six months of age in Holstein calves.* Arquivo Brasileiro de Medicina Veterinária e Zootecnia, 2018. **70**: p. 1823-1832.
135. Price, S.J., et al., *Workshop cluster 1+ gammadelta T-cell receptor T cells from calves express high levels of interferon-gamma in response to stimulation with interleukin-12 and -18.* Immunology, 2007. **120**(1): p. 57-65.
136. Sims-Mourtada, J.C., et al., *In vivo expression of interleukin-8, and regulated on activation, normal, T-cell expressed, and secreted, by human germinal centre B lymphocytes.* Immunology, 2003. **110**(3): p. 296-303.
137. Dentener, M.A., et al., *Involvement of CD14 in lipopolysaccharide-induced tumor necrosis factor-alpha, IL-6 and IL-8 release by human monocytes and alveolar macrophages.* J Immunol, 1993. **150**(7): p. 2885-91.
138. Baggiolini, M., A. Walz, and S.L. Kunkel, *Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils.* J Clin Invest, 1989. **84**(4): p. 1045-9.
139. Lafleur, R.L., M.S. Abrahamsen, and S.K. Maheswaran, *The biphasic mRNA expression pattern of bovine interleukin-8 in Pasteurella haemolytica lipopolysaccharide-stimulated alveolar macrophages is primarily due to tumor necrosis factor alpha.* Infect Immun, 1998. **66**(9): p. 4087-92.
140. Yang, J., et al., *Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases.* Biomarker Research, 2014. **2**(1): p. 1.
141. Altstaedt, J., H. Kirchner, and L. Rink, *Cytokine production of neutrophils is limited to interleukin-8.* Immunology, 2011. **59**(4): p. 563-568.
142. Gesser, B., et al., *Interleukin-8 Induces Its Own Production in CD4+ T Lymphocytes: A Process Regulated by Interleukin 10.* Biochemical and Biophysical Research Communications, 1995. **210**(3): p. 660-669.
143. Caccamo, N., et al., *Differentiation, phenotype, and function of interleukin-17-producing human Vgamma9Vdelta2 T cells.* Blood, 2011. **118**(1): p. 129-38.
144. Pilsczek, F.H., et al., *A novel mechanism of rapid nuclear neutrophil extracellular trap formation in response to Staphylococcus aureus.* J Immunol, 2010. **185**(12): p. 7413-25.
145. Gerszten, R.E., et al., *MCP-1 and IL-8 trigger firm adhesion of monocytes to vascular endothelium under flow conditions.* Nature, 1999. **398**(6729): p. 718-23.
146. Krieger, M., et al., *Activation of human basophils through the IL-8 receptor.* J Immunol, 1992. **149**(8): p. 2662-7.
147. Erger, R.A. and T.B. Casale, *Interleukin-8 is a potent mediator of eosinophil chemotaxis through endothelium and epithelium.* Am J Physiol, 1995. **268**(1 Pt 1): p. L117-22.
148. Rogers, A.N., et al., *T Cell Function Varies with the Expressed WC1 Coreceptor.* The Journal of Immunology, 2005. **174**(6): p. 3386-3393.
149. Gasch, M., et al., *Generation of IL-8 and IL-9 producing CD4(+) T cells is affected by Th17 polarizing conditions and AHR ligands.* Mediators Inflamm, 2014. **2014**: p. 182549.
150. Li, L., et al., *Functional imaging of interleukin 1 beta expression in inflammatory process using bioluminescence imaging in transgenic mice.* BMC Immunology, 2008. **9**(1): p. 49.
151. Duffy, D., et al., *Functional analysis via standardized whole-blood stimulation systems defines the boundaries of a healthy immune response to complex stimuli.* Immunity, 2014. **40**(3): p. 436-50.

152. Antoniak, S. and N. Mackman, *Multiple roles of the coagulation protease cascade during virus infection.* Blood, 2014. **123**(17): p. 2605.
153. Migale, R., et al., *Specific Lipopolysaccharide Serotypes Induce Differential Maternal and Neonatal Inflammatory Responses in a Murine Model of Preterm Labor.* The American Journal of Pathology, 2015. **185**(9): p. 2390-2401.
154. Pisetsky, D.S. and A.-M. Fairhurst, *The origin of extracellular DNA during the clearance of dead and dying cells.* Autoimmunity, 2007. **40**(4): p. 281-284.
155. de Buhr, N., et al., *How Neutrophil Extracellular Traps Become Visible.* Journal of Immunology Research, 2016. **2016**: p. 13.
156. Ene, C.D., et al., *25-OH Vitamin D and Interleukin-8: Emerging Biomarkers in Cutaneous Melanoma Development and Progression.* Mediators Inflamm, 2015. **2015**: p. 904876.
157. Olsen, H.G., et al., *Fine mapping of a QTL on bovine chromosome 6 using imputed full sequence data suggests a key role for the group-specific component (GC) gene in clinical mastitis and milk production.* Genet Sel Evol, 2016. **48**(1): p. 79.
158. Annweiler, C., et al., *Vitamin D and survival in COVID-19 patients: A quasi-experimental study.* J Steroid Biochem Mol Biol, 2020. **204**: p. 105771.
159. Ohaegbulam, K.C., et al., *Vitamin D Supplementation in COVID-19 Patients: A Clinical Case Series.* American journal of therapeutics, 2020. **27**(5): p. e485-e490.
160. Wang, Z., et al., *Vitamin D improves immune function in immunosuppressant mice induced by glucocorticoid.* Biomed Rep, 2017. **6**(1): p. 120-124.
161. Olliver, M., et al., *Immunomodulatory Effects of Vitamin D on Innate and Adaptive Immune Responses to Streptococcus pneumoniae.* The Journal of Infectious Diseases, 2013. **208**(9): p. 1474-1481.
162. Mulligan, J.K., et al., *Dietary vitamin D3 deficiency exacerbates sinonasal inflammation and alters local 25(OH)D3 metabolism.* PLoS One, 2017. **12**(10): p. e0186374.
163. Laaksi, I., et al., *An association of serum vitamin D concentrations < 40 nmol/L with acute respiratory tract infection in young Finnish men.* The American Journal of Clinical Nutrition, 2007. **86**(3): p. 714-717.
164. Boylan, M., et al., *1,25(OH)D vitamin D promotes NOS2 expression in response to bacterial and viral PAMPs in primary bovine salivary gland fibroblasts.* Veterinary Research Communications, 2020. **44**(2): p. 83-88.
165. Whiston, R., et al., *A dual targeted beta-defensin and exome sequencing approach to identify, validate and functionally characterise genes associated with bull fertility.* Sci Rep, 2017. **7**(1): p. 12287.
166. Merriman, K.E., et al., *Multiple beta-defensin genes are upregulated by the vitamin D pathway in cattle.* J Steroid Biochem Mol Biol, 2015. **154**: p. 120-9.
167. Haynes, L.M., et al., *Involvement of Toll-Like Receptor 4 in Innate Immunity to Respiratory Syncytial Virus.* Journal of Virology, 2001. **75**(22): p. 10730.
168. Yousefzadeh, P., S.A. Shapses, and X. Wang, *Vitamin D Binding Protein Impact on 25-Hydroxyvitamin D Levels under Different Physiologic and Pathologic Conditions.* International Journal of Endocrinology, 2014. **2014**: p. 6.
169. Carpenter, T.O., et al., *Vitamin D binding protein is a key determinant of 25-hydroxyvitamin D levels in infants and toddlers.* J Bone Miner Res, 2013. **28**(1): p. 213-21.
170. Carlberg, C., *Vitamin D Signaling in the Context of Innate Immunity: Focus on Human Monocytes.* Front Immunol, 2019. **10**: p. 2211.

171. Wong, K.L., et al., *Gene expression profiling reveals the defining features of the classical, intermediate, and nonclassical human monocyte subsets.* Blood, 2011. **118**(5): p. e16-31.
172. Zhang, Z., et al., *An analysis of the association between the vitamin D pathway and serum 25-hydroxyvitamin D levels in a healthy Chinese population.* J Bone Miner Res, 2013. **28**(8): p. 1784-92.
173. Ahn, J., et al., *Vitamin D-related genes, serum vitamin D concentrations and prostate cancer risk.* Carcinogenesis, 2009. **30**(5): p. 769-76.
174. Yoon, B.N., et al., *Induction of interleukin-8 from nasal epithelial cells during bacterial infection: the role of IL-8 for neutrophil recruitment in chronic rhinosinusitis.* Mediators Inflamm, 2010. **2010**: p. 813610.
175. Davidson, S., et al., *Fibroblasts as immune regulators in infection, inflammation and cancer.* Nat Rev Immunol, 2021.
176. Green, B.B., S.D. McKay, and D.E. Kerr, *Age dependent changes in the LPS induced transcriptome of bovine dermal fibroblasts occurs without major changes in the methylome.* BMC Genomics, 2015. **16**: p. 30.
177. Murata, E., et al., *Differential expression of serum amyloid A1 and A3 in bovine epithelia.* J Vet Med Sci, 2020. **82**(6): p. 764-770.
178. Hosokawa, Y., et al., *Increase of CCL20 expression by human gingival fibroblasts upon stimulation with cytokines and bacterial endotoxin.* Clin Exp Immunol, 2005. **142**(2): p. 285-91.
179. Hosokawa, Y., et al., *TLR3 agonist enhances CC chemokine ligand 20 production in IL-1beta-stimulated human gingival fibroblasts.* Cell Immunol, 2013. **283**(1-2): p. 8-11.
180. Walch-Rückheim, B., et al., *Stromal Fibroblasts Induce CCL20 through IL6/C/EBPβ to Support the Recruitment of Th17 Cells during Cervical Cancer Progression.* Cancer research, 2015. **75**(24): p. 5248-5259.
181. Buskermolen, J.K., S. Roffel, and S. Gibbs, *Stimulation of oral fibroblast chemokine receptors identifies CCR3 and CCR4 as potential wound healing targets.* J Cell Physiol, 2017. **232**(11): p. 2996-3005.
182. Rostkowska-Nadolska, B., et al., *Vitamin D derivatives: calcitriol and tacalcitol inhibits interleukin-6 and interleukin-8 expression in human nasal polyp fibroblast cultures.* Advances in Medical Sciences, 2010. **55**(1): p. 86-92.
183. Nakashyan, V., et al., *Effect of 1,25(OH)2 D3 and 20(OH)D3 on interleukin-1beta-stimulated interleukin-6 and -8 production by human gingival fibroblasts.* J Periodontal Res, 2017. **52**(5): p. 832-841.
184. Kelly, P., K.G. Meade, and C. O'Farrelly, *Non-canonical Inflammasome-Mediated IL-1beta Production by Primary Endometrial Epithelial and Stromal Fibroblast Cells Is NLRP3 and Caspase-4 Dependent.* Front Immunol, 2019. **10**: p. 102.
185. Yao, C., et al., *Toll-like receptor family members in skin fibroblasts are functional and have a higher expression compared to skin keratinocytes.* Int J Mol Med, 2015. **35**(5): p. 1443-50.
186. Pegu, A., et al., *Human lymphatic endothelial cells express multiple functional TLRs.* J Immunol, 2008. **180**(5): p. 3399-405.
187. Skovdahl, H.K., et al., *Expression of CCL20 and Its Corresponding Receptor CCR6 Is Enhanced in Active Inflammatory Bowel Disease, and TLR3 Mediates CCL20 Expression in Colonic Epithelial Cells.* PLoS One, 2015. **10**(11): p. e0141710.

188. Liston, A., E.J. Carr, and M.A. Linterman, *Shaping Variation in the Human Immune System.* Trends in Immunology, 2016. **37**(10): p. 637-646.
189. Galligan, C.L. and B.L. Coomber, *Effects of human IL-8 isoforms on bovine neutrophil function in vitro.* Vet Immunol Immunopathol, 2000. **74**(1-2): p. 71-85.
190. Nissen, J., et al., *Common variants in CYP2R1 and GC genes predict vitamin D concentrations in healthy Danish children and adults.* PLoS One, 2014. **9**(2): p. e89907.
191. van den Bemd, G.J., et al., *A central dinucleotide within vitamin D response elements modulates DNA binding and transactivation by the vitamin D receptor in cellular response to natural and synthetic ligands.* J Biol Chem, 2002. **277**(17): p. 14539-46.