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Structural and Functional Characterisation of Interleukin 8 Haplotypes in Cattle

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Abbreviations

ACTB	Actin β
ANOVA	Analysis of variance
AP1	Activating protein 1
BEND	Bovine endometrial epithelial cells
Bp	Base pairs
C/EBP	CAAT/Enhancing binding protein
C3	Complement 3
CD14	Cluster of differentiation 14
CD3	Cluster of differentiation 3
CR1 and CR2	Complement receptor 1 and 2
Ct	Cycle threshold
CXCR1	CXC receptor 1
CXCR4	CXC receptor 4
dNTP	Deoxyribonucleotide triphosphate
dUTP	Deoxyuridine triphosphate
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal-regulated kinase
ESL-1	E-selectin ligand
Fcγ receptor	Fc (Fragment, crystallisable) γ receptor
FITC	Fluorescein isothiocyanate
FAO	Food and Agriculture Organization of the United Nations
GAPDH	Glyceraldehyde3-phosphatedehydrogenase
GCP-2	Granulocyte chemotactic protein 2
G-CSF	Granulocyte-colony stimulating factor
H3F3A	H3-histone family 3A
HF	Holstein-Friesian
ICAM-1	Intercellular adhesion molecule 1
IFNγ	Interferon γ

IL17	Interleukin 17
IL23	Interleukin 23
IL8	Interleukin 8
IκB	Inhibitor of κB
JNK	JUN-N-terminal protein kinase
Kb	Kilobase
LFA-1	Limphocyte function-associated antigen 1
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
MAC-1	Macrophage-1 antigen
MAPK	Mitogen-activated protein kinase
MDM	Monocyte-derived macrophage
MKK	MAPK kinase
MRPS6	Mitochondrial ribosomal protein S6
NFκB	Nuclear factor κB
nm	Nanometer
NRF	NFκB repressing factor
OCT-1	Octamer-binding protein
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
Pen/Strep	Penicillin/Streptomycin
PI	Propidium iodide
PPIA	Peptidylprolyl isomerase A
PSGL-1	P-selectin glycoprotein ligand 1
RT-QPCR	Quantitative Real Time PCR
RIN	RNA Integrity Number
SCC	Somatic cell count
SNP	Single nucleotide polymorphism
TLR	Toll-like receptor
TNFR1	TNF receptor 1
TNFSF1A and TNFSF1B	TNF ligand superfamily member 1A and 1B
TNFα	Tumor necrosis factor α
UCSC	University of California, Santa Cruz

UNG	Uracil-N-Glycocylase
uORF	Upstream open reading frame
VCAM1	Vascular cell adhesion molecule 1
VLA-4	Very late antigen 4

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Abstract

Accumulation of leukocytes in the tissue is one of the hallmarks of inflammation. In bovine mastitis and uterine disease neutrophils are rapidly recruited to either the mammary gland or the uterus. This leads to an increase in the number of somatic cells in milk as well as uterine tissue damage and infertility problems, both of which constitute massive constraints to global agriculture. Interleukin 8 (IL8) is a chemokine specifically involved in neutrophil recruitment and activation of anti-microbial function. It is secreted by a range of cell types including monocytes, epithelial and endothelial cells, as well as neutrophils. IL8 plays a major role in the immunopathology of mastitis and uterine disease and genetic polymorphisms within *IL8* have been associated with susceptibility to a number of infectious diseases in both humans and cattle.

Previously, analysis by our group, of a 2.1 kilobase spanning the upstream region of the *IL8* gene transcription start site, uncovered 29 novel polymorphisms in the promoter region of *IL8*, which segregate into two distinct blocks, namely *IL8* haplotype 1 and *IL8* haplotype 2. The aim of this project was to characterize the population genetic variation present at the *IL8* gene, in a range of cattle breeds from both dairy and beef production systems, as well as to test an association between the *IL8* haplotype and both somatic cell count and calving interval, traits of great agricultural importance for the Irish dairy sector. A functional analysis was then performed in order to compare the ability of both *IL8* promoter haplotypes to drive IL8 expression, both *in vitro*, in bovine endometrial epithelial cells and monocyte-derived macrophages; and *in vivo*, in LPS challenged Holstein-Friesian (HF) calves. Finally, the role of IL8 dose and *IL8* haplotype on neutrophil recruitment and activation was investigated.

Population genetic analysis of the *IL8* haplotype distribution revealed significant differences in frequencies among breeds. *IL8* haplotype 1 was more prevalent in Shorthorn, whereas haplotype 2 was dominant in Charolais. The Holstein-Friesian and Brahman population showed balanced frequencies of both *IL8* haplotypes.

Highly significant associations between the *IL8* haplotype and both somatic cell count and calving interval were identified. The results suggest that artificial selection might have played a role in shaping the genetic structure of the bovine *IL8* gene.

Functional analysis of IL8 expression in endometrial epithelial cells showed significantly higher responsiveness of the *IL8* haplotype 2 promoter, to LPS and TNFα. Additionally, the critical regions in the bovine *IL8* promoter responsible for *IL8* regulation, as well as the number of potential transcription factor binding sites in both *IL8* haplotypes, were identified. Haplotype-specific IL8 expression was then confirmed in HF calves *in vivo*, demonstrating higher IL8 gene and protein expression in whole blood and serum, in animals of *IL8* haplotype 2. Interestingly, this haplotype was also associated with higher somatic cell count, suggesting that increased IL8 expression could be responsible for attracting more neutrophils to the mammary gland. This hypothesis was tested *in vitro*, using different doses of recombinant IL8 to drive neutrophil chemotaxis and a dose dependent effect of recombinant IL8 on neutrophil migration was identified. However, neutrophil incubation with serum collected from calves of the two *IL8* haplotypes produced comparable levels of neutrophil migration and killing of bacteria, possibly due to low systemic IL8 levels. This suggests that alternative methods, using a local infection model should be employed in order to characterise neutrophil activation profiles in the two *IL8* haplotypes.

In conclusion, functional characterization of the genetic polymorphisms in the bovine *IL8* gene has identified differences in the proinflammatory responsiveness between the two *IL8* haplotypes, both *in vitro* and *in vivo*. Additionally, significant associations of the *IL8* promoter variants with somatic cell count and calving interval were also identified. These data strongly suggest that differential IL8 signalling profiles may confer differential ability to clear mastitis or post-partum infection, which could be exploited via selection for enhanced immunity in cattle.

Chapter I General introduction

I. 1. Innate immunity

The innate immune system is a highly conserved component of the host defence to pathogens that can be found in all multicellular organisms. During an encounter with microbes, the innate immune system is initially activated and responds to eliminate the source of infection. The innate immune response begins with the recognition of pathogen- or damage-associated molecular patterns (PAMPs and DAMPs) by the receptors on the surface of resident immune and epithelial cells, known as pattern-recognition receptors (PRR). Pattern-recognition receptors are usually located on the cell surface but can also reside in the cytosol or they can be embedded in the membrane of the intracellular vesicles. They can also be present in soluble form in blood and extracellular fluids. PAMPs are molecules, found in microbes and include viral double stranded RNA, bacterial unmethylated CpG DNA and bacterial cell wall compounds such as lipopolysaccharide (LPS), lipoteichoic acid and mannose-rich oligosaccharides. Different PAMPs shared by classes of microbes are recognized by different receptors ensuring an efficient and optimized response to the invading pathogen. In contrast, DAMPs are produced from stressed, damaged or dying host cells during infection or injury, and they initiate tissue repair activation [1].

One of the most important classes of PRRs is the family of proteins known as the Toll-like receptors (TLRs). Toll-like receptors are located on the cell surface and in the endosomal membranes and are associated with adapter molecules that transmit the signal into the cell which leads to the activation of host defence mechanisms [2]. There are two domains in the TLR protein: the cytoplasmic domain and the extracellular domain. In contrast to the cytoplasmic domains, which are highly conserved between species, extracellular domains show greater divergence reflecting their involvement in diverse PAMP recognition. Ten TLR receptors have been identified in both humans and cattle, showing specificities for different ligands of bacterial, synthetic and viral origin (Table I-1) [3, 4]. The interaction between the PRRs and pathogenic ligands results in signal transduction via different protein kinases and the activation of transcription factors. Two adapter proteins play a key role in TLR signalling: MyD88 and TIR

domain-containing adaptor inducing interferon β (TRIF). MyD88 is an adaptor protein involved in the signal transduction initiated by diverse TLRs, except TLR3. This adaptor protein promotes the formation of IL1R-associated kinase (IRAK) and TNFR-associated factor 6 (TRAF6), which catalyses the formation of the free polyubiquitin chain and the activation of the TGFβ-activated kinase (TAK) and TAK binding protein (TAB) complex. This complex phosphorylates IκB kinase which then phoshorylates IκBa. This protein is bound to the NFκB transcription factor, inactivating it. The phosphorylation of IκBa leads to its disintegration and removal, which in turn allows NFκB to translocate into the nucleus and activate the transcription of multiple cytokine genes. Alternatively, TAK/TAB complex phosphorylates MAP kinase (MAK), which activates the AP-1 transcription factor and the transcription of cytokine genes. TLR3 signaling employs an alternative pathway mediated via the TRIF adaptor protein. TLR4 can utilise both MyD88 and TRIF signalling pathways. In the TRIF signalling pathway, a complex between TRIF and TRAF is formed, which activates NFκB and interferon regulatory factor 3 (IRF3). These transcription factors then induce the expression of proinflammatory cytokine genes and type I interferons (Figure I-1) [4]. In addition to the transcription of cytokine genes, TLR7 and TLR9 signalling also involves the activation of type-1 intereferons, which induce the anti-viral response [4]. The activation of these pathways triggers a series of events that leads to increased vascular permeability, leukocyte adhesion and migration to the site of infection or injury, also known as inflammation.

The principal cell types involved in the innate immune response are epithelial and endothelial cells, and myeloid phagocytes such as neutrophils and macrophages.

Epithelial cells form physical barriers facing the external environment at sites such as the mucosal respiratory and intestinal surface, skin and uterine tissue. These cells are packed closely to prevent microbes from penetrating through the epithelial layer. In addition, these cells produce a number of cytokines, chemokines and antimicrobial peptides that further amplify the host reaction to the presence of pathogens. This effect is mainly manifested in the attraction of the immune cells such as phagocytes to the site of infection [1].

Phagocytes are an important group of cells which are at the front line of the host defence. Once activated, these cells migrate to the site of infection where they encounter the microbes and engulf them in a process known as phagocytosis. Phagocytes are

equipped with various mechanisms to kill the microbes which have been internalised. They can be classified into sub-groups based on their morphology and function: neutrophils, macrophages and dendritic cells.

Neutrophils are the most abundant circulating leukocytes and they are characterised by a segmented nucleus composed of multiple lobuli interconnected with short filaments. These cells are the first to respond to infection by migrating in large numbers to encounter and eliminate the pathogen. Individuals with a reduced blood neutrophil count are severely immunocompromised. On the other hand, an excessive neutrophil infiltration can cause prolonged tissue damage which is a basis of a number of autoimmune diseases. Therefore, tight regulation of neutrophil response to infection is critical for achieving an optimal response to infection. A number of chemoattractants produced during infection are known to promote neutrophil recruitment along the concentration gradient. Chemokines that belong to the CXC class of chemokines, such as interleukin 8, growth-related gene product (GRO) α, GRO-β, GRO-γ, neutrophil-activating peptide (NAP) 2, epithelial cell-derived and neutrophil-activating 78-amino acid peptide (ENA-78) are the principal neutrophil chemoattractants [5]. These chemokines activate neutrophils, upregulating the expression of the cell surface adhesion molecules which allows neutrophils to slowly roll along the endothelial wall. Activated neutrophils are able to change their shape in order to squeeze through the endothelial wall and reach the infected tissue.

Macrophages are mononuclear phagocytes of the myeloid lineage that originate in the bone marrow and constantly circulate in the blood as monocytes, scanning the environment for proinflammatory signals. Once these signals are present, in the form of chemoattractants and cytokines produced during infection, monocytes get activated and, similarly to neutrophils, upregulate the expression of various selectins and integrins which allows them to bind firmly to the endothelial wall and migrate into tissues. The primary chemokines involved in the monocyte recruitment are CCL2 and CCL7. These chemokines regulate recruitment of monocytes from the bone marrow into the circulation and they accomplish their function via CCR2 receptor on the monocyte surface [6]. In addition, CCL2 and CCL7 also mediate the monocyte migration to the site of bacterial encounter [7]. Here, they differentiate into macrophages which are capable of phagocytosing the microbes and dead or apoptotic cells, eliminating the threat to the host. In addition to eliminating pathogens through phagocytosis, monocytes

are also important regulators of the immune response as they produce a number of cytokines.

Table I-1. Human Toll-like receptors.

A list of TLR molecules characterised in humans with the corresponding localisation, ligand specificity and origin. Reproduced from Takeuchi *et al.* 2010 [4].

PRRs	Localization	Ligand	Origin of the Ligand
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7, TLR8	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa

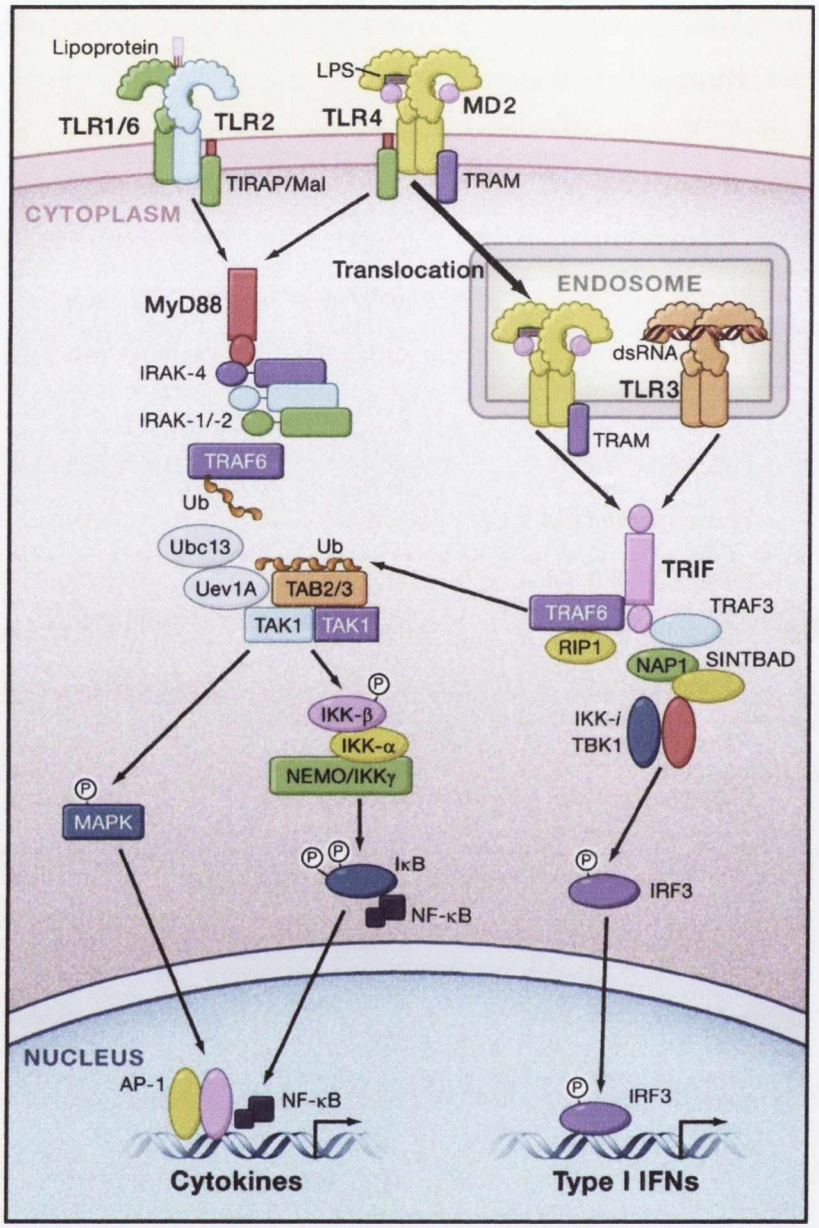

Figure I-1. TLR2, 3 and 4 signalling pathway.

MyD88 and TRIF adaptor proteins mediate a cascade of events that begins with the recognition of PAMPs on a cell surface, followed by signal transduction in the cytoplasm and activation of proinflammatory genes and type I IFN expression, in the nucleus. Reproduced from Takeuchi *et al.* 2010 [4].

I. 2. The overview of neutrophil attracting chemokines

Accumulation of leukocytes in the tissue is one of the hallmarks of inflammation, but it also occurs in a number of pathological conditions such as trauma, ischemia and cancer [8]. Key molecules responsible for the attraction and activation of leukocytes to the inflamed or damaged tissue are chemokines. They are secreted by the immune cells in response to pathogens, creating a concentration gradient towards the site of infection. Chemokines are usually produced at low levels (picograms per ml) and this is sufficient to carry out their biological function. Chemokines are small proteins, made of 70 to 80 amino acids, characterised by four conserved cysteines which form two disulfide bonds. Chemokines are divided into four subfamilies depending on the arrangement of the first two cysteines. CXC chemokines (CXCL) have one amino acid separating two cysteines whereas in CC subfamily (CCL) they are adjacent [5], where C is cysteine and X is any amino-acid. Two other groups are C (XCL) and the CX_3C (CX_3CL) family. C chemokines are missing cysteine 1 within the first pair of cysteines [9], whereas CX_3C chemokines exhibit 3 amino-acids between the two cysteines and are also the only membrane-bound chemokines [10]. The chemokines are recognised by receptors named after the corresponding ligands such as CXCR, CCR, CX_3CR and XCR which recognise CXCL, CCL, CX_3CL or CL, respectively.

A number of chemoattractants are involved in neutrophil recruitment, and they mainly belong to the CXC class of chemokines, particularly a subgroup of CXC chemokines that have glutamic acid-leucine-arginine (ELR) amino-acid motif, before the first cysteine. This region is critical for the chemokine interaction with the receptor and subsequent activation. The members of ELR chemokines are: CXCL 1-3 and CXCL 5-8. It has been demonstrated that blocking of CXCL1 and CXCL5 significantly reduces peritoneal neutrophil recruitment in mice immunised with methylated bovine serum albumin (BSA). In addition, the concentration of these chemokines in peritoneum was elevated upon imunisation [11]. A similar study by De Fillipo *et al.* have demonstrated that inactivating CXCL1 and CXCL2, in mice challenged with LPS, results in significantly reduced neutrophil counts in peritoneal fluid [12]. Similarly to CXCL1 and CXCL2, CXCL3 also promotes human neutrophil movement and activation [13]. CXCL6, also known as granulocyte chemotactic protein 2 (GCP-2) is a potent neutrophil activator in both human and mouse, even though its ability to induce

chemotaxis is higher in mouse than in human [14]. CXCL8 (IL8) is the first CXC chemokine to be characterised and is considered one of the most potent neutrophil chemoattractants in human.

Neutrophil mobilisation is a complex process which requires orchestrated action of multiple chemokines and cytokines. Vieira *et al.* demonstrated that CXCL1 and CXCL5 cooperatively induce neutrophil chemotaxis via TNFα [11]. Struyf *et al.* have shown that CC chemokine regakine 1, complement 5a (C5a) and CXCL8 induce human neutrophil movement and the change in cellular appearance, *in vitro*, and these effects were more pronounced when proteins were added together than when they were used individually [15].

The bovine chemokines have been less studied as compared to the human or murine chemokines. CXCL1 (GROα) was one of the first bovine CXC chemokines to be characterised, showing positive dose-dependent effect on neutrophil migration and activation [16]. Bovine CXCL1-3 chemokines have been investigated extensively in bovine mastitis models. *Staphylococcus aureus* lipoteichoic acid injection into bovine mammary gland induced rapid upregulation of CXCL1, CXCL2, CXCL3 and CXCL8, which was accompanied by significant influx of neutrophils. Interestingly, the basal levels of CXCL1 and CXCL8 were low or undetectable. However, CXCL2 and CXCL3 were present at high concentration in milk, in unchallenged mammary gland [17]. In addition, Rainard *et al.* have demonstrated that the main agent responsible for chemotactic ability of non-mastitic milk is CXCL3 [18]. On the other hand, CXCL8 appears to be an important neutrophil chemoattractant in mastitic milk as shown by the reduced neutrophil chemotaxis upon CXCL8 inactivation in mastitic milk [19]. In addition, Watanabe *et al.* have shown that CXCL8 infusion in the mammary gland, at dry-off period, leads to an elevated neutrophil counts in milk [20]. Similarly, Takahashi *et al.* demonstrated a significant increase in the number of milk cells upon recombinant CXCL8 injection into mammary gland [21]. In contrast, a number of other studies have failed to report an *in vivo* effect of IL8 infusion into mammary gland on neutrophil mobilisation, despite the evident *in vitro* chemotactic effect, suggesting possible limited ability of chemokines to penetrate the mammary epithelium and/or the importance of other chemotactic agents. In addition to chemokines, a complement factor C5a is considered an important end-target neutrophil chemoattractant in cattle [22, 23]. This protein has strong chemotactic properties for bovine neutrophils and it is capable of desensitising these cells making them irresponsive to CXCL8 and platelet-activating

factor [24]. In contrast to humans, it has recently been shown that bovine neutrophils express CCR1 on their surface, which is an important chemokine receptor involved in the recognition of CCL5 – CCL10. Interestingly, this receptor is recognised by a specific *Staphylococcus aureus* virulence factor, which binds it and induces the formation of pores making neutrophils susceptible to infection [25].

I. 3. Genetic control of innate immunity

A common approach for identifying the genetic variants with significant impact on the phenotypic trait of interest is by performing a gene association study. In the first stage, hundreds/thousands of animals are genotyped for the gene of interest using SNP chip. Then, the specific trait or performance measurements of animals with different gene variants are compared using complex statistical methodology. The most commonly used genetic variants are known as the single nucleotide polymorphisms (SNPs), which are single nucleotide mutations in the genome. SNPs are very useful for association studies due to their high density and even distribution across the genome.

The completion of the bovine genome sequencing project in 2009 [26] has generated valuable DNA sequence information which allows comparative analysis of the genetic diversity within innate immune genes. The innate immune response is under genetic control and the alteration in the sequence of the genes controlling innate immune response could impact the ability of an individual to mount an effective immune response to a pathogen. A missense mutation in the *TLR4* gene encoding the extracellular domain of the TLR4 receptor has been associated with LPS hyporesponsiveness in humans [27]. In addition, Khor *et al.* reported that a variant in a gene encoding Mal adapter protein, involved in TLR2 and TLR4 signaling, protects against a number of infectious diseases such as pneumococcal disease, malaria, tuberculosis and bacteremia [28].

Innate immune genes in cattle are characterised by a high level of genetic polymorphism. Relative to the bovine genome-wide SNP density of 1 SNP every 714 base pairs [29], the *TLR* gene family shows a high level of diversity. Seabury *et al.* reported an average *TLR* polymorphism density of 1 SNP every 219 bp, with up to 31% of the variants predicted to potentially influence the protein activity [30]. A number of

recent studies characterised additional SNPs in the *TLR* family, increasing the estimated SNP density to 1 SNP per 100 bases [31]. A number of papers have identified an association between *TLR* variants and susceptibility to infection in cattle. An association study between *TLR* genetic variants and bovine tuberculosis has demonstrated a higher frequency of the two *TLR1* polymorphisms in the infected animals, suggesting a potential role in TLR-mediated susceptibility to this disease [32]. Similarly, significant differences in genotype frequencies between healthy cows and cows infected with *Mycobacterium avium* ssp. *paratuberculosis* infection have been reported at three positions in the *TLR4* gene [33].

Downstream proinflammatory genes are also characterised by significant levels of variation in cattle. A study on DNA sequence diversity in the genes encoding six chemokines (*GRO1, GRO3, GROX, SDF1, ECIP1, IL8*), two cytokines (*TNF*, *IFNG*) and a chemokine receptor (*IL8R*) has revealed an average of one SNP per 143 bp of sequence in the reference beef cattle population [34, 35]. Polymorphisms in the coding region of the bovine IL8 receptor (*CXCR1*) and *IL8* have been associated with the incidence of clinical mastitis and resistance to mastitis, respectively [36, 37]. The *IL8* locus associated with the susceptibility of mastitis also shows different *IL8* mRNA levels between the genotypes [37], suggesting the functional role of IL8 in the response to mastitis infection. Finally, Leyva-Baca *et al.* have demonstrated the association of a genetic variant in the coding region of bovine IL8 with udder depth, a conformational trait related to mastitis [38].

Over the course of evolution, variants in immune genes have been under selection due to their critical role in pathogen recognition and elimination [39]. Divergent selection pressures in different breeds and environments might induce breed- and region-specific structure of these genes. Screening the bovine immune genes for selective signatures may help identify the variants that have been of critical importance for the development of disease resistance [40]. The existence of positively selected genetic variants in the extracellular domains has been demonstrated in *TLR2* [41] and *TLR4* [42]. Better understanding of these differences and identifying advantageous genetic variants could potentially enable breeding for disease resistance, and tailored vaccination strategies facilitating improved disease control.

I. 4. Breeding for resistance to infectious disease in cattle

Selection of animals with desirable production characteristics has been used for centuries, leading to the significant improvement of overall animal performance. Average milk production per lactation in US Holstein cows has almost doubled since 1960, which can mainly be attributed to an average increase in the genetic trend for milk production of approximately 1.5% per year [43, 44]. The Irish dairy industry has experienced a rapid breed replacement of British Friesian by North American Holstein. In the 1977, less than 10% of the Irish dairy herd was Holstein, whereas that proportion increased to about 80% in 1998 [45]. This was accompanied by a significant increase in milk gain, from 5429 kg in 1991 to 5884 kg of milk per cow per year, in 2000 [46].

In addition to milk gain, milk quality is also monitored, and an important indicator of milk quality is the somatic cell count (SCC), which represents the total concentration of cells in milk and is routinely measured in dairy cows. The SCC cut off for collecting milk produced in Ireland is regulated by the EU with a maximum permissible level of 400,000 cells per ml of milk [47]. Elevated level of SCC in milk is used as an indicator of the prevalence of clinical and subclinical mastitis within the dairy herd. Mastitis is a chronic mammary gland infection which leads to reduced milk yield and milk quality, and causes substantial damage to the dairy industry. The cost of mastitis for Irish dairy sector ranges between €45 and €185 per case, depending on the infection severity, including the cost of veterinary treatment, labour and milk loss [48].

Due to a negative correlation with fertility traits, intensive selection for milk yield has resulted in serious fertility problems in the Irish national herd [49]. The cost of infertility, combined with production losses and treatment costs, is estimated at €292 per cow per pregnancy [50]. If we take into account the size of the Irish dairy herd of approximately 1.3 million cows and disease incidence ranging from 20% to 75%, we can calculate that uterine infection costs the Irish dairy industry between €76 and €284 million annually. The cost in other dairy regions is even higher. An Irish Delphi study has identified both fertility and udder health as the most important animal health issues facing Irish livestock industries [51].

Antagonistic genetic correlation between mastitis incidence and a number of production traits such as milk yield, fat yield and fat content, has been demonstrated

previously [52, 53]. In addition, excessive use of antibiotics in the treatment of infectious diseases is costly and favours the emergence of antibiotic resistant pathogens [54]. Improving animal health through selection is advantageous and permanent because the beneficial SNPs will persist in the population for many generations [55].

The outcome of selection for a trait depends on the number of animals and the proportion of the phenotypic variation attributable to genes, known as the heritability. Heritability is usually expressed as a value between 0 and 1. Heritability of 0 indicates that only non-genetic factors play a role in the total phenotypic variation, whereas heritability of 1 signifies that a trait is completely genetically controlled. If the heritability for a trait of interest is low, selecting for another trait that shows higher heritability and a strong correlation with the original trait is required for a beneficial response to selection.

Since the heritability of susceptibility to clinical mastitis is low (~0.03) [56], selecting for a trait that shows higher heritability and a strong correlation with mastitis is a preferred approach. One trait that meets these criteria is the SCC. Moderate to high positive correlation (0.37-0.98) has been reported between mastitis and SCC which is considered to be a good indicator of udder infection [57], suggesting that by selecting for low SCC we indirectly select the animals with low susceptibility to mastitis. Importantly, the heritability of the SCC is significantly higher than that of mastitis and is estimated to be close to 0.10 [58]. This notion has been previously investigated and it was concluded that it is possible to improve resistance to mastitis by selecting for a low somatic cell count [59]. It has been shown that selecting for low mastitis frequency results in a decreased incidence in not only mastitis but also other diseases common in dairy cattle such as ketosis and retained placenta [56]. However, low number of cells in milk might impair an animal's ability to combat other infections – either in the mammary gland or in another tissue. Kulberg *et al.* have demonstrated that the cows selected for low mastitis incidence show reduced numbers of total white blood cells and neutrophils [60], which might impair animals' ability to clear the infection at sites where a neutrophil response is critical for success, such as the uterus. Also, selection for mastitis resistance is accompanied by a decreasing trend for milk protein content which is a trait of high economic importance [56]. Therefore, to achieve a balance between improving both health and performance, multiple traits need to be included in the evaluation. Multiple traits can be selected simultaneously using what is known as a

selection index. Appropriate weight is given to each trait based on its relative economic importance, its heritability and the correlations between different traits. The component traits are combined into a total score or index which is considered as a single character to which selection is applied [61]. The pace of the genetic improvement by index selection is slower but the holistic approach employed in this strategy will result in the steady and sustainable enhancement of the overall performance including both health and production traits.

The selection index used for animal evaluation in Irish dairy system was initially launched in 2001 and was composed of production traits such as milk yield, fat yield and protein yield as well as survival and calving interval. In an effort to reverse the antagonistic effects of selection solely on milk production, a new selection index has been established in 2006, which includes SCC [48]. Currently, milk processors in Ireland apply a penalty to the farmers when the SCC exceeds 400,000 cells per ml [48], encouraging the culling of animals with high SCC. This may lead to selection of animals with low neutrophil response, which will have negative effects on the long-term immunocompetence of Irish national herd.

In addition to mastitis, selection for natural disease resistance can potentially be applied in a number of other bovine infectious diseases such as tuberculosis, paratuberculosis, bovine respiratory disease and nematode infection [62].

I. 5. Genomic selection

Conventional methods of selection are based on the existence of extensive phenotypic records for each animal as well as the pedigree information on the progeny. There are several limitation to the methodologies currently used in animal breeding including [55]:

- Error in the phenotypic measurements
- Some phenotypes cannot be measured in both genders (e.g., mastitis)
- Some phenotypes cannot be measured at young age delaying the time of selection

- Unfavourable genetic correlations between traits of interest
- The interaction between the genotype and environment is not taken into account

Currently, new and powerful genotyping and computational technologies are emerging, replacing the conventional phenotypic selection through the use of genomic selection. In this approach, thousands of SNPs across the whole genome are screened for their association with all the traits of interest which constitute the appropriately weighted selection index. Having established these associations, the SNPs are then used to select the best individuals based solely on their DNA profile. This approach has a number of advantages over the traditional phenotype-driven selection including [63]:

- Increase in accuracy of the genetic evaluation of selection candidates
- The age at which an animal is evaluated is significantly reduced as each animal is genotyped at birth
- The number of potentially available selection candidates is significantly increased
- Applicable to both male and female selection candidates

Genomic selection in Ireland was launched in 2009 when over 900 Holstein-Friesian bulls were genotyped in order to estimate the optimal DNA signature for the Irish production system, significantly increasing the reliability of genetic prediction. Genomic evaluation service has recently became available to Irish farmers and breeding organisations, providing genomic testing of new-born dairy calves [63].

Despite the great potential of genomic selection, the lack of currently available health phenotypes limits its application to the selection of disease resistance. This is partly because host-pathogen interactions are complex and involve many factors, both genetic and non-genetic that influence the outcome of exposure to pathogens [64]. Infections are often accompanied by the collateral damage of host tissues which underlines a number of immunopathological conditions. For example, the lung pathology of a calf infected with bovine respiratory syncytial virus is believed to be induced by an excessive neutrophil immune reaction [64], as it has been shown that *in vitro* infection of human airway epithelial cells with respiratory syncytial virus is not accompanied by cytopathological effects [65]. Therefore, a certain level of tolerance to

pathogen is beneficial for the host as it prevents the tissue damage induced by the host immune cells. The balanced interaction between genes controlling resistance and tolerance to pathogen will determine the outcome of infection.

Another problem with genomic selection is that the vast majority of SNPs that are being genotyped are not the causative mutations for the trait of interest but are rather in close proximity, linked with the gene of interest and inherited as a single unit known as the haplotype. The length of haplotypic blocks differs between cattle breeds and populations. It has been shown that identifying the association between a SNP and a trait of interest that persist across divergent breeds such as Holstein-Friesian, Jersey and Angus, would require approximately 300,000 SNPs [66]. Over time, and with recombination, the physical relationship between a linked allele and an economically important phenotype can decay. It is therefore important to understand the biology of disease and characterise the causative polymorphisms in the genes mediating the resistance to infectious diseases in cattle.

I. 6. Interleukin 8 and neutrophils

I.6.1. Molecular properties of interleukin 8

Interleukin 8 (IL8) is a CXC chemokine specifically involved in neutrophil recruitment and activation of anti-microbial function. IL8 was originally purified from the medium of LPS stimulated human monocytes [67], but its production was subsequently demonstrated in a wide variety of immune and non-immune cells such as fibroblasts [68], epithelial [69], endothelial cells [70] and even neutrophils themselves [71]. Under normal conditions, circulating levels of IL8 are almost undetectable. However, IL8 in human serum is rapidly induced by endotoxin [72]. Oxidative stress is also a potent inducer of IL8 secretion which has been demonstrated in the human hepatoma cell line [72, 73].

The human IL8 protein is localised in the cell cytoplasm before being released into the extracellular environment [74]. IL8 exists in a dynamic equilibrium between monomer and dimer forms. The monomer interacts with neutrophils, while the dimeric

form functions as a negative regulator for this interaction [75]. The soluble IL8 protein dimer structure consists of two anti-parallel alpha helix motifs, lying on top of a six-strand beta-sheet [76] (Figure I-2 A). The disulphide bonds that are formed between IL8 cystein residues (Figure I-2 B) are important for the chemokine stability and functional integrity, as it has been shown that altering the cysteine groups in the CXC domain of IL8 leads significantly reduces the protein ability to activate the receptor [77]. The precursor IL8 protein is 99 amino acids long and is subjected to the cleavage of a leader 20 amino acid sequence prior to being secreted from the cell. After secretion, the N-terminus of the mature protein is further processed generating four biologically active IL8 isoforms - 77, 72, 70 and 69 amino acids long [78]. Most cell types produce all variants, but at varying concentrations. The predominant form secreted by activated endothelial cells is 77 amino acids in length [79], whereas most other cell types produce 72 amino acid long IL8 [78]. These two isoforms differ in their ability to activate neutrophils. Neutrophils exhibit a two-fold preference for the 72 amino acid isoform and this isoform is also 2-3 fold more potent in the activation of neutrophil degranulation [80]. In addition to different isoforms, dimerization of proteins plays a role in IL8 function.

Bovine IL8 has been originally purified from LPS stimulated monocytes and chemotactic activity of the bovine IL8 on neutrophils has been demonstrated [81]. It is a 7.8 kDa protein with 76% amino acid sequence identical with human IL8 [82]. Human recombinant IL8 has been shown to induce chemotaxis and degranulation of bovine neutrophils in a dose-dependent manner [83]. The IL8 gene structure is similar in human and bovine and shows 76% identity. IL8 gene is composed of 5' and 3' regulatory regions and the gene body that has 4 exons and 3 introns (Figure I-2 C). The gene encoding bovine *IL8* is 4.8 kb long, whereas the corresponding spliced open reading frame is 303 bp long, coding for a 101 amino acid protein [84]. Unlike in human studies, multiple IL8 protein isoforms have not been identified in cattle to date.

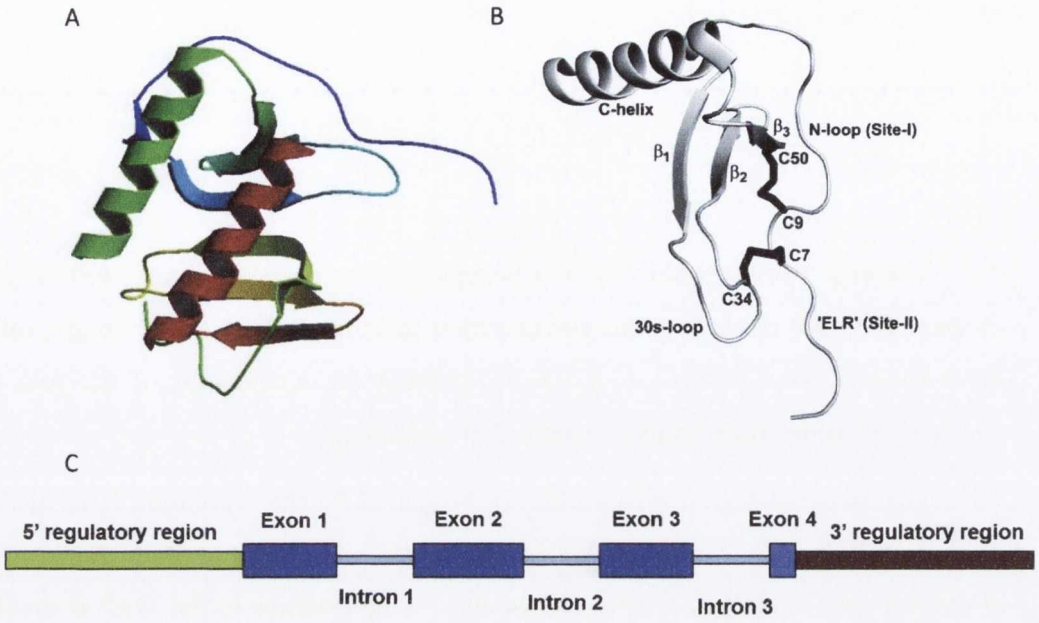

Figure I-2. Human interleukin 8 protein and gene structure.

A) The IL8 homodimer protein structure is characterised by two alpha helices (red and green) and a six strand beta sheet located underneath. Reproduced from Clore *et al* 1990 [76]. B) The IL8 monomer structure indicating two disulphide bonds (highlighted in black) near ELR domain. Reproduced from Joseph *et al.* 2010 [85]. C) IL8 gene structure showing two regulatory regions and the gene body composed of 4 exons and 3 introns.

I.6.2. Regulation of IL8 gene expression

The efficient *IL8* on/off system is regulated by complex intracellular regulatory mechanisms, and has been well characterised in humans [86]. Interestingly, mice and other rodents do not have an *IL8* gene, making the elucidation of the role of this chemokine in non-model organisms more of a challenge.

The essential region responsible for human *IL8* gene regulation is located in the proximal 150 bp upstream of the transcription start site (TSS), in the 5' flanking region of the *IL8* gene. The region between -94 and -71 bp (relative to the TSS) is involved in the activation of *IL8* expression in response to the proinflammatory stimuli such as TNFα and IL1. This region contains a binding site for the nuclear factor kappa B (NFκB), which is the most important transcription factor for driving *IL8* gene activation, as well as containing binding sites for CCAAT/Enhancer-binding protein (C/EBP) [87] and activating protein 1 (AP1) [88]. The latter two transcription factors are additional elements required for maximal IL8 gene expression (Figure I-3).

NFκB belongs to a family of dimeric transcription factors and it is involved in the regulation of expression in a number of proinflammatory cytokines, including IL8. NFκB is composed of five members: p65 (REL-A), REL-B, cytoplasmic REL, p50 and p52 [2]. Under basal conditions, NFκB is retained in the cytoplasm in an inactive form by binding to the inhibiting factors (IκB). Upon microbial or proinflammatory stimulation, IκB gets phosphorylated by IκB kinases which results in rapid degradation of IκB enabling NFκB to translocate to the nucleus and interact with the cis-regulatory elements in the *IL8* promoter, which results in *IL8* gene activation [86].

Unlike NFκB, the activation of AP-1 involves mitogen-activated protein kinases (MAPK), such as extracellular-regulated protein kinase (ERK), JUN-N-terminal protein kinase (JNK), and p38 kinase [89]. The activation of MAPKs is accomplished by phosphorylation through dual-specificity MAPK kinases (MKK) [86].

Two key regions within *IL8* gene promoter are responsible for the inhibition of *IL8* expression under basal conditions: NFκB repressing element (NRE), located

adjacent to the NFκB binding site, and POU-homeodomain transcription factor (Oct-1) binding site, overlapping that of C/EBP, on the complementary strand in the opposite direction. NRE is an element that binds NFκB repressing factor (NRF), which has a dual role. In the absence of stimulation, NRF acts as a repressor, resulting in *IL8* transcriptional silencing. In cells stimulated with IL1, NRF is also required for full induction of the *IL8* promoter activity [90]. Oct-1 is a member of octamer transcription factors which belong to a highly conserved family of transcription factors that recognize a specific DNA sequence (ATTTGCAT) in the promoter region of various genes. They share the DNA binding domain, also known as POU domain, which consists of 75 amino acid N-terminal subdomain and 60 amino acid carboxy terminal subdomain. This domain is responsible for the sequence specific DNA-protein interaction between the octamer transcription factors and DNA promoter domain. Oct-1 is ubiquitously expressed in a variety of tissues [91] and performs various functions in development and immunity. The effects of Oct-1 transcription factor binding on the target gene transcription can be either positive or negative. Yang *et al.* have shown that PMA enhances the interaction between the Oct-1 and the lingual and tracheal antimicrobial peptide (LAP and TAP, respectively) promoters, in bovine mammary epithelial cells [92]. In addition, binding of Oct-1 transcription factor to the *IL8* promoter abolishes the basal and inducible *IL8* promoter activity, upon IL1β stimulation [93]. Zhang *et al.* have demonstrated that mutating the Oct-1 binding site in the *IL8* promoter leads to an increased *IL8* expression in the human bladder carcinoma cell line [94].

Another molecule involved in the basal state IL8 repression is splicing factor proline/glutamine-rich (SFPQ) protein, which interacts with the *IL8* promoter, downstream from the TATA box, inhibiting the promoter function. Upon inflammatory stimulation, this factor interacts with the long non-coding RNA known as nuclear enriched abundant transcript (NEAT), which results in removal of SFPQ and *IL8* transcriptional activation [95] (Figure I-3).

It has been also shown that epigenetic mechanisms are involved in the transcriptional silencing of human *IL8*. In unstimulated cells, the *IL8* promoter is repressed by deacetylation of histone proteins. Inhibition of histone deacetylase (HDAC) activity causes hyperacetylation of the *IL8* promoter around the NFκB binding site leading to an enhanced and prolonged expression of the *IL8* gene [96]. Histone acetylation is also involved in *IL8* upregulation by oxidative stress agents such as H_2O_2

and environmental particles [97]. DNA methylation has also been linked with *IL8* transcriptional control. Metastatic tumour cells are known to produce higher amounts of IL8 than non-metastatic cells and this difference is induced by different DNA methylation profiles in the distal *IL8* promoter between these two types of tumour cells [98].

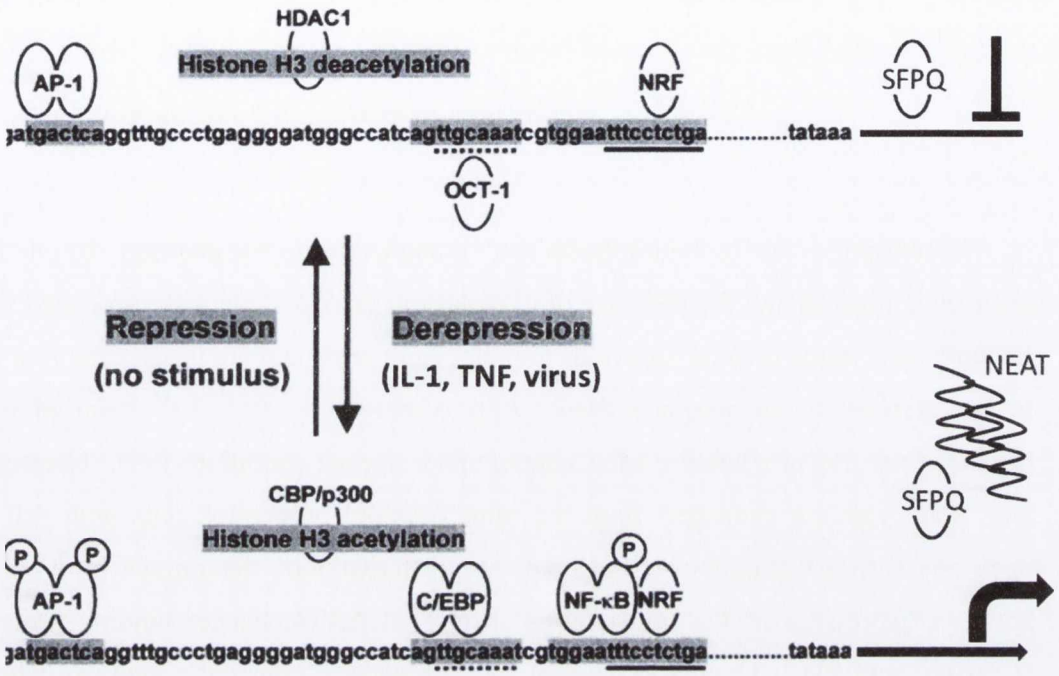

Figure I-3. Human interleukin 8 gene regulation.

In the absence of stimulus, AP-1 dephosphorylation along with HDAC-1 deacetylation and the repressive action of Oct-1, NRF and SFPQ inhibit *IL8* expression. Upon IL1, TNF or viral stimulation, AP-1 phosphorylation, binding of C/EBP and NFκB to the core IL8 promoter and the removal of Oct1 and SFPQ by NEAT non-coding RNA, leads to *IL8* transcriptional activation. Adapted from Hoffmann 2002, and Imamura *et al* 2014 [86, 95].

I.6.3. Neutrophil biology and IL8 pathway in neutrophil activation

I.6.3.1. Neutrophil generation in bone marrow

Neutrophils are polymorphonuclear granulocytes, characterised by a large segmented nucleus and the presence of cytoplasmic granules. They are produced in the bone marrow in the process known as granulocytopoiesis, and are usually the first cells to be recruited to an inflamed tissue. Approximately 1 - 2 x 10^{11} neutrophils are produced per day in a healthy adult human under normal conditions [99]. Neutrophils and monocytes are generated from the same myeloid progenitor cells with cell fate decisions between granulocyte and monocyte differentiation determined by a balance between expression of the transcription factors CCAAT/enhancer binding protein α (C/EBPα) and PU.1. Overexpression of PU.1 induces a monocytic pathway whereas C/EBPα drives differentiation of granulocytes [100]. The hallmark of neutrophil maturation is the progressive aggregation of granules and the segmentation of the nucleus. The progenitor cell of the granulocyte lineage is the myeloblast which then progress to a promyelocyte, that is characterised by the appearance of granules in the cytoplasm. These primary granules are azurophil granules, mainly characterised by the presence of the enzyme myeloperoxidase. Differentiation of the promyelocyte into a myelocyte is accompanied by the synthesis of secondary granules containing lactoferrin. In the next stage myelocytes are transformed into band cells which are characterised by tertiary granules containing gelatinase [99]. Finally, band cells differentiate into the mature neutrophil whose nucleus is composed of multiple lobes connected by filaments.

The key molecule regulating the output of neutrophil production in the final stages of granulocytopoiesis is granulocyte colony stimulating factor (G-CSF), a glycoprotein that stimulates hematopoietic progenitors at low concentrations [99]. Granulocyte colony stimulating factor induces colony formation by mouse bone marrow cells. At lower G-CSF doses, all colonies are granulocytic but with higher concentrations of G-CSF, some mixed granulocyte-macrophage and macrophage colonies also develop [101]. In addition, *in vivo* study has shown that G-CSF increases the number of circulating granulocytes 2h after administration in hamsters [102]. The production of G-

CSF depends on the rate of apoptosis of neutrophils in tissues. When dead or apoptotic neutrophils become phagocytosed by residential macrophages and dendritic cells, the production of interleukin 23 (IL23) in these cells is decreased. IL23 is known to stimulate the production of interleukin 17A (IL17A) which in turn induces the production of G-CSF [103]. Hence, the increase in the number of tissue neutrophils inhibits the production of G-CSF. The complete maturation of neutrophils is accompanied by their translocation from the bone marrow into the systemic circulation. The key molecule preventing neutrophils from leaving the bone marrow is CXC chemokine receptor type 4 (CXCR4). Its ligand, CXCL12, is produced by osteoblasts and endothelial cells and triggers the signals that maintain neutrophils in the bone marrow [104]. Knocking out of *CXCR4* in mice leads to the redistribution of neutrophils from the bone marrow to the blood and spleen [105]. G-CSF has the ability to block the expression of both CXCR4 [106] and CXCL12 [107], thus initiating the release of neutrophils into the circulation.

I.6.3.2. Neutrophil migration to sites of infection

In order to carry out their microbicidal function at the site of infection, neutrophils need to move from systemic circulation, cross the vascular wall between the endothelial cells, and reach the site of infection. This process is coordinated by a set of molecules, expressed on both endothelial cells and neutrophils, whose expression is regulated by proinflammatory cytokines, which are generated during infection. Endothelial cells begin to express P- and E-selectins, and chemokines such as IL8 and GRO-α [99, 108]. They bind P-selectin ligand 1 (PSGL-1), E-selectin ligand (ESL-1) and L-selectin, which are expressed on neutrophils. Interaction between the selectins on endothelial cells and their ligands on neutrophils is responsible for the loose adhesion and slow rolling of neutrophils along the blood vessel. The key surface molecules responsible for firm adhesion of neutrophils to the vascular wall are integrins expressed on neutrophils and their ligands expressed on endothelial cells: β1 integrins LFA-1 and Mac-1 and their ligands, ICAM-1 and ICAM-2, as well as β2 integrin VLA-4 and its ligand VCAM1 [109]. Neutrophils can cross through the endothelial wall via paracellular or transcellular routes. The paracellular route is the predominant method for neutrophil

migration and involves neutrophils squeezing between cells and migrating through the endothelial-cell junctions. Key regulators of paracellular transmigration are ICAM-1 and VE-cadherin. Upon endothelial activation, ICAM-1 clusters around the cell junctions forming ring-like structures, whereas VE-cadherin forms gaps through which neutrophils can migrate [110]. Transcellular migration requires the formation of pores through the cell membrane and the displacement of cytoplasmic organelles. This route is considered to be taken by a minority of emigrating cells [109].

The final barrier neutrophils must cross before they can reach the site of infection is the basal membrane and pericytes surrounding the endothelial wall. Neutrophils are equipped with a wide repertoire of proteolytic enzymes, such as elastases and matrix metaloproteinases. They are stored in the granules and capable of breaking down the basal membrane proteins [109, 111]. Neutrophils tend to migrate through certain regions in the basal membrane that show considerably lower expression of the membrane constituents and are also associated with gaps between pericytes [112]. Neutrophil arrival at the site of infection is accompanied by the significant increase in their phagocytic ability [99] and the secretion of chemokines, including IL8 and Groα [113].

I.6.3.3. Role of IL8 in neutrophil trafficking and activation

IL8 secreted from the tissue resident cells is internalized by the endothelial cells and transported to the vascular lumen where it is presented to the neutrophils. The concentration gradient created by IL8 serves to guide neutrophils from the blood to the site of infection [114]. The interaction between IL8 and neutrophils is accomplished via interleukin 8 receptors located on the neutrophil surface. Neutrophils express two distinct receptors for IL8: CXCR1 and CXCR2, which belong to a G protein-coupled family of receptors. The two IL8 receptors show overlapping but different ligand specificities. CXCR1 binds IL8 and granulocyte chemotactic protein 2 (GCP-2) whereas CXCR2 in addition to IL8 also interacts with other CXC chemokines such as GROα, GROβ, GROγ, CXCL5, CXCL7 and neutrophil-activating peptide 2 (NAP-2) [115-117] (Figure I-4). IL8 binding to the CXCR1 and CXCR2 results in the activation of

phosphatidyl-inositol-3-kinase (PI3-K), or phospholipase C and D pathways (PLC and PLD, respectively) [118, 119], promoting the MAPK signalling cascade, calcium accumulation in the cytosol, and activation of a number of proteins such as previously discussed: NFκB, AP-1 transcription factors, as well as hypoxia-inducible factor 1 (HIF-1), androgen receptor (AR), signal transducer and activator of transcription 3 (STAT3) and β-Catenin [120]. This signal cascade eventually leads to the activation of CD11b/CD18 and CD11c/CD18 integrins and neutrophil chemotaxis [83, 119, 121]. IL8 alone does not activate the oxidative burst of neutrophils but it "primes" them for greater response to agonists [83, 122-124]. In addition, it has recently been demonstrated that IL8 possesses the ability to bind to the membrane of *Mycobacterium tuberculosis*, thereby facilitating its uptake and killing by neutrophils [125].

Neutrophils also possess the ability to produce IL8 when activated by a number of stimuli [126] such as microbial ligands and endogenous cytokines (TNFα, IL1β), thus amplifying the recruitment of additional neutrophils to the site of infection [113].

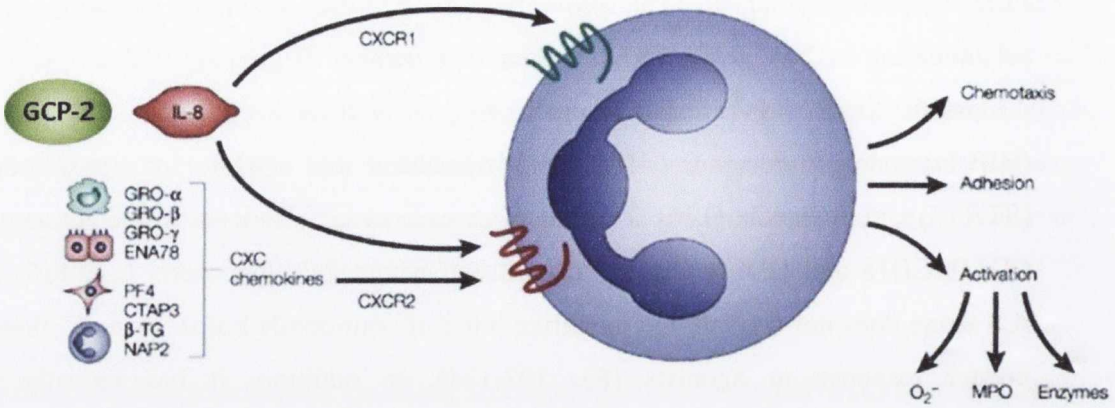

Figure I-4. CXCL signalling mediated via CXCR1 and CXCR2 receptors.

CXCR1 receptor has only two ligands: IL8 and GCP-2, whereas CXCR2 can also bind a number of other chemokines. The binding of a CXCL chemokine to the CXCR1 or CXCR2 receptor results in neutrophil chemotaxis adhesion and enhanced superoxide and MPO production and secretion of elastase. Adapted from Barnes 2002 [127].

I.6.3.4. Antimicrobial functions of neutrophils

Neutrophils are equipped with multiple mechanisms for degrading microbes and defects in these mechanisms are associated with impaired ability to fight infection [128]. Neutrophils kill bacteria both inside and outside the cell. In order to be recognized by neutrophils, the bacterial cell wall is first coated with proteins of the complement system or antibodies, in the process known as opsonization. The key complement proteins involved in opsonization are the cleavage products of the complement C3. They are recognized by the complement receptor 1 and 3 (CR1 and CR3) expressed on neutrophils [129]. Opsonisation is mainly mediated by IgG isotype antibodies and corresponding Fcγ receptors expressed on neutrophils [130]. Receptor binding of the opsonized microbial pathogen triggers phagocytosis. In this process, neutrophils form two cell projections, surrounding the microbe from both sides. When the two projections meet they form a vesicle known as the phagosome, which results in the internalization of the microbe.

Once encapsulated inside the cell, microbes can be killed using oxygen-dependent or oxygen-independent mechanisms, mediated by reactive oxygen species or antimicrobial peptides, respectively. These compounds are stored in the cytoplasmic granules, mainly primary (azurophilic) and secondary (specific) granules. They merge with the phagosome to form the phagolysosome in which the microbial pathogens are destroyed. Killing via reactive oxygen species plays a pivotal role in host defence. This is clearly evident in the conditions that result from defects in reactive oxygen species production such as chronic granulomatous disease. This condition is a result of the genetic mutations in the genes encoding subunits of NADPH oxidase and is manifested by the persistent infections in epithelial surfaces [131]. NADPH oxidase is the key enzyme responsible for the oxygen-dependent killing and it is mainly stored in the secondary and tertiary granules [132]. NADPH oxidase is a membrane-bound protein that catalyses a transfer of electrons from the cytosol to the molecular oxygen located in the phagosome, generating superoxide anion (O_2^-). O_2^- participates in a series of reactions producing other reactive oxygen species such as hydroxyl radical (HO°) and hydrogen peroxide (H_2O_2) [131, 133]. Primary azurophilic granules are rich in

myeloperoxidase, an enzyme responsible for converting hydrogen peroxide into hypochlorous acid, and exhibits potent microbicidal activity [134].

Oxygen-independent mechanisms are mediated via production of microbicidal enzymes. Upon neutrophil activation, granules containing these enzymes are discharged into the phagosome or extracellular environment, where the killing of microbes occurs. Primary effector functions are located in the azurophilic granules which are rich in antimicrobial proteins: defensins and proteolytic enzymes such as elastase, cathepsin G and proteinase 3 [132]. Defensins are the dominant proteins in azurophilic granules [135]. They are a family of antimicrobial peptides with a characteristic β-sheet fold and six cysteines interlinked with disulfide bonds. They possess a broad spectrum of antimicrobial activity against fungi, bacteria and viruses, mediated mainly via permeabilization of target membranes [136]. A predominant protein in the secondary granules of neutrophils is lactoferrin. The antimicrobial activity of lactoferrin is accomplished through the blocking of iron making it unavailable for bacteria thus inhibiting their growth [137]. Lactoferrin can also kill bacteria by inducing the LPS release from the bacterial surface which alters the outer membrane permeability [138].

In addition to their role as professional killer cells, neutrophils are also capable of performing more sophisticated functions important for the successful activation of adaptive immune response such as promoting B-cell survival and antibody production, as well as antigen presentation to T-cells [139].

I.6.3.5. Neutrophil apoptosis

Neutrophils are short-lived cells with a half-life in circulation of approximately 5-10 hours. Under the physiological conditions, neutrophils are removed from circulation in the liver, spleen and bone marrow [140]. However, during inflammation their lifespan is significantly prolonged. The exocytosis of proteolytic enzymes into the extracellular matrix is associated with collateral tissue damage. Therefore, once the infection is cleared, it is important that neutrophils are efficiently removed from the tissue [141]. Apoptosis or programmed cell death, plays a major role in the removal of the neutrophil aggregates after microbial clearance [142]. Apoptotic neutrophils are engulfed and degraded by macrophages preventing the release of the hazardous content

[143]. The cell surface transmitters of apoptotic signals are known as "death receptors". They belong to the tumor necrosis factor (TNF) receptor gene superfamily with conserved cysteine-rich extracellular domains. CD95 (also called Fas or Apo1) and TNFR1 are the most studied death receptors [144]. Neutrophils express higher levels of both Fas receptors and Fas ligands, and are more sensitive to Fas mediated apoptosis compared to other phagocytes (monocytes and eosinophils) [145]. Cytokines play an important role in the regulation of neutrophil apoptosis. One of the key mediators of neutrophil apoptosis is TNFα [146].

Downstream signalling pathways responsible for the activation of apoptotic machinery are different between spontaneous and stress-induced apoptosis. It has been shown that p38 MAP kinase is involved in the stress-induced neutrophil apoptosis [147] whereas protein kinase C is the mediator of preference in the neutrophil apoptosis under basal conditions [148]. Both pathways seem to be activated by caspase 3, a proteolitic enzyme essential for the programmed cell death. In the final stage of apoptosis, cellular DNA is fragmented, the nucleus is condensed and the neutrophil cell is phagocytosed before releasing any hazardous component within.

I.6.3.6. Characteristics of bovine neutrophils

In terms of general appearance bovine and human neutrophils show similar characteristics. Bovine neutrophils are characterised by polymorphic segmented nucleus with lobes connected by short filaments. The cytoplasm contains isles of glycogen and numerous cytoplasmic granules that are employed during infection [149].

However, the granules and enzyme composition within neutrophils are significantly different between humans and cattle. Peroxidise activity in bovine neutrophils is reduced by 80-85% compared to the human neutrophils [150]. In addition to the azurophilic and specific granules, a third type of peroxidise-negative cytoplasmic granules has been identified in cattle. These organelles are larger, denser and more numerous than both azurophilic and specific granules [150]. The majority of the antimicrobial activity is localised within these granules. They contain a group of highly cationic antimicrobial proteins known as bactenecins, with two members identified, called Bac7 and Bac5 [151]., now known to belong to the cathelicidin antimicrobial-

peptide family [152]. These granules also store β-defensins [149], a group of antimicrobial peptides showing particularly high diversity in cattle [153]. One of the first β-defensins to be characterised was tracheal antimicrobial peptide, which was obtained from the extracts of the bovine tracheal mucosa and showed anti-bacterial and anti-fungal activity [154]. Subsequently, 13 β-defensins have been characterised in bovine neutrophils, with most of them showing bactericidal activity against *E. coli* and *S. aureus* [155].

In addition to the circulating and tissue neutrophils, bovine milk is another site of high-level neutrophil migration. Milk neutrophils are characterised by the presence of phagocytosed casein micelles, fat globules and fat globule membrane material. Another important difference between the blood and milk neutrophil is the lack of membrane protrusions in milk neutrophils, due to membrane internalisation during the phagocytosis of casein and fat globules (Figure I-5). Also, milk neutrophils are characterised by reduced responsiveness to bacteria and lower capacity to produce reactive oxygen species and perform phagocytosis, upon bacterial encounter, possibly due to the fact that milk neutrophils are pre-stimulated with milk components before confronting bacteria [149].

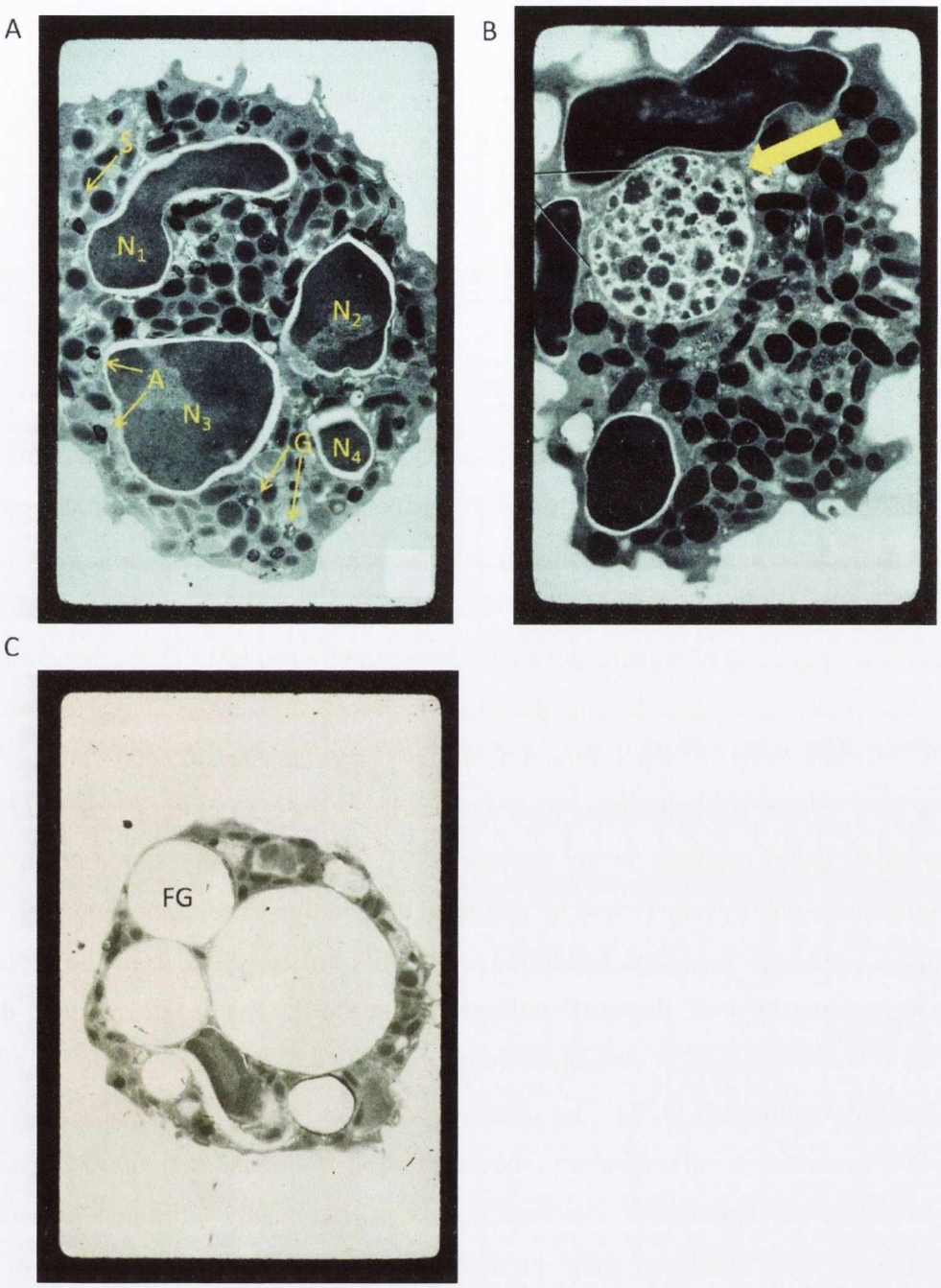

Figure I-5. Transmission electron micrograph of bovine polymorphonuclear neutrophils (PMN) from blood and milk.

(A) Blood PMN are characterised by multilobular nuclei (N_1, N_2, N_3, N_4), specific granules (S), and azurophilic granules (A); (B) Milk PMN with a large vacuole, containing casein granules, indicated with an arrow, (C) a milk PMN that lost the pseudopodes due to membrane internalisation during formation of vacuoles containing milk fat globles (FG). Reproduced from Paape *et al.* 2003 [149].

I.6.4. Role of interleukin 8 and neutrophils in fighting infectious diseases in cattle

I.6.4.1. Relevance of IL8 and neutrophils to mastitis infection

Bovine mastitis is an infectious disease induced by bacteria that penetrate into the lumen of the mammary gland through the teat canal. Mastitis is the most prevalent infectious disease in the dairy industry and causes substantial economic losses due to reduced milk quality, veterinary treatment and potential early culling. It is estimated that the average cost of mastitis per cow is between €95 and €211 [156]. In addition, the net Irish farm profit decreases as the SCC increases, from €31,252 per annum at the SCC baseline to €11,748 per annum at the SCC greater than 400,000 cells per ml of milk [47]. One of the hallmarks of bovine mastitis is the migration of leukocytes to the mammary gland resulting in an increased SCC. In the healthy condition, milk cell population is mainly represented by epithelial cells and macrophages. However, during acute *E. coli* and *S. aureus* infection, neutrophils are recruited from the blood, and comprise over 80% of the milk cell population [157]. The innate immune defence against invading mastitis pathogens relies heavily on the number and viability of circulating neutrophils [158]. The milk concentration of haptoglobin, an acute-phase protein involved in inflammation, shows strong correlation with the SCC, and the elevation of the haptoglobin concentration is predominately attributed to neutrophil recruitment to the mammary gland during mastitis [159].

Despite the large numbers of neutrophils recruited to the mammary gland during mastitis, the efficiency of bacterial killing in mastitis is very low. This could be due to the reduced killing ability of neutrophils in the milk compared to the blood, or due to invasion of bacteria into neutrophil cells. Shortage of oxygen and the lack of glucose are limiting factors influencing the bactericidal capacity of neutrophils in milk [160, 161]. Also, various milk compounds such as casein and fat globules are known to inhibit the microbicidal function of neutrophils [160]. Finally, milk neutrophils are incapable of active chemotaxis towards bacteria and are dependent on random collision, which

further reduces phagocytic ability [161]. Nevertheless, the importance of neutrophils in coping with the mammary gland infection is clearly reflected through the conditions in which neutrophil numbers or functions are compromised. During early lactation, the recruitment of neutrophils is impaired and the production of reactive oxygen species is reduced, and this has been linked with increased susceptibility of the mammary gland to intramammary infections [162].

Within the mammary gland, the main sources of IL8 are macrophages, epithelial cells lining the alveoli and ducts, endothelial cells and migrating leukocytes. Neutrophil chemotaxis is induced by mastitic mammary secretions and is blocked by anti IL8 antibodies indicating a major role of IL8 in neutrophil recruitment during mastitis [19]. Somatic cells from mastitis-affected cows show elevated *NFκB* expression which is positively correlated with the increased level of IL8 [163]. *In vitro* studies on the bovine mammary epithelial cells demonstrated an upregulation of *IL8* gene expression when the cells are obtained from mastitic cows [164] or co-cultured with *E. coli* [165], and the magnitude of *IL8* gene upregulation depends on the infecting concentration of *E. coli* particles [166]. Finally, IL8 concentration in milk increases markedly at 2h upon the intramammary infusion of endotoxin from *E. coli* [167], which suggests that IL8 plays a major role in the early immune response to infection, in the mammary gland.

I.6.4.2. Relevance of IL8 and neutrophils to uterine disease

Uterine disease is an inflammation of the uterine lumen or wall after calving. It is one of the most significant disorders in cattle leading to high economic costs due to prolonged inter-calving intervals and reduced fertility [168]. The first stage in the onset of uterine disease is bacterial contamination of the uterine lumen after parturition. Bacteria are present in the uterine lumen after calving in almost all animals. Most animals successfully eliminate the microbes within two weeks of calving. However, infection can still be detected in up to 40% of animals as the clinical metritis which is manifested by the infection of both endometrium facing the cavity as well as layers above the endometrim. Disease persists in up to 20% of animals as clinical endometritis, which is a localised infection of the endometrial layer [169].

Uterine epithelial cells provide a physical barrier between the uterus and the outside environment and represent the first line of defence. In addition, they produce a number of antimicrobials including lactoferrin, defensins and lysozyme as well as the numerous cytokines and chemokines such as GM-CSF, TNFα, IL1, IL6, IL8 [170]. Secreted cytokines are involved in the communication between epithelial cells and leukocytes recruiting more cells to the site of uterine infection. Neutrophils are the primary effector cells involved in the defence against bacteria contaminating the uterus. Typically, timing of activation and function of neutrophils is different between cows that develop uterine disease and healthy cows postpartum. The uterus of healthy cows is characterised by an infiltration of neutrophils around calving, followed by their rapid clearance. In the cows that develop uterine disease, neutrophil mobilization occurs latter in lactation and persists for a prolonged period of time causing chronic inflammation [171]. These changes can also cause the damage to the endometrium which eventually leads to the sub-fertility and infertility manifested in the prolonged interval between successive calvings or conceptions [169].

An association between the impaired neutrophil function and uterine health disorders have been documented in the literature. Retained placenta has been associated with reduced neutrophil counts [172] and suboptimal chemotactic ability [173]. In addition, several studies have demonstrated that the neutrophils from cows with metritis or subclinical endometritis exhibit decreased myeloperoxidase activity that reached minimum the day of calving [174, 175]. Also, reduced mobility and myeloperoxidase activity of neutrophils in cows with retained placenta is associated with lower IL8 concentrations throughout the periparturient period [174]. On the other hand, *IL8* mRNA shows significant upregulation in inflamed or subclinical endometritic uterus compared to healthy endometrium [176-178]. The principal source of IL8 in the uterus are endometrial epithelial cells which upregulate IL8 expression in response to bacterial stimulation [179]. Not only is IL8 involved in the neutrophil recruitment in the state of uterine infection, but it is also plays a role in the normal reproductive cycle in cattle. It has been shown that IL8 concentration increases during the course of the follicular development stimulating the progesterone production by the ovarian and luteal cells [180, 181]. In addition, IL8 impacts luteal function by promoting progesterone production from luteal cells in a dose dependent manner [181]. Finally, in healthy cows,

serum IL8 is elevated 15 days post-partum followed by a return to normal levels by day 60. This is accompanied by the clearance of uterine inflammation [182].

I. 7. Hypothesis

Multiple studies have reported the association between the genetic polymorphisms in the human *IL8* promoter and the infectious disease susceptibility [183-185]. Moreover, cooperative interaction between the adjacent genetic mutations indicates the importance of studying promoter haplotypes rather than the individual SNPs. This was demonstrated in *IL6* where it was shown that individual SNPs in the promoter of this gene interact with one another in a complex manner influencing IL6 expression cooperatively rather than additively [186].

Previously, our group has identified 29 novel polymorphisms in the promoter region of bovine *IL8* spanning 2.1 kb upstream of the transcription start site which segregate into two distinct haplotypic blocks, namely *IL8* haplotype 1 and *IL8* haplotype 2 (Figure I-6) [187]. It is hypothesised that the genetic differences between the two IL8 haplotypes identified in the bovine *IL8* promoter affect *IL8* expression via creation or abrogation of transcription factor binding sites with significant downstream consequences for the neutrophil function/responses.

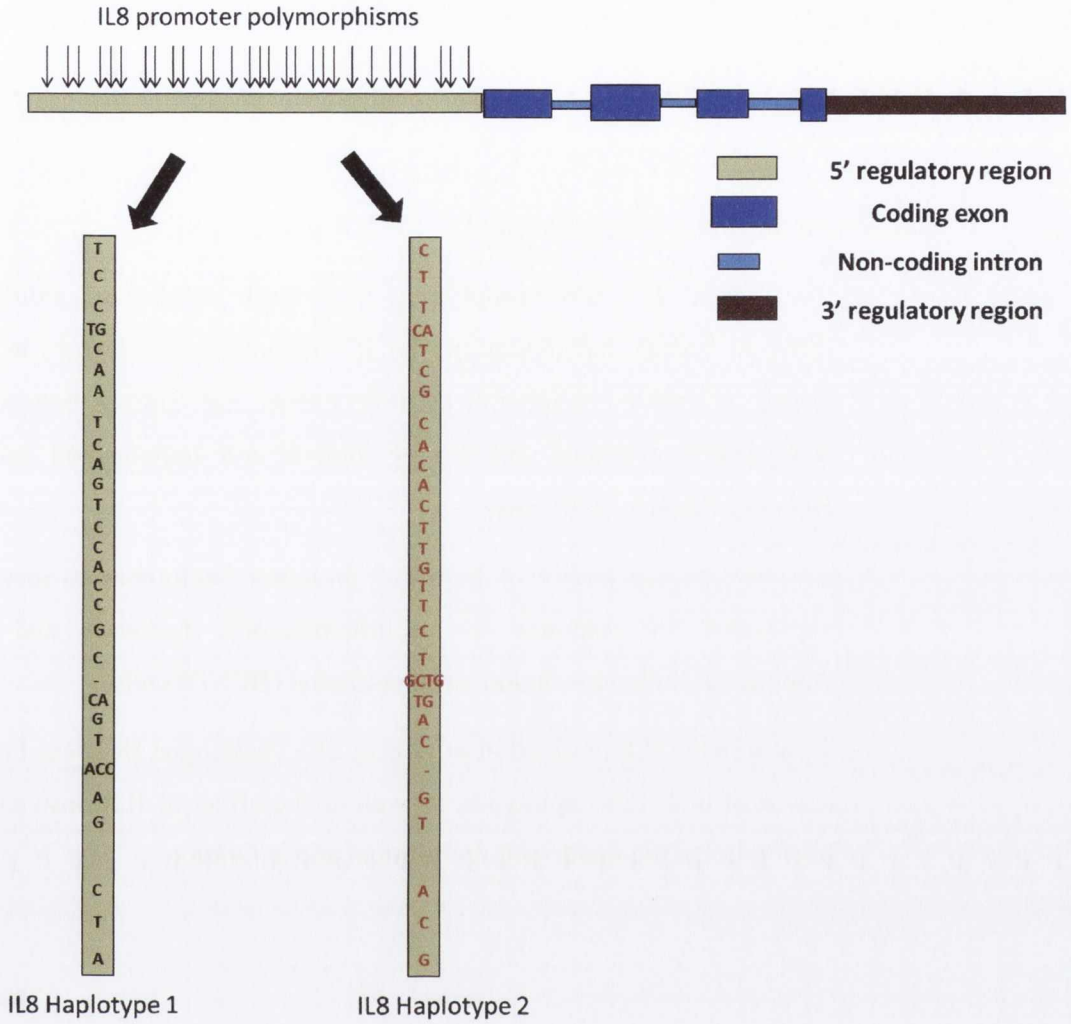

Figure I-6. Highly polymorphic bovine IL8 promoter structure in Irish cattle.

IL8 promoter variants (29 polymorphic sites), spanning 2.1 kb region, are indicated by arrows. They segregate into 2 IL8 promoter haplotypes. All the genetic differences (26 SNPs, 1 dinucleotide polymorphism and 2 insertions/deletions) are shown in the two IL8 promoter haplotypes. Adapted from Meade *et al.* 2012 [187].

I. 8. Aims

The aims of the project were three fold:

1. To perform *IL8* SNP genotyping in a large number of animals of different breeds and determine the *IL8* haplotype frequencies in those breeds, as well as to identify possible genetic associations between the *IL8* promoter variants and calving interval and somatic cell count in Holstein-Friesian (HF) cows.
2. To compare the ability of both *IL8* promoter haplotypes to drive IL8 expression in response to proinflammatory, bacterial and viral stimulation, in bovine endometrial epithelial (BEND) cells.
3. To assess the IL8 production *in vivo* in LPS challenged Holstein-Friesian calves of both IL8 haplotypes, as well as the effect of IL8 dose and *IL8* haplotype on the neutrophil recruitment and activation.

Chapter II Materials and Methods

II. 1. Animals

All the blood sampling was performed from both research and commercial farms under license from the Department of Health and Children to Dr. Kieran Meade. All the animals used for the separation of macrophages and neutrophils were healthy Holstein-Friesian cows from Teagasc Grange research center or a local commercial farm. Animals used for the IL8 promoter genotyping were of five different breeds: Holstein-Friesian, Jersey, Charolais, Shorthorn and Brahman, all sampled on commercial farms. The LPS challenge was conducted on the four week old Holstein-Friesian bull calves from a local farm. The licence for the procedure was approved by Health Products Regulatory Authority under the licence number AE19132/P008 and case Number 7016023.

II. 2. Genomic DNA extraction

Blood (8 ml) was collected from the jugular vein, in the vacutainers containing acid citrate dextrose and centrifuged at 3000 rpm for 15 min at 4°C. Buffy coat was collected from each sample and stored at -20°C. DNA was extracted on the Maxwell® DNA extraction instrument according to manufacturers instructions (Promega UK Ltd., Southampton, UK). Briefly, 300 µl of the buffy coat was added into the first well of the DNA extraction cartridge containing lysis buffer, followed by five washing steps. DNA was eluted in 400 µl of elution buffer. The concentration of the purified DNA was measured by absorbance at 260 nm and the purity was confirmed by measuring the A260/A280 ratio which was typically greater than 1.7. The eluted samples were then transferred to new 1.5 ml tubes and stored at -20°C.

II. 3. PCR and Sanger sequencing

The 442 bp *IL8* promoter region spanning 3 SNPs of interest was amplified by PCR using 500 nM of both forward and reverse primer (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland) (Table II-1). The primer annealing temperature was optimized on the

±10°C temperature gradient using 1.5 or 2.5 mM $MgCl_2$ concentration. Following optimization, the region of interest was amplified in all DNA samples using 100 ng DNA or water as a negative control, 2.5 mM $MgCl_2$, 1× PCR reaction buffer, 500 nM of each primer, 25 units/ml Platinum Taq Polymerase, 250 µM of each dNTP (Invitrogen, Paisley, UK) in the 55 µl reaction volume. The amplified PCR products were commercially cleaned up and Sanger sequenced (Source Bioscience, Dublin, Ireland), and the sequences were analysed using the BioEdit package [188].

II. 4. Taqman *IL8* genotyping

A TaqMan assay was carried out on the Applied Biosystems 7500 Fast Real-Time PCR System, following the instructions from the manufacturer (Applied Biosystems, Paisley, UK). Primers and probes were designed to amplify the region within which the tag *IL8* promoter SNP is located: chr6:91739266-91739367. Forward and reverse primer sequences are shown in Table II-1. The total TaqMan PCR reaction volume was 25 µl. Each DNA sample was diluted to deliver a final DNA mass of 10 ng. DNA samples, negative or positive control (11.25 µl) were added into each well of a 96-well optical reaction plate. The reaction master mix (13.75 µl) used for genotyping is shown in Table II-2. TaqMan Universal PCR Master Mix (Applied Biosystems, Paisley, UK) contained: AmpliTaq Gold DNA polymerase, dNTPs with dUTPs, passive reference and optimized buffer components. SNP Genotyping Assay Mix (Applied Biosystems, Paisley, UK) contained: Sequence-specific forward and reverse primers to amplify the polymorphic sequence of interest and two TaqMan probes: one probe labelled with VIC dye to detect the Allele 1 sequence and the other probe labelled with FAM dye to detect the Allele 2 sequence.

A standard 2h program was used for TaqMan PCR (Table II-3), as recommended by the manufacturer (Applied Biosystems, Paisley, UK). After PCR amplification, an end-point plate read was performed. The Sequence Detection System (SDS) software uses the fluorescence measurements made during the plate read to plot fluorescence values based on the signals from each well. The plotted fluorescence signals indicate which alleles are in each sample.

The genotype and allelic frequencies, deviations from Hardy-Weinberg equilibrium and inter- breed comparisons of allelic frequencies were analysed by Chi-square test, as implemented in GraphPad Prism (Version 6). The Ewens-Waterson test of neutrality was performed using Arlequin (Version 3.0) [189].

Table II-1. The sequences of primers used for *IL8* promoter sequencing and the TaqMan assay

IL8 primer application	Forward primer sequence 5' – 3'	Reverse primer sequence 5' – 3'
Promoter sequencing	TGATGGTGCCATAAAAGAACA	TCTGCTGTCCCAGAGGACTT
TaqMan assay	ATGACTCAGATGTGCTCTCAAAGG	GCTTGCATCATGTCAGAGGAAATTC

Table II-2. Components used in the TaqMan Genotyping Assay

Component	Volume (µl/well in 96 well plate)
TaqMan Universal PCR Master Mix (2X), No AmpErase UNG	12.5
20X working stock of SNP genotyping assay	1.25
DNA	11.25
Dnase – free water	-
Total volume per well	25

Table II-3. Cycling conditions used in the TaqMan Genotyping Assay

Pre PCR stage	AmpliTaq Gold Enzyme Activation	PCR (40 cycles)		Post PCR read
HOLD	*HOLD*	*Denature*	*Anneal/Extend*	*HOLD*
30 sec at 60°C	10 min at 95°C	15 sec at 92°C	1 min at 60°C	30 sec at 60°C

II. 5. Genome-wide association study [1]

II.5.1. Genotypes

IL8 promoter SNP rs110291328 (A/G) was selected for genotyping as this mutation is a potential causative variant with multiple regulatory elements located in close proximity to the SNP. This SNP was added to the research component of the custom International Dairy and Beef (IDB) SNP chip for cattle breeding [190]. Genotypes were obtained from the Irish Cattle and Breeding Federation (ICBF). A total of 3310 Holstein-Friesian cows with IDB SNP call rates $\geq$ 90% were available for analysis.

II.5.2. Phenotypes

The phenotypes for somatic cell score (SCS) were expressed as predicted transmitting abilities (PTA) and were based on genetic evaluations carried out by the Irish Cattle Breeding Federation (ICBF). These PTA phenotypes for average SCS are estimated in Ireland using a repeatability animal model across the first five lactations according to the protocol of Berry *et al.* [48]. The SCS phenotype was checked for normality using the R statistical software [191]. Prior to analysis phenotypes were deregressed and parental contributions removed following the previously described protocol [192].

II.5.3. Statistical analysis

The associations between the SNP and calving interval and somatic cell score were quantified using a single SNP regression approach. The dependent variable (i.e., the trait expressed as a PTA) was regressed on each SNP individually in a mixed model (EMMAX) [193]. Each SNP was fitted as a fixed, continuous effect. The individual animal was fitted as a random effect with relationships among animals accounted for using the genomic relationship matrix (GRM) based on 16,488 SNP genotypes on the IDB [194]. Correction for multiple testing was performed using the Bonferroni correction.

[1] Performed by Dr. Michael Mullen

II. 6. Monocyte separation and macrophage differentiation

Blood (60 ml) was collected from the jugular vein, in tubes containing acid citrate dextrose anticoagulant. Peripheral blood mononuclear cells (PBMC) were separated using Leucosep tubes (15 ml of blood per tube) and Histopaque 1077 (15 ml per tube) (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland), as a separation medium. The blood was centrifuged at 1200 × g for 20 min, the buffy coat was collected and transferred to a new 50 ml tube. Cells were pelleted by centrifugation at 400 × g for 10 min. Any red blood cells carried over during the separation process were lysed by resuspending the cells in 0.25% NaCl. Two washing steps in 15 ml of 1× phosphate-buffered saline (PBS) (Gibco®, Paisley, UK) were performed to eliminate any impurities. The cells were resuspended in 15 ml complete medium.

Twenty microliters of the cell suspension was added to 180 µl of PBS (20 fold dilution) and 10 µl was added to the haemocytometer slide for counting. The cells were counted in all four squares and the average count was calculated across the four squares, multiplied by 20, and then multiplied by 10,000 to obtain the number of cells per mililiter. The cells were placed in 75 cm^2 flasks (15 ml), and incubated at 37°C and 5% CO_2. Culture medium consisted of RPMI 1640, 10% foetal calf serum, 1% Penicillin/Streptomycin and 1% non-essential amino acids (Gibco®, Paisley, UK). Cells were incubated for 3h to let monocytes adhere. Then, all the contaminating lymphocytes were removed by washing the flasks twice with 1× PBS. Adhered monocytes were cultured for 7 days to allow monocytes to differentiate into macrophages. Culture medium was changed every 2 days. At day 7, fully differentiated monocyte-derived macrophages were detached from the flask bottom by treating them with 5 ml 0.25% Trypsin-EDTA (Gibco®, Paisley, UK) for 5 min followed by the addition of 10 ml complete medium in order to stop the action of trypsin. Cells were centrifuged at 400 × g for 10 min and resuspended in 5 ml complete medium. Then, the cells from each animal were counted on the haemocytometer and plated in 12-well plates at the density of 5 x 10^4 cells per well and left overnight.

The following day, the cells were treated with lipopolysacharide (LPS) (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland), lipoteichoic acid (LTA) (Sigma-Aldrich Ireland

Ltd, Wicklow, Ireland), and recombinant bovine tumour-necrosis factor alpha (TNFα) (Kingfisher Biotech, Inc., Oxfordshire, UK). In the time-course experiment, three concentrations of all three stimuli were tested: 0.05, 0.2 and 1 μg/ml LPS, 1, 5 and 10 μg/ml LTA and 25, 50 and 100 ng/ml TNFα and sampled at multiple time points (0, 2, 6, 10 and 24h), over 24h time-course. The stimulations were carried out by adding the media supplemented with LPS, LTA and TNFα. At the end of each time point, a sample of medium from each treatment (1 ml) was collected and stored at -20°C. Trizol (Invitrogen, Paisley, UK) (500 μl per well) was used to lyse the adhered macrophages, the lysates were vortexed and stored at -80°C.

II. 7. Flow cytometry

Purity and viability of the bovine monocyte-derived macrophages were analysed by flow cytometry, on the Attune® Acoustic Focusing Cytometer (Applied Biosystems, Paisley, UK). Cell suspension (1 million cells in 300 μl) was aliquoted into round-bottom wells of a 96-well plate and washed twice in ice cold 1% bovine serum albumin (BSA) (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland) in PBS by centrifugation at 500 × g for 3 min and resuspending in 300 μl in PBS (1% BSA). Monocyte specific mouse anti-bovine CD14 (1/500), lymphocyte specific mouse anti-bovine CD3 (1/500) and the isotype control mouse anti-bovine IgG_1 (1/500) (Washington State University, Pullman, WA, USA) were used as primary antibodies. The cells in three wells were left unlabeled and were used for propidium iodide (PI) (Molecular Probes, Paisley, UK) viability staining, negative unstained control and negative control treated with the secondary antibody only. Cells were incubated for at least 30 min at 4°C in the dark. Following incubation with the primary antibodies, cells were washed three times in 300 μl in PBS (1% BSA). Goat anti-mouse secondary antibody labelled with fluorescein isothiocyanate (FITC) (AbD Serotec, Kidlington, Oxford, UK) (1/100) was added to cells and incubated for at least 30 min at 4°C in the dark. After incubation, the cells were washed three times in 300 μl in PBS (1% BSA) and analysed by collecting 10,000 events from each sample.

II. 8. RNA extraction, cDNA synthesis and primer design

RNA was extracted using Trizol and RNeasy Plus Mini Kit with on-column DNA digestion (Qiagen Ltd, Crawley, UK). Chloroform (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland) (200 µl per ml of Trizol) was added into the cell lysates and mixed vigorously before centrifuging at 12 000 × g for 15 min, at 4°C. The aqueous layer containing RNA was transferred to another tube and mixed with the same volume of ethanol (≈500 µl). This solution was then added to the RNeasy column and processed according to manufacturer's instructions (Qiagen Ltd, Crawley, UK). The quantity of the purified RNA was measured using NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). The quality of RNA was assessed on Agilent 2100 BioAnalyzer using Agilent RNA 6000 Nano Kit according to the manufacturer's instructions (Agilent, Wharfedale Road, Wokingham, Berkshire, UK). The samples were stored at -80°C.

The High-Capacity cDNA Reverse Trancription Kit (Applied Biosystems, Warrington, UK) was used to convert 500 ng of RNA into cDNA as per manufacturer's instructions. The reverse transcription process was carried out under the following conditions: 10 min at 25°C, 120 min at 37°C and 5 min at 85°C. The stock cDNA was stored at -20°C.

The reference gene DNA sequences were extracted from the UCSC Genome Browser and the primers were designed using Primer 3. All the primers were checked to span introns in order to prevent amplification of contaminating genomic DNA. The specificity was confirmed by Primer Blast (NCBI) and the primers were commercially synthesised and resuspended in Rnase, Dnase free water to obtain 100 µM concentration (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland). A list of all primers used for RT-qPCR is presented in Table II-4. The primer mix was made using 225 µl Rnase, Dnase free water and 12.5 µl of both forward and reverse primers, to obtain 5 µM concentration of each primer.

Table II-4. List of genes and corresponding primer pairs used in the RT-qPCR

Gene symbol	Forward primer sequence 5' – 3'	Reverse primer sequence 5' – 3'
PPIA	CCACCGTGTTCTTCGACAT	TCTGTGAAGCAGGAACCTTT
H3F3A	CATGGCTCGTACAAAGCAGA	ACCAGGCCTGTAACGATGAG
GAPDH	CTCCCAACGTGTCTGTTGTG	TGAGCTTGACAAAGTGGTCG
ACTB	AGATGACCCAGATCATGTTCGA	TGACCCCGTCACCGGAGTCCATCACGAT
MRPS6	CGCACGCTTCCCTATAAGAT	TTCACTTCCTGGGTCAGAGG
TLR2	GGTTTTAAGGCAGAATCGTTTG	AAGGCACTGGGTTAAACTGTGT
TLR4	CTTGCGTACAGGTTGTTCCTAA	CTGGGAAGCTGGAGAAGTTATG
TNFRSF1A	GTGGAGATTTCGCCTTGTGT	TTGGCGCCTTTAAGAAAGAA
TNFRSF1B	CAGGCTGTGTTTACCCCCTA	TGTCCAAGGTCATGTTGCAT
IL8	TGCTCTCTGCAGCTCTGTGT	TGTGGCCCACTCTCAATAACT
IFNG	TTGAATGGCAGCTCTGAGAAA	TCTCTTCCGCTTTCTGAGGTT
IL1B	CCCTGCAGCTGGAGGAAGTA	CTTCGATTTGAGAAGTGCTGATGT
TNFA	CCATCAACAGCCCTCTGGTT	TCACACCGTTGGCCATGA
CD62L	GCTCATCATGGCACCGATTG	TCAGGTATTCGATCTCTCCCT
CD18	AGCGACCTCAGGGAGTACCAT	GTCGTGGTGGCACTCTTGAAA
ELANE	CCTGAACTTTCGATCGGTGC	GGCCATCCTATTGAGCTGGA
CXCR2	GTCGTCATCTATGCCCTGGT	GCCAGGTTCAGCAGGTAGAC
CXCL6	GGACCCAGAAGCTCCTTTGA	TGCTGAAGAACTTGGGCAAC
CXCL3	GCTTGTCTCAACCCTGAAGC	CCTCTAGGTCAGTTGGTGCT

II. 9. Quantitative Real Time PCR (RT-qPCR)

Quantitative RT PCR (RT-qPCR) was performed on Applied Biosystems 7500 Fast Real-Time PCR System, using SYBR Green intercalating DNA dye, according to the manufacturer's instructions (Applied Biosystems Ltd, Warrington, UK). The reaction mix consisted of: 10 µl SYBR Green (Applied Biosystems Ltd, Warrington, UK), 1.5 µl of the primer mix (375 nM), 6.5 µl Rnase, Dnase free water and 2 µl cDNA. Cycling parameters of the RT-qPCR program were: 95°C for 20 sec, 40 cycles of 95°C for 3 sec, 60°C for 30 sec followed by dissociation steps (95°C for 15 sec, 60°C for 60 sec, 95°C for 15 sec and 60°C for 15 sec). The melt curve produced for each PCR product was examined for the amplicon specificity represented by the correct melting temperature and a single peak. A negative control was included on each reaction plate.

The reaction efficiency for each primer pair was calculated using a two-fold dilution series on a pooled cDNA sample, obtained by pooling 2 µl of cDNA from each sample in a single eppendorf tube. The standard curves were represented as the semi-log regression line plot of Ct value vs. log of the relative input cDNA concentration. Then, the efficiencies were calculated using the following formula:

$$E = (10^{-1/Slope}-1) \times 100$$

The efficiencies between 85% and 110% were considered acceptable and primers with efficiencies within these limits were included. Before calculating the relative expression levels on the samples, a validation experiment was carried out in order to validate that the efficiency of the target amplification and the efficiency of the reference amplification are approximately equal. The Ct values generated from both target and reference gene standard curves were used in the ΔCt calculation ($C_{t\ target} - C_{t\ reference}$), and were plotted against the log input amount. In a validation experiment that passes, the absolute value of the slope of ΔCt vs log input is <0.1. The software package GenEx 5.2.1.3 (MultiD Analyses, Gothenburg, Sweden) was used for RT-qPCR analyses. The expression of five potential normaliser genes (*PPIA*, *H3F3A*, *GAPDH*, *ACTB* and

MRPS6) was analysed using geNorm tool implemented in GenEx software and ranked according to the expression stability across treatments [195]. The amount of target, normalized to an endogenous reference and relative to a calibrator, was: $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in Ct between target and reference and $\Delta\Delta Ct$ is the difference in ΔCt between all the samples and the calibrator, the sample with the lowest expression [196].

The differences in expression levels between treatments were analysed by two-way ANOVA and Student's t-test as implemented in GraphPad Prism (Version 6).

II. 10. ELISA

Supernatants from treated cells were used for protein detection by ELISA according to manufacturer's instructions (R&D Systems, Abingdon, Oxon, UK). Levels of IL8 were measured using human IL8 ELISA kit (R&D Systems, Abingdon, Oxon, UK), previously validated in cattle [83, 197] or bovine IL8 ELISA kit (USCN Business Co., Ltd.; Wuhan, China). Levels of other cytokines were quantified using bovine specific ELISA antibodies, according to the manufacturer's instructions: TNFα, IL6, IL1β (Thermo Scientific, Paisley, UK) and IL17 (Kingfisher Biotech, Inc., Oxfordshire, UK). The absorbance levels were measured on a GloMax Multiplate Reader (Promega UK Ltd., Southampton, UK). The absorbance level at 560 nm was used to normalize the target specific absorbance at 450 nm. The data was analysed using a 4-parameter logistic model and the differences between treatments were analysed using a two-way ANOVA and the Sidak post-test or Student's t-test as implemented in GraphPad Prism (Version 6).

II. 11. *IL8* promoter cloning

Full (2.1 kb upstream from transcription start site) and 4 deletion (1.2 kb, 883 bp, 400 bp and 196 bp, upstream from transcription start site) *IL8* promoter fragments from both IL8 haplotypes were previously cloned into pGL4.17 luciferase vector (Promega

UK Ltd., Southampton, UK) and purified [187]. The plasmids were transformed into OneShot chemically competent *E. coli* cells following the manufacturer's instructions (Invitrogen Ltd., Paisley, UK). Plasmids were purified from overnight cultures using a Plasmid Endo-free Mini Kit (Invitrogen Ltd., Paisley, UK) and eluted in endotoxin free TE buffer. The DNA concentrations were determined by spectrophotometry (Nanodrop Technologies Inc., Wilmington, DE, USA).

Plasmids were run on 1% agarose gel to confirm that the size was correct and then sequenced to verify the correct insertion (Source Bioscience, Dublin, Ireland). Forward primer (RV primer 3) anneals at the 5' upstream region of the *IL8* construct insertion position: 5548 bp – 5567 bp in the pGL4.17 luciferase vector, whereas the reverse primer anneals at the 3' end of the inserted *IL8* promoter.

II. 12. Bovine endometrial epithelial cell transient transfection, cell stimulations and luciferase assay

The bovine endometrial cell line (BEND) was purchased from ATCC (LGC Standards, U.K.) and cultured in 75 cm^2 flasks in 5% CO_2, in a 37°C incubator. Complete growth medium consisted of 1:1 mixture of Hams F12 medium and minimal essential medium MEM Glutamax™ (Gibco®, Paisley, UK), with 34 mg/ml D-Valine (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland), 10% heat-inactivated foetal calf serum (Gibco®, Paisley, UK), 10% heat-inactivated horse serum (Gibco®, Paisley, UK) and 1% Pen/Strep (Gibco®, Paisley, UK).

BEND cells were subcultivated 2-3 days before seeding. Cells were seeded at a density of 3×10^4 in 12-well plates, and allowed to adhere for 24-48 hours. All transient co-transfections were carried out in 6 replicates using 100 ng of each of the *IL8* promoter constructs together with 10 ng of a renilla control vector (pGL4.74) (Promega UK Ltd., Southampton, UK), using 1.5 µl of GeneJuice transfection reagent (Novagen, Merck Biosciences Ltd., Nottingham, UK) according to the manufacturer's instructions. The transfection mixture was removed after 24h and replaced with either complete growth media or media supplemented with serial dilutions of LPS (0.2, 2 and 20 µg/ml), LTA (1, 5 and 20 µg/ml) and TNFα (20, 50 and 100 ng/ml), for 5h. Medium was removed, cells were washed with PBS and lysed by addition of 250 µl per well of 1×

Passive Lysis Buffer (Promega UK Ltd., Southampton, UK)) and left on the horizontal shaker for 15 min. Luciferase and renilla levels were measured using Dual Luciferase Reporter Assay System on a GloMax Multiplate Reader (Promega UK Ltd., Southampton, UK), with automatic injectors using 20 µl cell lysate and set to inject 100 µl of luciferase assay and Stop and Glo reagent (Promega UK Ltd., Southampton, UK). The expression levels were expressed as the ratio between luciferase and renilla signals. The data was log transformed and analysed using a two-way ANOVA and the Sidak post-test, as implemented in GraphPad Prism (Version 6).

II. 13. Octamer-binding protein 1 gene knockdown using RNA interference

II.13.1. Oct-1 siRNA transfection

One day before transfection, BEND cells (2×10^4 per well) were plated in 12 well plates, in 1 ml growth medium without antibiotics, as they reduce transfection efficiency. On the following day, siRNA (Santa Cruz Biotechnology, Heidelberg, Germany) - Lipofectamine 2000® (Life Technologies, Paisley, UK) complexes were prepared; the same procedure was carried out for bovine Oct-1 siRNA and scramble control transfection:

- Forty picomoles of siRNA (4 µl) was diluted in 100 µl Opti-Mem® I Reduced Serum Medium without serum (Life Technologies, Paisley, UK), and mixed gently.
- Lipofectamine 2000® (2 µl) was diluted in 100 µl of Opti-Mem® I Reduced Serum Medium without serum. The solution was mixed gently and incubated for 5 min at room temperature.
- After the 5 min incubation, the diluted oligomer was combined with the diluted Lipofectamine 2000®. The solution was mixed gently and incubated for 20 min at room temperature.
- Oligomer- Lipofectamine 2000® complex (206 µl per well) was added to each well containing cells and medium and the plate was rocked back and forth.

- The cells were incubated at 37°C for 24h.
- The medium (1 ml) was sampled, 250 µl of Trizol was added to each well to lyse the cells. The RNA was extracted and *Oct-1* gene knockdown was assessed using RT-qPCR.

II.13.2. *Oct-1* siRNA and *IL8* haplotype construct co-transfection

One day before transfection, BEND cells (5×10^4 per well) were plated in 12 well plates, in 1 ml growth medium without antibiotics. On the following day, siRNA (Santa Cruz Biotechnology, Heidelberg, Germany) – Plasmid - Lipofectamine 2000® (Life Technologies, Paisley, UK) complexes were prepared; the same procedure was carried out for bovine *Oct-1* siRNA and scramble control transfection:

- Plasmid (200 ng) and siRNA (20 pmol) were diluted in 100 µl Opti-Mem® I Reduced Serum Medium without serum and mixed gently.
- Lipofectamine 2000® (3 µl) was diluted in 100 µl of Opti-Mem® I Reduced Serum Medium without serum. The solution was mixed gently and incubated for 5 minutes at room temperature.
- After the 5 minute incubation, the diluted plasmid-siRNA was combined with the diluted Lipofectamine 2000®. The solution was mixed gently and incubated for 20 minutes at room temperature.
- Oligomer- Lipofectamine 2000® complex (210 µl per well) was added to each well containing cells and medium and the plate was rocked back and forth.
- The cells were incubated at 37°C for 24h and the luciferase assay was performed.

The effect of the *Oct-1* knockdown on the *IL8* promoter activity in the two *IL8* haplotypes was investigated using the Student's t-test as implemented in GraphPad Prism (Version 6).

II. 14. *In vivo* LPS challenge

The experimental design is presented in Figure II-1. The potency of the experimental LPS dose was tested on a single calf first, before a panel of (n=87) four week old Holstein-Friesian bull calves on a local farm were genotyped using *IL8* TaqMan assay and 10 animals of each haplotype were selected for LPS challenge. At

each sampling time-point, two 9 ml vacutainers of blood were taken by neck venipuncture from each animal: one vacutainer for serum separation and haematology; and one Tempus® tube for RNA extraction. The first tube was left 30 minutes to allow clotting, centrifuged at 1000 × g for 10 min, serum was separated and stored at -20°C. The haematology analysis was carried out using an ADVIA 2120 and included both absolute blood cell numbers and percentages. Tempus tubes were inverted 5 times, stored at -20°C and RNA was extracted according to the manufacturer's instructions (Applied Biosystems Ltd, Warrington, UK).

The data was log transformed when necessary. All the data from the *in vivo* LPS challenge, such as leukocyte abosolute counts, cytokine gene expression data and cytokine protein concentrations, was analysed using repeated measures ANOVA with hour post-challenge, *IL8* haplotype and the interaction between the hour post-challenge and *IL8* haplotype used as explanatory variables. Pair-wise comparisons between the haplotypes, at each time-point were carried out using the Sidak post-test.

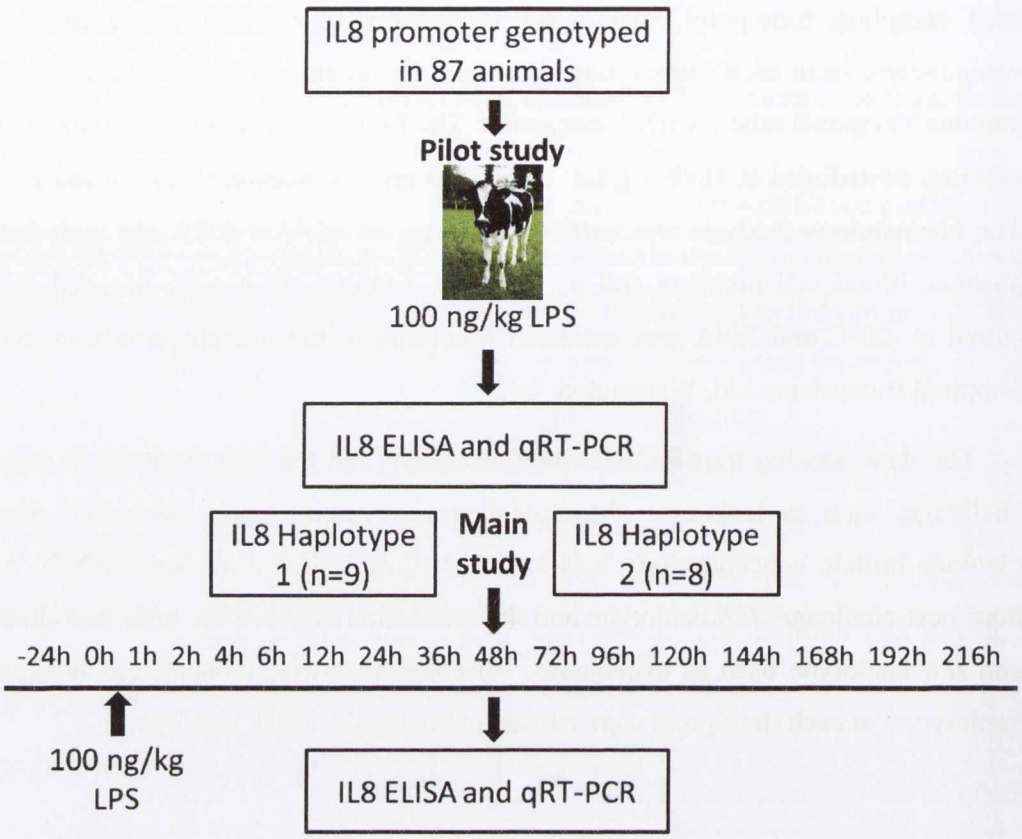

Figure II-1. Experimental design for the *in vivo* LPS challenge of Holstein Friesian calves.

In the pilot study, a calf was challenged with 100 ng/kg LPS, followed by haematology analysis. Then, a larger group of animals was challenged with 100 ng/kg LPS and serum and whole blood samples were collected over a 216h time-course, followed by IL8 gene and protein expression analysis.

II. 15. Bovine neutrophil separation using magnetic-activated cell sorting (MACS)

II.15.1. Magnetic labeling

Blood was obtained by jugular venipuncture and collected in acid citrate dextrose vacutainers. The content from 2 vacutainers (16 ml) was transferred to a 50 ml tube and a high-yield lysis buffer (Life Technologies, Paisley, UK) was added to make up 50 ml. Blood was incubated at room temperature for 10 min, with occasional mixing to facilitate the lysis. Then, the blood was centrifuged at 300 × g for 10 min and washed once in 5 ml of MACS buffer containing PBS, pH 7.2, 0.5% bovine serum albumin (BSA), and 2 mM EDTA (Sigma-Aldrich Ireland Ltd, Wicklow, Ireland). The cells were counted using an Attune® Acoustic Focusing Cytometer (Applied Biosystems, Paisley, UK). Granulocyte-specific mouse anti-bovine G1 primary antibody of IgG1 isotype (Washington State University, Pullman, WA, USA), 500× diluted; was added and incubated for 15 min at 4°C. Following incubation with the primary antibody, cells were washed using the MACS buffer and resuspended in 80 µl of buffer per 10^7 total cells. Twenty microliters per 10^7 total cells of anti-mouse IgG1 Microbeads (Miltenyi Biotec, Bisley, Surrey, UK) was added, and the cells were incubated for 15 min at 4°C. Goat anti-mouse secondary antibody labelled with fluorescein isothiocyanate (FITC) (AbD Serotec, Kidlington, Oxford, UK), 100× diluted was added to cells and incubated for an additional 5 min at 4°C in the dark. After incubation, the cells were washed in MACS buffer and resuspended in 500 µl of MACS buffer.

II.15.2. Magnetic separation

Columns were placed in the magnetic field of the magnetic separator and prepared by rinsing with 3 ml of MACS buffer. The cell suspension (500 µl) was added onto the column and the flow-through containing unlabelled cells was collected. The column

was washed three times with the 3 ml of MACS buffer, removed from the separator and placed in a suitable collection tube. Finally, 5 ml of MACS buffer was added onto the column and magnetically labelled cells were flushed out by pushing the plunger into the column. Both positively and negatively selected cells were analysed by collecting 10 000 events in the granulocyte gate. The cells were resuspended in RPMI at 1×10^7 cells/ml.

II. 16. Neutrophil functional assays

II.16.1. Chemotaxis assay

The cell migration was quantified in the 24 well plate using hanging inserts (Boyden chamber) with 3 μm pore-sized filters (Merck Millipore, Tullagreen, Ireland). The following solutions were used to induce chemotaxis: RPMI alone, RPMI supplemented with the increasing recombinant bovine IL8 (Kingfisher Biotech, Inc., Oxfordshire, UK) concentration (1, 10, 50, 100 ng/ml) or blood serum. The test solution (300 μl) was pipetted into each well and neutrophils (250 μl, 2.5×10^6 cells) were added to the hanging insert. The plates were incubated for 1h at 37°C. The media from the bottom wells were collected; the adhered neutrophils were removed by trypsin and pooled with the media. The cells that migrated through the filter were counted using flow cytometry. The data was initially expressed as the number of neutrophils per μl and then transformed to fold change, obtained by dividing the number of cells that migrated towards the chemoattractant by the number of cells that migrated towards the RPMI alone.

The statistical analysis was conducted using the Student's t-test as implemented GraphPad Prism (Version 6).

II.16.2. Neutrophil killing assay

Two strains of *Staphylococcus aureus* were used in the assay: human *S. aureus* SH1000 and bovine *S. aureus* MOK032. Bacteria were streaked on an agar plate a day before the assay. Neutrophils were resuspended at 1×10^7 cells per ml. Using a sterile inoculating loop a small amount of bacteria was scraped from the agar plate and dispersed in 6 ml of PBS. The suspension was vortexed and 600 µl was transferred to a cuvette to check the OD at 600 nm. The bacteria concentration was adjusted until OD reached 0.45 which is the equivalent of approximately 4×10^8 CFU/ml. Log 5 and log 6 dilution of the suspension were plated on agar plates to confirm the bacterial concentration. Neutrophils were resuspended at 1×10^7 cells per ml and infected using the following mix:

- 250 ul neutrophils (2.5×10^6 cells)
- 50 ul bovine serum
- 50 ul bacteria
- 150 ul RPMI

Also, tubes with no neutrophils were prepared to allow calculation of the percentage of killing in the presence of neutrophils. Each treatment was performed in duplicates. The tubes were incubated at 37°C for 2h in the shaking incubator. Finally, each tube was diluted in distilled water to allow neutrophil lysis, a five point serial dilution was made and log 3, 4 and 5 samples were spread on agar plates. Plates were left overnight at 37°C and colony forming units (CFU) were counted. The percentage killing of bacteria was determined by comparing the number of total CFU in the presence of neutrophils to the number of CFU in medium only.

The statistical analysis was conducted using the Student's t-test as implemented in GraphPad Prism (Version 6).

Chapter III Population genetic analysis of *IL8* promoter haplotype in a range of bovine breeds and association with the somatic cell score and calving interval

III. 1. Introduction

Different economic traits of interest in dairy and beef cattle have driven divergent selective pressure resulting in breed-specific allele frequencies. Breed-specific signatures of selection have been detected across the whole bovine genome [198]. Natural selection is also involved in shaping the population genetic structure. In particular, the genes encoding molecules involved in the immune response are under constant selective pressure by pathogens which can create differences in immune gene diversity among bovine breeds. The evidence of selection has been reported in a number of loci within genes regulating the immune response in cattle, such as *IL13, IL5, IL2, TLR4* [40, 42]. The promoter regions of immune genes are of particular importance as mutations in these regions can affect transcription factor binding, the rate of transcription and ultimately influence the amount of cytokine being produced. The promoter region of the human *IL8* gene has been well studied. The European human population is characterised by an unusual *IL8* haplotype distribution which is dominated by only two haplotypes and displays a possible signature of selection at this locus [199]. A common variant at position -251 bp has previously been shown to affect the level of *IL8* expression and has been associated with susceptibility to diarrhea [184], tuberculosis [183] and bronchiolitis [185], in human. A fewer studies have investigated bovine IL8 promoter compared to the human ortholog. Meade *et al.* sequenced the bovine IL8 promoter region and demonstrated a high level of variation, with 29 polymorphic sites within 2.1 kb upstream of the transcription start site. A high level of linkage among these variants was also found, as demonstrated by the existence of only two IL8 promoter haplotypes in the Irish cattle population. In addition, these two haplotypes showed differential promoter activity in response to the proinflammatory stimuli. *In silico* analysis of the two IL8 promoter haplotypes has identified a number of tracription factors binding in a haplotype-specific manner. The SNP rs110291328 (A/G), located in the core IL8 promoter region, is a putative causative variant as this variant is in a close proximity to a number of transcription factor binding sites [187].

IL8 is a likely target for selection in cattle as this gene is involved in neutrophil recruitment to mammary gland and uterus, sites of particular economic importance in

cattle. The mammary gland and the uterus in dairy cows are particularly susceptible to bacterial infection. Neutrophil driven tissue damage is a regular consequence, as mammary gland and uterus form interfaces with the external environment. In addition, the time around calving and early lactation is a period of increased susceptibility to infection when these tissues are exposed to diverse microbes including possible pathogens. Neutrophil migration into the mammary gland, accompanied by the release of lysosomal enzymes and oxygen species can damage mammary epithelium which has negative consequences on milk yield [200, 201]. Neutrophil recruitment is also an important component of the immune response in endometritis, which is associated with reduced cow fertility [202]. Therefore the genes involved in initiating the early immune response and neutrophil recruitment are likely to have an impact on phenotypic traits related to mammary health and fertility, such as somatic cell count and calving interval. These two traits are of great importance for the Irish dairy industry and they have been included in the most recent version of the economic breeding index. Calving interval has the highest trait emphasis in the overall index of 24%, whereas SCC has the third highest negative economic weight of -€43.49, after mastitis and lameness [203]. *IL8* is a good candidate gene for association with these traits, as this chemokine is the crucial factor in mastitis driven neutrophil recruitment to the mammary gland [19]. In addition, the neutrophil migration that occurs upon bacterial contamination and tissue damage of uterus postpartum is mediated via IL8 [204, 205].

A number of *IL8* association studies using phenotypic traits such as milk yield and composition, and SCC have been carried out to date. Genetic variants in the *IL8* gene body and promoter have been linked with SCC in two studies, using Chinese HF population [37, 206]. In addition, a number of studies have identified associations between quantitative trait loci in the *IL8* chromosomal region and mastitis [207-209], a condition that displays moderate to high correlation with SCC. In contrast, the association between *IL8* and fertility traits such as calving interval, has not been extensively explored. Previous studies using a genome wide approach to target genomic regions that have an impact on calving interval did not report any associations in the region surrounding *IL8* locus [210, 211].

A previous study characterising the two IL8 promoter haplotypes in Irish cattle, has identified significant breed differences in haplotype frequencies using populations of HF, Norwegian Red, Montbelliarde, Angus Aberdeen and Charolais [187]. In the

present study, a larger sample was employed to validate these findings on an additional population of HF and Charolais, as well as to quantify the IL8 promoter haplotype frequencies in additional dairy and dual purpose breed populations such as Jersey and Shorthorn. Also, the IL8 promoter was genotyped in the Brahman breed population, which is of *Bos indicus* origin, in contrast to all the other breeds included in the present and the previous study, which are of *Bos taurus* origin.

Also, the objective was to conduct an association study between the IL8 promoter haplotypes and SCC and calving interval, the traits of great economic importance for Irish dairy sector. Even though the genetic association between IL8 promoter variants and SCC has been demonstrated in the Chinese HF populations, this is the first IL8 promoter targeted association study using SCC in Ireland.

It is hypothesised that the variants in the *IL8* promoter display divergent allele frequencies between breeds and are associated with the somatic cell count and calving interval in Irish HF cattle.

III. 2. Aims

The aims of these studies are two-fold:

1. To investigate the frequency of *IL8* promoter haplotypes across the range of economically important breeds, including both dairy and beef cattle breeds.
2. To characterise the association between the *IL8* promoter variant and calving interval and somatic cell count in Irish HF cattle.

III. 3. Results

III.3.1. Pedigree analysis

Before carrying out the IL8 promoter genotyping, it was necessary to perform a pilot two-generation pedigree analysis, to ensure that the animals used in the study originated from multiple sires. This is of particular importance as the Irish HF herd is characterised by an extensive use of a small number of sires which has led to a significant reduction of the effective population size and an increase of inbreeding over time [212]. Therefore, in order to preclude the analysis of a large sub-group of related individuals, sires were identified in the HF population. The analysis showed that 138 HF cows and 68 HF bulls originated from 41 and 22 sires, respectively. In addition, a single sire has 1-18 daughters in the HF cow population and 1-4 sons in the HF bull population. The pedigree analysis was also conducted in the Jersey population and it was shown that 66 Jersey cows originate from 20 sires with 1-10 daughters per sire (Table III-1). The pedigree analysis has not been conducted in the Charolais as the mean inbreeding level for Irish Charolais is the lowest of all the breeds. Also, the effective number of founders in this breed is the highest among the Irish breeds [212]. The pedigree information for Shorthorn and Brahman breeds was not available.

Based on these results, it was concluded that animals used in the study originate from multiple sires. The number of progeny per sire in HF as determined in the present study is lower than previously reported [212] so it was concluded that the population used in the study is representative of the overall HF population structure in Ireland and therefore suitable for *IL8* promoter genotyping.

Table III-1. The pilot two-generation pedigree analysis in Holstein Friesian and Jersey cattle.

Breed	Sex	Number of sires in the population	Number of progeny per sire
Holstein-Friesian	Male	22	1-18
	Female	41	1-4
Jersey	Female	20	1-10

III.3.2. *IL8* promoter genotyping

In order to carry out the genotyping of the *IL8* promoter, a pair of TaqMan probes were designed (Table II-1), specific for the *IL8* promoter SNP rs110291328 (A/G), located 104 bp upstream from the transcription start site (Figure III-1). This variant is within the same haplotypic block as all other *IL8* promoter SNPs as previously demonstrated [187], and it was used to determine the haplotype of each animal. The haplotypic structure was also confirmed by Sanger sequencing the *IL8* promoter region from the transcription start site to 400 bp upstream, in 10 HF and 10 Jersey animals (n=20 chromosomes). This polymorphism was genotyped in a number of animals of 5 bovine breeds (Table III-2): two dairy breeds (HF and Jersey), one beef breed (Charolais) and two dual purpose breeds (Shorthorn and Brahman). The HF breed was represented by both cow and bull populations, whereas only cows were present in all the other breeds. All breeds except Brahman are of taurine origin. A typical TaqMan allelic discrimination plot with three genotype clusters, representing two *IL8* homozygotic groups and the heterozygotes, is shown in Figure III-2. Finally, *IL8* haplotypes were assigned to each animal and haplotype frequencies were calculated for all populations.

IL8 haplotype frequencies in all breeds are shown in Table III-3. The Jersey breed was monomorphic, with only *IL8* haplotype 2 present in the population. Both *IL8* haplotypes were observed in all other breeds. Haplotype 1 was more prevalent in Shorthorn (73%), whereas haplotype 2 was dominant in Charolais (64%). HF and Brahman population showed balanced frequencies of both *IL8* haplotypes, 43% and 57% for *IL8* haplotype 1 and 2 in HF, respectively; and 58% and 42% for *IL8* haplotype 1 and 2 in Brahman, respectively.

After calculating *IL8* promoter genotype and allele frequencies for each breed, two population genetic parameters were estimated: the deviation from Hardy-Weinberg equilibrium and the signatures of positive selection at the *IL8* promoter locus. The first parameter was estimated by comparing observed and expected genotype frequencies and conducting the chi-square test as described in Materials and Methods. Results from a chi-square test for the deviation from Hardy-Weinberg equilibrium showed non-significant *P* values in all breeds. A trend towards a significant deviation was observed

in HF bulls (P=0.059) and Charolais (P=0.052). The Ewens-Watterson test of neutrality was used to determine if the IL8 promoter locus is under positive selection. No significant effect of selection was found in any of the breeds under investigation. However, a trend towards significance was observed in the HF cow population (P=0.056) and Brahman (P=0.085), as shown in Table III-4.

Finally, the IL8 haplotype frequencies were compared between each pair of breeds and breed-specific differences were observed for comparisons: Jersey vs. all other breeds (P<0.001), HF vs. Shorthorn (P<0.001), HF vs. Brahman (P<0.05), Charolais vs. Shorthorn (P<0.001) and Charolais vs. Brahman (P<0.005) (Figure III-3). The *IL8* haplotype frequencies between males and females within the HF breed were also compared. In HF bulls, haplotype 1 was dominant (67%) whereas in HF cows, haplotype 2 was more prevalent (57%). These frequencies were significantly different (P<0.01) (Figure III-4). Animals in all the other breeds were only cows.

Taken together, the breed- and sex-specific IL8 promoter haplotype frequencies as demonstrated in this study suggest possible contrasting selection pressures for health and production traits in these breeds. They also suggest a genetic association between the IL8 variants and economically important traits.

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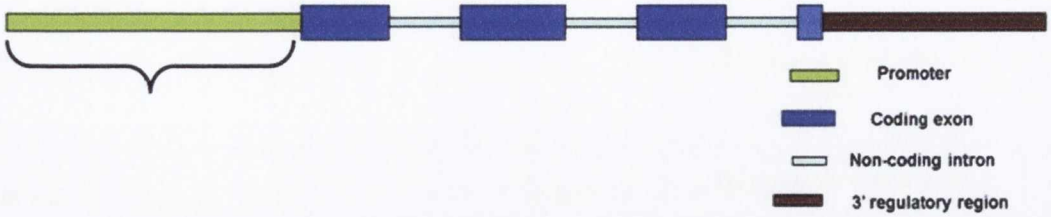

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gtgcacttgttccctcttcttacgatgatataaaagccacaggagttctctgcccaac[illegible]gtcctc

rs110291328

Figure III-1. The genetic structure of the IL8 promoter in cattle

(A) A schematic diagram illustrating the structure of the gene encoding bovine IL8, including the promoter region (green), 4 exons (dark blue), 3 introns (light blue) and the 3' regulatory region (dark red). (B) Full 2.1 kb *IL8* promoter sequence showing 29 polymorphic sites highlighted in yellow within brackets, the first nucleotide in each bracket forms *IL8* haplotype 1, the second *IL8* haplotype 2, an asterisk indicates deleted bases in that haplotype. The TaqMan primer annealing sites are highlighted in green.

Table III-2. List of the bovine breeds genotyped for the IL8 promoter haplotype.

Breed/Herd	Purpose	Number of animals	Subspecies
Holstein-Friesian (HF) bulls	Dairy	68	*Bos taurus*
Holstein-Friesian (HF) cows	Dairy	138	*Bos taurus*
Jersey cows	Dairy	66	*Bos taurus*
Charolais cows	Beef	58	*Bos taurus*
Shorthorn cows	Dual	30	*Bos taurus*
Brahman cows	Dual	50	*Bos indicus*

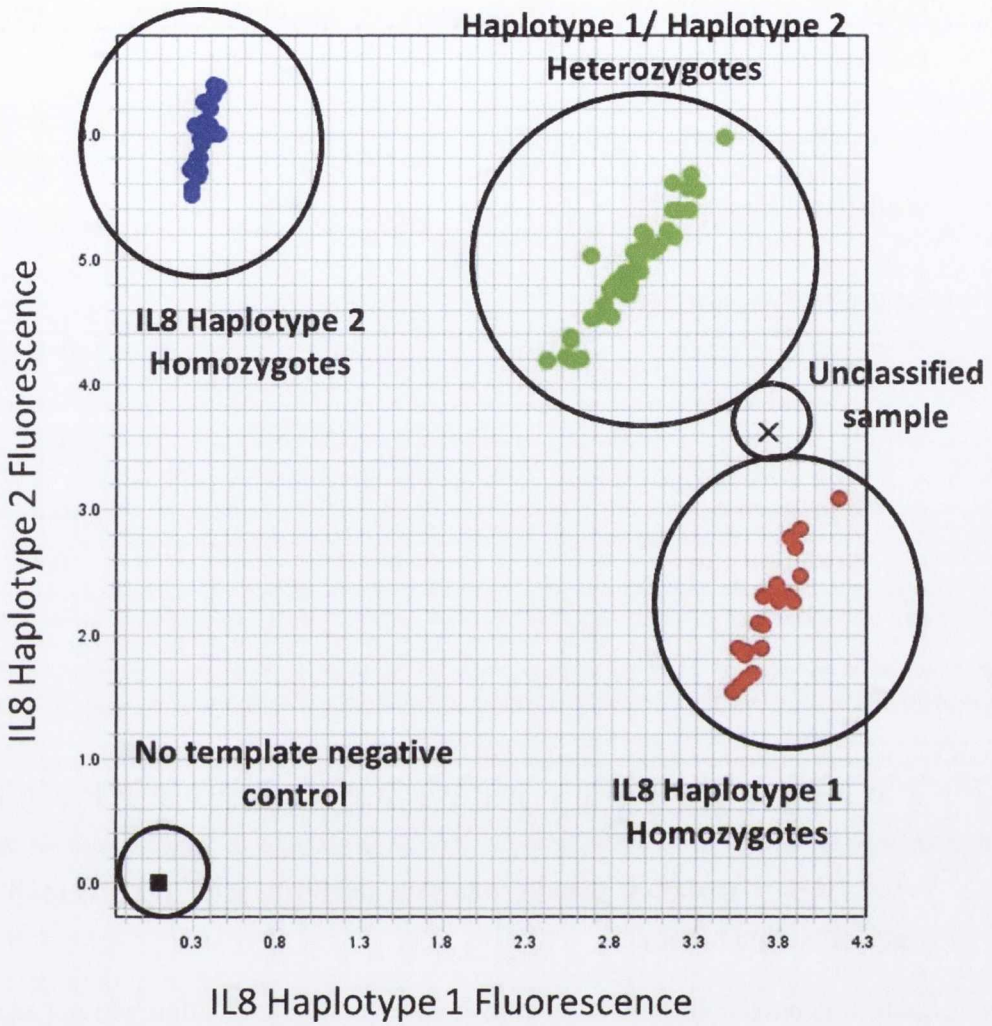

Figure III-2. Representative TaqMan *IL8* haplotype discrimination plot.

A total number of 410 animals from 5 breeds were genotyped for a SNP within the *IL8* promoter which is used to infer the *IL8* promoter haplotype. Haplotypes were assigned based on the fluorescence signals between the probes specific for *IL8* haplotype 1 and 2, shown on the horizontal and vertical axis, respectively. The animals clustered as *IL8* haplotype 1 homozygotes (red), *IL8* haplotype 2 homozygotes (blue) or heterozygotes (green). The genotype of one sample was undetermined. No signal was detected from the negative control (bottom left corner of the graph).

Table III-3. *IL8* Haplotype frequencies in the bovine breeds as determined by Taqman Assay.

Breed/Herd	*IL8* Haplotype 1 Frequency	*IL8* Haplotype 2 Frequency
Holstein-Friesian (HF) bulls	0.67	0.33
Holstein-Friesian (HF) cows	0.43	0.57
Jersey cows	0	1
Charolais cows	0.36	0.64
Shorthorn cows	0.73	0.27
Brahman cows	0.58	0.42

Table III-4. Population genetic analyses performed on rs110291328 (A/G) in a range of bovine breeds.

Each cell contains the corresponding *P*-value. Jersey population was not analysed as the *IL8* promoter locus was monomorphic in this breed.

	HF bulls	**HF cows**	**Jersey cows**	**Charolais cows**	**Shorthorn cows**	**Brahman cows**
Hardy-Weinberg equilibrium test	0.06	0.63	N.A.	0.05	0.29	0.63
Ewens – Waterson test	0.2	0.06	N.A.	0.12	0.64	0.08

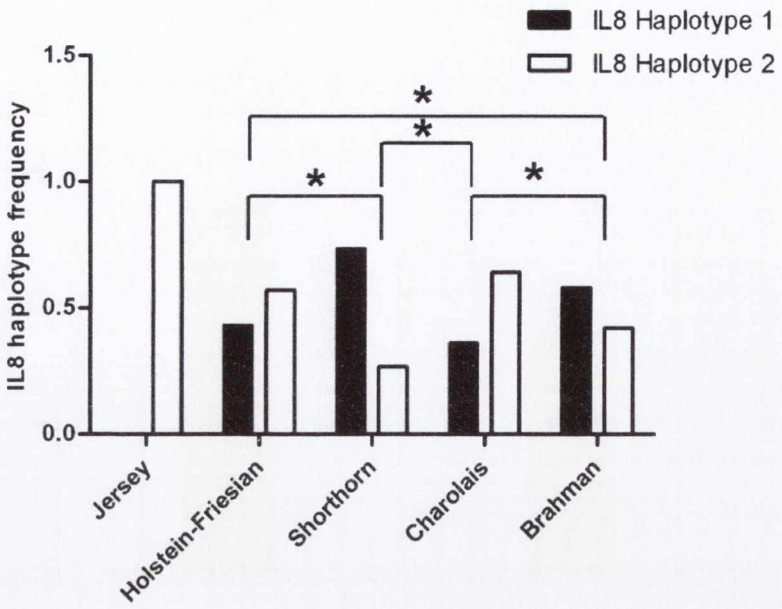

Figure III-3. Breed-specific *IL8* promoter haplotype frequencies.

A number of cows from Jersey (n=66), Holstein-Friesian (n=138), Shorthorn (n=30), Charolais (n=58) and Brahman (n=50) breeds, were genotyped for the *IL8* haplotype. The Chi-square test was used to investigate the significance of differences in the distribution of haplotypes between breeds, * $P<0.05$. Haplotype frequency distribution in Jersey was significantly different from all other breeds ($P<0.05$).

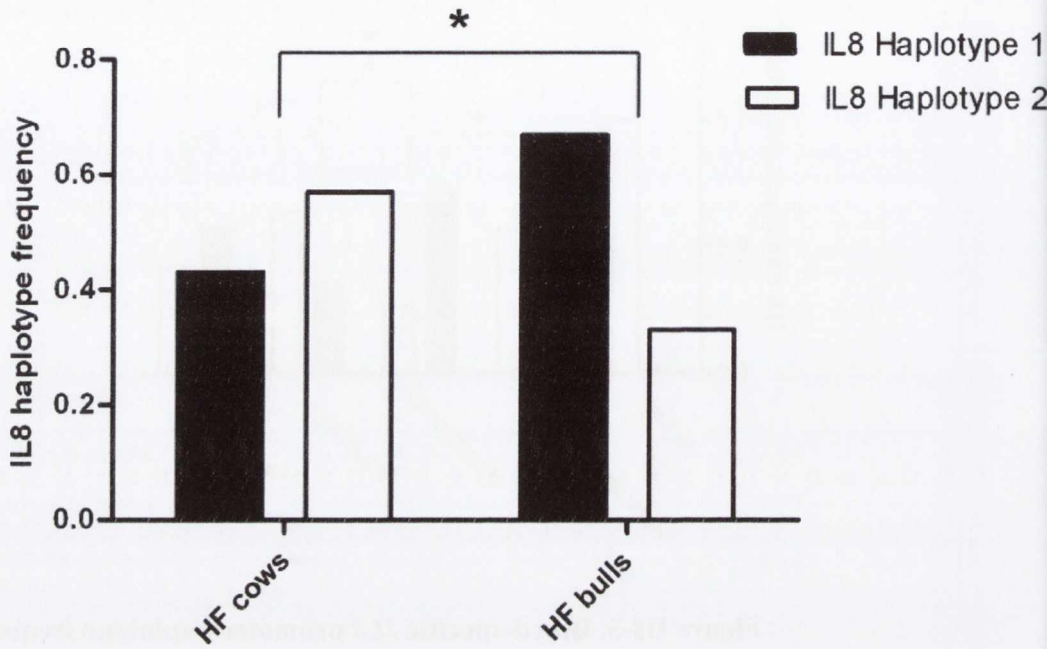

Figure III-4. Sex-specific *IL8* promoter haplotype frequencies.

A number of Holstein-Friesian (HF) cows (n=138) and bulls (n=68) were genotyped for the *IL8* haplotype and the Chi-square test was used to investigate difference in the distribution of haplotypes between sexes; * $P<0.05$.

III.3.3. Association of *IL8* promoter variant with somatic cell count and calving interval

Due to the selection trend at the *IL8* promoter in HF breed, as shown in the previous section, it was hypothesised that there is an association between the *IL8* promoter haplotype and traits of economic importance in the dairy sector, SCC and calving interval. The SNP rs110291328 (A/G), previously used for genotyping, was selected for the association study and added on to the SNP chip. Significant associations between the *IL8* promoter SNP were detected with both somatic cell score and calving interval (CIV) (adjusted $P<0.01$), with an estimated effect size of -4.956 days and 0.048 (logarithmic scale), for the calving interval and SCC, respectively (Table III-5). The allele substitution from A to G was associated with increased somatic cell score and reduced calving interval. This suggests that animals of *IL8* haplotype 1 (tagged with the A allele) show lower SCC and longer calving interval compared to animals of *IL8* haplotype 2 (tagged with the G allele). In addition, the *IL8* allele frequencies were balanced, confirming the previous results obtained using TaqMan genotyping, on a smaller HF population.

Table III-5. The summary of results from the IL8 promoter association study.
CIV – calving interval, SCC – somatic cell count

	P value	Regression Beta	Beta Standard Error	Proportion of Variance Explained	Call Rate	Minor Allele Frequency
CIV	0.003	-4.956	1.655	0.003	0.999	0.490
SCC	0.002	0.048	0.015	0.003	0.999	0.481

III. 4. Discussion

The results of this study demonstrated that the two *IL8* promoter haplotypes show divergent frequencies between bovine breeds. In addition, significant association between the *IL8* haplotype and SCC and calving interval was found. Our results suggest the existence of selection in the *IL8* promoter, particularly in the Irish HF breed, for which SCC and calving interval are economically important traits and are incorporated in the selection index.

HF population showed balanced frequencies of both *IL8* haplotypes whereas the *IL8* haplotype 2 was predominant in Charolais. The *IL8* haplotype frequencies observed for HF and Charolais are in accordance with the previous study, which demonstrated lower *IL8* haplotype 1 promoter activity in mammary epithelial cells and higher frequency of *IL8* haplotype 1 in dairy breeds [187]. It had been hypothesised that lower IL8 production could result in a lower SCC in animals of *IL8* haplotype 1, which results in a positive selection for this haplotype. This is in agreement with our findings, as *IL8* haplotype 1 was more prevalent in HF (dairy breed) than Charolais (beef breed). Further support for this hypothesis is the association of *IL8* haplotype 1 with lower SCC in Irish HF cattle, as demonstrated in our study. However, if the *IL8* haplotype 1 is beneficial in HF, why are the frequencies of the two haplotypes balanced in this breed? The balanced *IL8* promoter haplotype frequencies observed in the HF could be the consequence of the directional selection for either IL8 haplotype in different contexts. In economically important bovine breeds there is an antagonistic effect of a genetic variant on health and production traits, as a variant can be advantageous for one trait but detrimental for another trait. The evidence of balancing selection has been demonstrated at the human interleukin 10 promoter in the European population [213]. Kadri *et al.* have shown that a 660 kb deletion has a dramatic negative effect on fertility but it is still present in Nordic Red cattle at a high frequency due to its positive effect on milk yield [214]. Also, the long-range haplotype in the close proximity to the bovine *CXCL* gene cluster, where *IL8* is located, has been associated with both increased milk protein content and increased susceptibility to mastitis in Norwegian Red cattle [209]. In our study, it was shown that *IL8* haplotype 1 was associated with the increased calving interval, which is unfavourable trait in terms of reproduction and fertility. The calving interval is particularly important in the Irish dairy production system in which the time of calving

has to be synchronised with maximal grass growth and delay in calving results in significant economic loss. Therefore, the *IL8* haplotype 2, which is associated with reduced calving interval is beneficial and could be under a strong positive selection. The divergent selective pressure favouring different *IL8* haplotypes for different traits could result in the retention of both haplotypes in the population, as demonstrated in the Irish HF breed in this study.

Selecting for the low SCC could have detrimental long term effects on the Irish national herd immunocompetence, Kulberg *et al.* have demonstrated that selection for the animals with low SCC leads to a long-term reduction in the number of circulating leukocytes [60]. Suriyasathaporn *et al* have demonstrated increased odds of clinical mastitis in cows with low SCC [215]. In addition, Kremer *et al.* have identified a highly significant negative correlation between the neutrophil chemotactic response, circulating neutrophil count and the severity of clinical mastitis [216]. Animals with high milk SCC are currently culled in Ireland, which means that we could be selecting for animals with weakened immune response, inadvertently reducing the immunocompetence of the national herd. Therefore, selection for a particular trait must be complemented by the full understanding of the biology behind it and studies on the polymorphisms in the genes such as *IL8* can provide some insight.

The predominance of different *IL8* haplotypes among breeds might be manifested in the breed-dependent differences in IL8 activation and neutrophil recruitment. These differences have been demonstrated before between Jersey and HF. One finding of particular interest is the fixation of *IL8* haplotype 2 in Jersey breed, as demonstrated in our study. Bannerman *et al.* compared innate immune response between HF and Jersey and reported an earlier induction of IL8 in HF, after intra-mammary infection with *E. coli* [217]. Also, HF cows show higher number of neutrophils post-infection with *E. coli* or *S. aureus* [217, 218]. In addition, Jersey cows have higher milk SCC and lower incidence of mastitis than HF cows [219], which might suggest the possible involvement of IL8 as the *IL8* haplotype associated with an increased SCC is fixed in Jersey. However, the divergent SCC between the two breeds was not found in other publications [220, 221], which clearly suggests that other factors are also involved in neutrophil recruitment to the mammary gland. In addition to IL8, a number of other CXC chemokines, such as CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL7, also play a role in attracting neutrophils. The genes encoding these chemokines in cattle

are located in the common chromosomal region with *IL8* forming a *CXCL* cluster [222]. However, the functions of these molecules as well as the polymorphisms in their genes have not been studied extensively in cattle. Sipka *et al.* have shown that intramammary *E. coli* challenge upregulates CXCL5 expression [223], possibly recruiting neutrophils to the site of infection. Further studies on these chemokines and their genes are necessary in order to fully understand biology behind the neutrophil influx that occurs during mammary infection. One of the future studies is the characterisation of polymorphisms in all the *CXCL* genes in the cluster. Given that the haplotype length in cattle can extend for thousands of base pairs [66], it is possible that the *IL8* promoter haplotype extends further in the surrounding genes comprising a longer *CXCL* haplotype, with even more dramatic effects on the neutrophil recruitment than IL8 alone.

The genetic association between the *IL8* variants and somatic cell score has been identified in the previous studies. Chen *et al.* conducted an association study using genetic variant in the *IL8* coding region and SCC records from 610 Chinese HF cows and identified significant association [37]. In addition, the same group carried out an association study in which an *IL8* promoter SNP identical to the one used in the present study (rs110291328 (A/G)) was genotyped in 810 Chinese HF cattle and was significantly associated with SCC [206]. However, in contrast to the present study, the allelic substitution resulted in decreased SCC. Also, the frequency of this allele in the population was 61%, whereas the frequencies of the two alleles in Irish HF population as determined in the present study are balanced. As the animals in the two studies originate from geographically distant and isolated populations, the haplotypic phase identified in the Irish herd may not persist in the Chinese HF population. This means that different haplotypes with different combination of *IL8* promoter polymorphisms might exist in the Chinese population, which could result in a different effect on SCC. Therefore, the knowledge obtained from the *IL8* association with the SCC in the Irish HF should only be exploited in the context of an Irish population, and cannot be applied to other, geographically isolated populations such as Chinese HF.

In the present study, *IL8* haplotype 2 was also associated with the reduced calving interval. No other *IL8* targeted association studies using calving interval have been conducted to date. Two genome-wide association studies aiming to identify variants affecting a number of fertility traits in cattle found no putative association

between the SNPs in the *IL8* region and the calving interval [210, 211]. However, as these studies aimed at genome-wide association without targeting *IL8* region, it is possible that the SNP coverage was not sufficiently dense to include any variants in the proximity to the *IL8* promoter. Other possible reasons for inconsistency in the SNP associations between different studies is the low heritability of traits, which is particularly true for calving interval, with the estimate of heritability around 0.05 [210]. Importantly, a number of functional studies suggest that elevated IL8 is associated with reduced cow fertility [176, 177], which makes *IL8* promoter mutation a possible causative variant and a good candidate for association with the calving interval, which was confirmed in our study.

A sex-specific difference in *IL8* promoter haplotype frequency within the HF breed was also demonstrated. Bulls were shown to have the higher frequency of *IL8* haplotype 1 compared to the cows. The concept of the sex-specific haplotype distribution in the immune-related genes has only been investigated in humans. Tripputi *et al.* demonstrated the sex-specific difference in the allele frequency of a SNP located in the human beta T-cell receptor [224]. A possible explanation for the sex-specific pattern of *IL8* haplotype distribution in HF is the divergent selective pressure between the sexes. If the recruitment of neutrophils to the udder and eventually the SCC are affected by the *IL8* haplotype of an animal, this could create a divergent selective pressure between bulls and cows, as SCC is only quantified in cows. The difference in *IL8* haplotype frequencies between bulls and cows could potentially result in a divergent IL8 response between sexes and may cause a sexually-dimorphic innate immune response to infection. Caroll *et al.* reported differences in the proinflammatory reaction to intravenous LPS in cattle manifested in divergent physiological responses to LPS as well as different levels of various cytokines such as TNFα, IFNγ and IL6, although IL8 was not quantified in the study [225]. However, further functional studies as well as the sex-specific determination of the *IL8* haplotype frequencies in other dairy and beef breeds would help to better understand the *IL8* promoter population genetic structure.

In conclusion, this study demonstrates a significant impact of the *IL8* promoter haplotype on SCC and calving interval. From an agricultural point of view, the *IL8* haplotype 1 is advantageous for SCC while the *IL8* haplotype 2 is favourable for the calving interval, suggesting the existence of selection for either IL8 haplotype in

different contexts, which is supported by the uniform *IL8* haplotype frequencies in the HF breed and the predominance of IL8 haplotype 2 in Charolais breed, as demonstrated in our study.

Chapter IV Functional characterisation of the *IL8* promoter haplotypes in bovine endometrial epithelial (BEND) cells

IV. 1. Introduction

The uterus is a complex organ equipped not only with mechanisms for embryo implantation but also with efficient immunocompetent structures. Bacterial influx during calving compromises the uterus. Most animals clear the bacteria but the infection persists in up to 40% of animals in the form of endometritis, which often results in inflammation and fertility problems [169]. The two most common bacterial species isolated from uterine swabs are *Escherichia coli* (37%) and *Arcanobacterium pyogenes* (49%) [226]. In addition to bacteria, viral infections are also associated with uterine post-partum complications. Bovine herpes virus 4 (BoHV-4) has been identified in the uterus early post-partum and has been associated with endometritis, mainly complementing the pre-existing bacterial infection caused by *E. coli* [227]. BoHV-4 normally infects epithelial cells, where it resides in a latent state until it gets reactivated by stress, usually associated with the periparturient period. This is accompanied by viral replication, mainly induced by TNFα, and viral dissemination throughout the body using lymphocytes as the carriers of viral particles [228]. The mechanism of TNFα driven viral replication involves NFkB translocation and binding to the BoHV-4 (Immediate Early 2 (*IE2*)) gene promoter and induction of its expression [229]. The *IE2* gene product, known as ORF50/Rta not only activates viral replication but also induces IL8 production in bovine endometrial stromal cells [230].

Neutrophil mobilisation from the peripheral blood and migration into the uterus is an important early defensive mechanism against bacteria. Neutrophils in cows with endometritis show impaired myeloperoxidase and phagocytic activity relative to the healthy controls [175, 231]. However, as much as an early neutrophil response is critical for the successful elimination of the source of infection, an excessive neutrophil oxidative burst and secretion of proteolitic enzymes can cause tissue damage [232]. It has been demonstrated that IL8 can promote neutrophil migration into uterus [205]. In order to achieve optimal response, IL8 expression must be tightly regulated.

Endometrial epithelial cells are competent innate immune cells and they represent the first line of defence during the uterine infection postpartum. The gene expression of *TLR 1-6* and *TLR 9* has been shown in the primary human endometrial epithelial cells

[233]. In addition, Davies *et al* have demonstrated the expression of *TLR 1-7* and *TLR 9* in primary bovine endometrial epithelial cells [234]. Importantly, uterine epithelial cells produce IL8 in both humans [235] and cattle [179, 236, 237], under basal condition and upon exposure with bacteria i.e. *E. coli*, and inflammatory stimuli i.e. LPS, IL-1 and TNFα.

Uterine *IL8* gene regulation has been mostly characterised in the human myometrium whereas little is known about the control of *IL8* expression in the endometrium, in either human and cattle. Physical stress and proinflammatory cytokines such as IL1β and TNFα induce *IL8* expression in human myometrial cells via NFκB [238, 239] and to a lesser extent through AP-1 and C/EBP [238]. Donofrio *et al.* have shown that the critical *IL8* promoter element responsible for BoHV-4 induction of *IL8* expression in the bovine endometrial stromal cells is located between -260 bp and -124 bp [230].

After revealing the population genetic structure of *IL8* promoter in a range of bovine breeds as well as the genetic association with important health traits, it was proposed to carry out a functional analysis of the two *IL8* promoter haplotypes *in vitro*. Functional characterisation of the *IL8* promoter from both haplotypes has been previously conducted in the bovine mammary epithelial cells [187]. These cells are important component in mammary gland immunity and the response to mastitis. It has been shown that *IL8* promoter haplotype 2 is more responsive to both LPS and TNFα stimulation of bovine mammary epithelial cells than *IL8* promoter haplotype 1, but the activity of the two *IL8* promoter haplotypes in bovine endometrial epithelial cells is unknown and is investigated in this study. In addition to gram-negative (LPS) and cytokine (TNFα) stimulation, the effects of gram-positive (LTA) and viral (BoHV-4) stimulation on the two *IL8* promoter haplotype activity was also studied in the present study. The critical DNA domains within the 2.1 kilobase *IL8* promoter responsible for *IL8* induction in response to the LPS and TNFα stimulation have been previously characterised in the bovine mammary epithelial cells [187], but the *IL8* promoter regions critical for *IL8* expression have not been studied in the bovine endometrial epithelial cells. Consequently, by using serial deletion, *in silico* and RNA interference analysis, critical *IL8* promoter domains and transcription factors responsible for *IL8* expression in the two *IL8* haplotypes were examined in the present study.

IV. 2. Aims

The aims of these studies are two-fold:

1. To investigate if the two *IL8* promoter haplotypes differ in their ability to drive the *IL8* expression, by bovine endometrial epithelial cells (BEND), in response to cytokine, bacterial and viral stimulation.
2. To identify the DNA domains in both distal and proximal *IL8* promoter which are critical for the effective induction of *IL8* expression, by BEND cells, in response to the cytokine and bacterial stimulation.

IV. 3. Results

IV.3.1. *IL8* expression in bovine endometrial epithelial cells

IL8 gene expression in BEND cells was examined, both basally and in response to inflammatory stimuli LPS, LTA and TNFα. In addition, expression of the genes encoding receptors involved in detection of those three stimuli was also examined. Bovine endometrial epithelial cells (BEND) were grown to 80% confluency and then transferred to 12-well plates (Figure IV-1). The medium was removed after 24h and replaced with either complete growth media or media supplemented with 2 μg/ml LPS, 20 μg/ml LTA or 100 ng/ml TNFα. The cells were stimulated for 5h. Optimal time and dose parameters were based on previous study [187]. After 5h of stimulation, media was removed. Adherent cells were lysed with Trizol and subjected to the RNA extraction, cDNA synthesis and RT-qPCR as described in the Materials and Methods. The expression of *IL8* as well as the expression of other genes involved in *IL8* signaling were investigated: Toll-like receptors *TLR4* and *TLR2* involved in the recognition of LPS and LTA, respectively; and TNFα receptors *TNFRSF1A* and *TNFRSF1B* involved in the recognition of TNFα. *PPIA*, previously validated as stably expressed reference genes in bovine epithelial cells [240], was used for data normalization.

The mRNA products of all the analysed genes were successfully detected. Several of the genes encoding TLR receptors showed a different pattern of expression following inflammatory stimulation. *TLR4* expression remained unchanged upon stimulation with proinflammatory stimuli (Figure IV-2 A). *TLR2* expression was significantly upregulated in response to LPS and TNFα, but remained unchanged in response to LTA (Figure IV-2 B). *TNFRSF1A* expression was stable in response to all the treatments (Figure IV-2 C). *TNFRSF1B* was significantly upregulated in response to TNFα, and unchanged in response to LPS and LTA (Figure IV-2 D). Finally, *IL8* mRNA levels were significantly elevated when stimulated with all proinflammatory molecules (Figure IV-2 E).

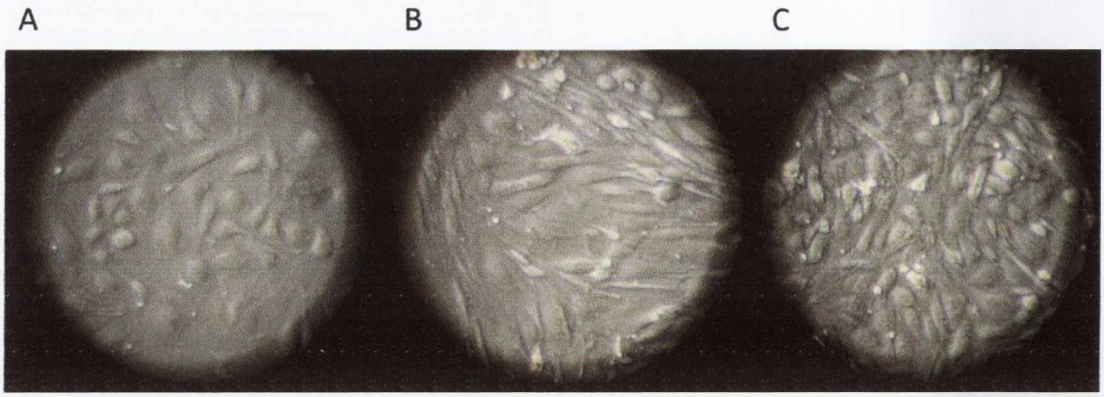

Figure IV-1. BEND cells in the three successive stages of confluency.

The cells were cultured in the complete growth medium and photos were taken at 40% (A), 80% (B) and 100% (C) confluency.

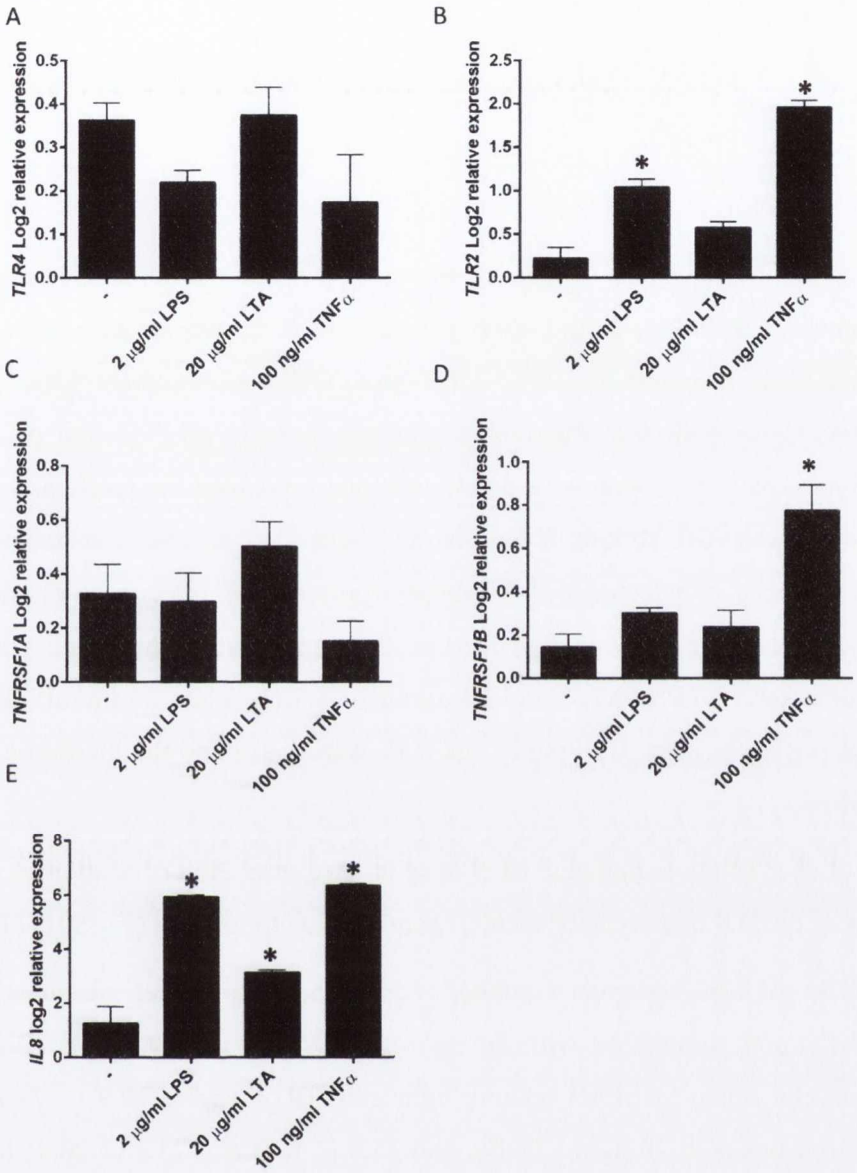

Figure IV-2. *TLR4, TLR2, TNFRSF1A, TNFRSF1B* and *IL8* expression, in response to LPS, LTA and TNFα, in bovine endometrial epithelial cells (BEND).

BEND cells were stimulated with 2 µg/ml LPS, 20 µg/ml LTA and 100 ng/ml TNFα for 5h, lysed and the expression of target genes: *TLR4* (A), *TLR2* (B), *TNFRSF1A* (C), *TNFRSF1B* (D) and *IL8* (E), was quantified by RT-qPCR. mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method; Gene expression was normalized with *PPIA*. Data is expressed as $\log_2$ fold change relative to the sample with the lowest level of expression. * $P<0.05$ for the comparison between the given treatment and the control (-), biological significance was >2 fold, n=4.

IV.3.2. *IL8* promoter response to bacterial and proinflammatory stimuli

Following analysis of the expression of *IL8* and the molecules involved in *IL8* signalling, in BEND cells, the activity of the full *IL8* promoter of both haplotypes was examined, in response to LPS, LTA and TNFα. A reporter gene plasmid was constructed for both *IL8* promoter haplotypes, namely pIL8-h1 and pIL8-h2 for *IL8* haplotype 1 and 2, respectively. Each construct contained the luciferase reporter gene and an upstream 2.1 kb long *IL8* promoter. Plasmids were then transfected into BEND cells. The cells transfected with an empty (promoterless) vector (EV) were used as a negative control. Following transfection, the cells were then treated with increasing concentrations of LPS (0.2, 2 and 20 μg/ml), LTA (1, 5 and 20 μg/ml) and TNFα (25, 50 and 100 ng/ml), for 5h. Finally, the cells were lysed and the luciferase signals were quantified.

In the TNFα stimulated BEND cells, two-way ANOVA showed a significant effects of the IL8 haplotype ($P<0.0001$) and TNFα stimulation ($P<0.01$) (Table IV-1).

The pIL8-h2 construct exhibited 4.5 and 5.9 fold higher promoter activity than pIL8-h1, upon stimulation with 50 ng/ml ($P<0.05$) and 100 ng/ml TNFα ($P<0.01$), respectively. Mean (± SEM) relative light units (RLU) were 19.9 (± 7.4) and 89.3 (± 27.5) in response to 50 ng/ml TNFα. and 15.8 (± 6.7) and 93.3 (± 26.4) in response to 100 ng/ml TNFα for pIL8-h1 and pIL8-h2, respectively (Figure IV-3 A).

In the LPS stimulated BEND cells, two-way ANOVA showed a significant effect of the IL8 haplotype ($P<0.01$) (Table IV-2).

A pair-wise comparison between the haplotypes showed a trend towards significantly higher pIL8-h2 activity upon 2 μg/ml LPS stimulation ($P=0.07$). In response to 2 μg/ml LPS, pIL8-h2 induced 2.4 fold higher luciferase expression than pIL8-h1. Mean (± SEM) RLU were 44.7 (± 16.1) and 107.3 (± 22.7), in pIL8-h1 and pIL8-h2, respectively (Figure IV-3 B).

In the LTA stimulated BEND cells, two-way ANOVA showed a significant effect of the LTA stimulation ($P<0.01$) (Table IV-3).

In contrast to LPS and TNFα, LTA stimulation did not produce significantly different response in two IL8 haplotypes (Figure IV-3 C).

Table IV-1. Analysis of variance (ANOVA) of TNFα stimulated BEND cells transfected with pIL8-h1 and pIL8-h2.

Source of Variation	% of total variation	*P* value	*P* value summary
Interaction	4.833	0.2885	ns
TNFα stimulation	18.09	0.0056	**
***IL8* Haplotype**	27.42	< 0.0001	****

Table IV-2. Analysis of variance (ANOVA) of LPS stimulated BEND cells transfected with pIL8-h1 and pIL8-h2

Source of Variation	% of total variation	*P* value	*P* value summary
Interaction	1.350	0.8478	ns
LPS	11.86	0.0860	ns
***IL8* Haplotype**	19.75	0.0014	**

Table IV-3. Analysis of variance (ANOVA) of LTA stimulated BEND cells transfected with pIL8-h1 and pIL8-h2

Source of Variation	% of total variation	*P* value	*P* value summary
Interaction	7.657	0.2311	ns
LTA	22.93	0.0085	**
***IL8* Haplotype**	1.039	0.4403	ns

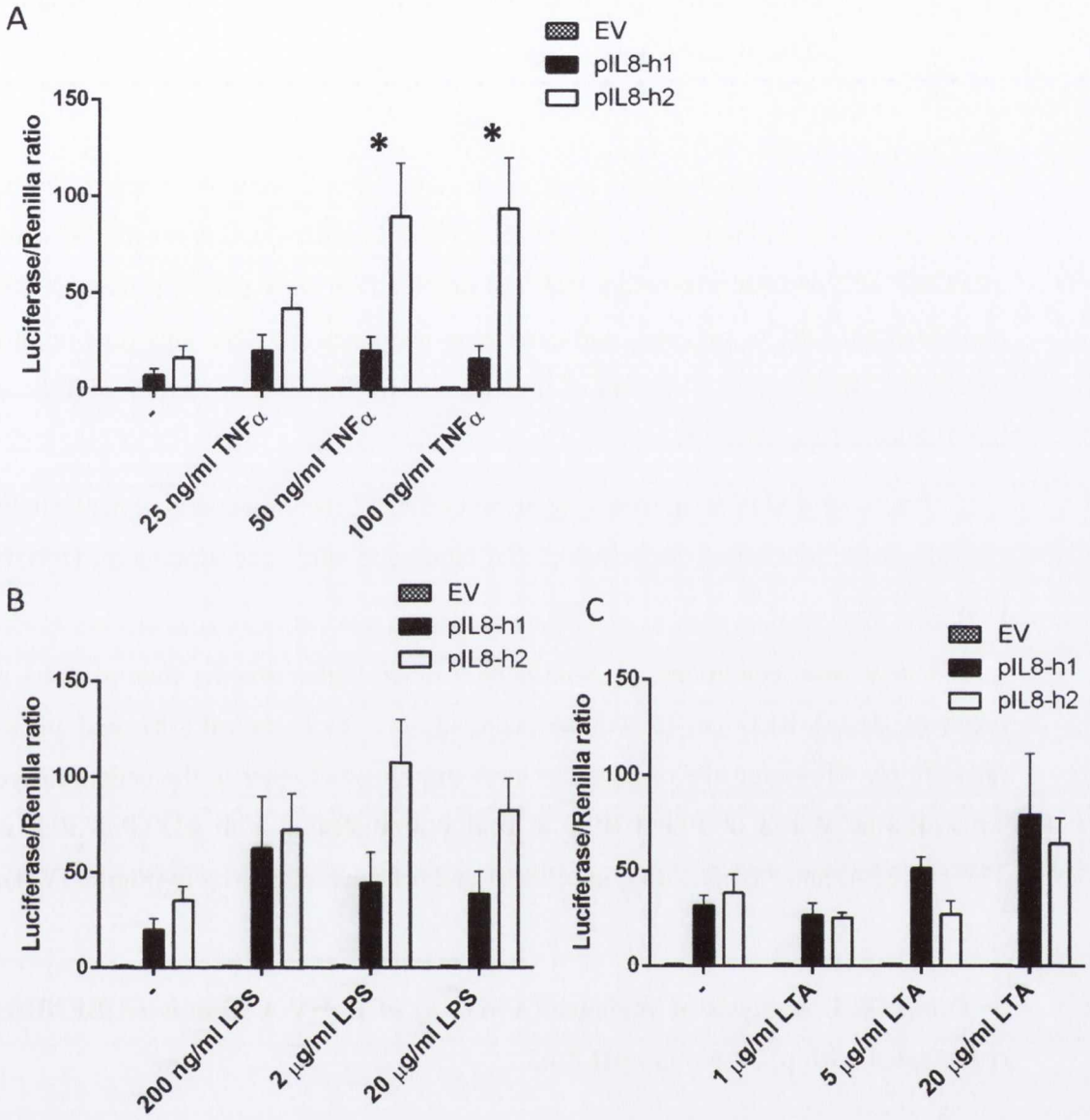

Figure IV-3. *IL8* haplotype 1 and *IL8* haplotype 2 promoter activity in response to increasing concentration of TNFα, LPS and LTA, in bovine endometrial epithelial (BEND) cells.

BEND cells were transfected with the full *IL8* haplotype 1 or *IL8* haplotype 2 constructs (pIL8-h1 and pIL8-h2, respectively) and treated with TNFα (A), LPS (B), LTA (C) for 5h; Relative luciferase activity was calculated as a ratio of luciferase signal (driven by *IL8* promoter) over the renilla control. * $P<0.05$ for pIL8-h1 vs. pIL8-h2, EV - empty vector; Data expressed as mean (± SEM), n=6.

IV.3.3. Full *IL8* promoter activity in BEND cells, in response to viral stimulation

In addition to the bacterial compounds, viral stimuli were also used to drive *IL8* response in both haplotypes. For this purpose, BEND cells were co-transfected with the p2XCMV IE2 plasmid expressing BoHV-4 intermediate early gene 2 product (ORF50) and either pIL8-h1 or pIL8-h2, and cells were incubated for 24h. This time-point was chosen as 24h is the least amount of time needed for the co-transfection with the viral and IL8 haplotype plasmids.

Two-way ANOVA showed a significant effect of the viral stimulation ($P<0.0001$) as well as the interaction between the *IL8* haplotype and viral stimulation ($P<0.001$) (Table IV-4).

Under basal conditions, pIL8-h2 demonstrated higher activity than pIL8-h1 with mean (± SEM) RLU of 7.2 (± 1.6) and 35.5 (± 14.1) in pIL8-h1 and pIL8-h2, respectively. However, the response to viral stimuli was higher in the cells transfected with pIL8-h1. Mean (± SEM) RLU in cells co-transfected with p2XCMV *IE2* were 137.7 (± 25.4) and 72.1 (± 20.1), in pIL8-h1 and pIL8-h2, respectively (Figure IV-4).

Table IV-4. Analysis of variance (ANOVA) of BoHV-4 stimulated BEND cells transfected with pIL8-h1 and pIL8-h2

Source of Variation	% of total variation	*P* value	*P* value summary
Interaction	15.95	0.0015	**
BoHV4	58.83	< 0.0001	****
IL8 Haplotype	1.491	0.2755	ns

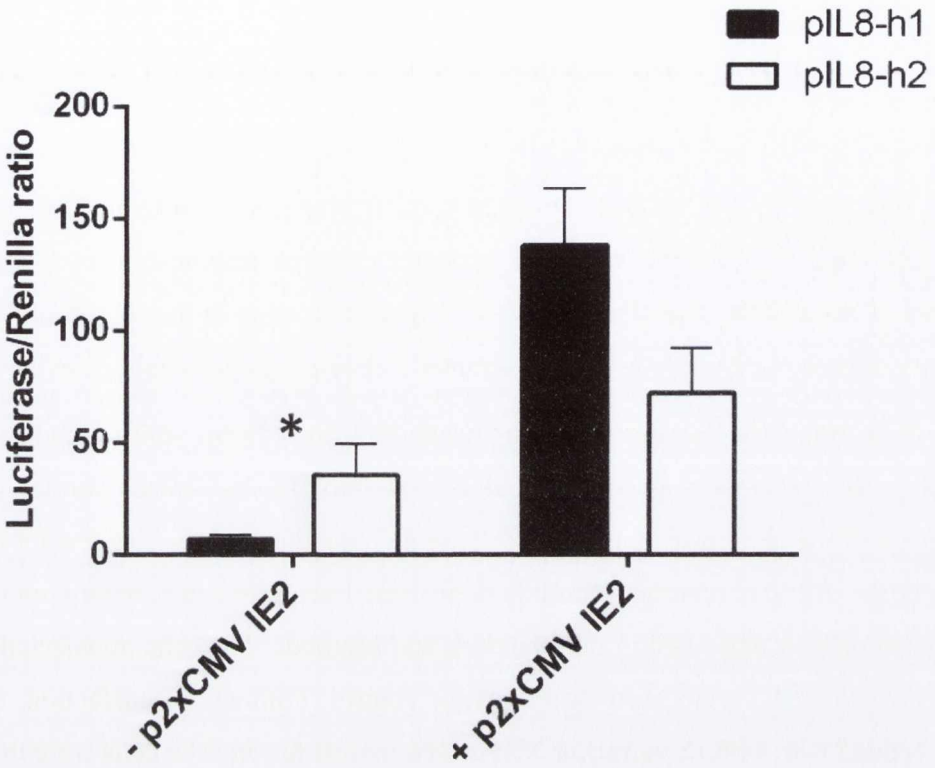

Figure IV-4. *IL8* haplotype 1 and *IL8* haplotype 2 promoter activity in response to BoHV-4 stimulation, in bovine endometrial epithelial cells (BEND).

BEND cells were co-transfected with the full *IL8* haplotype 1 or *IL8* haplotype 2 constructs (pIL8-h1 and pIL8-h2, respectively), and p2XCMV *IE2* plasmid expressing BoHV-4 intermediate early gene 2 product (ORF50), and incubated for 24h; relative luciferase activity was calculated as a ratio of the luciferase signal (driven by *IL8* promoter) over the renilla control. * $P<0.05$ for pIL8-h1 vs. pIL8-h2, Data expressed as mean (± SEM), n=6.

I.1.1. Serial deletion analysis of *IL8* promoter in BEND cells

Having observed the *IL8* haplotype-specific response to inflammatory stimuli, it was important to characterise critical promoter domains responsible for the observed pattern of expression. The effects of serial fragment deletion in the *IL8* promoter on the activity of both *IL8* haplotypes were examined in bovine endometrial epithelial cells.

Four truncated *IL8* promoter fragments: 883 bp, 803 bp, 400 bp and 196 bp in length, were previously generated and ligated into the luciferase reporter plasmid, upstream of the luciferase gene [187]. The same procedure was carried out for both *IL8* haplotypes. All *IL8* reporter plasmids were then transfected into bovine endometrial epithelial cells as described in the Materials and Methods. The cells transfected with an empty vector (EV) were used as a negative control. Following transfection, the cells were treated with LPS (2 μg/ml) or TNFα (100 ng/ml) for 5h. The cells were then lysed and the luciferase signals quantified (Figure IV-5). Basal and induced promoter activity were analysed separately using a two-way ANOVA with the effects of *IL8* haplotype, promoter deletion and the interaction between the two included in the statistical model.

ANOVA analysis showed that interaction between the deletion and *IL8* haplotype effect was significant in the untreated cells (P=0.0002) (Figure IV-6 A), as well as in the cells stimulated with TNFα (P=0.0296) (Figure IV-6 B) and LPS (P=0.0004) (Figure IV-6 C). In the untreated cells, deleting the distal 1.26 kb region caused a significant increase in the *IL8* promoter activity, in *IL8* haplotype 1 only (P<0.0001). Deleting the region between 400 bp and 196 bp caused a significant decrease in the *IL8* promoter activity, in *IL8* haplotype 1 only (P<0.0001) (Figure IV-6 A). In the TNFα treated cells, deleting the distal 1.26 kb region caused a significant increase in the *IL8* promoter activity, in *IL8* haplotype 1 only (P<0.0067). However, deleting the region between 400 bp and 196 bp had no significant effect on the *IL8* promoter activity (Figure IV-6 B). In the LPS treated cells, deleting the distal 1.26 kb region caused a significant increase in the *IL8* promoter activity, in *IL8* haplotype 1 only (P<0.0001) Deleting the region between 400 bp and 196 bp caused a significant decrease in the *IL8* promoter activity, in *IL8* haplotype 1 only (P=0.0003) (Figure IV-6 C).

Due to a lack of the *IL8* haplotype-specific activity in response to the increasing concentration of LTA, the activity of the truncated constructs in the LTA stimulated BEND cells was not investigated.

These experiments suggest the existence of repressive elements in the distal *IL8* promoter region (between 2100 bp and 883 bp upstream of the transcription start site), and activating elements in the proximal *IL8* promoter region (between 400 bp and 196 bp upstream of the transcription start site). Furthermore, it appears that these elements are haplotype- and stimuli- specific.

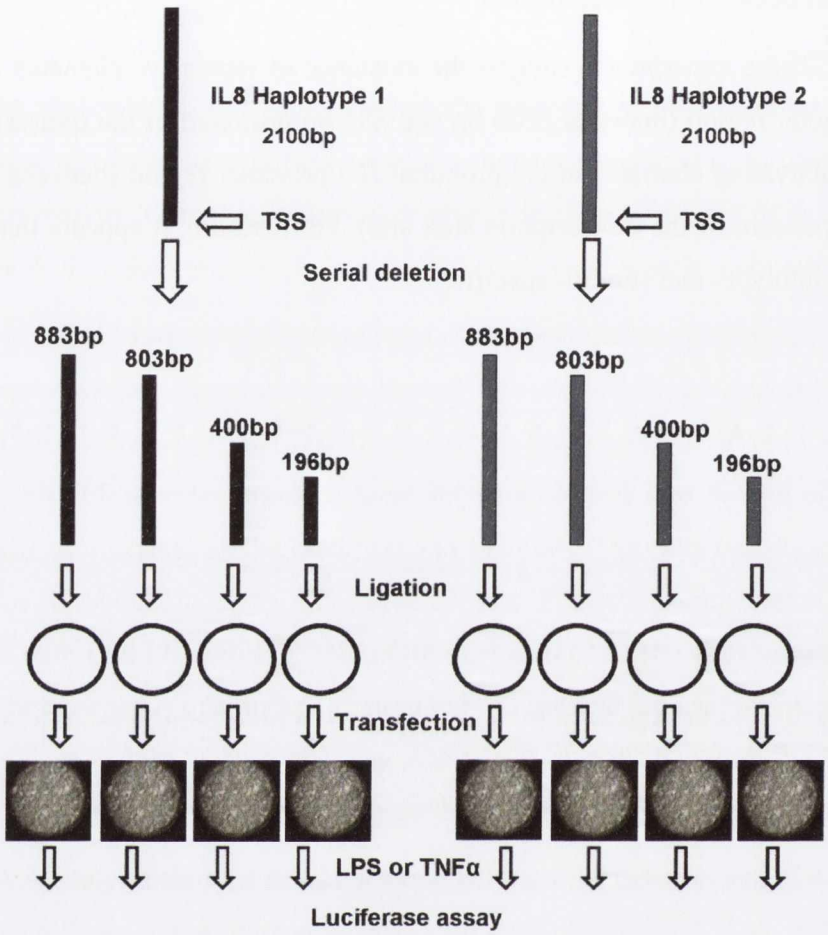

Figure IV-5. The effect of *IL8* promoter serial deletions on *IL8* expression in BEND cells – experimental design.

2.1 kb of the upstream genomic region of the bovine *IL8* gene was PCR amplified, PCR products were digested generating 4 truncated *IL8* promoter fragments (883 bp, 803 bp, 40 bp and 196 bp), ligated into the reporter plasmid and transfected into BEND cells. The cells were stimulated with 2 μg/ml LPS or 100 ng/ml TNFα and the luciferase assay was performed as described in Materials and Methods. The same procedure was carried out for both *IL8* promoter haplotypes. TSS – transcription start site, bp – base pairs.

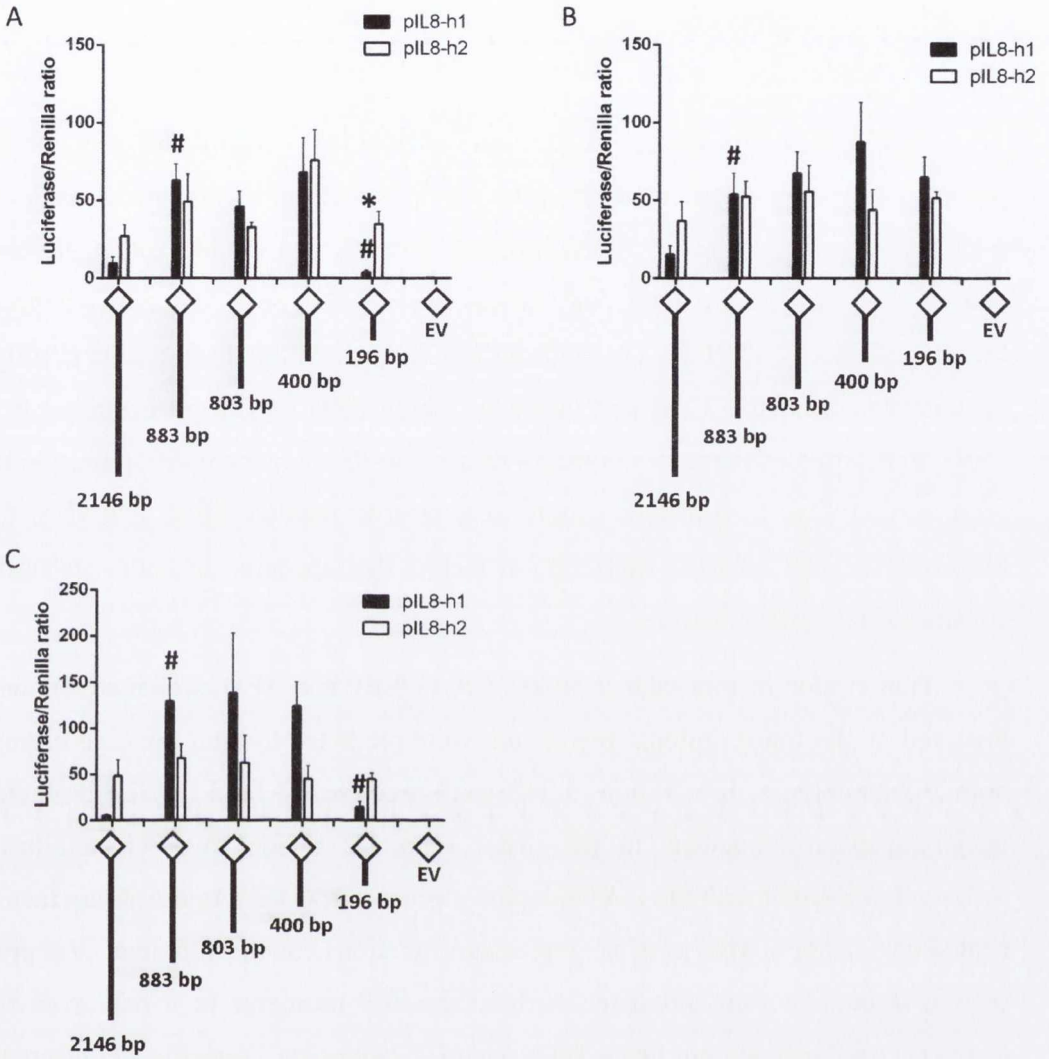

Figure IV-6. *IL8* promoter activity of the full construct (2.1 kb) and deletion constructs (883 – 196 bp) in response to TNFα and LPS.

BEND cells were transfected with the full or serially deleted *IL8* constructs and left untreated (A) or treated with 100 ng/ml TNFα (B) or 2 μg/ml LPS (C) for 5h. Relative luciferase activity was calculated as a ratio of luciferase signal (driven by *IL8* promoter) over the renilla control. * $P<0.05$ between pIL8-h1 vs. pIL8-h2, # $P<0.05$ between the marked construct and the previous construct for the same haplotype. EV- empty vector. Data expressed as mean (± SEM), n=6.

IV.3.1. *In silico* analysis of the bovine *IL8* promoter in both haplotypes

As *IL8* promoter deletions of certain domains had a significant effect on the *IL8* promoter activity, as demonstrated in the previous section, it was important to identify putative transcription factors (TFs) in these domains that could explain the observed pattern of expression. *In silico* analysis was conducted in order to identify transcription factor binding sites (TFBS). The analysis was focused on the distal region (2100 to 883 bp upstream from the TSS) and proximal region (400 to 196 bp upstream from the TSS), as deletion of these two domains resulted in the functional differences in the *IL8* promoter activity. Two different tools were used to identify TFBS, TRANSFAC and Matinspector, and only the transcription factors that appeared in both databases were considered for further analysis.

Trancription factors such as NFkB, CAAT/EBP and AP-1, which are known to be involved in *IL8* transcriptional regulation, were predicted to bind the core promoter in both *IL8* haplotypes. In addition, 5 TFs were predicted to bind the *IL8* promoter in a haplotype-specific manner. In the distal promoter region, two TF families were predicted to interact with the *IL8* haplotype 1 promoter: GATA transcription factors and heat-shock factors, with possible repressive functions. In the proximal *IL8* promoter region, three TFs were predicted to bind the *IL8* promoter in a haplotype-specific manner. Fork-head domain factor D3 was an *IL8* haplotype 2 specific TF, whereas Pou-domain transcription factor and Octamer-binding protein 1 (Oct-1) were predicted to interact with *IL8* haplotype 1 only (Table IV-5).

Table IV-5. The list of transcription factors predicted to bind the bovine *IL8* promoter in the two *IL8* promoter haplotypes

Transcription Factor	Binding position (bp upstream from TSS)	*IL8* haplotype binding preference	Sequence (SNP is highlighted)
Nuclear factor kappa B/c-rel	89-99	Both	tggaaTTTCC*tct*gac
Octamer-binding protein 1	97-112	Haplotype 1	tggttGCATAttgtg tggttGCGTAttgtg
CCAAT/Enhancer Binding Protein	154-165	Both	agaaaCCAATcactagag
Activating protein 1	131-142	Both	gagtcagaTGACTcaga
POU domain transcription factor	202-218	Haplotype 1	agcaatAGAATtatttgaa agcaatAGAATtacttgaa
Forkhead domain factor D3	258-270	Haplotype 2	aaaaaCATATtttaaaa aaaaaAATATtttaaaa
GATA transcription factor	868-896	Haplotype 1	aacagtgtccCTATCtagaaggtgttct aacagtgtccTTATCtagaaggtgttct
Heat shock factors	1443-1458	Haplotype 1	agaagatTCTTGaga agaagacTCTTGaga

IV.3.2. RNA interference analysis of the Octamer binding protein 1 in the two *IL8* haplotypes

IV.3.2.1. Oct-1 knockdown optimisation

Of all the transcription factors suggested by the computer analysis of the *IL8* promoter region in two haplotypes, Oct-1 was chosen for further investigation, as Oct-1 is previously known to be involved in both basal and inducible IL8 repression [93, 94]. Interestingly, the *in silico* analysis has suggested that Oct-1 interacts with the core promoter in *IL8* haplotype 1 only, which could partly explain a lower promoter activity in this haplotype. Therefore, it was hypothesised that Oct-1 knock down would have a greater impact on *IL8* promoter activity in haplotype 1 than haplotype 2.

RNA interference method was employed in order to knock down *Oct-1* gene expression and the effect of the knock down was studied using RT-qPCR. Initially, the duration of transfection was optimised. BEND cells were incubated with the recommended concentration (40 pmol/well) of Oct-1 siRNA for 24h and 48h following which RNA was extracted from cell lysates and *Oct-1* mRNA expression was examined. Downregulation of *Oct-1* RNA was observed after both 24h and 48h incubation with siRNA and it is expressed as the percent expression relative to the control sample with no siRNA treatment. Upon 24h incubation, the *Oct-1* expression was reduced to 20.5%, while 48h incubation caused *Oct-1* knock down to 37.5% of the control expression. Twenty-four hour incubation was used in all subsequent experiments (Figure IV-7 A).

In order to rule out the possibility of unspecific RNA interference, siRNA with an altered sequence (scrambled siRNA) was used as a negative control in the transfection experiment. *Oct-1* was knocked down to 50.9%. In contrast, scrambled siRNA incubation had no effect on the *Oct-1* gene expression (Figure IV-7 B).

As Oct-1 is known to repress the IL8 transcription [93, 94], the impact of *Oct-1* knockdown on the basal and LPS induced *IL8* expression was investigated. The scramble siRNA was only used in the control group due to a very low amount of

scramble siRNA available for the experiment. As Oct-1 is primarily involved in the basal state repression [86], it was decided that the use of scramble Oct-1 in the control group was sufficient.

In the sample untreated with siRNA, LPS stimulation upregulated *IL8* expression by 48.7 fold. The *Oct-1* knockdown using *Oct-1* siRNA caused an increase in *IL8* expression by 14.2 and 57.7 fold in the control and LPS treated sample, respectively. However, the treatment with scrambled siRNA also induced *IL8* expression by 22.9 fold in the control group, which suggests the existence of an unspecific innate immune response to siRNA treatment (Figure IV-8 A). To test this further, the expression of *IL1β*, a cytokine that is not regulated by Oct-1, was quantified by RT-qPCR. Similarly to *IL8* gene expression, LPS treatment caused 33.6 fold induction in *IL1β* expression in untransfected cells and *Oct-1* and scramble siRNA treatments resulted in a 7.2 and a 3.5 fold increase in *IL1β* expression in the control group, respectively (Figure IV-8 B).

An additional scrambled siRNA control was tested for its ability to cause an unspecific *Oct-1* knockdown and *IL8* upregulation. Similarly to the previous scramble siRNA control, there was no effect on the *Oct-1* expression but *IL8* was induced 3.4 fold, confirming the existence of an innate immune response to siRNA transfection (Figure IV-9).

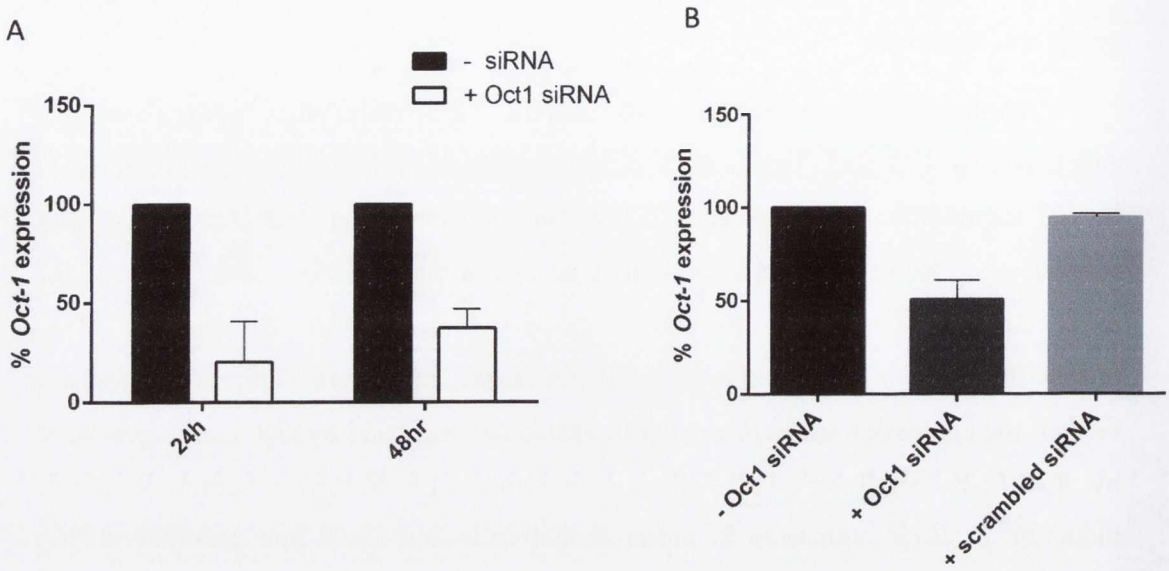

Figure IV-7. *Oct-1* gene knockdown.

BEND cells were incubated with *Oct-1* siRNA for 24h and 48h and *Oct-1* gene expression was quantified using RT-qPCR (A); BEND cells were incubated with *Oct-1* or scrambled siRNA for 24h and *Oct-1* gene expression was measured by RT-qPCR (B). mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method, *PPIA* gene was used for normalization. Data is expressed as a mean (± SEM) percent expression relative to the - siRNA untreated control, n=2.

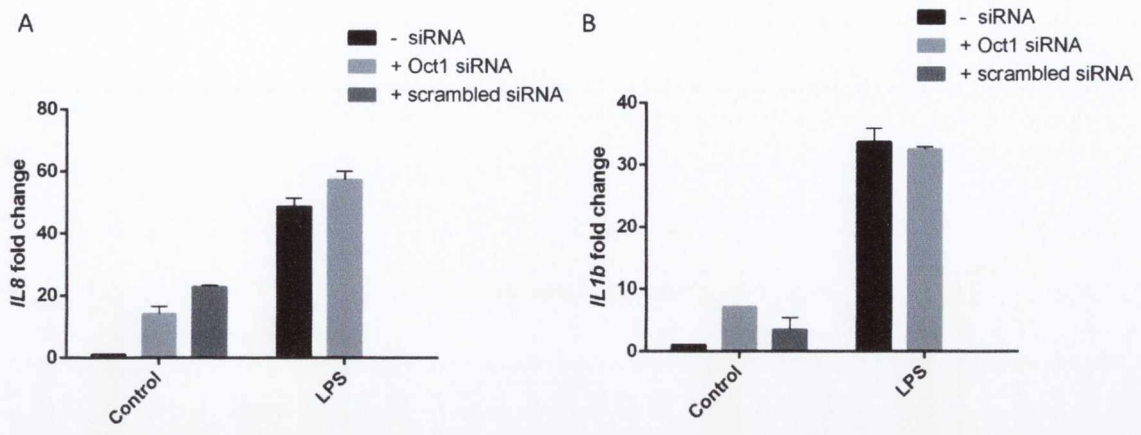

Figure IV-8. The effect of *Oct-1* knockdown on *IL8* and *IL1β* gene expression in BEND cells

The cells were transfected with 40 pmol *Oct-1* siRNA (+ *Oct1* siRNA), scrambled siRNA (+ scrambled siRNA) or left untransfected (- siRNA), for 19h, before LPS (2 μg/ml) was added for another 5h. The cells were lysed and the expression of target genes: *IL8* (A) and *IL1β* (B), were quantified using RT-qPCR. mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method; data is expressed as a mean (± SEM) fold change relative to - siRNA control sample, *PPIA* gene was used for normalization; n=2.

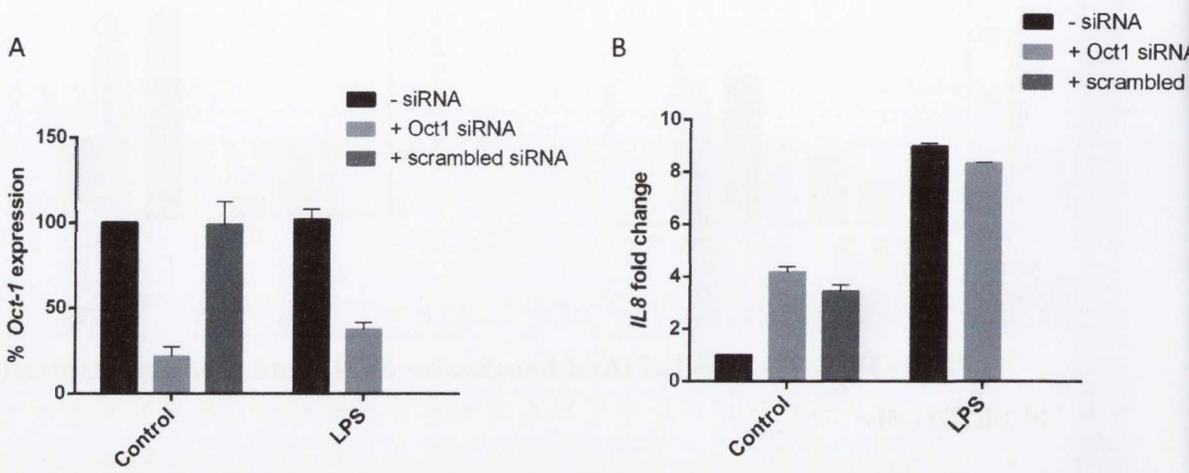

Figure IV-9. The effect of *Oct-1* knockdown on *Oct-1* and *IL8* gene expression in BEND cells.

The cells were transfected with 40 pmol *Oct-1* siRNA (+ *Oct1* siRNA), scrambled siRNA (+ scrambled siRNA) or left untransfected (- siRNA), for 19h before LPS (2 μg/ml) was added for another 5h. The cells were lysed and the expression of target genes: *Oct-1* (A) and *IL8* (B), was quantified using RT-qPCR. mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method; data is expressed as a mean (± SEM) percent relative to the siRNA untreated control (A) or a mean (± SEM) fold change relative to - siRNA control sample (B), *PPIA* gene was used for normalization; n=3.

IV.3.2.2. The effect of the *Oct-1* knockdown on the *IL8* promoter activity in IL8 haplotype 1 and IL8 haplotype 2

After establishing the siRNA transfection conditions, the aim was to characterise the effect of the *Oct-1* knockdown on the *IL8* promoter activity in the two haplotypes. *Oct-1* siRNA was cotransfected with either pIL8-h1 or pIL8-h2 plasmid and the luciferase expression was quantified in both haplotypes, basally and in response to LPS. The overall promoter activity was higher in *IL8* haplotype 2, as expected (Figure IV-10 A). In order to quantify the effect of the *Oct-1* knockdown on the *IL8* promoter activity on both haplotypes, the data was then expressed as the percent increase of the luciferase expression in the *Oct-1* siRNA treated sample, relative to the internal haplotype control. In the *IL8* haplotype 1, *Oct-1* knockdown increased *IL8* promoter-driven luciferase expression by 164.1% and 79.5%, in the control and LPS treated group, respectively. In the *IL8* haplotype 2, *Oct-1* knockdown increased *IL8* promoter-driven luciferase expression by 73.4% and 37.2%, in the control and LPS treated group, respectively. The effect of the *Oct-1* knockdown was greater in the *IL8* haplotype 1, as expected, but there was no statistically significant difference. A trend towards significance was observed for the comparison between the *Oct-1* knockdown in LPS treated *IL8* haplotype 1 and LPS treated *IL8* haplotype 2 (Figure IV-10 B).

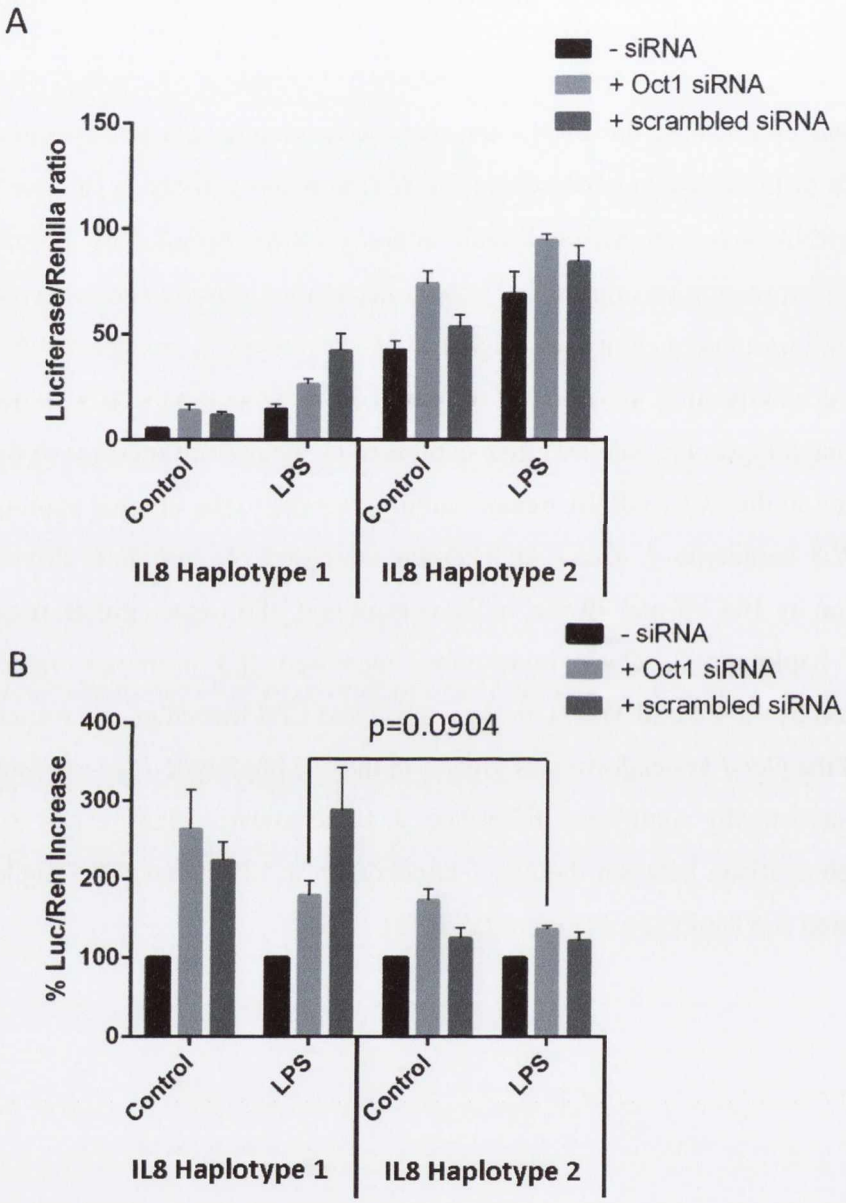

Figure IV-10. The effect of *Oct-1* knockdown on *IL8* promoter activity in the two *IL8* haplotypes.

The cells were transfected with 40 pmol *Oct-1* siRNA and 200 ng pIL8-h1 or pIL8-h2 for 19h before LPS (2 μg/ml) was added for 5h. Data is expressed as a ratio of luciferase signal (driven by *IL8* promoter) over the renilla control (A) or the percent luciferase/renilla increase relative to the – siRNA control (B), n=3.

IV. 4. Discussion

In the present study, it was shown that genetic polymorphisms in the bovine *IL8* promoter have a significant impact on the *IL8* promoter-driven transcriptional activity in BEND cells. It was demonstrated that the two *IL8* promoter haplotypes differ in their ability to activate luciferase expression. Critical regions responsible for *IL8* promoter activity were identified, as distal region between 2.1 kb and 883 kb and the proximal region between 400 and 196 bp, upstream from the transcription start site. In addition, a number of candidate transcription factors were predicted for the haplotype-specific pattern of expression. The role of octamer-binding protein 1 in *IL8* promoter repression was functionally characterised and it was shown that this transcription factor could be responsible for the lower *IL8* promoter activity in the IL8 haplotype 1.

As *IL8* promoter activity was analysed using the reporter gene construct which is an artificial system, it was important to show that BEND cells naturally express *IL8* under basal conditions and upon stimulation with cytokine (TNFα), gram-negative bacterial endotoxin (LPS) and gram-positive bacterial (LTA) compounds. Similar studies comparing *IL8* response to different stimuli in bovine endometrial cells have not been conducted before. In our study, *IL8* gene product was successfully detected in BEND cells and it was shown that *IL8* gene expression is significantly upregulated by all three stimuli, and LTA was the least potent inducer. Lower potency of LTA in inducing the inflammatory response has been reported before, in both human and bovine. LTA induces lower TNFα protein expression than LPS, in murine macrophages [241]. In addition, Gilbert *et al* have demonstrated that LPS induced the expression of a higher number of genes and with greater fold change, compared to stimulation with *Staphylococcus aureus* supernatant. Among these genes, a number of *CXCL* chemokines were upregulated, and *IL8* fold change was higher in response to LPS than in response to *S. aureus* supernatant [242]. Also, Strandberg *et al*. have demonstrated that *IL8* gene upregulation in response to LPS is higher and more sustained than in response to LTA, in bovine mammary epithelial cells [243]. IL8 was detected in the milk following an intramammary infection with *E. coli*, whereas milk IL8 was undetectable after the intramammary infection with *S. aureus* [244].

Having demonstrated *IL8* gene expression in BEND cells, the aim was to investigate the *IL8* promoter activity in both *IL8* haplotypes, in stimulated BEND cells, using a luciferase reporter gene assay. *IL8* haplotype 2 shows higher promoter activity compared to *IL8* haplotype 1, in BEND cells following both LPS and TNFα treatment. This observed profile of *IL8* promoter activity in the BEND cells is similar to the one previously observed by our group, in bovine mammary epithelial cells [187]. The difference in responsiveness to LPS between the two IL8 haplotypes in BEND cells could have important consequences for the response to bacterial infection, especially at sites where neutrophil response is critical, such as the uterus. Retained placenta (RP) is a failure of a cow to expel the placenta after calving and it has a negative impact on fertility as many RP cows develop metritis. Kimura *et al.* have demonstrated that neutrophil chemotaxis and myeloperoxidase activity is significantly lower in RP cows compared with normal controls. Importantly, the neutrophil functional impairment was accompanied by a significantly lower plasma IL8 concentration in RP cows relative to healthy controls [174]. Therefore, IL8 haplotype 2 could be advantageous for improved uterine health around calving.

On the other hand, full *IL8* haplotype 1 promoter was more responsive to the BoHV-4 viral stimulation. Upon stimulation with BoHV-4 product ORF50, *IL8* haplotype 1 promoter induced higher luciferase expression than *IL8* haplotype 2. Differential responsiveness to viral stimulation could have important implications for neonatal health. Newborns are particularly susceptible to bacterial (*E. coli,* Salmonella) and viral infections (respiratory syncytial virus, bovine viral diarrhoea, herpesvirus, rotavirus), which results in a high mortality rate in the Irish calf population of approximately 10%. Unlike the neutrophil function in bacterial infections, the role of neutrophils and IL8 in coping with viral infections is not fully understood. Positive effects of neutrophils in fighting viral infections have been reported mainly in influenza viral infection models [245]. Tate *et al.* have demonstrated that granulocyte depletion causes a severe disease in mice infected with H3N2 influenza, manifested in reduced animal survival upon viral infection, significant weight loss and increased virus titre [246]. Murayama *et al.* reported negative consequences of increased IL8 levels in humans on cytomegalovirus clearance by promoting viral replication and inhibiting the antiviral function of IFNα [247].

IL8 gene regulation has mostly been studied in human and very little is known about the regulation of *IL8* transcription in other mammalian species, as IL8 is absent in mouse [248]. The proximal promoter region, spanning 150 bp upstream from the transcription start site, has been identified as an essential element in *IL8* activation in humans [87], but no information is available on the role of distal promoter domains in *IL8* gene regulation. It was shown in the present study that distal *IL8* promoter elements can also modulate *IL8* expression. A deletion of the region between 2.1 kb and 883 bp upstream of the transcription start site resulted in a significant increase in the *IL8* promoter activity, suggesting that the transcriptional repressors are acting in this area. One of the protein families predicted to bind in this region belongs to GATA transcription factors. These proteins share a DNA binding domain that consists of the zinc finger and they target a DNA element characterised by GATA sequence. These transcription factors are mainly involved in the development of various cell types such as blood cells, cardiocytes and epithelial cells [249]. The repressive function of GATA transcription factor has been demonstrated before. Schwenger *et al.* reported the repressive effect of GATA3 transcription factor in the distal *IL4* promoter, in both unstimulated and stimulated murine T-cell line [250].

A deletion of the proximal region between 400 bp and 196 bp upstream of the transcription start site caused a significant reduction in the *IL8* haplotype 1 promoter activity, indicating the presence of the activating factors in this region. This *IL8* promoter region contains only two SNPs located 209 bp and 267 bp from transcription start site. Further functional studies including techniques such as site-directed mutagenesis and electrophoretic mobility shift assays, are needed to fully characterise the role of these SNPs in haplotype-specific *IL8* expression.

One of the TFs predicted to bind the core *IL8* promoter is Octamer-binding protein 1 (Oct-1). Interestingly, the Oct-1 TFBS overlaps the SNP located 104 bp upstream from the transcription start site. The *in silico* analysis predicted that Oct-1 only binds the *IL8* haplotype 1 promoter. Wu *et al.* reported the repressive role of the Oct-1 TF on the *IL8* expression in both unstimulated and IL1β stimulated cells [93]. As IL8 haplotype 1 was showing consistently lower transcriptional activity, it was hypothesised in the present study that Oct-1 might be involved in *IL8* promoter repression in the haplotype 1. RNA interference was used to knock down the *Oct-1* mRNA in BEND cells transfected with both *IL8* haplotypes. The *Oct-1* transcription

was successfully inhibited, and accompanied by an increased *IL8* expression. There was a trend towards a significantly higher effect of the *Oct-1* knockdown on *IL8* haplotype 1 promoter activity, which was expected, based on *in silico* and functional analysis. However, an important limitation of the study was the fact that there was an unspecific *IL8* upregulation that was most likely the product of cell transfection and siRNA recognition by the innate immune system. This notion is further supported by the data showing an unspecific *IL1β* upregulation, upon transfection with both *Oct-1* and scrambled siRNA. As siRNA is a double-stranded RNA, it is possible that this molecule is recognised by TLR3 receptors which trigger the proinflammatory reaction. Therefore, further optimisation of the RNAi protocol is necessary in order to obtain more information on the role of Oct-1 on *IL8* supression in both haplotypes. A possible approach is the use of inhibitors in order to block TLR pathways responsible for the dsRNA recognition followed by the siRNA transfection. An important limitation of the RNAi approach is the incomplete and transient gene knockdown which means that the targeted gene is still showing low levels of expression even after the knock down has been performed, which could hinder the detection of differences between the two *IL8* haplotypes. A technique that can overcome this limitation is known as the gene editing or gene knockout. By using a specific type of bacterial enzyme, known as CRISPR-associated protein-9 nuclease, it is possible to perform precise and targeted excision of the gene of interest completely switching off its expression. These changes are stable; enabling the creation of the cell line with the *Oct-1* gene completely deleted from the genome and available for the further transfection with the two *IL8* haplotypes.

In conclusion, higher responsiveness of *IL8* haplotype 2 promoter to LPS and TNFα were demonstrated in the present study. In contrast, *IL8* haplotype 1 displayed stronger activity in response to a viral treatment. In addition, critical repressive and activating elements were identified in the *IL8* promoter. Preliminary study suggests that Oct-1 binding to the *IL8* haplotype 1 promoter could be responsible for the reduced promoter activity of this haplotype. However, due to study limitations, further examination is necessary in order to confirm this finding.

Chapter V The effect of *IL8* promoter haplotype on IL8 expression in primary monocyte-derived macrophages and Holstein-Friesian calves challenged with LPS

V. 1. Introduction

It was shown in previous chapters that the IL8 haplotype has a significant impact on somatic cell count and calving interval in HF cows, which suggested functional differences between the two haplotypes. These differences were then confirmed *in vitro*, showing that the *IL8* haplotype 2 promoter is more potent in driving *IL8* expression, in response to inflammatory treatment. A cloned cell line system, employed and presented in the previous chapter to study *IL8* promoter activity in both haplotypes is a convenient method to perform initial characterisation of the two *IL8* haplotypes. However, in order to fully confirm the *IL8* haplotype-specific activity, it was necessary to move on to using primary cell culture and an *in vivo* model. Therefore, following the *in vitro* functional characterisation of the bovine *IL8* promoter in a cell line transfected with both haplotypes, the next step was to examine the *IL8* promoter activity in primary immune cells obtained from animals of both haplotypes as well as the *in vivo IL8* promoter activity in Holstein-Friesian calves of both haplotypes.

Genetic polymorphisms in the regulatory regions of innate immune genes have been shown to affect both gene and protein expression in primary immune cells both *in vivo* and *ex vivo*. This has been most extensively studied in the human *TNFα* and *IL1β* promoters. Lu *et al.* have demonstrated a significant effect of a genetic variant in the human *TNFα* promoter on *TNFα* gene expression in patients with ankylosing spondylitis [251]. The same mutation also drives higher TNFα protein production in LPS stimulated whole blood culture obtained from patients with aggressive periodontitis [252]. Additionally, a haplotype in the human *IL1β* promoter is associated with higher IL1β expression in the whole blood stimulated with LPS [253] and in monocytes treated with *Mycobacterium tuberculosis* [254]. Moreover, an elevated IL1β expression is accompanied by an increased granulocytic infiltration to the lung [254]. An *ex vivo* study investigating the effect of SNPs in the human *IL6* promoter on LPS driven IL6 expression in the whole blood has revealed a significant difference in IL6 activation between promoter haplotypes [255]. However, the effect of *IL8* promoter variants on the IL8 expression *in vivo* has not been investigated before, in either human or cattle, and it is examined in this study. In addition, it is hypothesised that the

divergence in IL8 production between the *IL8* promoter variants will have significant downstream consequences for neutrophil function, resulting in different neutrophil activation profiles between the two *IL8* haplotypes. IL8 is primarily considered to be important for neutrophil recruitment, but it is also involved in neutrophil activation. The evidence of a dose-dependent effect of IL8 on neutrophil activation has been reported before in both human and cattle. IL8 dose-dependent priming of human neutrophils to enhanced oxidative burst, following stimulation with N-formylmethionine leucyl phenylalanine, has been demonstrated before [123, 256]. The IL8 concentration also has a significant impact on the phospholipase D formation in human neutrophils [119]. A dose-dependent effect of human IL8 on the *in vivo* neutrophil recruitment in the mouse lung has also been reported [257]. Abu-Harb *et al.* have shown that an increased serum IL8 concentration correlates with the elevated neutrophil elastase levels in the respiratory tract of infants with bronchiolitis [258]. A number of papers have reported a dose-dependent effect of IL8 on neutrophil recruitment in cattle. An increasing human IL8 concentration causes an increase in the *in vitro* bovine neutrophil chemotaxis through endothelial/epithelial barrier [83, 259], and an elevated release of alkaline phosphatase from bovine neutrophils [83]. Caswell *et al.* have shown that recombinant bovine IL8 promotes dose-dependent bovine neutrophil migration, both *in vitro* and *in vivo*, upon intradermal injection of IL8 [82]. In addition, an IL8 dose-dependent enhancement of oxidative burst and phagocytosis of *S. aureus* have been reported in the bovine neutrophils [122]. The haplotype-specific neutrophil recruitment could have significant consequences on an animal's ability to mount an early response to infection. Strong and early neutrophil recruitment to mammary gland is crucial for successful defence against invading bacteria, while reduced neutrophil migration is accompanied by severe forms of mastitis [161]. Youngerman *et al.* have demonstrated that the cows carrying a particular variant in the *CXCR2* coding region are more susceptible to subclinical mastitis and have an impaired bovine neutrophil function, manifested by the reduced migration and downregulation of CD11b and CD18 [260, 261]. Also, suboptimal bovine neutrophil chemotaxis and reduced myeloperoxidase activity have been linked with retained placenta and susceptibility to metritis [173, 175]. Therefore, animals from two different *IL8* haplotypes could exhibit different neutrophil response to pathogens.

V. 2. Aims

The aims of these studies are three fold:

1) To quantify the IL8 expression by primary bovine monocyte-derived macrophages obtained from animals of both *IL8* haplotypes.
2) To quantify the IL8 expression in Holstein-Friesian calves of both *IL8* haplotypes, in response to the intravenous LPS challenge.
3) To characterise the potential effects of IL8 dose and *IL8* haplotype on the neutrophil activation, migration and ability to eliminate bacteria.

V. 3. Results

V.3.1. IL8 promoter haplotype effect on IL8 expression in the bovine monocyte-derived macrophages

V.3.1.1. Time-course and dose response to LPS, LTA and TNFα stimulation in bovine monocyte-derived macrophages

Following functional characterisation of the *IL8* promoter haplotypes in the bovine endometrial cell line, the objective was to assess the ability of both *IL8* haplotypes to drive IL8 expression in primary immune cells. Monocyte-derived macrophages (MDM) were used as the cell culture model, as they are among the most potent producers of IL8 [5]. PBMC were extracted from bovine peripheral blood and MDM were obtained, using selective monocyte adherence and 7 days differentiation into macrophages (Figure V-1).

The purity and viability of the MDM on day 7 were investigated by flow cytometry, using the macrophage-specific surface marker CD14 and lymphocyte-specific surface marker CD3, to assess purity; and propidium iodide (PI) staining to determine cell viability. Of the total cell population, 80.1% stained positive for CD14 surface marker, 4.3% stained positive for CD3 surface marker, and 15.6% were uncharacterised (Figure V-2). Cell viability was shown to be greater than 90% as measured by PI staining (Figure V-3).

In order to identify the optimal concentration of the proinflammatory stimuli as well as the post-stimulation time-point at which the IL8 response is maximal, MDM were stimulated with LPS (0.05 μg/ml, 0.2 μg/ml and 1 μg/ml), LTA (1, 5 and 10 μg/ml), and TNFα (25, 50 and 100 ng/ml); and the cells were collected at multiple time points (0h, 2h, 6h, 10h and 24h).

RNA was extracted and the RNA quantity was determined by spectrophotometry. The quality of RNA was assessed on Bioanalyser and typical profiles showing 18 S and

28 S rRNA traces are presented on Figure V-4. RNA Integrity Numbers (RIN) were automatically calculated and used to determine the quality of RNA. RIN can take any value between 1 and 10, with 1 being the most degraded profile, and 10 being the most intact. All RNA samples used in the study had RIN values greater than 7 which signify good quality RNA.

Five candidate normaliser genes were selected in order to normalize the *IL8* gene mRNA expression across treatments: *PPIA*, *H3F3A*, *GAPDH*, *ACTB* and *MRPS6*; and their expression was analysed using GenEx. In accordance with the previous study [262], peptidylprolyl isomerise A gene (*PPIA*) was the most stably expressed gene, followed by *H3F3A*, *GAPDH*, *ACTB* and *MRPS6* (Figure V-5). Therefore, *PPIA* and *H3F3A* were used as reference genes.

After the most stably expressed pair of reference genes was determined (*PPIA* and *H3F3A*), RT-qPCR analysis was performed in order to establish the kinetics of *IL8* gene expression in response to LPS, LTA and TNFα, in MDM. All three stimuli induced *IL8* expression at all time-points under investigation The *IL8* mRNA fold change in MDM, in response to LPS, was greater at earlier time points (2h and 6h) than at later time points (10h and 24h). There was no clear dose response to the increasing concentration of LPS, at any of the time points. Maximal *IL8* expression (108.6 fold change) was observed at 6h, after the stimulation with 1 μg/ml LPS (Figure V-6 A). A similar *IL8* gene expression profile in MDM was observed upon LTA stimulation. LTA was shown to be a less potent inducer of *IL8* expression in MDM, compared to LPS. The *IL8* mRNA fold change in MDM, in response to LTA, was greater at earlier time points (2h and 6h) than at later time points (10h and 24h). There was no clear dose response to the increasing concentration of LTA, at any of the time points. Maximal *IL8* expression (30.5 fold change) was observed at 6h, after the stimulation with 5 μg/ml LTA (Figure V-6 B). In response to TNFα, the difference in *IL8* gene expression in MDM, across the time-points was not as dramatic as after LPS and LTA treatment. Also, the *IL8* fold change in response to TNFα was lower overall compared to the LPS and LTA stimulated *IL8* responses in MDM. There was a clear dose response to the increasing concentration of TNFα, at all of the time points. Maximal *IL8* expression (6.9 fold change) was observed at 6h, after the stimulation with 100 ng/ml TNFα (Figure V-6 C).

As it has been reported before that quantifiying bovine IL8 using human IL8 specific antibodies can largely underestimate the IL8 protein levels [263], the

comparison of IL8 concentration on a random set of MDM and PBMC supernatant samples was conducted using both human and bovine specific IL8 ELISA. After examining the levels of IL8 obtained using both methods, it was concluded that they are comparable (Figure V-7), and human IL8 ELISA kit was further used.

IL8 protein expression was upregulated in response to all three stimulations, at all 4 time-points. IL8 protein expression in MDM, in response to LPS, showed a minor increase at 2h (~50 pg/ml) followed by a substantial induction at 6h, 10h and 24h (>100 pg/ml). Maximal IL8 protein expression in MDM, in response to LPS (194 pg/ml) was observed at 10h, after stimulation with 1 μg/ml LPS (Figure V-8 A). IL8 protein levels in response to LTA were lower than those observed upon LPS stimulation, which was consistent with the *IL8* gene expression data. Similar kinetics of IL8 expression was observed for LTA stimulated MDM. There was a consistent increase in IL8 protein level reaching a peak at 24h. Maximal IL8 protein expression in MDM, in response to LTA (109 pg/ml) was observed at 24h, after stimulation with 5 μg/ml LTA (Figure V-8 B). IL8 protein expression levels for TNFα stimulated cells were lower compared to those observed in response to LPS and LTA In response to TNFα, MDM showed maximal IL8 protein level at 24h (42 pg/ml) after stimulation with 50 ng/ml TNFα (Figure V-8 C).

Taken together this data suggests that IL8 is upregulated at both gene and protein level, in bovine MDM, in response to all three stimuli. Based on the fact that *IL8* mRNA levels started decreasing after 6h of LPS, LTA and TNFα stimulation, this time-point was chosen for the subsequent experiments. Additionally, since the clear dose response was only observed in the TNFα stimulations while all three concentrations of LPS and LTA induced similar levels of IL8, the highest concentrations, 1 μg/ml LPS, 10 μg/ml LTA and 100 ng/ml TNFα, were chosen for subsequent experiments.

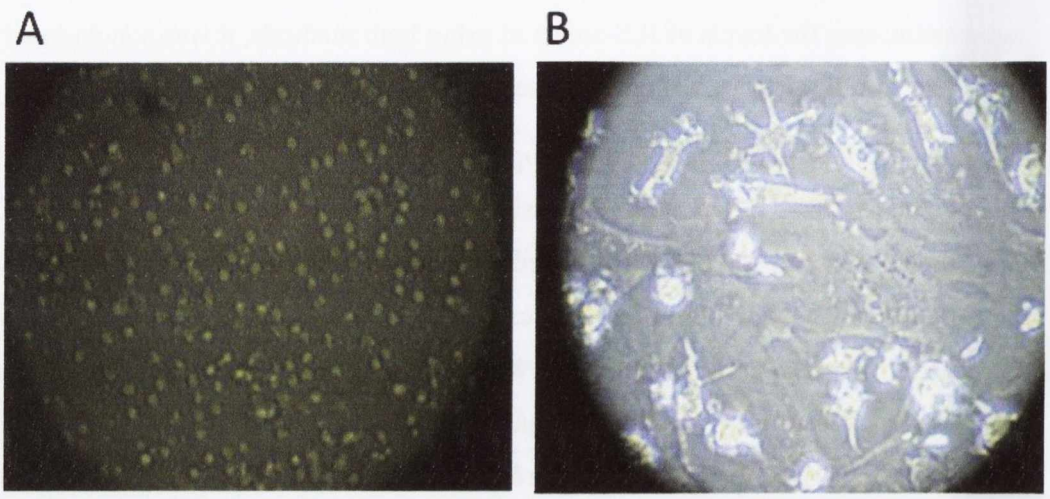

Figure V-1. Bovine leukocytes visualized under the light microscope.

(A) Peripheral blood mononuclear cells (PBMC) were separated from the blood and visualized under 200 × magnification. (B) A population of monocyte-derived macrophages (MDM) was generated by allowing monocyte adherence and differentiation in culture for 7 days; cells were visualized under 400 × magnification.

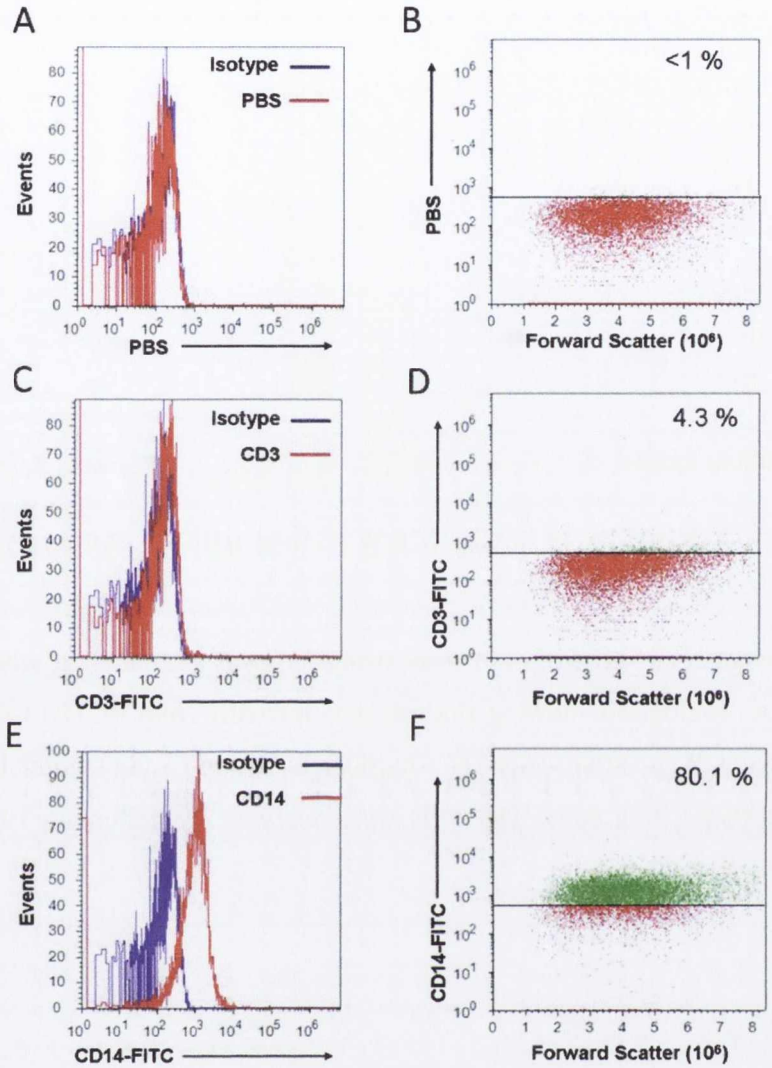

Figure V-2. Purity of bovine MDM stained with $CD14^+$ and analysed by flow cytometry.

MDM were removed from the flask using Trypsin and labelled with PBS (unstained control), anti-bovine IgG1 (isotype control), anti-bovine CD3 or anti-bovine CD14 primary antibody, followed by incubation with the FITC-labelled secondary antibody. Histograms showing fluorescence intensity from PBS (unstained control) (A), CD3 (C) and CD14 (E) positive cells. Corresponding dot plots showing PBS (unstained control) (B), CD3 (D) and CD14 (F) positive cells. Numbers in top right corners indicate percentage of positive cells. The cells were analysed by collecting 10,000 events. Representative of n=6 animals.

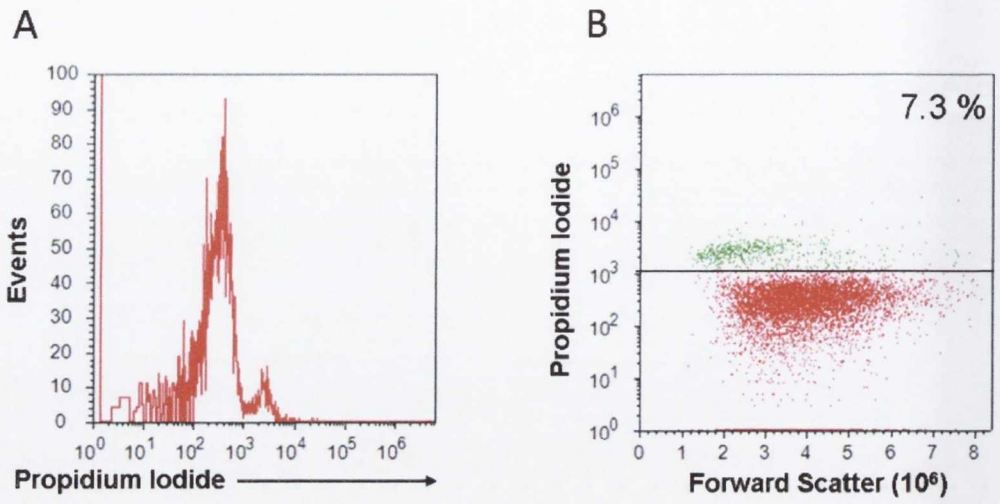

Figure V-3. Viability of bovine MDM stained with PI and analysed by flow cytometry.

MDM were removed from the flask using Trypsin and labelled with propidium iodide (PI). (A) Histogram showing fluorescence intensity from PI, (B) Corresponding dot plot showing PI positive cells. The number in the top right corner indicates the percentage of PI positive cells. The cells were analysed by collecting 10,000 events. Representative of n=6 animals.

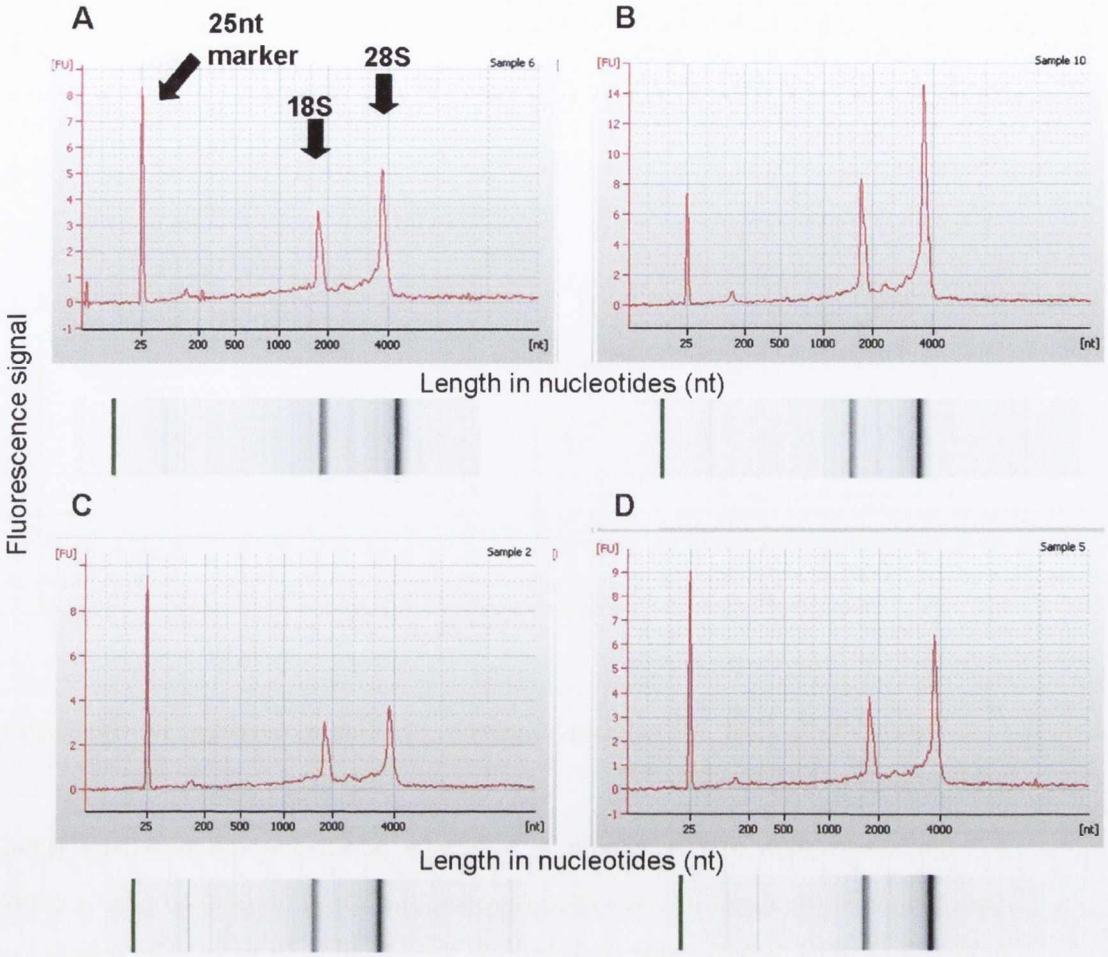

Figure V-4. Profiles of four representative RNA samples extracted from the bovine MDM.

The quality of RNA was examined on the Bioanalyser. Typical profiles of four individual samples are shown (panels A, B, C and D). Horizontal axis shows the length (number of nucleotides) of the RNA and the vertical axis shows the intensity of the fluorescence signal. Typical profile of the non-degraded RNA is represented by two bends on the gel (18 S and 28 S rRNA), quantified by two peaks used to calculate the RNA Integrity Number (RIN).

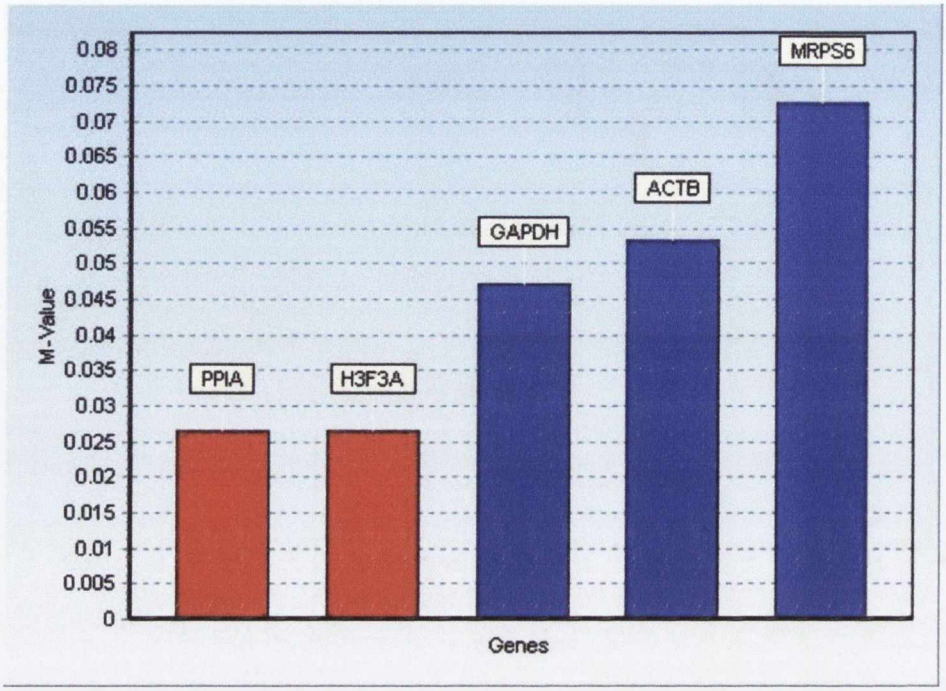

Figure V-5. Plot of reference genes, ranked according to the expression stability in bovine MDM.

The expression of five candidate reference genes was quantified by RT-qPCR. Then, the GeNorm algorithm was used to assess the variation of each gene's expression compared to other genes, expressed through the M-value (y axis). The optimal pair of reference genes is the pair with the lowest M-values: *PPIA* and *H3F3A*. n=2.

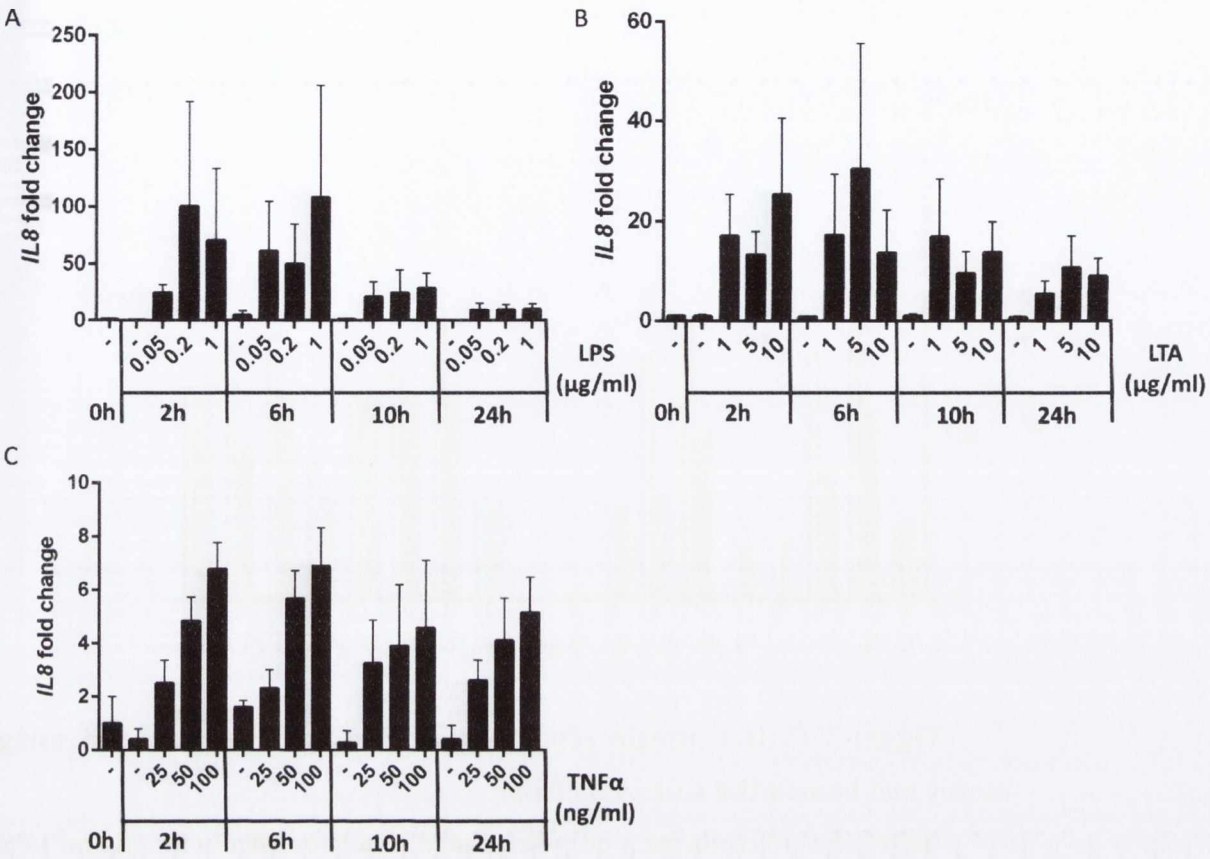

Figure V-6. Time course and dose response of *IL8* mRNA expression in the bovine monocyte-derived macrophages (MDM) following stimulation with LPS, LTA and TNFα.

Bovine macrophages were stimulated with increasing concentrations of LPS (0.05 µg/ml, 0.2 µg/ml and 1 µg/ml) (A), LTA (1, 5 and 10 µg/ml) (B), and TNFα (25, 50 and 100 ng/ml) (C); and *IL8* mRNA expression was assessed at multiple time points (0h, 2h, 6h, 10h and 24h), using RT-qPCR. mRNA expression levels were calculated using $2^{-\Delta\Delta Ct}$ method; data is expressed as a fold change relative to the non-treated sample collected at 0h; *PPIA* and *H3F3A* were used for normalization; n=2.

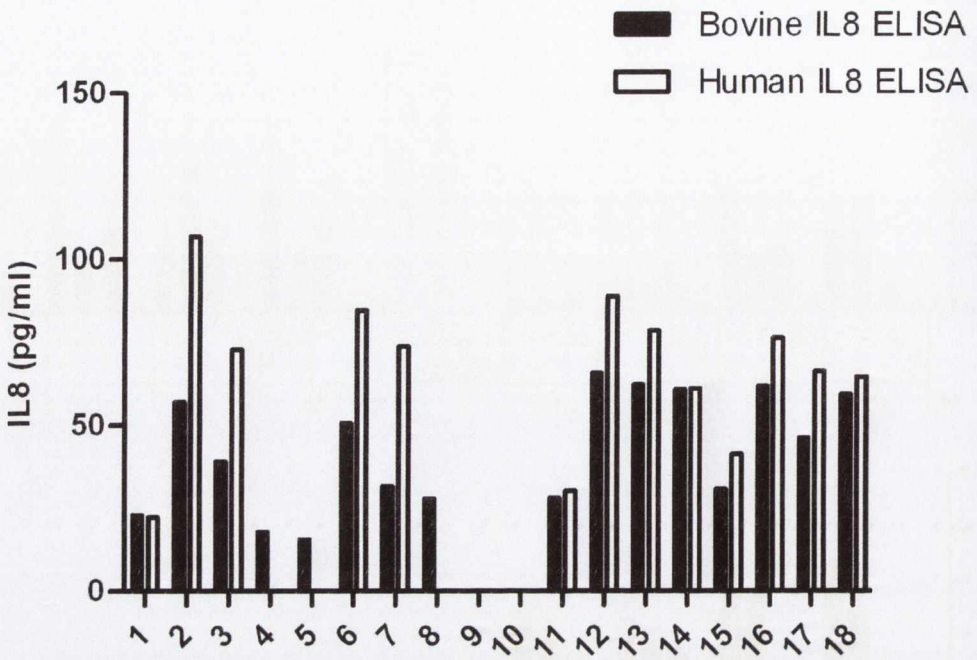

Figure V-7. IL8 protein concentration measurements obtained using bovine and human IL8 ELISA method.

MDM (5 x 10^4 cells per well) were plated, and stimulated with 1μg/ml LPS or left untreated for 24h. Supernatant samples were then collected and IL8 protein concentration was measured in randomly selected samples (1-18) using ELISA Kit specific for both bovine and human IL8 and ELISA data was analysed using 4-parameter logistic model.

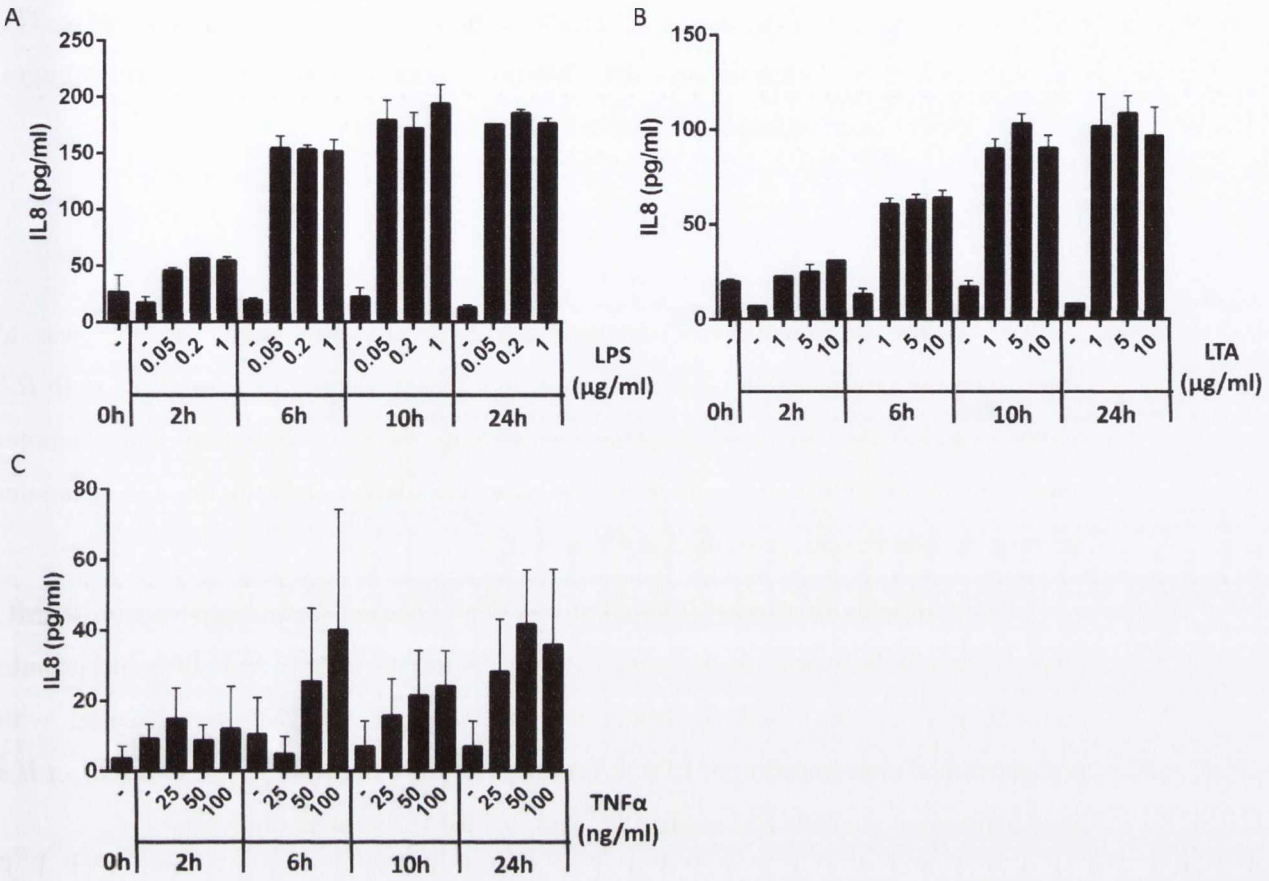

Figure V-8. Time course and dose response of IL8 protein secretion by the bovine monocyte-derived macrophages (MDM) following stimulation with LPS, LTA and TNFα.

Bovine macrophages were stimulated with increasing concentrations of LPS (0.05 µg/ml, 0.2 µg/ml and 1 µg/ml) (A), LTA (1, 5 and 10 µg/ml) (B), and TNFα (25, 50 and 100 ng/ml) (C); and IL8 protein concentration was quantified in the media at multiple time points (0h, 2h, 6h, 10h and 24h), using ELISA. Normalized OD values were analysed using 4-parameter logistic model; n=2.

V.3.1.2. Characterization of the haplotype-specific IL8 expression in bovine monocyte-derived macrophages, stimulated with LPS, LTA and TNFα

Following optimization of the dose and time of stimulation, the aim was to investigate the bovine MDM IL8 expression in a larger number of animals of both *IL8* haplotypes. Initially, we used TaqMan Genotyping Assay to determine *IL8* promoter haplotype in 138 HF cows, which is the same population used in the *IL8* promoter genotyping experiment, presented in Chapter 3.

Eight animals from both *IL8* haplotypes were selected for the experiment. PBMC were separated from the blood, monocytes were left to adhere and differentiate into macrophages as described in Materials and Methods. MDM were treated with predetermined concentration of LPS, LTA and TNFα for 6h, RNA was extracted for *IL8* gene expression analysis and media was sampled for IL8 protein analysis.

All three stimuli induced significant increase in both IL8 gene and protein expression ($P<0.001$), as compared to unstimulated MDM. TNFα was the least potent inducer of IL8 gene and protein expression, whereas LPS and LTA induced similar levels of both IL8 gene and protein expression. None of the stimulations resulted in a significantly different response between the two *IL8* haplotypes in either basal state or in response to any of the stimuli (Figure V-9), suggesting that the two *IL8* haplotypes show comparable activity in MDM. However, as MDM cell culture does not reflect the complex *in vivo* environment, it was decided to follow up the study of *IL8* haplotypes in MDM, with the *in vivo* characterisation of IL8 expression in HF calves of the two haplotypes.

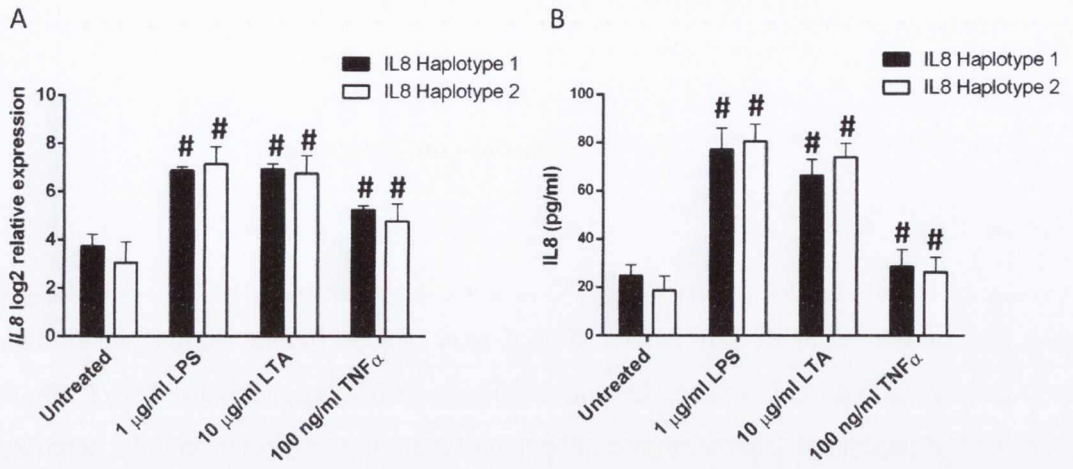

Figure V-9. IL8 gene and protein expression in bovine monocyte-derived macrophages (MDM), from animals of the two *IL8* haplotypes.

MDM were isolated from the two different *IL8* haplotypes and stimulated with 1 µg/ml LPS, 10 µg/ml LTA and 100 ng/ml TNFα, cell lysates and media were sampled after 6h of incubation and *IL8* gene (A) and protein levels (B) were measured by RT-qPCR and ELISA, # $P<0.05$ for the comparison between the untreated and LPS, LTA or TNFα stimulated group for a given haplotype; n=8.

V.3.2. The effect of *IL8* haplotype on IL8 expression in Holstein-Friesian calves challenged with LPS

V.3.2.1. LPS dose optimization

Following the *in vitro* analysis using the bovine MDM model, IL8 expression was then quantified in HF bull calves *in vivo*, upon LPS challenge. Eighty-two animals were genotyped for the *IL8* promoter and the genotype frequencies were 0.28, 0.13 and 0.59 for *IL8* haplotype 1 homozygotes, *IL8* haplotype 2 homozygotes and *IL8* heterozygotes, respectively. The corresponding allele frequencies were 0.57 and 0.43, for *IL8* haplotype 1 and *IL8* haplotype 2, respectively (Table V-1).

Before conducting the LPS challenge on animals from the two haplotypes, it was necessary to identify an optimal LPS concentration needed to induce an inflammatory response in calves. Previous experiments, aimed to characterise clinical, cellular and blood biochemical responses following an intravenous LPS challenge in cattle, have used doses of LPS ranging from 10-1000 ng/kg [264, 265]. In a pilot study, a single HF bull calf was challenged with 100 ng/kg LPS and haematology analysis[2] was carried out in order to assess the leukocyte response to LPS. There was an early decrease in neutrophil count, at 1h post-stimulation, followed by the gradual increase in neutrophil numbers which returned to the baseline level 48h post-stimulation (Figure V-10 A). There was also an early decrease in the number of circulating monocytes, at 1h post-stimulation, followed by the return to baseline monocyte cell count at 12h post-challenge. The number of circulating monocytes continued to increase and reached a plateau at 36h post-challenge, followed by the return to baseline levels 96h post-stimulation (Figure V-10 B). This analysis confirmed that 100 ng/kg LPS was sufficient to induce haematological changes, and did not have adverse effects on the health of the animal.

[2] Performed by Margaret Murray

Table V-1. IL8 promoter genotype and allele frequencies in 87 HF bull calves

	Genotype Frequencies		Allele Frequencies
***IL8* Haplotype 1**	0.28	***IL8* Haplotype 1**	0.57
***IL8* Haplotype 2**	0.13	***IL8* Haplotype 2**	0.43
Heterozygote	0.59		

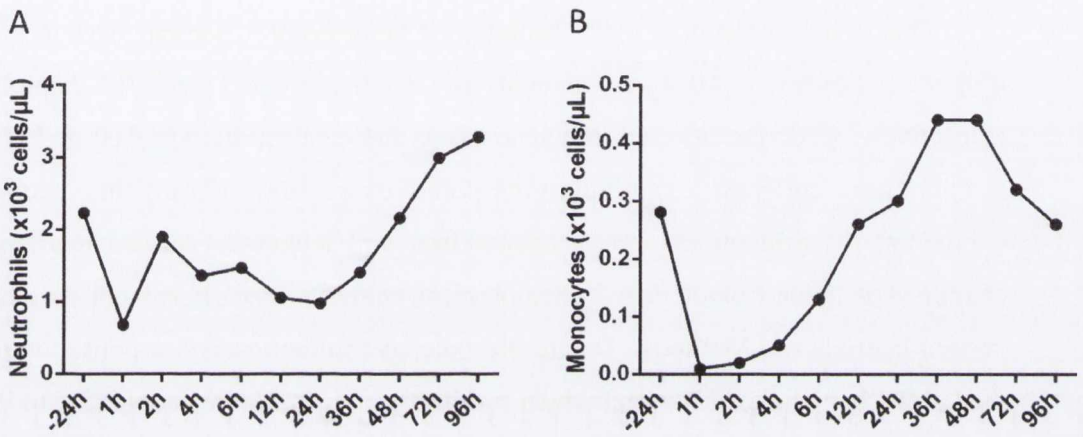

Figure V-10. Haematology profiles in a Holstein-Friesian calf stimulated with 100ng/ml LPS

A single healthy HF calf was challenged with 100 ng/kg LPS, peripheral blood samples were collected over 96h time-course and haematology analysis was carried out using ADVIA. Data is expressed as absolute cell count of circulating neutrophils (A) and monocytes (B).

V.3.2.2. LPS challenge of Holstein-Friesian calves of two *IL8* promoter haplotypes

V.3.2.2.I Cytokine gene expression in the two *IL8* promoter haplotypes

Due to low frequency of *IL8* haplotype 2 homozygotes, sample sizes in the main trial were limited to 10 and 9 animals of *IL8* haplotype 1 and *IL8* haplotype 2, respectively. LPS was injected intravenously at the concentration of 100 ng/kg. Blood samples were collected at multiple time points over 9 days (216h) time-course. The expression of different cytokines, chemokines and leukocyte surface markers were examined in whole blood and haematological analysis was carried out as described under Materials and Methods. Due to the excessive inflammatory response, two calves died at the beginning of the trial which resulted in a decrease in sample size to 9 and 8 animals of *IL8* haplotype 1 and 2, respectively.

Only a subset of samples were analysed for the gene expression due to practical and time limitations, especially due to the high cost of performing RNA extraction, cDNA synthesis and RT-qPCR on all the samples and analysing multiple genes. Therefore, five critical time-points were selected, 0h, 2h, 6h, 12h and 24h, keeping in mind that most genes under investigation influence early innate immune response.

The same group of reference genes used in the MDM model were tested for their expression stability in whole blood RNA samples from the *in vivo* LPS challenge: *PPIA*, *GAPDH*, *ACTB*, *MRPS6* and *H3F3A*. The GeNorm analysis has shown that *PPIA* was again the most stably expressed gene, followed by *GAPDH*, *ACTB*, *MRPS6* and *H3F3A*. Therefore, *PPIA* and *GAPDH* were used to normalise the target gene expression.

Gene expression data was analysed using the repeated measures ANOVA with hour post-challenge, *IL8* haplotype and the interaction between the hour post-challenge and *IL8* haplotype used as explanatory variables. *IL8* gene expression in whole blood demonstrated significant effects of time post-challenge ($P<0.0001$) and *IL8* haplotype

(P=0.0051) (Table V-2). The pair-wise comparison at individual time-points has demonstrated a significantly elevated *IL8* gene expression in *IL8* haplotype 2 compared to *IL8* haplotype 1 at 2h and 24h post-challenge (Figure V-11 A). In addition, a number of other cytokine genes were analysed, such as *TNFα* (Figure V-11 B), *IL1β* (Figure V-11 C) and *INFγ* (Figure V-11 D), and none of these were differentially expressed between the two *IL8* haplotypes, suggesting that the difference in gene expression is *IL8* haplotype-specific.

Table V-2. Analysis of variance (ANOVA) of *IL8* gene expression in HF calves stimulated with LPS.

Source of Variation	% of total variation	*P* value	*P* value summary
Interaction	0.4800	0.8521	ns
Time post-challenge	21.04	< 0.0001	****
***IL8* Haplotype**	23.84	0.0051	**

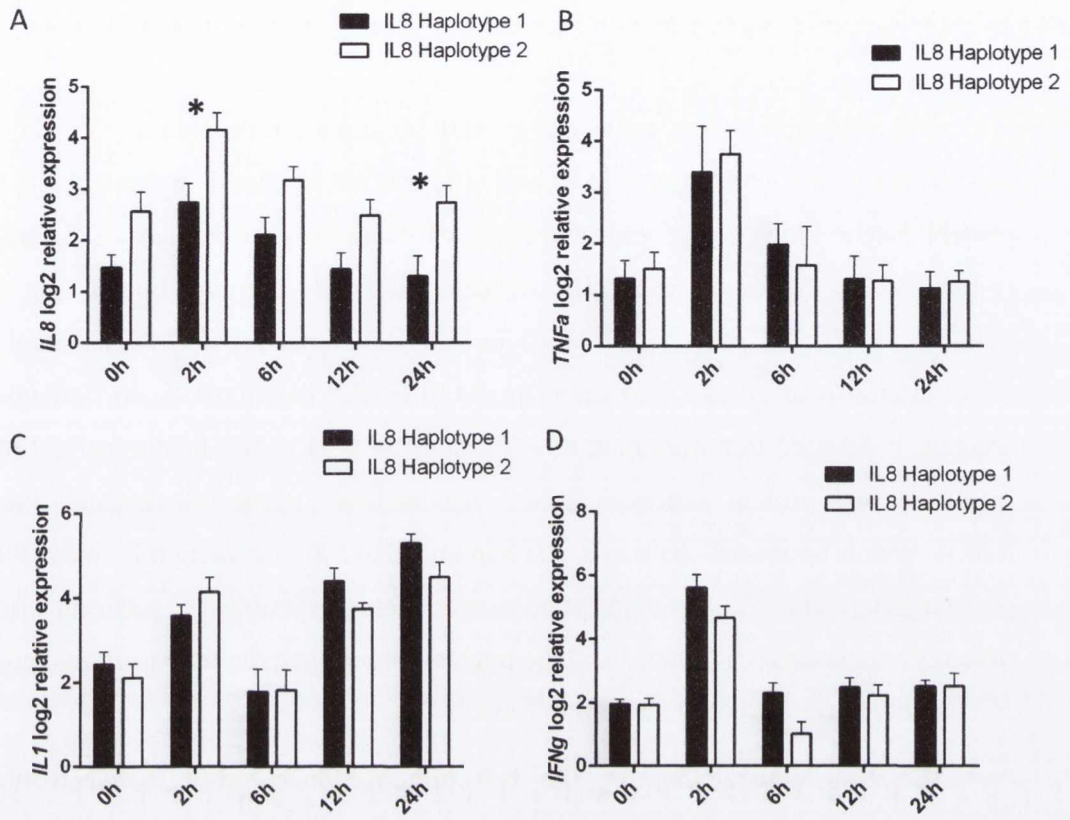

Figure V-11. *IL8*, *TNFα*, *IL1β* and *IFNγ* gene expression in whole blood from Holstein-Friesian calves challenged with 100 ng/kg LPS.

Upon LPS challenge, whole blood samples were collected from HF calves of both *IL8* haplotypes, over a 216h time-course, RNA was extracted and converted to cDNA which was used for RT-qPCR analysis of *IL8* (A), *TNFα* (B), *IL1β* (C) and *IFNγ* (D) gene expression. Samples collected up to 24h post-challenge were used in the study. Gene expression was normalized with the two reference genes, *PPIA* and *GAPDH*. Data is expressed as $\log_2$ fold change relative to the sample with the lowest level of expression. * $P<0.05$ for the comparison between the two haplotypes at the given time-pont. n=9 for IL8 haplotype 1, n=8 for IL8 haplotype 2.

V.3.2.2.II IL8 and IL6 protein expression in the two *IL8* promoter haplotypes

Serum samples were collected at multiple time-points over a nine-day time-course, upon LPS challenge, and cytokine levels were quantified using ELISA. IL8 protein levels showed a divergent pattern of expression in the two *IL8* haplotypes (Figure V-12). Mean (± SEM) IL8 concentration before stimulation averaged 73.4 pg/ml (± 18.7) and 68.9 pg/ml (± 9) in IL8 haplotype 1 and 2, respectively. IL8 concentration rose up to 108.4 pg/ml (± 34.9) in IL8 haplotype 2, 4h post-injection, whereas it dropped to a minimum of 25.2 pg/ml (± 5.9) in IL8 haplotype 1, 12h post-injection. The protein expression data was analysed using the repeated measures ANOVA with hour post-challenge, *IL8* haplotype and the interaction between the hour post-challenge and *IL8* haplotype used as explanatory variables. Significant effects of time post-challenge ($P<0.0001$) and the interaction between the time post-challenge and *IL8* haplotype ($P<0.006$) were found (Table V-3).

Pair-wise comparisons of the IL8 protein concentration between the two haplotypes, at individual time points, showed higher IL8 expression in haplotype 2 at 12h post LPS injection (Figure V-12). Finally, in order to confirm that the haplotype specific profile of protein expression was exclusive to IL8, an IL6 ELISA was performed on samples obtained 0h, 2h, 6h and 12h post-stimulation. Maximal induction of IL6 expression was observed at 2h post LPS injection, with the mean (± SEM) IL6 concentration of 742 pg/ml (± 191) and 656 pg/ml (± 295.5) in IL8 haplotype 1 and 2, respectively. No significant difference between the *IL8* haplotypes was detected at any of the analysed time-points (Figure V-13). The concentration of a number of other proinflammatory cytokines such as TNFα, IL1, IL17 and IFNγ were also measured, but the analysis was precluded by the fact that the presence of these cytokines could not be detected in all the samples due to low concentration and/or low sensitivity of ELISA kits.

Table V-3. Analysis of variance (ANOVA) of IL8 protein expression in HF calves stimulated with LPS.

Source of Variation	% of total variation	*P* value	*P* value summary
Interaction	5.652	0.0060	**
Time post-challenge	15.17	< 0.0001	****
***IL8* Haplotype**	5.587	0.1438	ns

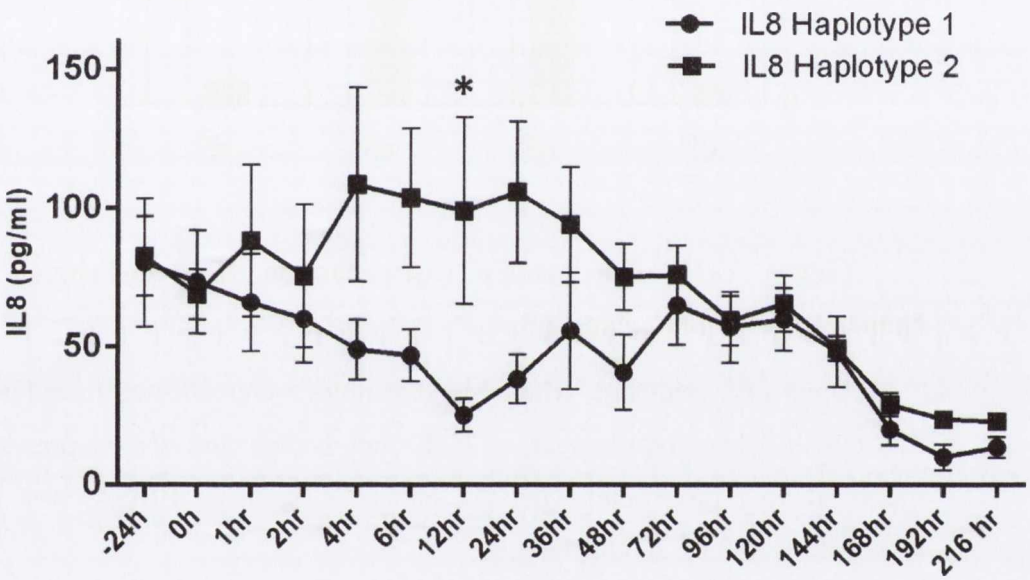

Figure V-12. IL8 protein expression in Holstein-Friesian calves challenged with 100 ng/kg LPS.

Upon LPS challenge, whole blood samples were collected from HF calves of both *IL8* haplotypes, over a 216h time-course and IL8 expression was measured by ELISA. * $P<0.05$ for the comparison between the two haplotypes at the given time-point; n=9 for *IL8* haplotype 1, n=8 for *IL8* haplotype 2.

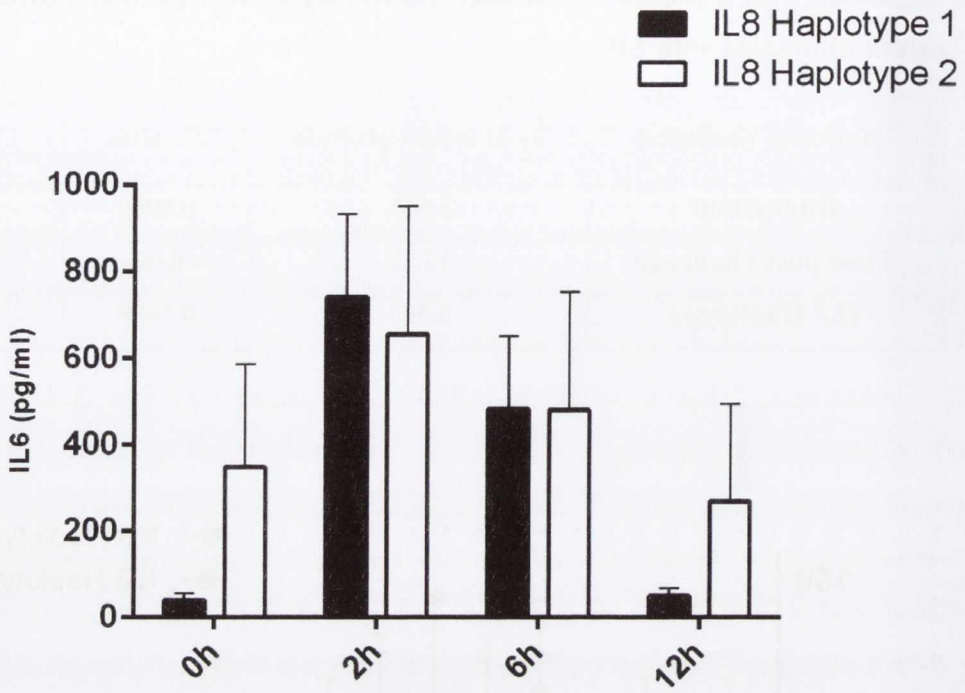

Figure V-13. IL6 protein expression in Holstein-Friesian calves challenged with 100 ng/kg LPS.

Upon LPS challenge, whole blood samples were collected from HF calves of both *IL8* haplotypes, over a 216h time-course and IL6 expression was measured at 4 time points: 0h, 2h, 6h and 12h; using ELISA; n=9 for *IL8* haplotype 1, n=8 for *IL8* haplotype 2.

V.3.2.2.III Circulating leukocyte counts at multiple time-points after LPS challenge, in calves of two *IL8* haplotypes

Peripheral blood samples were collected at multiple time-points post-challenge, and absolute leukocyte counts were obtained using ADVIA[3]. The haematology data was analysed using the repeated measures ANOVA with hour post-challenge, *IL8* haplotype and the interaction between the hour post-challenge and *IL8* haplotype used as explanatory variables. The neutrophil cell count showed an early decrease at 1h, followed by an increase in cell count, which reached basal levels 12h post-stimulation, in both *IL8* haplotypes. The effect of time post-challenge on neutrophil cell count was highly significant ($P<0.0001$), and there was no significant difference between the two *IL8* haplotypes (Figure V-14 A). The monocyte cell count showed an early decrease at 1h, followed by an increase in cell count, which reached basal levels 72h post-stimulation in *IL8* haplotype 1 and 12h post-stimulation, in *IL8* haplotype 2. The effect of time post-challenge on monocyte cell count was highly significant ($P<0.0001$), and there was no significant difference between the two *IL8* haplotypes (Figure V-14 B). The lymphocyte cell count showed an early decrease at 4h, followed by an increase in cell count, which reached basal levels 24h post-stimulation, in both *IL8* haplotypes. The effect of time post-challenge on lymphocyte cell count was highly significant ($P<0.0001$). Also, there was a significant effect of *IL8* haplotype on the lymphocyte cell count ($P=0.0091$). The pair-wise comparison in lymphocyte cell count between the two haplotypes, at individual time-points showed a significantly higher cell count in *IL8* haplotype 2; at 12h post-challenge (Figure V-14 C).

Collectively, these data suggest that LPS treatment induced a significant inflammatory response in calves, manifested in early and transient leukopenia. The neutrophil and monocyte counts were comparable between the haplotypes, whereas lymphocyte count showed consistently higher numbers in *IL8* haplotype 2 and the difference was statistically significant 12h post-challenge.

[3] Performed by Margaret Murray

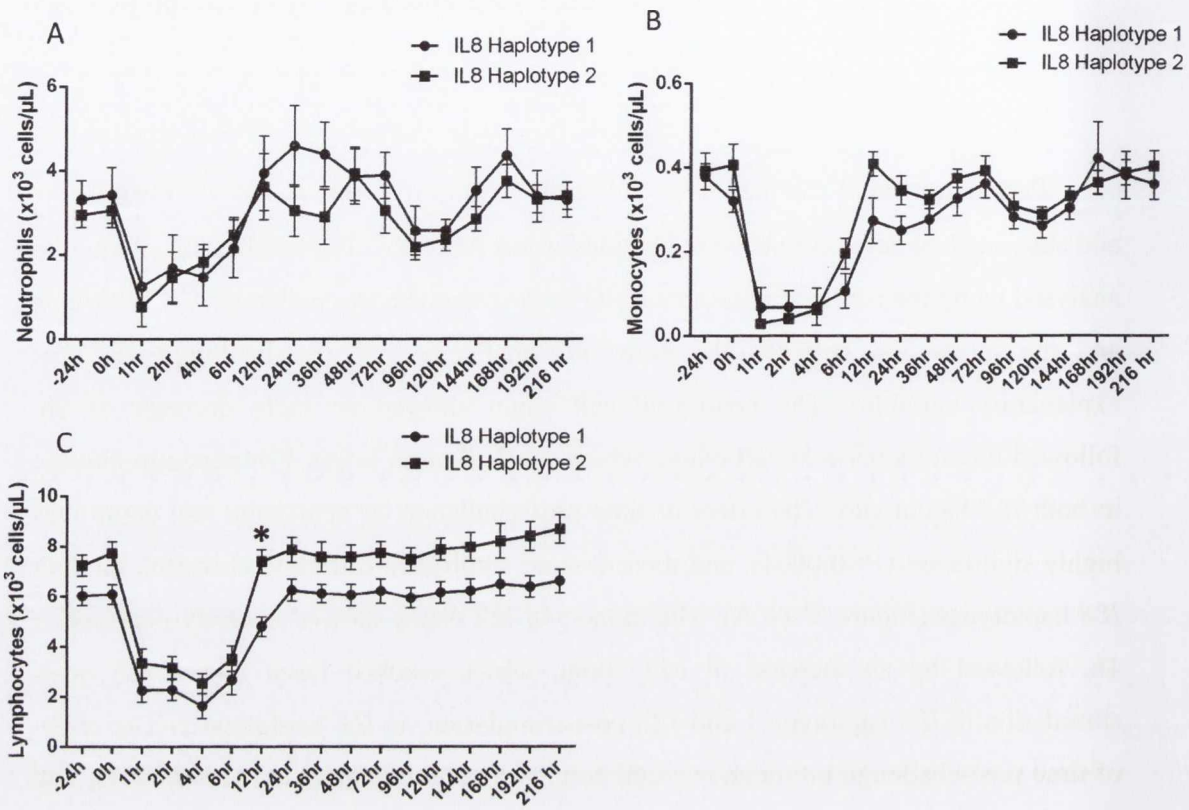

Figure V-14. Haematology profiles in Holstein-Friesian calves stimulated with 100ng/ml LPS.

Upon LPS challenge, whole blood samples were collected from HF calves of both *IL8* haplotypes, over a 216h time-course, and haematology analysis was carried out on neutrophil (A), monocyte (B), and lymphocyte (C) counts; data is expressed as mean (±SEM) absolute leukocyte count; * $P<0.05$ for the comparison between the two haplotypes at the given time-point, n=9 for *IL8* haplotype 1, n=8 for *IL8* haplotype 2.

V.3.2.2.IV Neutrophil activation marker gene expression

Serum IL8 protein levels, after LPS stimulation, were higher in animals of *IL8* haplotype 2, compared to animals of *IL8* haplotype 1, as presented in the previous section (Figure V-12). This means that the circulating neutrophils of the calves of *IL8* haplotype 2 are exposed to higher IL8 levels compared to the neutrophils of calves of *IL8* haplotype 1. A number of papers have reported the ability of IL8 to enhance the expression of neutrophil intergrins and bactericidal enzymes, in a dose dependent-manner [119, 121, 122]. Therefore, it was important to establish if the higher serum IL8 levels in calves of *IL8* haplotype 2, result in the higher gene expression of neutrophil activation markers in these animals, compared to the calves of *IL8* haplotype 1. RNA was extracted from the whole blood of calves of the two *IL8* haplotypes and four genes expressed by neutrophils were studies by RT-qPCR, at multiple time-points after LPS challenge: *CD18*, *CD62L* (L-Selectin), *Lactoferrin* and *CXCR2*. Data was analysed using the repeated measures ANOVA with hour post-challenge, *IL8* haplotype and the interaction between the hour post-challenge and *IL8* haplotype used as explanatory variables. The effect of time post-challenge was significant for all four genes ($P<0.0001$). However, there was no significant effect of *IL8* haplotype and the expression levels of all the four genes were comparable between the two *IL8* haplotypes, at all the time-points (Figure V-15).

This data suggests that the difference in the serum IL8 protein concentration between the calves of two haplotypes did not induce haplotype-specific systemic upregulation of genes encoding neutrophil functional markers.

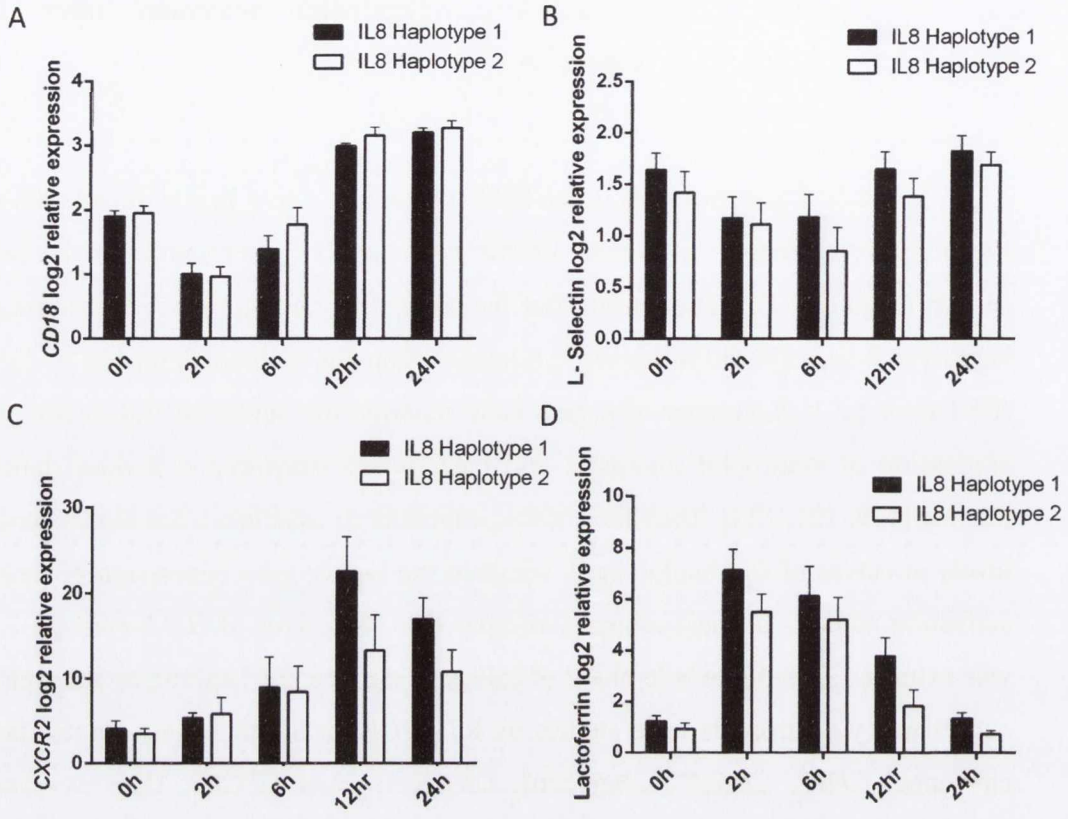

Figure V-15. *CD18*, *CXCR2*, *Lactoferrin* and *L-Selectin* gene expression in whole blood from Holstein-Friesian calves challenged with 100 ng/kg LPS.

Upon LPS challenge, whole blood samples were collected from HF calves of both *IL8* haplotypes, over a 216h time-course, RNA was extracted and converted to cDNA which was used for RT-qPCR analysis of *CD18* (A), *L-Selectin* (B), *CXCR2* (C), *Lactoferrin* (D) gene expression. Samples collected up to 24h post-challenge were used in the study. Gene expression was normalized with 2 reference genes, *PPIA* and *GAPDH*. Data is expressed as Log2 fold change relative to the sample with the lowest level of expression; n=9 for *IL8* haplotype 1, n=8 for *IL8* haplotype 2.

V.3.2.2.V *CXC* chemokine gene expression in the two *IL8* promoter haplotypes

The *IL8* gene is located in the region of chromosome 6 *CXCL* cluster with genes encoding other CXC chemokines, such as *CXCL2*, *CXCL3*, *CXCL6* and *GRO1*, downstream from *IL8*. The close proximity to other chemokine encoding genes suggests that that the *IL8* haplotype could extend beyond *IL8* and cover other *CXCL* genes. Therefore, gene expression of *CXCL3* and *CXCL6* was quantified. Both chemokines showed early upregulation in response to LPS, while there was no significant difference in expression between the two *IL8* haplotypes, suggesting that the haplotype-specific chemokine response is specific to *IL8* (Figure V-16).

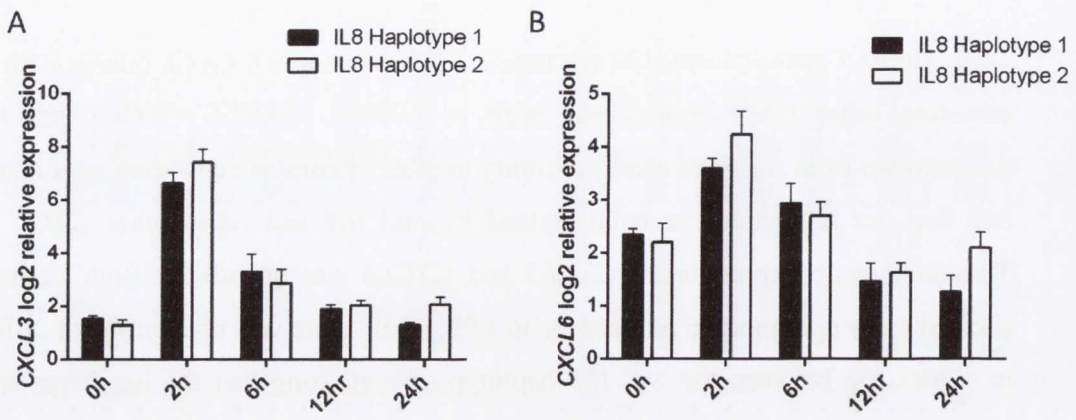

Figure V-16. *CXCL3* and *CXCL6* gene expression in whole blood from Holstein-Friesian calves challenged with 100 ng/kg LPS.

Upon LPS challenge, whole blood samples were collected from HF calves of both *IL8* haplotypes, over a 216h time-course, RNA was extracted and converted to cDNA which was used for RT-qPCR analysis of *CXCL3* (A) and *CXCL6* (B) gene expression. Samples collected up to 24h post-challenge were used in the study. Gene expression was normalized with 2 reference genes, *PPIA* and *GAPDH*. Data is expressed as Log2 fold change relative to the sample with the lowest level of expression. n=9 for *IL8* haplotype 1, n=8 for *IL8* haplotype 2.

V.3.2.3. The effect of IL8 dose and *IL8* haplotype on neutrophil function

V.3.2.3.I Neutrophil purity and viability

IL8 haplotype 2 can drive more than two fold higher IL8 production on average than IL8 haplotype 1 in response to LPS challenge *in vivo*. Consequently, it was important to determine if this difference in IL8 production between haplotypes has any impact upon neutrophil function. To establish this, a series of *in vitro* experiments was conducted in which the serum obtained during *in vivo* LPS challenge from calves of the two *IL8* haplotypes was used to induce neutrophil activation. For the purpose of these experiments, granulocytes were used instead of neutrophils as bovine neutrophil specific antibody was not available. However, given that neutrophils represent majority of bovine granulocyte population, it was decided that the cell population used in this study is representative of the bovine neutrophil population. For each neutrophil functional assay, cells were first purified using MACS from the peripheral blood of a single cow, as described under Materials and Methods. The cell purity and viability were determined using anti-bovine G1 antibody and PI staining, respectively. The typical purity was always above 90% and viability above 95% (Figure V-17).

Neutrophils were separated from a single healthy HF cow, incubated with serum from a number of animals of both haplotypes and the ability of neutrophils to migrate and kill the pathogens was assessed *in vitro*.

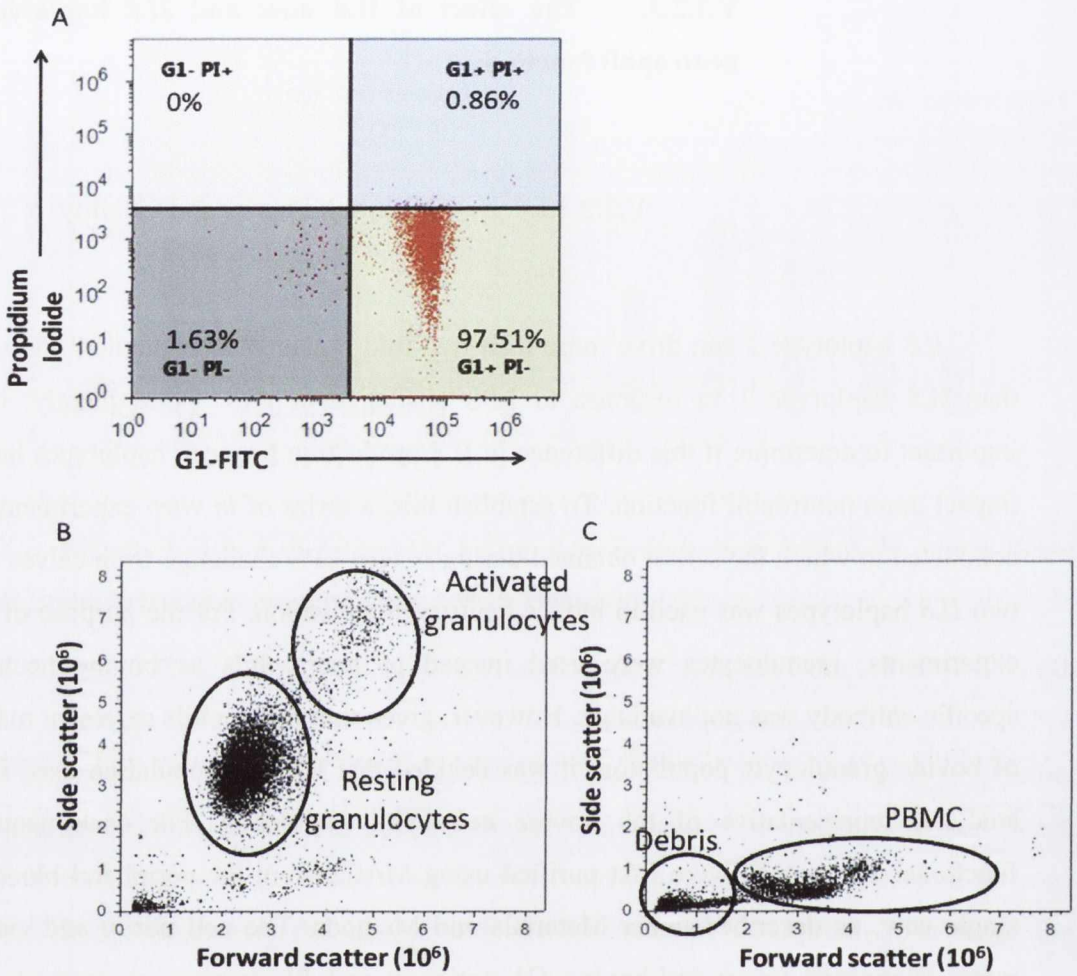

Figure V-17. FACS analysis of the purity and viability of bovine neutrophils

Bovine neutrophils were isolated from peripheral blood of a single healthy HF cow using MACS, anti-bovine G1+ primary antibody and magnetic anti-mouse IgG1 microbeads, followed by staining with goat anti-mouse IgG-FITC secondary antibody and propidium iodide. (A) A dot plot demonstrating purity and viability of neutrophils. (B) A dot plot showing side and forward scatter profile of the positively selected cells using MACS. (C) A dot plot showing side and forward scatter profile of the negatively selected cells using MACS.

V.3.2.3.II The effect of IL8 dose and *IL8* haplotype on neutrophil chemotaxis

The aim of this experiment was to assess the effect of IL8 dose on the neutrophil chemotaxis, and to determine if serum collected from *IL8* haplotype 2 animals, during the *in vivo* LPS challenge, is more potent in driving neutrophil chemotaxis as compared to the serum from *IL8* haplotype 1 animals. Neutrophils were separated from peripheral blood of a single healthy HF cow and incubated for 1h in the Boyden chamber nested inside the well, in the presence of the medium alone (control) or medium supplemented with IL8 (1 ng/ml, 10 ng/ml, 50 ng/ml and 100 ng/ml). The cells in the chamber were separated from the media by a porous membrane that allowed neutrophils to migrate towards the chemokine gradient. Cells that migrated towards recombinant IL8 were collected by removing the media from the bottom wells. The remaining adhered neutrophils were removed by trypsin and pooled with the media. The cells were then counted using flow cytometry. The data is expressed as fold change migration relative to control, which was obtained by dividing the number of cells that migrated towards recombinant IL8 by the number of cells that migrated towards the medium alone.

IL8 induced neutrophil chemotaxis and the magnitude of this increase was dose dependent (Figure V-18 A and B). To confirm that the chemotaxis was IL8 driven, medium supplemented with 50 ng/ml IL8 was preincubated with 30 μg/ml mouse anti-IL8 antibody for 1h in order to block IL8 activity. The anti-IL8 antibody treatment reduced the neutrophil chemotaxis to 26% of the migration driven by 50 ng/ml IL8 (Figure V-18 C).

Having established that IL8 can dose dependently induce neutrophil chemotaxis, it was necessary to characterise the effect of the *IL8* haplotype on neutrophil migration. Blood serum samples collected in the *in vivo* LPS challenge experiment were tested for their ability to induce neutrophil migration. Serum samples obtained 12h post LPS injection were selected as the difference in IL8 concentration between the *IL8* haplotypes was most pronounced at this time-point (Figure V-12). Neutrophils were isolated from the peripheral blood of a single, healthy HF cow, and incubated with RPMI alone as the negative control, or undiluted serum from the two *IL8* haplotypes. The chemotaxis induced by the sera was significantly higher than the chemotaxis

induced by RPMI alone, in both *IL8* haplotypes ($P<0.01$). However, there was no significant difference between the level of chemotaxis induced by sera from calves of *IL8* haplotype 1 and *IL8* haplotype 2 (Figure V-19).

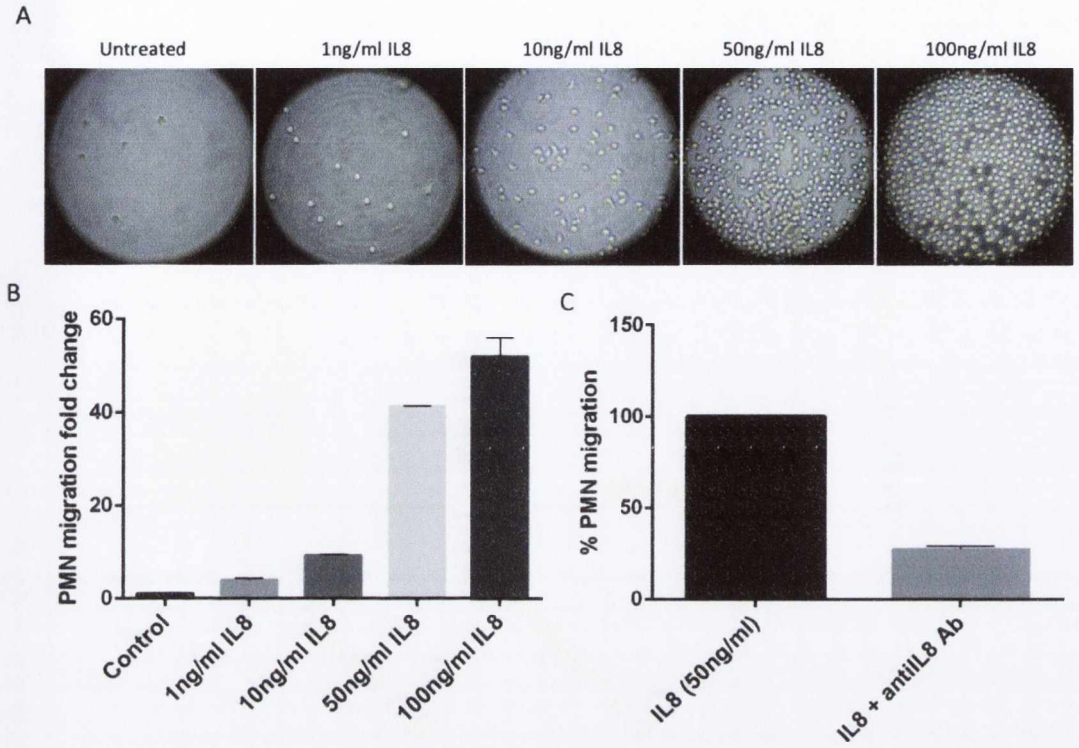

Figure V-18. IL8 induces bovine granulocyte chemotaxis

Bovine neutrophils were isolated from the peripheral blood of a single healthy HF cow, 2.5×10^6 cells were added to the Boyden chamber, placed in a 24 well plate with wells containing 300 µl RPMI supplemented with 1, 10, 50 or 100 ng/ml IL8, and incubated for 1h at 37°C. The cells were visualised under the microscope to confirm migration (A) and collected by removing the media from the bottom wells. The remaining adhered granulocytes were removed by trypsin and pooled with the media. The cells were then counted using flow cytometry and data was initially expressed as the number of cells per µl of medium. Data was then transformed to mean (±SEM) fold change migration relative to control, which was obtained by dividing the number of cells that migrated towards recombinant IL8 by the number of cells that migrated towards the medium alone (B). Blocking of IL8 was carried out by adding 30 µg/ml anti-IL8 antibody to the RPMI media supplemented with 50 ng/ml IL8, and incubating for 1h, before performing the chemotaxis assay. Data is expressed as the percentage of migration (C), n=2.

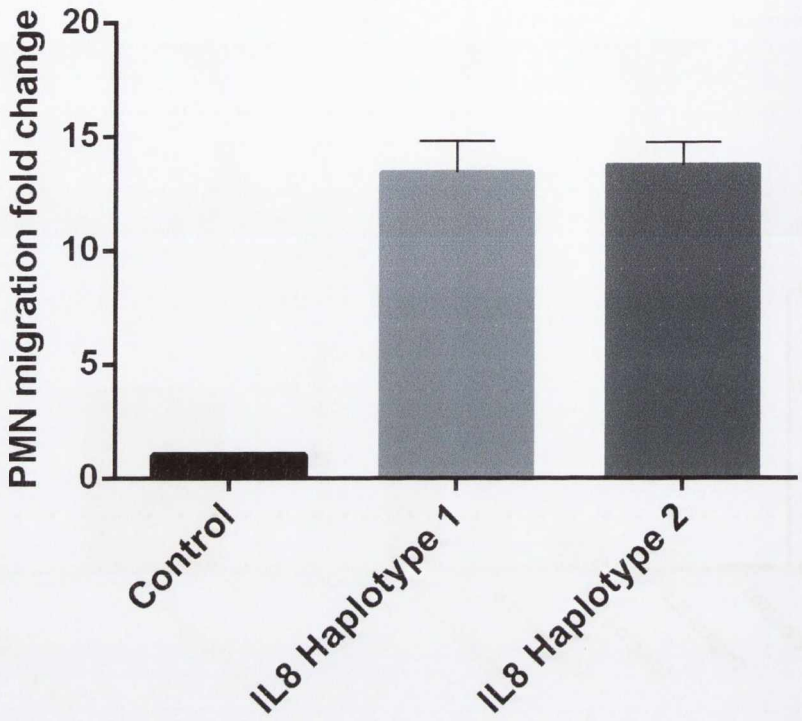

Figure V-19. Bovine neutrophil chemotaxis in response to the serum collected from calves of both IL8 haplotypes, stimulated with LPS.

Bovine neutrophils were isolated from peripheral blood of a single healthy HF cow, 2.5×10^6 cells were added to the Boyden chamber, placed in a 24 well plate with wells containing 300 µl of undiluted serum collected from calves of both *IL8* haplotypes, 12h upon LPS challenge. The neutrophils were incubated for 1h at 37°C, and then collected by removing the media from the bottom wells. The remaining adhered neutrophils were removed by trypsin and pooled with the media. The cells were then counted using flow cytometry and data was initially expressed as the number of cells per µl of medium. Data was then transformed to mean (±SEM) fold change migration relative to control, which was obtained by dividing the number of cells that migrated towards sera from both haplotypes by the number of cells that migrated towards RPMI (control), n=9 for *IL8* haplotype 1, n=8 for *IL8* haplotype 2.

V.3.2.3.III The effect of IL8 dose and *IL8* haplotype on neutrophil killing of *Staphylococcus aureus*

IL8 has been previously shown to promote neutrophil phagocytosis and killing of bacteria [122, 266]. As IL8 concentration in *IL8* haplotype 2 animals was significantly higher than in *IL8* haplotype 1 animals, it was hypothesised that serum collected from *IL8* haplotype 2 animals, during the *in vivo* LPS challenge, would be more potent in promoting neutrophil killing of bacteria as compared with the serum from *IL8* haplotype 1 animals. The bacterial species used in the experiment was *Staphylococcus aureus*, as this species is the most common cause of mastitis in Ireland [267].

Before assessing the neutrophil killing by the two IL8 haplotypes, it was necessary to optimize the concentration of serum used to opsonise bacteria. As the protocol employed in the present study has only been previously used to kill human *S. aureus* SH1000, this strain was initially used as a positive control for neutrophil killing. This strain of *S. aureus* has been described previously [268, 269]. In addition, a common bovine strain of *S. aureus*, MOK032, was also used in the study. This strain of *S. aureus* has been described previously [270]. Neutrophils were separated from a single healthy HF cow. The cells were infected with live *S. aureus*, at the multiplicity of infection 10 (MOI 10), meaning that the ratio between the number of bacterial and neutrophil cells was 10 to 1. The bacteria were opsonised using undiluted or 10 fold diluted bovine serum, which was obtained from the same animal used to source the neutrophils, on the same day. The infected cells were incubated for 2h before the neutrophils were lysed, bacteria were spread on agar plates and colony forming units (CFU) were counted the following day. Neutrophils killed SH1000 more efficiently that MOK032, irrespective of bacterial opsonisation. In the absence of opsonisation, 75% of SH1000 and 41.7% of MOK032 were killed. Bacterial opsonisation improved killing of both strains. When SH1000 was opsonised with undiluted and 10 fold diluted bovine serum, neutrophils killed 95.8% and 96.9% of bacteria, respectively. When MOK032 was opsonised with undiluted and 10 fold diluted bovine serum, neutrophils killed 75.6% and 72.3% of bacteria, respectively (Figure V-20). Undiluted bovine serum was

used for bacterial opsonisation in all the subsequent experiments. Only the bovine strain of *S. aureus* (MOK032) was used in the subsequent experiments.

Having optimised the bacteria opsonisation conditions, the influence of IL8 on neutrophil killing ability was investigated. A previous study has shown that 18h bovine neutrophil pre-stimulation with 40 ng/ml IL8 and 400 ng/ml IL8, results in a IL8 dose-dependent increase in neutrophil oxidative burst and phagocytosis upon subsequent bacterial stimulation [122]. Therefore, these time and dose parameters were used in the experiment. Before infecting the neutrophils with bacteria, the neutrophils were incubated in RPMI supplemented with 40 ng/ml and 400 ng/ml IL8 for 2h and 18h. The neutrophils killed 79.3% and 77.4% bacteria, when stimulated with 40 ng/ml and 400 ng/ml IL8 for 2h, respectively. A similar pattern was observed upon 18h IL8 pre-treament, after which 72.9% and 67.1% of bacteria were killed upon 40 ng/ml and 400 ng/ml IL8 stimulation, respectively (Figure V-21). There was no significant effect of IL8 on neutrophil ability to kill *S. aureus* suggesting that perhaps other more sensitive methods should be used in order to elucidate the impact of IL8 on neutrophil bactericidal capacity.

In order to characterise the effect of *IL8* haplotype on neutrophil ability to kill bovine *S. aureus*, the serum collected during the *in vivo* LPS challenge, from the calves of the two haplotypes, was used. Four serum samples from each haplotype, collected 12h post-challenge, were used in the experiment. Neutrophils were separated from a single healthy HF cow, infected with live *S. aureus* (MOK032), at the MOI 10, 100 µl serum was added and the infected neutrophils were incubated for 2h. Neutrophils were lysed, bacteria were spread on agar plates and colony forming units (CFU) were counted the following day. Neutrophils treated with *IL8* haplotype 1 and *IL8* haplotype 2 sera killed 74.25% and 70.37% of bacteria, respectively. There was no significant difference in the bacterial killing ability between the two groups (Figure V-22). This data suggests that the sera from the two haplotypes induced comparable levels of neutophil killing of *S. aureus*.

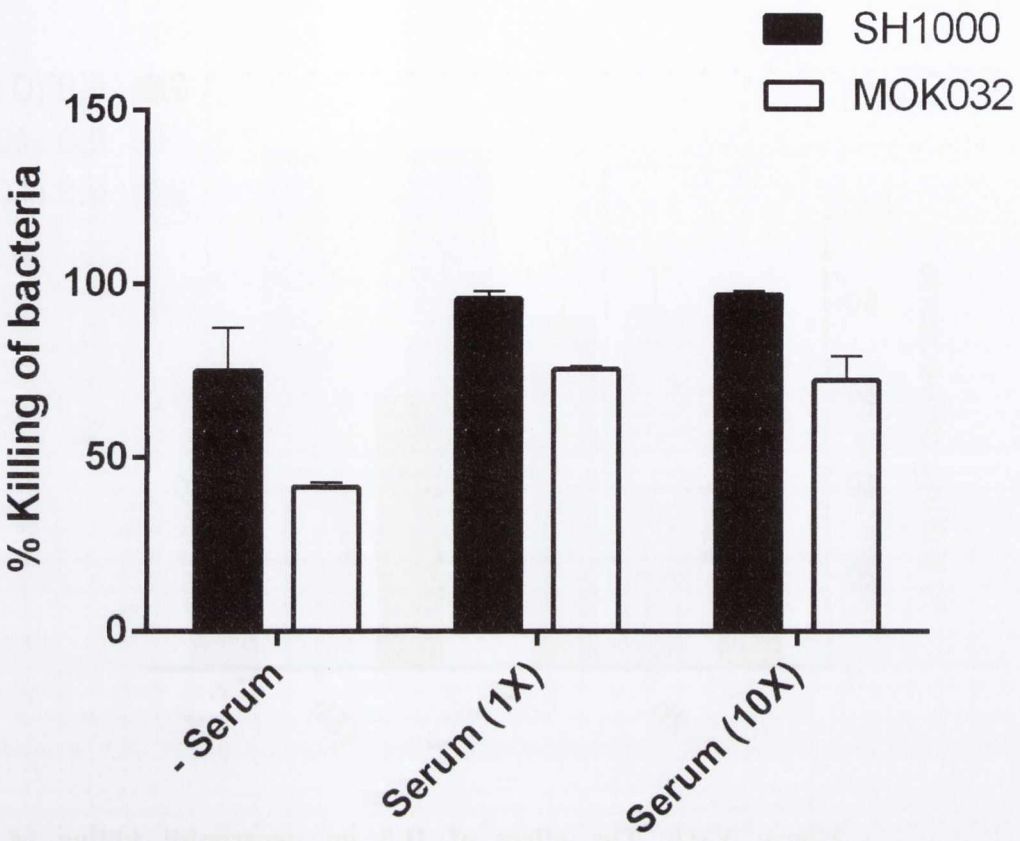

Figure V-20. The effect of bacteria opsonisation on the bovine neutrophil ability to kill *Staphylococcus aureus*.

Bovine neutrophils were separated from peripheral blood of a single healthy HF cow, infected with human (SH1000) or bovine (MOK032) strain of *Staphylococcus aureus* at MOI 10 and incubated at 37°C for 2h. Bacteria were left unopsonised (- Serum) or were opsonised with the bovine serum which was undiluted (Serum 1×) or diluted 10× (serum 10×). Bacteria were plated and CFU were counted after 24h. The percentage of killing of bacteria was determined by comparing the number of total CFU in the presence of neutrophils to the number of CFU in medium only. n=2.

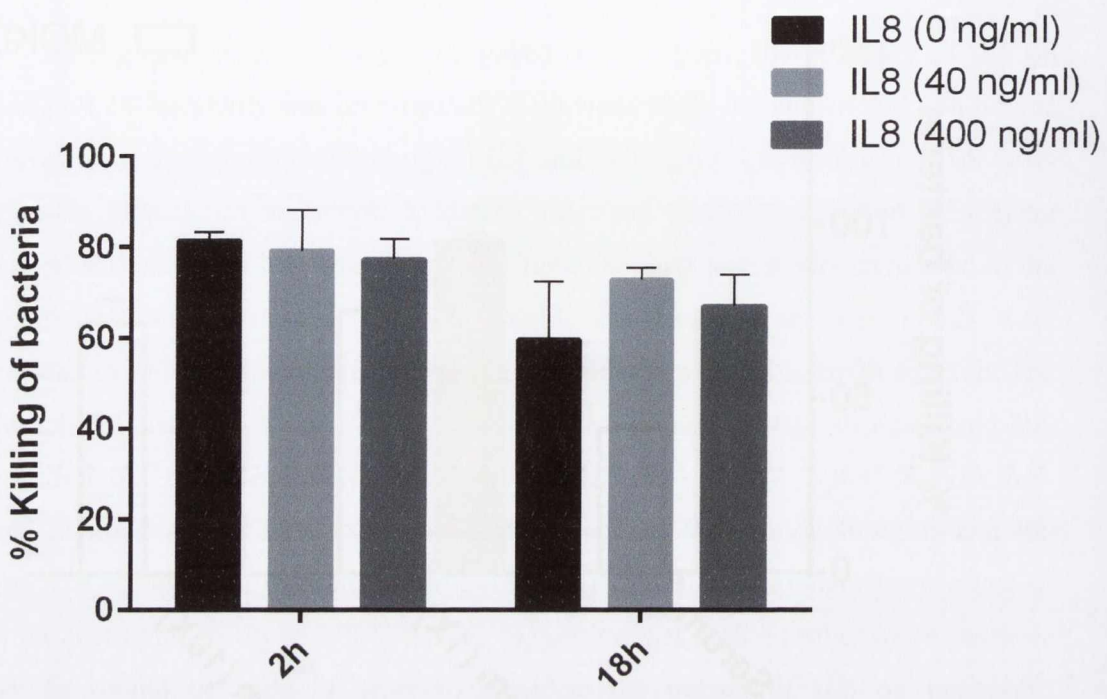

Figure V-21. The effect of IL8 on neutrophil killing of bovine *Staphylococcus aureus.*

Bovine neutrophils were isolated from peripheral blood of a single healthy HF cow, pre-treated with 40 and 400 ng/ml IL8 for 2h or 18h, infected with *Staphylococcus aureus* (MOK032) bacteria at MOI 10 and incubated for 2h at 37°C. Bacteria were plated and CFU were counted after 24h. The percentage of killing of bacteria was determined by comparing the number of total CFU in the presence of neutrophils to the number of CFU in medium only, n=4.

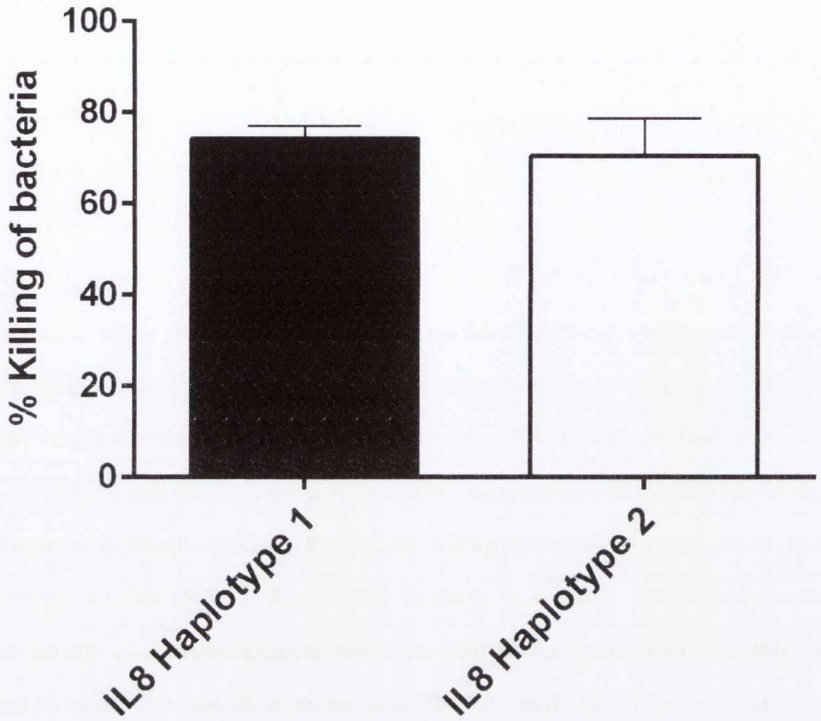

Figure V-22. The effect of *IL8* haplotype on neutrophil killing of bovine *Staphylococcus aureus.*

Bovine neutrophils were isolated from peripheral blood of a single healthy HF cow, infected with *Staphylococcus aureus* (MOK032) bacteria at MOI 10 and incubated for 2h at 37°C, in the presence of sera from the two *IL8* haplotypes. Bacteria were plated and CFU were counted after 24h. The percentage of killing of bacteria was determined by comparing the number of total CFU in the presence of neutrophils to the number of CFU in medium only, n=4.

V. 4. Discussion

In the present study, macrophages obtained from animals of both *IL8* haplotypes produced comparable levels of IL8 at both gene and protein level. On the other hand, a haplotype-specific IL8 response to LPS challenge was shown, in HF calves *in vivo*. Systemic IL8 gene and protein levels in animals of *IL8* haplotype 2 were significantly higher. The same haplotype showed higher activity under *in vitro* conditions using *IL8* promoter transfected bovine mammary epithelial cells [187], and endometrial epithelial cells, as described in the previous chapter, confirming the higher potency of this haplotype in driving IL8 expression. The demonstration of the higher *IL8* haplotype 2 potency *in vivo*, has not been reported before. The *IL8* haplotype-specific differences were detected at both gene and protein level and confirmed as specific to IL8 by analysing the expression of a number of other proinflammatory genes such as *TNFα*, *IL1β* and *INFγ*, as well as IL6 protein expression. None of these cytokines showed differential expression between the two haplotypes. The lack of haplotype-specific response observed in MDM *in vitro* could be due to limitations associated with bovine MDM culture. Even though these cells are among the most dominant IL8 producers, the primary MDM cell culture has a number of disadvantages. The proportion of monocytes in the bovine blood is less than 5% of whole blood leukocytes, which means that large amounts of blood are necessary in order to obtain sufficient number of cells, making it difficult to analyse a large number of animals. Before macrophages can be analysed for IL8 expression, the monocyte are cultured for 7 days which is a minimal time needed for differentiation of monocytes into macrophages. This time is associated with reduced proliferative ability of primary macrophages and phenotypical changes that might not reflect the events that occur *in vivo*.

The difference in IL8 production between the two haplotypes, observed following LPS challenge, may have serious implications in animals' ability to fight infection. More than a two fold difference in the IL8 secretion 12h post-challenge, between the two *IL8* haplotypes, suggests that the circulating neutrophil function will also be different between the haplotypes. To investigate this, a number of neutrophil activation markers were quantified at the gene expression level. These include CD62L, L-selectin involved in neutrophil rolling along the endothelial wall; CD18, an integrin subunit involved in firm neutrophil adhesion and migration through the endothelial wall;

CXCR2, CXCL chemokine receptor and lactoferrin, an antimicrobial protein. No significant gene expression differences were detected between the two *IL8* haplotypes for any of the neutrophil activation markers under investigation. As gene expression was investigated in whole blood, it is possible that the lack of haplotype-specific expression of *CD18* and *CD62L* could be due to the presence of monocytes in whole blood. In addition to neutrophils, monocytes also express *CD18* and *CD62L* [271], but they do not respond to IL8 so it is possible that the inter-haplotype difference in neutrophil *CD18* and *CD62L* expression is abolished due to monocyte expression of these genes. Also, circulating levels of IL8 are very low compared to local chemokine levels and might not be sufficient to induce the difference *in vivo*. Therefore, a series of neutrophil functional assays were conducted *in vitro*, using higher doses of recombinant bovine IL8, in order to assess the physiological significance of the different IL8 levels between the haplotypes. Neutrophil chemotaxis in response to the increasing concentration of recombinant bovine IL8 demonstrated a clear dose-response upon stimulation with IL8 ranging from 1 ng/ml to 100 ng/ml. A number of studies have demonstrated a dose-dependent effect of IL8 on the bovine neutrophil migration. Lee and Zhao have tested the ability of recombinant human IL8, ranging from 0.1 to 100 ng/ml, to induce bovine neutrophil migration and they have demonstrated that the lowest IL8 concentration, inducing significant neutrophil chemotaxis was 1 ng/ml [259], which is in accordance with our study. Caswell *et al* used recombinant IL8 to induce bovine neutrophil migration and they also showed that 1 ng/ml was the minimum IL8 needed to promote bovine neutrophil migration [82].

On the other hand, the effect of different recombinant bovine IL8 doses on neutrophil killing was not significant in the present study. Mitchell *et al.* have reported a dose-dependent effect of bovine IL8 on neutrophil bactericidal mechanisms such as oxidative burst and phagocytosis [122]. The lack of the difference in neutrophil killing in response to high and low IL8, as shown in the present study, could be due to a low sensitivity of the method which was used. The method used in this study is advantageous over measurements of the oxidative burst or phagocytosis, as it eliminates the possibility of bacterial escape, and quantifies the actual reduction in bacterial burden due to the neutrophil activity. However, as the quantification is performed by manually counting CFU, it is also associated with high inter-plate variation. Therefore, increasing the sample size and complementing the killing assay with measurements of neutrophil

phagocytosis and/or oxidative burst could provide additional information regarding the IL8 dose-dependent activation of neutrophil bactericidal function.

In addition to the increasing dose of the bovine recombinant IL8, the serum collected from animals of the two *IL8* haplotypes upon LPS challenge, was also used as a chemoattractant, in order to compare the effect of *IL8* haplotype on neutrophil function. The data showed that all the neutrophil parameters including chemotaxis and ability to kill bacteria were comparable between the two haplotypes. As the largest difference in the serum IL8 concentration between the two haplotypes was approximately 75 pg/ml, it is possible that this concentration is insufficient to drive detectable neutrophil functional differences between the two haplotypes. This is supported by previous studies showing that minimal dose of IL8 neeeded to induce significant bovine neutrophil chemotaxis is 1 ng/ml [82], which is over 10 fold higher than the difference between the two *IL8* haplotypes. Also, the serum contains a number of other CXC chemokines, which are capable of inducing the neutrophil response, thus hindering the detection of IL8 haplotype differences. One of those factors is C5a, which is considered to be the primary chemoattractant in the bovine plasma, serum and milk [272]. A very high concentration of this protein in serum could mask the chemotaxis differences that are driven by IL8 in the two haplotypes, as C5a is capable of desinsitising neutrophils to respond to IL8 [24]. Even though the systemic IL8 concentration was relatively low, it is possible that it does not fully reflect the IL8 concentration gradient that occurs during localised infections, such as intra-mammary or intra-uterine infection. Person Waller *et al* have investigated the bovine cytokine secretion in milk upon intra-mammary infusion of *E. coli* LPS and have reported strong early response manifested by high concentration of IL8, which is over hundred fold higher than the IL8 serum concentration reported in the present study [167]. Also, Shimizu *et al.* have reported high basal level of bovine IL8 in ovarian follicular fluid (>1 ng/ml) and an increase in local IL8 during the follicular development [180]. Also, a local intradermal IL8 injection results in a dose dependent infiltration of neutrophils in calves, *in vivo* [82]. Further studies using local rather than systemic infection model are necessary in order to fully characterise neutrophil function in the two *IL8* haplotypes. These studies will involve the intra-mammary bacterial challenge of cows of the two haplotypes and subsequent measurements of local IL8 production in milk and neutrophil recruitment to the mammary gland.

To conclude, in the present study, the *in vivo* difference in IL8 production has been demonstrated for the first time, between the LPS challenged HF calves of the two haplotypes, confirming the previous results obtained *in vitro*. In addition, it was shown that IL8 induces bovine neutrophil migration, in a dose-dependent manner, *in vitro*, which suggests that the neutrophil recruitment at the local infection sites, such as mammary gland and uterus, could be divergent between the two IL8 haplotypes. This study provides the foundation for future investigation of the downstream consequences of the haplotype-specific IL8 responsiveness on neutrophil function and ability to fight infection.

Chapter VI Final discussion

VI. 1. Evidence of selection in the *IL8* promoter, in the Irish Holstein-Friesian cattle

Molecular genetic research has made a significant contribution in livestock improvement. The identification of genes and genetic variants that influence economic traits of interest has facilitated selection of animals with superior production characteristics. However, intensive selection for high production has negative effects on animal health, such as increased incidence of mastitis and reduced fertility [49, 273]. Therefore, a comprehensive functional characterisation of genetic variants that influence immune responses is warranted.

In the present study, the two bovine *IL8* promoter haplotypes were closely examined. An analysis of the population genetic distribution of the two haplotypes in Irish cattle, and the study of the genetic association of the haplotypes with important health traits were conducted. Also, functional characterisation of the two *IL8* haplotypes was carried out both *in vitro* and *in vivo*. A significant association of *IL8* promoter haplotypes with both somatic cell score and calving interval in a population of Irish HF cattle was identified for the first time. The substitution from haplotype 1 to haplotype 2 was associated with an increased SCC, suggesting that *IL8* haplotype 2 animals have a higher SCC. As IL8 is directly involved in neutrophil migration to the mammary gland during mastitis [19], and neutrophils comprise the majority of the cells present in milk during udder infection [157], it was hypothesised that the *IL8* promoter haplotype is a causative variant that contributes to the genetic control of SCC and has an impact on *IL8* promoter activity. The haplotypes were then functionally characterised in bovine endometrial epithelial cells and it was shown that *IL8* haplotype 2 exhibits higher transcriptional activity than *IL8* haplotype 1, in response to LPS and TNFα. This finding is in accordance with the previous study comparing *IL8* haplotype activity in bovine mammary epithelial cells [187]. Together, this data suggests that *IL8* haplotype 2, showing significantly higher activity *in vitro*, is also associated with higher SCC. It is possible that the higher IL8 production in the *IL8* haplotype 2, along with other factors, is responsible for elevated SCC observed in the cows of this haplotype (Figure VI-1). This is further supported by previous studies demonstrating that milk IL8 levels

increase during mammary gland infection and are accompanied by an elevation in milk SCC [274, 275]. Furthermore, this data suggests that the *IL8* haplotype 1, associated with the lower SCC, will be advantageous in dairy breeds, as low SCC is desirable in the dairy industry. The comparison of *IL8* haplotype frequencies between HF (dairy) and Charolais (beef) cows, demonstrated significantly higher proportion of *IL8* haplotype 1 in HF, which is consistent with the previous study [187]. As HF cows are strongly selected for the low SCC, *IL8* haplotype 1 could be under indirect selection. However, as the frequencies of the two *IL8* haplotypes are uniform in HF, it is possible that both haplotypes are under selection in the HF breed. The evidence of balancing selection has been demonstrated before, in human immune gene promoters. High sequence variation and heterozygosity, maintained through the action of balancing selection, have been previously identified in the human defensin β-1 and interleukin 10 promoters [213, 276]. Also, a 660 kb deletion is maintained at high frequency in Nordic Red Cattle, despite its detrimental effect on fertility, due to a strong positive influence on milk yield [214]. Interestingly, the substitution from haplotype 1 to haplotype 2 was associated with reduced calving interval, suggesting that the calving interval in *IL8* haplotype 2 animals is shorter than in *IL8* haplotype 1 animals. The calving interval is an important trait for the dairy industry, and it is included in the economic breeding index [203]. Shorter calving interval is highly desirable in the Irish dairy sector, as the calving needs to be synchronised with maximal grass growth, as grass is the main food supply for Irish cattle. Therefore, it is possible that *IL8* haplotype 2, associated with the shorter calving interval, is indirectly selected and maintained in the population. In addition, the higher promoter responsiveness of *IL8* haplotype 2 could reduce the risk of retained placenta, as this condition has been associated with the reduced neutrophil count and low IL8 levels around calving [172, 174]. As retained placenta has a negative effect on cow fertility, it is possible that *IL8* haplotype 2 is under indirect selection in HF. The haplotype frequency analysis in the Jersey breed has shown that the *IL8* promoter region is monomorphic in this breed with *IL8* haplotype 2 being fixed in this population. As Jersey is not under a strong selection for milk and low SCC, it is expected that the *IL8* haplotype 1 is less prevalent in Jersey as compared with HF, as observed in the present study. However, the explanation for the fact that this haplotype is completely absent from the population is unknown, and further studies are needed in order to fully understand the haplotype frequency differences between the HF and Jersey breeds. The strong divergence in *IL8* haplotype population genetic structure

between HF and Jersey cattle could have significant consequences on the SCC profiles in the two breeds.

VI. 2. Octamer-binding protein 1 could be responsible for IL8 haplotype 1 transcriptional repression

The bovine *IL8* promoter region in the two haplotypes was further investigated *in silico*, in order to identify the transcription factors responsible for the haplotype-specific *IL8* promoter activity. Five haplotype-specific transcription factors were identified and Oct-1 was further studied, as Oct-1 is involved in both basal and inducible human *IL8* repression [93] and tracheal and lingual antimicrobial peptide gene regulation in cattle [92]. The *in silico* analysis has shown that the Oct-1 binding site overlaps a core *IL8* promoter variant and predicted that Oct-1 only binds *IL8* haplotype 1 promoter, which could contribute to lower promoter activity in this haplotype. The preliminary study, using RNA interference technology, has shown that knocking down *Oct-1* partly removed its repressive effect which resulted in an increased *IL8* promoter activity, and this effect was greater in *IL8* haplotype 1, which suggests that this transcription factor could be partially responsible for the lower *IL8* expression observed in haplotype 1 (Figure VI-1). However, an important limitation of the RNA interference technology employed in the present study, is the unspecific activation of the immune response, which was manifested by the upregulation of the *IL8* and *IL1β* expression upon treatment with the control siRNA. As double-stranded RNA is characteristic of a number of viruses, siRNA can be recognised by the TLR3 receptor [277]. In addition, separated strands of the siRNA molecule can also trigger an immune response as single stranded RNA via TLR7, followed by signal transduction and eventually type I interferon activation [278]. In order to rule out the possibility of unspecific *IL8* activation, a possible approach is to inhibit TLR3 or TLR7 pathways, thus preventing the initial step in siRNA recognition. Another powerful method that could be employed in future studies is gene editing using genome-specific nucleases to precisely excise the *Oct-1* DNA coding sequence, stably knocking-out its expression. In addition to these methods, a mobility shift assay can also be used to study an interaction between a specific transcription factor and a DNA fragment of interest. In order to investigate the

Oct-1 interaction with the two *IL8* haplotype promoters, a prelabelled DNA probe must be designed, that includes a mutation of interest. The probes are then incubated with the nucleic proteins and visualised on a gel. The formation of a complex between the Oct-1 and a DNA probe from both *IL8* haplotypes can be distinguished from the free DNA probe, as the DNA-protein complex is heavier and travels slower on a gel. An Oct-1 specific antibody can then be used to label the complex in order to confirm the specificity of the DNA-protein interaction. These additional approaches would help to clarify the specific role of Oct-1 in IL8 transcriptional repression, in the two IL8 haplotypes.

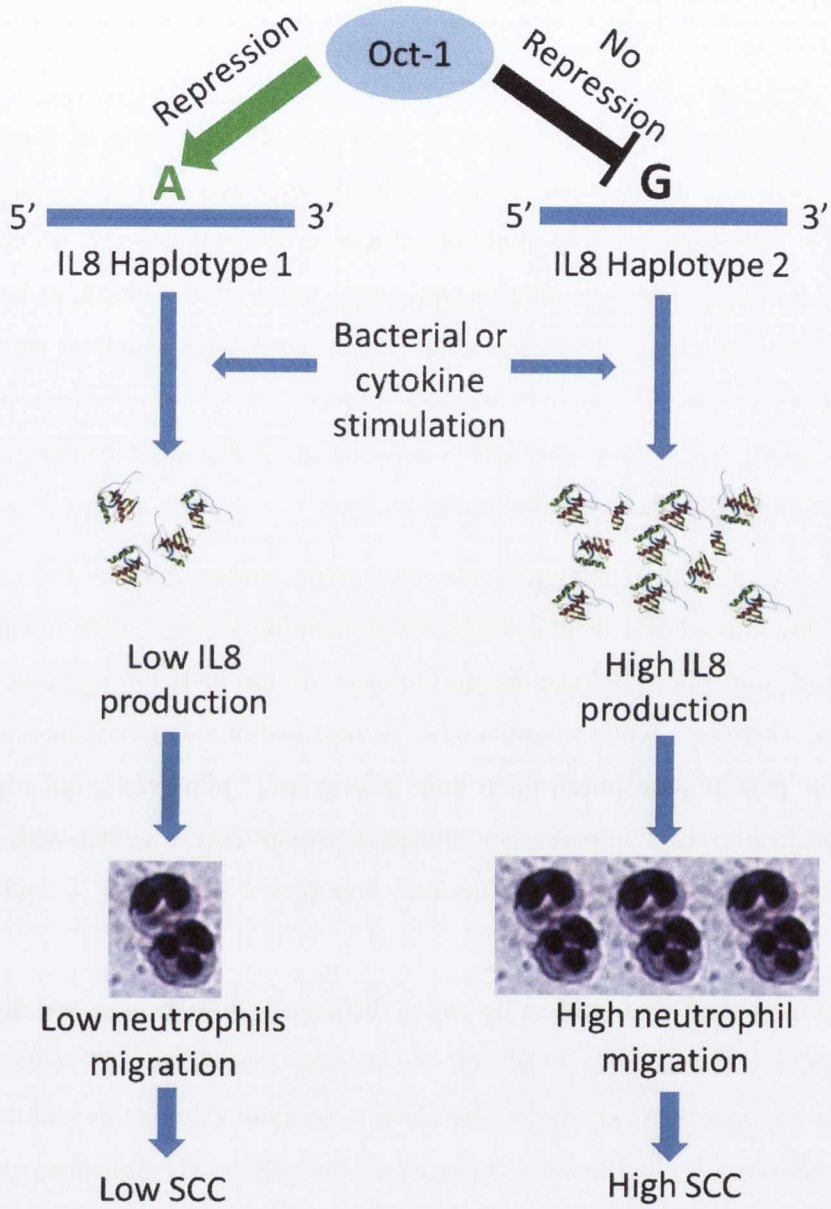

Figure VI-1. A simplistic model of the suggested mechanism responsible for the haplotype-specific IL8 expression and downstream consequences on neutrophil recruitment.

Preliminary data suggests that Oct-1 repressive action on the *IL8* promoter is higher in haplotype 1, which results in lower haplotype 1 expression. Consequently, divergent levels of IL8 result in a different rate of neutrophil migration and ultimately have an impact on SCC.

VI. 3. Haplotype-specific IL8 expression *in vivo* and downstream neutrophil recruitment *in vitro*

In order to validate the *in vitro IL8* haplotype-specific mode of expression, an *in vivo* LPS challenge of HF calves of the two haplotypes was carried out, and IL8 mRNA and protein were quantified in whole blood and serum, respectively. It was shown that calves of *IL8* haplotype 2 produce significantly more IL8 in blood, at both gene and protein level, which is consistent with the *in vitro* data obtained on endometrial epithelial cells. The *in vivo* data showing higher IL8 levels in haplotype 2 animals further supports the genetic association between the *IL8* promoter haplotype and SCC and calving interval, also presented in this study.

A large portion of genome-wide association studies are not fully reproducible [279], as the variant that demonstrates the association is most often not the causative variant itself but it is in linkage disequilibrium with the mutation that controls the trait of interest. Therefore, when a similar study is repeated in a different, genetically distant population, genetic association often does not persist. Collectively, our results suggest that the *IL8* promoter haplotype is a causative variant that, together with other genes, has a direct impact on traits of economic importance such as SCC and the calving interval.

The most likely mechanism by which different IL8 levels between the haplotypes could induce differences in SCC and the calving interval is a divergent neutrophil activation between the haplotypes. Therefore, neutrophil chemotaxis and their ability to kill bacteria were studied *in vitro*, in order to characterise the IL8 dose and haplotype effects on neutrophil function. The data showed that IL8 dose has an effect on chemotaxis, but no impact on the neutrophil killing function, *in vitro*. In addition, both neutrophil migration and killing were comparable between the haplotypes, *in vitro*. There was no significant difference in the number of circulating neutrophils or the level of neutrophil activation *in vivo*, as demonstrated by the gene expression analysis of *CD18*, *CD62L*, *CXCR2* and *Lactoferrin*. The lack of haplotype-specific systemic *in vivo* or *in vitro* neutrophil differences is most likely due to low IL8 serum levels in the circulation. It was demonstrated in the present study that higher doses of IL8 (>1 ng/ml) have an impact on neutrophil recruitment *in vitro*. Even though the systemic level of

IL8 was approximately 10 fold below 1 ng/ml, it is likely that in a localised infection IL8 concentration would be significantly higher. A number of papers suggest that the systemic cytokine levels do not fully reflect the local levels in tissues where the infection occurs and that the cytokine concentration is normally higher locally than systemically. Holzheimer *et al.* have demonstrated that the peritoneal concentrations of IL6 and TNFα were significantly higher than the plasma levels of these cytokines, in patients of severe secondary peritonitis [280]. Also, significantly higher levels of peritoneal IL1 and TNFα, as compared with the systemic concentration of these cytokines have been demonstrated in rats [281]. A previous study quantifying both circulating and local levels of IL8 in psoriasis patients has found a lack of correlation between the systemic IL8 concentration and the regression of the disease. In contrast, local IL8 production in the skin, as assessed using imunohistochemical staining, showed gradual reduction as the disease regressed upon hospital treatment [282]. Therefore, the immunological significance of the inter-haplotype difference in IL8 production can be fully characterised in studies using local pathogenic challenge. A limitation of the local IL8 analysis is that the methods are often not fully quantitative as they involve procedures such as tissue staining and scoring rather than measuring IL8 concentration. Therefore, future studies aiming to compare the local IL8 response in the two haplotypes should target the tissues where IL8 levels can be accurately quantified. The bovine mammary gland is a tissue with an abundant population of IL8 producing cells, such as resident macrophages, mammary epithelial cells and neutrophils. In addition, milk samples are easy to collect and a standardised ELISA assay can be used to quantify IL8 protein concentration accurately. A previous study has reported high milk IL8 protein levels in cattle, in response to intramammary injection of *E coli* endotoxin [167]. Watanabe *et al* have demonstrated that bovine milk contains a high level of IL8 in response to naturally occurring *S. aureus* mastitis, or upon intramammary challenge using recombinant bovine IL8 [283]. In addition to IL8 quantification, the intramammary pathogenic challenge of cows from the two *IL8* haplotypes can be followed by the quantification of the SCC in the two haplotypes, aiming to identify significant differences in milk neutrophil recruitment profiles between the two haplotypes.

VI. 4. *IL8* haplotype 1 promoter is more responsive to bovine herpes virus stimulation

In contrast to the higher *IL8* haplotype 2 response to LPS and TNFα stimulation, *IL8* haplotype 1 promoter activity was significantly higher in response to the viral stimulation *in vitro*, using BoHV-4 treatment of endometrial epithelial cells. The data presented in this study suggests that the stimulatory effect of BoHV-4 on *IL8* haplotype 1 transcriptional activity is stronger that the Oct-1 repressive action on the same haplotype, as *IL8* haplotype 1 activation in response to BoHV-4 exceeds the *IL8* haplotype 2 response. The critical BoHV-4 responsive element in the bovine *IL8* promoter is located between 260 and 124 bp, upstream from the transcription start site [230]. As this region contains only one polymorphism it is likely that this mutation is responsible for the differential responsiveness of the two *IL8* haplotypes to BoHV-4 stimulation. In order to confirm this notion, further studies need to be conducted. These will involve a transfection experiment, using 400 bp and 196 bp *IL8* promoter constructs, from the two haplotypes. As the shorter of these two constructs does not contain the mutation that is predicted to be responsible for the *IL8* haplotype-specific response to BoHV-4, the promoter activity using this construct is expected to be comparable between the two haplotypes. In contrast, the 400 bp *IL8* promoter construct is expected to produce a BoHV-4 response similar to the full *IL8* promoter, in both haplotypes.

The higher responsiveness of *IL8* haplotype 1 to BoHV-4 viral stimulation could have significant implications in animal health as BoHV-4 particles have been isolated from various tissues and BoHV-4 has been linked with a number of disorders in cattle, such as peritonitis, metritis, mastitis, respiratory disease, infertility, encephalitis, diarrhoea [284]. The role of chemokines and neutrophils in viral infections is not entirely clear and a large portion of evidence regarding the role of chemokines in viral infection comes from human studies. The chemokine expression during viral infections is mostly represented by CC chemokines, while CXC chemokines seem to be less expressed [285]. A previous study has shown that human cytomegalovirus promotes IL8 secretion from human monocytes, and it was demonstrated that the transcription factors NFκB and AP-1 are responsible for this induction [286]. There is a limited amount of information regarding the effect of IL8 on viral replication and pathogenesis.

Murayama *et al.* have demonstrated that IL8 promotes human cytomegaloviral production in a dose-dependent manner, stimulating the transcription of the viral genome [287]. It is possible that increased *IL8* haplotype 1 response to BoHV-4 acts to promote viral infection, which could have a negative effect on animal health, particularly in the uterus, where BoHV-4 can be present and cause metritis. An alternative explanation for the balanced frequencies of the two haplotypes, as determined in the HF breed, could be due to different IL8 haplotypes being advantageous in bacterial and viral infections. IL8 haplotype 1 showed stronger response to BoHV-4 stimulation and could be beneficial in viral infection. On the other hand, IL8 haplotype 2 demonstrated higher activity in response to bacterial ligand and could be advantageous in bacterial infections. A divergent selective pressure for both haplotypes under different pathogens could be responsible for maintaining both haplotypes in the population, as observed in HF breed. However, further studies are needed in order to fully characterise the immunological significance of the haplotype-specific *IL8* response to BoHV-4.

VI. 5. Conclusion

A comprehensive analysis of the two *IL8* promoter haplotypes has been conducted in the present study. Novel associations between the *IL8* promoter variants and SCC and calving interval have been demonstrated in the Irish HF population. In addition, these associations have been further dissected in order to better understand the underlying biology of the genetic association. It was shown that the *IL8* haplotype 2 promoter exhibits higher activity than the *IL8* haplotype 1 promoter, in response to LPS and TNFα stimulation, in bovine endometrial epithelial cells. This finding was validated *in vivo*, showing that HF calves of *IL8* haplotype 2 produce more IL8, when challenged with intravenous LPS. Finally, it was demonstrated *in vitro* that bovine neutrophils migrate towards the IL8 gradient in a dose-dependent manner. As IL8 is a critical chemokine in early response to infection, the significant difference in the responsiveness between the two *IL8* haplotype promoters could result in a divergent susceptibility to pathogens in cattle, particularly at sites where neutrophil response is crucial, such as the mammary gland and the uterus. The knowledge of the haplotype-specific association with economically important traits, and the characterisation of the

underlying biology, presented in this study, could potentially aid the selection of animals with superior health characteristics and ultimately improve the immunocompetence of Irish cattle.

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