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Bovine P-selectin mediates leukocyte adhesion and is highly polymorphic in dairy breeds

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ABSTRACT

Bovine P-selectin (SELP) mediates leukocyte rolling and primes leukocyte adhesion to endothelium, both essential for leukocyte recruitment to an infection site. We investigated SELP-mediated adhesion between bovine peripheral blood leukocytes (PBLs) and cultured bovine aortic endothelial cells pre-activated with lipopolysaccharide (LPS). We examined gene polymorphism for bovine selectins SELP, L-selectin (*SELL)* and E-selectin (*SELE)* and compared their SNP frequency between five dairy breeds (Holstein, Friesian, Jersey, Ayrshire and Brown Swiss*)*. LPS treatment caused a rapid (10 min) and slower (4 h) enhancement of PBL adhesion ($P<0.01$). Antibody blocking of SELP inhibited LPS induced cell adhesion. *SELP* was highly polymorphic, with 9 of the 13 SNPs in its exons, whereas only three synonymous SNPs in *SELL* and one in *SELE*. The resulting amino acid changes for the three missense *SELP* SNP were located in the lectin domain and in two consensus repeat (CR) regions, CR2 and CR5. The Val475Met variant locus in the CR4 and CR5 linking region was very close to a predicted *N*-acetyl-D-glucosamine glycosylation site, which is likely to influence SELP function. The AA genotype was under-represented, only being found in 1% of 373 heifers genotyped from the 5 breeds ($P=0.056$), suggesting that AA homozygous animals carrying the Val475Met substitution for SELP may have compromised development. Our study thus confirmed that SELP mediates the attachment of PBL to endothelium and provides novel evidence that its high polymorphism is likely to affect biological function. This may potentially influence leukocyte migration and fertility, both key to successful performance in dairy cows.

Keywords: Cows, Selectin, Adhesion molecules, Leukocyte, Polymorphism.

1. Introduction

Mastitis is an inflammatory disease which causes significant economic losses to the dairy industry (Geary et al., 2012; Kossaibati and Esslemont, 2000). The efficiency of the host defense system plays a major role in the ability to deal with mastitis-causing pathogens, and thus ultimately in determining the severity of infection (Burvenich et al., 2003; Rainard and Riollet, 2006). We recently undertook a combined analysis of data from seven published genome-wide association studies (GWAS) on milk somatic cell count (SCC) together with two experiments reporting changes in gene expression in the mammary gland following experimental infection with either *E. coli or S. uberis* (Chen et al., 2015; Mitterhuemer et al., 2010; Moyes et al., 2009) to determine key pathways involved in the host response. In support of previous work, this approach again highlighted the importance of the leukocyte adhesion/diapedesis signaling pathway in combatting mammary gland infection, by assisting a timely influx of polymorphonuclear leukocytes (PMN) to the infected udder (Paape et al., 2002).

Circulating granulocytes go through a multistep process to reach the site of inflammation by the process of extravasation (Ley et al., 2007). The process is initially triggered by increased release of TNFα, IL1β, C5a and histamine in response to the pathogen, subsequently leading to an increase in expression of adhesion molecules such as P-selectin (SELP) and vascular cell adhesion molecule (VCAM)1 (Asako et al., 1994; Gotscha et al., 1994). SELP is a glycoprotein transported to the surface of the endothelial cells where it can interact with its ligand P-selecting glycoprotein ligand 1 (PSGL1; CD162) on the surface of PMN. This interaction results in neutrophil rolling and subsequent activation of integrin expression, necessary for their adhesion to the endothelium (Norman et al., 2000; Wang et

al., 2007). Increased expression of L-selectin (SELL) by the circulating PMN facilitates their secondary tethering to an already rolling neutrophil (Bargatze et al., 1994; Sperandio et al., 2003). PSGL1 and SELL form a complex, which triggers integrin activation (Stadtmann et al., 2013). SELE like SELP is found in cytokine-stimulated endothelial cells and is also thought to mediate the adhesion of cells to the vascular lining at sites of inflammation (Bevilacqua et al. ,1989).

All three bovine selectin genes (*SELP*, *SELL* and *SELE*, also known as *CD62P, L* and *E*) are located near each other on BTA 16 in a putative mastitis-related quantitative trait locus (QTL) (Klungland et al., 2001; Sorensen et al., 2008; Sodeland et al., 2011). This observation potentially supports their importance in combatting bovine mastitis. Indeed, it has been shown that the absence or dysfunction of SELP can lead to recurrent bacterial infections and persistent disease (Ley, 2003). Furthermore, a polymorphism in the human *SELP* gene is associated with the development of the autoimmune disease systemic lupus erythematosus (Morris et al., 2009).

In addition to mastitis, poor fertility is considered the next most important factor for early culling of dairy cows (Bell et al., 2010; Brickell and Wathes, 2011). The condition is caused through a variety of contributing factors, such as endometritis (Sheldon et al., 2009), irregularities in the oestrous cycle (Bulman and Lamming, 1978) and high rates of early embryo mortality (Diskin and Morris, 2008). Similar to mastitis, there is evidence for the involvement of selectins in all of these processes. Both SELP and SELL are significantly up-regulated in endometrial cells stimulated with the bacterial product lipopolysaccharide (LPS) (Oguejiofor et al., 2015). SELP is also crucial for the migration of PMN into the bovine corpus luteum during luteolysis (Shirasuna et al., 2012). Persistent corpora lutea are commonly present in cows with uterine infection (Opsomer et al., 2000). Both SELL and SELP have also

been implicated in the process of bovine conceptus attachment during which SELL mRNA and protein are both up-regulated on uterine epithelial cells and uterine SELP expression also increases from Day 20 (Bai et al., 2015). In humans, SELL ligand appears to play a key role in blastocyst attachment (Wang et al., 2008) while low levels of uterine expression are associated with implantation failure and infertility (Foulk et al., 2007; Margarit et al., 2009). *SELP* polymorphisms are also associated with recurrent pregnancy losses in women (Dendana et al., 2012). These studies provide an initial indication that selectins are also important regulators of fertility.

Given the potential importance for selectins in the pathogenesis of a variety of disease conditions, we investigated in the present study the potential importance of SELP in cattle using two complementary approaches. Firstly, we examined its role in innate immune response through influencing adhesion of bovine peripheral blood leucocytes (PBL) to endothelium under static conditions *in vitro*. Secondly we sought to provide evidence regarding the frequency and potential functionality of identified polymorphisms in the exons of SELP in different breeds of dairy cows to determine whether this is a potentially promising candidate gene relating to the functions of innate immune response and/or fertility.

2. Material and methods

2.1. Reagents and animals

All reagents were purchased from Sigma-Aldrich Company Ltd (Dorset, UK) unless otherwise stated. All procedures involving animals were carried out under the Home Office Animals (Scientific Procedures) Act 1986, Home Office Project license and approved by the

Royal Veterinary College's (RVC) Ethics and Welfare Committee.

Blood samples for isolation of bovine peripheral blood leukocytes (PBL) were collected from healthy Holstein-Friesian cattle housed at Bolton Park farm of the RVC into 50 ml falcon tubes containing 10 U/ml heparin. PBL were isolated by lysis of erythrocytes as described by Werling et al. (1998). In brief, 3 ml of anti-coagulated blood was added to 25 ml ammonium chloride lysing solution (8.29 g NH_4Cl, 1.00 g $KHCO_3$, and 0.372 g Na_2EDTA in 1 L distilled water, pH 7.2) and incubated at room temperature for 10 min. PBL were harvested by centrifugation and washed twice with phosphate buffered saline (PBS). The trypan blue dye exclusion method revealed that >95% cells were viable.

Whole blood samples for SNP identification (10 ml into heparinized vacutainers, BD Vacutainer System, Devon, UK) were collected from 336 Holstein heifers at 6 months of age from 19 different commercial farms and stored at -20^{o}C. A further 53 samples were collected in the same way from pre-weaned dairy calves of different breeds (30 Friesian, 14 Jersey, 6 Ayrshire, 3 Brown Swiss), with each breed sampled on a different farm.

2.2. Culture of bovine aortic endothelial cells (BoAEC) and PBL adhesion assays

BoAEC were purchased from Cell Applications, Inc. (San Diego, CA, USA) and used at passage 4 to 7. Cells were grown initially at 37 °C in 5% CO_2 in 75 cm cell culture flasks (Corning, NY 14831, USA) with 15 ml bovine endothelial cell (EC) growth medium (Cell Applications, Inc.). To investigate PBL-BoAEC adhesion characteristics *in vitro*, BoAEC were first grown to confluence in 96-well plates (Corning). Thereafter, BoAEC were cultured with or without 0.5 µg/ml ultra-pure LPS from *E. coli* 0111:B4 strain (Sigma-Aldrich Company Ltd.) in 100 µl bovine EC cell growth medium for different time periods (0, 10 min, 1 h, 2 h, 3 h, 4

h and 6 h). The medium was then discarded and the cells washed with 200 µl PBS to remove any LPS residue before the addition of PBL. Each treatment was replicated in four wells. Freshly isolated PBL were initially stained with 5 mM Calcein-AM (a fluorescent cell permeable derivative of calcein) in Dulbecco Modified Eagle Medium (DMEM) containing 10% FBS for 10 min at room temperature. After washing three times with 10 ml PBS, the stained PBL were added to each well (10^5 cells per well in 100 µl RPMI-1640 medium (VWR International, Lutterworth, Leics, UK) containing 10% FBS and co-incubated with the BoAEC for 30 min at 37 °C with 5% CO_2. After incubation, non-attached cells were removed by careful washing three times with 150 µL PBS containing 2% FBS. Thereafter, 100 µL of NP40 lysis buffer (VWR, Lutterworth, Leics, UK) was added to each well for 10 min at room temperature. The numbers of adherent PBL were estimated by fluorescent measurement of the remaining Calcein-AM using a Tecan Infinite m200 Pro plate reader (Tecan, Männedorf, Switzerland) at 485 nm and 528 nm wavelengths. The mean fluorescence intensity (MFI) of four wells was used for statistical analysis. At least 3 separate experiments were performed, each using freshly isolated PBL.

To further investigate the role of SELP in PBL adhesion, BoAEC were stimulated as above with 0.5 µg/ml LPS for either 10 min or 4 h. Following LPS treatment, BoAEC were pre-incubated with or without a SELP monoclonal antibody (100 µL of 5 mg/ml per well (AbD Serotec, Kidlington, Oxford, UK) in BoAEC growth medium for 20 min. PBL labelled with Calcein-AM were then added and the adhesion assay performed as described above. Each treatment was replicated in four wells and 2 independent experiments were performed. Anti-P-selectin antibody treated groups were compared with the untreated control groups.

2.3. Western blotting for SELP protein

To confirm that SELP protein was indeed expressed in the BoAEC, cells were cultured to 90% confluence in 24 well plates (Thermal Scientific, Waltham, MA, USA) and stimulated with 1 ml 0.5 µg/ml LPS in BoAEC growth medium for different time periods (0, 10 min, 30 min, 1 h, 3 h and 6 h), then washed three times with 200 µL PBS. Cells were then lysed by passage through a syringe in 150 µL cold generic lysis buffer (GLB) (83 ml 76.5 mM Tris-base pH 6.8, 10% glycerol, 4% SDS made up to 100 ml using diH_2O). Protease inhibitor cocktail (Calbiochem, Watford, UK), supplemented with 2 mM sodium orthovanadate, 0.2 mM sodium flouride were added to GLB immediately before use. Lysates were centrifuged at 13,000 *g* for 7 min. Total protein in the supernatant was measured using a bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, UK [#23225]) following the manufacturer's instructions and the supernatant was stored at -80^{o}C. For analysis, samples were adjusted to 20 µg total protein and boiled at 100^{o}C for 5-10 min after addition of loading buffer [v/v= 1/4] (1ml Glycerol + 0.25 ml β-ME + 0.005gr Bromophenol blue). Samples were loaded on BioRad 10% Mini-PROTEAN® precast SDS-PAGE gels (BioRad, UK) for 70 min at 120 V. Gels were transferred to PVDF Hybond P 0.45 (GE Healthcare Life Sciences, Little Chalfont, Bucks, UK) for 75 min at 100 V on ice. Membranes were blocked overnight at 4^{o}C in 10% Skim Milk (GE Healthcare Life Sciences) dissolved in PBS containing 0.05% TWEEN 20 (PBS-T). After a 10 min wash in PBS-T, membranes were incubated with primary antibody shaking at room temperature (RT) for 2 h. SELP protein with an expected molecular weight (MW) of 140 kDa was detected using a polyclonal antibody (Biorbyt Ltd, Cambridge, UK, 1:200 dilution). This antibody has previously been validated for use on bovine endothelium (Maliba et al. 2008). Beta-actin (ACTB) with a predicted MW of 40 kDa was assessed as a housekeeping protein using a polyclonal antibody (Cell Signaling Technology, Danvers, MA, USA, 1:1000 dilution).

After 6 washes (15 min each) in PBS-T, membranes were incubated with secondary antibody (horseradish peroxidase-conjugated anti-rabbit IgG, DAKO, Ely, UK, 1:4000 dilution) at RT for 2 h. After final washes (as above), the signal was detected using an ECL Western Blotting Detection system (GE Healthcare Life Sciences). Images were captured using a G:BOX Gel Imaging System (Syngene, Cambridge, UK).

2.4. RNA extraction and cDNA synthesis

BoAEC were stimulated with 0.5 µg/ml LPS for different time periods (0, 30 min, 1 h, 3 h and 6 h). The medium was aspirated then procedures for RNA isolation and reverse transcription (RT) were carried out following the protocol described previously (Swangchan-Uthai et al., 2012) with some modifications. Briefly, total RNA was extracted directly from the cell culture plate using an RNA extraction kit (RNeasy mini-kits; Qiagen, Hilden, Germany), and contaminating genomic DNA was deleted using an RNase-free DNase kit (Promega UK Ltd., Southampton, UK). Subsequently, total RNA was transcribed using an RT system kit (PCRBiosystems Ltd., London, UK). All procedures were carried out following the suppliers' protocols.

2.5. Quantitative real-time reverse transcription-polymerase chain reaction (qPCR)

Primers used for qPCR were designed the Primer3 web-based software based on gene sequences obtained from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Gene symbols, accession numbers, primer sequence information, optimized annealing temperature and expected product lengths are provided in

supplementary Table S1. The qPCR amplification was performed following the method described previously (Cheng et al., 2013) on a CFX 96 real-time PCR detection system (BioRad) using a Sygreen mix kit (PCR Biosystems Ltd.) following the protocol supplied. A melting curve analysis was included to confirm the specificity of amplification and avoid primer dimers. *GAPDH* was initially quantified using qPCR via an absolute quantification approach following the method described previously (Cheng et al., 2013). As GAPDH expression was not affected by LPS treatments (P>0.05) (data not shown) it was used as an appropriate reference gene

2.6. SNP Identification in selectin transcripts (SELP, SELL, SELE)

DNA from blood samples was extracted using a FlexiGene DNA Kit (Qiagen) following the protocol supplied and quantified with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc. Wilmington, USA). A DNA pooling sequence strategy was initially used to identify the SNP in the exons of the three bovine selectin genes *SELP, SELL,* and *SELE* by direct sequencing of PCR amplification products. A total of 37 primers were designed using Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA) based on the published gene sequences from the Ensembl database (ENSBTAG00000007307, ENSBTAG00000011515 and ENSBTAG00000020755 respectively) (Supplementary Table S2), with NCBI genbank accession numbers of NM_174181.2, NM_174182.1 and NM_174183.2, respectively. To construct a DNA pool, 1 µl of 50 ng/µl DNA samples from each of 10 British Holstein cows were pooled. PCR was performed using a Qiagen Multiplex PCR kit in a 40 µl reaction volume containing 50 ng DNA. The PCR reaction procedures were performed according to the manufacturer's instructions, and resulting PCR products were sequenced by

Source Bioscience (Nottingham, UK). Sequence files were assessed using the Chromas Pro software package (release 2.33; Technelysium Pty Ltd, South Brisbane QLD 4101, Australia). All SNP found in the exon area of the three selectin genes are presented in Table 1.

2.7 SELP genotyping by *PCR-RFLP*

Five SNPs from different exon in *SELP* among different breeds of dairy cow (Holstein, Friesian, Jersey, Ayrshire and Brown Swiss) were genotyped using PCR-RFLP. The appropriate enzymes were designed using the WatCut online tool (http://watcut.uwaterloo.ca/template.php?act=snp_new). Primers for PCR-RFLP were designed using Primer Premier 5.0. PCR was performed using a Qiagen multiplex PCR kit and the PCR reaction procedures were performed according to the manufacturer's instructions. Thereafter, 10 µl PCR products were digested with 2 U restriction enzyme (New England Biolabs Inc, UK) in a 20 µl reaction volume at an appropriate temperature for each enzyme according to the instructions supplied. The digested products were detected by electrophoresis with 1.5% agarose gel at 80 Volts for 40 min. Alleles were indicated by different bands. The primers and restriction enzymes used for genotyping of SNPs by PCR-RFLP are given in Supplementary Table S3.

2.8. Bioinformatics analysis of SNP in SELP

The sequence for bovine SELP (NP_776608.1) was compared against sheep (NP_001009295.1), mouse (NP_035477.1) and human (NP_002996.2) sequences to determine functional domain conservation and similarity among species with ClustalW2

(http://www.ebi.ac.uk/Tools/msa/clustalw2/). Human SELP contains 5 regions: (i) an N-terminal C-type Lectin (Lec) domain, (ii) an epidermal growth factor (EGF)-like module, (iii) nine consensus repeats (CRs), (iv) a trans-membrane segment and (v) a cytoplasmic tail domain (Ushiyama et al., 1993). Schematic diagrams of the protein domains of the other species were made according to homology compared with human SELP. Bovine *SELP* SNPs were labeled according to the relevant region. Three regions of bovine SELP (Lec and EGF; CR1 and CR2; CR5 and CR6) included the amino acid mutations Asn107Lys, Glu297Ala and Val475Met respectively. These regions were analyzed individually using homology modeling software (Swiss-PdbViewer, http://spdbv.vital-it.ch/) (Biasini et al., 2014) against the reported crystal structure of human SELP (1g1q.1.B) and SELE (4c16.1) (Protein Data Bank, http://www.rcsb.org) as the model. Structural superposition was performed between the protein variants and wild-type protein of these three regions using the Match-Align subroutine of Chimera to determine whether the amino acid change caused a change in protein structure (Pettersen et al., 2004). The amino acid sequences used for the structural predictions are given in Supplementary Table S4.

2.9. Statistical and Linkage Disequilibrium (LD) analysis

Data are presented as the mean ± SD. The differences between treatments were examined using analysis of variance (ANOVA) with repeated measurement design via a linear mixed effect model built in SPSS 22 (Chicago, IL, USA), in which the treatment was taken as the fixed effect, cow as subject and time as repeat factor. Statistical significance was confirmed using a p-value <0.05. When ANOVA showed significance, Fisher's LSD multiple comparisons based on the least square means were carried out to test the differences

between the treatment pairs.

Genotypic frequencies were tested for Hardy-Weinberg equilibrium (HWE) using a chi-squared or Fisher's Exact test as appropriate. In addition, breed differences in genotypic frequencies were tested using a 3 x 2 Fishers Exact test or chi-squared test as appropriate.

The LD of SNP in *SELP* as measured was performed with the HAPLOVIEW software (Version 4.2) using r^2 measure (Barrett et al., 2005).

3. Results

3.1. LPS induction of increased PBL adhesion to BoAEC

We first investigated the time course for adhesion of PBL to BoAEC pre-treated with 0.5 µg/ml LPS. LPS treatment caused a significant rapid, short increase in PBL-BoAEC adhesion compared to baseline values within 10 min, which returned to basal levels within 1 h. After 2 h, the adhesion started to increase again, reaching a peak value at 4 h and remaining greater at 6 h post LPS treatment (Fig. 1). The more stable second phase of adhesion suggested that different regulatory mechanisms could be involved compared with the brief initial phase.

3.2. LPS treatment increased mRNA expression of SELP, SELE, VCAM1 and ICAM1 in BoAEC

As the above data suggested that LPS increased adhesion of PBL to BoAEC, the effect of LPS treatment on mRNA expression of four different adhesion molecules in BoAEC was examined to determine if other molecules could be involved. Indeed, LPS treatment increased the expression of *SELP, ICAM1, VCAM1* and *SELE* mRNA within 1 h, peaking at 3 h

(Fig. 2). However, 6 h after stimulation, only *SELE* mRNA was still significantly increased. At the transcription level, *ICAM1* showed the greatest increase (5 fold) compared to *VCAM1* and *SELE* (both about 3.5 fold), and *SELP* (2.5 fold).

3.3. Blocking SELP inhibits LPS induced PBL-BoAEC adhesion

We next used Western blot analysis to confirm that SELP protein was expressed by BoAEC at all the time points studied (unstimulated and from 10 min to 6 h after stimulation with LPS) by identifying a band at the predicted MW of 140KD (Fig. 3A). The effect of blocking SELP on PBL adhesion by pre-treating the endothelial cells with anti-SELP antibody was next assessed. Whereas pre-treatment did not alter the adhesion to non-stimulated cells, it reduced significantly the enhancement of adhesion caused by LPS at both 10 min and 4 h (Fig. 3B).

3.4. Polymorphism of the bovine selectin genes (SELP, SELL, SELE)

We identified 13 SNPs potentially present in the exons of selectin genes in British Holstein cows investigated in our study (Table 1). *SELP* was found to have the highest level of polymorphism as 9 of the 13 SNPs were located in *SELP* exons, including three missense mutations. The resulting amino acid changes for these were located in the lectin, CR2 and CR5 domains, respectively (Fig. 4A). Three synonymous SNP existed in *SELL* and only one synonymous SNP in *SELE*. Recent data have suggested differences in the innate immune response between different breeds of cow (Gibson et al., 2016). Genotyping was therefore also performed to detect five of the 9 *SELP* SNP in 4 other breeds of dairy cows: Friesian, Jersey, Ayrshire and Brown Swiss. Polymorphisms were present for all the *SELP* SNPs tested

in all breeds (Table 2).

The distribution of genotypes between the various breeds was tested to see if the breed samples were in HWE. BP-SNP2 and BP-SNP3 in exons 4 and 5 were in complete linkage disequilibrium (LD) with each other ($r^2 = 1$). These two SNP were in HWE (P = 0.9) and there were no discernable difference between the five breeds. The SNP BP-SNP4 in Exon 6 showed a similar pattern. The SNP BP-SNP7 in Exon 8 almost differed from HWE (P = 0.056) with the AA genotype being under represented. No breed differences were detected in this SNP. Finally, the SNP BP-SNP9 in Exon 13 was found not to be in HWE (P = 0.011) with an under representation of heterozygotes. Also there was complete reversal in the breed distribution of genotypes for the SNP in Exon 13 between the Holstein and both the Friesian and Jersey animals. In Holsteins the TT genotype was predominant in 57% of individuals tested, whereas the TT genotype was not found in either the Friesian or Jersey cows, in which the AA genotype predominated (present in 83% and 86% of individuals, respectively, P < 0.001).

3.5. Structural analysis of bovine SELP

To assess whether the identified SNPs may have an impact on the function of the SELP molecules, the sequence of bovine SELP was compared against that of mouse, human, and sheep proteins by homologous alignment. Bovine SELP has only 6 CR, compared with 8 (mouse, sheep) and 9 (human), resulting in a shorter molecule. The lectin, EGF and cytoplasmic tail domains of these four species had high similarity (all >70% homology). Three dimensional structural models of three bovine SELP domains (i) Lectin and EGF, (ii) CR1 and CR2 and (iii) CR5 and CR6 were successfully generated using human SELP and SELE as templates (Fig. 4B). These regions included the variants Asn107Lys, Glu297Ala and Val475Met respectively. The variant protein sequences were then superimposed on to the

consensus bovine SELP sequence. Amino acid substitution caused a minor influence on protein structure (RMSD <0.009 angstroms). There was a conserved Ca^{2+} binding site in the lectin domain (121Glu, 123Asn, 124Asn, 146Asn and 147Asp). Asn107Lys substitution introduced a new hydrogen bond into the protein structure (Fig. 4B). Two N-acetyl-D-glucosamine glycosylation sites (GlcNAc) were located at the interface of the CR1/CR2 and CR4/CR5 domains. The mutation in Val475Met was very close to a predicted GlcNAc site (455Gly, 457Gln, 476Arg and 506Arg).

4. Discussion

A bacterial infection of the udder is initially detected by pattern recognition receptors (PRRs) including toll-like receptors (TLRs), which bind pathogen-associated molecules such as LPS (Rainard and Riollet, 2006; Schukken et al., 2011). Activation of PRRs triggers an inflammatory response characterized by downstream signaling and transcription of both pro-inflammatory and chemotactic cytokines as well as chemokines. These are pleiotropic proteins that modify vascular endothelial permeability, recruit and activate inflammatory cells and induce the production of antimicrobial proteins and acute-phase proteins (Kawai and Akira, 2010; Takeuchi and Akira, 2010). Achieving a rapid immune response is beneficial to eliminate pathogens and so protect the body from damage.

Selectins play an important role as part of this process. Cell adhesion initiated by SELP is crucial in leukocyte trafficking through their interaction with activated endothelium. In this study, we first confirmed using bovine cells that SELP can respond rapidly (within 10 min) to an LPS stimulus and so mediate adhesion of PBL. SELP can be stored for a long time in Weibel-Palade bodies of resting cells ready for immediate translocation to the plasma

membrane on activation (Lowenstein et al., 2005). This provides a more efficient faster response than for other adhesion molecules, which require *de novo* synthesis. Our results also confirmed that LPS increased the mRNA expression of *SELP, SELE, VCAM1* and *ICAM1* in BoAEC. At the level of transcription regulation, *SELP* up-regulation was slower and weaker than for the other three molecules, which could in part be due to the fact that it can be stored as a pre-formed molecule in Weibel-Palade bodies for immediate translocation once required. Previous study has indicated that intra-mammary infection with *E. coli* causes a fast, short term increase of leukocytes numbers in milk before a more stable subsequent increase (Shuster et al., 1997). Our data suggest that SELP is responsible for the fast response, helping to provide the cow with early protection against bacterial invasion.

In addition to its direct role in adhesion, SELP activates the integrin molecule αLβ2 (also called LFA-1) via the SELP-PSGL1-SYK signaling pathway (Abbal et al., 2006; Mocsai et al., 2010). Activated integrin binds to its ligand ICAM1 on endothelial cells and promotes firm adhesion of leukocytes. In this study, we found that *ICAM1* showed the greatest transcriptional regulation in BoAEC in response to LPS in comparison with the other adhesion genes measured. A spatiotemporal synergistic effect of SELP and ICAM1 is probably vital in early innate immunity and contributes to the acute reaction to *E. coli* induced mastitis.

Within the sequences of Holstein cows analyzed, a higher degree of polymorphism was identified in *SELP* compared with *SELE* and *SELL*. Furthermore, there were three missense SNPs located in the lectin domain and CRs of SELP, potentially impacting on the function of SELP and so PMN extravasation in carrier cows. The N-terminal lectin domain contains the carbohydrate binding site which is crucial for SELP interaction with PSGL1 or other ligands (Revelle et al., 1996). Subsequent protein model analysis of bovine SELP showed a conserved

Ca^{2+} binding site in the lectin domain (121Glu, 123Asn, 124Asn, 146Asn, 147Asp). The Ca^{2+} ion plays a key role in conformational changes associated with ligand binding (Preston et al., 2016). The Asn107Lys substitution in the lectin domain introduced a new hydrogen bond into the protein structure. Further work is required to determine whether this change influences the interaction between SELP and its ligands.

SELP is an extended molecule and it is thought that the length provided by the repeating CRs helps to project the lectin domain above the endothelial cell membrane to facilitate attachment of flowing leukocytes. Removal of CR domains resulted in decreased strength of the SELP–ligand bond which reduced the ability of neutrophils to remain attached under shear stress (Patel et al., 1995). Another important feature of the SELP molecule is the two *N*-linked glycosylation sites which are predicted at the interface of the CRs. SELP is a highly glycosylated protein and it has been suggested that this glycosylation arrangement helps to protect the exposed CR domain linking regions against proteolytic cleavage (Preston et al., 2016). On the other hand, Ghoshal et al. (2014) investigated the importance of glycosylation in mediating the adhesion of leukocytes to endothelial cells. They found that treatment of endothelial cells with *N*-glycosylation inhibitors suppressed the expression of SELP on the cell surface and reduced leukocyte adhesion. Furthermore, a mutation Thr715Pro in human *SELP* located in CR9 impairs terminal glycosylation of SELP in the Golgi, leading to reduced amounts of mature SELP and subsequently less surface expression and secretion (Subramanian et al., 2012). It was therefore of particular interest that the Val475Met variant loci of bovine *SELP* was very close to the predicted *N*-acetyl-D-glucosamine glycosylation site in the CR4 and CR5 linking region. This variant showed a very unbalanced distribution within the population of dairy breeds investigated. Only 4 cows, all Holstein, from the total population of 373 cows from five breeds genotyped showed the AA genotype. This was

fewer than would be predicted from the allele frequencies in this population using the HWE. One possible cause of such a difference is the presence of a lethal or semi-lethal allele. It may also be worth noting that there were breed differences in the genotype frequencies of the 3' UTR SNP rs211179622 in Exon 13. The distribution in the Holstein animals differed from those of both the Friesian and Jersey animals. It remains to be seen whether this difference translates into differences in the health status of these breed groups. Different cow breeds have, however, been reported to show higher or lower genetic resistance to mastitis (Rupp and Boichard, 2003).

These results suggested that AA animals carrying the Val475Met substitution may rarely survive to adulthood. The Holstein blood samples used were taken from animals at 6 months of age whereas the other breeds were genotyped from samples collected from pre-weaned calves. Calf mortality rates in the UK from birth to 6 months are of the order of 5-7%, mainly due to infectious diseases in particular pneumonia and diarrhoea (Brickell et al., 2009; Johnson et al., 2011). The potential loss of animals homozygous for the Val475Met mutation highlighted here is, however, more likely to have occurred during pre-natal development. Around 40% of bovine embryos are lost by day 21 while a further 7-20% die between 4 to 12 weeks of gestation (Diskin and Morris, 2008). Endometrial SELP expression in the cow increases from Day 20 of gestation (Bai et al., 2015) and *SELP* polymorphisms in women are associated with recurrent pregnancy losses (Dendana et al., 2012). The precise role of SELP during embryo and placental development remains to be determined.

In summary, we found that bovine SELP mediates the attachment of PBL to the endothelium, with a rapid response time to LPS stimulation. We also identified three missense SNPs in *SELP* exons. Bioinformatics analysis indicated that these polymorphisms may alter the ability of SELP to bind ligands and/or influence protein expression at the cell

surface via altered glycosylation. Further work is required to investigate whether these polymorphisms confer differential innate immunity or influence fertility by affecting the ability of bovine embryos to develop successfully to term.

Competing interests

The authors declare that they have no competing interests.

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Table1 Polymorphisms identified in the exons of bovine selectin genes in UK.

Gene	SNP name	dbSNP ID	Alleles	Exon	Chr	Gene position#	SNP type	Amino acid change
SELP	BP-SNP1	rs478953454	C/A	2	16	38,078,133	Missense	Asn/Lys
	BP-SNP2	rs110033243	C/T	4	16	38,075,512	Synonymous	
	BP-SNP3	rs42312260	A/C	5	16	38,074,580	Missense	Glu/Ala
	BP-SNP4	rs137027551	A/G	6	16	38,068,897	Synonymous	
	BP-SNP5	rs109373658	A/G	8	16	38,057,172	Synonymous	
	BP-SNP6	rs109571103	C/T	8	16	38,057,157	Synonymous	
	BP-SNP7	rs378218397	A/G	8	16	38,057,153	Missense	Val/Met
	BP-SNP8	rs208474389	A/G	13	16	38,049,882	3ʾUTR	
	BP-SNP9	rs211179622	A/T	13	16	38,049,712	3ʾUTR	
SELL	BL-SNP1	rs109354144	C/T	3	16	38,170,720	Synonymous	
	BL-SNP2	rs109966956	A/G	3	16	38,170,687	Synonymous	
	BL-SNP3	rs41803917	C/T	4	16	38,168,866	Synonymous	
SELE	BE-SNP1	rs110045112	C/G	14	16	38,184,844	Synonymous	

SNP location based on UMD 3.1 genome assembly.

Table 2 Polymorphisms in SELP exons detected in different breeds of dairy cows.

Breed	Holstein	Friesian	Jersey	Ayrshire	Brown Swiss	Total genotyped	Genotype frequency
n	268-336	30	14	6	3	n	
	rs110033243 Exon 4					389	
TT	73	0	4	2	1	80	20.6%
TC	162	16	4	4	2	188	48.3%
CC	101	14	6	0	0	121	31.1%
	rs42312260 Exon 5					389	
AA	73	0	4	2	1	80	20.6%
AC	162	16	4	4	2	188	48.3%
CC	101	14	6	0	0	121	31.1%
	rs137027551 Exon 6					321	
GG	126	7	2	2	1	138	43.0%
AG	118	15	5	4	2	144	44.9%
AA	24	8	7	0	0	39	12.1%
	rs378218397 Exon 8					373	
GG	214	17	13	5	3	252	67.5%
AG	102	13	1	1	0	117	31.4%
AA	4	0	0	0	0	4	1.1%
	rs211179622 Exon 13					385	
TT	190	0	0	0	3	193	50.1%
AT	131	5	2	3	0	141	36.6%
AA	11	25	12	3	0	51	13.2%

Figure legends

Fig. 1. Time course of adhesion of peripheral blood leucocytes (PBL) to monolayers of bovine aortic endothelial cells (BoAEC*)* which had been pre-stimulated with 0.5 µg/ml LPS for different time periods from 10 min to 6 h . PBL were pre-stained by Calcein-AM before adding to the BoAEC and fluorescence intensity, representing numbers of adherent PBL, was measured at 485 nm/528 nm. Time 0 represents the numbers of PBL which adhered to BoAEC in the absence of any added LPS (control). The results are presented as the ratio of the flourescence measured at each time point following LPS treatment relative to the unstimulated control wells. All values are the mean ± SD. There were 4 replicate wells per time point for each cow and all treatments were repeated in PBL isolated from 3 different cows. ** P<0.01 versus time 0.

Fig. 2. Relative expression of *SELP, SELE, VCAM1* and *ICAM1* mRNAs in LPS-stimulated bovine aortic endothelial cells (BoAEC). BoAEC were incubated with 0.5 µg/ml LPS for the indicated times. Time 0 represents the expression level in the absence of any added LPS (control). The mRNA was detected by qPCR using *GAPDH* as the reference gene. All values are the mean ± SD. There were 4 replicate wells per time point in each batch of cell culture and all treatments were repeated with 4 batches of cells. The cells from 4 replicate wells per treatment were pooled for RNA extraction. Compared with time 0, * P<0.05 and ** P<0.01.

Fig. 3. A. Western blot analysis of SELP expression in bovine aortic endothelial cells (BoAEC) before and after treatment with 0.5 µg/ml LPS showing that SELP protein was present at all times investigated. ACTB was used as a reference protein. Samples were pooled from 4

replicate wells. **B**. Effect of LPS and SELP on adhesion of peripheral blood leucocytes (PBL) to BoAEC. BoAEC were pre-incubated in the absence or presence of LPS (100 µl, 0.5 µg/ml) for 10 min or 4 h then incubated in BoAEC growth medium with or without anti-SELP monoclonal antibody (5 mg/ml) for 20 min. Leukocytes were pre-stained with calcein-AM and added to the BoAEC. Fluorescence was measured at 485 nm/528 nm as an indicator of the number of adhered PBL. All values are the mean ± SD. There were 4 replicate wells per time point for each cow and all treatments were repeated in PBL isolated from 3 cows. ** $P<0.01$ compared with the group without SELP Ab treatment.

Fig. 4. Bioinformatics analysis of bovine SELP protein structure and polymorphisms. A. Multi-alignment of SELP amino acids among different species. Polymorphisms of bovine SELP are labeled in the relevant domain. Underscore denotes a missense SNP. B. Homology modeling of three regions of bovine SELP. The wild type is shown in tan, the mutated type is sky-blue, the green ball indicates Ca^{2+}, the green arrow indicates a hydrogen bond, the red arrow an amino acid mutation and the yellow arrow a site of N-acetyl-D-glucosamine glycosylation (**GlcNAc**).

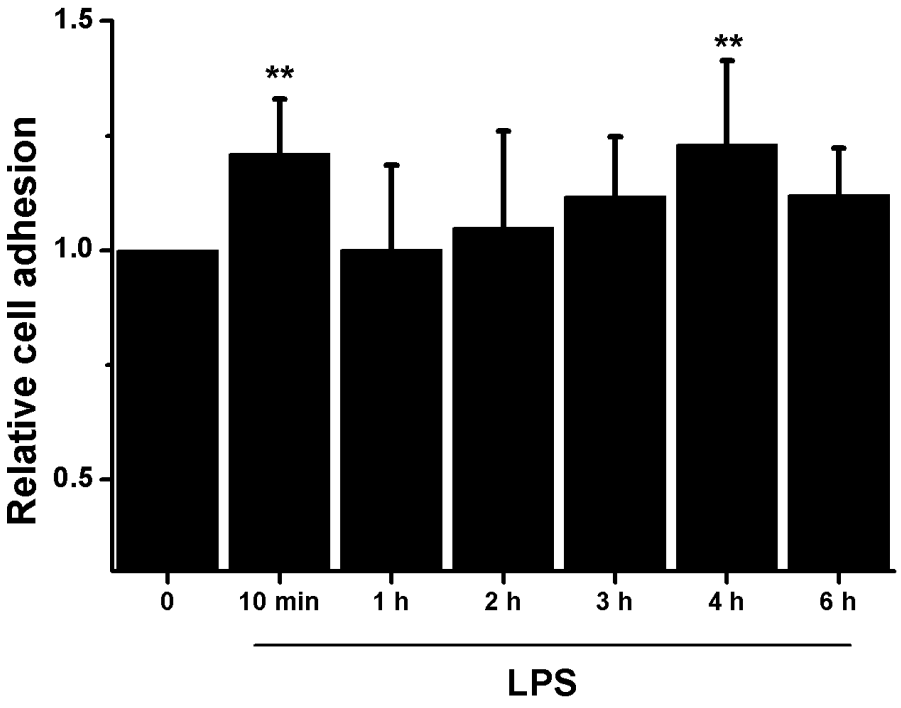


Fig. 1

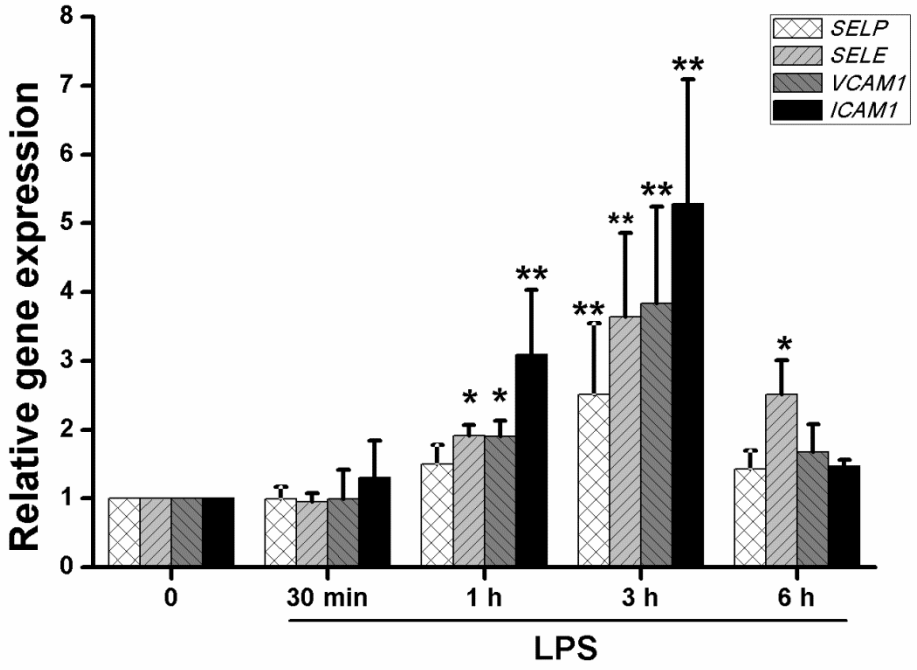
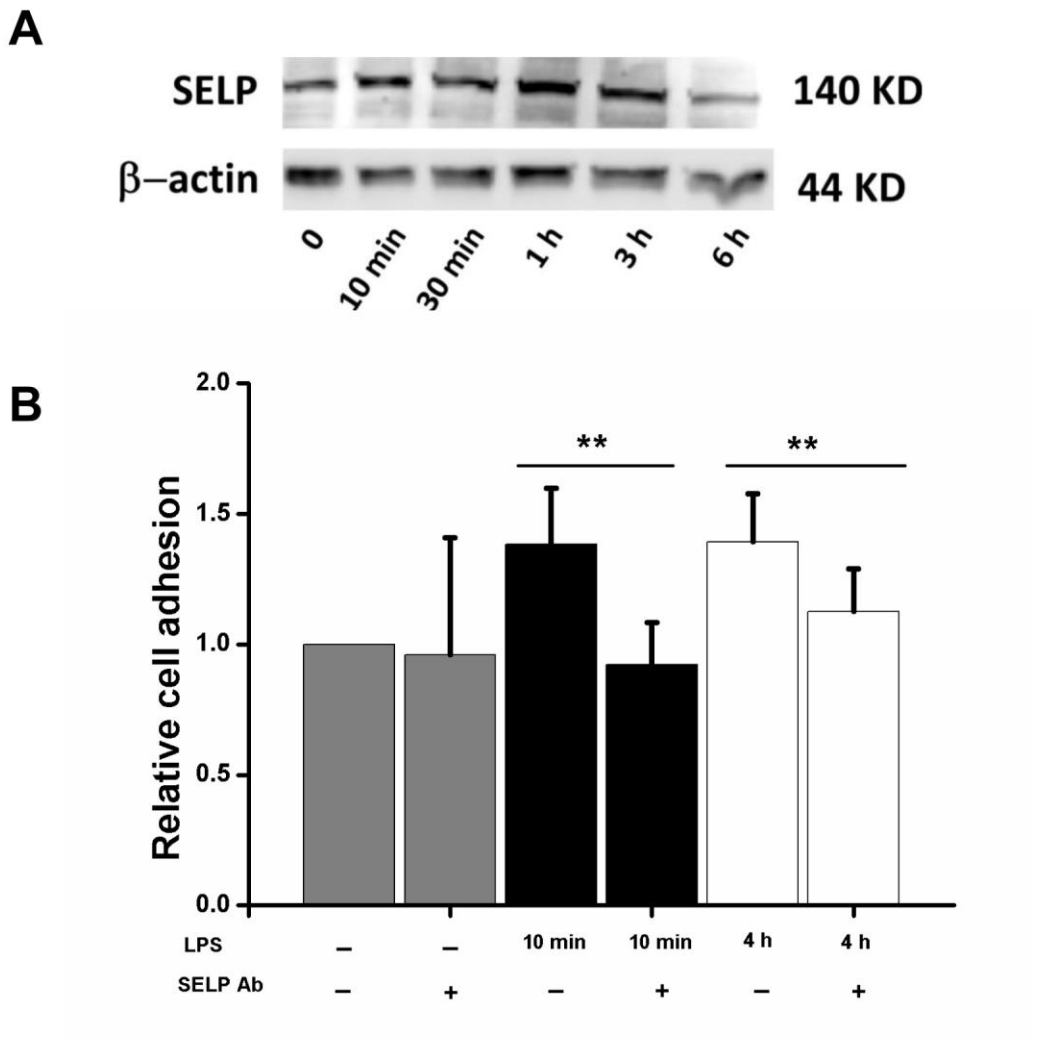


Fig. 2


A
SELP
140 KD
β–actin
44 KD
0
10 min
30 min
1 h
3 h
6 h
B
Relative cell adhesion
2.0
1.5
1.0
0.5
0.0
**
**
LPS
SELP Ab
–
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10 min
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4 h
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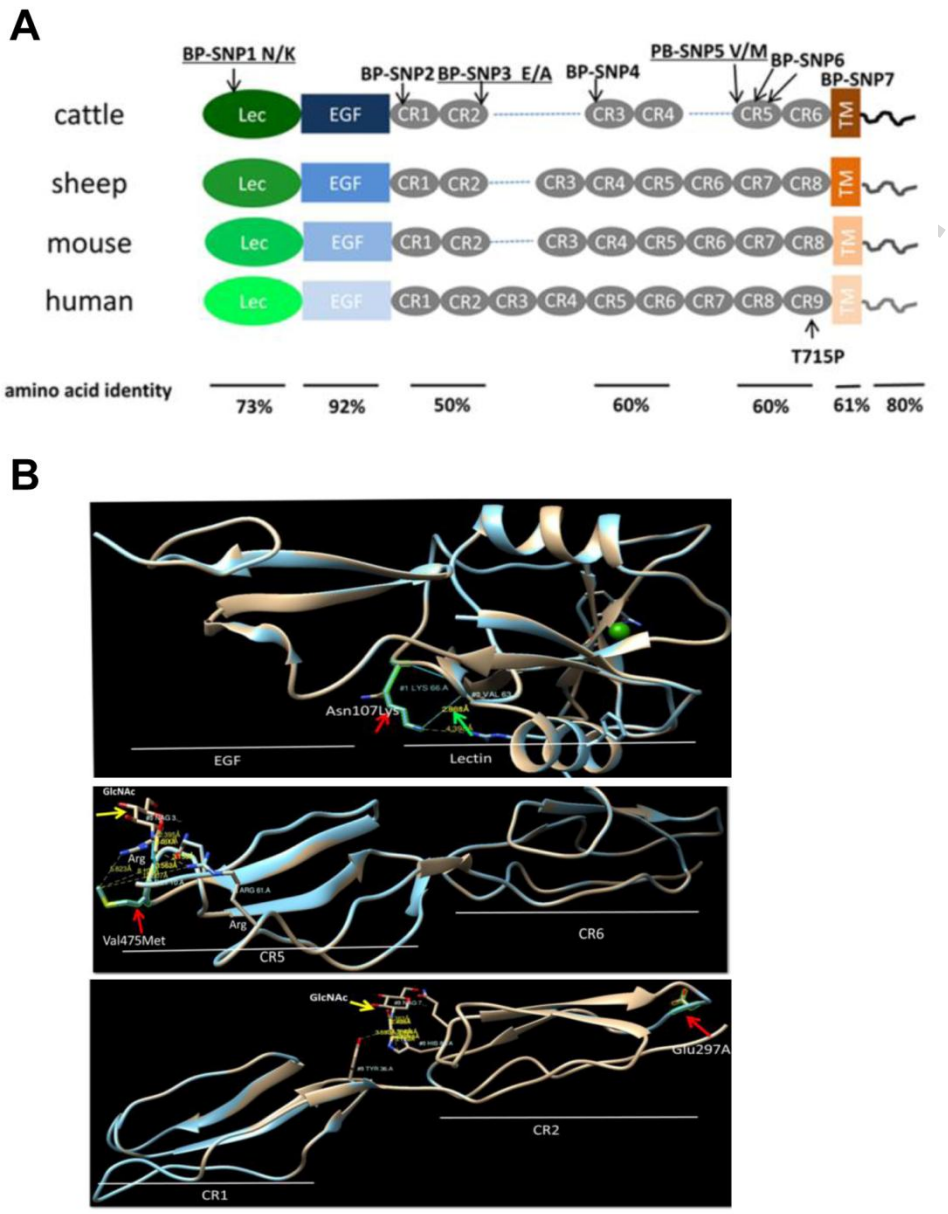
+
–
+
–
+

Fig. 3


A
BP-SNP1 N/K
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PB-SNP5 V/M
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BP-SNP7
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human
Lec
EGF
CR1
CR2
CR3
CR4
CR5
CR6
CR7
CR8
CR9
TM
T715P
amino acid identity
73%
92%
50%
60%
60%
61%
80%
B
Asn107Lys
EGF
Lectin
GlcNAc
Arg
Val475Met
CR5
CR6
Glu297A
CR2
CR1

Fig. 4

Highlights

- LPS caused both a rapid and slower enhancement of PBLs adhesion to endothelium.
- Antibody blocking of SELP inhibited the above LPS induced cell adhesion.
- SELP mediates the attachment of PBL to the endothelium.
- SELP is highly polymorphic with 13 SNPs located in its exons.
- This polymorphism may influence its function and performance traits in dairy cow.