**EXPRESSION OF COMPLEMENT RECEPTORS 1 (CR1/CD35) AND 2 (CR2/CD21)
AND CO-SIGNALING MOLECULE, CD19 IN CATTLE**

A Thesis

Presented to

The Faculty of Graduate Studies

of

The University of Guelph

by

ERIC S. PRINGLE

In partial fulfillment of requirements

for the degree of

Masters of Science

June, 2011

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ABSTRACT

EXPRESSION OF COMPLEMENT RECEPTORS 1 (CR1/CD35) AND 2 (CR2/CD21) AND CO-SIGNALING MOLECULE, CD19 IN CATTLE

Eric S. Pringle
University of Guelph, 2011

Advisor: Patricia Shewen
Program: M.Sc. Immunology

C3d is a sub-fragment of the C3 component of the complement system. Covalent binding of multiple C3ds to antigen reduces the activation threshold of cognate B lymphocytes by one thousand fold through co-ligation of the BCR and complement receptor 2 (CR2/CD21). Reverse transcriptase polymerase chain reaction (RT-PCR), revealed that, in cattle, four distinct complement receptors are produced from the *Cr2* gene by alternative splicing. Cattle express two major variants of the *Cr2* gene representing homologues of murine CR1 and CR2, each of which are expressed in both a long and a short form. Expression of CR1 was detected in splenocytes but not in splenic mononuclear cells or monocyte derived macrophages. CR2 was detected only on IgM⁺ blood cells and unsorted splenocytes but not in CD3⁺ cells, CD14⁺ cells or neutrophils. Additionally, the coding sequence of CD19, the CR2 co-signaling molecule was found.

Acknowledgements

This work would not have been possible without grants from the following funding agencies: Natural Sciences and Engineering Research Council of Canada, the Ontario Cattlemen's Association, the Canadian Cattlemen's Association, the Dairy Farmers of Canada, the Alberta Beef Producers and the Ontario Ministry of Agriculture, Food and Rural Affairs.

I have had a wonderful time in this program and I am grateful to have met some of the many kind, interesting and/or peculiar students, staff and faculty in Pathobiology. I hope future students in the department will enjoy their time there as much as I did; it's hard to believe it's time to move on.

Thanks to my committee members, Dr. Sarah Wootton for never being too busy to offer direction and a friendly ear, and Dr. Doug Hodgins for his seemingly limitless patience, immunological know-how and guidance. I need to thank Dr. Shayan Sharif and Dr. Bonnie Mallard for their excellent teaching and conversation through my coursework, Betty-Anne McBey, our amazing lab technician and the tireless molecular biologists of the University of Guelph Genomics Facility. I also need to thank my parents, Mark Rodford, the Cornerstone and all the friends and family there who watched me grow up over the years. While I'm sure my partner, Katherine Ross, will be happy to know that this leg of the journey is finally over, her love and patience have been invaluable and vitalizing.

Dr. Patricia Shewen deserves more gratitude than I can offer here. She offered me the rare opportunity to make a real change in my life and for that I am forever grateful. Her advice and care has been invaluable; I'm sure I'll be seeking more for many years to come.

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Introduction

Our group has worked on the development of molecular adjuvants for cattle using physiological ligands of receptors on B cells. We have specifically investigated how components of the complement system, such as C3d, could interact with complement receptor 2 (CR2) on the surface of B cells to improve antibody responses to vaccination. In our work we have also been interested in how the expression of CR2 changes with age in cattle. While CR2 expression has been confirmed in cattle by monoclonal antibodies, at the beginning of this work was known about the gene which expressed it.

CR2 is known to be an alternatively spliced transcript in mice. Complement receptor 1 (CR1) and CR2 are products of a single gene in mice; some murine anti-CR2 monoclonal antibodies react with both gene products. In humans CR1 and CR2 are products of two distinct genes. As CR1 has not yet been identified in cattle it is unknown if cattle express CR1 and if so, whether CR1 is a product of a distinct gene or is an alternatively spliced transcript of the *Cr2* gene.

From studies in mice and humans we know that CR2 signaling depends on the presence of CD19, the co-signaling molecule of CR2. Almost nothing is known about bovine CD19; in fact, the hypothetical gene encoding CD19 is predicted to be a non-functional pseudogene in the latest build of the bovine genome.

This work examined the sequence of the *Cr2* gene in cattle to clarify whether it encodes both CR1 and CR2 as is seen in mice. The bovine gene encoding CD19 was sequenced to support or discredit a role in bovine immune function.

Literature Review

The Complement Cascade

The complement system comprises a phylogenetically ancient family of serum proteins and membrane receptors that contribute to innate immune defenses and modulate adaptive responses. The soluble complement components are a series of proteases that function in an amplifying activation cascade which can be initiated by any one of three recognized pathways. All pathways culminate in the common terminal pathway leading ultimately to formation of the membrane attack complex (MAC). MACs are promiscuous and form pores in neighbouring lipid membranes causing loss of osmoregulation and lysis of eukaryotic cells, Gram negative bacteria and enveloped viruses. In addition, cleaved components of the complement cascade have a role in regulating immune responses. Cleaved products can mediate opsonization, chemotaxis of phagocytes, mast cell degranulation, antigen transportation to germinal centers (Phan *et al.*, 2007; Phan *et al.*, 2009), as well as regulating signaling by Toll-like receptors (Hajishengallis & Lambris, 2010). These cleaved products also affect activation of the adaptive immune system in jawed vertebrates.

The three major activation pathways of the complement cascade are the classical pathway, the alternative pathway and the mannose binding lectin (MBL) pathway (Figure 1.1). Although the three pathways begin with different stimuli, they intersect in the formation of one of two complement component 3 (C3) convertases, both of which split complement component C3 into C3a and C3b. Many molecules of C3 can be cleaved by a single C3 convertase complex making this a

Figure 1.1. Complement Cascade. Classical pathway: C1q and C1r/C1s proteases bind to IgM or IgG antibodies that are complexed with antigen (immune complexes), then cleave C4 and C2 to form C3 convertase. Mannose binding lectin (MBL) pathway: MBL binds to pathogen-associated molecular patterns (PAMP), activating serine proteases MBL associated proteins 1 and 2 (MASP1/2) that cleave C4 and C2 to form C3 convertase. Alternative pathway: spontaneous hydrolysis of C3 leads to formation of C3b. C3b can interact with Factor B and Factor D to form a C3 convertase (C3bBb). C3 convertases bind to (additional) C3b and become C5 convertases, which initiate completion of the complement cascade leading to

significant amplification step of the cascade. C3 cleavage exposes an unstable thioester bond in the C3b fragment that is quickly reduced by covalent attachment via transacylation to a nucleophile. If the nucleophile is water then the C3b will remain in fluid phase; if the nucleophile is on a surface of a cell, organism or immune complex, the C3b will remain surface bound. C3 convertases may combine with a molecule of C3b to form C5 convertases leading ultimately to formation of the MAC (Figure 1.1).

The classical pathway is initiated by the binding and activation of collagen-like complement component C1q. C1q is slightly cationic and binds to a variety of anionic substrates including bacterial lipopolysaccharide (LPS), DNA and some viruses and bacteria. C1q can also bind to exposed carbohydrate moieties on the Fc region of IgM and IgG in immune complexes. At least two exposed carbohydrate moieties are needed to bind C1q, requiring either two monomeric IgG molecules in close proximity or one pentameric IgM molecule. IgG sub-isotypes differ in their ability to bind C1q. C1q is associated with the serine proteases C1r and C1s to form complement component C1. When C1q is bound, C1r cleaves C1s which in turn cleaves soluble complement protein C4 into C4a and C4b. C4b, like C3b contains a reactive thioester bond that allows binding to nucleophiles. C4b then binds C2 which is then cleaved by C1s into C2a and C2b. C4bC2a functions as a C3 convertase. C4b2a can bind C3b to create C4b2a3b, a C5 convertase. The MBL pathway is similar to the classical pathway except that in place of C1q activation, MBL binds to pathogen associated molecular patterns (PAMP). MBL then associates with serine proteases MBL-associated protein (MASP) -1 and MASP-2 to cleave C4 and C2.

The alternative pathway begins with low level spontaneous hydrolysis of the C3 thioester bond generating C3(H₂O). C3(H₂O) can then bind complement Factor B which is then cleaved by complement factor D, forming the C3(H₂O)Bb C3 convertase. This complex is unstable until it binds to the serum protein properdin. The C3(H₂O)Bb C3 convertase can then cleave other C3 molecules into C3a and C3b augmenting production of C3bBb C3 convertase. This in turn may join with another molecule of C3b to generate the C3bBbC3b, a C5 convertase.

Activation by classical and MBL pathways requires the presence of either an antibody response or a PAMP and, outside of a pathological state, would not be expected to affect host tissues. However, the potential for spontaneous activation of complement through the alternative pathway and the proximity of host tissues to activated complement components during infection necessitates mechanisms by which the host can halt the cascade. This is accomplished by expression of membrane proteins in the regulation of complement activity (RCA) gene family. These include decay accelerating factor (CD55), protectin (CD59), complement receptor 1-related protein y (Crry) in mice and complement receptor 1 (CR1), among others (Carroll *et al.*, 1988). All of these bind activated complement components and catalyze the cleavage of complement components into inactive fragments.

Complement Receptors

Complement receptors are membrane bound proteins whose ligands are products of the complement cascade (Table 1.1). Complement receptors mediate

Table 1.1 - Complement Receptors

Complement Receptor	Species	Ligand	Cellular Expression
CR1	Human	C3b ^{1,2}	FDC ³ , B cells ^{4,5} , Erythrocytes ⁵ , Neutrophils ⁵ , Monocytes ⁵
		C4b ^{1,2}	
		MBL ⁶	
	Mouse	C3b ⁷	B cells ⁸ , FDC ⁹
		C4b ⁷	
	Cattle	N.D.	N.D.
CR2	Human	C3d ^{10,11,12}	B cells ¹⁰ , FDC ^{3,13} , T cells (?) ¹⁴ , Epithelial cells ¹⁵
		iC3b ^{11,12}	
		FcεRII ^{12,15,16}	
		IFN-α ^{12,17}	
	Mouse	iC3b ¹⁸	B cells ²⁰ , Mast cells ^{21,22} , FDC ⁹ , T cells ²³ (?)
		C3d ¹⁸	
	Cattle	C3d ²³	B cells ^{25,26,27} , FDC ²⁷ , Veiled dendritic cells ²⁸
CR3	Human	iC3b ²⁸	FDC ³ , Neutrophils ^{30,31} , Macrophages ²⁹ , Monocytes ²⁹
	Mouse	iC3b ³¹	Granulocytes ³³ , Macrophage ^{32,33}
	Cattle	iC3b (?)	Neutrophils ³⁴ , Monocytes ³⁴ , Dendritic cells ³⁵
CR4	Human	iC3b ^{35,36,37}	Macrophage ^{36,37} , Activated B cells ^{36,37} , Granulocytes ^{36,37} , Monocytes ^{36,37}
	Mouse	iC3b ³⁸	Dendritic cells ³⁹ , Natural Killer Cells ⁴⁰
	Cattle	iC3b (?)	Neutrophils ³⁴ , Monocyte ³⁴

References: 1. Furtado *et al.*, 2008, 2. Smith *et al.*, 2002, 3. Reynes *et al.*, 1985, 4. Jozsi *et al.*, 2002, 5. Fearon 1980, 6. Ghiran *et al.*, 2000, 7. Kalli & Fearon, 1994, 8. Kinoshita *et al.*, 1988, 9. Fang *et al.*, 1998, 10. Weis *et al.*, 1984, 11. Sarrias *et al.*, 2001, 12. Gilbert *et al.*, 2006, 13. Liu *et al.*, 1997, 14. Braun *et al.*, 1998, 15. Birkenbach *et al.*, 1992, 16. Aubry *et al.*, 1992, 17. Hibbert *et al.*, 2005, 18. Asokan *et al.*, 2006, 19. Kalli *et al.*, 1991, 20. Fingerioth *et al.*, 1989, 21. Gommerman *et al.*, 2000, 22. Andrásfalvy *et al.*, 2002, 23. Molnár *et al.*, 2008, 24. Firth, 2010, 25. Naessens *et al.*, 1990, 26. Sopp, 1996, 27. Chattha *et al.*, 2009, 28. Howard *et al.*, 1997, 29. Wright *et al.*, 1983, 30. Eden *et al.*, 1973, 31. Schleiffenbaum *et al.*, 1989, 32. Beller *et al.*, 1982, 33. Springer *et al.*, 1979, 34. Splitter & Morrison, 1991, 35. Miyazawa *et al.*, 2006, 36. Bilsland *et al.*, 1994, 37. Schwarting *et al.*, 1985, 38. Myones *et al.*, 1988, 39. Metlay *et al.*, 1990, 40. Burt *et al.*, 2008.

cellular responses to products of the complement cascade. There are four receptors for the covalently bound portion of fragments of complement C3 and C4, CR1 (CD35), CR2 (CD21), CR3 (CD11b/CD18) and CR4 (CD11c/CD18). There are also receptors for the anaphylatoxins (C3a, C4a and C5a) released from the cascade. CR1 and CR2 are both type 1 transmembrane proteins encoded in the RCA locus, which contain multiple highly conserved complement control protein domains (CCP) whereas CR3 and CR4 are heterodimers of integrin beta-2 (CD18) and CD11b or CD11c, respectively. CR3 and CR4 are primarily expressed on granulocytes and mediate extravasation of leukocytes and phagocytosis of C3b or iC3b opsonized targets.

Human CR1 binds C3b (Eden *et al.*, 1973; Ross *et al.*, 1973; Smith *et al.*, 2002) and C4b (Furtado *et al.*, 2008; Smith *et al.*, 2002), and acts as co-factor for the RCA protein Factor I that cleaves C3b and C4b to iC3b and iC4b, respectively, halting further progression of the complement cascade towards MAC formation. iC3b can be cleaved again by Factor I to yield C3dg. Human CR1 has also been identified as a receptor for MBL, leading to phagocytosis, (Ghiran *et al.*, 2000) and plays an inhibitory role in human adaptive immunity by reducing B lymphocyte proliferation following cross-linking of surface IgM by anti-IgM antibodies (Józsi *et al.*, 2002).

In humans, CR1 expressed on erythrocytes has an important function in clearance of immune complexes from blood. Experiments using transgenic mice expressing human CR1 on murine erythrocytes demonstrated increased adherence and clearance of opsonized pneumococci (Li *et al.*, 2010). Clearance is mediated by

cleavage of CR1 and transfer of the complex to macrophages in the liver and spleen, in an IgG Fc receptor dependent manner (Li *et al.*, 2010; Reinagel & Taylor 2000).

As CR1 and CR2 are products of the same gene in mice (Kurtz *et al.*, 1990; Molina *et al.*, 1990) it is impossible to discern the functions of CR1 in the current *Cr2* knockout model. As a result, little is known about the function of murine CR1. However, it has been demonstrated that the six N-terminal domains of murine CR1 bind C3b and C4b (Kalli & Fearon, 1994). Murine CR1 can still bind C3d though with less affinity than CR2 (Molina *et al.*, 1994). This is likely due to the additional N-terminal domains interfering with the C3d binding site of CR2 which is located in the first and second N-terminal domains of CR2 (Elsen & Isenman, 2011).

CR2 (CD21), in both mice and humans, is the receptor for complement products iC3b, C3dg and C3d (Eden *et al.*, 1973; Ross *et al.*, 1973; Sarrias *et al.*, 2001). There are two isoforms of CR2 in humans differing by one 58 amino acid domain; only the shorter isoform of CR2 is expressed in mice (Fingerroth *et al.*, 1989; Ling *et al.*, 1998; Liu *et al.*, 1997). C3dg is the bound remnant of C3b, resulting from its cleavage and inactivation, and can be further reduced to C3d by trypsin. In humans, CR2 also binds to the low-affinity Fc ϵ receptor II (CD23) (Aubry *et al.*, 1992; Hibbert *et al.*, 2005) and to IFN- α (Asokan *et al.*, 2006), and acts as the obligate receptor for the Epstein Barr virus (EBV) (Fingerroth *et al.*, 1984). In both mice and humans, CR2 is expressed on B lymphocytes (Fingerroth *et al.*, 1989; Jacobson & Weis, 2008; Weis *et al.*, 1984) and follicular dendritic cells (Reynes *et al.*, 1985; Liu *et al.*, 1997; Ling *et al.*, 1998); CR2 is also expressed on human epithelial cells (Birkenbach *et al.*, 1992). In mice, CR2 has also been detected on mucosal and

peritoneal mast cells (Andrásfalvy *et al.*, 2002; Gommerman *et al.*, 2000). There are conflicting reports concerning the presence and significance of CR2 on T cells. mRNA for CR2 has been detected in both T cells and B cells when CR2 protein was not detected by monoclonal antibodies (mAb) (Braun *et al.*, 1998). The functional implications of CR2 expression on T lymphocytes are unknown. However, CR2 ligation on T lymphocytes leads to nuclear translocation of the transcription factor NF- κ B, implying a change in gene expression (Molnár *et al.*, 2008).

In B lymphocytes, cross-linking CR2 with surface IgM leads to calcium flux despite suboptimal B cell receptor complex (BCR; membrane bound immunoglobulin with Ig α /Ig β) ligation (Lyubchenko *et al.*, 2005) and stimulates increased antigen presentation (Cherukuri *et al.*, 2001). CR2 has a short cytoplasmic domain and signaling is dependent upon the presence of the co-signaling molecule CD19. Ligand bound to CR2 is endocytosed in a manner dependent upon motifs in its cytoplasmic tail (Barrault & Knight, 2004; Carel *et al.*, 1990; Tessier *et al.*, 2007). *Cr2*^{-/-} mice have normal basal levels of circulating immunoglobulins but have greatly reduced IgG responses to antigen (Molina *et al.*, 1996), an accelerated decline in antibody titers and a concurrent loss of antibody forming cells in the bone marrow (Chen *et al.*, 2000).

Complement receptors are important for both antigen retention in germinal centers and transport of antigen to the germinal center. CR1 and/or CR2 expression is required for transport of immune complexes from subcapsular sinus macrophages to the light zone of the lymph node germinal center by non-cognate B lymphocytes. Failure of this transport in *Cr2*^{-/-} mice is associated with decreased affinity

maturation (Phan *et al.*, 2007; Phan *et al.*, 2009). Adoptive transfer experiments in *Cr2*^{-/-} mice have also shown that CR2 and/or CR1 expression on follicular dendritic cells (FDCs) is essential for strong IgG antibody responses (Fang *et al.*, 1998). CR2 on B cells has been found to bind a CR2-ligand present on the surface of FDCs (Qin *et al.*, 1998).

Complement Component C3d and CR2

Antigen with bound C3d can cross-link the BCR and CR2 on a cognate B lymphocyte leading to a 1 000 fold reduction in the amount of antigen required to activate the cell (Carter & Fearon, 1992; Dempsey *et al.*, 1996). However, this C3d adjuvancy appears to be dependent on the antigen to which it is bound (Bergmann-Leitner *et al.*, 2006). There is also evidence that C3d can have adjuvant effects in the absence of CR2. C3d has been shown to act as an adjuvant in CR1-/CR2- knockout mice in which HIV gp120/C3d streptavidin tetramers induced a greater IgM and IgG antibody response than gp120 alone (Haas *et al.*, 2004). One proposed mechanism involves helper T cells. C3d contains a high concentration of computationally-predicted MHC II epitopes, ten- to one hundred-fold greater than other serum proteins, such as albumin, the immunoglobulin Fc region and transferrin. (Knopf *et al.*, 2008). Incubation of peripheral blood mononuclear cells (PBMCs) with these MHC II-binding peptides generated a greater IFN- γ response from CD4⁺ cells than controls in two of the four human subjects examined (Knopf *et al.*, 2008). Adjuvancy, in this model, may require that the MHC II genotype of the individual is capable of binding the predicted T cell epitopes and that auto-reactive CD4⁺ T cells are present.

Physiologically, C3d might serve as a second signal from the innate immune system during B cell activation. Co-ligation of CR2 and IgM rescues murine B cells from apoptosis induced by cross-linking only surface IgM (Kozono *et al.*, 1995). Anergic B lymphocytes up-regulate Fas (Lyubchenko *et al.*, 2007; Rathmell *et al.*, 1995;) while CD40-CD40L interaction induces Fas expression on activated B cells (Garrone *et al.*, 1995; Schattner *et al.*, 1995). In the absence of concurrent survival signals, CD95 expression predisposes B cells to Fas-ligand induced apoptosis following interaction with CD4+ T cells (Rathmell *et al.*, 1995). This apoptotic signal can be overridden when C3d binds to CR2 by up-regulation of FLIP, which competes with Fas-activated death domains for caspase 8 (Barrington *et al.*, 2005). B cell anergy can also be overcome if cognate antigen is bound to C3d leading to the formation of extra-follicular antibody producing cells (Lyubchenko *et al.*, 2007). The requirement for complement activation in addition to BCR ligation reduces the probability of aberrant B cell activation. BCR co-ligation with CD21 leads to reduced engagement of inhibitory molecules, such as CD22 and SHP-1 further enhancing activation (Lyubchenko *et al.*, 2005). Antigens associated with complement are also subject to improved processing and presentation on MHC-II (Cherukuri *et al.* 2001). In experiments using athymic mice, C3d bound antigens also induced class switching from IgM to IgG for T independent antigens (Test *et al.*, 2001).

Genes Encoding Complement Receptors 1 and 2 (CR1, CR2)

In mice, CR1 and CR2 are products that result from alternate splicing of the *Cr2* gene. Alternative splicing is a complicated process regulated by both *cis*-acting

RNA elements as well as *trans*-acting protein elements including expression of tissue specific splicing factors as well as histone modification and chromatin architecture (reviewed in Luco *et al.*, 2011). Murine CR2 is comprised of fifteen CCP domains while CR1 is identical to CR2 with an additional six CCPs at the N-terminus of the protein (Kurtz *et al.*, 1990; Molina *et al.*, 1990). The additional N-terminal domains of CR1 are similar to the CCP domains comprising the rest of the protein but share greater homology with CCP encoded in the murine gene *Crry* (Jacobson & Weiss, 2008). In mice, CR1 and CR2 have a restricted expression pattern; both are found as maturation markers on B lymphocytes and FDCs whereas CR1 is also expressed on neutrophils and macrophages (Kinoshita *et al.*, 1988).

In mice, *Crry* acts as a co-factor for Factor I and can accelerate the decay of the C4b2a C3 convertase (Kim *et al.*, 1995). *Crry* is crucial for protecting the placenta from maternal complement component C3 activation at the feto-maternal interface. *Crry* ^{-/-} mice are non-viable, unless they develop in a C3 ^{-/-} doe (Xu *et al.*, 2000). Although murine *Crry* and CR1 are paralogous they have different receptor and regulatory properties. *Crry* reduces the amount of C3 products deposited on the cell membrane, whereas murine CR1 does not affect the amount. *Crry* will not rosette C3 sensitized erythrocytes while murine CR1 will (Molina *et al.*, 1992).

In humans the *Cr2* gene has lost its ability to produce CR1 and only CR2 is encoded (Holguin *et al.*, 1990). The function of CR1 in humans appears to have been fulfilled by the paralogous *Crry* gene in mice. *Crry* has expanded in humans, as a result of gene duplication, to produce a protein with a similar size and function to

murine CR1. Human CR1 retains the near ubiquitous tissue expression of Cr1 in mice (Jacobson & Weiss, 2008).

CD19 - the Co-signaling Molecule of CR2

CD19 is a type I transmembrane protein present in a quaternary structure with CR2, Leu 13 (CD225) and a tetraspanin protein, TAPA-1 (CD81) (Bradbury *et al.*, 1992; Matsumoto *et al.*, 1991; Matsumoto *et al.*, 1993). CD19 is a co-signaling molecule for CR2 and amplifies signaling from the BCR. The presence of CD19 on the cell surface is essential for B lymphocyte activation; under-expression of CD19 is associated with fewer peritoneal B cells, decreased B cell proliferative responses to mitogen, greatly reduced concentrations of serum immunoglobulin (Engel *et al.*, 1995), decreased responses to T-cell-dependent B cell antigens, absent germinal center formation and affinity maturation (Rickert *et al.*, 1995). CD19 provides tonic survival signals to recirculating naive B lymphocytes; the B cell compartment in CD19 ^{-/-} mice can be partially rescued by over-expression of apoptosis survival genes (Otero *et al.*, 2003).

In humans CD19 is expressed only on B cells (Tedder & Isaacs, 1989). In mice CD19 is expressed on B cells and some dendritic cells (Baban *et al.*, 2005). CD19 is the first B cell lineage surface antigen expressed on B cell-committed common lymphoid progenitor cells and persists until plasma cell differentiation. (Mansson *et al.*, 2008; Nadler *et al.*, 1983).

Although the structure of CD19 has not been determined by X-ray crystallography, the translated product is estimated to be 550 amino acids in length

with a mass of 95 kD. Predicted structures include a 16-residue N-terminal leader sequence, an extracellular region containing many glycosylation sites, a transmembrane region and an acidic cytoplasmic domain. The extracellular and cytoplasmic domains are approximately equal in length. Even though six cysteines are present in the extracellular region of CD19 sequences isolated to date, other residues required to form an immunoglobulin super-family domain are lacking (Tedder & Isaacs, 1989). The cytoplasmic tail of CD19 contains the nine highly conserved tyrosine residues that are crucial for signal transduction (Wang *et al.*, 2002).

Tyrosines in the cytoplasmic tail of CD19 are phosphorylated by Src family kinases when the B cell antigen receptor (BCR) is cross-linked or when the BCR and CR2 are cross-linked by antigen complexed with C3d. In human CD19, tyrosine Y482 and Y513 are the first tyrosines to be phosphorylated and are essential for CD19 signaling by recruiting PI3-kinase. Mutating the CD19 sequence to include phenylalanine residues in place of these two tyrosines reproduces the CD19 knockout phenotype (Engel *et al.*, 1995; Tueveson *et al.*, 1993; Wang *et al.*, 2002).

Polymorphisms, which affect the quantity of protein expressed on the cell surface, have been found in human sequences of CD19. These include a single nucleotide polymorphism (SNP) in the promoter region, associated with an increased risk of systemic sclerosis (Tsuchiya *et al.*, 2004), and two SNPs and long GT repeats, associated with systemic lupus erythematosus in Japanese populations (Kuroki *et al.*, 2002).

CR1 and CR2 Expression in Cattle

Protein expression of CR2 has been confirmed in cattle and monoclonal antibodies are commercially available (Naessens *et al.*, 1990; Sopp, 1996). These antibodies were assigned to the cluster of differentiation 21 (CD21) due to similarity to human CD21 tissue staining patterns and immunoprecipitation of human CR2 (Sopp, 1996). The first bovine CD21 monoclonal antibody, clone CC-21, was generated from spleen cells of a mouse inoculated with bovine alveolar macrophages (Naessens *et al.*, 1990) and immunoprecipitated two bands of 180 kDa and 145 kDa under reducing conditions (Sopp, 1996). However, the tissue from which the CD21-like antigen was derived in Sopp's (1996) study was not disclosed.

In cattle, CD21 is detected on veiled cells (dendritic cells) isolated from pseudo-afferent lymphatic vessels (Howard *et al.*, 1997). Over 90% of IgM⁺ B cells in the peripheral blood are also CD21⁺ from birth until at least six months of age (Chattha *et al.*, 2010a); over 95% of IgM⁺ cells in lymph nodes and greater than 90% of IgM⁺ cells in the spleen of young calves are also CD21⁺ (Chattha *et al.*, 2010b). In the peripheral blood of one-week-old calves, 20-40% of the CD21⁺ cells in the lymphocyte gate are IgM⁻; the number of CD21⁺/IgM⁻ cells decreased with age. These cells were not classified further (Chattha *et al.*, 2009). It is unlikely these cells are class-switched B cells as 39% of cells are CD21⁺/IgM⁻ in one-week-old animals and only 23% of B cells are CD21⁺/IgM⁻ after six weeks of age.

Immunohistochemical studies show that IgM⁺ and CD21⁺ cells co-localize in the cortex of lymph nodes and the marginal zone of the spleen. CD21⁺ cells are also observed in the subcapsular sinus of lymph nodes (Chattha *et al.*, 2010c). The

monoclonal antibody CC-21 also labels cells in tonsil follicles in a manner consistent with human follicular dendritic cells (Naessens *et al.*, 1990).

There is a partial nucleotide sequence available for bovine *Cr2* on the National Center for Biotechnology Information website (NCBI) from an unpublished manuscript (NCBI GenBank Accession Number: AJ535317). There is also a computationally predicted sequence for *Cr2* (Accession: XM_613039.4/XP_613039.4) representing a bovine homolog of murine or human CR2.

There is no predicted nucleotide sequence for bovine CR1 and the CR1 protein has not been identified in cattle. There is a mouse anti-human CR1 antibody that labels bovine monocytes, but it is not thought to be binding CR1 (Sopp & Howard, 1997).

Bovine CD19

Neither a protein nor an mRNA transcript with CD19 homology has been identified in cattle. There is a computationally predicted CD19 pseudogene sequence available (Accession: XM_028027) that contains two premature stop codons in the putative extracellular region of the protein.

There are no polyclonal or monoclonal antibodies reported which are specific for bovine CD19. For comparison, there are currently thirty-three available for CD19 in humans. There is one published account of a CD19+ bovine thymic lymphosarcoma identified using monoclonal antibody CC-55 (Da Costa *et al.*, 1992). However, the reference provided, Naessens and Howard (1991), does not support the use of CC-55 as an anti-CD19 antibody.

There have been several efforts to characterize heterologous monoclonal antibodies that cross-react with bovine leukocytes (Lewin *et al.*, 1985; Sopp & Howard, 1997; Sopp *et al.*, 2001; Sopp *et al.*, 2007) but they have not identified antibodies that bind to bovine CD19. Screening reported by Sopp *et al.*, 2001, found that one mouse anti-human antibody, clone BU12, stained bovine CD21+ lymphocytes and some monocytes but the weak staining pattern was not consistent with that for human CD19.

Rationale

It is unknown if cattle express CR1 and CR2 from the *Cr2* gene as is seen in mice or if they only express CR2 as is seen in humans. The predicted bovine *Cr2* gene (Accession: XM_613039) has a length similar to both the human and the murine *Cr2* genes, however, the translated product of this gene (Accession: XP_613039) is over two hundred base pairs longer than human (Accession: NP_001006659/NP_001868) or mice CR2 (NP_031784). A sequence alignment of these sequences shows that these extra residues are at the N-terminus of the hypothetical bovine CR2 sequence. It has also been noted that the monoclonal antibody, CC-21, immunoprecipitates two proteins of 145 and 180 kDa (Sopp, 1996), similar to the sizes of murine CR1 and CR2 (Kinoshita *et al.*, 1985; Kurtz *et al.*, 1990; Molina *et al.*, 1990). Taken together, it is feasible that cattle, like mice, also express two protein products from the *Cr2* gene.

It is especially important to discover whether multiple CC-21 targets are expressed on B cells since this would alter the interpretation of previous studies using this antibody. It is also of interest whether the bovine gene is most similar to

that of mice or humans as this would influence cross-species translation of results. It is possible that cattle have a unique translational arrangement of *Cr2*; if so, that would limit extrapolation of information learned in other species.

Given that CD19 is known to be an integral part of BCR signaling in mice and humans and that CD19 has been identified in many species, it is unlikely that cattle lack a functional CD19. Presence of a functional CD19 is also essential for proper signaling of CR2. The latest build of the bovine genome, Btau_4.0 still contains some ambiguities and areas of low coverage (The Bovine Genome Sequencing and Analysis Consortium *et al.*, 2009); this includes a gap in the genomic sequence (Accession: NC_007326.4) from which the predicted CD19 pseudogene was derived. As there is no other locus in cattle with greater homology to CD19, there is likely an error in the current predicted sequence. Clarification of this sequence would also be a useful first step in the generation of anti-bovine CD19 antibodies. If cattle express CD19 in a manner similar to what is observed in mice and humans, availability of a bovine-specific anti-CD19 antibody could provide a much needed tool to assess B cell lineage development in cattle. The goal of this thesis is to clone and sequence bovine *Cr2* mRNA transcripts and to determine whether cattle express a functional CD19 protein.

Materials and Methods

Animals and Tissues

All samples were obtained from Holstein cattle, housed at the University of Guelph research facilities in Elora and Ponsonby, Ontario, Canada. Animals were handled with the approval of the University of Guelph Animal Care Committee following the procedures outlined by the Canadian Council on Animal Care. Spleen samples were obtained from healthy 7 month old bull calves. Blood was collected from the coccygeal vein of adult cows into vacutainer collection tubes (Becton Dickinson and Company, Mississauga ON, Canada) with acid citrate dextrose anticoagulant (ACD). Spleen sections used to determine the full sequences of CD19 and CD21 were placed in an aqueous RNA stabilization solution, RNAlater® (Ambion, Streetsville ON, Canada) as directed by the manufacturer and stored at -20°C until RNA extraction. Spleen samples were held in a solution of RPMI 1640 (Sigma-Aldrich, St. Louis MO, USA) containing 0.01M HEPES buffer with 200 µg/ml gentamycin (Intervet/ Schering-Plough, Kirkland QC Canada) and 20 µg/ml ampicillin (Sigma, St. Louis MO, USA) on ice before further processing. Medium for spleen samples also included ACD to prevent coagulation.

Cell Isolation

Blood mononuclear cells were isolated by density-gradient centrifugation (1000 g for 30 minutes at room temperature) using Histopaque-1077 (Sigma-Aldrich). Tissue samples were collected at necropsy and processed by Kuldeep Chattha for use in a flow cytometry study (Chattha *et al.*, 2010c). Briefly, tissues

were diced into 1 mm cubes, then passed once through a Collector™ tissue sieve (Sigma, size 60 mesh), twice through 4 layers of cheesecloth, and finally through a 40 µm cell strainer (BD Biosciences) to yield a single cell preparation. Mononuclear cells were separated by density gradient centrifugation, as described above. Erythrocyte contamination was removed from all samples by hypotonic lysis using sterile water. Single cell suspensions were stained with trypan blue and live cells were counted on a haemocytometer. Neutrophils were isolated from the erythrocyte fraction of centrifuged whole blood following hypotonic lysis of the erythrocytes using sterile water.

IgM+, CD3+, and CD14+ cells were obtained from blood mononuclear cells by positive selection using the MACs magnetic bead separation system (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's directions. Monoclonal antibodies (mAbs) used for selection included B1g73A (mouse anti-bovine IgM), MM1A (mouse anti-bovine CD3), and M-M8 (mouse anti-bovine CD14), all from VMRD Inc., Pullman WA, USA. Each separation was conducted with 7.5×10^6 blood mononuclear cells in a volume of 750 µL of MACs buffer (phosphate buffered saline, pH 7.2, 0.5% bovine serum albumin, 2mM ethylenediaminetetraacetic acid [EDTA]) using 7.5 µL of undiluted mAb and incubating at 4°C for 5 minutes. Cells were washed once in MACs buffer, resuspended in 80 µL of MACs buffer, then incubated at 4°C for 15 minutes with 20 µL rat anti-mouse IgG coated magnetic beads (Miltenyi Biotech). Cells were washed again before separation on MS magnetic columns (Miltenyi Biotech) and a MiniMACSTM separator (Miltenyi Biotech). IgM+ cells were isolated from the spleen by labeling approximately 10^8

mononuclear cells with BM-23 (mouse monoclonal antibodies, anti-bovine IgM, Sigma) and separated magnetically as described above.

The purity of the sorted populations was assessed by flow cytometry. A 50µL aliquot from each population (approximately 150 000 to 200 000 cells) was labeled with 20 µL rat anti-mouse IgG conjugated with peridinin chlorophyll protein complex (PerCP) (BD Biosciences, Mississauga ON, Canada), washed, and 10 000 events were analyzed using a Becton-Dickinson fluorescence activated cell scanner (FACScan, BD Biosciences).

Monocyte derived macrophages were obtained by culture of 3 mL of blood mononuclear cells at a concentration of 1×10^7 cells per mL in RPMI 1640 supplemented with fetal bovine serum (Sigma-Aldrich), 100 IU/mL penicillin and 100 µg/mL streptomycin in a 25cm² polystyrene cell culture flask (Sigma-Aldrich). Medium and non-adherent cells were aspirated after 24 hours and medium was refreshed. After 72 hours of culturing, RNA was extracted from the resultant cell population following aspiration of the medium and non-adherent cells.

RNA Isolation

RNA was isolated from both sorted and unsorted mononuclear cells using RNeasy Mini Kit spin columns (Qiagen, Mississauga ON, Canada) following homogenization with QIAshredder columns (Qiagen) as per the manufacturer's directions. RNA from splenocytes was isolated from an approximately 3 mm³ sample with RNeasy Mini Kit spin columns (Qiagen) after homogenization with a rotor-stator homogenizer. RNA was quantified by spectrophotometry (Nanodrop,

Thermo Scientific, Wilmington DE, USA) using DNase/RNase-free water (Invitrogen) as a reference. Each sample was incubated with RQ-1 DNase (Promega, Madison WI, USA) at 37°C for 30 minutes followed by 5 minutes at 65°C (inactivation step) to remove contaminating genomic DNA. All RNA isolates were stored at -20°C in DNase/RNase-free water until further processing.

General Reverse-Transcriptase Polymerase Chain Reaction

All gene specific primers were designed using NCBI Primer-BLAST (Altschul *et al.*, 1990; Rozen & Skaletsky, 2000, available at <http://www.ncbi.nlm.nih.gov/tools/primer-blast/>), produced by Sigma-Genosys, Oakville ON, Canada, and used at a concentration of 2 ng/μL. All reverse transcriptase (RT) and polymerase chain reaction (PCR) reactions were incubated in a Bio-Rad MyCycler Thermocycler (Bio-Rad, Hercules CA, USA). Primer sequences used are listed in Table 2.1.

All RT reactions were performed using Thermoscript Reverse-Transcriptase (RT) (Invitrogen, Burlington, ON, Canada). All RT reactions were treated with RNase H (Invitrogen) for 20 minutes at 37°C to remove the RNA template. All PCR reactions were performed using either Platinum Taq (Invitrogen) or Platinum Taq High Fidelity (Invitrogen), each in a reaction volume of 50 μL. Deoxynucleotide triphosphate (dNTP) was obtained from Promega, Madison WI, USA and was used at a final concentration of 1.2 mM. All PCR reactions employing Platinum Taq used a final MgCl₂ concentration of 2 mM and all reactions with Platinum Taq High Fidelity used a final MgSO₄ concentration of 2 mM. All reactions used 1 μL of the RT product as template, and were conducted in parallel with a template negative

Table 2.1 - Primer Sequences

Primer	Sequence	Primer	Sequence
CD21-1F	TCTAGCCCTGTCTTCTCGGA	CD21B	TCCTGGTCCAAAAAGTACGC
CD21-RF	TTCAACTTCTGGAATTGGGC	CD21C	ACCGACTGGGTGAAAGTGTC
CD21-2F	TGTCCACACACCTCAGGGTA	CD21D	AATCCTTTGGAACCAAACCC
CD21-2R	GGAGCTACCACACGACCATT	CD21E	TTCAACTTCTGGAATTGGGC
CD21-3F	GCCCAATTCCAGAAGTTGAA	CD19AF	CCACAAAGTCCCAGCTGAAT
CD21-3R	TCCTGGTCCAAAAAGTACGC	CD19AR	GGGGTCCCAGATTTCAAAGT
CD21-4F	AAGGGTACCGGTAAATGGC	CD19BF	AGGACCAACTCTCCCAGGAT
CD21-4R	AGGGGCTTGACAATCCTTTT	CD19BR	GGGGTCCCAGATTTCAAAGT
CD21-5F	GCGTACTTTTTTGGACCAGGA	CD19-5Ro	GCTGCGTGGGTTTCCGTAGG
CD21-5R	AATCCTTTGGAACCAAACCC	CD19-5Ri	CATGGAGAGGGAAAGCATGT
CD21-6F	TACCATGAAGGGTAGCAGGG	CD19-3Ro	TATCGTTGTAACCACGGCAA
CD21-6R	GAGATTTTAAGCACTGCGGG	CD19-3Ri	AGGACCAACTCTCCCAGGAT
CD21-7F	ACCGACTGGGTGAAAGTGTC	GAPDHF	CUUAUGACCACUGUCCACGC
CD21-7R	AATCCTTTGGAACCAAACCC	GAPDHR	AUCCACAACAGACACGUUGG
CD21-8F	CTCGGTGTGAAGAAGCACAA	M13F	GTAAAACGACGGCCAG
CD21-8R	TTGACAGCCAAAGAAAGCCT	M13R	CAGGAAACAGCTATGAC
CD21-9F	GGGTTTGGTTCCAAAGGATT	Qo	CCAGTGAGCAGAGTGACG
CD21-9R	CTTGCCACTCACGTTCTCTG	Qi	GAGGACTCGAGCTCAAGC
CD21-5Ro	TGTTGCTGCCTTTCATGGTA	Qt	CCAGTGAGCAGAGTGACGAGGACT CGAGCTCAAGCTTTTTTTTTTTTTT TTTT
CD21-5Ri	GATGTGTCACCAATGAGTCGAT		
CD21-3Ro	CGTCCACTTACAATCTCCCA		
CD21-3Ri	CTCTTTTCTGCTTGGGTGCT		
CD21A	TCTAGCCCTGTCTTCTCGGA		

control and with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Accession: NM_001034034.1) as a positive control.

Sequencing of PCR Products

Sequencing of all PCR products and plasmids was performed by the University of Guelph Genomics Facility where samples were sequenced by the Sanger method (Sanger *et al.*, 1977) using an Applied Biosystem BigDye Terminator v3.1 and an Applied Biosystems 3730 sequencer. PCR products were either sequenced directly using the primers that generated the product or cloned into a plasmid for sequencing. When sequencing plasmids, primers matching the M13 sequences flanking the insertion site, M13F and M13R, were used.

To insert a PCR product into a plasmid, the reaction mixture was first purified using a QIAquick PCR Purification Kit (Qiagen) then the product was ligated into a TOPO2.1 plasmid (Invitrogen) as directed by the manufacturer. The resulting plasmids were used to transform One Shot TOP10 chemically competent cells (Invitrogen). Cells were cultured overnight on Luria-Bertani (LB) agar plates supplemented with 40 µg/mL kanamycin (Invitrogen) to select successful transformants using 40 mg/mL X-Gal (Invitrogen). Colonies containing a plasmid with an insert stained white rather than blue. When the PCR product of interest contained multiple products, white colonies were screened for inserts of the predicted size by PCR, using reaction conditions identical to those that generated the original product. Colonies with the insert of interest and colonies generated from a PCR reaction yielding one product were sub-cultured in 7.5 mL of LB medium with

kanamycin at 37°C for 18 hours with 220 rpm horizontal shaking. Plasmids were extracted from 3 mL of culture suspensions using a QIAprep Spin Miniprep Kit (Qiagen) following the manufacturer's directions.

Agarose Gel Electrophoresis

PCR reactions were resolved on a 2.0% agarose gel made with Ultrapure Agarose (Invitrogen) suspended in TAE running buffer (Tris (hydroxymethyl amino methane), acetic acid, EDTA) diluted from a stock solution of 50x TAE buffer (Bio-Rad, Mississauga ON, Canada). 5 µL of sample were loaded into the gel with approximately 1 µL of loading buffer. All gels contained a 1kb Plus DNA ladder (Invitrogen) as a size reference. Gels were run in a Bio-Rad Mini-sub Gel GT (Bio-Rad) at 100V for 40 minutes in TAE. Gels were stained by either the inclusion of 3 µL of SYBR® Safe DNA gel stain (Invitrogen) in the gel prior to casting or stained for 15 minutes in a bath of 0.5 µg/mL ethidium bromide in reverse osmosis purified (Milli-Q) water. Gels stained with ethidium bromide were rinsed briefly with tap water before visualization by a Gene Genius Bio Imaging System (Syngene, Frederick MD, USA) using the GeneSnap software (Syngene).

Sequence Analysis

Sequence analysis was performed by the Geneious™ software suite. (Drummond *et al.*, 2010). Protein and amino acid alignments were performed using ClustalW (Thompson *et al.*, 1994) through the Geneious interface. Protein secondary structure was predicted by the Sanger Institute Pfam database (Finn *et*

et al., 2010). The exon structure of genes was predicted by NCBI Spidey (Wheelan *et al.*, 2001). Potential N-glycosylation sites were predicted using NetNGlyc (Gupta *et al.*, 2004. <http://www.cbs.dtu.dk/services/NetNGlyc/>) and potential tyrosine phosphorylation sites were determined using NetPhos (Blom *et al.*, 1999. Available at <http://www.cbs.dtu.dk/services/NetPhos/>).

Confirming the Sequence of the Cr2 Gene

To confirm the hypothetical sequence of Cr2, several overlapping PCR reactions were designed using the hypothetical gene for bovine Cr2 (Accession: XM_613039.4) as a template (Fig 2.1). cDNA was generated from splenocyte RNA with an oligo-dT primer following the manufacturer's directions, incubating at 60°C for 60 minutes followed by 5 minutes at 85°C to inactivate the polymerase. PCR reactions were performed using Platinum Taq polymerase (Invitrogen). Samples were incubated at 94°C for 5 minutes to activate the polymerase, followed by 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 1 minute, followed by a 5 minute final extension time at 72°C. The resulting products were cloned into plasmids for sequencing as described previously.

To obtain the full length mRNA sequence of Cr2, 5' and 3' rapid amplification of cDNA ends (RACE) was employed using protocols adapted from Scotto-Lavino *et al.*, (2007a and 2007b). In this technique, both 3' and 5' RACE use a hybrid primer, Qt, which contains an oligo-deoxythymidine sequence that anneals to polyadenosine sequences, and a known adapter sequence. In 3' RACE, Qt binds the poly-A tail of mRNA transcript. In 5' RACE, Qt binds to a poly-A sequence appended

Fig 2.1 - Primer binding sites on hypothetical bovine Cr2 (Accession: XM_613039). The binding sites of forward primers and reverse primers are depicted in dark green and light green, respectively. Primers are described in Table 2.1.

to the 5' end of the first strand cDNA with terminal deoxynucleotidyl transferase (TdT). The adapter sequence provides a known nucleotide sequence that can be used to anneal primers, Qo and Qi. This allows for a PCR reaction using one gene specific primer in a known region of an mRNA transcript and an adapter primer to amplify the unknown portions of an mRNA sequence. As only one gene specific primer is used in 3' and 5' RACE, two rounds of PCR are performed using two nested gene specific primers and two nested adapter primers to reduce non-specific transcript amplification.

For 3' RACE the RT reaction was performed on RNA from a section of spleen with Thermoscript RT (Invitrogen) using Qt. To generate the cDNA, the reaction was incubated at 25°C for 5 minutes followed by 55°C for 40 minutes, 65°C for 10 minutes then 85°C for 5 minutes to inactivate the polymerase. Two rounds of nested PCR were performed with Platinum Taq High Fidelity (Invitrogen) using gene specific forward primers. Primers CD21-3Ro and Qo were used for the first round of amplification and primers CD21-3Ri and Qi were used for the second. Both rounds of amplification were incubated at 94°C for 30 seconds to activate the polymerase followed by 35 rounds of amplification at 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute, followed by 68°C for 5 minutes of final extension. The resultant products were cloned into plasmids for sequencing as described above.

The RT for 5' RACE was performed on RNA from splenocytes using a gene specific primer CD21-5Ro incubated at 60°C for 60 minutes, then 65°C for 10 minutes, followed by 85°C for 5 minutes to inactivate the polymerase. cDNA from

the RT reaction was purified using the QIAquick PCR purification Kit (Qiagen) according to the manufacturer's instructions, with the exception of reducing the final elution volume from 50 μ L to 30 μ L to increase the final concentration of the first strand product. An oligo-deoxyadenosine tail was added to the 5' end of the RT products using recombinant TdT (Invitrogen). 15 μ L of purified RT product was added to 1 unit of TdT, 4 μ L of 100 mM dATP (Invitrogen), and 5 μ L of 5x TdT buffer (0.5 M potassium cacodylate, 10mM CoCl₂ and 1 mM of dithiothreitol). The reaction was incubated at 37°C for 5 minutes to append the tail, and the enzyme was inactivated by incubating at 65°C for 5 minutes.

PCR for 5' RACE was performed with Platinum Taq High Fidelity Polymerase (Invitrogen). The first round of nested PCR used three primers, Qt, Qo and CD21-5Ro. After incubation at 94°C for 30 seconds to activate the polymerase, the reaction was incubated at 48°C for 2 minutes to allow Qt to anneal to the poly-A tract on the 5' end of the first strand cDNA product, then at 68°C for 40 minutes. The unusually long extension time for CD21-5Ro and Qo to generate the second strand was recommended by Scotto-Lavino *et al.*, 2007b. The reaction continued with 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute, followed by 68°C for 5 minutes of final extension. The second round of amplification used primers Qi and CD21-5Ri, and was incubated at 94°C for 30 seconds to activate the polymerase followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute, followed by 68°C for 5 minutes of final extension. The resulting products were cloned into plasmids for sequencing as previously described.

Identifying the Tissue Distribution of Cr2 Gene Products

The nucleotide sequence for CD21 was used to design primers that amplified specific splice variants of CD21. The specificity of the primers was confirmed by sequencing initial test reactions in a sample of spleen cells. The locations of these primers and the splice variants amplified are depicted in the results section in Figure 3.1. These primers were used with Platinum Taq High Fidelity (Invitrogen) for samples from splenocytes and IgM+ splenocytes. The reaction was activated by incubating at 94°C for 30 seconds, followed by 35 cycles of 94°C for 15 seconds, 60°C for 30 seconds, 68°C for 1 minute, followed by 68°C for 5 minutes of final extension. Platinum Taq (Invitrogen) was used for analysis of samples from magnetically separated blood mononuclear cells and neutrophils. The reaction was activated by incubating at 94°C for 5 minutes, followed by 35 cycles of 94°C for 30 seconds, 60°C for 60 seconds, 72°C for 1 minute, followed by 72°C for 5 minutes of final extension.

Confirming the Sequence of the CD19 Gene

The hypothetical gene for bovine CD19 (Accession: XR_028074) is predicted to be a pseudogene containing premature stop codons. Working on the assumption that the predicted sequence was in error and that a functional *Cd19* gene exists in cattle, the hypothetical gene was used in a BLASTn query (NCBI) of the bovine expressed sequence tag (EST) database. BLASTn hits were then assembled into a contiguous sequence (contig) of the putative bovine CD19 sequence (Fig 2.2). This contig was aligned to published CD19 sequences for the human, mouse and pig to determine the putative start and stop codons and to confirm a productive CDS. PCR

using primer sequences CD19AF and CD19AR generated from the bovine CD19 EST contig as well as 5' and 3' RACE were employed on RNA from whole spleen as described above to confirm expression of CD19 in cattle. Primer CD19-5Ro was used to generate the 5' RACE RT product. Primers CD19-5Ro then CD19-5Ri were used in 5' RACE and primers CD19-3Ro then CD19-3Ri were used in 3' RACE. Primers CD19BF and CD19BR, which generate a smaller PCR product than CD19AF and CD19AR, were used to confirm the presence of CD19 in sorted blood mononuclear cell populations. RT reactions for CD19 included 5% dimethylsulfoxide (DMSO) to reduce secondary structure due to a high guanine-cytosine (GC) content in some regions of the CD19 EST contig.

Fig 2.2. Expressed sequence tag (EST) contiguous DNA (contig) aligned to hypothetical CD19 pseudogene sequence. The hypothetical CD19 pseudogene (Accession: XR_028074), found at the top of the alignment, was used in a BLASTn query (NCBI) of the bovine expressed sequence tag (EST) database. BLASTn hits were then assembled into a contiguous sequence (contig) of the putative bovine CD19 sequence. The identity of the ESTs with the hypothetical gene decreases in the region containing the premature stop codons. The contiguous sequence made in this alignment does not contain premature stop codons. Primers described in Table 2.1.

Results

Determination of the Full Length Sequence for Cr2 Derived mRNA

Cr2 derived mRNA sequences were determined using spleen cells of one seven-month-old male Holstein calf by assembling contiguous sequences from overlapping PCR reactions using primers designed from the hypothetical bovine CD21 sequence (Accession: XM_613039.4). The full mRNA sequence was completed using 5' and 3' RACE. Three of the primer sets used, CD21-5F/R, CD21-6F/R and CD21-7F/R, generated two PCR products differing in length by 177 base pairs (bp) suggesting the presence of alternatively spliced transcripts. The full length contig obtained by assembly of all overlapping PCR products measured 5 261 bp. The start codon was identified by translating the contig into the longest open reading frame with homology to murine CR2 (Accession: NM_007758).

It was noted that the bovine CD21 coding sequence (CDS) was much longer than the murine CR2 CDS, 4 419 bp versus 3 099 bp, respectively. This suggested that cattle might encode CR1 from the same gene as CR2 as is seen in mice (Kurtz *et al.*, 1990; Molina *et al.*, 1990; Jacobsen & Weis 2008). To test if cattle alternatively splice the *Cr2* gene to express both CR1 and CR2, PCR was used with a primer in the 5'-UTR (CD21A) and another primer near the middle of the CDS which was expected to be present in both CR1 and CR2 (CD21B, Fig 3.1). Two PCR products were observed and then sequenced to confirm that both were products of the same gene.

To determine the relationship between the CR1 and CR2 alternative splice variants and the 177 bp splice variant, primers were designed in the 5' and 3'-UTR to amplify the entire CDS of the gene. Unfortunately, the transcripts were too large

and too little difference in size to be accurately resolved by electrophoresis. An alternative strategy was pursued wherein a single reaction used a forward primer within the upstream alternative exons (primer CD21E) and a reverse primer downstream of the second alternative exon (primer CD21D) as shown in Figure 3.1. This reaction yielded a PCR product that appeared to be a single band when resolved by electrophoresis, which proved to be two distinct products following cloning and sequencing. This suggested that the upstream alternative exons may be present either with or without the downstream alternative exon. The presence of the 177 bp variant in CR2 was determined by examining tissues that did not express the upstream alternatively spliced exons (discussed below). As there is no sequence present in CR2 that is not present in CR1 it would be difficult to design a specific PCR reaction that would isolate CR2 without using a primer bridging the 5'UTR and the first CCP domains of CR2. However, this precludes making definite conclusions on whether CR2 exists in two sub-isoforms in the spleen or which single isoform may be present.

In all, four transcripts were identified in the spleen: 5 261 bp, 5 084 bp, 4 120 bp and 3 943 bp in length (Fig. 3.1). The identity of transcripts as products of the bovine CR2 gene was confirmed by sequencing. The CDS was identified by translating the sequences into the reading frame with the greatest homology to murine CR2 (Accession: NP_031784). A BLASTn query of the bovine genome revealed no other genes encoding these transcripts. The exon structure of the genes was determined by NCBI Spidey (Wheelan *et al.*, 2001).

The translated sequences were annotated using *Geneious* to identify the signal sequence and transmembrane regions (Fig. 3.2). CCP domains were identified by searching the sequences against the Sanger Institute Pfam Database (Fin *et al.*, 2010; Ichinose *et al.*, 1990). Potential glycosylation sites were determined using NetNGlyc (Gupta *et al.*, 2004) and potential tyrosine phosphorylation sites were determined using NetPhos (Blom *et al.*, 1999). Pairwise alignment of the four sequences revealed two primary isoforms, each with two sub-isoforms. The products of the 4 120bp transcript and the 3 943bp transcript were predicted to represent sub-isoforms of CR2 as these sequences were of a similar length to two human CR2 sub-isoforms. The products of the 5 261bp and the 5 084bp transcripts were predicted to represent sub-isoforms of CR1 (Kurtz *et al.*, 1990; Molina *et al.*, 1990; Jacobsen & Weis 2008). CR1 sub-isoforms differed from CR2 by the presence of an additional six CCP domains after the signal sequence. The two sub-isoforms of CR1 and CR2 differed in the presence or absence of the 16th CCP domain. All sequences have been deposited in GenBank and are available as CR1-Long (5 261 bp, CR1-L; Accession: HM461977.1), CR1-Short (5 084 bp, CR1-S; Accession: HM461978.1), CR2-Long (4 120 bp, CR2-L; GQ497281.2) and CR2-Short (3 943 bp, CR2-S; Accession: GQ497282.2). The complete CDS of the *Cr2* was confirmed using RNA extracted from the spleen of an additional seven-month-old animal (data not shown.)

Fig. 3.2. Amino acid alignment of four gene products of *Cr2*. CCP domains as predicted by Pfam are depicted in red and numbered from the N-terminus to the C-terminus. Each CCP domain is approximately 60 amino acids long. CR1 products differed from CR2 products by the inclusion of the first 6 CCP domains. Long products differed from short products by the inclusion of the 16th CCP domain. *Cr2* gene products had short cytoplasmic regions. Potential N-glycosylation sites are noted in blue and potential tyrosine phosphorylation sites are shown in green. Signal sequence – blue, transmembrane region – yellow, cytoplasmic region – green.

Tissue Distribution of Cr2 Splice Variants

Primers were used (CD21C and CD21D) to specifically amplify the long and short versions CR1 and CR2; these primers did not distinguish whether or not the additional CCP domain was present in CR1 or CR2. Primers were also designed to amplify CR1 but not CR2 (CD21A and CD21E) and to amplify both CR1 and CR2 (CD21A and CD21B). The binding sites of these primers are depicted in Figure 3.1. All products analyzed on agarose gels were the expected size.

The distribution of the CR1 and CR2 isoforms was assessed using RNA isolated from sorted blood mononuclear cells, IgM⁺ spleen mononuclear (B) cells, splenocytes, and blood monocyte derived macrophages. Blood cells were sorted using magnetic beads (Miltenyi Biotech) after incubation with specific monoclonal antibodies, into CD3⁺ (T cells), CD14⁺ (monocytes) and IgM⁺ (B cells). Neutrophils were isolated from the pellet of the erythrocyte fraction of centrifuged blood. The purity of the isolated cells was assessed by flow cytometry; over 96% of the sorted CD3⁺ population expressed CD3, over 92% of the sorted CD14⁺ population expressed CD14, and over 98% of the sorted IgM⁺ population expressed IgM. CR1 transcripts were not detected in earlier examinations of blood mononuclear cells (data not shown). CR2 transcripts were detected only in the IgM⁺ cells using primers CD21C and CD21D; both the long and the short isoforms were present (Fig. 3.3).

RNA was isolated from lysate of unsorted splenocytes as well as from IgM⁺ splenocytes isolated by magnetic bead separation (Miltenyi Biotech). Transcripts encoding both CR1 and CR2 were detected in the unsorted splenocytes while only

CR2 was detected in IgM⁺ spleen mononuclear cells (Fig. 3.4). Both the long and short isoforms were detected in unsorted splenocytes and the IgM⁺ splenocytes (data not shown). Blood mononuclear cells were cultured in plastic flasks to isolate monocyte derived macrophages. Neither CR1 nor CR2 could be detected in these cells (Fig. 3.5).

Fig. 3.4. Ethidium bromide stained agarose gel showing CR1 in splenocytes and IgM+ splenocytes. CR1 was detected only in splenocytes and not in IgM+ cells isolated from the spleen. Lane 1, 1kb Plus Ladder (Invitrogen). Lane 2, primers A and B in splenocytes. Lane 3, primers A and B in IgM+ splenocytes. Lane 4, primers A and E in splenocytes. Lane 5, primers A and E in IgM+ splenocytes. Lane 6, primers A and E, no template. Lane 7, housekeeping control GAPDH in splenocytes. Lane 8, GAPDH in IgM+ splenocytes. Lane 9, GAPDH, template negative. Included is an image from Fig. 3.1 demonstrating the binding locations of primers C and D.

Fig. 3.5. Ethidium bromide stained agarose gel showing CR2 in blood monocyte derived macrophages. Lane 1, 1kb Plus Ladder (Invitrogen). Lane 2, primers C and D in blood mononuclear cells. Lane 3, primers C and D in macrophages. Lane 4, housekeeping control GAPDH in blood mononuclear cells. Lane 5, GAPDH in macrophages. Lane 6, GAPDH with no template. CR1 was not detected (data not shown). Included is an image from Fig. 3.1 demonstrating the binding locations of primers C and D.

Coding Sequence of CD19

The consensus sequence assembled using bovine ESTs (Fig. 2.2) contained a 1 638 bp coding sequence encoding a protein 546 amino acid residues long without any premature stop codons, consistent with CD19. The CDS was found by analyzing splenocytes of a 7-month-old calf. The transcript obtained from spleen had a 1704 bp CDS encoding a 568 aa protein. The discrepancy between the EST contig and the sequence obtained from splenocytes was accounted for by a 66 bp gap in the EST contig (Fig 3.6). The inclusion of these 66 bp in cattle is supported by their presence in the predicted bovine CD19 sequence (XR_028074) and homologous inclusion in CD19 sequences of mice (Accession: NP_033974) and humans (Accession: NP_001761.3). A BLASTn query of the bovine genome revealed no other genes encoding these transcripts. The exon structure of the genes was determined by NCBI Spidey (Wheelan *et al.*, 2001). However, due to the gap in the genomic sequence between 626 and 909 bp, exons could not be predicted (Fig. 3.6). The complete nucleotide sequence is available on GenBank (Accession: GQ_497280.1).

The translated sequence was annotated using *Geneious* to identify the signal sequence and transmembrane regions. A search of the Pfam Database for domains revealed only two insignificant homologies to V-set type immunoglobulin domains in the extracellular region (Finn *et al.*, 2010). Potential glycosylation sites were determined using NetNGlyc (Gupta *et al.*, 2004) and potential tyrosine phosphorylation sites were determined using NetPhos (Blom *et al.*, 1999) (Fig. 3.7).

Fig. 3.6. Exon structure of *Cd19*. Exon prediction is incomplete due to a gap in the nucleotide sequence of the genomic region of bovine *CD19*.

The distribution of CD19 in blood mononuclear cells was assessed in RNA isolated from CD3+ (T cells), CD14+ (Monocyte), IgM+ (B cells) and neutrophils. CD19 was detected only in IgM+ cells (Fig. 3.8).

Fig. 3.8. SYBR® Safe DNA (Invitrogen) stained agarose gel showing CD19 transcripts in sorted blood cell populations. CD19 was detected only in IgM+ lymphocytes. Lane 1, 1kb Plus Ladder (Invitrogen). Lane 2, primers CD19BF and CD19BR in IgM+ cells. Lane 3, CD19BF and CD19BR in CD3+ cells. Lane 4, CD19BF and CD19BR in CD14+ cells. Lane 5, CD19BF and CD19BR in neutrophils. Lane 6, CD19BF and CD19BR, template negative. Lane 7, Blank. Lane 8, housekeeping control GAPDH in IgM+ cells. Lane 9, GAPDH in CD3+ cells. Lane 10, GAPDH in CD14+ cells. Lane 11, GAPDH in neutrophils. Lane 12, GAPDH, template negative. Primer bind sites are depicted in Figure 2.2 and primer sequences are depicted in Table 2.1.

Discussion

Cattle Express CR1 and CR2 from the Cr2 Gene

This research showed that four transcripts were encoded by the bovine *Cr2* gene. All the *Cr2* gene products were comprised of tandem complement control protein (CCP) domains (Ichinose *et al.*, 1990), also known as short consensus repeat domains, as is seen in other members of the regulation of complement activation (RCA) family. The frequent incorporation of CCP domains in the RCA locus is due to their common evolutionary origin (Krushkal *et al.*, 2000). CCP domains are approximately 60 - 75 amino acid residues in length with four highly conserved cysteine residues (Moore *et al.*, 1987). CCP domains are comprised of β -sheets with extensive hydrogen binding which creates a compact globular structure (Chou & Heinrichson, 1997). Cattle encoded CR1 and CR2 by alternative splicing of a single gene as is observed in mice (Kurtz *et al.*, 1990; Molina *et al.*, 1990). Cattle also alternatively spliced an exon encoding the 16th CCP domain, which corresponds to the alternatively spliced CCP 10a domain in humans (Braun *et al.*, 1998; Douglas *et al.*, 2009; Ilges *et al.*, 1997; Liu *et al.*, 1997). As the human *Cr2* gene has lost the ability to encode CR1 domains (Holguin *et al.*, 1990; Jacobson & Weis, 2008) and mice appear to have lost the ability to encode the alternatively spliced CCP 10a domain, it is likely that cattle have a more phylogenetically ancient *Cr2* gene. To date no stimuli has been observed to alter the rates of inclusion or exclusion of the CR1 associated CCP domains in mice or the 11th CCP domain in humans (Ilges *et al.*, 1997; Jacobson & Weis, 2008).

Given that cattle express CR1 in a manner similar to mice, it could be hypothesized that they would also express a gene similar to the murine *Crry*. In mice, *Crry* is closely linked to *Cr2* with less than 10 kilobases separating their CDS (Kurtz *et al.*, 1989). However, no nucleotide sequence homologous to *Crry* was found in a BLASTn search of the NCBI bovine genome databases with the exception of the first six CCP domains of bovine CR1 (data not shown). A search of the bovine EST database revealed three expressed sequences that are identical to, and likely products of, the first six CCP domains of bovine CR1.

In cattle, the *Cr2* gene is located on chromosome 16. There are several other complement-associated genes in this region including decay accelerating factor (CD55), factor H and the C4 binding protein (C4bp) alpha and beta chain precursors. This suggests that bovine *Cr2* might be located in a RCA locus as has been observed in other species. There are hypothetical proteins in this region that have been annotated as 'factor H-like' or 'C4bp-alpha chain-like', likely due to predominance of CCP domains in the RCA locus and proximity to either the factor-H and C4bp loci. None of the predicted genes are similar in size to murine *Crry* (441 amino acid residues) and thus are unlikely a bovine *Crry*. Further studies would be necessary to determine if cattle express a homologue of murine *Crry* or if another gene is replacing its function.

CR1 and CR2 are both Present in Long and Short Sub-isoforms

Both CR1 and CR2 were found in cattle, each with two sub-isoforms differing by the presence of the 16th CCP domain. The presence of an alternatively spliced

CCP domain has been recognized in human CR2. Initial findings indicated that in humans CR2L was comprised of 16 CCP and expressed only on FDCs, while CR2S was comprised of 15 CCP and expressed only on tonsillar B cells (Liu *et al.*, 1997). However, mRNA transcripts of both the long and short sub-isoforms have been detected in peripheral blood cells (Braun *et al.*, 1998; Douglas *et al.*, 2009) and in lymphoid tissues where both sub-isoforms were expressed in what appears to be equal proportions (Ilges *et al.*, 1997). Only the short form of CR2 has been detected in mice (Accession: NP_031784, Validated Reference Sequence). A role for CCP16 in cattle cannot be described at this time. The long and short sub-isoforms of CR1 have not been identified in other species and no clear functional differences between the long and short versions of CR2 have been determined in the human literature. The inclusion of the additional CCP domain in humans does not change ligand affinity (Kalli *et al.*, 1991). This is possibly because all known CR2 ligands bind to the first and second CCP domains (Asokan *et al.*, 2006; Gilbert *et al.*, 2006).

The crystal structure of the short version of human CR2 depicts a linear molecule with minimal proximity relationships between CCP domains with the exceptions of domains 1 and 2, as well as domains 10 through 13 (Gilbert *et al.*, 2006; RCSB Protein Data Bank Accession: 2gsx, available at <http://www.pdb.org/pdb>). It is possible that introducing an additional domain in the region of the protein containing domains 10 through 13 could disrupt tertiary protein interactions and lead to functional differences.

Support for functional differences has been found in studies of single nucleotide polymorphisms (SNPs) in the *Cr2* gene that are associated with an

increased risk of systemic lupus erythematosus (SLE). Two of these SNPs decreased inclusion of the 11th exon (corresponding to the 16th CCP domain in cattle) in *Cr2* mRNA transcripts (Douglas *et al.*, 2009). It has been suggested that this domain may play a role in activation-induced ectodomain shedding (Ling *et al.*, 1998; Masilamani *et al.*, 2003)

Many transmembrane proteins, including those associated with signaling in lymphocytes, undergo ectodomain shedding wherein the extracellular region of a transmembrane protein is cleaved by plasma or membrane proteases (reviewed in Dello Sbarba *et al.*, 2002). Ectodomain shedding generates soluble receptors, resulting in down-modulation of ligand dependent cell signaling by reducing the density of membrane bound receptors and removing potential ligands from the surrounding environment.

Soluble CR2 (sCR2) has been found in serum and plasma from healthy humans (Douglas *et al.*, 2009; Ling *et al.*, 1998; Masilamani *et al.*, 2003; Masilamani *et al.*, 2004a); decreased plasma concentration of sCR2 has been associated with rheumatoid arthritis, SLE, Sjögren's syndrome and pregnancy (Masilamani *et al.*, 2004a; Masilamani *et al.*, 2004b; Masilamani *et al.*, 2008). sCR2 is not a product of an alternatively spliced mRNA transcript that lacks the transmembrane domain (Illges *et al.*, 1997); it is derived from cleavage of the membrane protein's extracellular domains (Frémeaux-Bacchi *et al.*, 1996; Masilamani *et al.*, 2003) through proteolysis by matrix metalloproteases (Aichem *et al.*, 2006). The cleavage site is directly adjacent to the membrane (Illges *et al.*, 1997; Data from Masilamani *et al.*, 2002 and Masilamani *et al.*, 2003 as interpreted by Aichem *et al.*, 2006) and is

dependent on cysteine reduction and the opening of a disulphide bridge in the membrane proximal CCP domain to create a juxtamembrane stalk (Aichele *et al.*, 2006). No clear amino acid motif required for ectodomain shedding has been identified, but the presence of a juxtamembrane stalk seems to be important (Dello Sbarba *et al.*, 2002).

Ectodomain shedding of CR2 might be an important means of B cell regulation. sCR2 is shed from B cells after activation by anti-IgM and anti-CD40 mAb or with the mitogen phorbol 12-myristate 13-acetate (PMA) (Masilamani *et al.*, 2003). Activated B cells have lower membrane density of CR2 than resting B cells due to ectodomain shedding. Shedding is augmented when CR2 is engineered to have a truncated cytoplasmic domain (Hoefer *et al.*, 2008). This could imply that *cis*-acting elements are involved in shedding.

Ectodomain shedding leaves a portion of the transmembrane protein embedded in the cell membrane (the carboxy-terminal fragment), which might have residual or altered cell signaling capacity (Dello Sbarba *et al.*, 2002). Two such fragments, 8 kDa and 16 kDa in size, have been identified in activated human B cells. However, these fragments seem to be rapidly endocytosed and catabolized (Hoefer & Illges, 2009).

sCR2 is capable of binding iC3b, CD23 (Aubry *et al.*, 1992; Frémeaux-Bacchi *et al.*, 1996), IFN- α (Asokan *et al.*, 2006), and Epstein Barr Virus (EBV) (Moore *et al.*, 1991; Nemerow *et al.*, 1990). Binding sCR2 can lead to changes in gene expression. In humans soluble CR2 binds to CD23 on monocytes leading to increases in HLA-DR, nitric oxide synthase, CD40 and TNF- α gene expression, a phenotype consistent with

increased antigen presentation to helper T cells (Frémeaux-Bacchi *et al.*, 1998). sCR2 may also interfere with the binding of membrane bound CR2 on B cells to FDC-bound CR2 ligands resulting in diminished IgG response to T cell dependent antigens (Qin *et al.*, 1998).

Ling *et al.* (1998) have reported that sCR2 in humans is predominantly derived from FDCs that exclusively expressed the long form of CR2. However, spectrometric analysis of sCR2 in human plasma identifies peptides consistent with the CR2S. Even though no peptides corresponding to the alternatively spliced domain present in the CR2L were detected, its presence cannot be formally excluded (Masilamani *et al.*, 2003). Accordingly, Masilamani *et al.* (2003) attribute soluble CR2 to B cells, which Liu *et al.* (1997) describe as expressing only CR2S. sCR2 has also been detected from murine B cells which only express the short form of CR2 (Hoefer *et al.*, 2008). These conflicting data allow for no conclusion on the implications of the presence of a short and long CR2 in cattle. Likewise, soluble CR2 has not been detected in cattle. While the current literature suggests functional differences between CR2L and CR2S, there is insufficient evidence available to definitively conclude a role for this alternatively spliced domain in any species.

Tissue Distribution of CR1 and CR2

The tissue distribution of CR1 and CR2 in cattle was assessed by RT-PCR. CR1 mRNA was detected in RNA isolated from splenocytes but not blood mononuclear cells or IgM⁺ B cells isolated from the spleen. As both long and short forms of CR1 were detected in spleen cell homogenates, the cells expressing CR1 in cattle appear

to express both sub-isoforms. This is a different expression pattern than is observed in humans and mice. In humans, expression of CR1, (a product of a different gene), is ubiquitous. In mice, CR1 is present on B cells from the spleen as well as from lymph nodes and peripheral blood (Kinoshita *et al.*, 1988). The weak PCR product suggests that CR1 is present in very low copy number in cattle and it is tempting to speculate that CR1 might be expressed exclusively on FDCs. FDCs in the germinal centers of cattle identified with an FDC specific monoclonal antibody, also appear to label with mAb specific for bovine CD21 (clone CC21; Sopp, 1996). CC21 immunoprecipitates two bands (Sopp *et al.*, 1996), one similar in size to CR2 and a larger band similar in size to CR1. The presence of CR1 on FDCs in cattle may facilitate antigen retention of immune complexes containing iC3b as is observed in mice. Phan and colleagues (2007) used a monoclonal antibody specific for murine CR1 (that did not bind CR2) for confocal microscopy. Their figures indicate CR1 expression on FDCs within B cell follicles in lymph nodes.

CR1 was not detected in blood mononuclear cells. Using cells sorted by means of magnetic beads, transcripts for CR2 were detected in B cells but not in T cells, monocytes or neutrophils. This is consistent with what has been observed in humans and mice, however flow cytometric studies of CD21 expression in cattle have shown CD21 expression on non-B cell populations of young calves (Chattha *et al.*, 2010a; Chattha *et al.*, 2010b).

Neither CR1 nor CR2 RNA was found in monocyte-derived macrophages. Similarly neither protein was found in murine macrophages derived from cell lines, bone marrow, or the peritoneum (Martin & Weis, 1993) nor was CR1 present on

murine subcapsular sinus macrophages (Phan *et al.*, 2009). However, in cattle, since the CC21 mAb was derived by reaction with bovine alveolar macrophages, CR2 must be expressed on those macrophages (Naessens *et al.*, 1990).

Bovine CD19

A productive coding region for CD19 was discovered. The size of the expected gene product and its expression by B cells is similar to that observed in mice and humans and has similar features. While the results presented here do not formally preclude the presence of a CD19 pseudogene in cattle due to gene duplication, the presence of a coding copy of CD19 suggests that what has been learned in mice and humans is potentially applicable to cattle. Given that there is a gap in the Btau_4.0 *Cd19* genomic sequence it is likely that the prediction that CD19 is a pseudogene is incorrect.

Conclusion

This work has demonstrated that cattle express CR1 and CR2 from the *Cr2* gene as has been observed in mice and that cattle express both long and short forms of *Cr2* gene products as has been observed in humans. As a result there is a long form of CR1 in cattle that is not present in either mice or humans. This suggests that cattle might express a more phylogenetically ancient form of the *Cr2* gene than either mice or humans as it has not lost the ability to encode CR1 or the ability to encode long and short sub-isoforms. The tissue distribution of CR1 and CR2 in cattle is different from that observed in mice suggesting that there are differences in

Cr2 gene regulation between these species. It has also been demonstrated that cattle express a functional CD19 as is seen in other species and that the prior assumption that CD19 was a pseudogene in cattle was due to an error in gene prediction caused by a sequencing gap in the *Cd19* locus of the latest build of the bovine genome.

Given the possibility that long or short versions of CR2 might be involved in ectodomain shedding, it would be interesting to assess if a soluble version of CR2 or CR1 can be found in bovine plasma or in the supernatant of cultured bovine B cells or splenocytes. The sequences of both the CR1 gene and CD19 are important first steps in generation of monoclonal antibodies to these proteins. An anti-CR1 monoclonal antibody would be useful in confirming the suspicion that CR1 is expressed on splenic FDCs.

In cattle B cells are often sorted using anti-IgM or anti CD21 antibodies. An anti-CD19 monoclonal antibody would be useful for sorting or labeling B cells while including cells that have class-switched. As CD19 is the earliest expressed surface antigen on B cells in mice and humans an anti-CD19 antibody would a useful tool to assess B cell lymphopoiesis.

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