Characterising the Role of CR1 and CR2 in a Humanised Mouse Model

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Summary

The complement cascade is being increasingly implicated in development and disease. To understand these various roles, mouse models have been used and are proving to be an excellent tool. While they have helped to elucidate many roles of central cascade components, they do not adequately model complement regulators. Complement Receptors 1 (CR1) and 2 (CR2) have been implicated in modifying disease states, such as Alzheimer's disease and Systemic Lupus Erythematosus, but they are not well replicated in mice. This leaves a gap in knowledge about how these receptors are functioning. To overcome this, a mouse model was engineered to replace endogenous murine Cr2 with the human complement receptors, CR1 and CR2. This model will be an asset to the complement research community, but there is need for characterizing the expression of CR1. This project aims to establish and validate this model. CR1 has an array of allotypes in human populations, and using traditional recombination methods (FLP-FRT and Cre recombination) two of the most common alleles are replicated within this mouse, along with creating a CR1 knockout allele. To validate the model a variety of techniques were used to ensure the correct targeting of the genomic construct into the murine Cr2 locus. Once integration was confirmed, the allelic series was established. Expression patterns in blood-derived cells were probed at an RNA level, with the different isoforms of CR1 being identified at a protein level. These models accurately produce viable protein products. These findings ensure that an accurate mouse model will be available to the complement research community.

Abbreviations

AD – Alzheimer's disease

Aβ – Amyloid beta

Bb – Factor B, fragment B

B6 – C57BL/6J

$B6^{Tyr}$ - B6(Cg)-*Tyr*$^{c-2J}$/J

β-ME – β-mercaptoethanol

B220 - Protein Tyrosine Phosphatase, Receptor Type C

C1 – Complement Component 1

C1INH – C1 Inhibitor

C1q – Complement Component 1, subunit q

C1r – Complement Component 1, subunit r

C1s – Complement Component 1, subunit s

C2 – Complement Component 2

C2a – Complement Component 2, fragment a

C2b – Complement Component 2, fragment b

C3 – Complement Component 3

C3a – Complement Component 3, fragment a

C3b – Complement Component 3, fragment b

iC3b – Inactivate Complement Component 3, fragment b

C3dg – Complement Component 3, fragment d,g

C3d - Complement Component 3, fragment d

C4 – Complement Component 4

C4a – Complement Component 4, fragment a

C4b – Complement Component 4, fragment b

C5 – Complement Component 5

C5a – Complement Component 5, fragment a

C5b – Complement Component 5, fragment b

C6 – Complement Component 6

C7 – Complement Component 7

C8 – Complement Component 8

C9 – Complement Component 9

CCP – Complement Control Protein

CD3 – Cluster of Differentiation 3

CD11b – Cluster of Differentiation 11b

CD19 – Cluster of Differentiation 19

CD21 – Cluster of Differentiation 21

CD23 – Cluster of Differentiation 23

CD35 – Cluster of Differentiation 35

CD41 – Cluster of Differentiation 41

CD45.2 – Cluster of Differentiation 45.2

CD81 – Cluster of Differentiation 81

CR – Complement Receptor

CR1 – Complement Receptor 1

sCR1 – Soluble Complement Receptor 1

uCR1 – Urinary Complement Receptor 1

CR2 – Complement Receptor 2

Cr1l – Complement receptor like 1

Crry - Complement receptor 1-related gene/protein-y

CVID7 – Immunodeficiency, Common Variable 7

DAF – Decay Accelerating Factor

DC – Detergent Compatible

EBV – Epstein Barr Virus

ES – Embryonic Stem

FACS - Fluorescence Activated Cell Sorting

FDC – Follicular Dendritic Cells

GATA – GATA binding protein

HIR – Human Intergenic Region

HIV – Human Immunodeficiency Virus

HRP – Horseradish peroxidase

JAX – The Jackson Laboratory

KO - Knockout

LAB – Liberate antibody binding

LHR – Long Homologous Repeats

MAC – Membrane Attack Complex

MAP – Mitogen-Activated ProteinMAP-K – MAP Kinase

MASP1 – MBL Associated Protein 1

MASP2 – MBL Associated Protein 2

MBL – Mannose Binding Lectin

MCP - Membrane Cofactor Protein

MHC-II – Major Histocompatibility Complex II

MS – Multiple Sclerosis

PfEMP1 – *Plasmodium falciparum* Erythrocyte Membrane Protein 1

PBS –Phosphate buffered solution

PCR – Polymerase Chain Reaction

PI3 – Phosphoinositide 3

PI3-K – PI3-Kinase

PVDF - Polyvinylidene difluoride

RBC – Red Blood Cell fraction

RCA - Regulators of Complement Activation

RLT – RNA Later

RT – Room temperature

SCR – Short Consensus Repeats

sIgM – Surface Immunoglobulin M

SLE – Systemic Lupus Erythematosus

SNP – Single Nucleotide Polymorphism

TBS – Tris Buffered Solution

WBC – White blood cell and platelet fraction

WT – Wild Type

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Chapter 1

1. Introduction

The innate immune system is the first line of defense for a variety of insults, whether this is an infection or at the site of injury. It is capable of driving regeneration, remodelling and restoration in a variety of different tissues, along with protecting our systems as a whole from pathogen-mediated damage. The complement cascade is one of the oldest components of the innate immune system and therefore, an integral player in the body's defensive and developmental systems (Wyssokowitsch 1866; Nuttall 1888; Ehrlich and Morgenroth 1889; Ehrlich and Morgenroth 1899; Buchner 1889a; Buchner 1889b; Buchner 1899; Buchner 1900; Bordet 1898; Bordet 1896). Central components of the cascade are highly conserved throughout differing species, highlighting its evolutionary importance, with bacteria evolving to produce complement combating components to defend themselves against attack (van den Berg *et al.* 1996).

Complement has been shown to have a major influence in diseases such as glaucoma(Williams *et al.* 2016; Howell *et al.* 2013; Howell *et al.* 2011), Alzheimer's disease (AD)(Shen and Meri 2003; Jun *et al.* 2010; Mahmoudi *et al.* 2015; Crehan *et al.* 2012; Fonseca *et al.* 2016; Lambert *et al.* 2009; Killick, T. R. Hughes, *et al.* 2013; Fonseca *et al.* 2011; Strohmeyer *et al.* 2000; Tooyama *et al.* 2001; Corneveaux *et al.* 2010; Kolev *et al.* 2009; Brouwers *et al.* 2012; Van Cauwenberghe *et al.* 2013; Schjeide *et al.* 2011; Hazrati *et al.* 2012; Shen *et al.* 2001; Killick, T.R. Hughes, *et al.* 2013; Ma *et al.* 2014; Carrasquillo *et al.* 2010; Zhou *et al.* 2008; Hamilton *et al.* 2012; MM *et al.* 2010; Biffi 2012; Maier *et al.* 2008b; Chibnik *et al.* 2011; Keenan *et al.* 2012; Britschgi *et al.* 2012; Maier *et al.* 2008a), Schizophrenia (Arakelyan *et al.* 2011; Sekar *et al.* 2016), Systemic Lupus Erythematosus (SLE) (Iida *et al.* 1982; Dykman *et al.* 1984; Ross *et al.* 1985; Richardson *et al.* 1990; Corvetta *et al.* 1991; Levy *et al.* 1992; Bowness *et al.* 1994; Marquart *et al.* 1995; Moulds *et al.* 1996; Korb and Ahearn 1997; Wu *et al.* 2002; Arora *et al.* 2004; Nath *et al.* 2005; Asokan *et al.* 2006; Wu *et al.* 2007), and Rheumatoid arthritis (Wilson *et al.* 1986; Corvetta *et al.* 1991; Kumar *et al.* 1994; Jones *et al.* 1994; Arora *et al.* 1998; Okroj *et al.* 2007) among many others. While the complement cascade components can influence disease progression, some of the major factors influencing their efficiency in diseased environments are the complement regulators and receptors. These include Complement Receptor 1 (CR1), Complement Receptor 2 (CR2), Decay Accelerating Factor (DAF), and Membrane Cofactor Protein (MCP), that all have important roles in regulating the responses of the complement cascade (reviewed in Merle *et al.* 2015a; Merle *et al.* 2015b).

1.1 The Complement Cascades

The complement cascade has three known branches of activation: The Classical pathway (Ferrata 1907; Brand 1907), the Alternative pathway (Pillemer *et al.* 1954; Pillemer 1955) and the Lectin pathway (Kawasaki *et al.* 1978; Kozutsumi *et al.* 1980) (Fig 1). Each pathway is initiated via different triggers; the Classical pathway is traditionally thought to be initiated via antibodies (though recent studies suggest that they may not be the only instigating factor (Naito *et al.* 2012; Nayak *et al.* 2010; Korb and Ahearn 1997; Bergamaschini *et al.* 1999)), the Alternative pathway generally initiates via the hydrolysis of a single complement protein and finally the Lectin pathway is initiated by the binding of polysaccharides commonly seen on the surface of bacteria. Although each pathway is initiated in different ways, all three pathways converge to form a convertase that allows for the initiation of the terminal pathway (reviewed in Merle *et al.* 2015a). While this is the traditionally held view, studies are emerging to suggest that there are additional roles for the complement cascade proteins, along with a variety of elements that are able to interact at multiple different places within the cascade (Amara *et al.* 2008; Naito *et al.* 2012; Bergamaschini *et al.* 1999).

With these inherent capabilities and the role of complement cascade components ever expanding, from development to disease, there is an urgency to fully understand all of its capabilities. Much of the cascade is being delved into and dissected via the use of animal models. For instance, of relevance to these studies, the use of these animal models has helped to uncover C1q as an integral component of synaptic modelling in development (Stevens *et al.* 2007; Schafer *et al.* 2012), the impact of C1q and C3 on the development and progression of glaucoma (Howell *et al.* 2011; Williams *et al.* 2016), the involvement of a variety of complement components in tissue regeneration and growth (Rutkowski *et al.* 2010; Mastellos and Lambris 2002; Del Rio-Tsonis *et al.* 1998), and that regulation of C3 is essential to embryo survival (Xu *et al.* 2000; Mao *et al.* 2003). Along with this, models have shown that complement can play a hugely influential role in the progression of AD (Hazrati *et al.* 2012; Keenan *et al.* 2012; Maier *et al.* 2008a; Zhou *et al.* 2008). Although these breakthroughs have aided in furthering our understanding of the complement cascade, these models are limited in their genetic ability to mimic human systems accurately.

1.1.1 Classical Pathway:

The activation of the Classical pathway is initiated via the binding of antigens to complement component 1 (C1) (reviewed in Gál *et al.* 2004; Sarma and Ward 2011;

Merle *et al.* 2015a). C1 is made up of a subset of units; C1q, C1r and C1s (C1q6r2s2). Once antigen binding occurs structural changes in C1q, permit the activation of C1r and C1s. Activated C1s in the C1 complex causes the cleavage of 2 proteins, C4 and C2. These proteins are broken down into 'a' and 'b' fragments (C2a, C2b, C4a and C4b). C2b and C4a are released into the fluid phase and are rapidly degraded. C2a and C4b are able to bind to the antigen-presenting cell, whether this be self or foreign, to form the C4b2a complex which is considered a C3 convertase. This C3 convertase can now contribute to the continuation of the cascade. C3 is converted into its anaphylatoxin, C3a, and opsonin, C3b. The C3 convertase, C4b2a, is now able to interact with C3b and this interaction in turn is considered to be a C5 convertase. While the C5 convertase is being formed, C3a is signaling an immune response, and attracting a variety of immune cells to the area of activation.

1.1.2 Lectin Pathway:

The Lectin pathway instigates a similar chain reaction to the Classical Pathway but is initiated via the binding of Mannose Binding Lectin (MBL) or Ficolins to polysaccharide residues on the surface of pathogens (reviewed in Gál *et al.* 2004; Sarma and Ward 2011; Merle *et al.* 2015a). Once bound, MBL associated serine proteases (MASP) MASP-1 and MASP-2 are activated. Once activated both MASP-1 and MASP-2 undergo conformational changes and have the capability to cleave C2 and C4 into their respective 'a' and 'b' fragments (Sarma and Ward 2011). These then form the C3 convertase, C4b2a. Along with the ability to cleave C2 and C4, MASP-1 is also capable of cleaving C3 independently (Dahl *et al.* 2016; Rossi *et al.* 2001; Thiel *et al.* 1997).

1.1.3 Alternative pathway:

The Alternative pathway is, at a low level, always active in healthy individuals and constantly surveying for pathogens (reviewed in Sarma and Ward 2011; Merle *et al.* 2015a). This pathway is activated via the spontaneous hydrolysis of C3, causing a cleavage of a thioester bond to form C3(H_2O). This event enables a conformational change of C3, allowing the binding of Factor B. Upon binding the serine protease, Factor D, is able to cleave Factor B into the non-catalytic Ba and catalytic Bb. Bb remains integrated with C3(H_2O) to form a C3(H_2O)Bb complex. This complex is now considered a fluid phase C3 convertase and is able to instigate the cleavage of C3 into C3b and C3a. The C3(H_2O)Bb complex is relatively unstable, but binding of the serum protein properdin stabilizes the complex and continues to cleave additional C3. An

additional C3b will then bind to the C3(H_2O)Bb complex creating C3(H_2O)BbC3b, a C5 convertase.

1.1.4 The Terminal pathway:

Once each pathway has converged upon their individual C5 convertase, the terminal pathway can be initiated (Tedesco *et al.* 2004; Sarma and Ward 2011; Merle *et al.* 2015a). The presence of these convertases initiates the conversion of C5 into C5a and C5b. C5a is an anaphylatoxin while C5b is recruited to form the membrane attack complex (MAC). This complex is established with C5b, C6, C7, C8 and C9 coming together into a ring like structure. This structure is able to embed into the membrane of the targeted cell, creating a pore and enabling cell lysis.

1.2 Complement components within the cascade:

Along with their roles of being initiators and integral members of the complement cascade itself, recent studies have suggested complement-independent roles of cascade components. These roles are considered to be 'complement-independent' as they do not follow the traditionally assumed cascades. The three main contenders in the complement cascade are C1, C3 and C5. Each play an integral role within the various pathways, but both can be activated via alternative means.

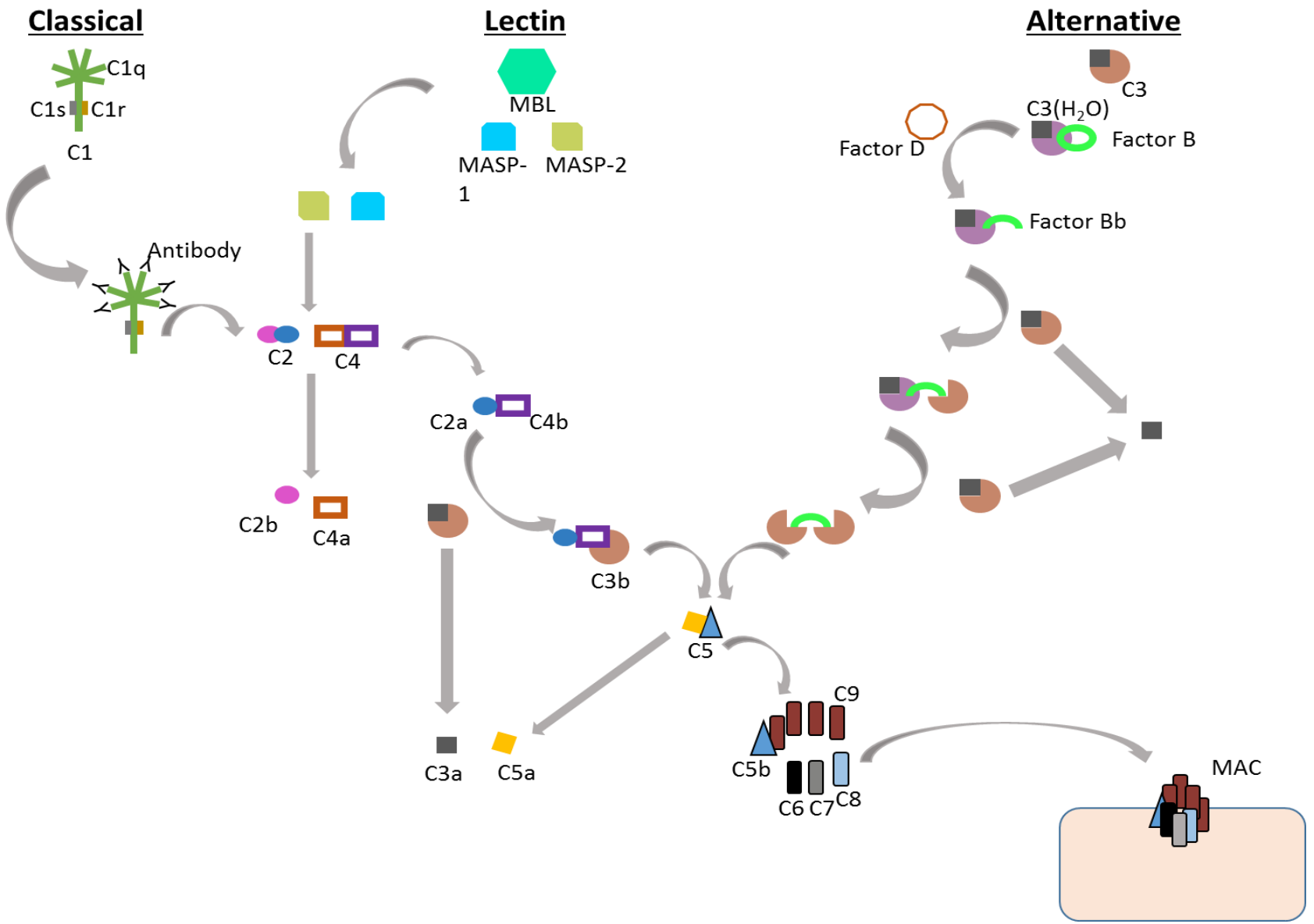

Classical
Lectin
Alternative
C1q
C1s
C1r
C1
MBL
MASP-1
MASP-2
C3
C3(H2O)
Factor D
Factor B
Factor Bb
Antibody
C2
C4
C2a
C4b
C2b
C4a
C3b
C5
C3a
C5a
C9
C5b
C6
C7
C8
MAC

Figure 1. The Three Complement Cascades *The complement cascade is comprised of multiple factors, traditionally defined by three major pathways. The classical pathway is generally activated by antibodies, and cleaves C4 and C2 to make a C3 convertase. This convertase is able to cleave C3, releasing an anaphylatoxin (C3a) and an opsonin (C3b). C3b interacts with the C3 convertase to form a C5 convertase. This convertase is then able to cleave C5 into C5a, an anaphylatoxin, and C5b, which recruits C6-C9 to form the membrane attack complex (MAC). The MAC creates a lytic pore in the membrane of a target cell, eventually leading to cell death. The Lectin pathway follows the same cascade as the classical, but is activated by the binding of MBL to cell surface polysaccharides, found on invading pathogens. The activation of the MBL causes conformational changes within MASP-1 and MASP-2, leading to the cleavage of C4 and C2, instigating the remainder of the pathway. The alternative pathway is activated by spontaneous hydrolisis of C3, which enables the binding of Factor B. Factor D is then able to cleave Factor B into its two fragments, Bb and Ba. Bb remains bound to C3(H_2O) and this is considered a C3 convertase. This C3 convertase is then able to cleave C3 into C3a and C3b. C3b is able to bind to this complex creating a C5 convertase and continuing to the terminal pathway, forming the MAC (as previously described).*

Reviewed in: Gál *et al.* 2004; Tedesco *et al.* 2004; Sarma and Ward 2011; Merle *et al.* 2015a

1.2.1 C1

The C1 complex is the initiating factor of the classical pathway and plays a pivotal role in the recognition of immune complexes. It is composed of six C1q molecules, two C1r molecules and two C1s molecules. The C1q molecule itself is built from 18 polypeptide chains (6A, 6B, 6C) (Kishore and Reid 2000). The C1r and C1s molecules interact to form a Ca^{2+} dependent complex (C1r2-C1s2) that in turn binds to the collagen domains of C1q. Once activated, via binding of IgG or IgM, conformational changes occur within the collagen region of C1q, this in turn activates C1r, which then activates C1s. This, now active, C1 complex is able to cleave C4 and C2, via C1s, into their fluid phase (C2b and C4a) and surface-bound (C2a and C4b) fragments, to form the C3 convertase. Ultimately, the C1 inhibitor (C1INH) binds to the C1 complex and dissociates the C1r2-C1s2 component, leaving the C1q molecule able to interact with cell surface receptors while remaining bound to an immune complex.

Although C1 is traditionally assumed to be activated by antibodies, in more recent years C1q has been show to also be activated through a variety of different ligands. These include the binding of soluble oligomers such as amyloid beta (Aβ) (Bergamaschini *et al.* 1999) and prion protein (Blanquet-Grossard *et al.* 2005); direct binding to apoptotic cells (Korb and Ahearn 1997); and cardiolipin (Rossen *et al.* 1994). Along with this, C1q has been demonstrated to activate canonical Wnt signaling (Naito *et al.* 2012) thus promoting age related phenotypes, as well as being implicated in the polarization of macrophages (Benoit *et al.* 2012).

1.2.2 C3

C3 is the most abundant complement protein (Rodriguez *et al.* 2015) and is an integral component of all three complement cascade pathways. Upon cascade activation, via C3 convertases or through spontaneous hydrolysis, C3 is cleaved into an anaphylatoxin (C3a) and an opsonin (C3b). C3a is released into the fluid phase surrounding the cell/target and mediates a potent inflammatory response, while C3b binds to glycoproteins across the surface of the targeted object. C3b in a C5 convertase will also bind C5, enabling C2a in the convertase to cleave C5. The production of C3b also creates a positive feedback loop, amplifying its own production through auto-activation. In the presence of properdin, the C3 convertase is stabilized and protected from cleavage by Factor I (Fearon and Austen 1975; Medicus *et al.* 1976; Kemper *et al.* 2010). C3b readily opsonizes target cell surfaces making them more attractive to phagocytes and giving additional binding site to allow for phagocytosis.

C3b and its breakdown products iC3b, C3dg, and C3d are important targets for complement receptors. Although C3b breakdown products can maintain a bond to a substrate they are unable to function within the C3 or C5 convertases (Jacobson and Weis 2008). While C3b plays a major role in continuing the complement cascade, C3a is creating a powerful inflammatory response through its anaphylactic activity. A recent review (Coulthard and Woodruff 2015) looked at the variety of roles C3a plays in the inflammatory response and found that it is a complex interaction which can be swayed by acute and chronic phase inflammation. In an acute setting, C3a is seen as an anti-inflammatory, preventing the mobilization of neutrophils that limits their accumulation within tissues, reducing the inflammatory response (Wu *et al.* 2013). Whereas in a chronic state of inflammation, C3a is deemed to be pro-inflammatory with signaling increasing cytokine release, increasing production of inflammatory mediators in monocytes/macrophages and modulating T-cell response (Lim *et al.* 2012; Strainic *et al.* 2013). The culmination of these findings is that C3a should be considered more of an "inflammatory modulator" rather than pro or anti-inflammatory. While C3a is readily released, it is rapidly cleaved to $C3a^{des\text{-}Arg}$, leaving it with less than 10% of its original biological activity (Sarma and Ward 2011). $C3a^{des\text{-}Arg}$ has not been seen to interact with the C3a Receptor, but studies have observed interactions with the C5a Receptor, C5aR2 (Kalant *et al.* 2003; Kalant *et al.* 2005; Chen *et al.* 2007), and this interaction is thought to contribute to the induction of triglyceride synthesis in adipocytes (Kalant *et al.* 2005).

While C3 is considered a lynch pin within the cascade, it also plays a complex role in development and disease. C3 deficient mice show protection against hippocampal decline associated with aging (Shi *et al.* 2015), while mouse models of AD develop an increased burden of Aβ plaques (Maier *et al.* 2008b). The increasing complexity of C3's influential function deems it important, and necessary, to fully understand the various associated regulators.

1.2.3 C5

C5 plays an important role for instigating the composition of the MAC. Once cleaved by its respective convertase its two derivatives, C5a and C5b, are released (Shin *et al.* 1968). C5a is considered to be a potent anaphylatoxin with powerful chemotactic abilities, more so than C3a. It has been shown to increase vascular permeability, influence cytokine and chemokine release, along with phagocytosis (reviewed in Guo and Ward 2005) and has also been shown to influence macrophage polarization (Ruan *et al.* 2015). C5b is the initiating factor for the construction the MAC, and is able to attract C6, C7, C8 and C9 molecules. These components come together to form a ring

like, porous structure (Podack *et al.* 1982; Tschopp *et al.* 1985). This configuration is then able to infiltrate the plasma membrane of cells, forming a lytic pore, and supports the destruction of the target cell. Although the majority of C5 activation occurs through the C5 convertases, there has been evidence to indicate an interaction, and activation, of this terminal component with the coagulation pathway (reviewed in Amara *et al.* 2008). One component of this pathway demonstrated to be able to locally activate C5a is Thrombin (Huber-Lang *et al.* 2006).

Along with being the final stage of all 3 complement pathways, C5 has been demonstrated to play a multitude of roles, including reassigning oligodendrocytes into a more precursor state (Rus *et al.* 1997), as well as being able to inhibit Caspase-3 activity (Mukherjee and Pasinetti 2001). While being incorporated into the membrane attack complex, a low copy number of C9 can create a functional pore rather than a rigid lysing one (Bhakdi and Tranum-Jensen 1991).

1.3 Regulating the complement cascade

While complement is a necessary tool for tackling infection and local tissue damage, if left unchecked it can be detrimental to the host. This requirement for regulation is provided by a group of regulatory proteins, which work by either competitively inhibiting, or degrading components of the C3 and C5 convertases (Fig 2, Table 1). Most of these regulators are located in a region defined as the Regulators of Complement Activation (RCA) locus, found on human Chromosome 1 at locus 1q32 (Fig 3) (Weis *et al.* 1987; Rodriguez de Cordoba *et al.* 1985; Rodriguez de Cordoba and Rubinstein 1986; Lublin *et al.* 1987; Lublin *et al.* 1988; Hourcade *et al.* 1992). The RCA is highly conserved throughout many organisms, and contains the majority of the genes currently understood to regulate the complement cascade. Each gene within the RCA is generally made up a varying number of short consensus repeats (SCRs, also known as complement control protein (CCP) repeat sequence), 60 amino acids in length (Hourcade *et al.* 1992). The presence of these SCRs is important as their main targets are the various convertases within the complement cascade. These regulatory proteins work in tandem to regulate the complement system, thus directing an immune response to invading pathogens, to clear potentially damaging debris, all while preventing aberrant damage to host tissue.

Table 1 – Regulators and Receptors of Complement

Regulators/Receptors of Complement	Alternative Names	Genomic Location	Target	SCRs
CR1	CD35, C3BR, C4BR	1q32	C3b, C4b, C1q, MBL	30 (varies depending on allotype)
CR2	CD21, C3DR	1q32	iC3b, C3d	15-16
DAF	CD55	1q32	C4b, C3b,	4
MCP	CD46	1q32	C3b, C4b	4
CD59	N/A	11p13	C5b-9, C8, C9	N/A
Factor H	CFH, FH, HF	1q32	C3b	20
Factor I	CFI, FI, IF	4q25	C3b, C4b	N/A
C4BP	C4BPA, C4BPB	1q32	C4b	N/A
Clu	APOJ	8p21-p12	C5b-, C7, C8, C9	N/A
CR3	CD11b/CD18, ITGAM/ITGB2	16p11.2/21q22.3	iC3b	N/A
CR4	CD11c/CD18, ITGAX	16p11.2	iC3b	N/A
C3aR1	C3AR	12p13.31	C3a, C4a	N/A
C5aR1	C5AR, C5R1, CD88	19q13.32	C5a	N/A
C1qR	CD93,C1QR1, C1qRP	20p11.21	C1q	N/A
C1 Inhibitor	SERPING1,C1IN, C1NH, C1INH	11q12.1	C1r, C1s, MASP-1, MASP-2	N/A

References: (Ripoche *et al.* 1988; Nicholson-Weller and Wang 1994; Andrews *et al.* 1985; Wong *et al.* 1989; Klickstein *et al.* 1988; Iida *et al.* 1983; Weis *et al.* 1984; Moore *et al.* 1987; Fujisaku *et al.* 1989; Toothaker *et al.* 1989)

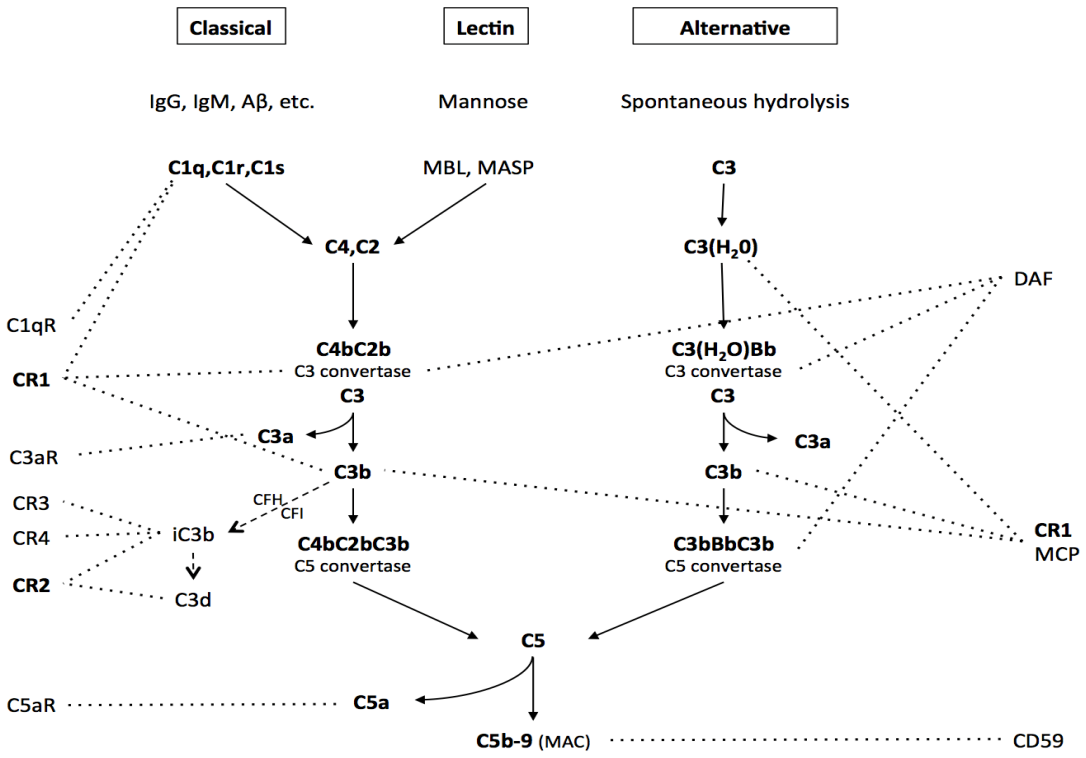


Figure 2. Interactions of Complement Regulators with the Cascades *There are many regulators and receptors put in place to ensure that the complement cascade does not go awry. A lot of these regulators/receptors are targeted towards the central component, C3, or the byproducts of its breakdown. CR1 (in bold) is integral to regulating C3, along with CR2 (in bold) being a receptor instigating immune cell response.*

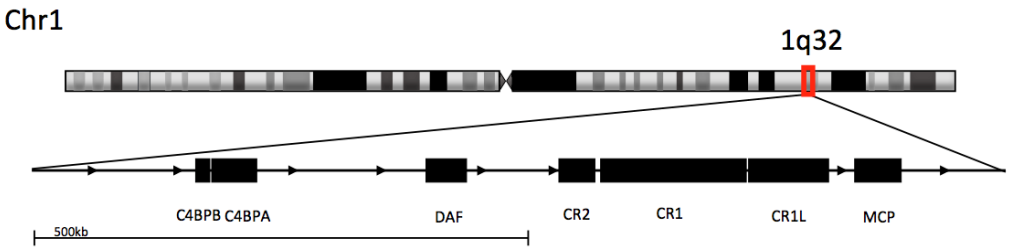


Figure 3. Regulators of Complement Activation *The regulators of complement activation (RCA) are an evolutionarily conserved family of genes. They are found on Chromosome 1 at 1q32. The genes that make up the RCA mostly contain short consensus repeats (SCR, also known as complement control protein repeats). Genes within this region tend to have C3 as a target.*

1.3.1 CR1

Complement Receptor 1 is a negative regulator of complement, and a known receptor for C3b, C4b, C1q and MBL proteins. CR1 works as a cofactor enabling Factor I to cleave and hence inactivate C4b and C3b (Zhang *et al.* 2013). CR1 is a type 1 transmembrane glycoprotein that is found on a multitude of immune cells and is considered an integral receptor for C3b and C4b (Iida *et al.* 1982). There are 4 known allotypes of CR1 in human populations, CR1-F (CR1-A) 220kDa in size, CR1-S (CR1-B) 250kDa in size, CR1-F' (CR1-C) 190kDa in size and CR1-D 280kDa in size (Dykman *et al.* 1983a; Dykman *et al.* 1983b; Dykman *et al.* 1984; Dykman *et al.* 1985; Van Dyne *et al.* 1987). Allotypes A and B are the most common in the human population (Fig 4) with a prevalence of A=0.87 and B=0.11 in Caucasians, A=0.82 and B=0.11 in African Americans, A=0.89 and B=0.11 in Mexicans (Moulds *et al.* 1996), and A=0.916 and B=0.084 in Asian Indians (Katyal *et al.* 2003). The differences between the 4 allotypes of CR1 is a result of the number of long homologous repeats (LHRs; each comprising a set of 7 SCRs) they consist of; this variation is thought to be due to insertion-deletion events that occurred via unequal crossing over (Holers *et al.* 1987). The unequal crossing over events have led to a variation of 1.3-1.5kb between each allotype, with this difference being equivalent to one LHR (Holers *et al.* 1987; Wong *et al.* 1989). Environmental or genetic stressors are thought to have perpetuated these CR1 polymorphisms (Nath *et al.* 2005; Cockburn *et al.* 2004). These LHRs are comprised of 7 SCRs, which tend to be repetitive in nature. The SCRs have an inherent binding affinity which allow for localization and immobilization of various complement components (McLure *et al.* 2004). Each SCR domain is oriented such that it is able to interact with adjacent domains, enabling the capture of the complement component it is targeted to (Lehtinen *et al.* 2004). Multiple SCR domains are required for interactions to occurs, as single SCR domains do not interact or immobilize potential targets (Adams *et al.* 1991; Coyne *et al.* 1992). The composition of the LHRs are repetitive and this is thought to facilitate the interaction of CR1 and its binding targets (Klickstein *et al.* 1987).

The most common allotype of CR1 (CR1-A) consists of 30 SCRs, with the first 28 being organized into 4 LHRs. Each LHR is comprised of 7 SCRs which are homologous across the LHRs (Wong *et al.* 1989). The first three SCRs are conserved in these LHRs and are the domains that contain the binding site for C3b and C4b (Klickstein *et al.* 1988; Wong *et al.* 1989; Merle *et al.* 2015a). Each LHR consists of sites able to bind a variety of molecules; LHR-A is able to interact with C4b and enables accelerated decay of C3 and C5 convertases (Klickstein *et al.* 1988; Krych-Goldberg *et al.* 1999),

while LHR-B and LHR-C are able to bind C3b along with C4b (Klickstein *et al.* 1988) and PfEMP1 (Rowe *et al.* 1997), and finally LHR-D is able to bind both C1q (Klickstein *et al.* 1997) and MBL (Krych-Goldberg and Atkinson 2001; Ghiran *et al.* 2000). LHR-B and C are also able to work together as a cofactor for Factor I, enabling the proteolytic cleavage of C3b and C4b into their inactivated forms; iC3b, C3c, C4c and C4d (Ross and Lambris 1982; Medof and Nussenzweig 1984; Sarma and Ward 2011; Merle, Church, *et al.* 2015; Krych *et al.* 1998; Klickstein *et al.* 1988; Smith *et al.* 2002). The inactive forms of C3b and C4b are now targets for other complement receptors such as CR2. LHR-D is a region generally associated with the various blood groups; Knops, McCoy, York and Swain-Langley/Villien (Moulds 1981; Moulds *et al.* 1991; Daniels *et al.* 1995). The variations within these blood groups are generated via a single nucleotide polymorphism (SNP) in exon 29 – within SCR 25 (of CR1-A) (Moulds *et al.* 2004).

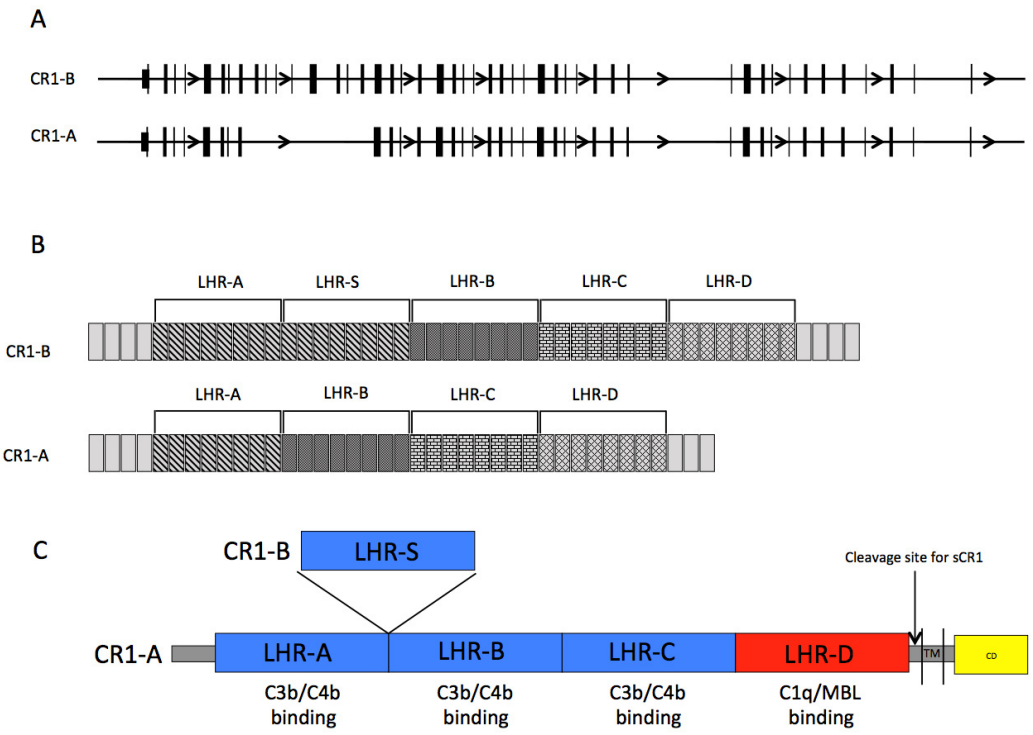


Figure 4. Complement Receptor 1 *(A) Complement Receptor 1 (CR1) has 4 allotypes, with the two most common being CR1-B and CR1-A. These genes contain a varying number of SCR (B) that influence the number of LHR (C) in the protein. CR1-B contains an extra LHR. CR1 is a membrane bound protein, containing extracellular LHR regions, a transmembrane (TM) region and an intracellular domain (CD) that is able to instigate intracellular signaling. The soluble form of CR1 (sCR1) is created via cleavage at the TM region by elastases and metalloproteinases.*

Although the precise expression pattern is still being determined, reports suggest CR1 is commonly, and in varying quantities, expressed on the plasma membrane of multiple blood derived cells (including erythrocytes, eosinophil, monocytes/macrophages, B-lymphocytes, dendritic cells and a sub-set of CD4+ T-cells (Fig 5) (Weiss *et al.* 1989; Rodgaard *et al.* 1995; Rodgaard *et al.* 1991; Fang *et al.* 1998; Pascual *et al.* 1993; Pascual *et al.* 1994; Merle, Church, *et al.* 2015)). In the brain, CR1 may also be expressed on microglia, neurons (Hazrati *et al.* 2012) and astrocytes (Morgan and Gasque 1996; Fonseca *et al.* 2016). Erythrocyte CR1 plays an integral role in the clearance of soluble immune complexes and is a mediator for transporting them to macrophages in the spleen and Kupffer cells in the liver (Cosio *et al.* 1990; Craig *et al.* 2002), allowing for these cells to engulf and phagocytose immune complexes (van Es and Daha 1984; Skogh *et al.* 1985). The levels of CR1 seen on erythrocytes is thought to differ due to a *HindIII* restriction fragment length polymorphism, which corresponds to a SNP in intron 27 of the *CR1* gene (Wilson *et al.* 1987). This variation is thought to affect the stability of mRNA, but the data for this is not clear and is deemed controversial.

Several exonic SNPs have also been suggested to potentially influence the stability of CR1 on erythrocytes, and thus mediate the high and low levels of expression (Xiang *et al.* 1999). While this variation is seen on erythrocytes, leukocyte expression does not seem to show the same variability (Wilson *et al.* 1986). While CR1 has been demonstrated on CD4+ and CD8+ T lymphocytes (Rodgaard *et al.* 1991), the functional significance has yet to be elucidated, but an increased level of expression has been seen in activated T-cells (Rodgaard *et al.* 1995). CR1 is also able to participate in T-cell regulation/activation by inhibiting proliferation if crosslinked (Lipp *et al.* 2014). CR1, activated by iC3b, on T-cells reduces their rate of proliferation and secretion of IL-2 (Wagner *et al.* 2006). It has also been shown to contribute to the generation of regulatory T-cells, through the co-ligation of MCP (Török *et al.* 2015). The presence of CR1 on B-cells is considered to be a controller of proliferation (Fingeroth *et al.* 1989), as, when bound to one of its ligands, B-cells are unable to instigate proliferation (Józsi *et al.* 2002; Erdei *et al.* 2003). This may be of importance for the prevention of auto-antigen mediated B-cell activation (Khera and Das 2009). While effects of CR1 have been observed on the fates of each of these cells, the precise mechanisms by which CR1 is acting is yet to be determined.

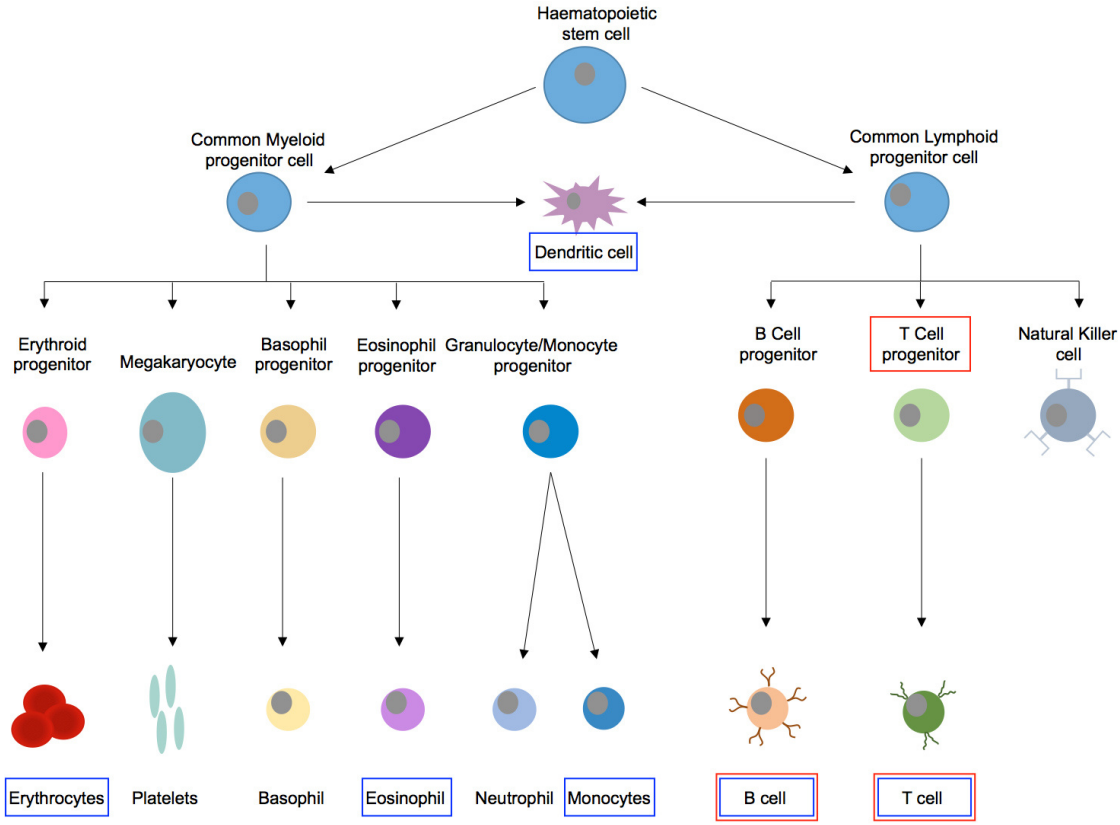


Figure 5. Complement Receptor Expressing Cells Present in the Blood *The various cell types in the blood are derived from haematopoietic stem cells. Each cell comes from a particular lineage with CR1 and CR2 expression differing among these cell types. Blue boxes denote CR1 expressing cells, such as erythrocytes, eosinophil, monocytes/macrophages, B-lymphocytes, dendritic cells and T-cells. Red boxes denote CR2 expressing cells such as T-cell progenitors, B-cells and mature T-cells.*

CR1 has been shown to have phagocytic properties that have been observed on neutrophils and macrophages (Schorlemmer *et al.* 1984), with C3b thought to be important for this process. Once an immune complex, or LPS, is bound to CR1 a phagocytic response is instigated (Griffin and Mullinax 1990). Although the presence of C3b is important for this process if C3b binds to CR1 without an immune complex attached, a phagocytic response is not induced (Griffin and Mullinax 1990). CR1 has also been shown to work synergistically with Fc-gamma receptors to promote the uptake of opsonized immune complexes with these particles being destroyed within the lysosome (Ehlenberger and Nussenzweig 1977). Along with these phagocytic capabilities of CR1, it has also been shown to exert decay accelerating abilities, similar to that of DAF (Noris and Remuzzi 2013).

The non-membrane bound, soluble form of CR1 (sCR1) found in plasma is derived from membrane bound CR1 through proteolytic cleavage at the transmembrane region via elastases and metalloproteinases (Danielsson *et al.* 1994; Hamer *et al.* 1998; Sadallah *et al.* 1999). While plasma levels of sCR1 are considered to be too low for functional significance (Pascual *et al.* 1993), it is suggested to be locally active (Khera and Das 2009) as it is released locally from leukocytes, predominantly polymorphonuclear leukocytes (Danielsson *et al.* 1994). This sCR1 is observed most commonly during potent complement activation (Weisman *et al.* 1990; Mulligan *et al.* 1992; Ramaglia *et al.* 2008). With the ability to be locally active, it enables a more immediate regulation of the complement cascade, allowing time for response cells to reach the site of activation and prevent untoward damage. CR1 is also commonly found in urine (uCR1), coating membrane vesicles released from the glomerular podocytes. The presence of this uCR1 was demonstrated when differences in the allotypes of erythrocyte CR1 and uCR1 of patients who had undergone renal transplant were seen (Pascual *et al.* 1994).

With CR1 playing a multifaceted and influential role on the regulation of the complement cascade, and being intertwined with the regulation of differing cell types, when studying the complement cascade in animal models, it is important to recapitulate the function of CR1 as closely as possible.

1.3.2 CR2

Complement Receptor 2 is a downstream receptor of the complement cascade, as well as being a bridge between the innate and adaptive immune systems. It is a 145kDa multi-functional glycoprotein receptor, primarily binding to the C3 fragments; iC3b, C3dg and C3d (Iida *et al.* 1983; Holers and Kulik 2007). Along with being the receptor for C3 fragments, it is also utilized as a receptor for the gp350/220 viral coat protein of the Epstein-Barr virus (EBV) (Fingeroth *et al.* 1984), CD23 (Aubry *et al.* 1992), Interferon-alpha (Asokan *et al.* 2006; Delcayre *et al.* 1991) and HIV-1 (Herrero *et al.* 2015). CR2 is commonly found on B-cells and follicular dendritic cells (FDC), and it plays an important role of bridging innate and adaptive immunity through engagement with the B-cell receptor (BCR). Once bound to an immune complex, CR2 will associate with CD19 and CD81, creating a B-cell specific signal transduction complex. This association results in enhanced calcium release, proliferation and activation of B-cells (Hannan *et al.* 2002). CR2 expression has also been found on thymocytes and a sub-population of T-lymphocytes (Fig. 5) (Levy *et al.* 1992; Watry *et al.* 1991; June *et al.*

1992). As well as this it has also been linked to intracellular signaling pathways such as the membrane phosphoprotein p53, nucleolin-mediated regulation of PI3-kinase (Barel *et al.* 2001; Barel *et al.* 2003) and antigen internalization and response (Barrault and Knight 2004).

CR2 consists of 15-16 SCR arranged into 4 LHR (Fig 6), containing 3 or 4 SCR each (Weis *et al.* 1984; Moore *et al.* 1987; Fujisaku *et al.* 1989; Toothaker *et al.* 1989), with a 28 amino acid transmembrane region and 34 amino acid cytoplasmic tail. The gene is comprised of 16 exons, with the variance in the number of SCR being down to alternate splicing of a single exon (exon 11). CR2 is able to function as a receptor for the various degradation products of C3b, regardless of whether they are bound to immune complexes or free, with most of this functionality occurring within SCR-1 and SCR-2 (Kalli *et al.* 1991).

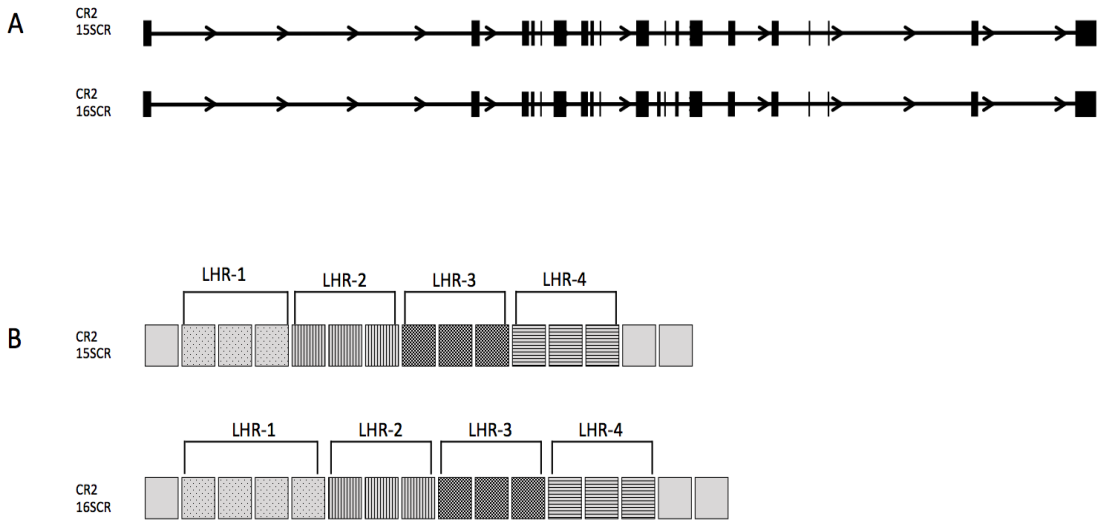


Figure 6. Complement Receptor 2 *(A) Complement Receptor 2 (CR2) has 2 common variants among human populations. The variation occurs in the number of Short Consensus Repeats (SCR) present at a protein level. This variation occurs at an alternate splicing level, with CR2 16 SCR containing an additional exon (exon 11). (B) The SCR form 4 long homologous repeats (LHR) within the protein, each having the capability to bind inactive and degraded fragments of C3b.*

Whilst the role of CR2 appears to be mostly as a receptor for C3b fragments, it was also discovered to be the receptor for EBV. CR2 was shown to bind EBV using MHC-II as a co-receptor, and thus enabling the virus to take advantage of CR2 to infect B-cells. Along with EBV, HIV also utilizes CR2s role on FDC, a receptor for C3b bound immune complexes that is able to retain them for a prolonged period (Qin *et al.* 1998).

HIV also takes advantage of this retention on naïve T-cells (Moir *et al.* 2000), and enables transfer, and thus infection, of HIV incorporated complexes. This ability of HIV to bind to CR2 on T-cells has also been suggested to be reflected in B-cells and potentially FDCs (Moir and Fauci 2009). The integral role CR2 has in the ability for HIV to infect has also been demonstrated through the blocking of this receptor, and therefore inhibiting HIV binding (Kacani *et al.* 2000).

CR2 is also able to make a complex with CR1 on B-cells, which is not fully understood. One theory suggests the role is for both CRs to bind and stabilize iC3 ($C3(H_2O)$) (Leslie *et al.* 2003; Nielsen *et al.* 2001). iC3 has a higher affinity for CR1, but is also able to bind to CR2. Once iC3 is bound to CR2, or the CR1/CR2 complex, it is stabilized and this enables the activation of the alternative pathway (Nielsen *et al.* 2001). The importance of this interaction, along with further potential roles this complex may play is yet to be fully determined.

CR2 has also been shown to have the ability to save peripheral B-cells from sIgM-mediated apoptosis (Kozono *et al.* 1998). The majority of research has focused on the role of membrane bound CR2, but more recent studies have suggested that soluble CR2 (sCR2) also plays various roles. This sCR2 is potentially derived from one of two origins; proteolytic cleavage (Ling *et al.* 1998) or alternate splicing. Although it has not been fully elucidated as to where sCR2 is derived from, it has been noted that there is an increased level in the cerebrospinal fluid of multiple sclerosis patients (Lindblom *et al.* 2016).

The signaling pathways CR2 has been shown to be integral in are with the activation and differentiation of B-cells (Roozendaal and Carroll 2007; Carroll 2008). This role is through the co-receptor complex with CD19 and CD81 (Bohana-Kashtan *et al.* 2004), reducing the threshold for B-cell activation and enhancing intracellular calcium influx (Dempsey *et al.* 1996; Carter *et al.* 1991). This in turn leads to MAP kinase (MAP-K) activation (Tooze *et al.* 1997). Once CR2 is bound to an antigen complex with iC3b, it associates with CD19 and CD81. Phosphorylation is initiated on CD19, which provides a docking site for Vav (O'Rourke *et al.* 1998) and PI3-K (Buhl and Cambier 1999). This interaction leads to the enhanced calcium flux and MAP-K activation. While CD19 activates this signaling at a low level when not associated with CR2 or CD81, when CR2 is present this signal is amplified (Dempsey *et al.* 1996). The binding of iC3b usually instigates this signaling pathway; but lipid rafts are also able to bind the CR2/CD19/CD81 receptor complex and prolong the signaling activity (Cherukuri *et al.* 2016; Pierce 2002). When CR2 is in excess, it can down regulate the calcium influx response (Chakravarty *et al.* 2002). Along with initiating signaling pathways in the

CR2/CD19/CD81 complex, CR2 is also able to signal independently. In addition, CR2 is able to directly trigger the binding of pp60src to nucleolin, initiating nucleolin phosphorylation that activates PI3-K which, in turn, phosphorylates AKT leading to activation of glycogen-synthase kinase activity (Barel *et al.* 2003; Bouillie *et al.* 1999).

Where there is much known about CR2, it is important to understand the full scope of its capabilities. While cell culture studies and animal models have been essential to understanding a wide variety of its function, they have not been able to elucidate the finer details of the inner workings of the interactions between CR2 and CR1.

1.4 Complement in disease

Historically, research has focused on the role of complement components in immune responses, infections and autoimmune diseases. However, more recently, the role of the complement cascade has been expanded to include a variety of additional responses including the remodelling and plasticity of neuronal synapses (Stevens *et al.* 2007; Schafer *et al.* 2012) and the dedifferentiation of cells to a more precursor-like state (Mastellos *et al.* 2013). These different functions of complement work on a finely balanced scale, and if there is any aberrant regulation of these components, then detrimental effects can occur. Most complement-associated diseases are related to defects within this regulation, potentially by modulating the levels and accessibility of different complement factors. The diseases associated with complement dysregulation include Age-Related Macular Degeneration, SLE, Rheumatoid Arthritis, AD, along with many others (Table 2).

1.4.1 CR1 as a risk factor for Alzheimer's disease (AD)

In 2009, a genome-wide association (GWA) study identified CR1 as a potential risk factor for AD (Lambert *et al.* 2009). This association was corroborated in 2010 (Carrasquillo *et al.* 2010; Jun *et al.* 2010; Corneveaux *et al.* 2010), 2012 (Brouwers *et al. 2012; Keenan et al.* 2012; Hazrati *et al.* 2012) and 2013 (Van Cauwenberghe *et al.* 2013). The exact nature of the association of CR1 with AD is not well understood. One study (Hazrati *et al.* 2012) identified the risk of AD is most likely associated with the B allele of CR1, with CR1-A/B allotypes carrying a 1.8x higher risk of disease over the CR1-A/A allele in their cohort. This risk was associated with a faster rate of cognitive decline. This study also identified differences in post-mortem neuronal morphology and distribution between the two carrier states, CR1-A/A and CR1-A/B. Carriers of

CR1-A/A appeared to have a more filiform like structure to neuronal CR1, with expression that associated more readily with the endoplasmic reticulum, whereas the CR1-A/B carriers tended to have a more vesicular like pattern to CR1 expression, associating more highly with lysosomes inside the neurons. Along with this difference in gross distribution, a reduced expression level of CR1-B was seen in comparison to that of CR1-A. Another study (Keenan *et al.* 2012) identified specific *CR1* SNPs (rs6656401 and rs4844609) that can influence the rate of cognitive decline in AD patients in combination with APOE status. These SNPs are associated with the C1q binding region in CR1, with the patients carrying both APOE4 and rs4844609 seeing a faster decline in episodic memory. While the functional implications of these SNPs are yet to be determined, it is suggested that they may be integral in the clearance of Aβ through C1q binding (Hazrati *et al.* 2012). Young adults who carry rs6656401 were seen to have reduced grey matter volume in the entorhinal cortex (Bralten *et al.* 2011), an area associated with atrophy in AD patients (Braak and Braak 1991; Hyman *et al.* 1984). Biffi *et al.* (2010) also saw drastic differences in entorhinal cortex volume of AD and MCI patients depending on their CR1 genotype. Although the expression of CR1 within these patients was seen in neurons, recently the Tenner group was unable to localize any CR1 expression to these cells, instead seeing a correlation with CR1 expression with astrocytes (Fonseca *et al.* 2016).

While variations in CR1 have been associated with AD through GWA and other genetic studies, the mechanisms by which they function is still elusive. With the expression patterns of CR1 varying between different studies, it is important to model these identified risk factor variants. Accurately modelling CR1 will enable a more precise definition to its influence in diseased states, along with allowing for the determination of interactions that result in potential compounding phenotypes.

Table 2 – Various Diseases Associated with Complement Components

Disease	Complement Association	References
Alzheimer's disease (AD)	CR1 has been associated via GWA studies as a potential risk factor. The implication of which is yet to be determined. Studies in mice have shown that a deficiency of C3 leads to a higher deposition of plaques.	Jun *et al.* 2010; Brouwers *et al.* 2012; Lambert *et al.* 2009; Keenan *et al.* 2012; Hazrati *et al.* 2012; Carrasquillo *et al.* 2010; Van Cauwenberghe *et al.* 2013; Corneveaux *et al.* 2010
Systemic Lupus Erythematosus (SLE)	Reduced levels of CR1 and CR2 are seen in patients with SLE. Polymorphisms in C2 have been identified in patients suffering from SLE.	Khera and Das 2009; Ross *et al.* 1985; Richardson *et al.* 1990; Wilson *et al.* 1986; Miyakawa *et al.* 1981; Walport *et al.* 1985; Corvetta *et al.* 1991; Birmingham *et al.* 2006; Holme *et al.* 1986; Moulds *et al.* 1996; Katyal *et al.* 2003; Kumar *et al.* 1994; Wu *et al.* 2007; Marquart *et al.* 1995
Rheumatoid Arthritis	CR1 has varying expression levels depending on cell type in RA patients. Studies have suggested an overall reduction in CR1 levels, additional work identified expression level are reduced among the B-cell and leukocyte populations, with neutrophils and monocytes seeing an increase in CR1.	Jones *et al.* 1994; Kumar *et al.* 1994; Arora *et al.* 1998
Malaria	CR1 is important to the process of rosetting, particularly on erythrocytes. Erythrocytes deficient in CR1 were shown to not perform rosetting behavior when infected with *P. falciparum,* and may play an important role in malarial virulence.	Khera and Das 2009; Birmingham *et al.* 2003; Xiang *et al.* 1999; Thomas *et al.* 2005
CVID	Mutations within the CR2 gene, whether homozygous or compound heterozygous, have been associated with CVID. While commonly this disorder is associated with mutations in CD19, CD81 and CD21, Thiel et al identified a CR2 deficient male in 2012 who had previously been undiagnosed with CVID.	Thiel *et al.* 2012
Schizophrenia	A recent study identified polymorphisms within C4 as a risk factor for schizophrenia.	Sekar *et al.* 2016
Epstein Barr Virus (EBV)	EBV utilizes CR2 via stabilization and internalization of immune complexes, to gain access to cells enabling it to carry out its life cycle.	Fingeroth *et al.* 1984
HIV	HIV hijacks the stabilization and internalization mechanisms of CR2. C3 and C2 both have increased expression levels in astrocytes of infected cultures.	Speth *et al.* 1997; Moir and Fauci 2009; Moir *et al.* 2000
Age Related Macular Degeneration	Variations within C2, C3, Factor I and C9 have been associated with an increased risk.	Seddon *et al.* 2013
Multiple Sclerosis	Increased levels of sCR2 observed in cerebrospinal fluid of patients	Lindblom *et al.* 2016
Bacterial Infections	C2, C6, C7, C8, C9. A deficiency or polymorphism in any of these complement components can leave a carrier at a profound risk to bacterial infections. With terminal components are most commonly susceptible to *Neisseria meningitidis* infections.	Orren *et al.* 2012; Egan *et al.* 1994; Friduss *et al.* 1992; Ross and Densen 1984; Joiner 1988; Nagata *et al.* 1989

1.4.2 Complement Roles in Systemic Lupus Erythematosus (SLE)

SLE is a complex autoimmune disorder that is caused by the production of autoantibodies against a variety of molecules that are not specific to an organ type. Autoantibodies deposit on a wide array of tissues, which induces inflammation and causes injury to these areas (Oishi *et al.* 2008). SLE is one of the most common diseases associated with complement dysregulation (Khera and Das 2009). CR1 levels are reduced in SLE patients, with a marked decline on erythrocytes (Ross *et al.* 1985; Richardson *et al.* 1990; Wilson *et al.* 1986; Miyakawa *et al.* 1981; Walport *et al.* 1985; Corvetta *et al.* 1991; Birmingham *et al.* 2006; Holme *et al.* 1986), along with leukocytes (Fyfe *et al.* 1987) and glomerular podocytes (Arora *et al.* 2004; Raju *et al.* 2001). Various influencing associations have been observed in CR1 and its role in SLE, which include; the *HindIII* polymorphism effecting CR1 levels on erythrocytes (Wilson *et al.* 1987), proteolytic cleavage of CR1 to create a stumped peptide, along with transcriptional and post-translational modifications. While the majority of these factors have withstood interrogation, the *HindIII* polymorphism has seen higher levels of variation in disease effects (Walport *et al.* 1985; Moulds *et al.* 1996; Katyal *et al.* 2003; Kumar *et al.* 1994). Often patients with SLE are seen to have increased deposition of complement C3 along with its degradation products, and this is frequently described as an inverse correlation with the levels of CR1.

Variations in CR2 have also been identified as a risk factor for SLE. Wu *et al.* (2007) identified a SNP within the untranslated region of CR2 that influenced the levels of transcriptional activity. Additional SNPs have been found within introns and exons between the studied populations, which also influence the inherent level of susceptibility (Douglas *et al.* 2009). The most strongly associated SNP increased CR2 expression, potentially promoting the development of autoimmunity and altering B-cell responses. Though Wu *et al* identifies this SNP as influencing an upregulation of CR2 expression, generally the levels of CR2 seen in SLE are decreased on the surface of B-cells (Marquart *et al.* 1995; Wilson *et al.* 1986).

While Complement Receptors 1 and 2 are regularly identified as influential factors in SLE studies, it still remains unclear how they are influencing disease state.

1.4.3 Malaria

Malaria is caused by infection with the *Plasmodium falciparum* parasite, transmitted by the Anopheles mosquito. Merozoites invade the erythrocytes of patients and multiply asexually, infecting the host further and enabling the uptake of the *P. falciparum* by

mosquitos to further their life cycle as well as continuing to infect a wider population. The role CR1 plays in malarial infection is not clear, but it is thought that plasmodium PfEMP1 is able to interact with CR1 specific sites on erythrocytes, leading to rosetting. This rosetting occurs when PfEMP1 binds to uninfected erythrocytes and enables the coupling of an infected erythrocyte. When this bond occurs, multiple erythrocyte rosettes are formed, which have the potential to block small capillaries. Many CR1 polymorphisms have been associated with malaria, with most having a connotation with the level of infection severity in *P. falciparum* malaria, such as the Q981H polymorphism (Birmingham *et al.* 2003; Xiang *et al.* 1999; Thomas *et al.* 2005).

1.4.4 Immunodeficiency, Common Variable 7 (CVID7)

CVID7 is an immunodeficiency disorder associated with mutations in the *CR2* gene. Mutations that occur, whether they are compound heterozygous or homozygous, result in a severe reduction of CR2 expression. Patients who present with CVID tend to have recurrent bacterial infections, starting from childhood, with diagnosis usually occurring in early adulthood. While uncommon, some patients with CVID will also present with an autoimmune disorder such as rheumatoid arthritis, immune thrombocytopenic purpura or autoimmune haemolytic anemia. One case was presented in 2012 (Thiel *et al.* 2012) of a 28-year-old male with CVID7, previously undiagnosed. Thiel *et al* describe the patient as having hypogammaglobulinemia and a severe reduction in $IgD^{-}CD27^{+}$ memory B-cells. Upon closer analysis, cells were devoid of CR2, internally and on the cell membrane, along with a lack of any sCR2 in the serum. While most other cases of CVID present due to mutations in CD19, CD81 or CD20, this is the first presentation of this disorder with a CR2 deficiency.

1.5 Modelling complement in disease

To be able to gain a more comprehensive view of the complement system mouse models are readily used (Howell *et al.* 2011; Zhou *et al.* 2008; Williams *et al.* 2013; Schafer and Stevens 2010; Chu *et al.* 2010; Stevens *et al.* 2007; Killick, T. R. Hughes, *et al.* 2013; Maier *et al.* 2008b; Stephan *et al.* 2013). The ability to use an organism with a comparable innate immune system enables a greater insight into the emerging complement-independent pathways, with complement components not following the traditional cascade route. Modelling this will allow for targeted therapies to be honed, causing minimal damage to unaffected areas. Mouse models have been an excellent tool for unraveling a variety of nuances surrounding complement, such as its role in

synaptic pruning in development (Schafer and Stevens 2010; Stevens *et al.* 2007), the importance of C3 regulators in development (Xu *et al.* 2000; Mao *et al.* 2003) and how a lack of certain complement factors can influence recovery from traumatic brain injury (Neher *et al.* 2014). These discoveries in mouse models have led to a much greater understanding of the inner workings of complement in a variety of scenarios, and have identified potential areas for therapeutics.

1.5.1 Evolutionary Divergence of Human and Mouse Complement Receptors

Elucidating the roles complement play in disease is key to designing therapeutics that target the cascade. Whilst our knowledge of complement is being vastly broadened through the use of mouse models, these models do not adequately recapitulate the scope of human complement receptors. Although the three central pathways of the complement cascade are highly conserved between mammals, the regulators differ (Farries and Atkinson 1988; Nonaka 2001; Jacobson and Weis 2008). It is thought that during evolution, branching occurred between primates and sub-primates leading to a duplication/deletion event (Jacobson and Weis 2008; Holers *et al.* 1992). While there are some similarities between the RCA of humans and mouse, a striking difference is that sub-primates carry no true orthologue of CR1 (Fig 7A). The closest mouse equivalent of the human CR1 gene is created through alternative splicing of the mouse *Cr2* (m*Cr2*) gene, or *Crry* (Paul *et al.* 1989; Kurtz *et al.* 1990). Given that CR2 and CR1 show differing expression patterns, these variations lead to differences in the expression pattern between human CR1 and the CR1-like mouse proteins. These differences greatly limit research into understanding the mechanisms by which CR1 and CR2 impact health and disease.

1.5.1.1 Mouse *Cr2* Produces the Closest Orthologue of Human CR1

Mouse *Cr2* (m*Cr2*) is considered a homolog to human *CR2*, but unlike human *CR2* which produces one protein containing either 15 or 16 SCR, m*Cr2* is alternatively spliced to create two mouse proteins CR2 (CD21, 145KDa) and CR1 (CD35, 190kDa) (Fig 7). This splice site lies directly after the signal sequence in the m*Cr2* gene, with the *Cd35*/*Cr1* (m*Cr1*) splice variant including all 19 exons, whereas *Cd21/Cr2* utilizes only 14. The supplementary exons in m*Cr1* encode for 6 additional SCRs, with binding domains available for C3b. Mouse Cr1 has also been demonstrated to be able to bind both C3b and C4b complexes while mCR2 is only able to bind the C3 fragment C3dg (Jacobson and Weis 2008). Both CR1 and CR2 have restricted expression patterns, only found in FDC and B-cells (Kaya *et al.* 2001; Kurtz *et al.* 1989; Qin *et al.* 1998), although recent reports have described expression of only the CR1 spliceform is

present in FDC (Donius *et al.* 2013). This FDC m*Cr2/Cr1* in mouse is not considered phagocytic but is an anchor for immune complexes enabling the generation of a strong antibody response, and aiding in the maturation of B-cells (Wu *et al.* 2000; Prodeus *et al.* 1998; Fang *et al.* 1998).

Mouse models completely deficient in m*Cr2/Cr1* have shown that m*Cr2/Cr1* is implicated in the prevention of the production of auto-reactive antibodies (Prodeus *et al.* 1998; Chen *et al.* 2000; Wu *et al.* 2002). Mice with restricted deficiencies in FDC and B-cells show depressed T-cell antibody response to low dose immunization, along with a heightened sensitivity to *S. pneumonia* infection (Molina *et al.* 1996; Ahearn *et al.* 1996; Haas *et al.* 2002). These deficient mice also display a heightened inflammatory response state in their spleens, compared to their WT counterparts (Jacobson and Weis 2008). Along with this, mCR2 deficient mice have been used extensively to study the role mCR2/CR2 plays in immune function and modulation, and are regularly used in research concerning autoimmune disease.

While researchers readily use mouse CR2 as a model for human CR2, they also use the alternate splicing of mCR2 to model human CR1. Restriction within the expression of mCR2 poses difficulties in modelling CR1 due to the wider array of expression seen in humans. Along with this restriction in expression, mouse CR1 and CR2 both possess the same C-terminal, giving each the same ability to interact with CD19 and CD81 (Barrington *et al.* 2009; Kalli and Fearon 1994) (along with fragilis/Ifitm proteins (Smith *et al.* 2006)), enabling co-accessory activation of B-cell response (Matsumoto *et al.* 1991; Bradbury *et al.* 1992; Matsumoto *et al.* 1993), an activity lost in human CR1 (Matsumoto *et al.* 1993).

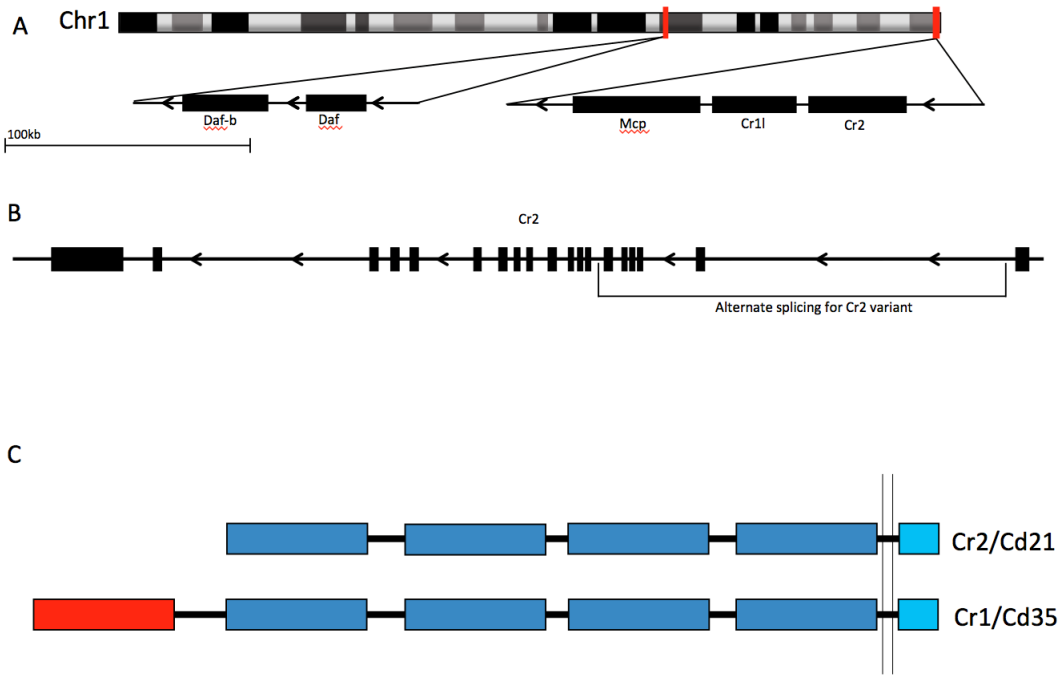


Figure 7. Murine Complement Regulation *(A) The Regulators of Complement Activation (RCA) are located in a similar region of the murine genome (Chromosome 1). The evolutionary divergence seen between human and mouse is with the presence/absence of CR1. (B) The Cr1 isoform seen in mice is derived from alternate splicing from the endogenous Cr2 gene. (C) The protein products from this alternate splicing have differing numbers of LHR, with an additional LHR in the Cr1 variant. This additional region is able to bind C3b, whereas the Cr2 protein is only able to bind iC3b and C3dg. While the binding of C3b and C4b is mostly carried out by Crry.*

1.5.1.2 Crry is Used as an Alternative Orthologue to CR1

Complement receptor 1-related gene/protein-y (Crry, Cr1l) is a rodent specific, 70 kDa, membrane bound regulator of the complement cascade. It is considered to be a CR1 like gene, and is able to inhibit C3b activity, while also mimicking the abilities of both human DAF and MCP (Li *et al.* 1993; Kim *et al.* 1995). CRRY is expressed ubiquitously and is highly important in the regulation of the alternative pathway (Paul *et al.* 1989). This is readily demonstrated by the perinatal lethality of *Crry* KO mice, with maternal C3 deposits seen on the placenta of *Crry* KO embryos giving rise to placental destruction by E10.5 (Xu *et al.* 2000; Mao *et al.* 2003). This lethal phenotype is rescued by the complete C3 or Factor B deficiency in the mothers (Xu *et al.* 2000; Mao *et al.* 2003).

CRRY has been essential in helping model human diseases, with the use of transgenic mouse models aiding in furthering our understanding. The role of CRRY has been studied in mouse models for a variety of diseases including MS (Ramaglia *et al.* 2012a; Davoust *et al.* 1999), AD (Killick, T. R. Hughes, *et al.* 2013; Maier *et al.* 2008a) and uveitis (Manickam *et al.* 2010). While CRRY has been a useful tool for studying gross complement regulation, it does not fully recapitulate CR1 in expression patterns or functionality, and so does not aid in the elucidation of CR1's function.

1.5.2 Current mouse models

The lack of ability to truly understand the nature of murine CR1 leaves a gap in knowledge and hampers the elucidation of its part in disease. While CRRY has been an important stepping stone in our understanding of the role complement modulators contribute to disease (Molina *et al.* 2002; Davoust *et al.* 1999; Ramaglia *et al.* 2012a; Killick, T. R. Hughes, *et al.* 2013; Manickam *et al.* 2010; Maier *et al.* 2008a), its multifunctional role, along with differential expression patterns, muddies the waters, leaving us unable to tease out the finer details of how human complement receptors are facilitating diseased states. It is also important to be able to differentiate the influence CR2 and CR1 (especially the various alleles) have in disease, along with understanding the impact various SNPs have in disease progression or protection. As much as human tissues and cell lines can help us gain a crude understanding, it does not allow for the finer details to be determined in a complete system.

While previous attempts have been made to further understand the roles of CR2 and CR1 in model systems they have not been wholly successful. Cr2 KO mice have helped to elucidate the importance of CR2 in humoral immune response, along with B-

cell maturation and prevention of auto-antibody production (Molina *et al.* 1996; Ahearn *et al.* 1996; Prodeus *et al.* 1998; Chen *et al.* 2000; Haas *et al.* 2002; Wu *et al.* 2002; Donius *et al.* 2014), but with the production of two proteins they do not reflect human complement regulators.

In 2000 Marchbank *et al.* developed a mouse model expressing hCR2 utilizing the P1 phage clone, containing all of the known transcriptional regulatory regions. When combined with a m*Cr2* KO background, the humoral immune function was restored and associations with CD19 were seen. While this is a useful model to understand the role of hCR2 in the B-cell and humoral immune function, especially when used in conjunction with a mCr2 knockout mouse, it has a reduced level of hCR2 expression. In 2002, Marchbank *et al.* developed a second mouse model carrying hCR2. This model utilizes the lambda promoter, giving B-cell specific expression. These mice showed premature expression in B-cell development, indicating that CR2 plays an integral function in the development and maturation of B-cells. Although these mice have been useful in identifying a potential role for CR2 in development, they showed a greatly diminished B-cell population, along with a reduced level of CR2 expression (~25% of the levels seen in humans). While these mice have opened up avenues for understanding the relationship between CR2 and B-cell development and immune function. However, they lack sufficient expression levels making it challenging to understand the roles of human CR2 in health and disease.

Along with mouse models for CR2, researchers have also worked to develop models for CR1. One group (Repik *et al.* 2005) has previously engineered a mouse model to carry the CR1 gene, expressed under the GATA promoter, but the expression pattern was restricted solely to erythrocytes. Although this was a first step to understanding a more comprehensive role of immune complex transportation to the spleen and liver, it has not allowed for the systemic importance of this regulator to be elucidated. Along with this, another group created a mouse model using the F-allele of CR1, under the lambda promoter (Pappworth *et al.* 2012). This mouse showed hCR1 expression restricted to B-cells, with diminished levels of expression. While the levels of CR1 expression were similar to those of the hCR2-int (previously reported in Marchbank *et al.* 2002s) it still does not reflect the levels seen in human populations. These mice were also bred to a mCR2 KO background and hCR1 was shown to not replace the role of CR2 in mice. When combined with hCR2 mice (under the lambda promoter,Marchbank *et al.* 2002), the presence of hCR1 was not able to rescue the effects of premature hCR2 expression. To date, no ‘humanized’ mouse models have

included the entire intergenic region between CR1 and CR2 and this may be a potential reason for the low expression level observed.

Despite, their limitations, humanised CR1 and CR2 models have provided some utility. For instance, CR2 mouse models have played an important role in understanding the importance of expression timing and humoral immune response activity, but they have not been able to replicate expression levels seen throughout the human population. The ability to model this is extremely important with regards to diseases such as SLE, which sees a decline in CR2. The same theme resonates with the models for CR1. CR1 is a widely expressed protein that has not been replicated in mouse models, with their expression patterns being restricted to Erythrocytes or B-cells. While these mice have been important to the understanding of immune complex transportation, they do not reflect what is seen in the general populous. Expression levels in these models are lower than those of a human system, so modelling diseases such as Rheumatoid Arthritis and SLE which see a general decline in CR1 levels.

With this in mind, more comprehensive research models need to be made readily available to the research community. This will allow the role of these complement regulators to be fully understood and potentially allow for more educated, targetable assets for human health and disease.

1.6 Aim of This Study

The aim of this thesis is to characterise a new mouse model for human CR2 and CR1 generated by JAX Genetic Engineering Technologies. This model was created by targeted insertion of a construct contain both hCR2, hCR1 along with their intergenic region. The construct was targeted at the endogenous mCr2, aiming to replace this genomic region in its entirety. With correct genomic placement within the RCA, this potentially enables tighter and more efficient regulation of both genes, and hopefully a more reflective expression pattern of hCR1 utilizing the intergenic region. Characterisation of this mouse model will include developing genotyping, creating an allelic series to mimic the more common alleles of CR1 in the population, determining whether the various protein products are produced and examining various cell types in whole blood for hCR2 and hCR1 expression. The development and characterisation of this mouse will aim to further the understanding of the regulation of the complement cascade, and its importance in development and disease.

Chapter 2

2. Materials and methods

2.1 Mouse husbandry

All mice were maintained on a 12/12 hour light/dark cycle. Mice were housed in 6 inch duplex wean cages with pine shavings and group-housed dependent on sex at wean. All mice were maintained on LabDiet ® 5K67. The Institutional Animal Care and Use Committee (IACUC) at The Jackson Laboratory approved all mice used in this study. Daily monitoring of mice via routine health care checks was carried out to determine the general wellbeing, with any mice considered to be unhealthy being euthanized with IACUC approved CO_2 euthanasia methods.

2.2 Humanising Complement Receptors CR1 and CR2

For the effective study of the role CR1 plays in health and disease JAX Genetic Engineering Technologies set developed a mouse model via vector targeted embryonic stem (ES) cells (Fig 8). A multi-staged approach was planned to create a construct of such a large caliber. *In silico* regions were designed to encompass the human mRNA transcripts of *Cr2* and *Cr1* along with their corresponding human intergenic region (HIR). In parallel to this design a retrieval vector for m*Cr2* was utilized, targeted with a Spectinomycin cassette, producing a vector with the 5' and 3' flanking regions of m*Cr2*. To ensure the integrity of the human genes, they were assembled in a linear manner. The human *Cr2* mini gene was excised from its vector and incorporated with the HIR gap repair vector. This was then targeted to the m*Cr2*/Spectinomycin vector, with successful integration being determined by selection with ampicillin and kanamycin. The *Cr1* mini gene containing vector was then targeted using *Apa1* and *AvrII* restriction enzymes, excising the fragment for integration into the multigene vector. Finally, this multigene vector was targeted with a neomycin cassette at synthetic intron 19 in the human CR2 mini gene. This neomycin cassette aids in the initial identification of successful integration of the construct into an ES cell line. Once the vector was confirmed, C57BL/6J (B6, Jax #664) ES cells were targeted with the multigene vector, with incorporation occurring at the genomic locus for *Cr2*. ES cells were then transferred to a blastocyst from a B6(Cg)-$Tyr^{c\text{-}2J}$/J ($B6^{Tyr}$, Jax #58) and implanted into pseudo-pregnant females. Litters contained a variety of chimeric pups of differing degrees of penetrance.

For this model to become an asset to research communities an initial level of characterization must be achieved. To ensure that this is achieved and that the model will be fruitful for complement research, the various alleles of CR1 will need to be

recreated, along with assessing if expression is present and where the expression might be seen within this mouse model.

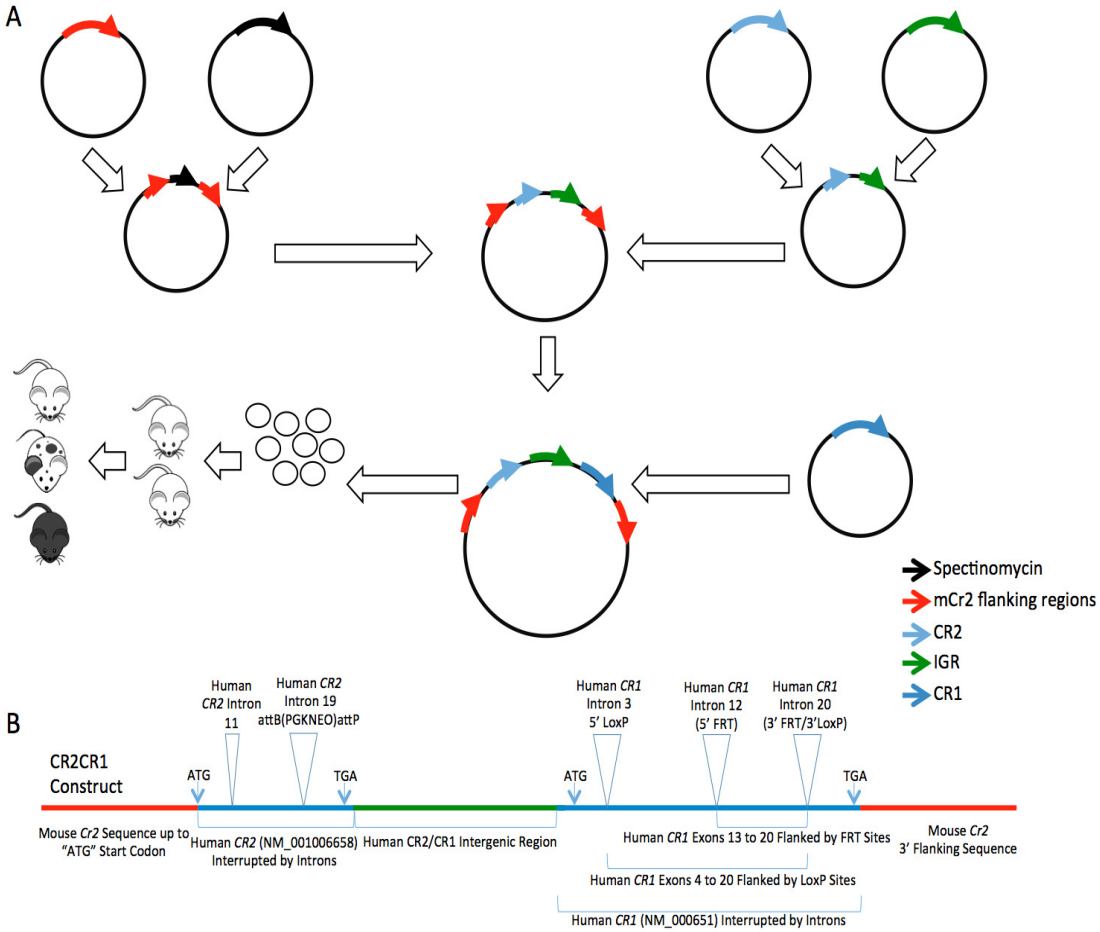


Figure 8. Design and Development of Humanising Complement Receptors 1 and 2 *(A) The development of the insertion vector used a multistage approach. First the Cr2 region was prepared with a spectinomycin cassette, and the CR2 and HIR vector is prepared in parallel. These vectors were integrated together, with the CR1 sequence being integrated into the vector after. Finally, a Neomycin cassette was insert into the synthetic intron 19 of CR2. Once the vector was confirmed to contain the whole sequence in the appropriate orientation, it was incorporated into embryonic stem cells from C57BL/6J mice. These were implanted into B6(Cg)-Tyr$^{c\text{-}2J}$/J blastocysts and introduced into pseudo-pregnant females. Chimeric mice were produced and utilized to create an allelic series. (B) A linear depiction of the final construct containing the Neomycin cassette in CR2 intron 19, LoxP sites in introns 3 and 20 of CR1, and FRT sites in introns 12 and 20 of CR1. The LoxP and FRT sites enable the development of an allelic series.*

2.3 Developing the allelic series

Chimeric mice were received from JAX Genetic Engineering Technologies and were used to create the subsequent mice throughout this study. The initial stage of developing the allelic series was to remove the neomycin cassette embedded within intron 19 of *CR2*. This was carried out using a breeding scheme involving B6.129S4-*Gt(ROSA)26Sor*$^{tm3(phiC31*)Sor}$/J (B6.ROSA-Phi, Jax #7743) mice. This particular strain of mouse targets the attB-attP region surrounding the Neomycin cassette. Removal was confirmed via genotyping targeting Neomycin. Once removal was confirmed a subset of mice were intercrossed to establish the CR2CR1$^{L/L}$ allele. Homozygous mice were intercrossed for a further generation to produce an experimental cohort and colony. The genotype for this colony represents the second most common allele in human populations the CR1-B.

Heterozygous CR2CR1$^{L/WT}$ mice were used to create the CR2CR1$^{S/WT}$ and CR2CR1$^{KO/WT}$ mice. To establish the CR2CR1$^{Short/WT}$ allele, CR2CR1$^{L/WT}$ mice were crossed to B6.129S4-*Gt(ROSA)26Sor*$^{tm1(FLP1)Dym}$/RainJ (B6.ROSA-Flp, Jax #9086) mice. The Flp recombinase is ubiquitously expressed and targets the removal of exons 13-20 via the flanking FRT sites in introns 12 and 20. The removal of these exons produces the shorter, more common, form of CR1 in the population – CR1-A. Mice that were successfully targeted, were used to establish a homozygous colony. These produced CR2CR1$^{S/S}$ mice which were then intercrossed for a further generation to produce a colony and cohort of mice for further characterization.

Finally, the CR2CR1$^{KO/WT}$ mice were created utilizing B6.Cg-*Tg(Sox2-cre)1Amc*/J (*Sox2*-cre Jax #8454). In this strain, Cre recombinase is ubiquitously expressed and excises the targeted region using the LoxP sites, located within introns 3 and 20, to create a null allele (knockout, KO). Female B6.*Sox2*-cre mice were bred to male CR2CR1$^{L/WT}$ mice, as the cre is active without necessarily needing to be inherited. Weanlings were genotyped to ensure the removal of the targeted sequence and were intercrossed to establish a homozygous colony.

2.4 Breeding schemes

Chimeric mice from the two targeted B6 ES cell lines, 5H4 and 5E2, were bred to B6^{Tyr} mice (Fig 9). From these, black pups were pursued for further breeding as they would have the construct integrated due to B6 ES cells being targeted initially. Mice determined positive through genotyping for the construct were then bred to B6 mice to confirm germline transmission by genotyping. After germline transmission was confirmed, male mice from each line were sent for sperm cryopreservation, with the

Neomycin cassette intact. Transgenic mice from the 5H4 line were also bred to B6.ROSA-Phi mice to remove the neomycin cassette. Once the cassette was removed mice were bred to establish the allelic series – [1] mice were intercrossed to establish the long allele ($CR2CR1^{Long}$.B6, $CR2CR1^{L}$), [2] $CR2CR1^{L}$ were crossed to B6.ROSA-Flp mice to create the short allele ($CR2CR1^{Short}$.B6, $CR2CR1^{S}$), and [3] $CR2CR1^{L}$ were crossed to Sox2-cre mice to create the knockout allele ($CR2CR1^{KO}$.B6, $CR2CR1^{KO}$). $CR2CR1^{S}$ mice were then intercrossed to establish the homozygous allele for each strain. $CR2CR1^{KO}$ mice were also intercrossed to produce a homozygote colony. Homozygotes from the original intercrosses were then bred for a further generation to establish cohorts for testing.

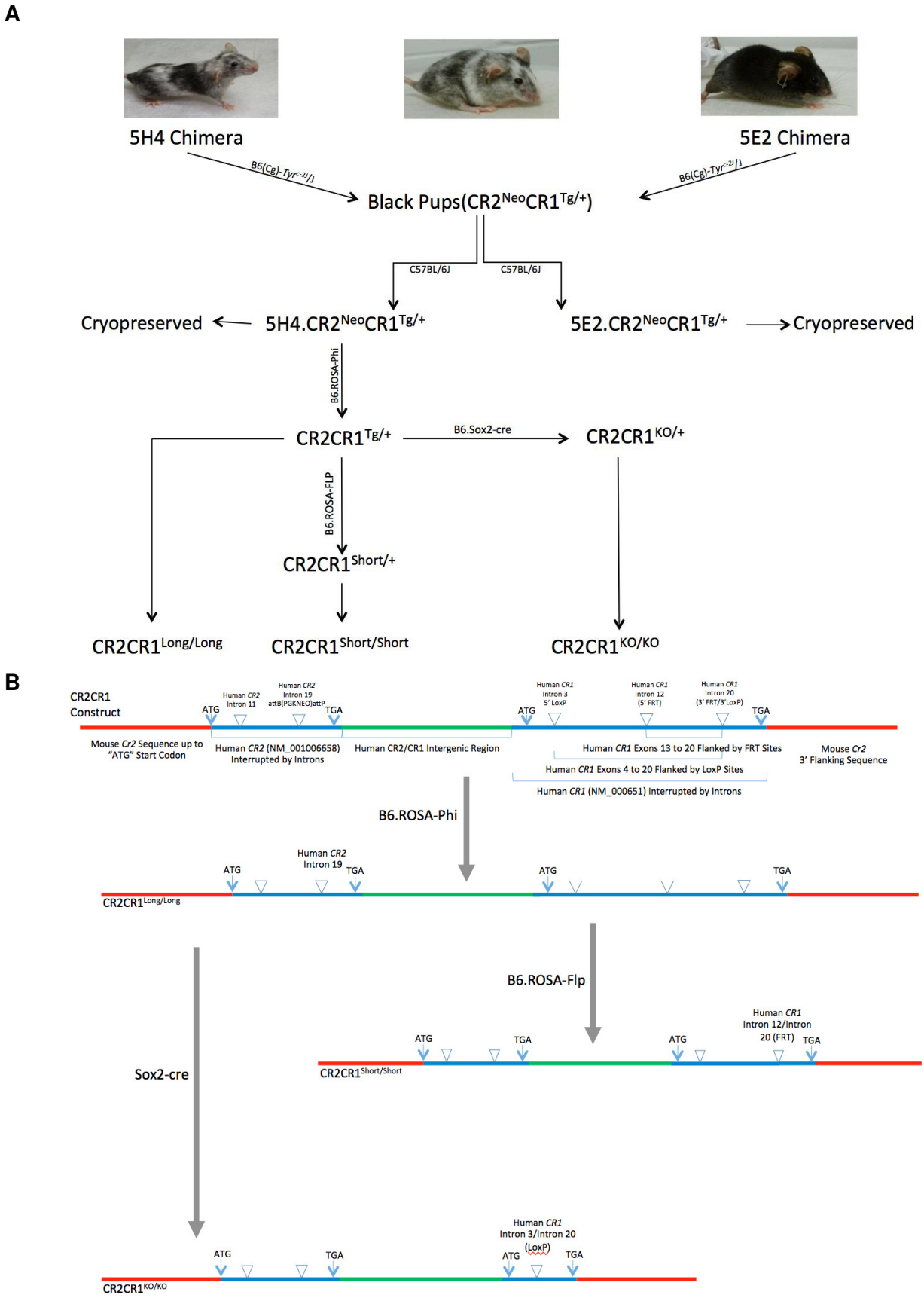

A
5H4 Chimera
5E2 Chimera
B6(Cg)-Tyr^c-2J/J
B6(Cg)-Tyr^c-2J/J
Black Pups(CR2^NeoCR1^Tg/+)
C57BL/6J
C57BL/6J
Cryopreserved
5H4.CR2^NeoCR1^Tg/+
5E2.CR2^NeoCR1^Tg/+
Cryopreserved
B6.ROSA-Phi
CR2CR1^Tg/+
B6.Sox2-cre
CR2CR1^KO/+
B6.ROSA-FLP
CR2CR1^Short/+
CR2CR1^Long/Long
CR2CR1^Short/Short
CR2CR1^KO/KO
B
CR2CR1 Construct
ATG
Human CR2 Intron 11
Human CR2 Intron 19 attB(PGKNEO)attP
TGA
ATG
Human CR1 Intron 3 5' LoxP
Human CR1 Intron 12 (5' FRT)
Human CR1 Intron 20 (3' FRT/3'LoxP)
TGA
Mouse Cr2 Sequence up to "ATG" Start Codon
Human CR2 (NM_001006658) Interrupted by Introns
Human CR2/CR1 Intergenic Region
Human CR1 Exons 13 to 20 Flanked by FRT Sites
Mouse Cr2 3' Flanking Sequence
Human CR1 Exons 4 to 20 Flanked by LoxP Sites
Human CR1 (NM_000651) Interrupted by Introns
B6.ROSA-Phi
Human CR2 Intron 19
ATG
TGA
ATG
TGA
CR2CR1^Long/Long
B6.ROSA-Flp
Sox2-cre
Human CR1 Intron 12/Intron 20 (FRT)
ATG
TGA
ATG
TGA
CR2CR1^Short/Short
Human CR1 Intron 3/Intron 20 (LoxP)
ATG
TGA
ATG
TGA
CR2CR1^KO/KO

Figure 9. Breeding Schemes for the Developing the Allelic Series *(A) The flow diagram represents the generation of the allelic series through the breeding with a variety of site-specific recombination strains. Each allele was eventually bred to be homozygous for the particular CR1 variants. Of the two-targeted ES cell lines; 5H4 was used to continue the development of the allelic series, whereas the 5E2 line was cryopreserved. (B) Mice containing the genomic construct were bred to a variety of strains to induce the allelic series for CR1. The breeding of the original construct to B6.ROSA-Phi mice enabled the removal of the Neo cassette, leaving a "Clean" CR2 and the* $CR1^{L}$ *allele. These mice were bred to either ROSA-FLP mice to yield the* $CR1^{S}$ *allele or the Sox2-cre mice to yield the* $CR1^{KO}$ *allele.*

2.5 Genotyping

Tail tip samples were incubated in lysis buffer (25mM NaOH, 200µM EDTA, pH12) at 95°C for 1 hour (hr) and then neutralized (40mM Tris HCl, pH5). Primers were designed to identify the presence of the specific parts of the CR1/CR2 construct, along with primers for the endogenous *Cr2* and *Crry* genes. These primers were designed using the Primer3 tool within MacVector using the genetic construct and through Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Once primers were identified they were checked against species (*Homo Sapiens* and *Mus Musculus*) with Primer-BLAST to ensure that the identified primer pairs were unique between species and within the genes of interest. All primers were diluted to 20µM. All genotyping assays were established using a temperature gradient to determine effective annealing temperatures.

The primer pairs were as follows:

For *Cr1*: Forward 5' – GTACTACGGGAGGCCATTCT – 3' and Reverse 5' – TGGCTTGGGGTACGCTC – 3' with a product size of 708bp at an annealing temperature of 58.1°C.

For the HIR Forward 5' – TCACTCACCTCGAGCCATCT - 3' and Reverse 5' – TCAGCAGGTCTTGGCTTCAG – 3' with a product size of 291bp at an annealing temperature of 59.3°C.

For the m*Cr2*/*Cr2* insertion site: Common primer forward primer: 5' - TCTTCCTCTCCTTGCTACAGG - 3' C Cr2 Reverse: 5' - AGAAGAGGTGGGGACGTTCT - 3' and CR2 Reverse: 5' - TACCAACAGCAATGGGGGTA - 3' with the m*Cr2* product size at 300bp and the *Cr2* product size at 198bp with an annealing temperature of 60°C.

For *Cr1* knockout (KO): Forward 5'- TCTTGTACTACAGGGCACCG – 3' and Reverse 5' – ACCTCTAGGATTAAACGGTGGGG – 3' with a product size of 150bp if cre recombination has not occurred, with an annealing temp 57.5°C. The absence of a band, with a CR1 positive genotype, indicated the removal of exons 4-20 and the knockout allele is present.

To identify the Short (S) allele, the protocol utilized the Forward primer from the KO allele with the following Reverse primer 5' – CGATCATGGCTCACTGCGAA-3'. A product size of 251bp was to be expected if Flp recombination had not occurred. The

absence of a band, with a CR1 positive genotype, indicated Flp recombination. The annealing temperature for this reaction was 57.8°C.

For *Crry*: Forward 5'- TTGCTAATTGGTAGTGAGGAAAGG -3' and Reverse 5'- TAAGTTGTTGTGAGGCTTGGGT -3' with a product size of 190bp and an annealing temperature of 55.4°C.

All samples and primer pairs were run on the following protocol:

1. 94°C 3minutes (min)
2. 94°C 30seconds (sec)
3. Annealing temp for 30sec
4. 72°C 1min
5. Repeat steps 2-4 39 times
6. 72°C for 5min
7. 10°C forever

2.6 Establishing Cohorts

Once homozygous mice of the various alleles were identified from the first stages of the intercross (CR2CR1$^{L/L}$ at generation N3F1, CR2CR1$^{S/S}$ at generation N4F1, CR2CR1$^{KO/KO}$ at generation N4F1), they were intercrossed for further generations and genotyped to confirm a homozygous population. Mice were genotyped and cohorts of 3 males and 3 females from each genotype in the allelic series were identified. A wild type cohort was established from a separate B6 colony, and aged alongside their transgenic counterparts. At 3 months old, all mice were bled via submandibular bleed and tissue harvested. The exception to these cohorts was with the CR2CR1$^{KO/KO}$ colony due to reduced numbers of homozygotes being produced at N4F1.

2.7 Timed matings and embryonic development of *CR2CR1$^{KO/KO}$* mice

To study the possible partial lethality in *CR2CR1$^{KO/KO}$* mice, timed matings were established and embryos collected at embryonic day 7.5 (E7.5) and 12.5 (E12.5). A total of 5 pregnant females were harvested with a total of 23 E7.5 pups and 14 E12.5 pups. Gross morphology was observed and posterior node and tail samples were taken for genotyping, respectively.

2.8 Isolation of specific blood-derived cells by Fluorescence Activated Cell Sorting (FACS)

For all mice in all cohorts (22 mice in total), 100µl of blood was collected via submandibular bleeding into tubes containing 0.5M EDTA to prevent coagulation (Fig 10). Each sample was diluted 1:1 with 1xPhosphate Buffered Solution (1xPBS) and the phases separated using Ficoll via centrifugation at 400g for 30min. The serum and leukocyte layer were collected into fresh tubes, while 10µl of the erythrocyte and granulocyte fraction were collected into an additional tube. The cells were washed with 3x volume of 1xPBS and centrifuged at 400g for 10min and this wash was repeated once. Cells were re-suspended in 3ml of FACS buffer (1% BSA in 1xPBS) and centrifuged at 400g for a further 6min. Cells were resuspended in 300µl of FACS buffer and incubated with the following antibodies for 30min on ice in the dark; leukocytes and serum samples - CD3e-APC (eBioscience clone: 17A2 #17-0032-82, 1:100), CD45.2-BV421 (BioLegend clone: 104 #109832, 1:100), B220-PECy7 (BioLegend clone: RA3-6B2 #103222, 1:100), CD41-FITC (eBioscience clone: eBioMWReg30 #11-0411-82, 1:240), CD11b-BV605 (BioLegend clone:M1/70 #101237, 1:200); Erythrocytes and granulocytes – CD45.2-BV421 (1:100). After incubation, 2ml of FACS buffer was added and samples were centrifuged for 6min at 400g. The supernatant was removed and cells were re-suspended in 250µl of FACS buffer. Samples were filtered before addition of propidium iodide. Cell sorting was carried out on the FACSAria II Sorter in collaboration with JAX Flow Cytometry Service. The erythrocyte and granulocyte fraction was sorted into CD45.2 negative and CD45.2 positive cells respectively. While the leukocyte fraction was sorted into CD3 positive (T-cells), B220 positive (B-cells), CD11b positive (Macrophages/Monocytes) and CD41 positive (Platelets) cells. Cells were sorted into RNA Later (RLT) buffer with 1% β -mercaptoethanol (β-ME) and stored at -80°C until RNA was extracted. Beads were used as single channel controls and to ensure cell viability, along with background intensities, a subsection of cells were used as unstained controls. For cell counts please see Appendix I.

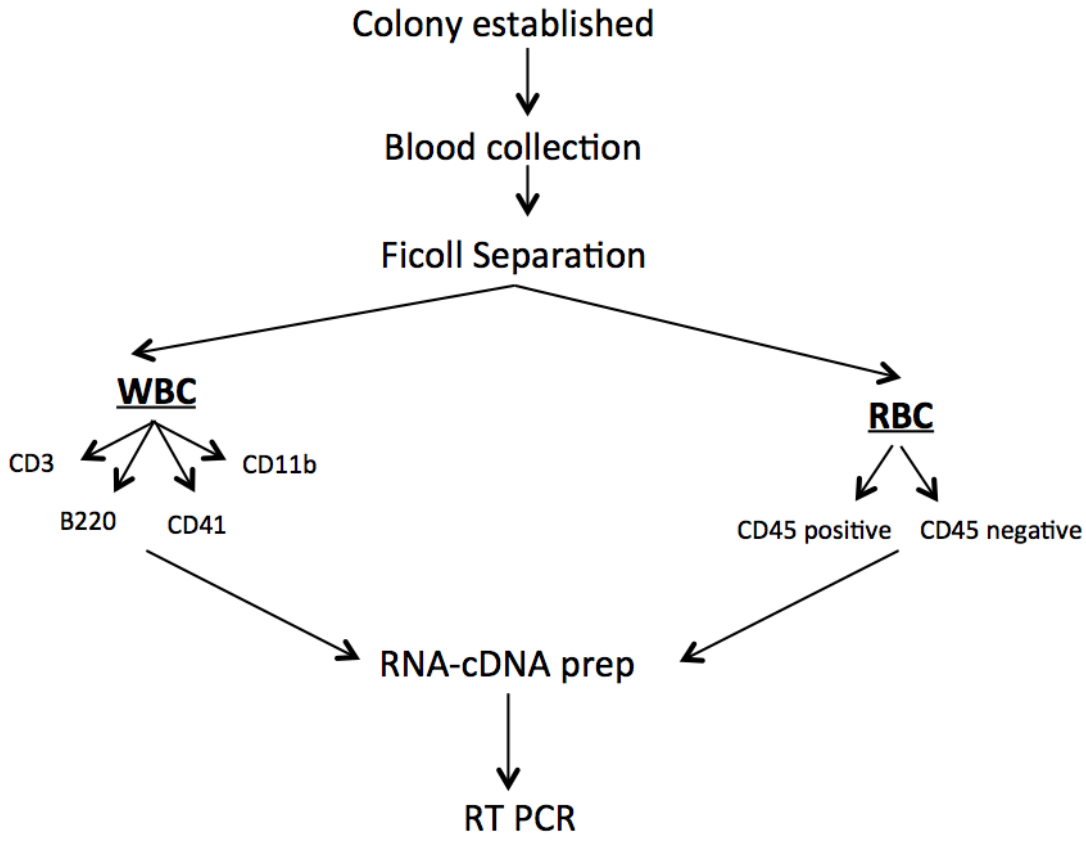


Figure 10. Experimental design for Blood-Derived Cell Sorting *To determine cell specific expression of each of the humanized genes FACS was utilized to crudely separate out a variety of cell types in peripheral blood. The white blood cell and platelet fraction was separated using Ficoll and cells sorted into T-cells, B-cells, Macrophages/Monocytes and Platelets. The red blood cell fraction was separated into Erythrocytes and Granulocytes. cDNA was synthesized from each division. WBC – White blood cell and platelet fraction, RBC - Red blood cell and Granulocyte fraction.*

2.9 Tissue harvesting and preparation

Mice were terminally anaesthetized using a Ketamine/Xylazine (99mg/kg Ketamine, 9mg/kg Xylazine) mix. They were transcardially perfused with 1XPBS. Spleen was harvested and half was cryopreserved in the following manner: submerged in 4%PFA overnight, rinsed in 1xPBS, submerged in a 10% sucrose solution overnight followed by submerging in a 30% sucrose solution overnight. Tissue was blocked in OCT and stored at -80°C until sectioning. Spleen samples were sectioned at 8µm and stored at -80°C for further testing. The other half was snap frozen and stored at -80°C for further use.

2.10 RNA and Protein preparation

RNA and protein were extracted from the snap frozen tissue using Trizol (Thermo Scientific #15596026) according to manufacturer's instructions. Briefly, samples were weighed and submerged into 1ml of Trizol per 0.1mg of tissue. Each sample was homogenized using the OmniTip^{TM} Homogenizer and dissociated for 5min at room temperature (RT). 200µl of chloroform was added per 1ml of Trizol, mixed for 15sec, incubated for 3min at RT and centrifuged for 15min at 12,000g. The aqueous phase was removed and RNA was precipitated out of solution with isopropanol for 10min at RT. Samples were centrifuged at 12,000g for 15min, after which the supernatant was removed and pellets were washed with 75% ethanol. Samples were centrifuged for a final time at 7,500g for 5min. The ethanol wash was removed and the pellets were air dried, and re-suspended in dH_2O (Sigma Aldrich #W3500).

From the remaining non-aqueous phase, DNA was precipitated out using 100% ethanol and centrifuged for 12min at 12,000g. The remaining supernatant was transferred into a clean tube, and protein was precipitated out of solution using isopropanol for 10min at RT. Samples were centrifuged at 12,000g for 15min, and the remaining supernatant removed. 0.3M Guanidine HCl in 95% ethanol was used to wash the protein pellets, incubated for 20min with a 5min centrifugation at 7,500g. This was repeated a further two times. Pellets were given a final wash in 100% ethanol and centrifuged at 7,500g for 5min. A 1:1 solution containing 8M urea and 1% SDS was used as a resuspension buffer for the protein. Samples were left overnight at +4°C before breaking down the pellet via sonication. Each sample was sonicated at least three times at 30sec intervals. Samples were centrifuged for 10min at 10,000g, the final supernatant was transferred to a new tube with protease inhibitor (Thermo Scientific #78440).

All RNA and protein samples were stored at -80°C before use. RNA Concentrations were determined via Nanodrop and protein concentrations via detergent compatible (DC) Protein Assay (Bio-Rad #5000112) respectively.

2.11 RNA extraction for sorted cells and cDNA synthesis

cDNA was synthesized using JAX Genome Technologies. Briefly, buffer RLT (Qiagen) containing 10 µl β-ME per 1 ml buffer was added to the cells. Cells were homogenized by vortexing. Total RNA was isolated using the RNeasy Mini kit (Qiagen) for samples containing >500,000 cells and the RNeasy Micro kit (Qiagen) for samples containing <500,000 cells, according to manufacturer's protocols, including the optional DNase digest step. RNA quality was assessed using the Agilent 2100 Bioanalyzer instrument and RNA Pico LabChip assay (Agilent Technologies). RNA was reverse transcribed with Random Decamers and M-MLV RT using the Message Sensor RT Kit (Invitrogen).

2.12 cDNA synthesis and RT-PCR from RNA extracted from spleen

RNA extracted via Trizol was treated with DNase at 37°C for 30min, the reaction was stopped on ice and 0.5M EDTA was used to deactivate the DNase. Samples were centrifuged and the supernatant was transferred to a new tube. A lithium chloride:Ethanol solution was used to precipitate the RNA overnight at -20°C. Samples were centrifuged at maximum speed for 20min at +4°C, the supernatant removed and remaining pellets were washed with 70% ethanol. RNA was resuspended in dH_2O and concentrations were read using the Nanodrop. 1µg of RNA was used to synthesize cDNA. Briefly, RNA was combined with random primers, dNTPs, RNase inhibitor, Multiscribe Reverse Transcriptase and made up to volume with dH_2O. The reaction was incubated at 25°C for 10min, 37°C for 2hr, 85°C for 5min and +4°C. Samples were diluted 1:4 and concentrations were read again on the Nanodrop to ensure that no degradation had occurred. Samples were stored at -20°C until required.

To assess transcription, primers were designed specifically to transcript sequence for m*Cr2*, *Crry*, *Cr1* and *Cr2*, with particular care taken to ensure that no repeat sequences were captured. This was determined by probing the whole construct sequence with a variety of primers and identifying pairs that were unique to the gene of interest. These primers were then run through the Primer3 program to ensure the primers were unique to species and gene. 100ng of cDNA was used to determine whether expression was present. The primers for each gene are as follows:

For *Cr2* at exon 11: Forward: 5'- TGGGGCAGAAGGACTCCAAT -3' and Reverse: 5'- GCTCCACCATGGTCGTCATA -3' with a product size of 148bp and an annealing temperature of 60°C.

For *Cr1* at exon 2: Forward: 5'- TCCATTTGCCAGGCCTACCA -3' and Reverse: 5'- TGCACCTGTCCTTAGCACCA -3' with a product size of 152bp and an annealing temperature of 60°C.

For *Cr1* covering exon 4 and 5: Forward: 5'- TGGTTCCTCGTCTGCCACAT -3' and Reverse: 5'- AGGATTGCAGCGGTAGGTCA -3' with a product size of 178bp and an annealing temperature 60°C.

For m*Cr2*: Forward: 5'- AATGCAAGAGAACCACTAAACAGAA -3' and Reverse: 5'- GCTTTTCGGTTCTTGTCACC -3' with a product size of 250bp and an annealing temperature of 60°C.

For *Crry*: Forward: 5'- GGAGGAGTCAAGCTAGAAGTTT -3' and Reverse: 5'- GTGTTGCAGCGGTAGGTAAC -3' with a product size of 521bp and an annealing temperature of 55.3°C.

All samples were run on a 2% agarose gel at 130V for 40min and images taken using the GeneSnap for Syngene program.

2.13 Western Blotting

6% gels were hand cast to determine the size difference between the $CR1^{L/L}$ and $CR1^{S/S}$ alleles. Protein was diluted to 80µg of total protein with 2x Laemmli buffer (BioRad). Samples were denatured at 95°C for 5min and loaded onto the gel. Gels were run for 1hr at 150V and transferred to nitrocellulose membrane via the iBlot for 13mins. Blots were incubated at RT for 1hr with blocking solution (5% skimmed milk powder block in 0.1% PBS-Tween). Blots were then washed with 0.1% PBS-Tween for three 15min incubations and then incubated using rabbit-anti CD35 (Abcam #ab126737, 1:100) for 48hrs in 0.1% PBS-Tween on an orbital shaker at +4°C. Blots were washed three times in 0.1% PBS-Tween and incubated with the appropriate secondary (Anti-Rabbit IgG HRP 1: 50,000 Millipore #AP132P) for 1.5hrs at RT. Blots were then washed an additional three times and detection was carried out using ECL detection regents (GE Healthcare RPN2109). Blots were treated with 0.25% sodium azide for 2hrs at RT and washed thoroughly in 0.1% PBS-Tween. These blots were re-blocked and re-probed with mouse anti-CD21 (Abcam #ab54253, 1:100) in 0.1% PBS-Tween overnight at +4°C. Blots were washed and incubated in the appropriate

secondary antibody (Anti-Mouse IgG HRP 1:40,000 Millipore #AP308P) for 2hrs at RT, washed and detected. Finally, blots were treated with 0.25% sodium azide and probed with a loading control, anti-Vinculin (Abcam# ab129002, 1:10,000) in 0.1% PBS-Tween overnight at +4°C, washed three times, incubated with the appropriate secondary antibody (Anti-Rabbit HRP 1:50,000) for 1hr at RT, washed and detected.

In addition to these antibodies, others were also tested to determine the expression of CR1 and CR2 in the spleen. A variety of mouse monoclonal antibodies were tested (Table 3). No signal was detected using the above method, so a variety of different testing paradigms were used. They included using: TBS instead of PBS, as some antibodies can have their binding inhibited using PBS; primary antibody incubation times were adjusted in an attempt to boost binding and increase signal; a variety of blocking buffers along with differing concentrations of block in PBS-Tween and TBS-Tween; and using a PVDF membrane instead of nitrocellulose, as this is deemed to be more sensitive. Although all these variables were tested, none of the mouse monoclonal antibodies showed specific binding to CR1 and CR2 (Appendix II).

Table 3 – Complement Receptor Antibodies used herein

Antibody	Host	Epitope target	Western blot	Immunofluorescence
CR1 ab126737	Rabbit	C-Terminus KGNNAHENPKEVAIHL HSQGG SSVHPRTLQT NEENSRVLP	Reactive with human CR1	Minimal staining – possibly macrophages
CR1 ab25	Mouse	E11 clone, target unknown	No detectable signal	No detectable signal
CR1 3E10	Mouse	Unknown	No detectable signal	No detectable signal
CR1 MB135	Mouse	Unknown	No detectable signal	No detectable signal
CR2 ab54253	Mouse	Unknown	Reactive with human and mouse CR2	N/A
hCR2	Mouse	Unknown	No detectable signal	N/A
1048	Mouse	Unknown	Reactive with human CR2	N/A
B Ly-4	Mouse	Unknown	Reactive with human CR2	N/A

2.14 Immunofluorescence

Sections of spleen were incubated with LAB solution (Polysciences #24310) for 20min, washed in PBT (1% TritonX-100 in 1xPBS) for 5min and incubated overnight in a humidified chamber at +4°C with goat anti-Iba1 (Abcam #ab5076, 1:300), rabbit anti-CD35 (Abcam #ab126737, 1:300) and Rat anti-B220 (Biolegend, 1:300) in PBT with 10% normal donkey serum. Sections were washed in PBT, 3x5min, and incubated with their respective Alexa Fluor secondary antibody at a concentration of 1:1000 for 2hrs in PBT: donkey anti-goat IgG 488 (Invitrogen #A11055), donkey anti-rabbit IgG 594 (Invitrogen #A21207), and donkey anti-rat IgG 647 (Abcam #ab150155). After incubating with the appropriate secondary antibodies, sections were washed 3x5min in PBT and counterstained with DAPI (Invitrogen #D1306, 1:1000) for 5min at RT in 1xPBS with a final wash in 1xPBS for 5min. Slides were mounted using Aqua Poly/mount (PolySciences, Inc. #18606) and stored at -20°C until visualized using the Zeiss Axio Observer.

Chapter 3

3. Results

Previous work carried out by JAX Genetic Engineering Technologies developed a construct carrying both human *Cr1* and human *Cr2*, along with the intergenic region. This construct was integrated into the mouse *Cr2* locus of B6 ES cells, with lines 5H4 and 5E2 demonstrating successful integration. These targeted cell lineages were implanted into $B6^{Tyr}$ blastocysts and produced 27 chimeras with >50% penetrance and 1 chimera with <50% penetrance. Chimeras from both lines successfully bred to $B6^{Tyr}$ mice with 30% of pups inheriting the construct.

3.1 Protein Products are Comparable in Size to the Human Counterparts

To estimate the predicted protein size of each protein product for CR2, $CR1^{L/L}$ and $CR1^{S/S}$ a combination of bioinformatics tools were utilized. First, to determine the mRNA sequence from the original construct, the NCBI tool Spidey was used (https://www.ncbi.nlm.nih.gov/spidey/). Spidey determines the mRNA regions within a query sequence by comparing a cDNA sequence to a genomic sequence. Briefly, for each gene, the predicted gene sequence from the CR2/CR1 construct was compared to the human genomic sequence (retrieved from NCBI Entrez gene; CR2: NM_001006658, CR1: NM_00651). Secondly, the predicted protein sequence for each gene was determined using Open Reading Frame finder from NCBI (https://www.ncbi.nlm.nih.gov/orffinder/). Finally, to determine the molecular weights of each predicted protein, a protein molecular weight predictor tool (http://www.bioinformatics.org/sms/prot_mw.html) was used. The predicted molecular weights are: CR2 – 148kDa, $CR1^{L/L}$ – 273kDa and $CR1^{S/S}$ – 223kDa.

To determine the most likely isoforms created by the CR2/CR1 mouse, protein sequences were aligned with previously reported sequences for the various alleles of CR1 and CR2 using ClustalW in the MacVector program. The predicted protein sequence for CR2 was aligned with the isoforms containing 15 (NP_001868.2) and 16 (NP_001006659.1) SCR sequences, and determined that the constructed sequence matched that of the 16 SCR variant commonly found in human populations. To align the $CR1^{L/L}$ and $CR1^{S/S}$, both sequences were compared with the known sequences for the CR1-A (NP_000564) and CR1-B (NP_000642) alleles (Fig 11). With the exception of the first few amino acids, the sequences aligned to their respective human counterparts precisely. This demonstrates that the CR1-A allele should be equivalently produced through the $CR1^{S/S}$ constructed sequence, and likewise the CR1-B allele should be produced through the $CR1^{L/L}$ sequence.

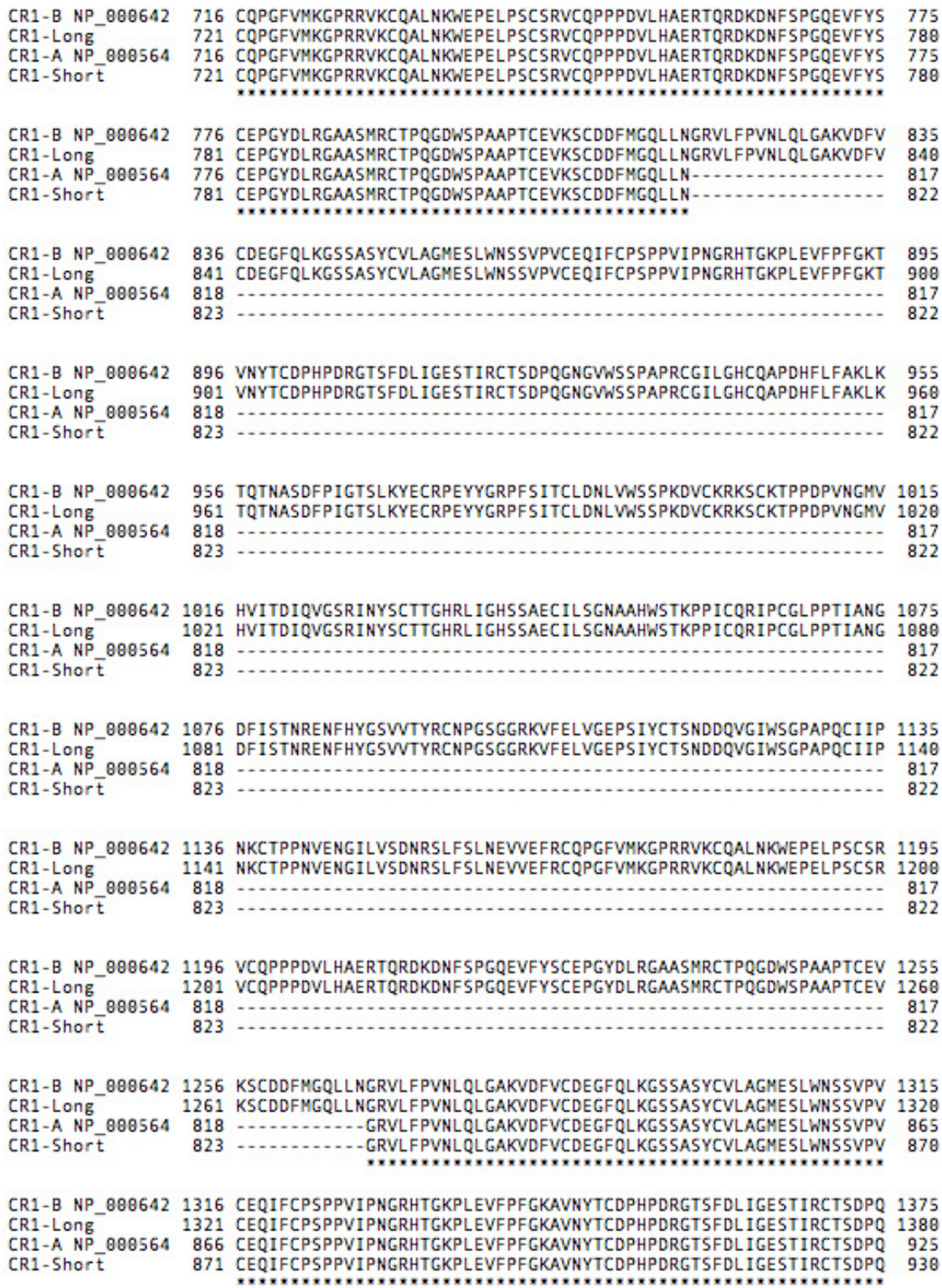


Figure 11. Alignments for Common Complement Receptor 1 Allotypes *From the construct, each viable protein sequence was aligned to the NCBI reference sequences for the most common CR1 variants. Both $CR1^{L}$ and $CR1^{S}$ align to their respective reference sequence. The image is a representative of the variation between the two most common isoforms. For whole sequence alignment please see Appendix (III and IV).*

3.2 Chimeras Produce Viable, Construct-Carrying Pups with Successful Germline Transmission

To determine the transmission of the CR2/CR1 construct, genotyping was designed to specific regions throughout each of the gene sets and confirmed in the first generation of $CR2^{Neo/WT}CR1^{L/WT}$ mice (Fig 12). Targeted mice, carrying the construct were bred to B6 mice to ensure germline transmission, and that no part of the insert would be lost due to recombination. Matings were established with male and female carriers to determine whether there was a sex bias to inheritance of the construct, but this was not seen. Both 5H4 and 5E2 lineages produced healthy pups which had successful transmission of the construct. Once germline transmission was identified and the development of the allelic series was instigated.

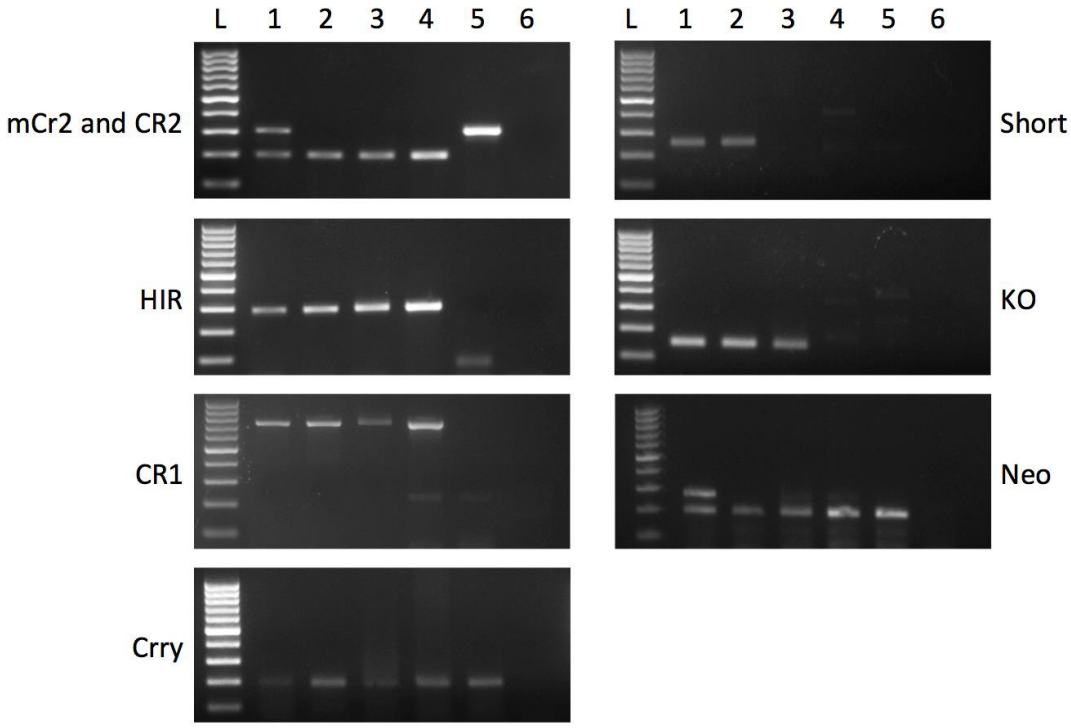


Figure 12. Confirmation of the Construct Integration and Transmission

Genotyping was developed to determine the inheritance of each portion of the construct. To ensure that the surrounding genes remained intact genotyping was developed to ensure Crry was not disrupted. Protocols were also developed to confirm the generation of the allelic series. Genotyping for Neomycin ensured that it was removed from the intronic region in CR2. Samples are labeled as such: [1] $CR2^{Neo}CR1^{L/WT}$ [2] $CR2CR1^{L/L}$ [3] $CR2CR1^{S/S}$ [4] $CR2CR1^{KO/KO}$ [5] WT and [6] Water, with [L] denoting the ladder. Neo - Neomycin

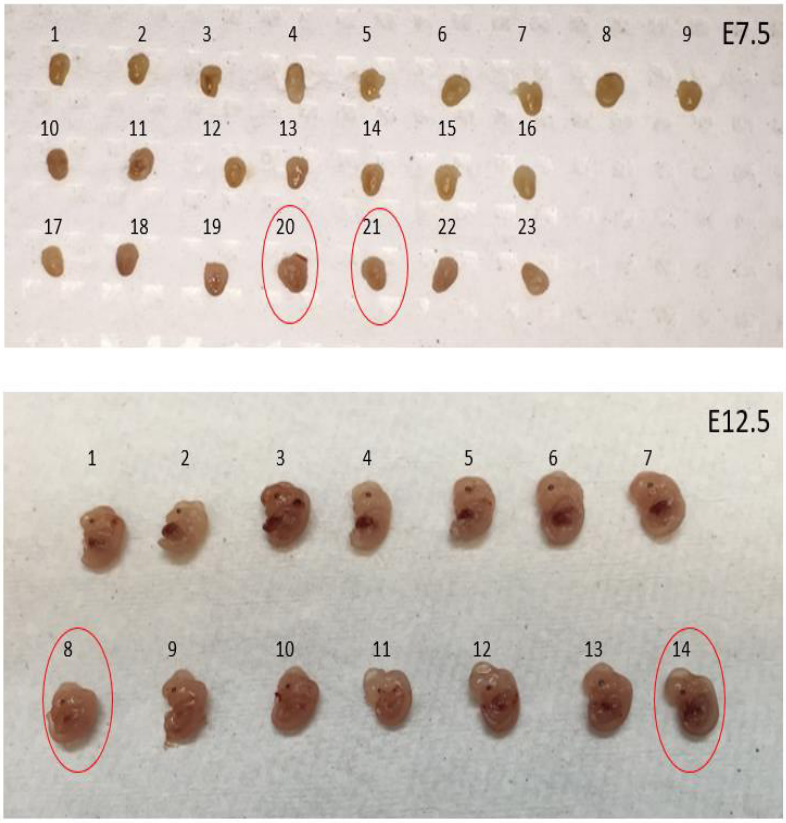
3.3 Possible Partial Homozygous Lethality in CR2CR1$^{KO/KO}$ Mice

While establishing a cohort for CR2CR1$^{L/L}$ and CR2CR1$^{S/S}$ mice produced homozygous mice, with 25% of the N3F1 and N4F1 pups being determined as homozygotes, the CR2CR1$^{KO/KO}$ cohort did not follow the same pattern. Upon an initial N4F1 intercross a markedly reduced number of CR2CR1$^{KO/KO}$ were seen, with 108 pups being born, and only 14 were homozygotes. From these, 10 were males and 4 were female. Therefore, I hypothesized that some CR2CR1$^{KO/KO}$ mice may fail to develop to term. To test this, timed mating's were established, sperm plugs observed, and embryos harvested at E7.5 and E12.5. Each uterine horn was inspected for signs of embryo resorption, or gaps between embryos indicating likely early resorption. No obvious signs were seen, and the embryos were removed from their amnions to identify any gross morphological changes (Fig 13), with posterior node and tail samples taken for genotyping at their respective time point. Of the 37 embryos studied at E7.5 and E12.5 only 4 were homozygous for CR2CR1$^{KO/KO}$, with no morphological differences observed between CR2CR1$^{KO/KO}$ and CR2CR1$^{WT/WT}$ or CR2CR1$^{KO/WT}$ embryos. Eventually, a breeding pair of homozygote CR2CR1$^{KO/KO}$ was established to produce a colony, and they produced homozygote pups at equivalent rates to the CR2CR1$^{L/L}$ and CR2CR1$^{S/S}$ colonies. This would suggest that while there is not total lethality of the CR1$^{KO/KO}$ mouse, there may be difficulties in establishing initial cohorts, due to associations that have been unexplored.

The insertion and subsequent removal of large portions of genomic material has the potential to interrupt regular transcription of genes surrounding the area of interest. As the RCA is an area that is tightly regulated, with a cluster of genes in close proximity, there is the potential for dysregulation. Determining whether there was the potential for lethality within the CR2CR1$^{KO/KO}$ mice is important due to the lethality seen within Crry-KO mice (Xu *et al.* 2000; Mao *et al.* 2003). Removal of the majority of the CR1 sequence has not disturbed *Crry* expression, enabling development to continue. As expected, the loss of endogenous m*Cr2* has not disrupted development.

In summary, using a combination of genomic recombineering and genotyping, these data show that the CR2/CR1 mouse is genetically viable. The loss of mouse Cr2 does not appear to disrupt development, and no surrounding regions were disturbed.

A



B

ID E7.5	Sex	Geno
1	F	HET
2	M	WT
3	F	WT
4	F	WT
5	F	WT
6	F	HET
7	F	HET
8	F	HET
9	F	HET
10	F	HET
11	F	HET
12	M	HET
13	F	HET
14	F	HET
15	F	HET
16	F	HET
17	F	HET
18	F	HET
19	F	HET
20	F	HOM
21	M	HOM
22	M	HET
23	F	HET

ID E12.5	Sex	Geno
1	F	WT
2	F	WT
3	F	WT
4	F	WT
5	M	HET
6	M	HET
7	M	HET
8	F	HOM
9	F	WT
10	M	WT
11	M	HET
12	M	HET
13	M	WT
14	M	HOM

Figure 13. Assessing $CR1^{KO/KO}$ Development *(A) While the expected results were seen in producing a cohort for the $CR1^{L/L}$ and $CR1^{S/S}$ alleles, $CR1^{KO/KO}$ proved to have difficulties. To investigate this, E7.5 and E12.5 were studied to determine if there were any developmental defects. No gross defects, or reabsorption, were seen and only 4 of the 37 mice showed a homozygous genotype (circled in red). (B) Tables identify the genotypes and sex of each embryo, M – male, F – Female, WT – Wild type, HET – heterozygous, HOM - homozygous.*

3.4 Splenic Expression of CR2 and CR1 in Homozygous Mice

To begin to assess the RNA expression of *Cr2* and *Cr1* we assessed spleen tissue. As a primary organ of complement processing the spleen is an ideal target to identify the expression of its receptors. cDNA was generated from 3 males and 3 females, from each cohort, from whole spleen to determine the successful expression of both *Cr2* and *Cr1*, along with ensuring that the expression of *Crry* was not disrupted. Targeted primers confirmed that *Cr2* and *Cr1* were present in the spleen and that m*Cr2* had been successfully removed (Fig 14). The presence of *Crry* was seen in all samples with no disruption observed between cohorts. Primers designed for *Cr1* targeted both exon 2 and a region spanning exon 4 and 5. This strategy identified that CR2CR1$^{KO/KO}$ mice produced a transcript containing only the first two exons but subsequent exons were excised.

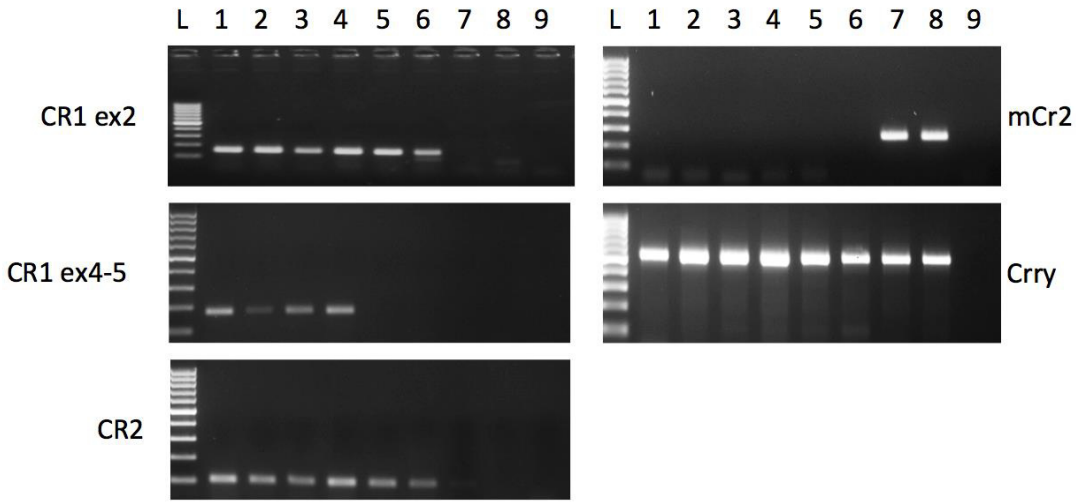


Figure 14. RNA Expression in Spleen of Transgenic Mice *To determine whether RNA was produced in the spleen, primers were designed to exon specific regions. All mice homozygous for each allele showed expression of the human genes, but no expression of the mouse Cr2. WT mice showed only Cr2 and Crry expression. CR2CR1$^{KO/KO}$ mice showed RNA expression of exons 2 for CR1, but did not have any further RNA present, as demonstrated with the CR1 ex4/5 protocol. All samples showed no disruption to Crry expression. Samples are labeled as such: [L] denotes the ladder, [1] CR2CR1$^{L/L}$ Female, [2] CR2CR1$^{L/L}$ Male, [3] CR2CR1$^{S/S}$ Female, [4] CR2CR1$^{S/S}$ Male, [5] CR2CR1$^{KO/KO}$ Female, [6] CR2CR1$^{KO/KO}$ Male, [7] WT Female, [8] WT Male, [9] Water.*

3.5 Western Blotting Confirmed the Presence of Human Protein in Spleen

To confirm that the predicted CR1 and CR2 proteins corresponded to their predicted sizes, western blotting was used to visualize each of the protein isoforms. Wild type samples were used as a negative control and to determine cross reactivity of antibodies. To assess the allelic variations a variety of antibodies were used, with one antibody showing specificity (See Material and Methods for details). Both CR2CR1$^{L/L}$ and CR2CR1$^{S/S}$ mice showed bands at their predicted sizes (Fig 15). The CR2CR1$^{KO/KO}$ mice showed no expression of a CR1 protein at any size. Minimal cross reactivity was observed within the wild type mice in the spleen. The presence of these bands confirms that these mice are translating the humanized genes and producing viable proteins.

Testing for CR2 showed that all CR2CR1 mice showed a single band at 148kDa. The wild type counterparts showed 2 bands, one at 150 kDa and another 190kDa. This is to be expected as the mouse *Cr2* gene produces two protein products via alternate splicing, in contrast to the human CR2 counterpart. These data suggest that the CR2 insert is being correctly translated and is able to be produced in cells within the spleen.

Therefore, the CR1$^{L/L}$, CR1$^{S/S}$ and CR1$^{KO/KO}$ alleles are created through recombineering and produce viable protein products. With protein expression confirmed, the expression patterns need to be identified in the mouse model.

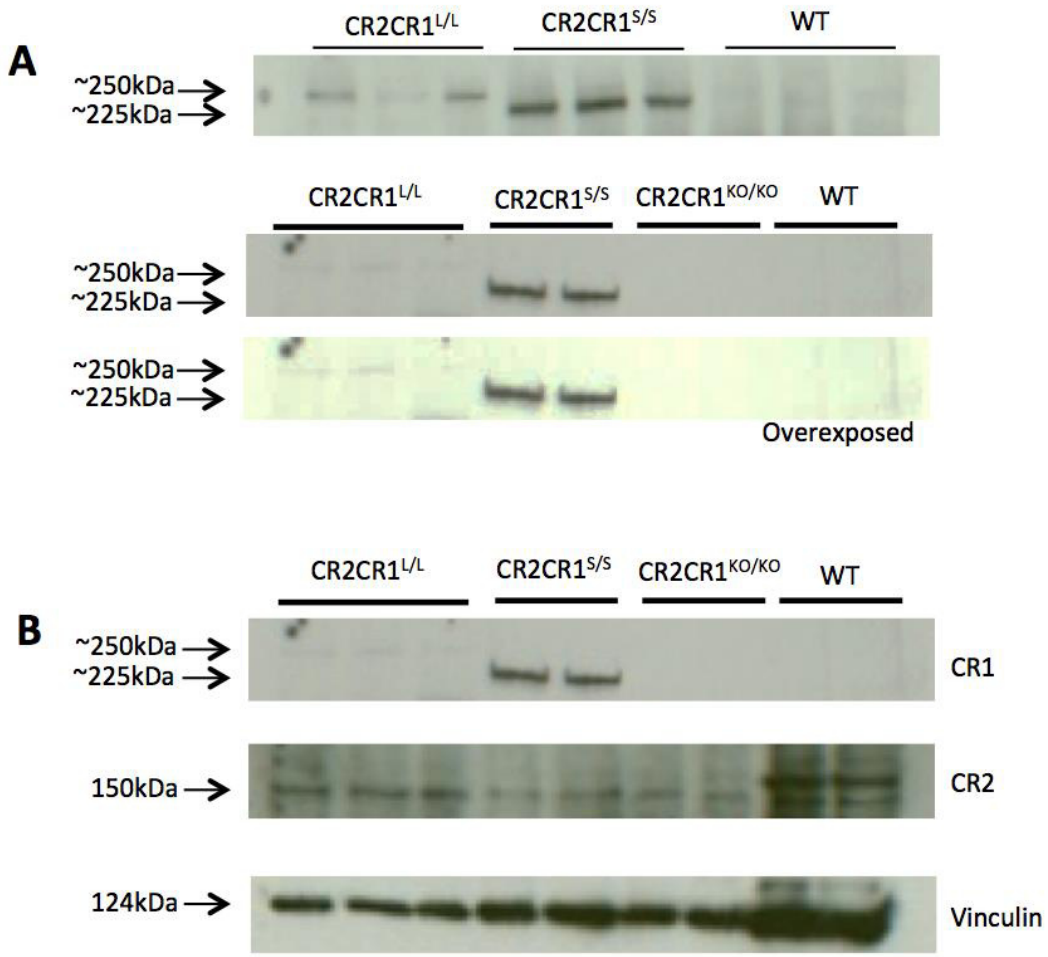


Figure 15. Protein Expression in the Allelic Series of Transgenic Mice *(A) Mice express CR1 at the expected sizes depending on their allotype. (B) CR2CR1$^{L/L}$ mice express a larger protein than the CR2CR1$^{S/S}$ mice. CR2CR1$^{KO/KO}$ do not express any form of CR1. CR2 expression is comparable between the three alleles, with a single protein product being produced. WT mice produce two protein products from the one Cr2 gene via alternate splicing. Vinculin is shown as the loading control.*

3.6 Localising Cell Specific Presence of CR1 and CR2

Two methods were selected to assess cell specific expression within this mouse model. First, antibodies were used to determine cellular location of the CR1 protein, and then blood-derived cells were sorted using FACS. RT-PCR was utilized to determine RNA expression in these cells.

3.6.1 Immunofluorescent Co-localization within the Spleen of Transgenic Mice

To assess the expression patterns of CR1 within the spleen of transgenic mice, immunofluorescence was used to determine cell specific expression. Sections were co-stained with Iba1 and B220 in an attempt to co-localize expression within the spleen. Although some staining was apparent in transgenic samples the WT counterparts showed similar staining. Although the endogenous mCR2 had been replaced, the appearance of this staining made it difficult to discern whether the signal was genuine or whether there was cross reactivity occurring (Fig 16). Pre-conjugated antibodies also showed no discernable signal preventing localization of expression to be effectively determined (data not shown). To be able to clearly define cell specific expression, and to determine antibody cross reactivity, a mCr2 knockout mouse will be used as a negative control in the future.

Initial staining identified that there was auto-fluorescence present in the spleen, seen with the secondary controls. In addition, non-specific binding was seen in WT samples with the CR1 antibody. To try to counter this, CR1 was stained with different secondary antibodies. This still showed non-specific staining in the WT controls. In an attempt to define whether this was due to experimental method a different approach was taken. Two paradigms were tested with just the CR1 antibody: one with LAB solution incubation and one without. Samples were incubated in either LAB solution for 20min or in PBT for 20min. All slides were washed in PBT for 5min and incubated for 30min in PBTB (3% BSA in PBT). CR1 antibody was diluted to 1:300 in PBTB and incubated at +4°C for 2 nights. After incubation, samples were washed in PBT 3x5min and the appropriate secondary antibody (Alexa Fluor 594) was applied 1:1000 for 2hrs at RT in PBT. Samples were washed for a further 3x5min in PBT and co-stained with DAPI. Slides were mounted using Aqua Polymount and visualized on the Zeiss Axio Observer (Fig 17). This was repeated with the addition of Goat anti-Iba1 in an attempt to localize expression to specific cell types (data not shown).

To test the mouse monoclonal antibodies in tissue, pre-conjugation was required to prevent any aberrant binding of secondary antibodies to the endogenous IgG's in the

spleen of these mice. Although these antibodies were pre-conjugated, no specific staining was observed.

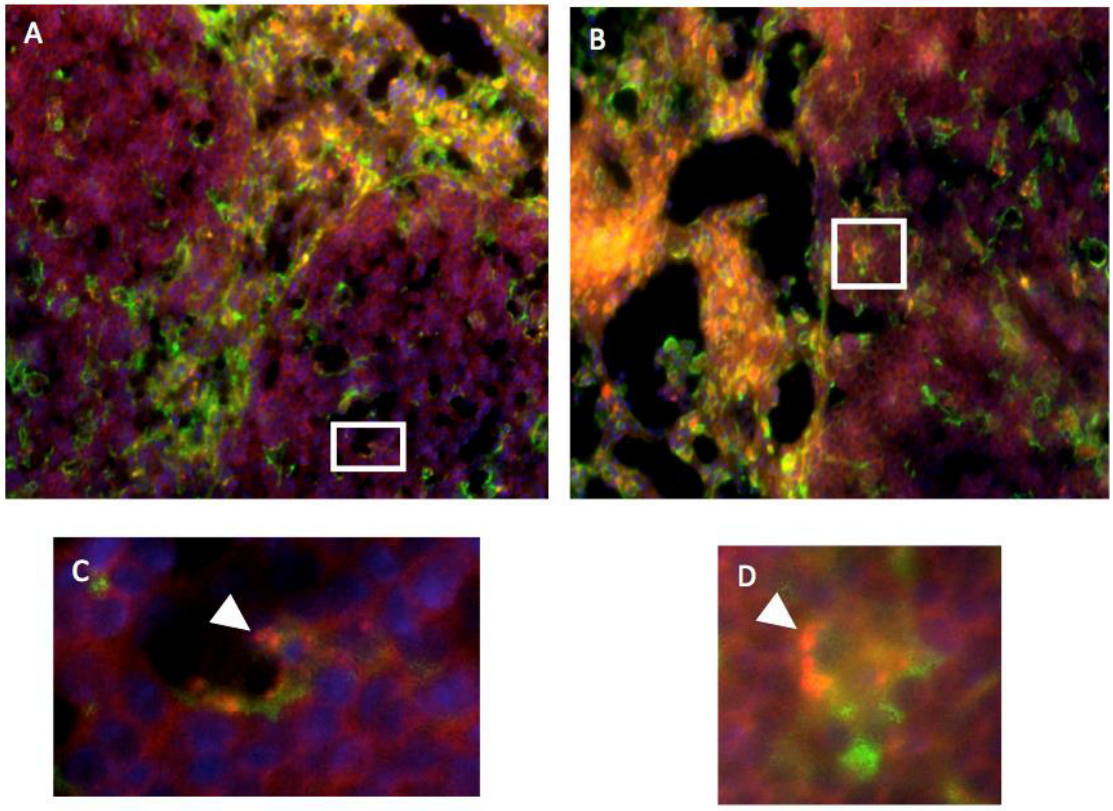


Figure 16. Localising Expression within the Spleen of Transgenic Mice

Immunofluorescent staining showed cross reactivity of the antibodies in both WT and Homozygous CR2CR1 mice. Macrophage staining (IBA1) green, CR1 red, DAPI blue. Images taken at 63x. [A] Is a transgenic mouse stained for CR1, [B] shows a WT spleen with similar staining. [C] and [D] represent the boxed area in the respective images above. White arrows show similar punctate staining between the transgenic and control.

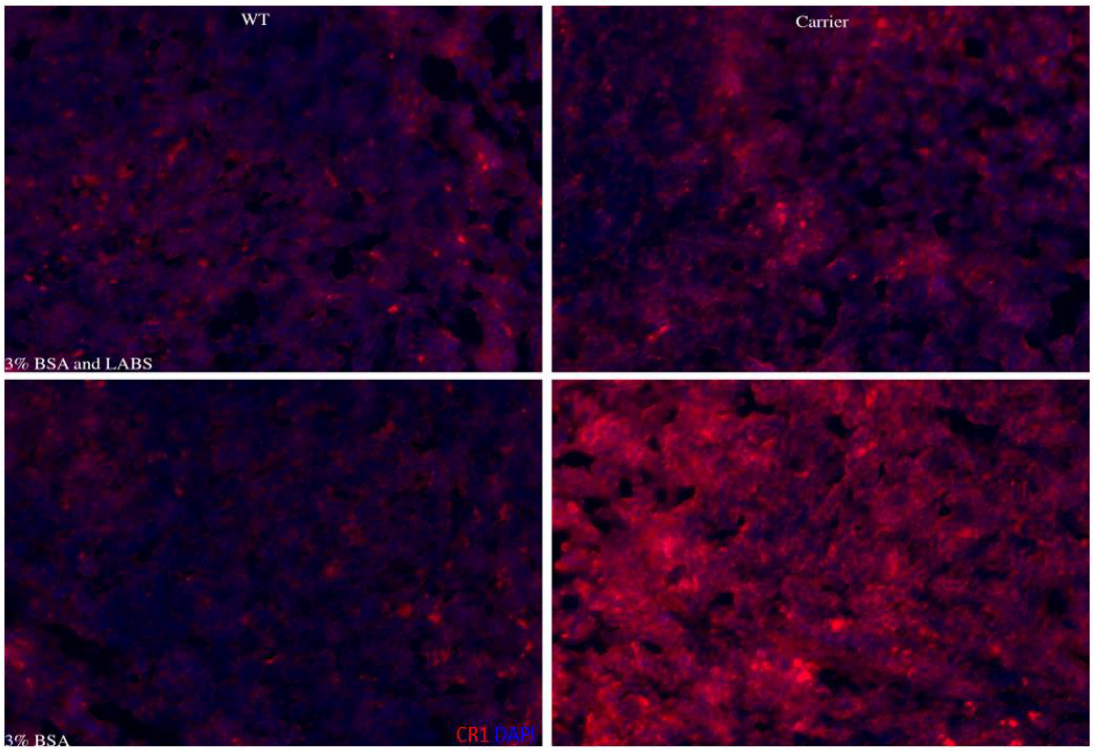


Figure 17. CR1 Staining Under Different Experimental Paradigms *Differing blocking conditions and the use of LAB solution was used to determine whether the cross reactivity was based on incubation conditions with the use of just the CR1 antibody (red) and DAPI (blue).*

3.6.2 Cell Specific Expression of CR2 and CR1 in Peripheral Blood

To determine the expression patterns of CR1, FACS was carried out on the peripheral blood of the allelic series and wild type mice. Cells were sorted into T-cells, B-cells, Monocyte/Macrophages, Platelets, Erythrocytes and Granulocytes using various antibodies. RNA was extracted, DNase treated and cDNA produced (Fig 10 and 18). To determine the cell specific expression B-cells were first targeted (Fig 19), as the human CR2 is driven by the mCr2 promotor this suggests expression will be identifiable in this cell type. Both *CR1* and *CR2* were seen to be expressed in B-cells, with the confirmation of a lack of m*Cr2* throughout the allelic series. These results also confirmed that the $CR1^{KO/KO}$ was a true null, but still expressed humanized *CR2*. From this both the $CR2CR1^{L/L}$ and $CR2CR1^{S/S}$ were screened to determine the cell specific expression on *CR1* in each sorted cell type (Fig 20). *CR1* expression was consistently present in B-cells and Granulocytes (Fig 20), but multiple other cells types gave inconsistent expression profiles (data not shown). Inconsistent expression was even observed within the erythrocyte population. This inconsistency is not entirely

unexpected and is thought to be due to the low levels of RNA generally found in mature erythrocytes.

In summary, this data has shown that the CR1/CR2 construct correctly integrated into the mouse genome, specific allotypes of CR1 can be produced through recombineering, and these allotypes are expressing in multiple cell types, including B-cells and Granulocytes.

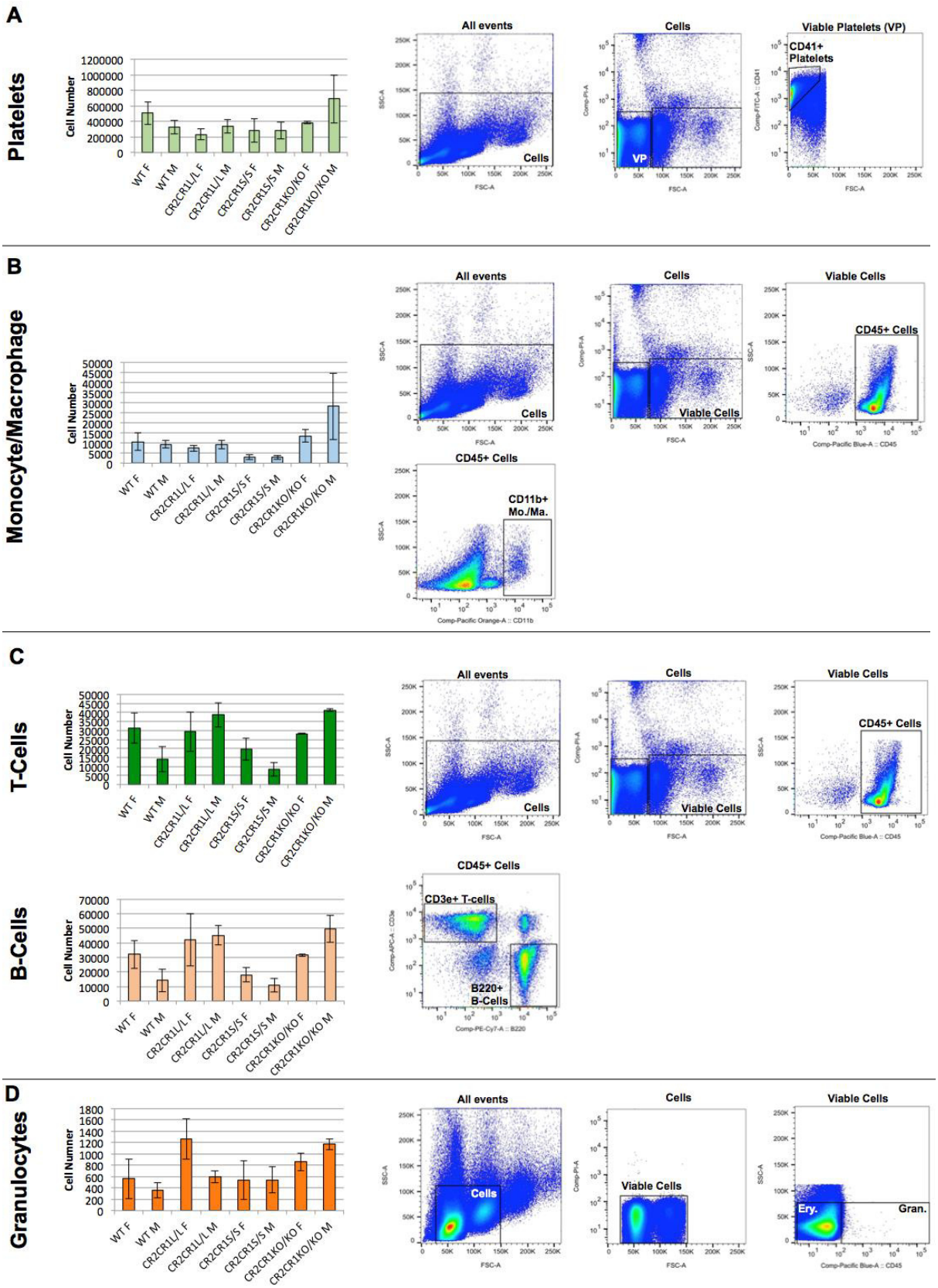

A
Platelets
Cell Number
1200000
1000000
800000
600000
400000
200000
0
WT F
WT M
CR2CR1L/L F
CR2CR1L/L M
CR2CR1S/S F
CR2CR1S/S M
CR2CR1KO/KO F
CR2CR1KO/KO M
All events
Cells
SSC-A
FSC-A
Cells
Cells
Comp-PI-A
VP
Viable Platelets (VP)
CD41+ Platelets
Comp-FITC-A :: CD41
B
Monocyte/Macrophage
Cell Number
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
All events
Cells
Cells
Viable Cells
Viable Cells
CD45+ Cells
Comp-Pacific Blue-A :: CD45
CD45+ Cells
CD11b+ Mo./Ma.
Comp-Pacific Orange-A :: CD11b
C
T-Cells
Cell Number
All events
Cells
Cells
Viable Cells
Viable Cells
CD45+ Cells
B-Cells
Cell Number
70000
60000
50000
40000
30000
20000
10000
0
CD45+ Cells
CD3e+ T-cells
B220+ B-Cells
Comp-APC-A :: CD3e
Comp-PE-Cy7-A :: B220
D
Granulocytes
Cell Nunmer
1800
1600
1400
1200
1000
800
600
400
200
0
All events
Cells
Cells
Viable Cells
Viable Cells
Ery.
Gran.

Figure 18. Cells sorted from Blood-Derived Cells *Each sorted cell type, had cell counts taken during sorting and were averaged for each cohort (for cell numbers refer to Appendix I Table A and B). Bars on the graph denote the standard error of the mean. Representative plots of various cells sorted from the white blood cell (WBC) and red blood cell fractions (RBC) from age and sex matched mice for all genotypes. [A] Platelets were sorted from the White blood cell (WBC) fraction and viable CD41-FITC+ cells were separated. [B] Monocyte/Macrophages (Mo./Ma.) were sorted from the WBC, CD45.2-BV421+ fraction and viable CD11b-BV605+ cells were separated. [C] T-cells and B-cells were sorted from the WBC, CD45.2-BV421+ fraction and viable CD3e-APC+ (B220-PECy7-) cells were sorted separately from B220-PECy7+ (CD3e-APC-) cells. [D] Granulocytes (Gran.) and Erythrocytes (Ery.) were sorted from the Red Blood Cell (RBC) fraction and separated based on their viable CD45.2-BV421 status; Erythrocytes were considered CD45.2-BV421- whereas Granulocytes were considered CD45.2-BV421+.*

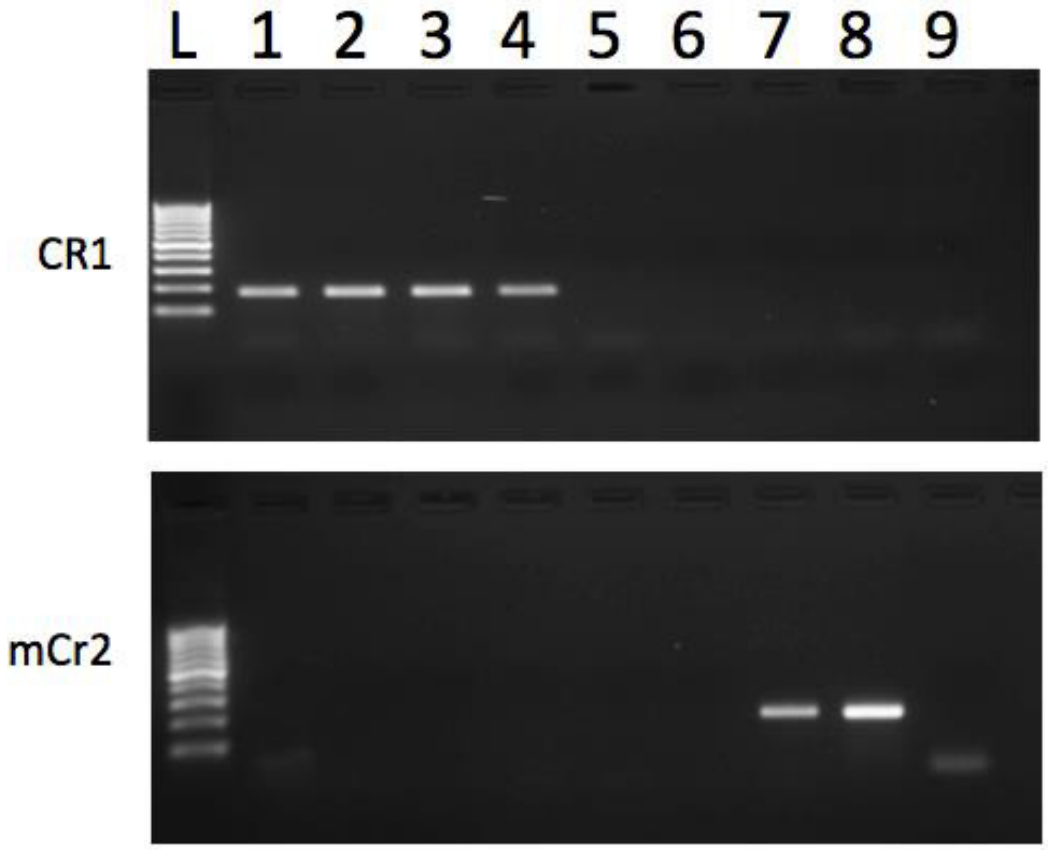


Figure 19. RNA Expression of CR1 in B-cells from Transgenic Mice *Primers designed to specific RNA sequences were utilized to determine cell specific expression of CR1 in peripheral blood cells. To begin screening, B-cells were initially assessed, as this was the cell type that was expected to express both genes. CR1 was expressed from the humanized mice and they were negative for mCr2. The samples in each lane are as follows, [L] denotes Ladder [1] CR2CR1$^{L/L}$ Male [2] CR2CR1$^{L/L}$ Female [3] CR2CR1$^{S/S}$ Male [4] CR2CR1$^{S/S}$ Female [5] CR2CR1$^{KO/KO}$ Male [6] CR2CR1$^{KO/KO}$ Female [7] WT Male [8] WT Female [9] Water.*

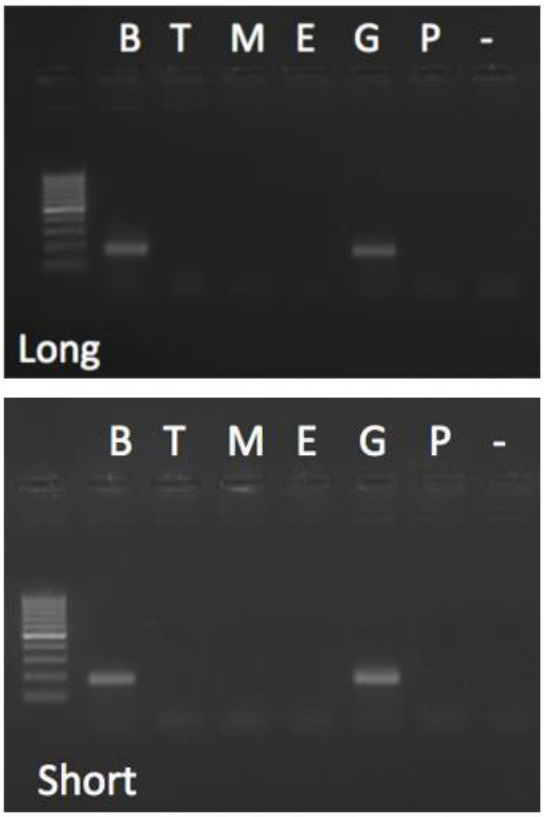


Figure 20. Blood-Derived Cell Specific Expression in CR1$^{L/L}$ and CR1$^{S/S}$ Mice *To assess the cell specific expression in both the CR2CR1$^{L/L}$ and CR2CR1$^{S/S}$ pooled samples from each cell type were tested for CR1 expression. B-cells and Granulocytes showed expression for CR1 in both CR2CR1$^{L/L}$ and CR2CR1$^{S/S}$ mice, showing no gross expression differences at initial characterization. B – B-cells, T- T-cells, M – Macrophages/Monocytes, E – Erythrocytes, G – Granulocytes, P - Platelets*

Chapter 4

4 Discussion

4.1 Mice Successfully Inherit Humanized Genes and Express Them

The very nature of creating a humanized mouse model is one fraught with difficulties. However, successful production of chimeras enabled the development of these humanized mice. Germline transmission was successful in both targeted cell lines, with no recombination or rearrangement occurring at any point throughout the construct. While there were initial hurdles in establishing the homozygous alleles, homozygosity was ultimately successful for all alleles, long, short and knockout. This enabled the characterization of all the alleles for CR1, including the more prevalent ones found in human populations. Western blotting was able to confirm the varying protein products produced by the CR1 allelic series. This work also confirmed that the homozygous CR1 knockout did not produce a viable protein product. When it came to determining the cell specific expression of each allele via immunofluorescence in the spleens difficulties were seen. These came from the potential cross-reactivity of antibodies with various other complement control proteins, as many of the complement receptor antibodies tend to target areas containing SCRs. These regions are replicated throughout many complement regulatory proteins, and due to the expansion and replication of CR1 through evolution (Farries and Atkinson 1988; Jacobson and Weis 2008), it is possible that there is non-specific binding throughout this family of genes across species. To counter this inability to be certain with immunofluorescent staining, an RNA based approach was utilized to determine the cell specific expression in whole blood. These RNA based techniques identified that there was definitive expression message of *Cr1* and *Cr2* in B-cells. Along with this, CR1 was also seen to be expressed in granulocytes at an RNA level. While expression levels in other cell types was not immediately identified, it cannot be ruled out, this is partly due to inconsistent results seen in a variety of cell types. To clarify the expression profile of these genes more cells will need to be sorted. RNA sequencing (a more sensitive approach than RT-PCR) will then be employed to determine the cell specific expression of CR1 and CR2. While one antibody showed specificity in western blotting, it was unclear whether it was specific in immunofluorescence. The inability to define specificity within this setting prevented having confidence in the antibodies ability to show specificity in a FACS setting, so it was excluded from the cell sorting panel. Along with this lack of specificity it also targeted the intracellular C-terminus, so may pose difficulties in giving a strong signal through FACS. Additional antibodies will also need to be identified, or created, to ensure specificity and protein expression, allowing for multiple techniques to be used to definitively define the expression patterns of CR1 and CR2.

4.2 Future Experiments Required for Comprehensive Validation of this Model

Although this mouse has been identified to express the human CR2 and various alleles of CR1 within the spleen and peripheral blood, it is important to identify whether this mouse model will interact with the complement system in the mouse and function as it does in the human system. To do this, both CR2 and CR1 will need to be rigorously tested for their functional ability to bind mouse C3b, C1q and other components. This will ensure that the mouse model will work in a comparative fashion to that of the human system. Along with determining cell specific expression in the blood and functional capabilities, additional work also needs to be carried out to determine cell specific expression within the brain. This would be carried out via primary cell culture of neurons, astrocytes and microglia to determine expression at an RNA level, as there are currently no definitive antibodies (Fonseca *et al.* 2016). These experiments will aim to bring a consensus to an inconsistently reported field and this knowledge will then be able to assist in understanding the mechanistic fundamentals of CR1 and CR2 roles in homeostasis, development and disease in a neural environment.

A further area that will require validation is identifying suitable antibodies that are readily available to the research community. While some of these antibodies worked in different contexts, they did not appear to work in this CR2/CR1 mouse. Currently available antibodies are generally inconsistent between assays and tissues samples, as identified by Fonseca *et al.* in 2016. As this inconsistency across antibodies has also proved difficult in defining this mouse clearly, it is important to test future antibodies with the appropriate controls. Two major controls for determining suitable antibodies are to use a m*Cr2* knockout mouse and a *Crry* knockout mouse. Both of these mice will eliminate the potential of cross reactivity due to similarities between the orthologous genes. Once these varying factors have been determined, a more effective model will be available, that does not rely on utilizing human samples and will allow the understanding of CRs in a complete system. This will enable new and more consistent antibodies to be developed and used to illuminate previously unknown aspects of both CR1 and CR2 in systemic research.

4.3 Implications of This Model

The development of this mouse model will open up a gateway to further understanding the complement cascade. With the discoveries made within the last decades a more expansive role of complement has emerged (Stevens *et al.* 2007; Schafer *et al.* 2012; Howell *et al.* 2011; Williams *et al.* 2016; Rutkowski *et al.* 2010; Mastellos and Lambris 2002; Del Rio-Tsonis *et al.* 1998). It is now more important than ever to understand

how the complement components are regulated. As mouse models are being used more readily to understand mechanisms, being able to better mimic the role of CR1 effectively will be of great benefit. The role CR1 plays in disease has previously been modeled in mice using m*Cr2* or *Crry* (Prodeus *et al.* 1998; Chen *et al.* 2000; Wu *et al.* 2002; Molina *et al.* 1996; Ahearn *et al.* 1996; Haas *et al.* 2002; Ramaglia *et al.* 2012b; Davoust *et al.* 1999; Killick, T. R. Hughes, *et al.* 2013; Maier *et al.* 2008b; Manickam *et al.* 2010), as they are the closest mouse orthologues to CR1. While this has led to some understanding of the role CR1 can have as a regulator in disease, it does not fully mirror the expression and functional capabilities of CR1. It is important to understand its role in modulating inflammation and other roles in a disease context, with this new mouse model it will hopefully open up undiscovered avenues of the role CR2 and CR1 that were not able to be elucidated via *in vitro* studies. The potential of this is that it could have a major impact on understanding autoimmune diseases such as SLE and rheumatoid arthritis, and what factors may influence this, along with the insights into the impact CR1 has on infectious disease, such as its influence on the rates of malarial infection, and understanding why CR1 is considered a risk factor for late onset AD and what role it may have in the progression of disease (Fig 21, Table 4).

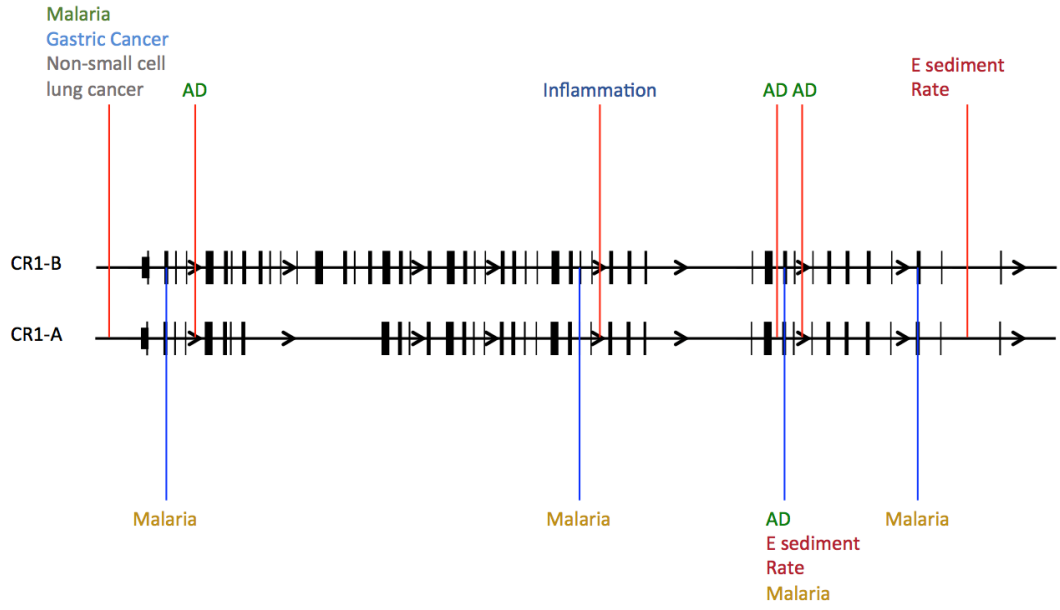


Figure 21. Locations of Disease Associated SNPs within CR1 *Various SNPs in CR1 have been associated with disease, with exon specific SNPs being targetable with this new mouse model. Red lines indicate an intronic SNP and blue lines indicate an exonic SNP. AD – Alzheimer's disease, E - Erythrocyte*

Table 4 – CR1 Relevant SNPs Associated with Disease

SNP	Position		Ref	Alt	Disease Association	Reference
rs6701713	Chr1:207612944	Intron, intron 31/39	A	G	AD	Naj *et al.* 2011
rs6656401	Chr1:207518704	Intron, intron 4	G	A	AD	Lambert *et al.* 2009
rs3818361	Chr1:207611623	Intron, intron 29/37	A	G	AD	Hollingworth *et al.* 2011; Lambert *et al.* 2009
rs4844609	Chr1:207609321	Missense, exon 29/37	T	A	AD	Keenan *et al.* 2012
rs9429942	Chr1:207495285	Upstream variant	C	T	Malaria Gastric cancer	Teeranaipong *et al.* 2008; Zhao *et al.* 2015
rs2274567	Chr1:207580276	Missense, exon 22/30	A	G	Malaria	Lan *et al.* 2015
rs2296160	Chr1:207621975	Missense, exon 36/44	A	G	Malaria	Lan *et al.* 2015
rs4844600	Chr1:207505962	Missense, exon 2	A	C,G	Malaria	Lan *et al.* 2015
rs7525160	Chr1:207495069	Upstream variant	G	C	Non-small cell lung cancer	Yu *et al.* 2014
rs6691117	Chr1:207609336	Exon 29/37	A	G	AD Erythrocyte sediment rate	Ma *et al.* 2014; Kullo *et al.* 2011
rs17047660	Chr1:207609511	Missense, Exon 29/37	A	G	Malaria	Apinjoh *et al.* 2014; Diakite *et al.* 2011; Toure *et al.* 2012; Kariuki *et al.* 2013
rs12034598	Chr1:207584170	Intron, intron 24/32	A	G	Inflammation	Naitza *et al.* 2012
rs12034383	Chr1:207630250	Intron 37/45	G	A	Erythrocyte sediment rate	Kullo *et al.* 2011

4.4 Long vs Short Forms of CR1

There are a variety of human CR1 isoforms and thus it is important to understand the functional implications of the more common allotypes. While selection pressures, such as malaria, have been a major factor in some geographical regions driving the prevalence of certain alleles, other regions do not have these pressures. Yet there is still a bias towards particular allele frequencies, with CR1-A and CR1-B remaining as the most common allotypes but varying in relative terms between populations. The prevalence within these different populations are: Caucasian A=0.87 and B=0.11, African Americans A=0.82 and B=0.11, Mexicans A=0.89 and B=0.11 and Asian Indians A=0.916 and B=0.084 (Moulds *et al.* 1996; Katyal *et al.* 2003). This bias may be due to undiscovered molecular functions of certain CR1 isoforms that could have major implications in our understanding of the complement cascade. With the development of this allelic series, the research community will be able to further decipher why there might be continued inheritance of multiple alleles of CR1. The research community can use these tools to gain insight into how each allele interacts within the complement cascade. These findings will be of importance in understanding and modelling human disease, and generating disease models.

4.5 Investigating the Role of CR1 in Alzheimer's disease (AD)

With the identification of CR1 as a major risk factor for AD (Jun *et al.* 2010; Brouwers *et al.* 2012; Lambert *et al.* 2009; Keenan *et al.* 2012; Hazrati *et al.* 2012; Carrasquillo *et al.* 2010; Van Cauwenberghe *et al.* 2013; Corneveaux *et al.* 2010), a more accurate model needed to be developed to understand how variation in CR1 increases susceptibility to this disease. While a variety of SNPs have been identified to link CR1 with AD (Corneveaux *et al.* 2010; Carrasquillo *et al.* 2010; Jun *et al.* 2010; Brouwers *et al.* 2012; Lambert *et al.* 2009; Keenan *et al.* 2012; Van Cauwenberghe *et al.* 2013), one study suggested that carrying both the CR1-A and CR1-B alleles represents a major risk factor (Hazrati *et al.* 2012). As these various alleles do not exist in any orthologue in mouse models of AD, it has not been possible to determine the importance of allotype in disease progression and prevalence. The influence of this heterozygous allotype could now be understood with this model. In addition, in 2012, Keenan *et al* identified a novel SNP within the C1q/MBL binding domain of CR1 in patients with AD. This SNP resulted in an increase in the decline of episodic memory. Further, co-inheritance with the ε4 allele of APOE compounded the effect of memory decline. The availability of the humanized CR2/CR1 mouse model will enable for the first time the functional consequences of the CR1 coding variants to be assessed. At JAX, we are

currently using gene editing by CRISPR to introduce the coding variant identified by Keenan and colleagues (Keenan *et al.* 2012) into our CR2/CR1 mouse. Additional work has seen the risk factor rs6656401 for AD also being correlated with reduced grey matter in the entorhinal cortex of young adults (Bralten *et al.* 2011), which suggests that CR1 may play a role in modelling the developing brain. While general associations of CR1 with AD are thought to be through the clearance pathways of Aβ (Hazrati *et al.* 2012), no true mechanism has been elucidated. With the availability of the allelic series within this mouse, and utilizing new gene editing technologies, it can be readily used to identify the strength and involvement of these risk factors on disease state.

To assess the role of CR1 in AD, we now plan to breed the various alleles into a mouse strain already carrying AD relevant mutations. These mutations are known early onset AD genes, such as APP (Goate *et al.* 1991; Murrell *et al.* 1991; Chartier-Harlin *et al.* 1991) and PSEN1 (Schellenberg *et al.* 1992). APP is a membrane bound protein that is cleaved via a variety of secretases (De Strooper and Annaert 2000). Mutations within these genes can affect the secretase cleavage sites, causing an imbalance in the level of Aβ40/Aβ42 produced. The imbalance favours the creation of Aβ42, a “stickier” protein than Aβ40, which is the main component of senile plaques, a hallmark feature of AD (Selkoe 1994). PSEN1 is a gamma secretase, which is able to cleave APP into these Aβ components. Mutations within this gene alter its ability to cleave APP in the correct ratio, leading to higher levels of Aβ42 being produced (Duff *et al.* 1996; De Strooper *et al.* 1998). One of the more common strains of mice that utilize mutations in these particular genes is the APP/PS1 strain (B6.Cg-Tg(APPswe,PSEN1dE9)85Dbo/Mmjax Jax#5864, MMRC# 34832-JAX (Jankowsky *et al.* 2004)). Utilizing this APP/PS1 strain, a model could be developed to understand the role of the various CR1 alleles in AD, along with being able to test the different GWAS identified SNPs.

While studying this model on an AD susceptible background will enable a deeper understanding of the influence CR1 has in disease status and progression, it is also important to be able to elucidate the genetic interaction APOEε4 and CR1 have. While both have been identified separately as major risk factors for AD, they have more recently been identified to have a compounding detrimental phenotype in late onset AD cases, specifically with the rs4844609 SNP (Keenan *et al.* 2012). Little has been uncovered about this potent effect, as it is difficult to fully dissect how each gene influences the other in human genetic studies. The use of this mouse model, in combination with a humanized APOEε4 model, while using CRISPR to introduce the disease associated SNPs, will enable the research community to fully elucidate the

molecular mechanisms that underpin how these two disease alleles work in concert to create a significantly higher risk factor for AD behavioural deterioration.

4.6 Conclusion

The work described in this thesis and the models created have the potential to profoundly impact the complement research community. The ability to adequately model CR1 and CR2 in a model system, such as the mouse, will give a greater understanding into why the complement cascade has been so heavily conserved throughout evolution along with its role in development and disease. A more complete understanding of the complement cascade, and its regulators, will lead to more targeted and personalized therapeutics.

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6. Appendix

Appendix I

Table A – Cell counts per sample and cell type

Cohort	Sex	CD3	B220	CD41	CD11b	CD45 +	CD45-
WT	F	16542	14808	217331	4083	1265	1*10^6
WT	F	45550	48423	689808	18780	289	1*10^6
WT	F	32069	33179	619628	9226	127	1*10^6
WT	M	8581	7548	372348	12619	602	1*10^6
WT	M	27942	29408	449116	9588	127	1*10^6
WT	M	5744	5375	151916	5838	354	1*10^6
$CR2CR1^{L/L}$	F	51000	78000	185000	10000	1434	1*10^6
$CR2CR1^{L/L}$	F	15751	24694	146000	4922	1777	1*10^6
$CR2CR1^{L/L}$	F	21348	23379	371947	7282	572	1*10^6
$CR2CR1^{L/L}$	M	45172	54383	295458	6940	481	1*10^6
$CR2CR1^{L/L}$	M	25349	32538	207249	6999	798	1*10^6
$CR2CR1^{L/L}$	M	45285	48806	512879	13238	503	1*10^6
$CR2CR1^{S/S}$	F	31297	25273	587000	2702	151	1*10^6
$CR2CR1^{S/S}$	F	16333	20259	157345	1123	244	1*10^6
$CR2CR1^{S/S}$	F	11031	8778	109886	5015	1210	1*10^6
$CR2CR1^{S/S}$	M	14538	17479	319093	4792	219	1*10^6
$CR2CR1^{S/S}$	M	9529	12652	77010	2891	411	1*10^6
$CR2CR1^{S/S}$	M	1358	1909	457812	1183	1002	1*10^6
$CR2CR1^{KO/KO}$	F	27914	30758	400669	16725	1019	1*10^6
$CR2CR1^{KO/KO}$	F	28374	32558	370961	10325	704	1*10^6
$CR2CR1^{KO/KO}$	M	42090	59187	378278	11725	1272	1*10^6
$CR2CR1^{KO/KO}$	M	40636	40333	1000000	44910	1077	1*10^6

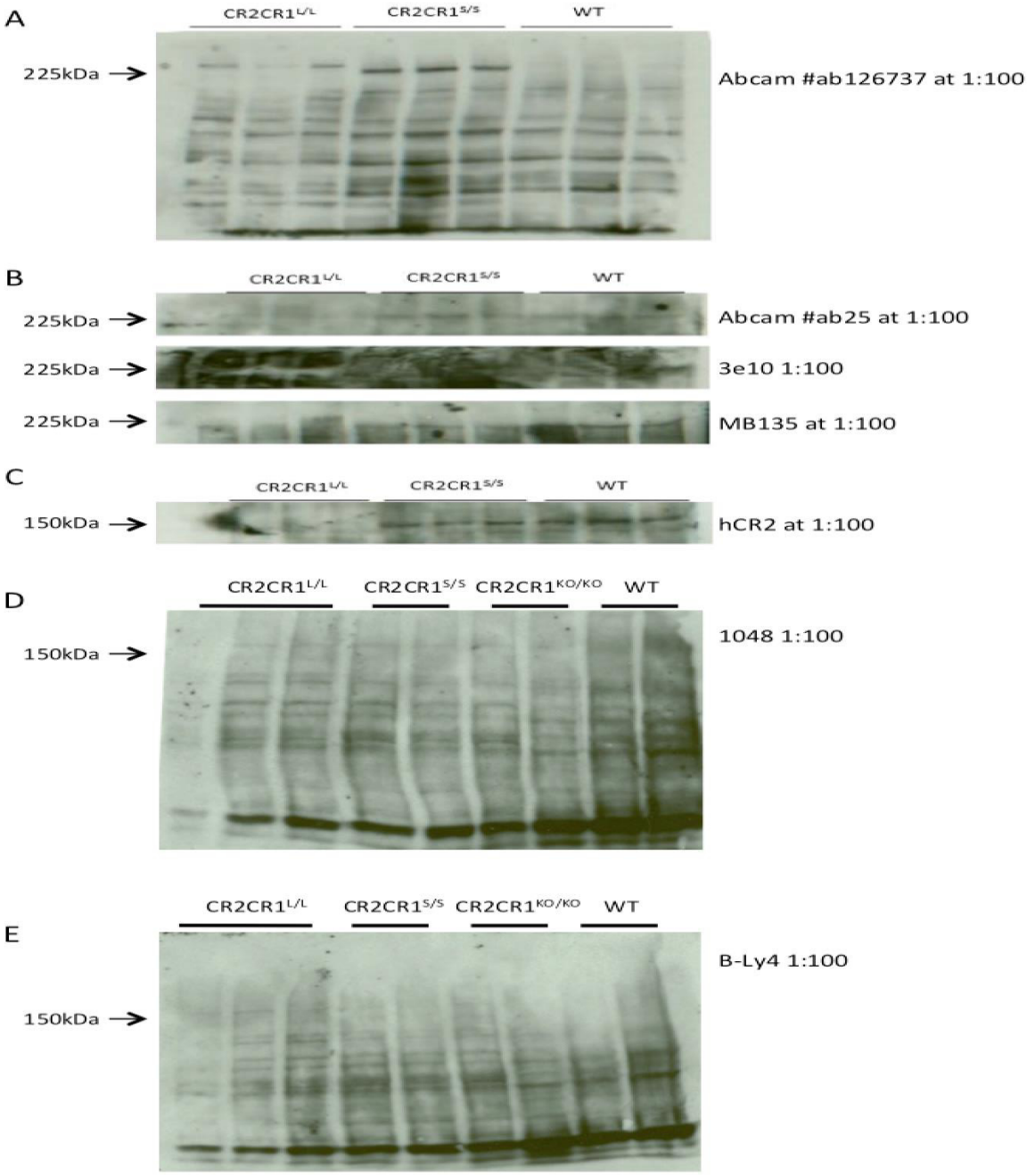
Appendix I

Table B – Average Cell counts per cohort per cell type

Cohort	Sex	CD3	B220	CD41	CD11b	CD45 +	CD45 -
WT	F	31387	32137	508922	10696	560	1*10^6
WT	M	14089	14110	324460	9348	361	1*10^6
$CR2CR1^{L/L}$	F	29366	42024	234316	7401	1261	1*10^6
$CR2CR1^{L/L}$	M	38602	45242	338529	9059	594	1*10^6
$CR2CR1^{S/S}$	F	19554	18103	284744	2947	535	1*10^6
$CR2CR1^{S/S}$	M	8475	10680	284638	2955	544	1*10^6
$CR2CR1^{KO/KO}$	F	28144	31658	385815	13525	862	1*10^6
$CR2CR1^{KO/KO}$	M	41363	49760	689139	28318	1175	1*10^6

Appendix II

Testing human complement receptor expression by Western blotting using various commercially available antibodies and antibodies provided by Prof. Paul Morgan. The antibodies tested produced a variety of results. Overall, antibodies gave inconclusive results. Some (e.g. B and C) showed no obvious specific binding, whereas the other antibodies (A, D and E) demonstrated expression of the hCR1 and hCR2 proteins - but with suspected non-specific staining also. Further work is required to validate CR1 and CR2 protein expression in the mouse, potentially using mouse Cr2 KO animals, additional antibodies and western blotting conditions.



Appendix III

Full sequence alignment of the CR1 proteins

```
CR1-B NP_000642     1      MGASSPRSPEPVGPPAPGLPFCCGGSLLAVVVLLALPVAWGQCNAPEWLPFARPT   55
CR1-Long            1 MCLGRMGASSPRSPEPVGPPAPGLPFCCGGSLLAVVVLLALPVAWGQCNAPEWLPFARPT   60
CR1-A NP_000564     1      MGASSPRSPEPVGPPAPGLPFCCGGSLLAVVVLLALPVAWGQCNAPEWLPFARPT   55
CR1-Short           1 MCLGRMGASSPRSPEPVGPPAPGLPFCCGGSLLAVVVLLALPVAWGQCNAPEWLPFARPT   60
                      ************************************************************

CR1-B NP_000642    56 NLTDEFEFPIGTYLNYECRPGYSGRPFSIICLKNSVWTGAKDRCRRKSCRNPPDPVNGMV  115
CR1-Long           61 NLTDEFEFPIGTYLNYECRPGYSGRPFSIICLKNSVWTGAKDRCRRKSCRNPPDPVNGMV  120
CR1-A NP_000564    56 NLTDEFEFPIGTYLNYECRPGYSGRPFSIICLKNSVWTGAKDRCRRKSCRNPPDPVNGMV  115
CR1-Short          61 NLTDEFEFPIGTYLNYECRPGYSGRPFSIICLKNSVWTGAKDRCRRKSCRNPPDPVNGMV  120
                      ************************************************************

CR1-B NP_000642   116 HVIKGIQFGSQIKYSCTKGYRLIGSSSATCIISGDTVIWDNETPICDRIPCGLPPTITNG  175
CR1-Long          121 HVIKGIQFGSQIKYSCTKGYRLIGSSSATCIISGDTVIWDNETPICDRIPCGLPPTITNG  180
CR1-A NP_000564   116 HVIKGIQFGSQIKYSCTKGYRLIGSSSATCIISGDTVIWDNETPICDRIPCGLPPTITNG  175
CR1-Short         121 HVIKGIQFGSQIKYSCTKGYRLIGSSSATCIISGDTVIWDNETPICDRIPCGLPPTITNG  180
                      ************************************************************

CR1-B NP_000642   176 DFISTNRENFHYGSVVTYRCNPGSGGRKVFELVGEPSIYCTSNDDQVGIWSGPAPQCIIP  235
CR1-Long          181 DFISTNRENFHYGSVVTYRCNPGSGGRKVFELVGEPSIYCTSNDDQVGIWSGPAPQCIIP  240
CR1-A NP_000564   176 DFISTNRENFHYGSVVTYRCNPGSGGRKVFELVGEPSIYCTSNDDQVGIWSGPAPQCIIP  235
CR1-Short         181 DFISTNRENFHYGSVVTYRCNPGSGGRKVFELVGEPSIYCTSNDDQVGIWSGPAPQCIIP  240
                      ************************************************************

CR1-B NP_000642   236 NKCTPPNVENGILVSDNRSLFSLNEVVEFRCQPGFVMKGPRRVKCQALNKWEPELPSCSR  295
CR1-Long          241 NKCTPPNVENGILVSDNRSLFSLNEVVEFRCQPGFVMKGPRRVKCQALNKWEPELPSCSR  300
CR1-A NP_000564   236 NKCTPPNVENGILVSDNRSLFSLNEVVEFRCQPGFVMKGPRRVKCQALNKWEPELPSCSR  295
CR1-Short         241 NKCTPPNVENGILVSDNRSLFSLNEVVEFRCQPGFVMKGPRRVKCQALNKWEPELPSCSR  300
                      ************************************************************

CR1-B NP_000642   296 VCQPPPDVLHAERTQRDKDNFSPGQEVFYSCEPGYDLRGAASMRCTPQGDWSPAAPTCEV  355
CR1-Long          301 VCQPPPDVLHAERTQRDKDNFSPGQEVFYSCEPGYDLRGAASMRCTPQGDWSPAAPTCEV  360
CR1-A NP_000564   296 VCQPPPDVLHAERTQRDKDNFSPGQEVFYSCEPGYDLRGAASMRCTPQGDWSPAAPTCEV  355
CR1-Short         301 VCQPPPDVLHAERTQRDKDNFSPGQEVFYSCEPGYDLRGAASMRCTPQGDWSPAAPTCEV  360
                      ************************************************************

CR1-B NP_000642   356 KSCDDFMGQLLNGRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV  415
CR1-Long          361 KSCDDFMGQLLNGRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV  420
CR1-A NP_000564   356 KSCDDFMGQLLNGRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV  415
CR1-Short         361 KSCDDFMGQLLNGRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV  420
                      ************************************************************

CR1-B NP_000642   416 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKTVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ  475
CR1-Long          421 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKTVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ  480
CR1-A NP_000564   416 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKTVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ  475
CR1-Short         421 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKTVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ  480
                      ************************************************************

CR1-B NP_000642   476 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT  535
CR1-Long          481 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT  540
CR1-A NP_000564   476 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT  535
CR1-Short         481 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT  540
                      ************************************************************

CR1-B NP_000642   536 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC  595
CR1-Long          541 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC  600
CR1-A NP_000564   536 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC  595
CR1-Short         541 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC  600
                      ************************************************************

CR1-B NP_000642   596 ILSGNAAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNPGSGGRKVF  655
CR1-Long          601 ILSGNAAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNPGSGGRKVF  660
CR1-A NP_000564   596 ILSGNAAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNPGSGGRKVF  655
CR1-Short         601 ILSGNAAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNPGSGGRKVF  660
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CR1-B NP_000642   656 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR  715
CR1-Long          661 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR  720
CR1-A NP_000564   656 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR  715
CR1-Short         661 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR  720
                      ************************************************************

CR1-B NP_000642   716 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPDVLHAERTQRDKDNFSPGQEVFYS  775
CR1-Long          721 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPDVLHAERTQRDKDNFSPGQEVFYS  780
CR1-A NP_000564   716 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPDVLHAERTQRDKDNFSPGQEVFYS  775
CR1-Short         721 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPDVLHAERTQRDKDNFSPGQEVFYS  780
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CR1-B NP_000642   776 CEPGYDLRGAASMRCTPQGDWSPAAPTCEVKSCDDFMGQLLNGRVLFPVNLQLGAKVDFV  835
CR1-Long          781 CEPGYDLRGAASMRCTPQGDWSPAAPTCEVKSCDDFMGQLLNGRVLFPVNLQLGAKVDFV  840
CR1-A NP_000564   776 CEPGYDLRGAASMRCTPQGDWSPAAPTCEVKSCDDFMGQLLN------------------  817
CR1-Short         781 CEPGYDLRGAASMRCTPQGDWSPAAPTCEVKSCDDFMGQLLN------------------  822
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CR1-B NP_000642 1256 KSCDDFMGQLLNGRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV 1315
CR1-Long        1261 KSCDDFMGQLLNGRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV 1320
CR1-A NP_000564  818 ------------GRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV  865
CR1-Short        823 ------------GRVLFPVNLQLGAKVDFVCDEGFQLKGSSASYCVLAGMESLWNSSVPV  870
                                ************************************************

CR1-B NP_000642 1316 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKAVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ 1375
CR1-Long        1321 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKAVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ 1380
CR1-A NP_000564  866 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKAVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ  925
CR1-Short        871 CEQIFCPSPPVIPNGRHTGKPLEVFPFGKAVNYTCDPHPDRGTSFDLIGESTIRCTSDPQ  930
                     ************************************************************

CR1-B NP_000642 1376 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT 1435
CR1-Long        1381 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT 1440
CR1-A NP_000564  926 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT  985
CR1-Short        931 GNGVWSSPAPRCGILGHCQAPDHFLFAKLKTQTNASDFPIGTSLKYECRPEYYGRPFSIT  990
                     ************************************************************

CR1-B NP_000642 1436 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC 1495
CR1-Long        1441 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC 1500
CR1-A NP_000564  986 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC 1045
CR1-Short        991 CLDNLVWSSPKDVCKRKSCKTPPDPVNGMVHVITDIQVGSRINYSCTTGHRLIGHSSAEC 1050
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CR1-B NP_000642 1496 ILSGNTAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNLGSRGRKVF 1555
CR1-Long        1501 ILSGNTAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNLGSRGRKVF 1560
CR1-A NP_000564 1046 ILSGNTAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNLGSRGRKVF 1105
CR1-Short       1051 ILSGNTAHWSTKPPICQRIPCGLPPTIANGDFISTNRENFHYGSVVTYRCNLGSRGRKVF 1110
                     ************************************************************

CR1-B NP_000642 1556 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR 1615
CR1-Long        1561 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR 1620
CR1-A NP_000564 1106 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR 1165
CR1-Short       1111 ELVGEPSIYCTSNDDQVGIWSGPAPQCIIPNKCTPPNVENGILVSDNRSLFSLNEVVEFR 1170
                     ************************************************************

CR1-B NP_000642 1616 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPEILHGEHTPSHQDNFSPGQEVFYS 1675
CR1-Long        1621 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPEILHGEHTPSHQDNFSPGQEVFYS 1680
CR1-A NP_000564 1166 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPEILHGEHTPSHQDNFSPGQEVFYS 1225
CR1-Short       1171 CQPGFVMKGPRRVKCQALNKWEPELPSCSRVCQPPPEILHGEHTPSHQDNFSPGQEVFYS 1230
                     ************************************************************

CR1-B NP_000642 1676 CEPGYDLRGAASLHCTPQGDWSPEAPRCAVKSCDDFLGQLPHGRVLFPLNLQLGAKVSFV 1735
CR1-Long        1681 CEPGYDLRGAASLHCTPQGDWSPEAPRCAVKSCDDFLGQLPHGRVLFPLNLQLGAKVSFV 1740
CR1-A NP_000564 1226 CEPGYDLRGAASLHCTPQGDWSPEAPRCAVKSCDDFLGQLPHGRVLFPLNLQLGAKVSFV 1285
CR1-Short       1231 CEPGYDLRGAASLHCTPQGDWSPEAPRCAVKSCDDFLGQLPHGRVLFPLNLQLGAKVSFV 1290
                     ************************************************************

CR1-B NP_000642 1736 CDEGFRLKGSSVSHCVLVGMRSLWNNSVPVCEHIFCPNPPAILNGRHTGTPSGDIPYGKE 1795
CR1-Long        1741 CDEGFRLKGSSVSHCVLVGMRSLWNNSVPVCEHIFCPNPPAILNGRHTGTPSGDIPYGKE 1800
CR1-A NP_000564 1286 CDEGFRLKGSSVSHCVLVGMRSLWNNSVPVCEHIFCPNPPAILNGRHTGTPSGDIPYGKE 1345
CR1-Short       1291 CDEGFRLKGSSVSHCVLVGMRSLWNNSVPVCEHIFCPNPPAILNGRHTGTPSGDIPYGKE 1350
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CR1-B NP_000642 1796 ISYTCDPHPDRGMTFNLIGESTIRCTSDPHGNGVWSSPAPRCELSVRAGHCKTPEQFPFA 1855
CR1-Long        1801 ISYTCDPHPDRGMTFNLIGESTIRCTSDPHGNGVWSSPAPRCELSVRAGHCKTPEQFPFA 1860
CR1-A NP_000564 1346 ISYTCDPHPDRGMTFNLIGESTIRCTSDPHGNGVWSSPAPRCELSVRAGHCKTPEQFPFA 1405
CR1-Short       1351 ISYTCDPHPDRGMTFNLIGESTIRCTSDPHGNGVWSSPAPRCELSVRAGHCKTPEQFPFA 1410
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CR1-B NP_000642 1856 SPTIPINDFEFPVGTSLNYECRPGYFGKMFSISCLENLVWSSVEDNCRRKSCGPPPEPFN 1915
CR1-Long        1861 SPTIPINDFEFPVGTSLNYECRPGYFGKMFSISCLENLVWSSVEDNCRRKSCGPPPEPFN 1920
CR1-A NP_000564 1406 SPTIPINDFEFPVGTSLNYECRPGYFGKMFSISCLENLVWSSVEDNCRRKSCGPPPEPFN 1465
CR1-Short       1411 SPTIPINDFEFPVGTSLNYECRPGYFGKMFSISCLENLVWSSVEDNCRRKSCGPPPEPFN 1470
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CR1-B NP_000642 1916 GMVHINTDTQFGSTVNYSCNEGFRLIGSPSTTCLVSGNNVTWDKKAPICEIISCEPPPTI 1975
CR1-Long        1921 GMVHINTDTQFGSTVNYSCNEGFRLIGSPSTTCLVSGNNVTWDKKAPICEIISCEPPPTI 1980
CR1-A NP_000564 1466 GMVHINTDTQFGSTVNYSCNEGFRLIGSPSTTCLVSGNNVTWDKKAPICEIISCEPPPTI 1525
CR1-Short       1471 GMVHINTDTQFGSTVNYSCNEGFRLIGSPSTTCLVSGNNVTWDKKAPICEIISCEPPPTI 1530
                     ************************************************************

CR1-B NP_000642 1976 SNGDFYSNNRTSFHNGTVVTYQCHTGPDGEQLFELVGERSIYCTSKDDQVGVWSSPPPRC 2035
CR1-Long        1981 SNGDFYSNNRTSFHNGTVVTYQCHTGPDGEQLFELVGERSIYCTSKDDQVGVWSSPPPRC 2040
CR1-A NP_000564 1526 SNGDFYSNNRTSFHNGTVVTYQCHTGPDGEQLFELVGERSIYCTSKDDQVGVWSSPPPRC 1585
CR1-Short       1531 SNGDFYSNNRTSFHNGTVVTYQCHTGPDGEQLFELVGERSIYCTSKDDQVGVWSSPPPRC 1590
                     ************************************************************

CR1-B NP_000642 2036 ISTNKCTAPEVENAIRVPGNRSFFTLTEIIRFRCQPGFVMVGSHTVQCQTNGRWGPKLPH 2095
CR1-Long        2041 ISTNKCTAPEVENAIRVPGNRSFFTLTEIIRFRCQPGFVMVGSHTVQCQTNGRWGPKLPH 2100
CR1-A NP_000564 1586 ISTNKCTAPEVENAIRVPGNRSFFTLTEIIRFRCQPGFVMVGSHTVQCQTNGRWGPKLPH 1645
CR1-Short       1591 ISTNKCTAPEVENAIRVPGNRSFFTLTEIIRFRCQPGFVMVGSHTVQCQTNGRWGPKLPH 1650
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CR1-B NP_000642 2096 CSRVCQPPPEILHGEHTLSHQDNFSPGQEVFYSCEPSYDLRGAASLHCTPQGDWSPEAPR 2155
CR1-Long        2101 CSRVCQPPPEILHGEHTLSHQDNFSPGQEVFYSCEPSYDLRGAASLHCTPQGDWSPEAPR 2160
CR1-A NP_000564 1646 CSRVCQPPPEILHGEHTLSHQDNFSPGQEVFYSCEPSYDLRGAASLHCTPQGDWSPEAPR 1705
CR1-Short       1651 CSRVCQPPPEILHGEHTLSHQDNFSPGQEVFYSCEPSYDLRGAASLHCTPQGDWSPEAPR 1710
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CR1-B NP_000642 2156 CTVKSCDDFLGQLPHGRVLLPLNLQLGAKVSFVCDEGFRLKGRSASHCVLAGMKALWNSS 2215
CR1-Long        2161 CTVKSCDDFLGQLPHGRVLLPLNLQLGAKVSFVCDEGFRLKGRSASHCVLAGMKALWNSS 2220
CR1-A NP_000564 1706 CTVKSCDDFLGQLPHGRVLLPLNLQLGAKVSFVCDEGFRLKGRSASHCVLAGMKALWNSS 1765
CR1-Short       1711 CTVKSCDDFLGQLPHGRVLLPLNLQLGAKVSFVCDEGFRLKGRSASHCVLAGMKALWNSS 1770
                     ************************************************************

CR1-B NP_000642 2216 VPVCEQIFCPNPPAILNGRHTGTPFGDIPYGKEISYACDTHPDRGMTFNLIGESSIRCTS 2275
CR1-Long        2221 VPVCEQIFCPNPPAILNGRHTGTPFGDIPYGKEISYACDTHPDRGMTFNLIGESSIRCTS 2280
CR1-A NP_000564 1766 VPVCEQIFCPNPPAILNGRHTGTPFGDIPYGKEISYACDTHPDRGMTFNLIGESSIRCTS 1825
CR1-Short       1771 VPVCEQIFCPNPPAILNGRHTGTPFGDIPYGKEISYACDTHPDRGMTFNLIGESSIRCTS 1830
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CR1-B NP_000642 2276 DPQGNGVWSSPAPRCELSVPAACPHPPKIQNGHYIGGHVSLYLPGMTISYICDPGYLLVG 2335
CR1-Long        2281 DPQGNGVWSSPAPRCELSVPAACPHPPKIQNGHYIGGHVSLYLPGMTISYICDPGYLLVG 2340
CR1-A NP_000564 1826 DPQGNGVWSSPAPRCELSVPAACPHPPKIQNGHYIGGHVSLYLPGMTISYICDPGYLLVG 1885
CR1-Short       1831 DPQGNGVWSSPAPRCELSVPAACPHPPKIQNGHYIGGHVSLYLPGMTISYICDPGYLLVG 1890
                     ************************************************************

CR1-B NP_000642 2336 KGFIFCTDQGIWSQLDHYCKEVNCSFPLFMNGISKELEMKKVYHYGDYVTLKCEDGYTLE 2395
CR1-Long        2341 KGFIFCTDQGIWSQLDHYCKEVNCSFPLFMNGISKELEMKKVYHYGDYVTLKCEDGYTLE 2400
CR1-A NP_000564 1886 KGFIFCTDQGIWSQLDHYCKEVNCSFPLFMNGISKELEMKKVYHYGDYVTLKCEDGYTLE 1945
CR1-Short       1891 KGFIFCTDQGIWSQLDHYCKEVNCSFPLFMNGISKELEMKKVYHYGDYVTLKCEDGYTLE 1950
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CR1-B NP_000642 2396 GSPWSQCQADDRWDPPLAKCTSRTHDALIVGTLSGTIFFILLIIFLSWIILKHRKGNNAH 2455
CR1-Long        2401 GSPWSQCQADDRWDPPLAKCTSRTHDALIVGTLSGTIFFILLIIFLSWIILKHRKGNNAH 2460
CR1-A NP_000564 1946 GSPWSQCQADDRWDPPLAKCTSRTHDALIVGTLSGTIFFILLIIFLSWIILKHRKGNNAH 2005
CR1-Short       1951 GSPWSQCQADDRWDPPLAKCTSRTHDALIVGTLSGTIFFILLIIFLSWIILKHRKGNNAH 2010
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CR1-B NP_000642 2456 ENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP 2489
CR1-Long        2461 ENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP 2494
CR1-A NP_000564 2006 ENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP 2039
CR1-Short       2011 ENPKEVAIHLHSQGGSSVHPRTLQTNEENSRVLP 2044
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Appendix IV

Full sequence alignment of the human CR2 sequences

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CR2 NP_00100665     1 MGAAGLLGVFLALVAPGVLGISCGSPPPILNGRISYYSTPIAVGTVIRYSCSGTFRLIGE   60
CR2 NP_001868.2     1 MGAAGLLGVFLALVAPGVLGISCGSPPPILNGRISYYSTPIAVGTVIRYSCSGTFRLIGE   60
CR2                 1 MGAAGLLGVFLALVAPGVLGISCGSPPPILNGRISYYSTPIAVGTVIRYSCSGTFRLIGE   60
                      ************************************************************

CR2 NP_00100665    61 KSLLCITKDKVDGTWDKPAPKCEYFNKYSSCPEPIVPGGYKIRGSTPYRHGDSVTFACKT  120
CR2 NP_001868.2    61 KSLLCITKDKVDGTWDKPAPKCEYFNKYSSCPEPIVPGGYKIRGSTPYRHGDSVTFACKT  120
CR2                61 KSLLCITKDKVDGTWDKPAPKCEYFNKYSSCPEPIVPGGYKIRGSTPYRHGDSVTFACKT  120
                      ************************************************************

CR2 NP_00100665   121 NFSMNGNKSVWCQANNMWGPTRLPTCVSVFPLECPALPMIHNGHHTSENVGSIAPGLSVT  180
CR2 NP_001868.2   121 NFSMNGNKSVWCQANNMWGPTRLPTCVSVFPLECPALPMIHNGHHTSENVGSIAPGLSVT  180
CR2               121 NFSMNGNKSVWCQANNMWGPTRLPTCVSVFPLECPALPMIHNGHHTSENVGSIAPGLSVT  180
                      ************************************************************

CR2 NP_00100665   181 YSCESGYLLVGEKIINCLSSGKWSAVPPTCEEARCKSLGRFPNGKVKEPPILRVGVTANF  240
CR2 NP_001868.2   181 YSCESGYLLVGEKIINCLSSGKWSAVPPTCEEARCKSLGRFPNGKVKEPPILRVGVTANF  240
CR2               181 YSCESGYLLVGEKIINCLSSGKWSAVPPTCEEARCKSLGRFPNGKVKEPPILRVGVTANF  240
                      ************************************************************

CR2 NP_00100665   241 FCDEGYRLQGPPSSRCVIAGQGVAWTKMPVCEEIFCPSPPPILNGRHIGNSLANVSYGSI  300
CR2 NP_001868.2   241 FCDEGYRLQGPPSSRCVIAGQGVAWTKMPVCEEIFCPSPPPILNGRHIGNSLANVSYGSI  300
CR2               241 FCDEGYRLQGPPSSRCVIAGQGVAWTKMPVCEEIFCPSPPPILNGRHIGNSLANVSYGSI  300
                      ************************************************************

CR2 NP_00100665   301 VTYTCDPDPEEGVNFILIGESTLRCTVDSQKTGTWSGPAPRCELSTSAVQCPHPQILRGR  360
CR2 NP_001868.2   301 VTYTCDPDPEEGVNFILIGESTLRCTVDSQKTGTWSGPAPRCELSTSAVQCPHPQILRGR  360
CR2               301 VTYTCDPDPEEGVNFILIGESTLRCTVDSQKTGTWSGPAPRCELSTSAVQCPHPQILRGR  360
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CR2 NP_00100665   361 MVSGQKDRYTYNDTVIFACMFGFTLKGSKQIRCNAQGTWEPSAPVCEKECQAPPNILNGQ  420
CR2 NP_001868.2   361 MVSGQKDRYTYNDTVIFACMFGFTLKGSKQIRCNAQGTWEPSAPVCEKECQAPPNILNGQ  420
CR2               361 MVSGQKDRYTYNDTVIFACMFGFTLKGSKQIRCNAQGTWEPSAPVCEKECQAPPNILNGQ  420
                      ************************************************************

CR2 NP_00100665   421 KEDRHMVRFDPGTSIKYSCNPGYVLVGEESIQCTSEGVWTPPVPQCKVAACEATGRQLLT  480
CR2 NP_001868.2   421 KEDRHMVRFDPGTSIKYSCNPGYVLVGEESIQCTSEGVWTPPVPQCKVAACEATGRQLLT  480
CR2               421 KEDRHMVRFDPGTSIKYSCNPGYVLVGEESIQCTSEGVWTPPVPQCKVAACEATGRQLLT  480
                      ************************************************************

CR2 NP_00100665   481 KPQHQFVRPDVNSSCGEGYKLSGSVYQECQGTIPWFMEIRLCKEITCPPPPVIYNGAHTG  540
CR2 NP_001868.2   481 KPQHQFVRPDVNSSCGEGYKLSGSVYQECQGTIPWFMEIRLCKEITCPPPPVIYNGAHTG  540
CR2               481 KPQHQFVRPDVNSSCGEGYKLSGSVYQECQGTIPWFMEIRLCKEITCPPPPVIYNGAHTG  540
                      ************************************************************

CR2 NP_00100665   541 SSLEDFPYGTTVTYTCNPGPERGVEFSLIGESTIRCTSNDQERGTWSGPAPLCKLSLLAV  600
CR2 NP_001868.2   541 SSLEDFPYGTTVTYTCNPGPERGVEFSLIGESTIRCTSNDQERGTWSGPAPLCKLSLLAV  600
CR2               541 SSLEDFPYGTTVTYTCNPGPERGVEFSLIGESTIRCTSNDQERGTWSGPAPLCKLSLLAV  600
                      ************************************************************

CR2 NP_00100665   601 QCSHVHIANGYKISGKEAPYFYNDTVTFKCYSGFTLKGSSQIRCKADNTWDPEIPVCEKG  660
CR2 NP_001868.2   601 QCSHVHIANGYKISGKEAPYFYNDTVTFKCYSGFTLKGNSQIRCKADNTWDPEIPVCEK-  659
CR2               601 QCSHVHIANGYKISGKEAPYFYNDTVTFKCYSGFTLKGSSQIRCKADNTWDPEIPVCEKG  660
                      ********************************** ********************

CR2 NP_00100665   661 CQSPPGLHHGRHTGGNTVFFVSGMTVDYTCDPGYLLVGNKSIHCMPSGNWSPSAPRCEET  720
CR2 NP_001868.2   660 ------------------------------------------------------------ET  661
CR2               661 CQSPPGLHHGRHTGGNTVFFVSGMTVDYTCDPGYLLVGNKSIHCMPSGNWSPSAPRCEET  720
                                                                              **

CR2 NP_00100665   721 CQHVRQSLQELPAGSRVELVNTSCQDGYQLTGHAYQMCQDAENGIWFKKIPLCKVIHCHP  780
CR2 NP_001868.2   662 CQHVRQSLQELPAGSRVELVNTSCQDGYQLTGHAYQMCQDAENGIWFKKIPLCKVIHCHP  721
CR2               721 CQHVRQSLQELPAGSRVELVNTSCQDGYQLTGHAYQMCQDAENGIWFKKIPLCKVIHCHP  780
                      ************************************************************

CR2 NP_00100665   781 PPVIVNGKHTGMMAENFLYGNEVSYECDQGFYLLGEKKLQCRSDSKGHGSWSGPSPQCLR  840
CR2 NP_001868.2   722 PPVIVNGKHTGMMAENFLYGNEVSYECDQGFYLLGEKKLQCRSDSKGHGSWSGPSPQCLR  781
CR2               781 PPVIVNGKHTGMMAENFLYGNEVSYECDQGFYLLGEKKLQCRSDSKGHGSWSGPSPQCLR  840
                      ************************************************************

CR2 NP_00100665   841 SPPVTRCPNPEVKHGYKLNKTHSAYSHNDIVYVDCNPGFIMNGSRVIRCHTDNTWVPGVP  900
CR2 NP_001868.2   782 SPPVTRCPNPEVKHGYKLNKTHSAYSHNDIVYVDCNPGFIMNGSRVIRCHTDNTWVPGVP  841
CR2               841 SPPVTRCPNPEVKHGYKLNKTHSAYSHNDIVYVDCNPGFIMNGSRVIRCHTDNTWVPGVP  900
                      ************************************************************

CR2 NP_00100665   901 TCIKKAFIGCPPPPKTPNGNHTGGNIARFSPGMSILYSCDQGYLLVGEALLLCTHEGTWS  960
CR2 NP_001868.2   842 TCIKKAFIGCPPPPKTPNGNHTGGNIARFSPGMSILYSCDQGYLLVGEALLLCTHEGTWS  901
CR2               901 TCIKKAFIGCPPPPKTPNGNHTGGNIARFSPGMSILYSCDQGYLLVGEALLLCTHEGTWS  960
                      ************************************************************

CR2 NP_00100665   961 QPAPHCKEVNCSSPADMDGIQKGLEPRKMYQYGAVVTLECEDGYMLEGSPQSQCQSDHQW 1020
CR2 NP_001868.2   902 QPAPHCKEVNCSSPADMDGIQKGLEPRKMYQYGAVVTLECEDGYMLEGSPQSQCQSDHQW  961
CR2               961 QPAPHCKEVNCSSPADMDGIQKGLEPRKMYQYGAVVTLECEDGYMLEGSPQSQCQSDHQW 1020
                      ************************************************************

CR2 NP_00100665  1021 NPPLAVCRSRSLAPVLCGIAAGLILLTFLIVITLYVISKHRERNYYTDTSQKEAFHLEAR 1080
CR2 NP_001868.2   962 NPPLAVCRSRSLAPVLCGIAAGLILLTFLIVITLYVISKHRERNYYTDTSQKEAFHLEAR 1021
CR2              1021 NPPLAVCRSRSLAPVLCGIAAGLILLTFLIVITLYVISKHRARNYYTDTSQKEAFHLEAR 1080
                      ******************************************* ****************

CR2 NP_00100665  1081 EVYSVDPYNPAS 1092
CR2 NP_001868.2  1022 EVYSVDPYNPAS 1033
CR2              1081 EVYSVDPYNPAS 1092
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Appendix V

Suppliers for consumables used throughout this thesis

Abcam

1 Kendall Square, Suite B2304
Cambridge, MA USA 02139

Agilent Technologies

5301 Stevens Creek Blvd
Santa Clara, CA USA 95051

BioLegend

9727 Pacific Heights Blvd
San Diego, CA USA 92121

Bio-Rad Technologies

Life Science Research, Education, Process Separations, Food Science
2000 Alfred Nobel Drive
Hercules, California USA 94547

eBioscience, Inc.

Headquarters
10255 Science Center Drive
San Diego, CA USA 92121

EMD Millipore

290 Concord Road
Billerica
Massachusetts 01821
United States of America

GE Healthcare Bio-Sciences
P.O. Box 643065 Pittsburgh,
PA 15264-3065
United States of America

Integrated DNA Technologies, Inc.

1710 Commercial Park
Coralville, Iowa USA 52241

Invitrogen

168 Third Avenue

Waltham, MA USA 02451

The Jackson Laboratory (JAX, JAX Genomic Engineering Technologies, JAX Flow Cytometry Service, JAX Genome Technologies)

600 Main Street

Bar Harbor, ME USA 04609

LabDiet
PO Box 19798
St. Louis, MO USA 63144

Polysciences, Inc.
400 Valley Road
Warrington, PA USA18976

Qiagen Inc.

27220 Turnberry Lane Suite 200

Valencia, CA USA 91355

Sigma-Aldrich
3050 Spruce St.
St. Louis, MO USA 63103

Thermo Fisher Scientific
168 Third Avenue
Waltham, MA USA 02451

Zeiss Microscopy

One Zeiss Drive
Thornwood, NY 10594
USA