A novel mouse model expressing human forms for complement receptors CR1 and CR2

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Abstract

Background: The complement cascade is increasingly implicated in development of a variety of diseases with strong immune contributions such as Alzheimer’s disease and Systemic Lupus Erythematosus. Mouse models have been used to determine function of central components of the complement cascade such as C1q and C3. However, species differences in their gene structures mean that mice do not adequately replicate human complement regulators, including CR1 and CR2. Genetic variation in CR1 and CR2 have been implicated in modifying disease states but the mechanisms are not known.

Results: To decipher the roles of human CR1 and CR2 in health and disease, we engineered C57BL/6J (B6) mice to replace endogenous murine Cr2 with human complement receptors, CR1 and CR2 (B6.CR2CR1). CR1 has an array of allotypes in human populations and using traditional recombination methods (Flp-frt and Cre-loxP) two of the most common alleles (referred to here as CR1long and CR1short) can be replicated within this mouse model, along with a CR1 knockout allele (CR1KO). Transcriptional profiling of spleens and brains identified genes and pathways differentially expressed between mice homozygous for either CR1long, CR1short or CR1KO. Gene set enrichment analysis predicts hematopoietic cell number and cell infiltration are modulated by CR1long, but not CR1short or CR1KO.

Conclusion: The B6.CR2CR1 mouse model provides a novel tool for determining the relationship between human-relevant CR1 alleles and disease.

Keywords: Complement cascade, Complement regulators, Immune cells, Alzheimer’s disease, Lupus, Hematopoietic cells, Immune cell infiltration

Background

The complement cascade is an integral component of our innate immune response and a first line of defense against bacterial infections. Various components of the complement cascade are constantly surveying for invading pathogens or debris, and tagging them for destruction. This system is composed of a number of plasma and membrane bound proteins and is tightly regulated. Circulating complement components are produced in the liver but can also be produced by specific cells in tissues.

In recent years, the recognized roles of the complement cascade have expanded. For example, the complement cascade is integral for the process of synapse pruning during development and disease [68, 70], for regulation of embryo survival [46, 84], and for tissue regeneration [19, 49, 66]. Many of these novel roles were
initially identified from animal models before being validated in human studies.

While animal models have proven fruitful in delivering greater understanding of the central components of the complement cascade such as C1q and C3, there are current limitations in studying human complement regulation in mice. In humans, the complement cascade is regulated in part by a series of genes on human chromosome 1 within the Regulators of Complement Activation (RCA) cluster [30, 42, 43, 63, 64, 76]. A major difference in the RCA cluster between humans and mice is in the locus encoding Complement Receptor 1 (CR1/CD35), that is absent in mice [25, 33, 54]. CR1 is both a receptor and a negative regulator of the complement cascade, binding to C3b, C4b, C1q, and MBL proteins. The interactions with C3b and C4b are considered to be the major function of this receptor [32, 39, 40, 80, 85].

Genetic variation in CR1 has been associated with a variety of diseases, such as Alzheimer’s disease [3, 8, 9, 12, 27, 34, 36, 41, 72], Malaria [4, 37, 71, 83], and Systemic Lupus Erythematosus [5, 13, 29, 35, 37, 48, 52, 53, 60, 65, 75, 78, 82]; however, in many cases the precise genetic variations have not been identified. CR1 has at least four allotypes: CR1-F, CR1-S, CR1-F’ and CR1-D (also known as CR1-A, CR1-B, CR1-C and CR1-D respectively) [20–23, 73]. These four allotypes differ in size through presence or absence of long homologous repeats (LHRs; each comprising seven short consensus repeats [SCRs]); the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs and is the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs [SCRs]; the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs; each comprising seven short consensus repeats [2, 3, 7, 27]. The difference between CR1long and CR1short is the number of LHR regions (Figs. 1c). These four allotypes differ in size through presence or absence of long homologous repeats (LHRs; each comprising seven short consensus repeats [SCRs]); the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs and is the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs; each comprising seven short consensus repeats (Figs. 1c). These four allotypes differ in size through presence or absence of long homologous repeats (LHRs; each comprising seven short consensus repeats [SCRs]); the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs and is the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs; each comprising seven short consensus repeats [2, 3, 7, 27]. The difference between CR1long and CR1short is the number of LHR regions (Figs. 1c). These four allotypes differ in size through presence or absence of long homologous repeats (LHRs; each comprising seven short consensus repeats [SCRs]); the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs and is the commonest form, CR1-F (gene frequency 0.87 in Caucasians), comprises four LHRs; each comprising seven short consensus repeats [2, 3, 7, 27].

To address this knowledge gap, we created a novel mouse model that enables the expression of different forms of human CR1 that we refer to as CR1short (equivalent to CR1-S) and CR1long (equivalent to CR1-F) (Fig. 1). Previous mouse models have relied on either the mouse Cr2 or Crry genes or on transgenically expressed forms of CR1 to study the function of human CR1 in mice [18, 38, 44, 45, 47, 55, 57–59, 81]. For instance, in the model created by Pappworth et al., human forms of CR1 and CR2 were expressed in mice using a transgenic approach and functional assessments made in a mouse Cr2-deficient background [55]. In the model we now present here, CR1 is expressed within the equivalent region of the mouse genome with human relevant promoter and regulatory sequences in conjunction with the expression of human CR2 driven by the mouse Cr2 promoter. Transcriptional profiling of the spleen and brain reveals significant differences in gene expression between mice carrying different allotypes of CR1 supporting the use of this new mouse model as a tool for studying CR1-dependent disease mechanisms.

### Results

**Chimeras produce viable, construct-carrying pups with successful Germline transmission**

To overcome species differences between mice and humans, we developed a new mouse model that, in the place of mouse Cr2 (mCr2), expresses human CR2 and CR1 (Figs. 1a-b and 2, see Methods). The B6.Cr2CR1 mouse model is capable of expressing two isoforms of CR1 (CR1long and CR1short, Figs. 1b) representing common allotypes predicted to be relevant to human disease [2, 3, 27]. The difference between CR1long and CR1short is the number of LHR regions (Figs. 1c). To maximize relevance to human CR1 regulation, we have incorporated the human intergenic region (HIR) between the CR2 and CR1 genes (Fig. 2). To create the B6.Cr2CR1 strain, B6 ES cells were targeted with a synthetic construct using recombineering (Fig. 2a, see methods). Twenty-eight chimeras, derived from two chimeric lines (5H4 and 5E2, Fig. 2b), were assayed for the presence of the construct. Chimeric mice carrying the synthetic construct were bred to B6.13y, and all black progeny were genotyped to confirm transmission (Fig. 2c). No sex bias was seen with regards to transmission of the construct. Once germline transmission, and no sex bias, was confirmed the development of the CR1 allelic series (CR1long, CR1short, CR1KO) was performed (Fig. 2c). All genotypes developed through the allelic series were successfully bred to homozygosity through brother/sister matings. Allele-specific genotyping was used to determine the presence or absence of specific regions that defined each strain (mCr2, CR2, CR1long, CR1short, CR1KO, HIR; Fig. 3, see methods). Of note, the initial establishment of the B6.Cr2CR1KO/KO line proved difficult, as low numbers of homozygous mice were generated. However, once a male and female B6.Cr2Cr1KO/KO were identified, a mating pair was established, and the litter sizes were comparable to those of the other strains.

**RNA and protein expression of CR1 and CR2 in spleens of B6.Cr2Cr1 mice**

To establish RNA and protein expression of CR1 and CR2, splenic tissue was assessed. As a primary organ of murine Cr2 expression and complement-dependent immune complex processing, the spleen was an ideal target to validate the expression of human CR1 and CR2. cDNA was generated from whole spleens of 3 males and 3 females from each genotype. Targeted primers confirmed the presence of human CR2 and CR1 in the spleen and the absence of mouse Cr2 (Fig. 4a-e). For
CR1, primers designed for CR1 targeted both exon 2 and a region spanning exons 4 and 5. This strategy enabled identification of B6.CR2CR1KO/KO mice that produced a transcript containing only the first two exons but not exons 4 and 5. As expected, Crry transcript, a mouse-specific gene that lies downstream of Cr2, was seen in all samples (Fig. 4e).

To evaluate expression of human CR1 and CR2 protein isoforms, first, western blotting using an anti-CR1 antibody was performed on spleen samples from B6.CR2CR1long/long, B6.CR2CR1short/short, and B6 mice. An approximately 250 kDa band was detected in B6.CR2CR1long/long mice, while an approximately 225 kDa band was detected in B6.CR2CR1short/short mice (Figs. 4f and S1). These data agree with the predicted sizes based on the amino acid sequences for CR1long (273 kDa, 2494 amino acids) and CR1short (223 kDa, 2044 amino acids) (see Supplement File 1). CR1 protein expression appeared greater B6.CR2CR1short/short compared to B6.CR2CR1long/long mice. No band was present at either of these sizes in B6 control mice. To quantify CR1 and CR2 protein expression in the spleen, western blots were performed on B6.CR2CR1long/long, B6.CR2CR1short/short, B6.CR2CR1KO/KO and B6 mice (Figs. 5a-c and S2). Samples from B6.CR2CR1long/long mice showed significantly lower levels of CR1 protein expression compared to samples from B6.CR2CR1short/short mice (Fig. 5b-c).

Fig. 1 The B6.CR2CR1 strain incorporates human CR2 and common isoforms of human CR1 into the mouse RCA cluster. a Humans and B6 mice differ in the genes they contain within the regulation of complement activation (RCA cluster). Human genes (blue boxes) include CR2, CR1 and CR1L whereas B6 mouse genes (red boxes) include Cr2 and Crry. b-c The B6.CR2CR1 strain contains the human CR2 gene and a long form of the human CR1 gene (containing multiple Long Homologous Repeats, LHR). Through recombination using the FLP/FRT system, one LHR (LHR') can be removed from the CR1 gene. Through recombination using the Cre/loxP system, the majority of the human CR1 gene can be deleted.
Mice of all genotypes showed a band of similar intensity at ~148 kDa—the expected size of the orthologous CR2 isoform in humans and mice (Figs. 5d-f Fig. S2). In addition, a second ~190 kDa band was observed that we expect to be the second CR2 isoform known to be expressed in mice. The 148 kDa band showed greatest expression in B6 mice, but very low levels of expression in some mice expressing human forms of CR2. This may be due to the fact that the human CR2 transcript expression is driven from the mouse Cr2 promoter, and includes the mouse Cr2 5’ UTR. Therefore, we cannot rule out a small amount of alternatively spliced human CR2 in the B6.CR2CR1 mice. A third band, a little smaller than 148 kDa, was only observed in B6 mice but not B6.CR2CR1 mice and may indicate an as yet uncharacterized CR2 isoform.

CR1<sub>long</sub> modifies expression of more genes in the brain and spleen compared to CR1<sub>short</sub>

Transcriptional profiling was performed to identify transcriptional differences between B6.CR2CR1 and B6 mice. Spleen and brain samples from three male and three female B6.CR2CR1<sup>long/long</sup>, B6.CR2CR1<sup>short/short</sup>, B6.CR2CR1<sup>KO/KO</sup> and B6 controls were assessed (24
samples in total). There were no differences between male vs female for each of the three comparisons, so samples were pooled by genotype to increase the quantity from three (three per sex) to six (pooled sexes). First, the spleen samples were analyzed (Fig. 6a-c). Compared to B6 controls, B6.CR2CR1long/long mice showed a greater number of differentially expressed (DE) genes than B6.CR2CR1short/short mice (104 and 38 genes respectively, Tables S1 and S2). Only ten genes were DE when comparing B6.CR2CR1KO/KO mice to B6 (Table S3) suggesting that, at least in the spleens of young, healthy mice, the human CR2 gene functions similarly to mouse Cr2.

Interestingly, the expression of the CR1short transcript was almost twice as high as the CR1long transcript (9.4 counts per million (cpm) compared to 5.6 cpm respectively) supporting our previous data that showed a greater amount of CR1short protein in comparison to CR1long protein (Figs. 4f, 5, S1 and S2).

To predict functional relevance of the 104 DE genes comparing spleen samples from B6.CR2CR1long/long compared to B6, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) term enrichment analyses were performed. Four KEGG pathways were significantly enriched (p < 0.05), with two of the four relevant to amylase related genes (starch and sucrose metabolism, carbohydrate digestion and absorption) and the other two pathways (African trypanosomiasis and malaria) driven by ‘heme’ related genes. Enriched biological processes included metabolic terms such as ‘cellular oxidant detoxification’ and ‘carbohydrate catabolic process’. Interestingly, ‘negative regulation of histone acetylation’ was significant, suggesting that expression of CR1 in B6.CR2CR1long/long mice may affect some epigenetic signatures when compared to B6 controls. For molecular function (MF), GO terms showed changes relating to binding, such as ‘haptoglobin binding’, ‘chloride ion binding’, ‘organic acid binding’ and ‘copper ion binding’, indicating that the CR1long gene may be playing a role in intracellular binding (Fig. 6c). None of these pathways or GO terms were enriched in the DE genes comparing samples from B6.CR2CR1short/short mice or B6.CR2CR1KO/KO to B6.

The number of DE genes was greater in the brain when compared to the spleen (Fig. 6d). There were 183 DE genes identified by comparing B6.CR2CR1long/long mice to B6 (Table S4), 58 DE when comparing B6.CR2CR1short/short with B6 (Table S5), and only 5 DE genes between B6.CR2CR1KO/KO and B6 (Table S6). This trend reflects the results seen in the spleen, indicating that expression of CR1long in mice had the greatest effect on gene expression in the brain compared with mice expressing either CR1short or CR1KO. The CR1long transcript was expressed at a much lower levels in the brain (0.5 cpm on average).
compared to the spleen, while no CR1<sub>short</sub> transcripts were detected in the brain.

To identify the biological relevance of the 183 genes DE in brains of B6.<sub>CR2CR1</sub><sub>long/long</sub> compared to B6, KEGG and GO term gene set enrichment was performed. Only one KEGG pathway, 'Fanconi anemia pathway' was significant (p < 0.05) in the DE gene set between B6.<sub>CR2CR1</sub><sub>long/long</sub> and B6 controls. Enrichment of this pathway was driven by the genes 'Wdr48', 'Atr', and 'Rev1' which respond to DNA damage. GO BP terms associated with differences between B6.<sub>CR2CR1</sub><sub>long/long</sub> and B6 controls identified 'regulation of transcription, DNA-templated', 'phosphorylation', and 'cellular response to DNA damage stimulus' indicating DNA-damage response genes may be affected by the CR1<sub>long</sub>. GO MF analysis identified terms involved in kinase activity and DNA binding, further suggesting potential involvement in DNA repair mechanisms. No pathways or GO terms were enriched in mice expressing either CR1<sub>short</sub> or CR1<sub>KO</sub> (compared to B6) further supporting
a model whereby CR1\textsuperscript{long} protein causes more changes to gene expression levels compared to CR1\textsuperscript{short}.

**CR1\textsuperscript{long}, but not CR1\textsuperscript{short}, is predicted to modulate hematopoietic cell quantity and cell infiltration**

To predict the functional consequences of the genes modified in B6.CR2CR1\textsuperscript{long/long}, B6.CR2CR1\textsuperscript{short/short} and B6.CR2CR1\textsuperscript{KO/KO} mice compared to B6, ‘Disease and Function Analysis’ was performed in IPA. This function predicts increases or decreases in downstream biological activities using the direction of change of the genes in each DE gene list. In the spleen, multiple functional terms were predicted to be increased with the DE genes comparing B6.CR2CR1\textsuperscript{long/long} with B6. These could be generally classified as being related to regulation of hematopoietic cell number and were predicted to be activated (Fig. 7a). Two terms considered significant were ‘quantity of erythroid precursor cells’ and ‘quantity of myeloid cells’. Interestingly, genes associated with these terms were generally downregulated, including Hba-a1 (~39.74 fold), Hba-a2 (~19.25 fold) and Hbb-bs (~20.00 fold) which all encode for hemoglobin subunits (Fig. 7b, c). These data suggest that there is an effect of the CR1\textsuperscript{long} allele on hematopoietic quantity, possibly specific to red blood cells, in the spleen. These functional consequences were not associated with DE genes when comparing samples from either B6.CR2CR1\textsuperscript{short/short} or B6.CR2CR1\textsuperscript{KO/KO} with B6.

In the brain samples, functional consequences associated with DE genes comparing B6.CR2CR1\textsuperscript{long/long} with B6 samples included ‘cellular infiltration by leukocytes’ and ‘infiltration by neutrophils’ (p < 0.05, Fig. 7d). DE genes associated with these terms were generally upregulated including Hyal1 (14.78 fold), Tlr9 (3.44 fold) and Cd276 (4.59 fold) (Fig. 7e,f). HYAL1, a lysosomal hyaluronidase that degrades hyaluronan (a major constituent
of extracellular matrix), has been shown to be involved in cell proliferation, migration and differentiation [1]. TLR9, a toll-like receptor, plays a critical role in pathogen recognition and activation of innate immunity [11, 67]. CD276, also known as B7-H3, a member of the immunoglobulin superfamily, plays a role in T cell-mediated immune responses [10, 15]. Collectively, these data predict CR1 long and CR1 short in the brain will differentially modulate immune-like cells such as resident microglia or the infiltration and functioning of peripherally derived immune cells – functional differences that may alter risk for diseases such as Alzheimer’s disease.

**Discussion**

Here, we present a new mouse model expressing the human proteins CR1 and CR2 that will be of value to study of an array of human diseases including Alzheimer’s disease [3, 8, 9, 12, 27, 34, 36, 41, 72], Systemic Lupus Erythematosus [5, 13, 29, 35, 37, 48, 52, 53, 60, 65, 75, 78, 82] and infections such as malaria [4, 37, 71, 83]. We show that the humanized B6.CR2CR1 strain expresses CR2 in place of mouse Cr2 and is capable of expressing two different isoforms of human CR1 – a long form (2494 amino acids) and a short form (2044 amino acids, lacking one long homologous repeat). CR1 and CR2 are important regulators of the complement cascade but their specific roles in human diseases have been difficult to study in mouse models due to species differences between humans and mice (Fig. 1) [18, 38, 44, 45, 47, 55, 57–59, 81].

The B6.CR2CR1 mouse model leaves the mouse Crry gene intact. Interestingly, while the murine CD21/Cr2 gene undergoes alternative splicing to encode the two complement receptors, CR1 and CR2; the additional N-terminal domains in CR1 represent duplications of sequences derived from Crry [33]. In primates, the CR2 gene has lost the ability to encode CR1. The human CR1 gene derives from the sub-primate Crry gene through amplification/duplication events; Crry was effectively sacrificed in primates as a consequence. It will be interesting to determine the functional relationship and any interactions between Crry and human forms of CR1 and CR2 in this model.

We anticipate this mouse model will provide an important resource for elucidating the functions of CR1 and CR2 in human diseases – however further work is required to assess its full potential. First, it will be necessary to validate that human forms of CR1 and CR2 regulate the mouse complement system in a similar fashion to those seen in human studies. To do this, both CR2

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**Fig. 6 Transcriptional Profiling of the Spleen and Brain.**

- **A** Number of differentially expressed (DE) genes (p < 0.05) in the spleen for i) B6.CR2CR1<sup>long/long</sup> compared to B6, ii) B6.CR2CR1<sup>short/short</sup> compared to B6, and iii) B6.CR2CR1<sup>KO/KO</sup> compared to B6.
- **B** Biological Process (BP) GO terms associated with the 104 DE genes between B6.CR2CR1<sup>long/long</sup> compared to B6 in the spleen.
- **C** Molecular Function (MF) GO terms associated with the 104 DE genes between B6.CR2CR1<sup>long/long</sup> compared to B6.
- **D** Number of DE genes between each previous comparison in the brain.
- **E** Biological Process (BP) GO terms associated with the 183 DE genes between B6.CR2CR1<sup>long/long</sup> compared to B6.
- **F** Molecular Function (MF) GO terms associated with the 183 DE genes between B6.CR2CR1<sup>long/long</sup> compared to B6. Dot size correlates to number of genes in the pathway associated with each term (see Table S7).
and CR1 (long and short forms) will need to be tested for their ability to bind mouse C3b, C4b and C1q, three central components of the cascade. Second, creating new antibodies that specifically recognize the human isoforms of CR1 expressed in this mouse is required to confirm tissue and cellular distribution patterns in health and disease. Commonly used mouse anti-human CR1 monoclonal antibodies were tested but were unsatisfactory in the B6.CR2CR1 mouse. Some previous studies show available anti-CR1 and anti-CR2 antibodies are inconsistent between assays and tissues samples [26]. Testing existing and new antibodies for human CR1 and CR2 in the B6.CR2CR1 strain as well as in strains deficient in mouse CR2 protein (B6.Cr2KO) and mouse CRRY protein (B6.CrryKO) will eliminate the antibody specificity issue and potential for cross-reactivity due to similarities between these homologous genes. Establishing the gene expression patterns of human CR2 and CR1 in specific cell types, particularly in the bone marrow, blood, spleen and blood, will be necessary. Human CR1 is broadly expressed, albeit in varying quantities, on the plasma membranes of blood derived cells, including erythrocytes, eosinophil, monocytes/macrophages, B-lymphocytes, dendritic cells and a sub-set of CD4+ T-cells [17, 24, 50, 51, 56, 61, 62, 77], on endothelia and numerous cell types in tissues. Erythrocyte CR1 plays an integral role in the clearance of soluble immune complexes, transporting them to macrophages in the spleen and Kupffer cells in the liver [14, 16], allowing for these cells to engulf and eliminate immune complexes [69, 74]. The levels of CR1 expression on erythrocytes can differ due to a HindIII restriction fragment length
polymorphism, which corresponds to a SNP in intron 27 of the CR1 gene [79]. Interestingly, CR1 transcript and protein levels were greater in spleens of mice expressing CR1\textsuperscript{short} compared to CR1\textsuperscript{long} (Figs. 4, 5). This may indicate reduced expression of CR1\textsuperscript{long} compared to CR1\textsuperscript{short} per cell and/or differences in the numbers of CR1+ cells in the spleen. Differences in cell number may indicate general baseline differences in hematopoietic cell numbers, or differences in the numbers of CR1+ cells being trafficked from the bone marrow and blood to the spleen. Differences in protein expression may also reflect efficiency of presentation of the different isoforms of CR1 protein at the cell membrane. However, transcript analysis indicated a similar reduction in expression levels of CR1\textsuperscript{long} compared to CR1\textsuperscript{short}. While the functional significance of these differences remains to be elucidated, the B6.CR2CR1 mouse model provides an ideal platform to determine the mechanisms by which the CR1\textsuperscript{long} and CR1\textsuperscript{short} isoforms impact health, the aging process and disease.

The B6.CR2CR1 mouse model also provides an important platform for studying the function of single nucleotide polymorphisms (SNPs) that have been shown to modify CR1 and CR2 function and increase risk for human disease. Several exonic SNPs have been suggested to influence the stability of CR1 on erythrocytes, and thus mediate the high and low levels of expression [83]. While this variation is seen on erythrocytes, leukocyte expression does not show the same variability [78]. Given the current focus on developing treatments for Alzheimer’s disease, we expect the B6.CR2CR1 model to be a key resource to advance our understanding of how CR1 risk alleles contribute to disease susceptibility. In 2009, a genome-wide association (GWA) study identified CR1 as a potential risk factor for Alzheimer’s disease [41]. This association was corroborated in 2010 [9], 2012 [8, 27, 36] and 2013 [72]. The exact nature of the association of CR1 with Alzheimer’s disease is not well understood. One study [27] proposed the risk for Alzheimer’s disease is most likely associated with the B allele of CR1 (CR1-B, equivalent to CR1\textsuperscript{long} in this study), with one copy of CR1-B carrying a 1.8x higher risk of disease over the CR1-A/A allele (equivalent to CR1\textsuperscript{short} in this study) and a faster rate of cognitive decline. These observations make determining the effects of CR1 variants specifically on brain health of key importance to fully elucidate its role in Alzheimer’s disease. Differences in neuronal morphology and distribution between CR1-A/A and CR1-A/B carriers were also reported, the former having a more filiform neuronal structure with CR1 expression that associated with the endoplasmic reticulum, whereas the latter had a more vesicular-like pattern of CR1 expression associated with lysosomes; reduced expression levels of CR1-B in comparison to that of CR1-A were also seen. Another study [36] identified specific CR1 SNPs (rs6656401 and rs4844609) that influenced rate of cognitive decline in Alzheimer’s disease in combination with APOE status. The latter SNP is associated with a single amino acid change in the C1q binding region of CR1; patients carrying both APOE4 and rs4844609 showed a faster decline in episodic memory. While the functional implications of this coding SNP is yet to be determined, it may impact clearance of A\textbeta through interfering with C1q binding [27]. Young adults who carry the CR1 SNP rs6656401 had reduced grey matter volume in the entorhinal cortex [7], an area associated with atrophy in AD patients [6, 31]. Biffi et al. [3] also saw drastic differences in entorhinal cortical volume in AD and MCI patients depending on their CR1 genotype. Precise gene editing in B6.CR1CR2 mice by methods such as CRISPR/CAS9 allows for the first time these putative risk SNPs to be studied in the context of the different CR1 isoforms.

Analysis of the transcriptional profiling data for the brain suggests that the CR1 isoforms differentially affect immune cell infiltration or immune cell activation, processes that have been shown to be important in disease susceptibility, onset and progression. Critically, the long form of CR1, CR1-B, the reported risk allele for Alzheimer’s disease, was associated with a DE gene signature indicating upregulation of pathways labelled ‘cellular infiltration by leukocytes’ and ‘infiltration by neutrophils’; we thus speculate that the association of CR1\textsuperscript{long} with AD might be explained at least in part, by altered immune cell infiltration into the brain. Interestingly, gene expression profiles of brain and spleen from mice expressing only human CR2 (KO for CR1) appeared very similar to B6 (Fig. 6). These data suggest that in the tissues (brain and spleen) and at the ages (approximately 3 mos) studied, the additional CR2 protein isoform encoded by the mouse C2 gene did not significantly affect gene expression profiles. Therefore, the presence of the additional C2 variant in the mouse genome may be more important during times of stress, during aging or in disease contexts. Further studies in aging mice and incorporating Alzheimer’s disease pathologies (such as amyloid and TAU) are required to further corroborate all of the predictions from our transcriptional profiling data.

Conclusion

The ability to more precisely study CR1 and CR2 in a model system such as the mouse will facilitate our understanding of the role that these receptors, and the complement cascade more generally, play in a wide variety of diseases that show a strong immune component. A more complete understanding of the complement cascade, and its regulators, will lead to more targeted and
personalized therapeutics for common diseases such as AD and lupus.

Materials and methods

Mouse husbandry
All mice were housed on a 12/12 h light/dark cycle. Mice were housed in 6-in. duplex wean cages with pine shavings, group-housed dependent on sex at wean, and maintained on LabDiet® 5 K67. The Institutional Animal Care and Use Committee (IACUC) at The Jackson Laboratory (JAX) approved all mice used in this study. Daily monitoring of mice via routine health care checks was carried out to determine general wellbeing, with any mice considered to be unhealthy being euthanized with IACUC approved CO2 euthanasia methods.

Humanizing complement receptors CR1 and CR2
The B6.CR2CR1 mouse model was created by Genetic Engineering Technologies at JAX via vector targeted embryonic stem (ES) cells. Due to the size, a multi-staged approach was used to create the targeting construct. Regions were designed in silico 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 mouse CR2 was utilized, targeted with a Spectinomycin (Spec) cassette, producing a vector with the 5′ and 3′ flanking regions of mouse Cr2 (mCr2). 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 within the HIR gap repair vector. This was then targeted to the mCr2/Spec vector. The CR1 mini gene-containing vector was then targeted using Apa1 and AvrII restriction enzymes, excising the fragment for integration into the multigene vector. Once the vector was confirmed, C57BL/6J (B6, Jax #664) ES cells were targeted, with incorporation occurring at the genomic locus for mCr2. ES cells were transferred to a blastocyst from a B6(Cg)-TyrC2/J (B6.1Cr2, Jax #568) and implanted into pseudo-pregnant females. Litters contained a variety of chimeric pups with differing degrees of penetrance.

Chimeric mice from two targeted B6 ES cell lines, 5H4 and 5E2, were bred to B6.1Cr2 mice (Fig. 2). From these, black pups were selected for further breeding as a consequence of B6 ES cells being targeted initially. Mice determined positive through genotyping for the CR2CR1 construct were then bred to B6 to confirm germline transmission (Fig. 2). After germline transmission was confirmed, male mice from each line were sent for sperm cryopreservation, with the Neomycin (Neo) cassette intact, and are available upon request. Mice from the 5H4 line were used throughout this study.

Developing the CR1 allelic series
The initial stage of developing the allelic series was to remove the Neo cassette within intron 19 of CR2. To achieve this, mice derived from the 5H4 ES cell line were bred to B6.129S4-Gt(ROSA)26Sortm3(Ft) (B6.ROSA-Phi, Jax #7743) mice, to target the attB-attP region surrounding Neo (Fig. 2). Mice negative for Neo were intercrossed to establish B6.CR2CR1long/long strain (Figs. 1 and 2). For CR1short, B6.CR2CR1long/+ mice were crossed to B6.129S4-Gt(ROSA)26Sortm1(Ft) (B6.ROSA-Ftp, Jax #9086) mice. The Flp recombinase is ubiquitously expressed and targets the removal of LHR1 (Fig. 1) encoded by exons 13–20 (via the flanking FRT sites) in synthetic introns 12 and 20; (Fig. 2). Mice carrying CR1short (B6.CR2CR1short/+) were intercrossed to establish the B6.CR2CR1short/short strain (Figs. 1 and 2). Finally, for CR1KO, B6.CR2CR1long/+ mice were crossed to 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 B6.CR2CR1long/+, mice, as Cre recombinase is active without necessarily needing to be inherited. Mice carrying CR1KO (B6.CR2CR1KO/+) mice were intercrossed to establish the B6.CR2CR1KO/KO strain (Figs. 1 and 2).

Genotyping
PCR assays for genotyping were as follows:
For mouse Cr2 or human CR2 (Fig. 3): Common primer forward primer: 5′-TCTTCCCTCCTTTGCCCTACAGG-3′ C. Cr2 Reverse: 5′-AGAAGAGGTTGGGACAGTCTT-3′ and CR2 Reverse: 5′-TACCAAACAGGAATGGGGTA-3′ with a product size at 300 bp and the hCR2 product size at 198 bp with an annealing temperature of 60°C.
For the HIR Forward 5′-TCACTCACCTCGAGGCATCT-3′ and Reverse 5′-TCAGCACCCATTGGCTTCAG-3′ with a product size of 291 bp at an annealing temperature of 59.3°C.
For CR1long (Fig. 3): Forward 5′-TGATTACCGGGAGGCCATTCT-3′ and Reverse 5′-TGACAGTGTTGGCTCAG-3′ with a product size of 708 bp at an annealing temperature of 58.1°C.
For CR1KO (Fig. 3): Forward 5′-TCTTGTACTACAGGGACCCATCCATTCT-3′ and Reverse 5′-ACCTGAGATTAACACGTTGGGG-3′ with a product size of 150 bp if cre recombination has not occurred, with an annealing temp 57.5°C. The absence of a band, with a CR1 positive genotype, indicates the removal of exons 4–20.
For CR1<sup>short</sup> (Fig. 3), forward primer from the KO allele with the Reverse primer 5’ – CGATCATGGGC TCACTGCGAA-3’. A product size of 251 bp is expected if Flp recombination had not occurred. The absence of a band in a combination with a CR1 positive genotype, indicated Flp recombination. The annealing temperature for this reaction was 57.8°C.

For Cry: Forward 5’- TTTGCTAATTTGATGTGAG GAAAGG – 3’ and Reverse 5’- TAAAGTTGTGGAG GCTTTGGGT – 3’ with a product size of 190 bp and an annealing temperature of 55.4°C.

Cohort generation
Homozogous mice of the three genotypes CR2CR1<sup>long/long</sup>, CR2CR1<sup>long/short</sup>, CR2CR1<sup>KO/KO</sup> were identified. A separate B6 colony was established for wild-type control samples. Cohorts of at least 4 males and 4 females were used for all assays except for transcriptional profiling where 3 males and 3 females per genotype were assessed. All mice were bled via submandibular bleed and tissue harvested at 3 months of age.

Tissue harvesting and preparation
Mice were terminally anaesthetized using a Ketamine/ Xylazine (99 mg/kg Ketamine, 9 mg/kg Xylazine) mix. They were transcardially perfused with 1xPBS (phosphate buffered saline pH 7.4). Spleens and brains were harvested, snap frozen and stored at 80 °C until required. 100 ng of cDNA was used to determine expression within the spleen. PCR assays for RT-PCR were as follows:

For CR2 at exon 11: Forward: 5’- TGGGGCAGAA GGACTCCAAT – 3’ and Reverse: 5’- GCTCCACCAG GGTCTGCTTA - 3’ with a product size of 148 bp and an annealing temperature of 60°C.

For CR1 at exon 2: Forward: 5’- TTCATTGGCC ACGCCCTACCA – 3’ and Reverse: 5’- TGCACCTGTCC TTAGCACCA – 3’ with a product size of 152 bp and an annealing temperature of 60°C.

Western blotting
To determine protein presence and size difference between the CR1<sup>long</sup> and CR1<sup>short</sup>, 6% SDS PAGE gels were hand cast. Protein was diluted to 80 μg of total protein with 2x Laemmli buffer (BioRad). Samples were denatured at 95°C for 5 min and loaded onto the gel. Gels were run for 1 h at 150 V and transferred to nitrocellulose membrane via the iBlot for 13mins. Blots were incubated at room temperature for 1 h (hr) with blocking solution (5% skimmed milk powder block in 0.1% PBS-Tween), washed with 0.1% PBS-Tween and then incubated with rabbit-anti-human CR1 (also known as CD35; Abcam #ab126737, 1:100) for 48 h 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) for 1.5 h at RT. Blots were then washed an additional three times and detection was carried out using ECL detection regents (GE Healthcare). When required, blots were stripped by treatment with 0.25% sodium azide for 2 h at RT and washed thoroughly in 0.1% PBS-Tween. Blots were re-blocked and re-probed with mouse anti-CD21 (anti-human CR2; 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), washed and detected. Finally, blots were treated with 0.25% sodium azide and probed with a loading
control, anti-Vinculin (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 1 h at RT, washed and detected.

**Transcriptional profiling**

**RNA isolation, library preparation and sequencing**

RNA was isolated from tissue using the MagMAX mirVana Total RNA Isolation Kit (ThermoFisher) and the KingFisher Flex purification system (ThermoFisher). Tissues were lysed and homogenized in TRIzol Reagent (ThermoFisher). After the addition of chloroform, the RNA-containing aqueous layer was removed for RNA isolation according to the manufacturer’s protocol, beginning with the RNA bead binding step. RNA concentration and quality were assessed using the Nanodrop (Thermo Scientific) and the RNA Total RNA Nano assay (Agilent Technologies). Libraries were prepared by the Genome Technologies core facility at The Jackson Laboratory using the KAPA mRNA HyperPrep Kit (KAPA Biosystems), according to the manufacturer’s instructions. Briefly, the protocol entails isolation of polyA-containing mRNA using oligo-dT magnetic beads, RNA fragmentation, first and second strand cDNA synthesis, ligation of Illumina-specific adapters containing a unique barcode sequence for each library, and PCR amplification. Libraries were checked for quality and concentration using the D5000 assay on the TapeStation (Agilent Technologies) and quantitative PCR (KAPA Biosystems), performed according to the manufacturers’ instructions. Libraries were pooled and sequenced by the Genome Technologies core facility at JAX, generating 100 bp paired-end reads on the HiSeq 4000 (Illumina) using HiSeq 3000/4000 SBS Kit reagents (Illumina).

**Sequence Alignment & Statistical Analysis Methods**

FASTQ files were trimmed using Trimmomatic v0.33 which removed adapters and sequences with more than 2 mismatches, a quality score < 30 for PE palindrome reads, or a quality score of < 10 for a match between any adapter sequence against a read. A sliding window of 4 bases was used with a required (average) minimum quality score threshold of 15. The leading and trailing minimum quality score thresholds were set to 3 to keep a base. Reads had to be 36 bases or greater in length. Sequence alignment was completed using the *Mus musculus* Ensembl v82 reference genome in addition to three custom references based on the strain. Samples from both brain and spleen tissue were edited to include human CR2 and one (or none) of three human sequences: CR1long, CR1short, or CR1 KO. Each of the three human sequences was appended to the *Mus musculus* reference genome individually; thus, three additional reference genomes were created to quantitate gene and isoform expression levels using RSEM v1.2.19. B6 samples were evaluated with the base *Mus musculus* reference genome. RSEM leveraged Bowtie2 alignment with strand-specific and paired-end parameters. The resulting gene/transcript (feature) count data were processed using edgeR v3.14.0 (R v3.3.1). For each of the four datasets (brain and spleen, gene and transcript), feature differential expression was evaluated in three pairwise comparisons per sex: 1.) CR1long vs. B6, 2.) CR1short vs. B6, and 3.) CR1KO vs. B6. Any feature that did not have at least 1 read per million for at least 2 samples in either sets of samples evaluated in the pairwise comparison was excluded from the differential expression analysis. The Cox-Reid profile-adjusted likelihood method was used to derive tagwise dispersion estimates based on a trended dispersion estimate. The GLM likelihood ratio test was used to evaluate differential expression in pairwise comparisons between sample groups. The Benjamini and Hochberg’s algorithm was used to control the false discovery rate (FDR). Features with an FDR-adjusted p-value < 0.05 were declared statistically significant.

**Gene set enrichment**

The Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.8) was used on each significant DE gene list for each pairwise comparison to identify enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and Gene Ontology (GO) terms. Background gene sets were all trimmed normalized gene reads. KEGG pathways and GO terms were considered enriched with a p-value less than 0.05 (p < 0.05). Ingenuity Pathway Analysis (IPA) was used for disease and functional analysis.

**Supplementary information**

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Authors’ contributions
HMI, BPM and GRH conceived the study. HMI performed experiments. ROR developed mouse cohorts. TMS and KEF performed analysis of transcriptional profiling data. DF validated and provided antibodies. HMI, KEF, GRH and BPM wrote the manuscript. All authors approved the submission of the manuscript.

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Availability of data and materials
The B6.CAxC57L/J mouse strain is available on request to gareth.howell@jax.org as well as from The Jackson Laboratory (https://www.jax.org/strain/027713) or MODEL-AD (http://model-ad.org). All RNA-seq data is being made available in GeoArchive (ID pending - data will be provided prior to acceptance of this manuscript and this statement will be amended accordingly).

Ethics approval and consent to participate
No human subjects or data was used in this study. All experiments involving mice were approved by the Animal Care and Use Committee at The Jackson Laboratory in accordance with guidelines set out in The Eighth Edition of the Guide for the Care and Use of Laboratory Animals. All euthanasia methods were approved by the American Veterinary Medical Association.

Consent for publication
Not applicable.

Competing interests
No financial or competing interests declared.

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