UC Irvine
UC Irvine Previously Published Works

Title
Peripheral complement interactions with amyloid β peptide in Alzheimer's disease: Polymorphisms, structure, and function of complement receptor 1

Permalink
https://escholarship.org/uc/item/83f1h262

Journal
Alzheimer's & Dementia, 14(11)

ISSN
1552-5260

Authors
Johansson, Jenny U
Brubaker, William D
Javitz, Harold
et al.

Publication Date
2018-11-01

DOI
10.1016/j.jalz.2018.04.003

Peer reviewed

eScholarship.org Powered by the California Digital Library
University of California

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

HHS Public Access

Author manuscript

Alzheimers Dement. Author manuscript; available in PMC 2019 November 01.

Published in final edited form as:

Alzheimers Dement. 2018 November ; 14(11): 1438–1449. doi:10.1016/j.jalz.2018.04.003.

Peripheral complement interactions with amyloid β peptide (Aβ) in Alzheimer's disease: polymorphisms, structure, and function of complement receptor 1

Jenny U. Johansson[a], **William D. Brubaker**[a], **Harold Javitz**[b], **Andrew W. Bergen**[a,1], **Denise Nishita**[a], **Abhishek Trigunaite**[a], **Andrés Crane**[a,2], **Justine Ceballos**[a], **Diego Mastroeni**[c], **Andrea J. Tenner**[d], **Marwan Sabbagh**[e], and **Joseph Rogers**[a,*]

[a]Biosciences Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025 USA

[b]Education Division, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025 USA

[c]The Biodesign Institute, Arizona State University, 727 East Tyler Street, Tempe, AZ 85287

[d]University of California, Irvine, 3205 McGaugh Hall, Irvine, CA 92697

[e]Alzheimer's and Memory Disorders Division, Barrow Neurological Institute, 350 West Thomas Road, Phoenix, AZ 85013

Abstract

INTRODUCTION—Genome-wide association studies consistently show that single nucleotide polymorphisms (SNPs) in the complement receptor 1 (*CR1*) gene modestly but significantly alter Alzheimer's disease (AD) risk. Follow-up research has assumed that CR1 is expressed in human brain, despite a paucity of evidence for its function there. Alternatively, erythrocytes contain >80% of the body's CR1, where, in primates, it is known to bind circulating pathogens.

METHODS—Multidisciplinary methods were employed.

RESULTS—Conventional Western blots and qPCR failed to detect CR1 in human brain. Brain immunohistochemistry revealed only vascular CR1. By contrast, erythrocyte CR1 immunoreactivity was readily observed and was significantly deficient in AD, as was CR1-mediated erythrocyte capture of circulating Aβ. *CR1* SNPs associated with decreased erythrocyte CR1 increased AD risk, whereas a *CR1* SNP associated with increased erythrocyte CR1 decreased AD risk.

*CORRESPONDING AUTHOR: Joseph Rogers, Ph.D., Biosciences Division, BN111, SRI International, 333 Ravenswood Avenue, Menlo Park, CA 94025 USA. Phone: 650-678-1422. joseph.rogers@sri.com.

[1]Present address: BioRealm LLC, 6101 W Centinela Avenue, Culver City, CA 90230; Oregon Research Institute, 1776 Millrace Drive, Eugene, OR 97403

[2]Present address: Department of Brain and Cognitive Sciences, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139

CONFLICTS OF INTEREST: None

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

DISCUSSION—SNP effects on erythrocyte CR1 likely underlie the association of CR1 polymorphisms with AD risk.

Keywords

complement receptor 1; CR1; complement C3b/C4b receptor 1; erythrocyte; red blood cell; amyloid β peptide; single nucleotide polymorphism; immune adherence; clearance

1.0 Background

Multiple genome-wide association studies (GWAS) have shown that single nucleotide polymorphisms (SNPs) in the complement receptor 1 (*CR1*) gene have a small but highly consistent effect on risk for Alzheimer's disease (AD) [1–6]. Efforts to elucidate physiologic reasons for this linkage have perhaps understandably begun with the assumption that CR1 is expressed in brain [7–11], particularly in microglia [12–14] where complement and neuroinflammatory mechanisms are most clearly evident in AD. However, there is no well-established role for CR1 in human brain and direct evidence even for its presence there is limited and conflicting. Gasque et al. [15], for example, reported CR1 immunoreactivity in astrocytes, but not neurons or microglia, in normal human adult and multiple sclerosis brain samples. Hazrati et al. [16], on the other hand, reported CR1 immunoreactivity in neurons, but not astrocytes or microglia in normal human adult brain samples, the exact opposite result. Singhrao et al. [17] were unable to detect CR1 protein in any of these cell types in adult human brain. Fonseca et al. [9] observed clear astrocyte staining and an absence of specific neuronal or microglial CR1 immunoreactivity with two novel antibodies from the laboratory of John Atkinson at Washington University [18]. Although the immunoreactivity was appropriately abolished after soluble CR1 preabsorption, neither antibody detected CR1 immunoreactivity in Western blots of brain homogenates, and the immunohistochemical staining in brain did not differ among AD and nondemented elderly control (ND) patients. Two recent studies have suggested that *CR1* mRNA expression is detected in AD cortical homogenates [19,20], but another report found that the only brain regions in which *CR1* mRNA could be detected in AD patients were frontal cortex white matter and cerebellum, and then only in very low amounts [21].

In contrast to the conflicting reports in brain, CR1 is known to play several critical roles in the periphery, the two most important of which are capture and clearance of complement-opsonized pathogens by erythrocyte and monocyte/macrophage CR1, and inhibition of spontaneous complement activation by soluble CR1 in the plasma. The vast majority of the body's CR1 (>80%) is, in fact, dedicated to the erythrocyte compartment [reviewed in 22,23]. Here, circulating antibodies that have bound their respective pathogens or antigens activate complement, resulting in fixation of complement opsonins (e.g., C3b, C4b) to the antibody/antigen complex. CR1 on the erythrocyte surface captures the complex via its multiple C3b/C4b binding sites. The bound complex is then ferried to the liver, where it is stripped off by liver Kupffer cells and degraded. This pathway, termed "immune adherence", is unique to primates (subprimate species do not have erythrocyte CR1), has been studied for over a half-century, and is widely considered to be a major mechanism for removal of circulating antigens/pathogens in primates [22,23]. Complement-dependent monocyte/

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

macrophage capture of circulating pathogens is similar, except that degradation of the pathogen occurs within the monocyte/macrophage.

Our previous studies have demonstrated that peripheral amyloid β peptide (Aβ) is subject to these well-known processes [24–27] (Fig. 1). Aβ itself, without antibody mediation, activates complement [24–30], is opsonized by complement [24,25,29,30], and is bound by erythrocyte and monocyte/macrophage CR1 [24–26]. We have also shown that Aβ capture by erythrocytes is significantly deficient in AD patients [24,25], and that the presence of Aβ antibodies, as in Aβ immunotherapy, dramatically enhances Aβ clearance into the erythrocyte and monocyte/macrophage compartments *in vitro* and in living non-human primates [26]. These results [24–30] and the equivocal findings on CR1 expression by brain cells [9,15–21] open the possibility that the effects of CR1 polymorphisms on AD risk [1–6] may be due to alterations in the ability of erythrocytes and monocytes/macrophages to clear circulating Aβ via CR1-dependent mechanisms. Deficits in such clearance might then impact brain Aβ metabolism by impairing efflux of brain Aβ or enhancing influx of circulating Aβ [31–35].

In the present report, we have evaluated the presence or absence of CR1 in brain, and the effects of CR1 SNPs on erythrocyte CR1 expression, erythrocyte CR1 structure, erythrocyte CR1-mediated capture of Aβ, and risk for AD. Our results confirm the small but highly-replicable link between CR1 and AD, but strongly suggest that the association is due to extremely well-established erythrocyte CR1 mechanisms for pathogen clearance [22,23] and not to hypothetical brain mechanisms.

2.0 Methods

2.1 Subjects and samples

***2.11 CR1* genotype, CR1 levels, CR1 structural isoforms, erythrocyte capture of Aβ, and their relationships to AD risk—**These variables were assayed using prospectively-obtained, intravenous blood samples from well-annotated, well-matched AD and nondemented elderly (ND) subjects who had been evaluated and diagnosed at a National Institute on Aging (NIA) Alzheimer's Disease Center, the Arizona AD Research Center. The AD group (N = 80) had a mean age of 80.8 ± 0.7 years, a mean MMSE score of 18.5 ± 0.6, and consisted of 60.0% males and 40.0% females. The ND group (N = 117) had a mean age of 80.9 ± 0.6 years, a mean MMSE score of 28.4 ± 0.2, and consisted of 42.7% males and 57.2% females.

***2.12* CR1 immunohistochemistry, Western blots, and quantitative polymerase chain reaction (qPCR)—**These studies were conducted on short postmortem (<4 hours from death to autopsy) neocortex samples from 4 neuropathologically-confirmed AD cases (mean age of 83 ± 4 years) and 4 neuropathologically-confirmed ND cases (mean age of 77 ± 4 years) provided by the Arizona AD Research Center Tissue Bank.

***2.13* Microglial gene expression—**To assess *CR1* gene expression specific to microglia, single cell laser capture methods were employed to collect a total of 21,600 microglia from hippocampus CA1 and midbrain substantia nigra sections of an additional 6

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

neuropathologically-confirmed, short postmortem AD cases (mean age of 75 ± 7 years), 6 neuropathologically-confirmed, short postmortem ND cases (mean age of 74 ± 6 years), and 6 neuropathologically-confirmed, short postmortem Parkinson's disease (PD) cases (mean age of 75 ± 16 years). These samples also derived from the Arizona AD Research Center Tissue Bank.

2.14 **Association of *CR1* SNPs with AD risk—**These analyses were based on genotype and phenotype data from six large-scale GWAS datasets furnished by the National Institute on Aging Genetics of Alzheimer's Disease Data Storage Site (NIAGADS). The datasets were the University of Pittsburgh GWAS (NG00026), TGEN2 GWAS (NG00028), ROSMAP GWAS (NG00029), Washington University GWAS (NG00030), MIRAGE GWAS (NG00031), and ACT and Genetic Differences GWAS (NG00034), which collectively included 3,925 AD and 4,997 ND subjects.

2.15 **Institutional Review Board approval—**Sample procurement and experimental protocols for all phases of the research were conducted after Institutional Review Board approval.

2.2 Immunohistochemistry and immunocytochemistry

Postfixed, cryoprotected neocortex blocks from 4 AD and 4 ND cases were sectioned at 40 μm and reacted with mouse monoclonal anti-CR1 antibody (#376223, clone E11) from US Biologicals (Salem, MA) or mouse monoclonal anti-CR1 (H2) antibody from Santa Cruz Biotechnology (Dallas, TX) using previously described methods [24–26] (see also Supplementary Material). To confirm the results, the experiments were replicated using a second, conventional immunohistochemistry protocol (Supplementary Material) and additional CR1 antibodies: clone E11 (Ancell, Bayport, MN) and clone J3D3 (Beckman Coulter, Indianapolis, IN). Selected sections were allowed to develop with chromogen for as long as 30 minutes. As a positive control for viability of the CR1 antibodies and immunohistochemical methods, erythrocyte preparations and brain sections containing morphologically-distinct vascular cells were also immunoreacted with the same CR1 antibodies and methods as employed for the brain samples.

2.3. Quantitative real-time PCR (qPCR)

Frozen neocortical and whole blood samples from each of the above 4 AD and 4 ND subjects were homogenized and total RNA extracted using RNeasy Mini Kits (Qiagen, Valencia, CA). Extracted RNA (250 ng/sample) was subjected to cDNA conversion using Omniscript RT Kits (Qiagen) and 10 μM Random Primers (Promega, Thermo Fisher Scientific, Waltham, MA). From the resulting material, 7 ng of cDNA/sample was amplified by SsoFast EvaGreen-based qPCR in a CFX96 Real-Time PCR machine (Bio-Rad, Hercules, CA) using two different primers for *CR1*, as well as primers for hemoglobin subunit alpha 2 (*HBA2*), glial fibrillary acidic protein (*GFAP*), and aldehyde dehydrogenase 1 family member L1 (*ALDH1L1*) (Supplementary Table 1). Quantification and copy number determinations were derived from standard curves for each of the primer pairs.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

2.4 Microglial gene expression

Snap-frozen limbic hippocampal CA1 and snap-frozen substantia nigra samples were obtained from each of 6 additional AD, 6 additional ND, and 6 additional PD cases. After sectioning at 10 μm on a cryostat, the sections were processed for single cell laser capture of microglia. The collected microglia were then assayed by immunohistochemical and gene expression methods using protocols we have previously described [36] (see also Supplementary Material). A total of 1,200 microglia were individually collected for each subject, yielding a total sample of 21,600 microglia. The data were expressed as Fragments per Kilobase Million (FPKM), a standard measure for gene expression that normalizes for sequencing depth and gene length.

2.5 Western blotting

Neocortex and erythrocyte membrane homogenates from the 4 AD and 4 ND cases employed for immunohistochemistry were subjected to CR1 non-reducing, denaturing Western blots using CR1 clone E11 antibody and protein loads of 20 μg/ml and 100 μg/ml per lane. A parallel membrane was probed at the same concentrations with rabbit monoclonal platelet glycoprotein VI (GPVI) antibody (Abcam). In the larger cohort of 80 AD and 117 ND subjects prospectively recruited for the study, CR1 Western blots with CR1 clone E11 were also employed to evaluate levels of CR1 and its structural isoforms, as well as to identify the isoform genotype for each patient (Fig. 2). All methods were as previously described [25,26] and are covered in detail in the Supplementary Materials.

2.6 Flow cytometry

Aβ antibody 4G8 (BioLegend, San Diego, CA) and mouse isotype control IgG (Thermo Fisher Scientific) were biotinylated using the EZ-Link NHS-PEG4 Biotin kit (Thermo Fisher Scientific) following the manufacturer's instructions. Erythrocyte membrane samples from the prospectively-enrolled 80 AD and 117 ND cases were incubated with the biotinylated antibodies and assayed for Aβ using conventional flow cytometry methods we have previously published [26] (see also Supplementary Material). A minimum of 10,000 events/sample were recorded on a Becton-Dickinson LSR II flow cytometer and analyzed for Aβ capture with FlowJo v10.1 (FlowJo, Ashland, OR).

2.7. CR1 genotyping

Using the prospectively-obtained blood samples from 80 AD and 117 ND subjects, a total of 30 *CR1* SNPs (Table 1) were chosen for evaluation based on previous GWAS reports on AD, associations with other disorders, or haplotype coverage. As a positive control, two apolipoprotein E (*APOE*) SNPs known to be associated with AD risk (rs429358 and rs7412) were also evaluated. To assess the SNPs, genomic DNA from buffy coat of prospectively-enrolled AD and ND subjects was extracted using Gentra PureGene Blood kits (Qiagen) and quantified by densitometry (N = 46 samples). Buffy coat was unavailable for the remaining 151 samples, and DNA was extracted from 3–4 ml plasma using QIAamp Circulating Nucleic Acid kits (Qiagen). Here, DNA was eluted with 35 μl 1:10 Tris-EDTA buffer and 1 μl was quantified in triplicate in a 9 μl reaction volume with a TaqMan RNase P Detection Reagents kit (Thermo Fisher Scientific) and Taqman Universal Master Mix II on a ViiA7

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

TaqMan instrument to estimate yields. The average yield of DNA from plasma was 17.0 ± 1.1 ng. The remaining eluted DNA was concentrated by Speedvac, and whole genome amplification (WGA) was performed on 3 μl samples using the REPLI-g Single Cell Kit (Qiagen). Genotyping was evaluated on the TaqMan OpenArray SNP Genotyping System (32 SNP array) or ViiA 7 instrument and included HapMap DNA samples for controls (Coriell Cell Repositories, Camden, NJ). OpenArray samples were run at the University of California, San Francisco, Helen Diller Family Comprehensive Cancer Center Genome Core. ViiA7 samples were run at SRI International. Data were analyzed using Genotyper Software. SNP genotyping assays were checked for clustering, concordance with replicate samples, and completion rates. Two of the original 30 *CR1* SNPs from the OpenArray Panel, rs6696840 and rs2025935, had <95% genotyping completion and were dropped from further analysis. Although we recognize that genotyping samples from WGA DNA may cause allelic bias, the viability of the plasma DNA approach, which has ample precedent in the literature [reviewed in 37], was strongly suggested by the fact that the vast majority of samples met the criteria of having a ≥87% call rate (samples with <87% call rate were dropped from the analysis), none of the assayed SNPs showed significant loss of heterozygosity with respect to Hardy-Weinberg equilibrium ($P > 0.10$), the frequencies of SNPs in our cohort were similar to those in much larger GWAS cohorts, and the *APOE* SNPs employed as a positive control exhibited highly significant associations with AD risk even in our small sample (N = 197) (e.g., for *APOE* rs429358: $z = 4.48$, $P \ll 0.001$, Odds Ratio (OR) = 4.89 ± 1.73, 95% Confidence Interval (CI) = 2.44 – 9.80).

Associations of the various SNPs with CR1, F isoform, and S isoform expression were assessed in the 80 AD and 117 ND individuals in this cohort. In addition, associations of the SNPs with AD risk were evaluated over the six GWAS datasets furnished by NIAGADS. Data from these datasets were phased and imputed for SNPs throughout the *CR1* region using Impute2 [38] and the 1000 Genomes Project Phase 3 reference dataset [39].

2.8. Statistics

Because we evaluated 28 *CR1* SNPs on four different outcome measures (112 total tests), the criterion significance level for SNP analyses was set at $P \leq 0.009$ (two-tailed) (i.e., one false positive result by chance alone in 112 tests). The remaining non-SNP analyses (e.g., effect of erythrocyte CR1 levels on erythrocyte Aβ capture) did not entail multiple comparisons and used a conventional significance level of $P \leq 0.05$ (two-tailed). Neither *APOE* genotype nor age proved to be significant covariates in the SNP analyses and their inclusion or exclusion had no effect on the statistical conclusions reached here. Likewise, a dominant model gave nearly identical results as an additive model. The results presented are therefore with a dominant model and no covariates.

3.0 Results

3.1. Peripheral versus CNS localization of CR1

Immunohistochemistry using commercially-available CR1 antibodies (clone E11, H2, J3D3) readily labeled peripheral erythrocytes. A representative micrograph with CR1 clone E11 is shown in Fig. 3A. Under the same conditions, even in extremely short postmortem (<4

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

hours) samples, there was no detectable CR1 immunoreactivity in AD or ND brain parenchyma (Fig. 3B), with the notable exception of CR1-immunoreactive profiles in the brain vasculature (inset, Fig. 3B, and Fig. 3C). These data are consistent with those of Fonseca et al. [9], who also failed to find material brain immunoreactivity with CR1 clone E11, H2, or J3D3 commercial antibodies after appropriate preabsorption controls were applied. In Western blots using CR1 clone E11 antibody, erythrocyte membrane homogenates gave clear CR1 immunoreactivity with conventional, 20 μg protein/lane loads, whereas short postmortem neocortex homogenates showed only faint CR1 immunoreactive bands even at extremely high, 100 μg protein/lane loads (Fig. 3D) where immunoreactivity for vascular-specific proteins such as platelet GPVI [40,41] (Fig. 3E) and hemoglobin (not shown) were equally or more evident. Likewise, the presence of *CR1* mRNA by qPCR was only detectable in short postmortem AD and ND neocortex homogenates at extremely high amplification levels where mRNAs for *HBA2*, a low-expression variant of hemoglobin A in human blood [42], also began to be observed (Fig. 4A). Conversely, mRNAs for blood-specific markers such as *CR1* and *HBA2* were readily observed at conventional amplification levels in blood samples (Fig. 4B).

Because several studies have attributed effects of CR1 SNPs on AD risk specifically to brain microglial mechanisms [e.g., 12–14], we also evaluated *CR1* gene expression in single cell laser captured AD and ND microglia unadulterated by other CNS cell types that are perforce collected in brain homogenates. Microglia-specific *CR1* gene transcripts were essentially not detectable in hippocampus CA1 or midbrain substantia nigra, with FPKMs of 0 for the vast majority of samples (94%) and an overall mean FPKM of 0.04 ± 0.03 for all samples. By contrast, transcripts for proteins known to be expressed by microglia such as CD45 and TLR5 were readily detected, with values on the order of 15–45 FPKMs (Fig. 4C).

3.2. Erythrocyte CR1 and Aβ clearance

The vast majority of the body's CR1 is present in the erythrocyte compartment, where CR1 is employed to capture and clear circulating, complement-opsonized antigens/pathogens [22,23]. Our previous studies [24–26] have demonstrated that circulating Aβ is subject to this major mechanism for pathogen removal. We therefore next evaluated whether changes in erythrocyte CR1 might help explain the association of CR1 polymorphisms with increased AD risk.

Expression of erythrocyte CR1 protein was significantly deficient in AD compared to ND subjects ($t = -2.11$, $P = 0.018$) (Fig. 5A), and these low levels of erythrocyte CR1 significantly increased AD risk ($z = 2.07$, $P = 0.038$, OR = 1.14 ± 0.07, 95% CI = 1.01–1.28). In turn, erythrocyte capture of Aβ, which is mediated by erythrocyte CR1 [24,25], was significantly deficient in the AD compared to the ND group ($t = -2.94$, $P = 0.002$) (Fig. 5B), replicating three previous studies by our group [24–26], and this deficit also significantly increased AD risk ($z = 2.79$ $P = 0.005$, OR = 1.02 ± 0.01, 95% CI = 1.01–1.03). These small but consistent effects on erythrocyte CR1 are wholly in accord with the small but consistent effects of CR1 SNPs on AD risk in previous GWAS reports [1–6].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

3.3. Effects of CR1 structural isoforms

CR1 is produced in four different isoforms, F, C, D, and S, each of which varies in its capacity to bind opsonized pathogens. The F (fast migrating on electrophoresis) isoform is by far the most common, with a frequency of 83% in the general population, whereas the S (slow migrating on electrophoresis) isoform occurs with a frequency of 15%. The C and D isoforms are quite rare, exhibiting frequencies of 1% or less [43]. In our sample, 66% of subjects were homozygous for the F isoform (F/F), 4% were homozygous for the S isoform (S/S), and 30% were heterozygous for the F and S isoforms (F/S). F/C and F/D subjects (<1% each) were too few to consider statistically and were dropped from further analyses.

Although the F isoform has fewer binding sites for opsonized pathogens, our data and a previous report by Hazrati et al. [16] suggest that this is compensated for by increases in the expression of F isoform CR1. Thus, subjects in our study who were homozygous for the F isoform (F/F) exhibited significantly greater total CR1 levels than F/S ($t = 3.16$, $P = 0.002$) or S/S ($t = 2.40$, $P = 0.018$) subjects, and there was a significant effect of the F isoform overall on CR1 levels ($t = 2.40$, $P = 0.004$) (Fig. 5C). Notably, the CR1-enhancing F/F genotype was significantly under-represented in the AD compared to the ND group ($z = -2.03$, $P = 0.042$) (Fig. 5D), and F isoform expression overall was significantly less in the AD compared to the ND group ($t = -2.01$, $P = 0.023$) (Fig. 5E). Deficits in erythrocyte F isoform levels, in turn, significantly increased AD risk ($z = 1.98$, $P = 0.048$, OR = 1.25 ± 0.14, 95% CI = 1.00–1.567). Results for the CR1 S isoform were less conclusive, and most likely reflect the much lower prevalence of that isoform relative to F. As discussed below, this does not, by any means, exclude a contribution of the S isoform to CR1 levels or AD risk.

3.4 Effects of CR1 SNPs on erythrocyte CR1 levels and structure

Of the 28 *CR1* SNPs evaluated, 18 were associated with significant decreases, and two were associated with significant increases in erythrocyte CR1 levels (Table 2). Consistent with our results on the relationship of erythrocyte F isoform expression to erythrocyte CR1 levels, 10 of the CR1-reducing SNPs were significantly associated with decreased expression of the F isoform, whereas the two CR1-increasing SNPs were significantly associated with increased expression of the F isoform (Table 2). A *CR1* SNP, rs6656401, of particular interest because of prior reports of its effects on the S isoform and AD risk [4,7], was also significantly associated with decreased total erythrocyte CR1. This SNP clearly co-segregated with the S genotype in our cohort: all S/S subjects possessed rs6656401, 89% of F/S subjects possessed rs6656401, and only 8% of F/F subjects possessed rs6656401. In fact, many of the CR1 SNPs that had an effect on F isoform expression and CR1 levels tended to co-segregate with the S genotype (Table 2). One interpretation of this result is that the SNPs are associated with presence or absence of the F or S isoform, as opposed to modulating their expression levels. By increasing the probability of an S isoform genotype, the occurrence of the alternate, CR1-enhancing F isoform genotype is proportionately lowered. Erythrocyte CR1 levels are therefore reduced from normal by the relative absence of the CR1-enhancing F genotype or, alternatively, by the relative presence of the S isoform. Because the prevalence of the F genotype is some five-fold greater than that of the S genotype, however, such effects are more readily observed statistically in measures of the F isoform than in measures of the

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

S isoform. In Table 2, for example, only two *CR1* SNPs were associated with changes in S isoform levels using our stringent significance criterion of $P \leq 0.009$, although seven other *CR1* SNPs had an effect on S isoform levels at $P < 0.05$ (statistics in parentheses in Table 2).

3.5 Effects of CR1 SNPs on AD risk

Over the six GWAS datasets supplied by NIAGADS, 10 CR1 SNPs that significantly altered erythrocyte CR1 levels also significantly altered AD risk (Table 2). Five of these 10 SNPs were in significant linkage disequilibrium with rs6656401 (Table 2). Importantly, the one lone SNP that was significantly protective for AD risk was a SNP that significantly enhanced erythrocyte CR1, whereas the remaining nine SNPs significantly decreased erythrocyte CR1 and significantly increased AD risk.

4.0 DISCUSSION

The present results strongly suggest that the consistent association of *CR1* polymorphisms with AD risk is related to well-established CR1-mediated peripheral mechanisms for pathogen clearance [reviewed in 22,23]. As summarized in Fig. 1, Aβ, like other circulating pathogens, is captured by erythrocyte and monocyte/macrophage CR1 and is subsequently degraded [24–26]. Both here (Fig. 5B) and in three previous studies [24–26] we have shown that this mechanism is deficient in AD patients, as is erythrocyte CR1 (Fig. 5A). Moreover, *CR1* polymorphisms that decrease peripheral CR1 and Aβ clearance increase AD risk, whereas *CR1* polymorphisms that increase peripheral CR1 and Aβ clearance decrease AD risk (Table 2). Given this converging evidence and the known roles of CR1 in peripheral clearance of pathogens, it is challenging to believe that peripheral CR1 interactions with Aβ do not significantly impact pathogenesis or risk for AD.

By contrast, previous efforts to localize CR1 to human brain have had mixed results [9,12,13,15–17,19–21] and, in our hands, conventional, commercially-available CR1 antibodies did not detect material amounts of CR1 in brain by any technique—including immunohistochemistry, Western blots, or qPCR—under normal conditions. Only under the most extreme conditions, where vascular contaminants became apparent, did any trace of brain CR1 begin to be seen. Fonseca et al. [9] obtained similar results with the same or similar commercially-available antibodies (i.e., clone E11, H2, J3D3). Although they did observe significant astrocyte staining with two novel CR1 antibodies, neither showed differential CR1 immunoreactivity in AD and ND samples, neither showed immunoreactivity in Western blots of brain homogenates, and neither was able to immunoprecipitate CR1 from brain samples [9]. Fonseca et al. [9] therefore concluded that “it is unlikely that astrocyte CR1 expression levels or C1q or C3b binding activity are the cause of the GWAS identified association of CR1 variants with AD.” Our studies showing no material *CR1* gene expression in some 21,600 single cell laser captured microglia also clearly challenge current speculation about a role for CR1 in microglial Aβ clearance.

With respect to alternative, peripheral mechanisms, our data demonstrate that changes in erythrocyte CR1 structure, expression, and ability to capture circulating Aβ are significantly associated with AD risk. These findings extend beyond erythrocyte mechanisms, since a specific *CR1* SNP manifest in a patient’s erythrocytes will also be present in the patient’s

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

macrophages, B-lymphocytes, and other CR1-expressing vascular cell types. Thus, it is possible that the CR1 SNPs we have studied affect multiple blood cell types in addition to erythrocytes and, through these cells, contribute to the findings. Our previous studies [26], for example, demonstrate that peripheral macrophages also capture circulating Aβ through CR1-dependent mechanisms. Their capacity for binding and degrading Aβ may therefore also be impaired or enhanced by the same *CR1* polymorphisms that influence Aβ clearance by erythrocytes.

In addition, although prior studies [7–11] have identified *CR1* SNPs that are associated with AD brain pathologies such as Aβ plaques and Aβ angiopathy, the mechanisms by which brain CR1 might influence such pathologies remain untested. Our studies, on the other hand, confirm the well-established fact that CR1 is present on human erythrocytes, and provide a mechanistic link between CR1 SNPs, erythrocyte CR1, vascular Aβ levels, and AD risk. Though speculative, it is not difficult to see how altering peripheral levels of Aβ could have an effect on brain Aβ metabolism, since there is clear evidence [reviewed in 33–35] for both Aβ influx from blood to brain and Aβ efflux from brain to blood (i.e., the "peripheral sink" hypothesis) [31,32]. Changes in the concentration gradient for Aβ between brain and blood as a result of failure to adequately clear circulating Aβ could also impact Aβ angiopathy, which is associated with the brain vasculature and meninges, not the brain parenchyma [reviewed in 44].

Our studies may also extend the findings from previous GWAS analyses of *CR1* polymorphisms, which have uniformly shown a modest but significant alteration in AD risk for various *CR1* SNPs [1–6]. Likewise, our data demonstrate a modest but significant impact of *CR1* SNPs on erythrocyte CR1 isoforms and levels, as well as modest but significant effects of these same SNPs on AD risk in GWAS cohorts. The symmetry of these results, moreover, is challenging to discount: *CR1* SNPs that significantly increased AD risk significantly decreased erythrocyte CR1, whereas the one *CR1* SNP that significantly decreased AD risk significantly increased erythrocyte CR1. These results therefore also strongly support the hypothesis that the association of *CR1* polymorphisms with AD risk is related to their effects on erythrocyte CR1.

Although our analyses have focused on the CR1 F isoform, other studies [4,7] have suggested a link between the CR1 S isoform and AD risk. In our relatively small cohort, S isoform expression was not different in AD and ND subjects, nor was it associated with AD risk, whereas F isoform expression was significantly deficient in AD compared to ND subjects and this deficiency was significantly associated with increased AD risk. It should be recognized, however, that because the F isoform is some five-fold more common in the population [43], analyses of the S isoform will almost always be statistically underpowered relative to analyses of the F isoform, as was the case in our study with only 197 subjects. Moreover, given the extreme rarity of the C and D isoforms, it could be said that, for genotype evaluations of AD risk, absence of the F isoform in one or the other allele is essentially the same as presence of the S isoform. For these reasons, we do not discount the findings of Keenan et al. [4] or Mahmoudi et al. [7] on the S isoform. On the contrary, we recognize that they may simply represent "the other side of the coin" from our results on the F isoform, or that the cumulative effects of both isoforms should be considered.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Although the effects of *CR1* polymorphisms on AD risk are small in our studies, consistent with GWAS efforts [1–6], this does not necessarily mean that the mechanisms they influence are trivial. Deficits in CR1-mediated erythrocyte clearance of pathogens have been linked to such disorders as lupus and malaria [e.g., 22,23,45,46] and, conversely, it is possible that ameliorating such deficits could be therapeutic [47,48]. In a companion study [26], for example, we showed, *in vitro* and *in vivo*, that the presence of Aβ antibodies dramatically enhances erythrocyte clearance of circulating Aβ, a mechanism that may better account for the ability of Aβ immunotherapy to remove brain Aβ than the extremely small amounts of Aβ antibody that actually reach the brain in Aβ immunotherapy [49–51].

Several limitations to the present study may be worth noting. With regard to our negative results on presence of CR1 in brain, it remains possible that a novel form of the molecule exists in brain that is not immunoreactive with multiple commercial antibodies that readily label CR1 in the periphery, as suggested by the results of Fonseca et al. [9]. A second consideration is that our studies of *CR1* SNPs were mostly conducted on extracts from plasma samples, whereas buffy coat extracts are the conventional preparation. Nonetheless, as previously described, the samples met numerous criteria for viability, including a ≥87% call rate, absence of significant loss of heterozygosity with respect to Hardy-Weinberg equilibrium, similar frequencies of SNPs in our cohort with those in much larger GWAS cohorts, and significant effects on AD risk of *APOE* SNPs that were included as a positive control.

In conclusion, the present research, as well as our previous studies [24–26], provides substantial, converging evidence that *CR1* polymorphisms impact AD risk by altering peripheral CR1 expression, and that mechanisms for the disposal of circulating Aβ may play a role in AD pathophysiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Experiments on human erythrocyte Aβ uptake were supported by the National Institute on Aging of the National Institutes of Health under award number RO1AG07367. Experiments on human CR1 and its genetics were supported by the National Institute on Aging of the National Institutes of Health under award number RO1AG039750. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. We especially thank the Arizona AD Research Center Tissue Bank, Dr. Thomas Beach, Ms. Lucia Sue, and Dr. Geidy E. Surrano for the generous provision of well-annotated human tissues used in this research. Genotypic and phenotypic data used here in GWAS analyses of AD risk were stored at the National Cell Repository for Alzheimer's Disease (NCRAD) at Indiana University, funded by NIA. Associated data were provided by the NIA-funded Alzheimer's Disease Centers and the National Alzheimer's Coordinating Center (NACC), and were stored at NCRAD, and at the NIA Alzheimer's Disease Data Storage Site (NIAGADS) at the University of Pennsylvania, funded by NIA. Contributors to the NIAGADS genetic analysis data included principal Investigators on projects that were individually funded by NIA, other NIH institutes, private U.S. organizations, or foreign governmental or nongovernmental organizations.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

References

1. Lambert JC, Heath S, Even G, Campion D, Sleegers K, Hiltunen M, et al. Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. Nat Genet. 2009; 41:1094–9. DOI: 10.1038/ng.439 [PubMed: 19734903]
2. Harold D, Abraham R, Hollingworth P, Sims R, Gerrish A, Hamshere ML, et al. Genome-wide association study identifies variants at CLU and PICALM associated with Alzheimer's disease. Nat Genet. 2009; 41:1088–93. DOI: 10.1038/ng.440 [PubMed: 19734902]
3. Pedraza O, Allen M, Jennette K, Carrasquillo M, Crook J, Serie D, et al. Evaluation of memory endophenotypes for association with CLU, CR1, and PICALM variants in black and white subjects. Alzheimers Dement. 2014; 10:205–13. DOI: 10.1016/j.jalz.2013.01.016 [PubMed: 23643458]
4. Keenan BT, Shulman JM, Chibnik LB, Raj T, Tran D, Sabuncu MR, et al. A coding variant in CR1 interacts with APOE-epsilon4 to influence cognitive decline. Hum Mol Genet. 2012; 21:2377–88. DOI: 10.1093/hmg/dds054 [PubMed: 22343410]
5. Jin C, Li W, Yuan J, Xu W, Cheng Z. Association of the CR1 polymorphism with late-onset Alzheimer's disease in Chinese Han populations: a meta-analysis. Neurosci Lett. 2012; 527:46–9. DOI: 10.1016/j.neulet.2012.08.032 [PubMed: 22960360]
6. Corneveaux JJ, Myers AJ, Allen AN, Pruzin JJ, Ramirez M, Engel A, et al. Association of CR1, CLU and PICALM with Alzheimer's disease in a cohort of clinically characterized and neuropathologically verified individuals. Hum Mol Genet. 2010; 19:3295–301. DOI: 10.1093/hmg/ddq221 [PubMed: 20534741]
7. Mahmoudi R, Kisserli A, Novella JL, Donvito B, Drame M, Reveil B, et al. Alzheimer's disease is associated with low density of the long CR1 isoform. Neurobiol Aging. 2015; 36:1766.doi: 10.1016/j.neurobiolaging.2015.01.006
8. Shulman JM, Chen K, Keenan BT, Chibnik LB, Fleisher A, Thiyyagura P, et al. Genetic susceptibility for Alzheimer disease neuritic plaque pathology. JAMA Neurol. 2013; 70:1150–1157. DOI: 10.1001/jamaneurol.2013.2815 [PubMed: 23836404]
9. Fonseca MI, Chu S, Pierce AL, Brubaker WD, Hauhart RE, Mastroeni D, et al. Analysis of the Putative Role of CR1 in Alzheimer's Disease: Genetic Association, Expression, and Function. PLoS One. 2016; 11(2):e0149792.doi: 10.1371/journal.pone.0149792 [PubMed: 26914463]
10. Biffi A, Shulman JM, Jagiella JM, Cortellini L, Ayres AM, Schwab K, et al. Genetic variation at CR1 increases risk of cerebral amyloid angiopathy. Neurology. 2012; 78:334–41. DOI: 10.1212/WNL.0b013e3182452b40 [PubMed: 22262751]
11. Chibnik LB, Shulman JM, Leurgans SE, Schneider JA, Wilson RS, Tran D, et al. CR1 is associated with amyloid plaque burden and age-related cognitive decline. Ann Neurol. 2011; 69:560–9. DOI: 10.1002/ana.22277 [PubMed: 21391232]
12. Villegas-Llerena C, Phillips A, Garcia-Reitboeck P, Hardy J, Pocock JM. Microglial genes regulating neuroinflammation in the progression of Alzheimer's disease. Curr Opin Neurobiol. 2016; 36:74–81. DOI: 10.1016/j.conb.2015.10.004 [PubMed: 26517285]
13. Crehan H, Hardy J, Pocock J. Blockage of CR1 prevents activation of rodent microglia. Neurobiol Dis. 2013; 54:139–49. DOI: 10.1016/j.nbd.2013.02.003 [PubMed: 23454195]
14. Efthymiou AG, Goate AM. Late onset Alzheimer's disease genetics implicates microglial pathways in disease risk. Mol Neurodegener. 2017; 12:43.doi: 10.1186/s13024-017-0184-x [PubMed: 28549481]
15. Gasque P, Chan P, Mauger C, Schouft MT, Singhrao S, Dierich MP, et al. Identification and characterization of complement C3 receptors on human astrocytes. J Immunol. 1996; 156:2247–55. [PubMed: 8690915]
16. Hazrati LN, Van Cauwenberghe C, Brooks PL, Brouwers N, Ghani M, Sato C, et al. Genetic association of CR1 with Alzheimer's disease: a tentative disease mechanism. Neurobiol Aging. 2012; 33:2949.doi: 10.1016/j.neurobiolaging.2012.07.001
17. Singhrao SK, Neal JW, Rushmere NK, Morgan BP, Gasque P. Differential expression of individual complement regulators in the brain and choroid plexus. Lab Invest. 1999; 79:1247–59. [PubMed: 10532588]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

18. Nickells M, Hauhart R, Krych M, Subramanian VB, Geoghegan-Barek K, Marsh HC Jr, Atkinson JP. Mapping epitopes for 20 monoclonal antibodies to CR1. Clin Exp Immunol. 1998; 112:27–33. [PubMed: 9566786]
19. Karch CM, Jeng AT, Nowotny P, Cady J, Cruchaga C, Goate AM. Expression of novel Alzheimer's disease risk genes in control and Alzheimer's disease brains. PLoS One. 2012; 7:e50976.doi: 10.1371/journal.pone.0050976 [PubMed: 23226438]
20. Allen M, Kachadoorian M, Carrasquillo MM, Karhade A, Burgess JD, Wang C, et al. Late-onset Alzheimer's disease risk variants mark brain regulatory loci. Neurol Genet. 2015; :e15.doi: 10.1212/NXG.0000000000000012 [PubMed: 27066552]
21. Holton P, Ryten M, Nalls M, Trabzuni D, Weale ME, Hernandez D, et al. Initial assessment of the pathogenic mechanisms of the recently identified Alzheimer risk loci. Ann Hum Genet. 2013; 77:85–105. DOI: 10.1111/ahg.12000 [PubMed: 23360175]
22. Birmingham DJ, Hebert LA. CR1 and CR1-like: the primate immune adherence receptors. Immunol Rev. 2001; 180:100–11. [PubMed: 11414352]
23. Hess C, Schifferli JA. Immune adherence revisited: novel players in an old game. News Physiol Sci. 2003; 18:104–8. [PubMed: 12750445]
24. Rogers J, Li R, Mastroeni D, Grover A, Leonard B, Ahern G, et al. Peripheral clearance of amyloid beta peptide by complement C3-dependent adherence to erythrocytes. Neurobiol Aging. 2006; 27:1733–9. DOI: 10.1016/j.neurobiolaging.2005.09.043 [PubMed: 16290270]
25. Brubaker WD, Crane A, Johansson JU, Yen K, Garfinkel K, Mastroeni D, et al. Peripheral complement interactions with amyloid β peptides in Alzheimer's disease: Erythrocyte clearance mechanisms. Alzheimers Dement. 2017; 13:1397–1409. DOI: 10.1016/j.jalz.2017.03.010 [PubMed: 28475854]
26. Crane A, Brubaker WD, Johansson JU, Trigunaite A, Ceballos J, Bradt B, et al. Peripheral complement interactions with amyloid β peptide in Alzheimer's disease: Relationship to Aβ immunotherapy. Alzheimers Dement. 2018; 14:243–52. DOI: 10.1016/j.jalz.2017.04.015 [PubMed: 28755839]
27. Rogers J, Cooper NR, Webster S, Schultz J, McGeer PL, Styren SD, et al. Complement activation by beta-amyloid in Alzheimer disease. Proc Natl Acad Sci USA. 1992; 89:10016–20. [PubMed: 1438191]
28. Jiang H, Burdick D, Glabe CG, Cotman CW, Tenner AJ. beta-Amyloid activates complement by binding to a specific region of the collagen-like domain of the C1q A chain. J Immunol. 1994; 152:5050–9. [PubMed: 8176223]
29. Bradt BM, Kolb WP, Cooper NR. Complement-dependent proinflammatory properties of the Alzheimer's disease beta-peptide. J Exp Med. 1998; 188:431–8. [PubMed: 9687521]
30. Watson DM, Roher AE, Kim KS, Spiegel K, Emmerling MR. Complement labeling of aggregated Aβ1-42 by normal human serum involves the classical and alternative pathways. In: Iqbal K, Winblad B, Nishimura T, Takeda M, Wisniewski HM, editorsAlzheimer's Disease: Biology, Diagnostics and Therapeutics. San Francisco: John Wiley and Sons; 1997. 365–73.
31. DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. Peripheral A beta antibody alters CNS and plasma A beta clearance and decreases brain A beta burden in a mouse model of Alzheimer's disease. Proc Natl Acad Sci USA. 2001; 98:8850–5. DOI: 10.1073/pnas.151261398 [PubMed: 11438712]
32. DeMattos RB, Bales KR, Cummins DJ, Paul SM, Holtzman DM. Brain to plasma amyloid-beta efflux: a measure of brain amyloid burden in a mouse model of Alzheimer's disease. Science. 2002; 295:2264–7. DOI: 10.1126/science.1067568 [PubMed: 11910111]
33. Morrone CD, Liu M, Black SE, McLaurin J. Interactions between therapeutic interventions for Alzheimer's disease and physiological Aβ clearance mechanisms. Front Aging Neurosci. 2015; 7:64.doi: 10.3389/fnagi.2015.00064 [PubMed: 25999850]
34. Marques F, Sousa JC, Sousa N, Palha JA. Blood-brain-barriers in aging and Alzheimer's disease. Mol Neurosci. 2013; 8:38.doi: 10.1186/1750-1326-8-38
35. Morgan D. Immunotherapy for Alzheimer's disease. J Intern Med. 2013; 269:54–63. DOI: 10.1111/j.1365-2796.2010.02315.x

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

36. Mastroeni D, Nolz J, Sekar S, Delvaux E, Serrano G, et al. Laser-captured microglia in the Alzheimer's and Parkinson's brain reveal unique regional expression profiles and suggest a potential role for hepatitis B in the Alzheimer's brain. Neurobiol Aging. 2018; 63:12–21. DOI: 10.1016/j.neurobiolaging.2017.10.019 [PubMed: 29207277]
37. Luke JJ, Oxnard GR, Paweletz CG, Camidge DR, Heymach DG, Solit DB, Johnson BE. Cell Free Working Group. Realizing the potential of plasma genotyping in an age of genotype-directed therapies. J Nat Cancer Inst. 2014; 106:1–5. DOI: 10.1093/jnci/dju214
38. Howie BN, Donnelly P, Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. PLoS Genet. 2009; 5:e1000529.doi: 10.1371/journal.pgen.1000529 [PubMed: 19543373]
39. Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, et al. 1000 Genomes Project Consortium. A global reference for human genetic variation. Nature. 2015; 526:68–74. DOI: 10.1038/nature15393 [PubMed: 26432245]
40. Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific collagen receptor from the immunoglobulin superfamily. Blood. 2000; 96:1798–807. [PubMed: 10961879]
41. Induruwa I, Jung SM, Warburton EA. Beyond antiplatelets: the role of glycoprotein VI in ischemic stroke. Int J Stroke. 2016; 11:618–25. DOI: 10.1177/1747493016654532 [PubMed: 27312676]
42. Steinberg MH, Rodgers GP. HbA2: biology, clinical relevance and a possible target for ameliorating sickle cell disease. Br J Haematol. 2015; 170:781–7. DOI: 10.1111/bjh.13570 [PubMed: 26104837]
43. Krych-Goldberg M, Atkinson JP. Structure-function relationships of complement receptor type 1. Immunological Reviews. 2001; 180:112–122. [PubMed: 11414353]
44. Qi XM, Ma JF. The role of amyloid beta clearance in cerebral amyloid angiopathy: more potential therapeutic targets. Transl Neurodegener. 2017; 6:22.doi: 10.1186/s40035-017-0091-7 [PubMed: 28824801]
45. Ren N, Kuang YM, Tang QL, Cheng L, Zhang CH, Yang ZQ, He YS, Zhu YC. High incidence of malaria along the Sino-Burmese border Is associated with polymorphisms of CR1, IL-1A, IL-4R, IL-4, NOS, and TNF, but not with G6PD deficiency. Medicine (Baltimore). 2015; 94:e1681. doi: 10:1097/MD.0000000000001681. [PubMed: 26448013]
46. Bao L, Cunningham PN, Quigg RJ. Complement in Lupus Nephritis: new perspectives. Kidney Dis (Basel). 2015; 1:91–9. DOI: 10.1159/000431278 [PubMed: 27536669]
47. Lim NT, Harder MJ, Kennedy AT, Lin CS, Weir C, Cowman AF, et al. Characterization of inhibitors and monoclonal antibodies that modulate the interaction between Plasmodium falciparum Adhesin PfRh4 and its erythrocyte receptor complement receptor 1. J Biol Chem. 2015; 290:25307–21. DOI: 10.1074/jbc.M115.657171 [PubMed: 26324715]
48. Das N, Biswas B, Khera R. Membrane-bound complement regulatory proteins as biomarkers and potential therapeutic targets for SLE. Adv Exp Med Biol. 2013; 735:55–81. [PubMed: 23402019]
49. Levites Y, Smithson LA, Price RW, Dakin RS, Yuan B, Sierks MR, et al. Insights into the mechanisms of action of anti-Abeta antibodies in Alzheimer's disease mouse models. FASEB J. 2006; 20:2576–8. DOI: 10.1096/fj.06-6463fje [PubMed: 17068112]
50. Goure WF, Krafft GA, Jerecic J, Hefti F. Targeting the proper amyloid-beta neuronal toxins: a path forward for Alzheimer's disease immunotherapeutics. Alzheimers Res Ther. 2014; 6:42.doi: 10.1186/alzrt272 [PubMed: 25045405]
51. Golde TE. Open questions for Alzheimer's disease immunotherapy. Alzheimers Res Ther. 2014; 6:3.doi: 10.1186/alzrt233 [PubMed: 24393284]
52. Roit I, Brostoff J, Male D. Immunology. 3. London: Mosby; 1993. 1.7
53. Paccaud JP, Carpentier JL, Schifferli JA. Direct evidence for the clustered nature of complement receptor type 1 on the erythrocyte membrane. J Immunol. 1988; 141:3889–94. [PubMed: 2972779]
54. Jandrot-Perrus M, Busfield S, Lagrue AH, Xiong X, Debili N, Chickering T, et al. Cloning, characterization, and functional studies of human and mouse glycoprotein VI: a platelet-specific

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

collagen receptor from the immunoglobulin superfamily. Blood. 2000; 96:1798–807. [PubMed: 10961879]

55. Induruwa I, Jung SM, Warburton EA. Beyond antiplatelets: the role of glycoprotein VI in ischemic stroke. Int J Stroke. 2016; 11:618–25. DOI: 10.1177/1747493016654532 [PubMed: 27312676]

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

RESEARCH IN CONTEXT

Systematic Review

Relevant literature on complement receptor 1 (CR1) and Alzheimer's disease (AD) was identified using PubMed. A brief summary of the findings is as follows. Genome-wide association studies (GWAS) have consistently shown that single nucleotide polymorphisms (SNPs) in the *CR1* gene significantly alter AD risk. Follow-up research has assumed that CR1 is expressed in human brain, despite a paucity of evidence for its function there. Alternatively, erythrocytes contain >80% of the body's CR1, where, in primates, it is known to bind circulating pathogens.

Interpretation

Our findings may help clarify the mechanism by which *CR1* SNPs impact AD and AD risk. Namely, they do so by altering erythrocyte and macrophage CR1 expression and their ability to capture circulating pathogens, including amyloid-β peptide.

Future Directions

Replication in larger samples and *CR1* haplotype analyses of GWAS cohorts may further confirm a primary role of peripheral CR1 in AD risk.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

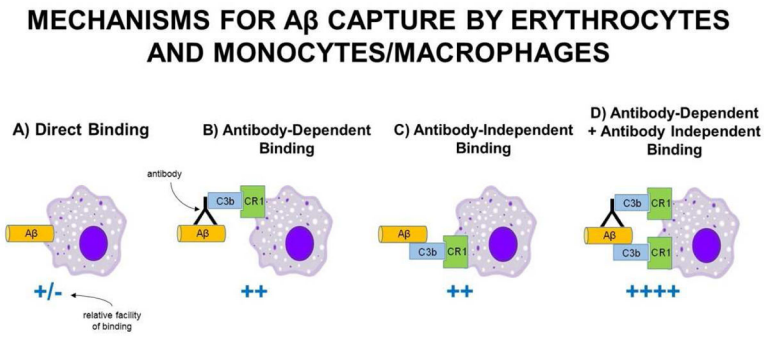

Fig. 1. Schematic of the different mechanisms by which cell-surface expression of CR1 mediates capture of circulating Aβ by monocytes/macrophages and erythrocytes [21,22,27] (adapted from Roit, Brostoff, and Male) [52]

A) Direct binding and degradation of pathogens such as Aβ can occur without CR1 mediation, but is typically less effective than CR1-dependent mechanisms [52]. **B)** Classical immune adherence occurs when pathogen/antibody immune complexes activate complement, resulting in fixation of the immune complex with complement opsonins, particularly C3b. C3b is a primary ligand for CR1 and therefore binds the immune complex to CR1 on monocyte/macrophage and erythrocyte surfaces. Such binding is considered to be more effective/facile than direct binding [52], as is the case for Aβ [26]. After capture of the pathogen/immune complex, monocyte/macrophages then internalize and degrade the pathogen. Erythrocytes, however, ferry the bound immune complex to the liver, where the pathogen is stripped off by Kupffer cells and degraded [22,23]. **C)** Aβ in its aggregated state is one of a handful of peptides that can activate complement without antibody mediation [24–30]. As a result, it is tagged with complement opsonins such as C3b [29,30] and becomes subject to capture by CR1. **D)** The most efficacious binding and degradation of pathogens occurs when both antibody-dependent (Panel B) and antibody-independent (Panel C) mechanisms of complement opsonization occur (Panel D) [52].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

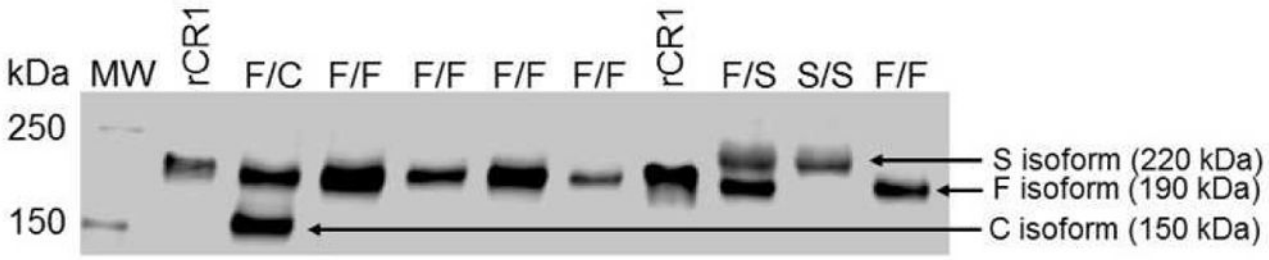

Fig. 2. Expression of CR1 and its structural isoforms

CR1 is synthesized in four different structural isoforms. The F (fast migrating on electrophoresis) isoform has a molecular weight of ~190 kDa; the S (slow migrating on electrophoresis) isoform has a molecular weight of ~220 kDa. The C (~160 kDa) and D (~250 kDa) isoforms are quite rare (<1% of the population) [43]. Western blots with CR1 clone E11 antibody reveal these structural isoforms. For example, the patient in lane 3 (F/C) has one allele that expresses the F isoform and one allele that expresses the C isoform, whereas the patients in lanes 4–6 (F/F) are homozygous for the F isoform, and the patient in lane 9 (S/S) is homozygous for the S isoform. In addition to defining the structural isoform genotype for each patient, Western blots of CR1 have the further advantage of providing separate expression measures for each isoform (i.e., densitometry of each isoform b and) as well as measures of total erythrocyte CR1 (i.e., sum of the levels for each band). (MW) Molecular weight standards. (rCR1) Recombinant CR1.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

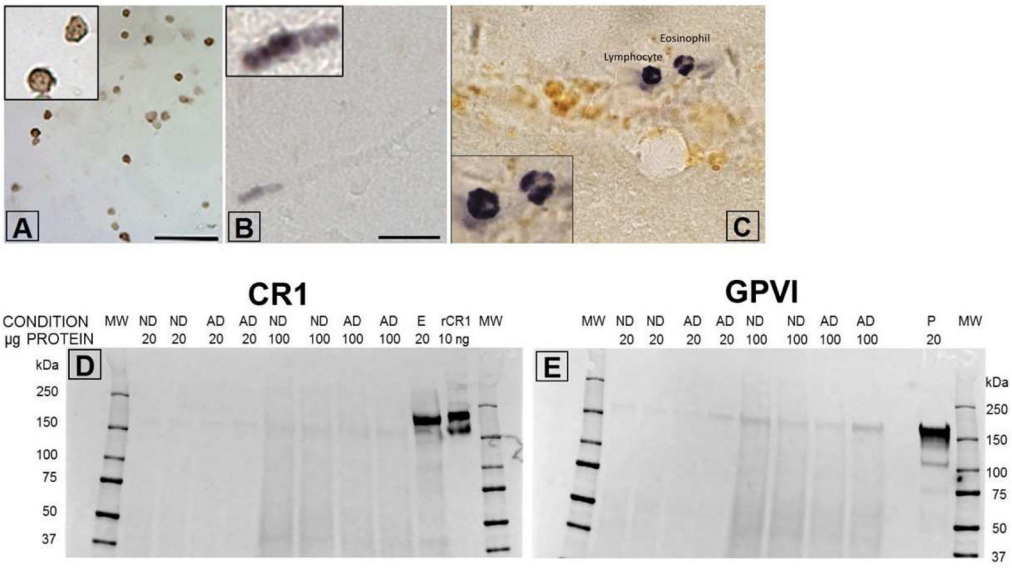

Fig. 3. CR1 immunocytochemistry, immunohistochemistry, and Western blots of blood and brain samples

A) Representative immunocytochemistry of erythrocyte ghosts from a normal elderly subject using a conventional, commercial CR1 antibody (#376223, clone E11, US Biologicals, Salem, MA) directed at an epitope in the C3b/C4b binding region of CR1. CR1 immunoreactivity was readily detected at the cell surface and in clusters, characteristics of this cell surface receptor [53]. **B)** Representative immunohistochemistry of a <4 hours postmortem cortical brain section from an AD case using the same antibody and conditions as in Panel A. No material staining of any cell type was observed in any of the 4 AD or 4 ND patients studied, except for staining of cells that clearly lay in the brain vasculature (inset). Similar results were obtained with the three other CR1 antibodies employed in the experiments. **C)** In addition to erythrocytes, other blood cells known to express CR1 were detected in the brain vasculature by these methods. For example, CR1 immunoreactive eosinophils could be identified based on their multi-nucleated morphology (inset). **D)** In Western blots with CR1 clone E11 antibody and conventional 20 µg/ml/lane protein loads, CR1 immunoreactive bands were readily detected in erythrocyte samples (E) (lane 10) at the same molecular weight as recombinant CR1 (rCR1) (lane 9). Under the same conditions, only faint traces of immunoreactive CR1 were observed for the neocortical samples even at extremely high, 100 µg/ml/lane loads. **E)** Nearly identical results were obtained for the same samples in parallel blots of GPVI, a platelet marker that is expressly not found in brain [54], strongly suggesting that it (and putative brain CR1) are simply vascular contaminants that are inherently trapped in brain homogenates. Autofluorescent and immunoreactive hemoglobin could also be observed at these high protein concentrations (not shown). (P) Plasma sample showing bands for GPVI, which appear as multimers of the 62 kDa monomer form [55].

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

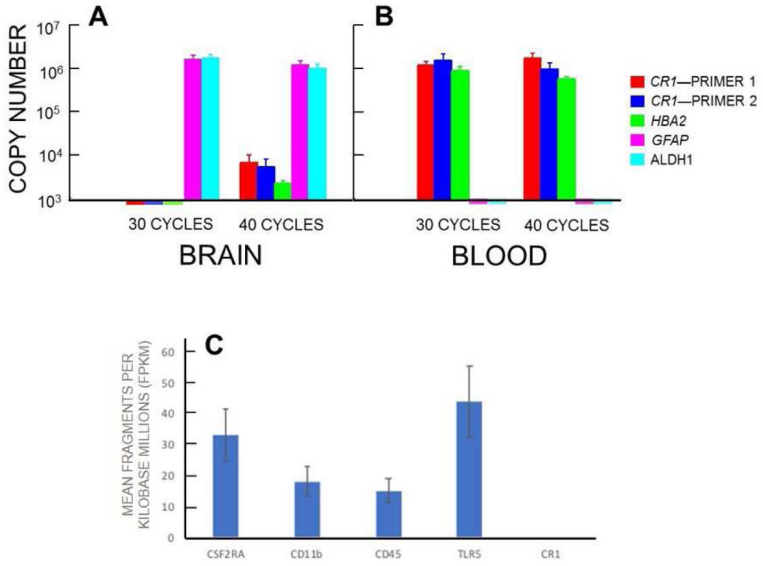

Fig. 4. *CR1* gene expression in cortical homogenates and microglia

A) Log plot of qPCR for *CR1* mRNA and mRNAs for other blood and brain proteins in brain samples. At 30 amplification cycles, mRNAs for brain-specific markers such as *GFAP* and *ALDH1L1* were readily detected in brain homogenates from four AD and four ND subjects, whereas, in the same samples, there was no detectable mRNA for *CR1* with either of two different primers. Likewise, mRNAs for the hemoglobin marker *HBA2* [42] exhibited only trace values. Nonetheless, in these same samples, small but consistent amounts of mRNAs for *CR1* and *HBA2* began to be observed after 40 amplification cycles, although their copy numbers were still some 2 orders of magnitude less than those for the brain-specific markers. **B)** As expected, using the same primers, mRNAs for *CR1* and *HBA2* were readily detected in blood samples at both 30 and 40 amplification cycles. By contrast, neither of the brain-specific markers had detectable mRNAs at 30 amplification cycles, and only trace amounts of astrocyte-specific *GFAP* mRNA were observed at 40 cycles. **C)** In single cell laser captured microglia (N = 21,600) from 6 AD, 6 ND, and 6 PD cases, *CR1* mRNA expression was essentially background (mean FPKM = 0.04 ± 0.03), with 0 values for 94% of samples, whereas mRNAs for other markers known to be expressed by microglia were readily observed in all samples.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

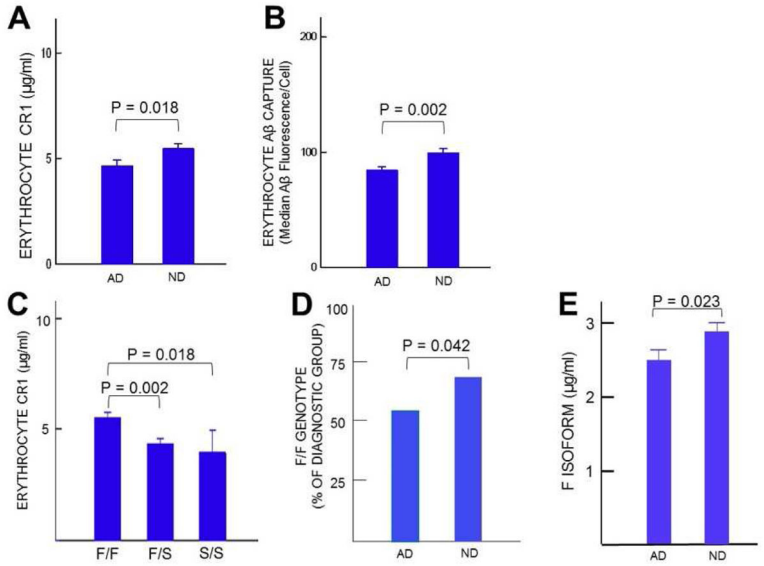

Fig. 5. Erythrocyte CR1, F isoform, S isoform, and Aβ levels in AD (N = 80) and ND (N = 117) subjects

A) Erythrocyte CR1 expression as measured by densitometry of Western blots using AD and ND erythrocyte membrane preparations. **B)** Erythrocyte Aβ levels as measured by flow cytometry. These data confirm our earlier reports of deficient erythrocyte Aβ capture using ELISA methods [24–26]. **C)** Relationship of erythrocyte CR1 expression to the structural isoforms of CR1. Possession of the F isoform in one or both alleles significantly increased total erythrocyte CR1 levels, in keeping with previous findings [16]. Homozygosity for the F allele (F/F) was particularly potent in this regard. **D)** Homozygosity for the CR1-enhancing F/F genotype was significantly under-represented in AD compared to ND subjects. **E)** Likewise, expression levels of the F isoform overall were significantly deficient in AD compared to ND subjects.

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

TABLE 1

CR1 and A*po E* SNPs evaluated in the present study for their relationships to erythrocyte clearance mechanisms and AD risk

Taqman Assay ID	Gene Symbol	Chromosome	NCBI SNP Reference
AHPAU5A	CR1	1	rs12734030
C__25598595_10	CR1	1	rs17259045
C____904973_10	APOC1;TOMM40;APOE	19	rs7412
C__16092063_10	CR2;CR1	1	rs2182911
C__30096371_10	CR2;CR1	1	rs9429781
C___8828478_10	CR2;CR1	1	rs1571344
C__12082954_10	CR1	1	rs2025935
C__12080026_10	CR1	1	rs646817
C__16092052_10	CR2;CR1	1	rs2182913
C__28011832_10	CR1	1	rs4310446
C__30033241_10	CR1	1	rs6656401
C__12080028_20	CR1	1	rs2274566
C__12080027_10	CR1	1	rs2274567
C__25472615_10	CR1	1	rs6691117
C__25598588_10	CR1	1	rs3818361
C__26971223_10	CR1	1	rs11803956
C__30510279_10	CR2;CR1	1	rs4618971
C__30213243_20	CR1	1	rs6701713
C__27978336_10	CR1	1	rs4844610
C___8828838_1_	CR1	1	rs1408077
C__31805093_10	CR1L;CR1	1	rs10779340
C___8828824_10	CR1L;CR1	1	rs1323720
C__25598594_10	CR1	1	rs41274768
C__30168451_10	CR2;CR1	1	rs9429940
C__26971293_10	CR1	1	rs10127904
C__26971230_10	CR1	1	rs12036785
C__26971198_10	CR1L;CR1	1	rs6696840
C__31805085_10	CR1L;CR1	1	rs12080578
C__26228458_10	CR1	1	rs12567990
C___8828813_10	CR1L;CR1	1	rs1323722
C__26971190_10	CR1L;CR1	1	rs6540437
C___3084793_20	APOC1;TOMM40;APOE	19	rs429358

Author Manuscript Author Manuscript Author Manuscript Author Manuscript

Alzheimers Dement. Author manuscript; available in PMC 2019 November 01.

Table 2

Effect of *CR1* SNPs on Erythrocyte CR1 Levels, Erythrocyte CR1 Structural Isoforms, and AD Risk

	SNP Effect on Erythrocyte CR1 Levels[2]		SNP Effect on Erythrocyte F Isoform Levels		SNP Effect on Erythrocyte S Isoform Levels[3]		% of Genotype Possessing SNP[4]			Effect on AD Risk (GWAS Cohorts)			
***CR1* SNP[1]**	**t**	**P**	**t**	**P**	**t**	**P**	**F/F**	**F/S**	**S/S**	**z**	**P**	**OR ± SEM**	**95% CI**
rs2274566	−6.80	0.000	−5.04	0.000	−3.51	0.001	49	92	100	+2.86	0.004	1.10 ± 0.04	1.03–1.17
rs12036785	−6.01	0.000	−3.93	0.000	−2.75	0.008	41	93	86	+3.46	0.001	1.12 ± 0.04	1.05–1.19
rs2182913	−4.63	0.000	−3.53	0.001	(−2.08	0.042)	40	84	100				
rs9429781	−4.62	0.000	−2.73	0.007			24	80	100	+2.76	0.006	1.12 ± 0.05	1.03–1.21
rs4310446	−3.39	0.001			(−2.04	0.045)	19	81	100	+2.63	0.009	1.13 ± 0.05	1.03–1.25
rs9429940	−3.34	0.001			(−2.04	0.045)	15	52	100				
rs4618971	−4.50	0.000	−2.72	0.007			23	80	57				
rs2182911	−3.70	0.000			(−2.29	0.025)	27	35	42				
rs6656401	−3.63	0.000					8	89	100	+3.30	0.001	1.14 ± 0.05	1.05–1.23
rs3818361	−4.07	0.000			(−2.60	0.012)	7	88	86	+3.22	0.001	1.14 ± 0.05	1.05–1.23
rs6701713	−4.07	0.000			(−2.63	0.011)	7	91	86	+3.26	0.001	1.14 ± 0.05	1.05–1.23
rs4844610	−3.60	0.000					7	80	86	+3.06	0.002	1.13 ± 0.05	1.05–1.22
rs1408077	−4.01	0.000			(−2.24	0.029)	7	85	86	+3.11	0.002	1.13 ± 0.05	1.05–1.23
rs10127904	+3.11	0.002	+3.37	0.001			58	45	14	−2.60	0.009	0.91 ± 0.03	0.85–0.98
rs17259045	+3.55	0.000	+3.25	0.001			25	18	0				
rs12567990	−3.72	0.000	−5.44	0.000			35	21	0				
rs12734030	−3.82	0.000	−5.14	0.000			26	20	0				
rs2274567	−3.95	0.000	−5.56	0.000			32	21	0				
rs646817	−3.97	0.000	−6.22	0.000			45	24	0				
rs6691117	−3.86	0.000	−6.16	0.000			44	22	0				

[1] Color-coding denotes SNPs found to be in significant ($R^2 > 0.40$) linkage disequilibrium (LD) with each other.

[2] Negative and positive t statistics indicate negative and positive effects of the SNPs on the various outcome measures. P values that were less than 0.001 are designated as 0.000.

[3] Statistics in parentheses met conventional levels of significance ($P \leq 0.05$), but did not meet our more stringent criterion ($P \leq 0.009$) that was imposed to account for multiple comparisons of the 28 SNPs tested (4 × 28 analyses). The additional statistics are included for the benefit of investigators who may have special interest in these particular SNPs.

[4] Percent of subjects with the F/F, F/S, or S/S genotype who carried a particular SNP (e.g., 100% of S/S subjects carried rs2274566).