Peripheral complement interactions with amyloid β peptide: Erythrocyte clearance mechanisms

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Abstract

Introduction: Although amyloid β peptide (Aβ) is cleared from the brain to cerebrospinal fluid and the peripheral circulation, mechanisms for its removal from blood remain unresolved. Primates have uniquely evolved a highly effective peripheral clearance mechanism for pathogens, immune adherence, in which erythrocyte complement receptor 1 (CR1) plays a major role.

Methods: Multidisciplinary methods were used to demonstrate immune adherence capture of Aβ by erythrocytes and its deficiency in Alzheimer’s disease (AD).

Results: Aβ was shown to be subject to immune adherence at every step in the pathway. Aβ dose-dependently activated serum complement. Complement-opsonized Aβ was captured by erythrocytes via CR1. Erythrocytes, Aβ, and hepatic Kupffer cells were colocalized in the human liver. Significant deficits in erythrocyte Aβ levels were found in AD and mild cognitive impairment patients.

Discussion: CR1 polymorphisms elevate AD risk, and 80% of human CR1 is vested in erythrocytes to subserve immune adherence. The present results suggest that this pathway is pathophysiologically relevant in AD.

Keywords: Alzheimer’s disease; Amyloid β peptide; Complement; Complement receptor 1; Immune adherence; Blood; Erythrocyte; Human

1. Introduction

Multiple studies have made clear that amyloid β peptide (Aβ) can move from the brain to the peripheral circulation [1–3] and from the peripheral circulation to the brain [4,5]. As such, the disposition of circulating Aβ may be pathophysiologically important. For example, failure to clear Aβ from blood could lead to an unfavorable concentration gradient for the movement of Aβ out of the brain [3]. Moreover, the propensity of fluid-phase Aβ to form insoluble fibrils and to activate complement and other inflammatory mediators could well play a role in the colocalization of inflammatory mediators with the vascular abnormalities that are observed in Alzheimer’s disease (AD), as
reviewed by Grammas et al. [6,7]. Mackic et al. [8,9] have provided critical data on serum and organ levels of Aβ after its intravenous (IV) inoculation into the bloodstream of nonhuman primates (NHPs). However, the mechanisms by which Aβ is purged from the circulation in primates still remain unclear.

Originally elucidated by Nelson et al. [10] in 1953, complement-dependent, erythrocyte-mediated clearance of circulating immune complexes (“immune adherence”) has been investigated in detail for more than 60 years and is now considered a primary mechanism for pathogen removal in humans, as reviewed by Birmingham et al. [11,12]. Fig. 1 illustrates some of the major steps in this pathway, several points of which may be worth emphasizing.

First, in humans, immune adherence hinges on the expression of complement receptor 1 (CR1) by erythrocytes, a phenomenon that is unique to primates. Subprimate erythrocytes do express complement receptors (e.g., Crry), but not CR1, so that their capacity to capture complement-opsonized immune complexes appears to be significantly limited compared with human immune adherence mechanisms [19].

Second, polymorphisms in the CR1 gene have been consistently shown to be among the top genetic risk factors for AD [20–24], and >80% of human CR1 is devoted to the erythrocyte compartment [11,12]. Taken together with the unique expression of CR1 by primate erythrocytes, these findings make erythrocytes perhaps the most parsimonious site for CR1 to impact AD risk.

Third, although human erythrocytes only express some 200 to 1500 CR1 molecules per red cell [11], the sheer number of erythrocytes (2–3 × 10¹³) in the bloodstream, compared with circulating and fixed macrophages, makes this an extremely powerful and efficient pathway for pathogen clearance. For example, pathogens experimentally infused into NHPs that have been immunized against the pathogen are typically eliminated by immune adherence mechanisms in 10 to 20 minutes [25].

Fourth, immune adherence research has focused on the clearance of immune complexes [11,12]. However, both our research [15,16] and that of others [14,17] have shown that Aβ, like certain bacterial and viral antigens [13], does not require immune complex formation to activate complement or to be bound by complement opsonins that serve as ligands for immune adherence. Thus, Aβ (and other antibody-independent complement activators) may have been overlooked as a substrate for immune adherence pathways.

Our laboratory first suggested that immune adherence might play a role in peripheral Aβ clearance and that

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**Fig. 1.** Simplified schematic of classical pathway complement activation and immune adherence. (A) An epitope on pathogens (gray tubes) is bound by circulating antibodies (YY) specific to it. C1, the first component of the classical complement pathway, then binds to closely apposed antibodies, forming an immune complex. Notably, like certain bacterial and tumor antigens [13], Aβ has been shown to bind C1 [14] and to induce activation of the C1r and C1s proteases without antibody mediation [14–17]. (B) C1s-mediated activation of the classical complement pathway ensues, including generation of C4b, C3b, and iC3b, which become covalently fixed to the antigen (black bars). C1q also remains bound to the antigen. The antigen and/or immune complex is therefore said to be “opsonized” by complement. (C) Primate (but not subprimate) erythrocytes (RBCs) express cell-surface CR1, which has C4b, C3b, and C1q as ligands. Antigen/complement complexes thus become bound to erythrocytes. (D) Erythrocytes then ferry the complex through the bloodstream until they reach specialized macrophages, Kupffer cells, lining the hepatic sinusoids. Kupffer cells recognize the complement tag via cell-surface CR1g receptors and strip off and degrade the opsonized antigen [11,12,18]. Abbreviations: Aβ, amyloid β peptide; CR1, complement receptor 1.
erythrocyte Aβ levels were significantly deficient in a small sample of AD and mild cognitive impairment (MCI) patients compared with nondemented elderly (NDE) controls [26]. In the present study, we provide new and more definitive, multidisciplinary evidence of immune adherence reactions with Aβ, as well as confirmation, in a 140-patient cohort, of AD and MCI deficits in erythrocyte Aβ capture.

2. Methods

2.1. Human subjects

Under institutional review board–approved protocols and consents, human IV blood samples were obtained prospectively from well-annotated, well-matched AD, MCI, and NDE subjects evaluated and diagnosed at a National Institute on Aging Alzheimer’s Disease Center, Banner Sun Health Research Institute, using standard National Institute on Aging Alzheimer’s Disease Center, Banner Sun Health Research Institute, and the National Institute on Aging Alzheimer’s Disease Center criteria. The AD group (N = 59) had a mean age of 80.1 ± 1.0 years (range 61–94 years) and consisted of 61% males and 39% females. The MCI group (N = 19) had a mean age of 80.1 ± 1.3 years (range 62–90 years) and consisted of 68% males and 32% females. The NDE group (N = 62) had a mean age of 80.4 ± 1.5 years (range 50–95 years) and consisted of 48% males and 52% females. Routine autopsy samples of AD, Parkinson’s disease, and control liver were obtained from the tissue bank at Banner Sun Health Research Institute.

2.2. Nonhuman primates

NHP IV blood samples were obtained from two 16-year-old male cynomolgus macaque monkeys and one 19-year-old cynomolgus macaque under Institutional Animal Care and Use Committee–approved protocols.

2.3. Initial processing of blood samples

Serum from human and NHP subjects was obtained by drawing blood into Becton-Dickinson (Franklin Lakes, NJ, USA) Serum Vacutainer tubes. After clotting for 30 minutes, the samples were centrifuged at 1100×g for 10 minutes at 4°C and the serum was withdrawn and stored at −80°C. Plasma and erythrocytes were derived from blood drawn into Becton-Dickinson ethylenediaminetetraacetic acid (EDTA) 2K Vacutainer tubes. The plasma/erythrocyte samples were immediately spun at 1100×g for 10 minutes at 4°C. Plasma and buffy coat were removed, and the remaining erythrocytes were washed with five volumes of tris-buffered saline (TBS). Plasma was stored at −80°C. Erythrocytes were used immediately or processed to erythrocyte membranes by lysis in five volumes of double-distilled (dd) H2O with 1× Protease Inhibitor Cocktail (PIC) (Roche, Basel, Switzerland) for 30 minutes at 4°C. Membranes were then pelleted in an ultracentrifuge by spinning for 40 minutes at 40,000×g, washed in five volumes of dd H2O with 1× PIC, centrifuged again at 40,000×g, and either used immediately or flash-frozen and stored at −80°C for subsequent experiments.

2.4. Aβ preparation

Human synthetic Aβ40 and Aβ42 (Bachem, Torrance, CA, USA or Genscript Biotech Corporation, Piscataway, NJ, USA) were solubilized in 100% dimethyl sulfoxide (DMSO) at 10 mg/mL, gradually diluted in dd H2O to 2 mg/mL, and then brought to a 1 mg/mL concentration in 0.1-M Tris buffer (pH 7.4). The 1 mg/mL Aβ42 stock solution was aggregated overnight at room temperature with agitation. The 1 mg/mL Aβ40 stock solution was aggregated for 3 days at 37°C without agitation.

2.5. Complement activation

To assess dose-dependent Aβ complement activation, Aβ40 or Aβ42 was diluted with 0.1-M Tris buffer to various concentrations and then incubated with an equal volume of normal human serum (NHS) (Complement Technology, Tyler, TX, USA) that had been diluted 1:5 in veronal-buffered saline containing calcium and magnesium (pH 7.4) for 1 hour at 37°C. To demonstrate specificity, 10-mM EDTA, which blocks complement activation, was added to Aβ/NHS control wells immediately before the incubation. To halt further activation, 10-mM EDTA was also added to all samples after incubation. Complement activation was assessed by the measurement of C3a or sC5b-9 formation using Quidel Human C3a or Human sC5b-9 enzyme-linked immunosorbent assay (ELISA) kits (San Diego, CA, USA). All assays were performed according to the protocols provided by the manufacturer. Conventional assays of complement activation, particularly with antibody-independent activators, typically require very high concentrations of activator under in vitro conditions, as here and in virtually all previous studies of complement interactions with Aβ c.f., [14–17,26,27].

2.6. Binding of Aβ by complement opsonins

NHS was incubated with Aβ42, as mentioned previously, to permit complement activation, generation of complement opsonins, and their covalent binding to Aβ. Aβ/NHS solutions were then run on conventional, reducing, sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) Western blots using anti-Aβ antibody 6E10 (BioLegend, San Diego, CA, USA) or an antibody directed at C3b or iC3b (Quidel Corporation). In our hands, the iC3b antibody also reacts with purified C3 and its two major chains, C3α and C3β, which are produced under SDS/reducing conditions. As a control to block complement activation and opsonization of Aβ, 10-mM EDTA was added to NHS before incubation with Aβ. Opsonization of Aβ was also studied in vivo. Here, a 19-year-old male cynomolgus macaque was anesthetized with Telazol (5 mg/kg, intramuscular) and infused intravenously with 61-µg/kg Aβ42 diluted in 0.9% sterile saline.
solution. Femoral artery blood samples were taken 20 minutes later and processed for plasma, as described previously. After a 1:1 dilution with sterile dd H₂O, the plasma preparations were incubated with either monoclonal anti-C₃ antibody (Abcam, Cambridge, MA, USA), which binds C3 and C3 split products (e.g., C3b, iC3b) or monoclonal anti-Aβ/amyloid precursor protein (APP) 4G8 antibody (Covance, Princeton, NJ, USA). Incubations were overnight at 4°C with gentle rocking. The samples were then subjected to immunoprecipitation (IP) using Thermo Scientific spin columns (Rockford, IL, USA). To prepare each column, 50 µL of Protein G Sepharose 4 Fast Flow (GE Healthcare Life Sciences, Pittsburgh, PA, USA) was added and the columns were prewashed with 200 µL of IP wash buffer (Thermo Fisher Scientific Inc.) and were additionally washed with 100 µL of 1× conditioning buffer (Thermo Fisher Scientific Inc.). Aβ and C3b protein complexes were eluted with 50 µL of elution buffer (Thermo Fisher Scientific Inc.) and incubated for 5 minutes at room temperature before centrifugation at 1000 x g, 4°C. To demonstrate complement opsonization of Aβ, the IP-bound fractions were boiled 5 minutes with Laemmli buffer containing 5% β-mercaptoethanol and then loaded into 4% to 20% Tris-glycine precast gels (Bio-Rad, Hercules, CA, USA). After electrophoresis, proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using the Trans-blot Turbo Transfer System (Bio-Rad) at 25 V for 20 minutes at room temperature before centrifugation at 1000 x g, 4°C. Membranes containing protein complexes that had been immunoprecipitated with the anti-Aβ antibody were immunoblotted with a 1:5000 dilution of mouse monoclonal anti-C3b antibody directed at a neoepitope specific to C3b (Quidel Corporation) or with mouse monoclonal anti-Aβ/APP antibody 4G8 at a 1:1000 dilution. Conversely, membranes with protein samples that had been immunoprecipitated with the anti-C₃ antibody were immunoblotted with a 1:1000 dilution of mouse monoclonal 4G8 or with mouse monoclonal anti-C3b neoepitope-specific antibody at a 1:5000 dilution. After four 5-minute washes with 1× phosphate-buffered saline (PBS) containing 0.1% Tween 20, the blots were incubated with a 1:10,000 dilution of anti-mouse Alexa Fluor 680 secondary antibody (Molecular Probes, Life Technologies, Grand Island, NY, USA) and imaged using the Odyssey Imaging System (LI-COR Biosciences, Lincoln, NE, USA). Blots were then stripped and incubated with a mouse monoclonal antibody specific for β-actin (Santa Cruz Biotechnology, Dallas, TX, USA) as a control.

2.7. Erythrocyte capture of Aβ in vitro

To assess the ability of erythrocytes to take up complement-opsonized Aβ in blood via CR1-dependent mechanisms, two strategies were used. First, 300 µL of NHS was incubated at 37°C for 1 hour with 300 µL of 20 µg/mL Aβ42 to permit complement activation and opsonization, then diluted to various concentrations in 1× TBS. The resulting NHS/Aβ42 solutions were incubated with 600 µL of packed erythrocytes (in TBS) at 37°C for 1 hour. After incubation, the mixtures were spun at 100 x g in a microfuge for 10 minutes, the supernatant was removed, and the erythrocytes were processed to erythrocyte membranes as described in Section 2.3. For ELISA, 200 µL of the erythrocyte membranes were solubilized in 160 µL of dd H₂O containing PIC and 40 µL of 1% SDS for 30 minutes at room temperature. The SDS-solubilized membranes were then mixed 1:5 with Wako (Richmond, VA) ELISA sample buffer and assayed using Wako Human Aβ40 ELISA kits (#298-62301) or Wako Human Aβ42 ELISA kits (#298-62401). The manufacturer’s protocols were followed throughout the process.

A second strategy for demonstrating specificity to complement mechanisms used a modified tip plate adhesion assay, previously used to characterize erythrocyte CR1 binding to its ligands [28]. Here, 96-well Costar high-binding microplates (Corning, Corning, NY, USA) were coated for 1 hour with aggregated Aβ42, diluted to various concentrations in 10-mM carbonate buffer (pH 9.6), and then blocked using 0.5% polyethylene glycol (PEG) 3350 in 2/3 tris-buffered saline/Tween 20 (TBST) (10-mM Tris pH 7.2, 100-mM NaCl, 0.05% Tween 20). Wells were subsequently exposed for 30 minutes, room temperature, to NHS to permit complement activation and binding (Complement Technology). To demonstrate that Aβ binding is mediated by complement opsonization, parallel wells were incubated with heat-inactivated NHS, C1q-depleted NHS (Complement Technology), C4-depleted NHS (Complement Technology), or EDTA + NHS, all of which block various stages of complement activation. Heat inactivation was for 30 minutes at 56°C. All sera were diluted 1:32 in veronal-buffered saline (with Mg²⁺ and Ca²⁺) (Complement Technology).

To evaluate binding, packed erythrocytes were diluted (1:2667) to 375 ppm with adhesion buffer (8-mM Tris pH 7.4, 100-mM NaCl, 140-mM dextrose, 0.45-mM CaCl₂, 0.17-mM MgCl₂). From the diluted erythrocyte sample, 200 µL (~700,000 erythrocytes) was added to each well and incubated for 60 minutes at room temperature. The wells were subjected to gentle, continuous washing/aspiration using 2 mL of adhesion buffer followed by 4 mL of PBS per well.

To further demonstrate that Aβ binding to erythrocytes is dependent on CR1, suspensions of erythrocytes were blocked, before exposure to Aβ-coated plates, with 2.0 µL of 0.2 mg/mL anti-CR1 antibody J3D3 (Beckman Coulter, Indianapolis, IN, USA) or with 25 µL of 1 mg/mL recombinant C3b (Complement Technology) for 30 minutes, room temperature. Erythrocytes that remained bound to the plate were imaged with bright-field illumination at 100× using an inverted Olympus IX71 microscope (Olympus, Center Valley,
PA, USA) and quantified using investigator-independent ImageJ software. Erythrocyte counts were normalized to wells coated with anti-CR1 antibody to adjust for any minor differences in the number of erythrocytes/samples.

2.8. Erythrocyte capture of Aβ in vivo

After Telazol (5 mg/kg) intramuscular anesthesia, two 16-year-old male cynomolgus macaques received 183 μg/kg or 366 μg/kg Aβ40, respectively, through an IV catheter inserted into the saphenous vein. The IV blood samples were taken from the same catheter at various intervals from baseline to 60 minutes after Aβ infusion. After Aβ infusion and after each withdrawal, the cannula was thoroughly flushed to prevent contamination of subsequent samples. The blood samples were centrifuged at 1100×g for 10 minutes at 4°C to isolate erythrocytes. The erythrocytes were then lysed and their membranes solubilized and assayed using Wako Human Aβ40 ELISA kits, as described previously.

2.9. Electron microscopy of liver samples

Liver samples from rapid (<4 hours) autopsies of AD, Parkinson’s disease, and NDE patients were dissected and processed using standard immunohistochemical and ultrastructural methods, as previously described in detail by our laboratory [27]. Antibodies directed at CD68 (Abcam, Cambridge, UK), a marker for Kupffer cells, and anti-Aβ antibody 4G8 (BioLegend) were used. Like most antibodies to Aβ, 4G8 also reacts with APP.

2.10. Aβ capture by AD, MCI, and NDE erythrocytes

Erythrocyte samples from AD, MCI, and NDE participants were processed to erythrocyte membranes, as mentioned previously, solubilized in SDS, and stored at −80°C. Plasma and the solubilized membranes were subsequently assayed for Aβ42 using a Covance (Princeton, NJ, USA) BetaMark Aβ42 ELISA kit (now marketed by BioLegend).

2.11. Statistics

Parametric (analysis of variance) and Pearson correlation statistics were used throughout the process. P values are two tailed.

3. Results

3.1. Aβ is an antibody-independent activator of complement

To be cleared by erythrocyte/CRI1-mediated mechanisms, pathogens must first activate the complement cascade. Here, we show that such activation occurs for Aβ and is significantly dose dependent and complete through C5b-9 (R = 0.93, P = .007), the terminal step of the classical and alternative pathways, as well as C3a (Aβ40: R = 0.96, P < .001; Aβ42: R = 0.87, P = .002), the step in which C3b/iC3b complement opsonins are generated (Fig. 2). Addition of 10-mM EDTA, a standard inhibitor of complement activation, reduced activation to background (i.e., serum only).

3.2. Complement activation by Aβ results in its opsonization

Substances that activate complement generate cleavage fragments of C3, including C3b, iC3b, and C4b, which bind back, covalently, to the activator and are said to “opsonize” it. Here, we show that Aβ intravenously infused into an NHP can be retrieved by IP with an antibody to C3b, and, conversely, that putative C3 opsonins can be retrieved by IP with an antibody to Aβ. In both cases, two major bands were observed for the opsonins and Aβ, both of which colocalized at ~75 kD and >250 kD (Fig. 3A and 3B). Importantly, these two bands were not observed with EDTA treatment, which abolishes complement activation and opsonization. These in vivo findings in an NHP extend two previous in vitro studies using human blood wherein colocalized bands for Aβ and C3b were also detected at high molecular weights on Western blots [17, 29], consistent with the fact that only Aβ aggregates, particularly Aβ fibrils, activate complement [16]. In addition, colocalization at the same molecular weights after IP and the reducing/denaturing conditions of the Western blot strongly suggest that Aβ and C3b were covalently bound, a characteristic feature of complement opsonization.

Binding of Aβ to a second complement opsonin, iC3b, was also demonstrated—here, in human blood samples exposed to Aβ in vitro. Under the reducing/denaturing conditions of the present experiment, iC3b is cleaved to 39, 63, and 75 kD fragments, and, like the C3b from which it derives, iC3b remains covalently bound to activators through a thioester bond. After incubation of Aβ42 with NHS to permit complement activation, Western blots for Aβ exhibited bands for Aβ monomer and multiple Aβ oligomer species (Fig. 3C, left lane), consistent with preaggregation of the peptide. Blots of the same solution that were immunoreacted for C3/3C3b (Fig. 3C, right lanes) showed bands parallel to those for Aβ at ~48 to 60 kD and >110 kD. These bands putatively represent iC3b fragments covalently bound to Aβ because (1) they are absent in samples treated with EDTA, which blocks iC3b formation and opsonization; (2) they remain present even under SDS/reducing conditions, consistent with the covalent binding of complement opsonins; and (3) they are shifted up from the normal molecular weights for iC3b fragments to match corresponding bands for Aβ. As expected, there was also heavy labeling of bands corresponding to fragments of C3, one of the most abundant proteins in blood. For example, under SDS/reducing conditions, the two disulfide-linked chains that comprise C3 (C3x and C3β) are observed (Fig. 3C, right lanes). Because C3 is endogenous in NHS and does
not require complement activation for its generation, immunoreactive bands at the normal molecular weights for C3 at and C3β remain present in the blot regardless of whether EDTA is used. A faint band at approximately 34 kD was also detected and may correspond to C3d, another C3 fragment generated by further cleavage of iC3b by the protease, factor I. The band is at the correct molecular weight for C3d and was abolished with EDTA treatment. Alternatively, the slow kinetics of C3d formation may not be consistent with the time scale of the present experiment [30]. Finally, the absence of any complement immunoreactivity associated with monomeric Aβ, despite the large amounts that were present, confirms our previous finding that Aβ monomer does not activate complement and that increasing fibrilization of Aβ enhances complement activation [16].

### 3.3. Erythrocytes capture Aβ through complement-dependent processes

Once opsonized by complement, circulating pathogens in primates are bound by erythrocytes via CR1 expressed at the erythrocyte surface. When incubated with erythrocytes, Aβ that had been exposed to NHS to permit complement activation and opsonization was captured by the erythrocytes in a significant dose-dependent fashion \( R = 0.99, P < 0.001 \) (Fig. 4A). To confirm this result using a second technique and to assess its specificity with respect to CR1 and the different potential ligands for CR1 (i.e., C1q, C4b, C3b), we next performed erythrocyte tip plate adhesion assays that have been previously used to assess CR1/ligand interactions [28]. Here, Aβ42 was coated to the bottom of wells, exposed to NHS (to permit complement activation and opsonization) or, as controls, exposed to C1-depleted NHS, C4-depleted NHS, EDTA-treated NHS, or heat-inactivated NHS, all of which inhibit complement activation and opsonization at different stages. Treated wells were then washed and incubated with erythrocytes, followed by washing to remove nonadherent cells. Incubation of Aβ42 with NHS produced significant dose-dependent adherence of erythrocytes to Aβ-coated wells \( R = 0.91, P = .03; \) Fig. 4B). By contrast, incubation of Aβ42 with heat-inactivated NHS or EDTA-treated NHS, which eliminate both alternative and classical pathway complement reactions and provide an estimate of non–complement-mediated mechanisms, reduced erythrocyte binding to Aβ-coated wells to background. C1-depleted serum, which abolishes classical complement pathway reactions but still permits C3b opsonization of Aβ via the alternative or lectin pathways, also reduced erythrocyte adherence to background. Similarly, C4-depleted serum, which eliminates classical pathway generation of
C4b and C3b opsonins, reduced erythrocyte adherence to background (Fig. 4B). These findings show that erythrocyte/Aβ binding is predominantly mediated by classical complement pathway–dependent mechanisms (i.e., immune adherence) and depends on the generation of appropriate CR1 ligands.

Specificity to CR1-mediated mechanisms was also explored by preincubating erythrocytes with anti-CR1 antibody J3D3 or recombinant C3b. J3D3 only blocks three of the four CR1 binding sites for complement-opsonized ligands (to our knowledge, no available antibody blocks all four sites), but nonetheless reduced erythrocyte/Aβ adhesion by 60% (F = 30.9, P = .03) (not shown). Similarly, recombinant C3b still permits CR1 binding to other complement opsonins (e.g., the CR1 long homologous repeat-A binding site preferentially captures C4b-opsonized substrates) but nonetheless reduced erythrocyte/Aβ adhesion by 62% (F = 52.1, P < .001; Fig. 4C).

3.4. Erythrocyte capture of Aβ in vivo

To extend the previous findings to in vivo conditions, two cynomolgus monkeys were infused with either 183 μg/kg or 366 μg/kg of Aβ40. Saphenous vein blood samples were taken at baseline and at intervals from 2 to 60 minutes thereafter. Plasma and erythrocyte Aβ40 levels were tightly correlated (R = 0.98, P < .001 and R = 0.85, P = .004 for the 186 μg/kg and 366 μg/kg Aβ doses, respectively; Fig. 5), with an immediate spike at 2.5 minutes and a return to near baseline within 20 minutes. These kinetics are comparable to previous studies of immune adherence with bacterial pathogens wherein >90% of plasma and erythrocyte clearance is observed within the first 10 to 20 minutes after intravenous injection [25]. Previous studies in monkeys by Mackic et al. [8,9] reported that some 97% of infused, radiolabeled Aβ40 was sequestered in other organs, including brain, with only ∼3% to 4% retrievable in plasma. Our studies, using a direct ELISA of Aβ40, gave almost identical results, including the spike and rapid fall in plasma Aβ in the first 20 minutes after infusion. Clearance of Aβ through the erythrocyte pathway appeared to operate on demand, such that the higher, 366 μg/kg dose of Aβ was reduced to near baseline as quickly as the lower, 188 μg/kg dose. Although erythrocyte Aβ40 levels were typically only ∼1% to 3% of plasma levels at any given

Fig. 3. Complement opsonization of Aβ in blood. (A) In blood samples from a nonhuman primate inoculated with Aβ, immunoprecipitation with an anti-Aβ antibody retrieved two major bands of Aβ immunoreactivity at ∼75 kD and >250 kD (left lane) and two major bands of putative C3b immunoreactivity at the same molecular weights (right lane). (B) Similarly, immunoprecipitation with an anti-C3b antibody retrieved two major bands of Aβ immunoreactivity at ∼75 kD and >250 kD (left lane) and two major bands of putative C3b immunoreactivity (right lane) at the same molecular weights. (C) Western blot of Aβ incubated with normal human serum using an antibody directed against Aβ (left lane) and a Western blot of the same solution using an antibody that reacts with C3 and iC3b (right two lanes). C3 is abundantly present whether complement activation has occurred or not. In sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) gels under reducing conditions, its two major, disulfide-linked chains, C3α and C3β, therefore dominate the gel and, as endogenous constituents, are not affected by EDTA. By contrast, generation and covalent binding of iC3b to activating substrates such as Aβ requires complement activation and is sensitive to EDTA. Thus, putative immunoreactivity for iC3b and its fragments (brackets) is present when complement activation is permitted (+EDTA) and absent when activation is inhibited (−EDTA). Abbreviation: Aβ, amyloid β peptide.
time, the erythrocyte immune adherence pathway is clearly capable of clearing normal circulating levels of Aβ. For example, from 2.5 to 20 minutes after infusion of 366 μg/kg Aβ, 3 ng/mL Aβ was removed from the erythrocyte compartment, which is some six-fold greater than typical blood Aβ levels in the monkeys (and humans).

3.5. Erythrocyte-mediated clearance of circulating Aβ to the liver

In primates, specialized macrophages called Kupffer cells line the hepatic sinusoids, where they recognize and strip off complement-tagged pathogens from erythrocytes [11,12]. Low power, high power, and electron micrographs of human AD, Parkinson’s disease, and NDE liver reveal Aβ immunoreactivity in Kupffer cells colocalized with Aβ-immunoreactive erythrocytes within the sinusoid (Fig. 6). Notably, Aβ clearance to Kupffer cells was observed in all patient diagnostic conditions, suggesting that this pathway is normally used for peripheral clearance of Aβ, as it is for other toxins.

3.6. Pathophysiologic significance of immune adherence in AD

We previously reported AD and MCI deficits in erythrocyte Aβ levels in a small (N = 36), retrospective set of AD, MCI, and NDE participants [26]. Here, we confirm this finding in a prospective set of samples from 140 well-annotated, well-matched AD, MCI, and NDE participants. As in our previous study [26], AD (F = 18.1, P < .001) and MCI (F = 7.5, P = .006) groups each exhibited significantly lower levels of erythrocyte Aβ compared with the NDE group (Fig. 7A), and there was a significant correlation of erythrocyte-captured Aβ with Mini–Mental State Examination scores (R = 0.216, P = .012) (Fig. 7B). A major point left unclear by the present replication, however, is whether MCI patients are developing or have fully evolved deficits in erythrocyte capture of Aβ. In our original study, MCI patients exhibited levels of erythrocyte Aβ that were significantly intermediate between AD and NDE patients [26], whereas in the present study, MCI deficits were similar to those of AD patients. In either case, the findings are consistent with the growing consensus
that AD treatment strategies need to be inaugurated as early as possible.

Finally, as in our previous research [26], plasma Aβ42 levels tended to be higher in the AD group (mean ± standard error of the mean = 1284 ± 190 pg/mL) compared with the NDE group (mean ± standard error of the mean = 995 ± 88 pg/mL) but did not differ significantly, consistent with the mixed results for plasma Aβ reported by other investigators [31–34]. By contrast, the fraction of Aβ42 captured in the erythrocyte compartment relative to the amount available in the plasma compartment was significantly lower for the AD group than the NDE group ($F = 13.2, P < .001$) (Fig. 8), again confirming an AD deficit in erythrocyte clearance. As shown in the figure, it may also be notable that the amount of Aβ42 in the erythrocyte compartment in all the patient groups was at least equal to or higher than ($P = .07$) the amount of Aβ42 in the plasma compartment, consistent with the hypothesis that erythrocyte clearance of Aβ, immune adherence, is a major player in handling circulating loads of Aβ.

4. Discussion

The present research demonstrates for the first time that all steps in the classical immune adherence pathway are fulfilled with respect to clearance of circulating Aβ and confirms our previous finding that this mechanism is deficient in AD and MCI patients compared with NDE patients [26]. Aβ inoculated into human serum dose-dependently activated complement, forming complement-opsonized complexes. When incubated with human erythrocytes, complement-opsonized Aβ was captured in a dose-dependent manner. Specificity to complement- and CR1-dependent mechanisms rather than to nonspecific binding was demonstrated by abolition of Aβ/erythrocyte adherence after heat inactivation of complement, EDTA treatment, or depletion of classical pathway components. Because these manipulations eliminate the formation of the ligands for CR1, they indirectly demonstrate that erythrocyte binding of Aβ is most likely to be dependent on CR1. This conclusion was directly demonstrated by inhibiting erythrocyte CR1 binding to Aβ by blocking CR1 binding sites with anti-CR1 antibody and recombinant C3b, a CR1 ligand. At the terminal end of the immune adherence pathway, Aβ-immunoreactive Kupffer cells and apposed erythrocytes could be localized to the hepatic sinusoids by electron microscopy. In primates, Kupffer cells are specialized to capture complement-opsonized complexes carried by erythrocytes [11,12].

Several lines of evidence suggest that erythrocyte CR1-dependent mechanisms play a pathophysiologic role in Aβ clearance. First, our experiments show that peripheral erythrocyte capture of Aβ is highly dependent on complement reactions with Aβ and, moreover, is dependent on interactions of complement-opsonized Aβ with the receptor for complement-opsonized ligands expressed on erythrocytes, CR1. Although these findings do not necessarily exclude other mechanisms for Aβ capture by erythrocytes, they strongly suggest that complement/CRI1 mediation is predominant.

Recent, nonoverlapping genome-wide association studies provide a second important connection of the erythrocyte immune adherence pathway to AD pathophysiology. Namely, multiple studies have found single-nucleotide polymorphisms in CR1 to be among the top genetic risk factors for AD [20–24]. Perhaps, because AD is a brain disorder, an underlying CR1 mechanism in brain has understandably been sought in previous studies [35–41]. Although the findings from these endeavors clearly confirm a role for CR1 in AD, the cell types expressing CR1 in brain—and even the presence of CR1 in brain—remain controversial. Although it is possible that the CR1 in brain is modified in some way that conceals, removes, or alters epitopes to conventional CR1 antibodies [42], the fact remains that the vast majority of human CR1 indisputably resides in the erythrocyte compartment, where CR1 expression is unequivocally and universally detected [11,12]. The most parsimonious underlying basis for CR1 as an AD risk factor is, therefore, likely to be its role in peripheral clearance of Aβ, where CR1 expression by erythrocytes has evolved to amplify pathogen clearance in primates and is most abundantly expressed toward that end. Consistent with this view, AD and MCI patients...
exhibited significant deficits in CR1-mediated erythrocyte capture of Aβ, a finding we previously reported [26] and confirmed in this report with a much larger sample. Deficits in erythrocyte clearance of pathogens have also been reported in several other human disorders, including leprosy, lupus, and malaria [11,12]. No consensus mechanism for this association with disease—whether by erythrocyte immunosenescence, decreased erythrocyte CR1 expression, impaired binding of CR1 to its ligands, or other mechanisms—has been accorded, and we are exploring these possibilities in the context of AD and the reported polymorphisms of CR1.

Fig. 6. Localization of Aβ42 to hepatic KCs, the final step in immune adherence. (A–C) Confocal microscopy of a 20-μm section of AD liver showing colocalization of CD68 immunoreactivity, a marker for KCs, and Aβ immunoreactivity. (B) Aβ-immunostained, toluidine blue–counterstained semi-thin section of PD liver, showing Aβ-immunoreactive KCs. (C) Electron micrograph of NDE liver, again showing typical KC localization with apoposed erythrocytes and cytoplasmic Aβ42 (arrowheads). Deletion of primary antibodies in all experiments gave uniformly negative results (not shown). We note that, like most anti-Aβ antibodies, the 4G8 antibody used here also reacts with APP. However, the punctate, granular, intracytoplasmic labeling in these micrographs appears to be more characteristic of Aβ than its precursor, amyloid precursor protein. Abbreviations: AD, Alzheimer’s disease; Aβ, amyloid β peptide; KC, Kupffer cell; PD, Parkinson’s disease.

Fig. 7. Pathophysiologic significance of erythrocyte capture of Aβ in AD. (A) Confirming our previous results [26], erythrocyte capture of Aβ42 is significantly deficient in both AD and MCI patients. (B) Also in concert with our previous findings [26], there was a significant correlation of erythrocyte Aβ42 levels with cognitive status (MMSE) score. Although the data clearly exhibit too much scatter to make erythrocyte Aβ a definitive prognostic for AD, they do strongly suggest that it has pathophysiologic relevance to clinical AD progression. Abbreviations: Aβ, amyloid β peptide; AD, Alzheimer’s disease; MCI, mild cognitive impairment; MMSE, Mini–Mental State Examination; NDE, nondemented elderly.
In addition to peripheral clearance of Aβ, complement opsonization of Aβ in the brain itself is also likely to have pathophysiologic significance—although not necessarily through CR1 mechanisms. Our laboratory, in fact, first reported colocalization of complement opsonins with Aβ in brain [15], a finding that has been recently extended by Hong et al. [43]. The latter demonstrated that opsonized Aβ associated with synapses helps target the synapses for microglial engulfment via microglial expression of another complement receptor, CR3. We have also shown direct attack of neurites in the vicinity of Aβ plaques by the terminal component of complement, C5b-9, the membrane attack complex [27].

Immune adherence has historically been studied in the context of immune complexes (i.e., antigen-antibody complexes) that activate and are bound by complement opsonins, as opposed to antigens, such as Aβ, that directly activate and are bound by complement in the absence of antibody. Although Aβ is a relatively potent antibody-independent complement activator [14–17], antibody-dependent classical pathway activation is typically much more powerful [18]. As such, circulating endogenous anti-Aβ antibodies, which are common in human subjects [44–48], and anti-Aβ antibodies introduced by immunotherapy, as reviewed in [49], should theoretically enhance complement activation and peripheral clearance of Aβ. We are also now exploring these possibilities as a means to better understand the putatively beneficial mechanisms and adverse consequences of Aβ immunization.

In summary, immune adherence, the clearance of pathogens through an erythrocyte CR1-mediated process, appears to be an important mechanism for removing Aβ from the circulation in humans, and deficiencies in this mechanism are likely to be pathologically relevant to AD.

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RESEARCH IN CONTEXT

1. Systematic review: Although there are data on peripheral amyloid β peptide (Aβ) levels after intravenous Aβ inoculation in monkeys, the mechanisms for removing Aβ from the blood in primates remain unclear.

2. Interpretation: Our findings demonstrate that circulating Aβ is subject to a highly efficient, well-studied pathway for clearing complement-opsonized antigens—immune adherence. This mechanism is unique to primates, deficient in Alzheimer’s disease, and dependent on erythrocyte complement receptor 1, single-nucleotide polymorphisms in which are a consistent risk factor for Alzheimer’s disease. Failure to remove peripheral Aβ is likely to provide an unfavorable concentration gradient for its clearance from brain and to have deleterious effects on the vasculature and other organ systems in which it becomes sequestered.

3. Future directions: Peripheral Aβ clearance by immune adherence should be enhanced by Aβ antibodies introduced in the course of Aβ immunotherapy, providing an additional explanation for why this treatment strategy helps remove Aβ in brain.

References

[1] Kanekiyo T, Liu CC, Shinohara M, Li J, Bu G. LRP1 in brain vascular smooth muscle cells mediates local clearance of Alzheimer’s amyloid-beta. J Neurosci 2012;32:16458–65.
[2] Castellano JM, Deane R, Gottesdiener AJ, Verghese PB, Stewart FR, West T, et al. Low-density lipoprotein receptor overexpression enhances the rate of brain-to-blood Aβ1-40 clearance in a mouse model of beta-amyloidosis. Proc Natl Acad Sci U S A 2012;109:15502–7.

[3] DeMattos RB, Bales KR, Cummins DJ, Dodart JC, Paul SM, Holtzman DM. Peripheral anti-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 U S A 2001;98:8850–5.

[4] Deane R, Du Yan S, Submarmary RK, LaRue B, Jovanovic S, Hogg E, et al. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. Nat Med 2003;9:907–13.

[5] Do TM, Bedussi B, Chasseigneaux S, Dodacki A, Yapo C, Chaunc H, et al. Oatp1a4 and an L-tyrosine-sensitive transporter mediate the mouse blood-brain barrier transport of amyloid-beta peptide. J Alzheimers Dis 2013;36:555–61.

[6] Grammas P. Neurovascular dysfunction, inflammation and endothelial activation: implications for the pathogenesis of Alzheimer’s disease. J Neuroinflammation 2011;8:26.

[7] Broussard GJ, Mytar J, Li RC, Klapstein GJ. The role of inflammatory mediators in the pathogenesis and progression of Alzheimer’s disease. J Alzheimers Dis 2001;4:285–93.

[8] Mackie JB, Bading J, Ghiso J, Walker L, Wisniewski T, Frangione B, et al. Circulating amyloid-beta peptide crosses the blood-brain barrier in aged monkeys and contributes to Alzheimer’s disease lesions. Vascul Pharmacol 2002;38:303–13.

[9] Mackie JB, Weiss MH, Miao W, Kirkman E, Ghiso J, Calero M, et al. Cerebrovascular accumulation and increased blood-brain barrier permeability to circulating Alzheimer’s amyloid beta peptide in aged squirrel monkey with cerebral amyloid angiopathy. J Neurochem 1998;70:210–5.

[10] Nelson RA Jr. The immunity-adherence phenomenon; an immunologically specific reaction between microorganisms and erythrocytes leading to enhanced phagocytosis. Science 1953;118:733–7.

[11] Birmingham DJ, Hebert LA. CR1 and CR1-like: the primate immune adherence receptors. Immunol Rev 2001;180:100–11.

[12] Hess C, Schifferli JA. Immunoadherence revisited: novel players in an old game. News Physiol Sci 2003;18:104–8.

[13] Cooper NR, Jensen FC, Welsh RM Jr, Oldstone MB. Lysis of RNA tumor viruses by human serum: direct antibody-independent triggering of the classical complement pathway. J Exp Med 1976;144:970–84.

[14] Jiang H, Burdick D, Glabe CG, Cotman CW, Tenner AJ, McGeer PL, Terai K, et al. Plasma amyloid beta-peptide 1–40 and 1–42 in Alzheimer disease. Arch Neurol 2000;57:100–5.

[15] Rogers J, Cooper NR, Webster S, Schultz J, McGeer PL, Styen SD, et al. Complement activation by beta-amyloid in Alzheimer disease. Proc Natl Acad Sci USA 1992;89:10016–20.

[16] Webster S, Bradt BM, Kolb WP, Cooper NR, Schultiz J, McGeer PL, Styen SD, et al. Complement activation by beta-amyloid in Alzheimer disease. Proc Natl Acad Sci USA 1992;89:10016–20.

[17] Webster S, Lue LF, Brachova L, Tenner AJ, McGeer PL, Terai K, et al. Molecular and cellular characterization of the membrane attack complex, C5b-9, in Alzheimer’s disease. Neurobiol Aging 1997; 18:415–21.

[18] Tas SW, Klückstein LB, Barbashov SF, Nicholson-Weller A. C1q and C4b bind simultaneously to CR1 and additively support erythrocyte adhesion. J Immunol 1999;163:5056–63.

[19] Webster 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: Igual K, Winblad B, Nishimura T, Takeda M, Wisniewski HM, eds. Alzheimer’s disease: Biology, diagnostics and therapeutics. John Wiley and Sons; 1997. p. 365–73.

[20] Vik DP, Fearon DT. Neutrophils express a receptor for iC3b, C3dg, and C3d that is distinct from CR1, CR2, and CR3. J Immunol 1985; 134:2571–9.

[21] Kuo YM, Kokjohn TA, Kalbach W, Luehrs D, Galasko DR, Chevallier N, et al. Amyloid-beta peptides interact with plasma proteins and erythrocytes: implications for their quantitation in plasma. Biochem Biophys Res Commun 2000;268:730–6.

[22] Rogers J, Cooper NR, Webster S, Schultz J, McGeer PL, Styen SD, et al. Complement activation by beta-amyloid in Alzheimer disease. Proc Natl Acad Sci USA 1992;89:10016–20.

[23] Taylor RP, Martin EN, Reinagel ML, Nardin A, Craig M, Choice Q, et al. Bispecific monoclonal antibody complexes facilitate erythrocyte binding and liver clearance of a prototype particulate pathogen in a monkey model. J Immunol 1997;159:4035–44.

[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.

[25] Taylor RP, Martin EN, Reinagel ML, Nardin A, Craig M, Choice Q, et al. Bispecific monoclonal antibody complexes facilitate erythrocyte binding and liver clearance of a prototype particulate pathogen in a monkey model. J Immunol 1997;159:4035–44.

[26] Taylor RP, Martin EN, Reinagel ML, Nardin A, Craig M, Choice Q, et al. Bispecific monoclonal antibody complexes facilitate erythrocyte binding and liver clearance of a prototype particulate pathogen in a monkey model. J Immunol 1997;159:4035–44.

[27] Webster S, Lue LF, Brachova L, Tenner AJ, McGeer PL, Terai K, et al. Molecular and cellular characterization of the membrane attack complex, C5b-9, in Alzheimer’s disease. Neurobiol Aging 1997; 18:415–21.

[28] Tas SW, Klückstein LB, Barbashov SF, Nicholson-Weller A. C1q and C4b bind simultaneously to CR1 and additively support erythrocyte adhesion. J Immunol 1999;163:5056–63.

[29] Webster 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: Igual K, Winblad B, Nishimura T, Takeda M, Wisniewski HM, eds. Alzheimer’s disease: Biology, diagnostics and therapeutics. John Wiley and Sons; 1997. p. 365–73.

[30] Vik DP, Fearon DT. Neutrophils express a receptor for iC3b, C3dg, and C3d that is distinct from CR1, CR2, and CR3. J Immunol 1985; 134:2571–9.

[31] Kuo YM, Kokjohn TA, Kalbach W, Luehrs D, Galasko DR, Chevallier N, et al. Amyloid-beta peptides interact with plasma proteins and erythrocytes: implications for their quantitation in plasma. Biochem Biophys Res Commun 2000;268:730–6.

[32] Mayeux R, Tang MX, Jacobs DM, Manly J, Bell K, Merchant C, et al. Plasma amyloid beta-peptide 1–42 and incipient Alzheimer’s disease. Ann Neurol 1999;46:412–6.

[33] Mehta PD, Pittilia T, Mehta SP, Sersen EA, Aisen PS, Wisniewski HM. Plasma and cerebrospinal fluid levels of amyloid beta proteins 1–40 and 1–42 in Alzheimer disease. Arch Neurol 2000;57:100–5.

[34] Tamaoka A, Fukushima T, Sawamura N, Ishikawa K, Oguni E, Komatsuzy T, et al. Amyloid beta protein in plasma from patients with sporadic Alzheimer’s disease. J Neurol Sci 1996; 141:65–8.

[35] Gasque P, Chan P, Mauger C, Schoultz MT, Singhroa S, Dierich MP, et al. Identification and characterization of complement C3 receptors on human astrocytes. J Immunol 1996;156:2247–55.

[36] Hazzrat LN, Van Cauwenberghe C, Brooks PL, Broussard GJ, Mytar J, Li RC, Klapstein GJ. The role of inflammatory mediators in the pathogenesis and progression of Alzheimer’s disease. J Alzheimers Dis 2001;4:285–93.

[37] Hauser RN, Hedges MR, Kurland LT, Chui DH, Ferris SH, Bowen JD, et al. Epidemiologic features of Alzheimer’s disease. Neurology 1987;37:419–23.

[38] Khan MA, Khan MA, Khan MA, Khan MA, Khan MA, Khan MA, et al. Complement activation in very early Alzheimer disease. Alzheimers Dis Assoc Disord 2005;19:55–66.

[39] 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.

[40] Allen M, Kachadoorian M, Carrasquillo MM, Karhade A, Manly L, Burgess J, et al. Late-onset Alzheimer’s disease risk variants mark brain regulatory loci. Neuron Genet 2015;1:e15.
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.

Fonseca MI, Chu S, Pierce AL, Brubaker WD, Haushart 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:e0149792.

Hong S, Beja-Glasser VF, Nfonoyim BM, Frouin A, Li S, Ramakrishnan S, et al. Complement and microglia mediated early synapse loss in Alzheimer mouse models. Science 2016;352:712–6.

Kim I, Lee J, Hong HJ, Jung ES, Ku YH, Jeong IK, et al. A relationship between Alzheimer’s disease and type 2 diabetes mellitus through the measurement of serum amyloid-beta autoantibodies. J Alzheimers Dis 2010;19:1371–6.

Maetzler W, Berg D, Synofzik M, Brockmann K, Godau J, Melms A, et al. Autoantibodies against amyloid and glial-derived antigens are increased in serum and cerebrospinal fluid of Lewy body-associated dementias. J Alzheimers Dis 2011;26:171–9.

Maetzler W, Langkamp M, Lerche S, Godau J, Brockmann K, Gaenslen A, et al. Lowered serum amyloid-β1-42 autoantibodies in individuals with lifetime depression. J Alzheimers Dis 2012;32:95–100.

Piazza F, Greenberg SM, Savoiardo M, Gardinetti M, Chiapparini L, Raicher I, et al. Anti-amyloid beta autoantibodies in cerebral amyloid angiopathy-related inflammation: implications for amyloid-modifying therapies. Ann Neurol 2013;73:449–58.

Sohn JH, So JO, Hong HJ, Kim JW, Na DR, Kim M, et al. Identification of autoantibody against beta-amyloid peptide in the serum of elderly. Front Biosci (Landmark Ed) 2009;14:3879–83.

Wisniewski T, Goni F. Immunotherapeutic approaches for Alzheimer’s disease. Neuron 2015;85:1162–76.