1-1-2022

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FEATURED ARTICLE

Leukocyte surface biomarkers implicate deficits of innate immunity in sporadic Alzheimer's disease

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Abstract

Introduction: Blood-based diagnostics and prognostics in sporadic Alzheimer's disease (AD) are important for identifying at-risk individuals for therapeutic interventions.

Methods: In three stages, a total of 34 leukocyte antigens were examined by flow cytometry immunophenotyping. Data were analyzed by logistic regression and receiver operating characteristic (ROC) analyses.

Results: We identified leukocyte markers differentially expressed in the patients with AD. Pathway analysis revealed a complex network involving upregulation of complement inhibition and downregulation of cargo receptor activity and Aβ clearance. A proposed panel including four leukocyte markers – CD11c, CD59, CD91, and CD163 – predicts patients' PET Aβ status with an area under the curve (AUC) of 0.93 (0.88 to

0.97). CD163 was the top performer in preclinical models. These findings have been validated in two independent cohorts.

Conclusion: Our finding of changes on peripheral leukocyte surface antigens in AD implicates the deficit in innate immunity. Leukocyte-based biomarkers prove to be both sensitive and practical for AD screening and diagnosis.

KEYWORDS

Alzheimer's disease, CD11c, CD163, CD59, CD91, complement system, innate phagocytosis, leukocyte biomarker, peripheral immune response, receptor for advanced glycation end products (RAGE), ROC curve

1 | NARRATIVE

1.1 | Contextual background

Alzheimer's disease (AD) is a neurodegenerative disease characterized by cognitive impairment, functional deterioration, and dementia. Pathologically, AD is defined by the presence of amyloid plaques and neurofibrillary tangles due to the accumulation of 42-amino-acid amyloid-β peptide ($A\beta_{42}$) and phosphorylated tau protein,[1] respectively.

The diagnosis of AD now relies on clinical assessment and on information from imaging and biofluid markers, which aimed at detecting the presence of $A\beta_{42}$ and phosphorylated tau protein.[2] Positron emission tomography (PET) neuroimaging and cerebrospinal fluid (CSF) measurements, the most commonly used biomarkers, can yield an accurate diagnosis of AD even in an asymptomatic group.[2] Mounting evidence indicates that the accumulation of amyloid in AD starts 20 to 30 years before clinically detectable cognitive impairment is observed, suggesting the presence of a long period of asymptomatic AD. Although the specific onset of this preclinical period is difficult to target, it is potentially significant to identify subjects in this asymptomatic preclinical stage of the disease. This is because newly developed disease modifying treatments may mainly be effective during this asymptomatic period. Currently, the preclinical phase of AD is identified in asymptomatic individuals as abnormal amyloid levels detected via neuroimaging or fluid biomarkers.[3] However, this approach is not feasible as a routine screening tool in a clinical environment. Therefore, there is a critical need to develop blood-based, cost-effective screening tools to detect AD in asymptomatic patients. Because of this, the search for preclinical blood-based biomarkers has become a major focus.[3–5]

While current biomarkers have conventionally focused on the central nervous system (CNS), emerging evidence from genetic, clinical, cell biological, and animal studies have shown abnormal systemic changes which are not only secondary to brain dysfunction but may also impact AD progression, suggesting that these bi-directional interactions between the brain and peripheral systems play a role in the natural history of AD.[6] Schwartz et al. previously described the presence of an active interchange of immune cells between the CNS and the periphery in the choroid plexus in animal models of AD.[7] More recent work from the same group demonstrated that inducing interferon (IFN)-γ-dependent recruitment of monocyte-derived macrophages to the brain resulted in the clearance of cerebral $A\beta$ plaques and improved cognitive performance in a mice AD model.[8] Thus, if these findings can be replicated in preclinical and symptomatic AD patients, this interaction between the CNS and the peripheral blood leukocytes may allow for the detection of potential immune changes in the periphery of AD. These immune changes could become blood-based biomarkers for AD. Early studies have demonstrated the feasibility of this approach, although the data are preliminary.[9]

1.2 | Study conclusions, disease implications, and therapeutic opportunities

We identified specific leukocyte markers that were differentially expressed in the patients with AD compared with cognitively normal (CN) individuals. Pathway analysis revealed a complex network involving upregulation of complement inhibition and downregulation of cargo receptor activity and $A\beta$ clearance. These findings may have important implications in our understanding of the etiology and progression of AD and become the basis for the development of new therapeutic approaches. Furthermore, the presence of a specific pattern of immune changes can constitute a blood-based biomarker panel for AD. Specifically, we proposed a panel including four leukocyte markers – CD11c, CD59, CD91, and CD163 to predict patients' PET $A\beta$ status with an area under the curve (AUC) of 0.93 (0.88 to 0.97). CD163 was the top performer in our preclinical models. In order to prove the concept, one best-performed biomarker, the MFI of CD163 on CD14$^-$ neutrophils (lnBM8), was added to the model. The AUC was improved to 0.94 (CI: 0.86 to 0.98) ($p = 0.063$).

Our findings demonstrated that leukocyte surface antigens exhibited differential expression in groups of older adults who met clinical criteria for CN, MCI, and AD-dementia and were associated with amyloid pathology confirmed by PET imaging. The importance of plasma biomarkers for AD has been suggested recently by Nakamura et al. and Fossati et al.[10–12] Our data may provide valuable insights suggesting the relevance of using the AD-associated leukocyte markers for the development of blood-based AD screening tools. However, the

RESEARCH IN CONTEXT

1. Systematic review: To put their findings into context, the authors reviewed the literature using traditional sources (e.g., PubMed), meeting abstracts and presentations. The importance of identifying individuals with preclinical Alzheimer's disease (AD) for implementing disease modifying therapies underlies the need for finding reliable biomarkers that can be easily obtained and administered at-scale. These data may provide the first evidence for the utility of leukocyte biomarkers for AD diagnosis. There have been several recent publications describing the microglial markers associated with AD. These relevant citations are appropriately cited.

2. Interpretation: Even in the absence of substantial passage of immune cells from the periphery to the brain, abnormalities of peripheral immune cells may reflect disease effects on brain-resident microglia. Our findings justify the use of blood cell biomarkers for a better understanding of the molecular pathogenesis of AD.

3. Future directions: The manuscript proposes leukocyte biomarkers for detecting AD. It would be important to design a longitudinal study to test whether these biomarkers could predict disease progression. Other leukocyte antigens, such as RAGE, may yet prove useful. Further studies are required to determine whether other cell surface antigens (i.e., RAGE) should be included in the development of a more sophisticated biomarker panel for AD diagnosis.

FCIP method has its limitations. For example, blood cells cannot be frozen and thawed in batches for testing; the FCIP method requires daily maintained laboratory personnel, equipment, and logistic services. Although these data have been validated in an independent sample, it is cross-sectional, not longitudinal. A longitudinal study of these biomarkers should be conducted. We acknowledge generalizability as limitation because AIBL is a convenience cohort, and we will cover this topic for future research.

2 | CONSOLIDATED RESULTS AND STUDY DESIGN

Our study was comprised of four stages. Stages I and II were exploratory and were aimed at investigating major leukocyte and CD markers. The scope of leukocyte surface antigens detected in Stage II was readjusted in Stage III. In Stage III, immune markers associated with AD risk alleles that potentially operate through immune cells, including microglia (thought to play a key role in AD pathogenesis), were included. Markers linked with newly identified changes in

leukocytes that may be associated with the onset of AD were also included.[2,13,14] These experiments resulted in the identification of three key biomarkers that together had high validity and reliability in identifying subjects presenting with preclinical or clinical AD and other brain biomarkers associated with the disease. We then validated them in an independent cohort, Stage IV.

From a methodological point of view, we performed flow cytometry immunophenotyping (FCIP) to identify which leukocyte markers were differentially expressed in AD compared with CN individuals. Here, we reported leukocyte surface antigens exhibited differential expression in groups of older adults who met clinical criteria for CN, MCI, and AD-dementia, and were also associated with amyloid burden measured via the PET Centiloid score (CL). We found that several markers were differentially expressed in patients with MCI/AD-dementia, including both significantly upregulated and downregulated markers. The upregulated proteins in patients with AD were mostly associated with negative regulation of activation of membrane attack complex or transcytosis, whereas the downregulated proteins were mostly involved in cargo receptor activity or Aβ clearance. After controlling for co-expression clusters, we found four leukocyte markers that were differentially expressed to mark the profile of patients with AD: CD11c, CD59, CD91, and CD163. The significance stayed after correction for age, gender, education, and APOE ε 4 allele status.

Further examination of the identified AD-associated leukocyte markers revealed a strong correlation with brain Aβ burden. For example, we found a positive correlation between the CD59 (or CD91) and PET Aβ score, such that a subject with a higher PET Aβ score had a higher expression level of CD59 (or CD91). The AD-associated leukocyte markers also showed tight correlation with cognitive scores. Episodic memory is one of the best performed cognitive tests and PACC is a comprehensive score, including MMSE and episodic memory. For example, CD59 (or CD91) was negatively correlated with both episodic memory and Preclinical Alzheimer's Cognitive Composite (PACC) scores.

3 | DETAILED METHODS AND RESULTS

3.1 | Methods

3.1.1 | Study design

This cross-sectional study comprised four stages. Stage I was exploratory research to investigate major leukocyte markers, Stage II was an update for Stage I, and Stage III was designed to prospectively confirm the findings from previous stages. Finally, Stage IV was designed to confirm the findings from Stage III.

3.1.2 | Subjects and ethical approval

Participants in Stages I, II, and III were randomly selected from the Australian Imaging, Biomarkers and Lifestyle study (AIBL). The AIBL study

TABLE 1 Demographic make-up of all stages

Demographics	Control (< 25CL)[a]	Case (> 25CL)
Number of participants (n)	197	221
Age (mean (SD))	74.2 (6.5)	75.7 (8.1)
Sex = male (%)	87 (44.2)	115 (52.0)
Years of education (mean (SD))	14.2 (3.1)	12.3 (3.1)
APOE genotype (%)		
E2/E2	1 (0.5)	0 (0.0)
E3/E2	28 (14.4)	3 (1.4)
E3/E3	126 (64.9)	81 (37.7)
E4/E2	7 (3.6)	7 (3.3)
E4/E3	32 (16.5)	92 (42.8)
E4/E4	0 (0.0)	32 (14.9)
Clinical classification (%)		
AD	10 (5.1)	61 (27.6)
CN	161 (81.7)	80 (36.2)
MCI	26 (13.2)	80 (36.2)
Image PET centiloid (mean (SD))	1.1 (11.7)	85.6 (32.8)
MMSE (mean (SD))	28 (2)	25 (4)
CDR (mean (SD))	0.1 (0.3)	0.5 (0.5)
Episodic memory (mean (SD))	0.27 (2.71)	-3.22 (3.66)
Composite PACC score (mean (SD))	0.31 (3.03)	-4.18 (4.55)

[a]Stratified by amyloid-PET (centiloid). The validation cohort is excluded.

is a longitudinal observational cohort of CN, MCI and AD participants recruited by specialist referral or in response to advertisement. AIBL clinical classification is generated blind to biofluid or imaging amyloid biomarkers. AD diagnosis is determined by a consensus panel using the DSM-IV (American Psychiatric Association, 1994) and ICD-10 (World Health Organization, 1992) diagnostic criteria and AIBL excludes major psychiatric illness and some cardiovascular risk factors including excessive drinking and untreated obstructive sleep apnoea.[15] The subject selection process was blind to any information about the subject. This study integrates data from neuroimaging, biomarkers, lifestyle, and clinical and neuropsychological evaluations. Classification on diagnosis and disease stage of the AIBL participants is based on both clinical and neuroimaging/CSF evidence of Aβ accumulation. For Stage IV, the samples were randomly recruited from subjects participating in the Anti-Amyloid Treatment in Asymptomatic Alzheimer's Disease (A4) Study. For this study, we recruited 76 subjects into Stage I, 142 subjects into Stage II, and 200 subjects into Stage III from AIBL study participants. Each Stage recruited a different group of subjects from the AIBL subject population. (Table 1; Break-up tables in S1). For Stage IV, we obtained whole blood from 112 participants who were blindly and randomly selected from the cohort of subjects seen at US sites as part of the screening process for the Anti-Amyloid in Asymptomatic AD (A4) clinical trial (e.g., A4 Biobank Addendum, PI Rissman).[16] The A4 participant samples included were those who passed initial assessments and had amyloid PET and CSF measures performed.

Excluding Stage IV, all datasets included a balanced number of individuals classified clinically as CN, MCI, or AD-dementia. Patient evaluation and diagnosis have been described elsewhere.[15] All samples had corresponding PET Aβ and cognitive data (PET Aβ imaging procedure and cognitive testing in Supporting information). This study was approved by the Human Research Ethics Committee, St Vincent's Hospital, Melbourne (Ref: HREC-A 028/06) and the Eastern Health Research and Ethics Committee (Ref: E05/1011). All participants and patient caregivers completed written informed consent before participation. All clinical and demographic information of AIBL participants was masked until the collection of all biological measurements. Stage IV subject samples were from those who consented to the A4 trial and the A4 biobank Addendum.

3.1.3 | Blood collection

For AIBL samples, peripheral whole blood was collected via venepuncture between 8:00am and 10:30am after overnight fasting. Whole blood was kept in EDTA anti-coagulant Vacutainer tube (Becton Dickinson Biosciences) and was kept on ice during transportation. Processing of whole blood was completed within 3 h after collection. For A4 samples, whole blood via venepuncture was collected from overnight fasted participants. Blood collected was shipped overnight on ambient temperature gel packs and processed the following morning for analyses.

3.1.4 | Flow cytometry immunophenotyping (FCIP)

Cell surface staining procedures were carried out using the BD standard protocol: aliquots of 100 μL fresh blood were added into fluorescence-activated cell sorting (FACS) tubes with pre-mixed antibody cocktails. Mouse anti-human IgG monoclonal antibodies (mAbs) conjugated with fluorophores, which were excited at FITC, PE, PerCP, and APC channels, were used to stain leukocytes (Table S2). An autofluorescence tube containing only whole blood and an IgG isotype control (BD Australia) tube were prepared for each AIBL sample. Optimal concentration for antibodies was determined by titration tests. For intracellular staining, cells were fixed and permeabilized before mixing with antibody cocktail according to the BD standard protocol. Flow cytometry was performed by using FACSCalibur (BD Biosciences). All flow data were stored in digital form and were analyzed using FlowJo software (V10, FlowJo, LLC).

3.1.5 | Statistical analysis

FCIP data were compared between CN, MCI, and AD-dementia groups using one-way analysis of variance (ANOVA). Pearson product-moment correlational analyses were conducted to examine the strength of relationships between fluorescent intensity of mAbs and PET Aβ centiloid, episodic memory and PACC score. Logistic regression models created with the rms R package to predict
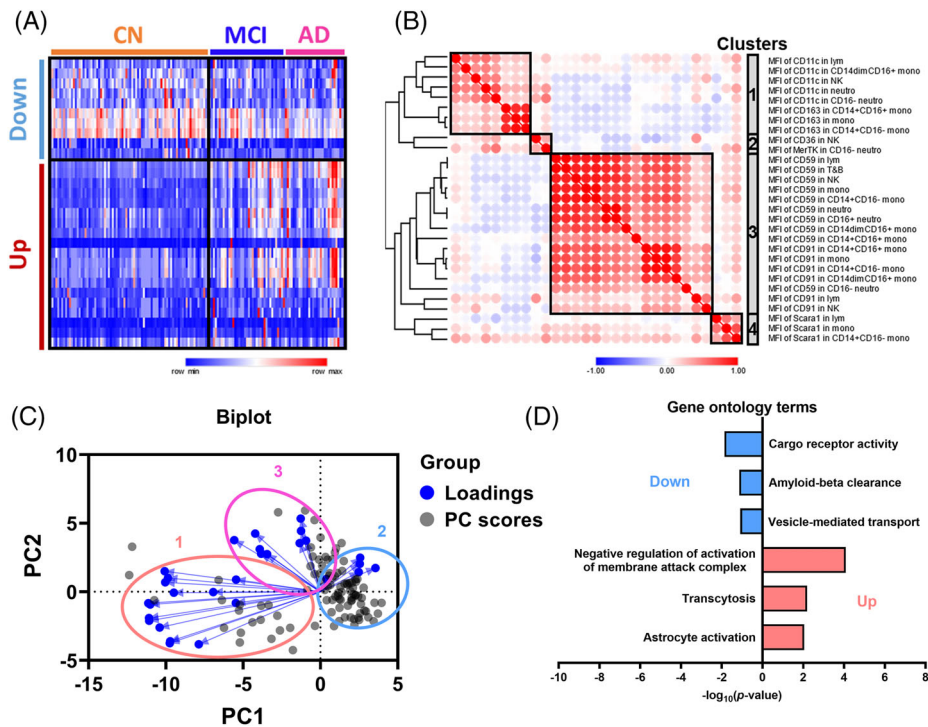
FIGURE 1 Differential expression of the leukocyte markers in AD. (A) A Heatmap of the expression levels of the top 27 down- and upregulated AD-associated leukocyte markers in CN, MCI, and AD-dementia. Blue and red represent a relative color scheme uses the minimum and maximum values in each row to convert values to colors. (B) A heatmap showing the pairwise correlations among AD-associated leukocyte markers. Each row and column represent one of the 27 AD-associated leukocyte markers. Red and blue indicate positive and negative correlations between pairs, respectively. Black squares denote the four clusters based on hierarchical clustering, and numbers in brackets on the right indicate the cluster number. (C) A biplot showing both the loadings and the PC scores. Variables in cluster 1 and 2 are strongly correlated (values close to 1 or -1) with PC1; while cluster 3 correlates strongly with PC2. (D) Representative GO term enrichment of the 12 AD-associated leukocyte markers. The GO terms of the down- and upregulated leukocyte markers are indicated in blue and red, respectively.

outcome were made for individual biomarkers, individual biomarkers adjusted for covariates and combinatorial sets of biomarkers. Detailed biomarker assessment can be found in Supporting information. Heat map and hierarchical clustering were created in Morpheus (https://software.broadinstitute.org/morpheus). Figures were plotted in GraphPad Prism for Windows (Version 8.4.2, San Diego, California USA).

4 | RESULTS

4.1 | Identification of differentially expressed leukocyte markers in patients with AD

We performed FCIP to identify which leukocyte markers were differentially expressed in AD. Among the 34 leukocyte markers assayed, 13 were differentially expressed in patients with clinically defined MCI/AD-dementia, including five upregulated markers and eight downregulated markers (Fold changes from 0.5 to 1.8, p-values from 7.9E-15 to 0.05; Heatmap in Figure 1A; Biological relevance in Table 2; Individual figures in Figure S3, S4 and S5). In Stage I, the levels of expression of CD11c (Figure 1) and P2X7 (Figure 2) were reduced in the MCI/AD-dementia compared with CN, in which CD11c decreased

by 19% to 34% and P2X7 decreased by 20-42% (Table S7.1 in Supporting information). Stage II confirmed the decreased expression of CD11c in the MCI/AD-dementia compared to CN (Figure 1), in which CD11c decreased by 14% to 23% (Table S7.2). It was also found that CD11b (Figure 2) and CD33 (Figure 3 A and B) decreased in the MCI/AD-dementia by 14% to 21% and 16% to 18%, individually (Table S7.2). However, P2X7 was not different in Stage II. In Stage III, 11 leukocyte markers differentially expressed, including five upregulated markers and six downregulated markers (Table S7.3 and S7.5). In patients with MCI and AD-dementia, CD35, CD59, CD91, RAGE, and Scara-1 increased by 40% to 47%, 27% to 78%, 44% to 59%, 31% and 32%, respectively (Figure 2A&E, Fig. S5.3, 5.4, 5.6, and S5.7); While CD11c, CD18, CD36, CD163, MerTK and P2X7 decreased by 15% to 19%, 20% to 26%, 45%, 11% to 16%, 12% and 5% to 15%, respectively (Figure 1, 5).

4.2 | Functionally classify the AD-associated leukocyte markers

We performed co-expression network analysis to examine the coregulation patterns of the 27 AD-associated leukocyte markers followed by hierarchical clustering analysis on the resultant correlation matrix. The

TABLE 2 Biological relevance and changes of potential biomarkers in AD

GO class (direct)	Potential biomarkers	Biological relevance to AD	Changes in AD
Amyloid-beta binding and microglial cell activation	CD11b	Integrin subunit alpha M; complement component receptor 3 alpha; phagocytosis; integrins; C3b binding.	Stage I, III Nil; Stage II ↓
Integrin binding	CD11c	Integrin subunit alpha X; complement component receptor 4 alpha; regulation of actin cytoskeleton.	Stage I, II, III ↓
Amyloid-beta binding and microglial cell activation	CD18/ITGB2	Integrin subunit beta 2; combines with different alpha chains, for example, CD11b and CD11c, to form different integrin heterodimers also referred to as CR3 and CR4.	Stage III ↓
Protein binding	CD33	GWAS associated gene for AD[17]; myeloid cell surface antigen; immunoglobulin superfamily cell adhesion molecule.	Stage II ↓
Complement component C3b/4b binding	CD35/CR1	GWAS associated gene for AD[18]; phagocytosis; integrins; complement receptor 1.	Stage III ↑
Amyloid-beta binding and antigen processing and presentation	CD36	Scavenger receptor class B member 1; phagocytosis; receptor for oxidized low-density lipoprotein (LDL)[19]; receptor for Aβ.[20]	Stage III ↓
Protein binding	CD59	Complement inhibitory protein; prevent formation of the complement membrane attack complex (MAC).[21]	Stage III ↑
Amyloid-beta binding and scavenger receptor activity	CD91	Genetically associated wi AD[22–24]; low-density lipoprotein receptor-related protein 1; mediates the endocytosis and degradation of secreted amyloid precursor protein.[25]	Stage III ↑
Scavenger receptor activity	CD163	Scavenger receptor.	Stage III ↓
Protein binding and protein phosphorylation	MerTK	Receptor protein-tyrosine kinase; mediates phagocytosis in microglial cells[26] and retinal pigment epithelium.[27]	Stage III ↓
Extracellularly ATP-gated cation channel activity	P2X7	Innate phagocytosis.[2]	Stage I, III ↓; Stage II Nil
Amyloid-beta binding and microglial cell activation	RAGE/AGER	Mediates Aβ transport across the blood-brain barrier and accumulation in brain[28,29].	Stage III ↑
Amyloid-beta binding and scavenger receptor activity	SCARA-1/MSR1	Macrophage scavenger receptor 1; phagocytosis; mediates uptake of fibrillar amyloid.[30]	Stage III ↑

GO: Gene ontology.
↑up-regulation.
↓down-regulation.
Nil: No change.

27 variables attributed to CD11c, CD36, CD59, CD91, CD163, MerTK, and Scara-1. CD18, CD35, P2X7, and RAGE were excluded because of extremely low sample number. This yielded four distinct clusters (Figure 1B). Different cell types had similar expression profiles for a specific leukocyte marker. For example, MFI of CD11c decreased in all cell types including lymphocytes, monocytes and neutrophils. Therefore, all five variables related to CD11c were classified into Cluster 1. The variables related to CD163 formed Cluster 2. Those related to CD36 and MerTK were joined to form Cluster 3. The variables related to CD59 and CD91 were joined to form Cluster 4. We also performed a principal component analysis (PCA) to project each data point onto the first two principal components to obtain lower-dimensional data while increasing interpretability. This yielded three distinct clusters (Figure 1C). Cluster 1 was composed of CD59 and CD91; Cluster 2 was dominated by CD163; Cluster 3 was dominated by CD11c; CD36, MerTK, and Scara-1 were scattered between Cluster 2 and 3. Taken together, the minimum number of AD-associated leukocyte markers required to sufficiently represent the leukocyte marker profile of patients with AD was four, including CD11c, CD59, CD91, and CD163.

Next, we identified enriched functional categories among the 13 AD-associated leukocyte markers by performing Gene Ontology (GO) analysis (Figure 1D). The upregulated proteins in patients with AD were associated with negative regulation of activation of membrane attack complex (CD35 and CD59; false discovery rate [FDR]-adjusted
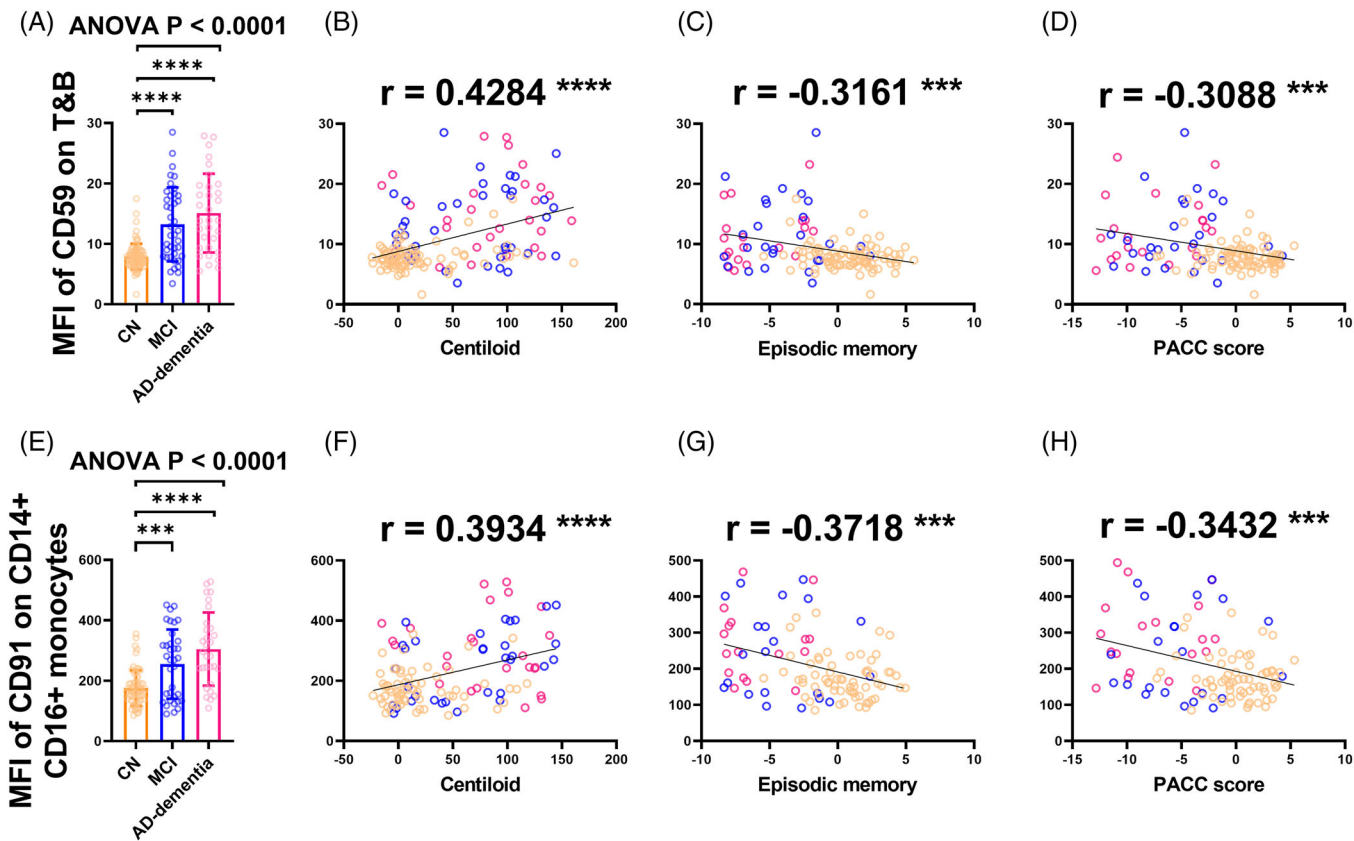
FIGURE 2 Associations between the top two AD-associated leukocyte markers and AD clinical stages, brain Aβ burden, and cognitive function. AD-associated leukocyte markers, CD59 and CD91, are presented in A-D and E-F, separately. The number of participants is 200 (CN = 124, MCI = 43, and AD = 33). Bar graphs are mean ± standard deviation of the mean and *p* value is decided by one-way ANOVA followed by multiple comparison using Tukey honest significant difference (HSD). Correlation r and *p* values are decided by Pearson product-moment correlational analysis. If the p-value is less than or equal to the significance level (alpha = 0.05), the correlation is deemed significantly different to zero, and the magnitude of the correlation is given to talk to the strength of the Pearson correlation R value (low absolute R value < 0.25, medium absolute R value of between 0.25 and 0.75, and high absolute R value of > 0.75. ***: *p* < 0.001; ****: *p* < 0.0001
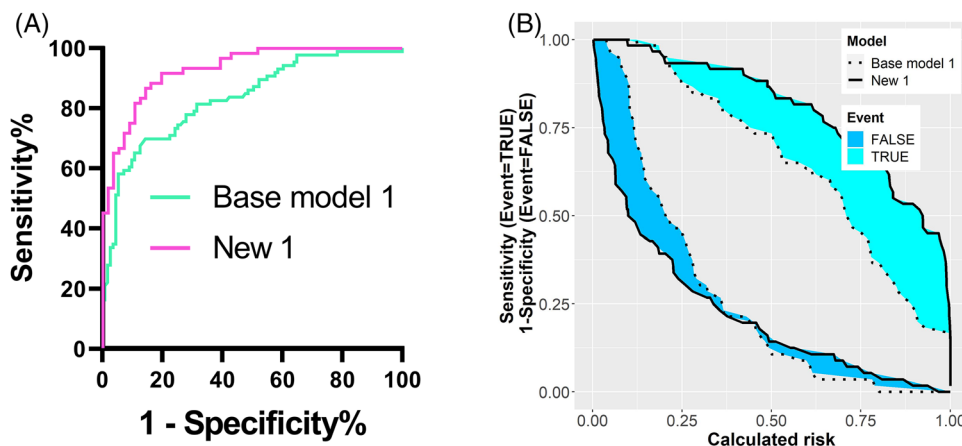

FIGURE 3 Performance assessment of the top four AD-associated leukocyte markers. (A) Paired sample area difference under the ROC curves between Base model 1 and a composite panel, New 1, when predicting diseased state from non-diseased state. Base model 1 comprises of age, sex, years of education, and APOE ε4 allele status. New 1 is formed by adding MFI of CD59 on T&B (BM4), MFI of CD163 on CD14$^-$ neutrophils (BM8), MFI of CD91 on CD14$^+$CD16$^+$ monocytes (BM10) and MFI of CD11c on CD14$^-$CD16$^+$ monocytes (lnBM2) to Base model 1. The New 1 panel proved an AUC of 0.93 (CI: 0.88 to 0.97), improved from the Base model 1 AUC of 0.88 (CI: 0.82 to 0.94) with a *p* value of 0.021 (DeLong test). (B) Assessing the improvement in risk prediction of New 1 model over Base 1 model. The difference between two models are shown by the filled areas. Light blue and dark blue represent the improvements with (TRUE) and without (FALSE) the event of interest.

p = 8.3E-5) or transcytosis (CD91 and RAGE; FDR-adjusted p = 6.4E-3), whereas the downregulated proteins were involved mostly in cargo receptor activity (CD11b, CD18, CD36, and CD163; FDR-adjusted p = 2.5E-6) or Aβ clearance (CD11b, CD18, and CD36; FDR-adjusted p = 5.5E-4).

4.3 | Differential leukocyte percentages for patients with AD

We repeatedly identified alterations in percentages of lymphocytes and monocytic subsets from Stage I, II, and III. We hereby use Stage III data as an example. The absolute percentage of CD14$^-$ lymphocytes in whole blood was lowered in MCI and AD-dementia groups compared with CN group by 11% (Figure S5.4) and correlated with CL, episodic memory, and PACC scores (Figure 15A and 5). The differences in the percentage of monocytes in whole blood between CN, MCI, and AD-dementia groups were trivial. However, when we divided the whole monocyte population into CD14$^+$CD16$^+$ (intermediate), CD14$^+$CD16$^-$ (classic), and CD14$^-$CD16$^+$ (nonclassic) subpopulations and evaluated their relative percentage from the whole monocyte population, the relative percentage of CD14$^+$CD16$^-$ monocytes was increased by 13% (p = 0.001) in AD-dementia and MCI groups compared with CN group (Figure 8B and Table S7) and negatively correlated with episodic memory and PACC (Figure 21C and D). Interestingly, compared to the CN group, the relative percentage of CD14$^+$CD16$^+$ monocytes decreased by 30% (p = 0.023) in MCI groups although the difference in AD-dementia group was minor (Figure 8C). A lower relative percentage of CD14$^-$CD16$^+$ monocytes in MCI group (-29%; p = 0.015) was also observed in another tube with smaller sample size (Figure 8D).

4.4 | Leukocyte markers correlated with brain Aβ burden and cognition

Further examination of the AD-associated leukocyte markers identified a strong correlation with brain Aβ burden, with a positive correlation between CD59 (or CD91) and PET Aβ score. Accordingly, a subject with higher CL had higher expression level of CD59 (Figure 2B and Figure S5.11) (or CD91 (Figure 2F and Figure S5.12)). The AD-associated leukocyte markers also showed a strong negative correlation with both cognitive scores. For example, CD59 (or CD91) was negatively correlated with both episodic memory and PACC (Figure 18) (or CD91 (Figure 19)).

4.5 | A biomarker panel comprising four leukocyte markers accurately distinguishes AD

Most of the AD-associated leukocyte markers were found in Stage III. AD-associated leukocyte markers were added to a logistic regression

model to evaluate their performance in predicting disease presence classified by PET Aβ imaging score, CL. We exercised extra caution by removing those leukocyte markers that did not have complete datasets (e.g., RAGE; see Table S7.5 in Supporting information), and sorted out ten representative variables, which were related to upregulated leukocyte markers, CD59 and CD91; downregulated leukocyte markers, CD11c and CD163; the percentages of CD14$^-$ lymphocytes (absolute) and CD14$^+$CD16$^-$ monocytes (relative) were included to make 12 candidates (Table S8 in Supporting information). However, leukocyte percentage was subject to alterations in many other diseases and therefore were not used for forming biomarker panel or composite in this study.

In Model 1 (Table S8.1), Base model 1 discriminated between < 25CL and > 25CL with an AUC of 0.83 (CI: 0.78 to 0.89). One leukocyte marker, the MFI of CD59 on T and B lymphocytes (BM4), had the best improved AUC of 0.9 (CI: 0.84 to 0.94) in predicting disease presence determined by PET Aβ imaging; Brier improvement of 19.4% to base model; IDI with events of 0.09 (CI: 0.06 to 0.14) and without events of 0.03 (CI: -0.01 to 0.05). Another leukocyte marker, the MFI of CD91 on CD14$^+$CD16$^+$ monocytes (BM10), had similar improved AUC and Brier score. Two other performing candidates, the MFI of CD163 on CD14$^-$ neutrophils (BM8) and the MFI of CD11c on CD14$^-$CD16$^+$ monocytes (lnBM2), were included to form a panel of biomarkers entitled New 1. The New 1 panel showed an AUC of 0.93 (CI: 0.88 to 0.97), improved from the Base model 1 AUC of 0.88 (CI: 0.82 to 0.94) with a p value of 0.021 (DeLong test, Figure 3A), Brier improvement 34.6%, IDI with and without events (Figure 3B) of 0.14 (CI: 0.08 to 0.18) and 0.05 (CI: 0.01 to 0.08), respectively. New 1 had sensitivity of 0.87 (CI: 0.75 to 0.94), specificity of 0.86 (CI: 0.74 to 0.94) and overall accuracy 0.86 (Table S11 in supporting information). Thus, we identified 4 leukocyte markers that associate both with AD clinical stages and brain Aβ accumulation.

4.6 | Results of Stage IV

Stage III identified CD11c, CD59, CD91, and CD163 as potential biomarkers to differentiate individuals who were PET Aβ positive (CI > 25) versus those who were PET Aβ negative (CL < 25) after correction for age, gender, education and APOE ε 4 allele status (Model 1, p = 0.021). We then validated them in an independent cohort (Stage IV, N = 112 for the demographic and clinical characteristics are shown in Table S1.7). Several leukocyte markers showed connection to clinical diagnosis (Figure S6.1) or association with amyloid burden (Figure S6.2), which were consistent with Stage III. A multivariable model for the validation cohort found that a combination of CD59 & CD91 discriminated Aβ status, CL > 25 or CL < 25, with an AUC of 0.91 (CI: 0.81 to 0.99). The AUC was 0.1 higher than for the mean base model AUC at 0.81 (CI: 0.72 to 0.9) after correction for age, gender, education and APOE ε 4 allele status (p = 0.072). A combination of CD11c, CD59, and CD91 had an AUC after correction at 0.95 (CI: 0.86 to 1), 0.14 above base model, p = 0.05, although this value could
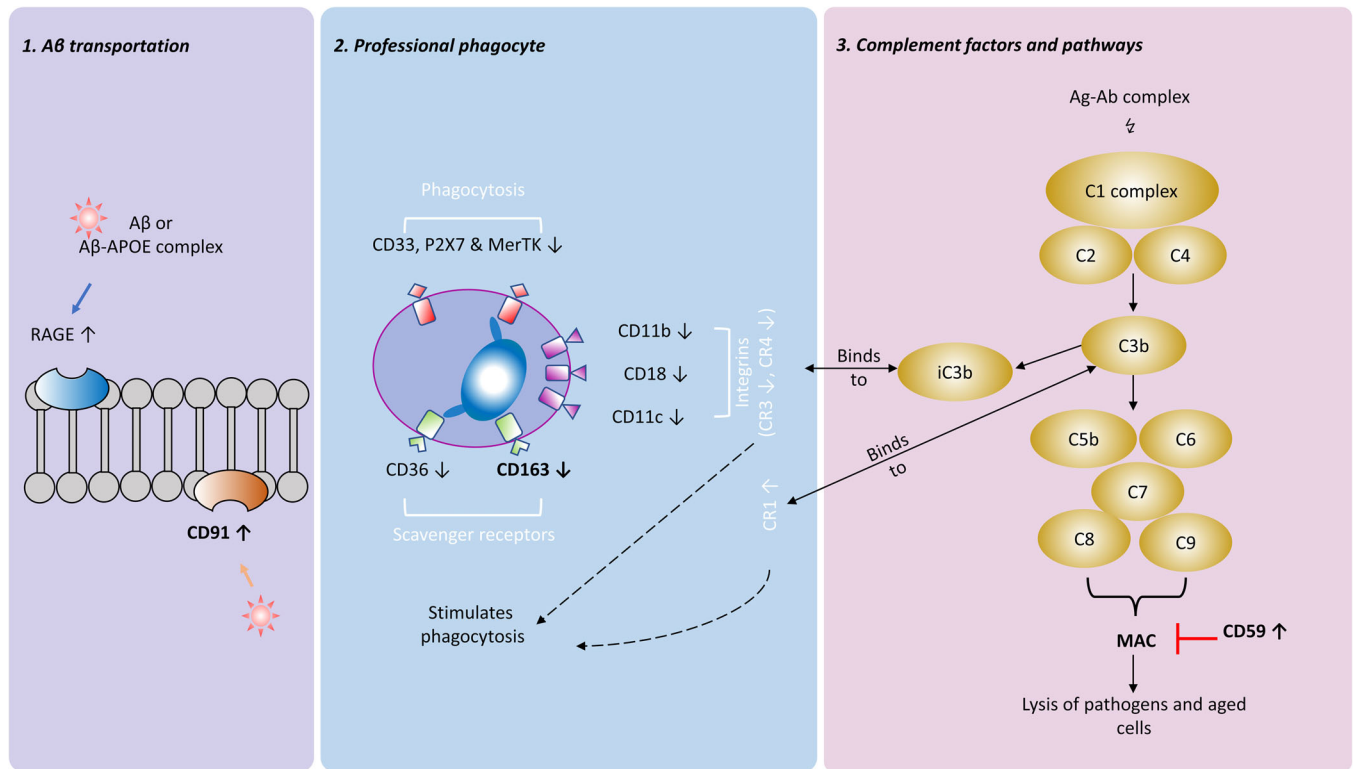
FIGURE 4 Scientific hypothesis and molecular pathways inhibiting phagocytosis and complement. Left. Aβ cross-membrane transportation is augmented given that both CD91 and RAGE increased in AD; Middle. A schematic phagocyte shows down-regulation in phagocytic receptors, MerTK and P2X7; scavenger receptors, CD36 and CD163; and cell adhesion integrins, CD11b, CD11c, and CD18, which can form CR3 and CR4; Right (adapted from KEGG map04610). Complement pathways are activated by antigen-antibody complex. It triggers cascade to form C3b. C3b binds to CR1 thatis upregulated in AD, and subsequentlystimulates phagocytosis. Whilst, iC3b binds to CR3 and CR4 thatare downregulated in AD, and subsequently inhibit phagocytosis. On the other hand, C5b, C6, C7, C8, and C9 form the membrane attack complex (MAC). CD59 prevents formation of MAC and lysis of target cells. Both inhibit opsonization, phagocytosis and clearance of pathogens which are involved in AD pathogenesis and development.

reflect an overfitting due to the relatively small sample size (detailed in Table S8.5).

4.7 | Potential diagnostic value of CD163 in preclinical AD

Next, we created several "preclinical" AD predicting models. In Model 2 (Table S8.2), Base model 2 discriminated between < 25CL and > 25CL and < 100CL with an AUC of 0.83 (CI: 0.76 to 0.9). The addition of the MFI of CD163 on CD14$^-$ neutrophils (BM8) to the model had improved AUC to 0.91 (CI: 0.84 to 0.95, $p = 0.079$). In Model 3 (Table S8.3), Base model 3 discriminated CN < 25CL and CN > 25CL & MCI with an AUC of 0.8 (CI: 0.73 to 0.87). Four biomarkers were added to the model. The AUC was improved to 0.9 (CI: 0.83 to 0.95) ($p = 0.034$). We also tried in a Model 4 (Table S8.4), which only had 30 cases. Base model 4 discriminated CN < 25CL and CN > 25CL with an AUC of 0.86 (CI: 0.79 to 0.93). In order to prove the concept, one best-performed biomarker, the MFI of CD163 on CD14$^-$ neutrophils (lnBM8), was added to the model. The AUC was improved to 0.94 (CI: 0.86 to 0.98) ($p = 0.063$).

Taken together, these results suggeste CD163 may prove to be useful in preclinical diagnosis.

5 | CONCLUSION

Our results suggest that leukocyte surface biomarkers involved in Aβ transportation, innate phagocytosis and completement mediated clearance pathways (Figure 4) are objective, measurable indicator of AD progression and could be valuable for early diagnosis. The main limitation of the current study is the cross-sectional nature of the study design. The next step to address this limitation is to perform a longitudinal study. A longitudinal study will be important not only to validate the presence of amyloid, but also more importantly, to establish the predictive value of the test to determine the odds of individuals for disease progression, especially from normal to MCI and from MCI to dementia. Furthermore, a longitudinal study will provide information about the potential changes in these biomarkers over time as the disease progresses in a given individual. Finally, it will be important to include these biomarkers in pharmacological trials of disease modifying

compounds to establish their potential value in predicting response to treatment.

These biomarkers could have a major impact on clinical practice by allowing primary care physicians to identify individuals at high risk of having amyloid burden in their brains with a simple blood test. Additionally, they could aid in the decision to proceed with more invasive assessments such as amyloid PET. They also could be critical in identifying asymptomatic patients that could be eligible for disease modifying interventions such as anti-amyloid treatments.

AUTHOR CONTRIBUTIONS

XH, YL, RR, FS: data collection, analysis, writing; CF, CD, VZ, KP, BT, KP, RR, FS, SAL: sample and data collection; JD, JP: analysis; YL, PM: cognitive tests; JM, YL, JF, RM, JW: results interpretation and writing; VD, VV, CR: brain imaging; CM&BG: design and writing.

CONFLICT OF INTERESTS

Stage I and II of this study were partially funded by NeuroQuest, Inc. (Israel). Stage III and IV were partially funded by Qiankang Life Science Melbourne R&D Centre. U.S. provisional patent application entitled, "Compositions, Kits, and Methods for Detecting Preclinical Alzheimer's Disease," (Thrive IP Ref. 1463.0002-P1, EFS ID:43461498) has been filed, followed by the PCT application (PCT/IL2022/050865).

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REFERENCES

1. Scheltens P, Blennow K, Breteler MMB, et al. Alzheimer's disease. *The Lancet*. 2016;388:505-517.
2. Jack CR Jr, Bennett DA, Blennow K, et al. A/T/N: an unbiased descriptive classification scheme for Alzheimer disease biomarkers. *Neurology*. 2016;87:539-547.
3. Dubois B, Hampel H, Feldman HH, et al, Proceedings of the Meeting of the International Working G. Preclinical Alzheimer's disease: definition, natural history, and diagnostic criteria. *Alzheimers Dement*. 2016;12:292-323. July, Washington Dc USA.
4. Sperling RA, Aisen PS, Beckett LA, et al. Toward defining the preclinical stages of Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7:280-292.
5. McKhann GM, Knopman DS, Chertkow H, et al. The diagnosis of dementia due to Alzheimer's disease: recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimers Dement*. 2011;7:263-269.
6. Wang J, Gu BJ, Masters CL, et al. A systemic view of Alzheimer disease - insights from amyloid-beta metabolism beyond the brain. *Nat Rev Neurol*. 2017;13:612-623.
7. Kunis G, Baruch K, Rosenzweig N, et al. IFN-gamma-dependent activation of the brain's choroid plexus for CNS immune surveillance and repair. *Brain*. 2013;136:3427-3440.
8. Baruch K, Deczkowska A, Rosenzweig N, et al. PD-1 immune checkpoint blockade reduces pathology and improves memory in mouse models of Alzheimer's disease. *Nat Med*. 2016;22:135-137.
9. Rezai-Zadeh K, Gate D, Szekely CA, et al. Can peripheral leukocytes be used as Alzheimer's disease biomarkers? *Expert Rev Neurother*. 2009;9:1623-1633.
10. Nakamura A, Kaneko N, Villemagne VL, et al. High performance plasma amyloid-beta biomarkers for Alzheimer's disease. *Nature*. 2018;554:249-254.
11. Fossati S, Ramos Cejudo J, Debure L, et al. Plasma tau complements CSF tau and P-tau in the diagnosis of Alzheimer's disease. *Alzheimers Dement (Amst)*. 2019;11:483-492.
12. Jiang Y, Zhou X, Ip FC, et al. Large-scale plasma proteomic profiling identifies a high-performance biomarker panel for Alzheimer's disease screening and staging. *Alzheimers Dement*. 2021.
13. Tansey KE, Cameron D, Hill MJ, et al. Genetic risk for Alzheimer's disease is concentrated in specific macrophage and microglial transcriptional networks. *Genome Med*. 2018;10:14.
14. Phongpreecha T, Fernandez R, Mrdjen D, et al. Single-cell peripheral immunoprofiling of Alzheimer's and Parkinson's diseases. *Sci Adv*. 2020;6.
15. Fowler C, Rainey-Smith SR, Bird S, et al. Fifteen years of the Australian imaging, biomarkers and lifestyle (AIBL) Study: progress and Observations from 2,359 Older Adults Spanning the Spectrum from Cognitive Normality to Alzheimer's Disease. *J Alzheimers Dis Rep*. 2021;5:443-468.
16. Sperling RA, Donohue MC, Raman R, et al. Team AS (2020) Association of factors with elevated amyloid burden in clinically normal older individuals. *JAMA Neurol*;77:735-745.
17. Naj AC, Jun G, Beecham GW, et al. Schellenberg GD (2011) Common variants at MS4A4/MS4A6E, CD2AP, CD33 and EPHA1 are associated with late-onset Alzheimer's disease. *Nat Genet*;43:436-441.
18. Lambert JC, Heath S, Even G, et al. Genome-wide association study identifies variants at CLU and CR1 associated with Alzheimer's disease. *Nat Genet*. 2009;41:1094-1099.
19. Endemann G, Stanton LW, Madden KS, et al. CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem*. 1993;268:11811-11816.
20. Stewart CR, Stuart LM, Wilkinson K, et al. CD36 ligands promote sterile inflammation through assembly of a Toll-like receptor 4 and 6 heterodimer. *Nat Immunol*. 2010;11:155-161.
21. Blom AM. The role of complement inhibitors beyond controlling inflammation. *J Intern Med*. 2017;282:116-128.
22. Lendon CL, Talbot CJ, Craddock NJ, et al. Genetic association studies between dementia of the Alzheimer's type and three receptors for apolipoprotein E in a Caucasian population. *Neurosci Lett*. 1997;222:187-190.

23. Hollenbach E, Ackermann S, Hyman BT, Rebeck GW. Confirmation of an association between a polymorphism in exon 3 of the low-density lipoprotein receptor-related protein gene and Alzheimer's disease. *Neurology*. 1998;50:1905-1907.

24. Baum L, Chen L, Ng HK, et al. Low density lipoprotein receptor related protein gene exon 3 polymorphism association with Alzheimer's disease in Chinese. *Neurosci Lett*. 1998;247:33-36.

25. Kounnas MZ, Moir RD, Rebeck GW, et al. LDL receptor-related protein, a multifunctional ApoE receptor, binds secreted beta-amyloid precursor protein and mediates its degradation. *Cell*. 1995;82:331-340.

26. Fourgeaud L, Traves PG, Tufail Y, et al. TAM receptors regulate multiple features of microglial physiology. *Nature*. 2016;532:240-244.

27. Gal A, Li Y, Thompson DA, Weir J, et al. Mutations in MERTK, the human orthologue of the RCS rat retinal dystrophy gene, cause retinitis pigmentosa. *Nat Genet*. 2000;26:270-271.

28. Deane R, Du Yan S, Submamaryan RK, et al. RAGE mediates amyloid-beta peptide transport across the blood-brain barrier and accumulation in brain. *Nat Med*. 2003;9:907-913.

29. Arancio O, Zhang HP, Chen X, Lin C, Trinchese F, Puzzo Det al, et al. RAGE potentiates Aβ-induced perturbation of neuronal function in transgenic mice. *EMBO J*. 2004;23:4096-4105.

30. Frenkel D, Wilkinson K, Zhao L, et al. Scara1 deficiency impairs clearance of soluble amyloid-beta by mononuclear phagocytes and accelerates Alzheimer's-like disease progression. *Nat Commun*. 2013;4:2030.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.