

Increased Number of Plasma B Cells Producing Autoantibodies Against A β ₄₂ Protofibrils in Alzheimer's Disease

Sofia Söllvander^a, Frida Ekholm-Pettersson^{a,1}, Rose-Marie Brundin^a, Gabriel Westman^b, Lena Kilander^a, Staffan Paulie^c, Lars Lannfelt^a and Dag Sehlin^{a,*}

^aDepartment of Public Health & Caring Sciences/Molecular Geriatrics, Rudbeck Laboratory, Uppsala University, Uppsala, Sweden

^bDepartment of Medical Sciences, Uppsala University, Uppsala, Sweden

^cMabtech AB, Nacka Strand, Sweden

Handling Associate Editor: Fabrizio Piazza

Accepted 26 May 2015

Abstract. The Alzheimer's disease (AD)-related peptide amyloid- β (A β) has a propensity to aggregate into various assemblies including toxic soluble A β protofibrils. Several studies have reported the existence of anti-A β antibodies in humans. However, it is still debated whether levels of anti-A β antibodies are altered in AD patients compared to healthy individuals. Formation of immune complexes with plasma A β makes it difficult to reliably measure the concentration of circulating anti-A β antibodies with certain immunoassays, potentially leading to an underestimation. Here we have investigated anti-A β antibody production on a cellular level by measuring the amount of anti-A β antibody producing cells instead of the plasma level of anti-A β antibodies. To our knowledge, this is the first time the anti-A β antibody response in plasma has been compared in AD patients and age-matched healthy individuals using the enzyme-linked immunospot (ELISpot) technique. Both AD patients and healthy individuals had low levels of B cells producing antibodies binding A β ₄₀ monomers, whereas the number of cells producing antibodies toward A β ₄₂ protofibrils was higher overall and significantly higher in AD compared to healthy controls. This study shows, by an alternative and reliable method, that there is a specific immune response to the toxic A β protofibrils, which is significantly increased in AD patients.

Keywords: Amyloid- β , amyloid- β protofibrils, anti-amyloid- β antibodies, enzyme-linked immunospot assay

INTRODUCTION

In Alzheimer's disease (AD) brains, extracellular amyloid deposits are found in the parenchyma as plaques and in vessel walls as congophilic amyloid angiopathy. These hallmarks of AD are composed of insoluble fibrils of amyloid- β (A β), a hydrophobic and self-aggregating peptide. Prior to plaque deposition,

A β gradually polymerizes from monomers into larger soluble molecular forms (oligomers/protofibrils) and eventually into insoluble fibrils. Several of the intermediate A β species have been shown to elicit adverse biological effects both *in vitro* and *in vivo* [1–5], suggesting that they play a central role in the pathogenesis. We have previously identified a pathogenic mutation in the amyloid- β protein precursor (A β PP) gene, the Arctic mutation (E693G). This mutation, causing early onset AD, is located within the A β domain and enhances the formation of A β protofibrils, suggesting that this A β species may be causative of the disease [6]. Moreover, it has been shown that A β protofibrils are present in cerebrospinal fluid (CSF) and that the levels

¹ Present address: Thermo Fisher Scientific, Uppsala, Sweden.

*Correspondence to: Dag Sehlin, Department of Public Health & Caring Sciences/Molecular Geriatrics, Rudbeck Laboratory, Uppsala University, Dag Hammarskjölds väg 20, 75185 Uppsala, Sweden. Tel.: +46 18 4715038; E-mail: dag.sehlin@pubcare.uu.se.

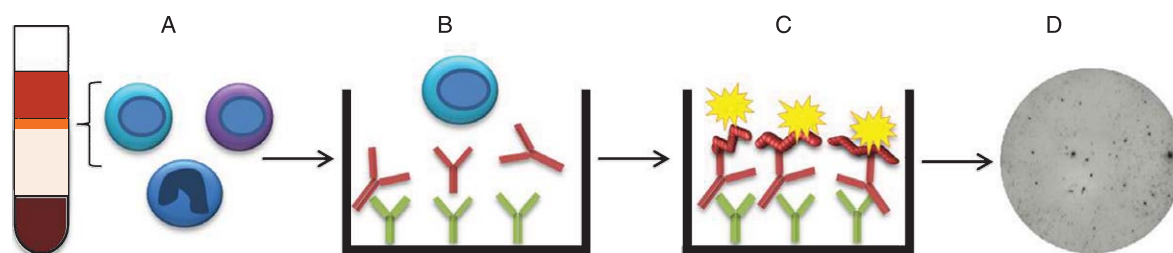


Fig. 1. B cell ELISpot. A) PBMCs were collected by Ficoll-Paque separation, stimulated with R848 and hIL-2 to start their antibody production and B) added to ELISpot wells coated with anti-IgG capture antibodies. C) Biotinylated A β binds to the captured anti-A β antibodies secreted from the activated B cells. D) The dots representing cells secreting anti-A β antibodies were visualized by Streptavidin-ALP.

of large soluble A β aggregates in CSF correlate with cognitive impairment, implying that the progression of the disease is dependent on A β protofibrils [7–10].

Autoantibodies, reactive to self-antigens, are often associated with pathological functions leading to autoimmune diseases. However, some autoantibodies play a physiological role in the body without leading to pathological processes and disease progression. These naturally occurring autoantibodies are believed to have a role in maintaining homeostasis and preventing inflammation [11]. Natural autoantibodies against A β (anti-A β antibodies) have been found in both AD and non-demented individuals [12, 13]. A vast part of anti-A β antibodies are believed to be produced in response to A β in the periphery and central nervous system. It has been proposed that anti-A β antibodies predominantly show affinity toward the toxic aggregated forms of A β , including oligomers and protofibrils, which may be considered nonself-antigens, and not to monomers [14–17]. The function of anti-A β antibodies is not understood but they have been implied to interfere with A β toxicity and the oligomerization and fibrillization of A β [1, 14, 16, 18, 19]. As a therapeutic approach, intravenous infusion of polyvalent IgG antibodies from healthy individuals (IVIG), proposed to contain anti-A β antibodies, has been studied in AD with positive effects on mouse behavior [14, 20] and AD cognition [21–23]. Nevertheless, to this date, clinical trials have failed to show any positive treatment effects of IVIG in AD [24, 25]. Aducanumab, originally derived from healthy aged individuals, makes up yet another therapeutic strategy for the use of anti-A β antibodies in AD and it has recently entered phase 3 clinical trials. Aducanumab recognizes aggregated forms of A β and appears to reduce A β deposits in patients with early and mild forms of the disease and to slow cognitive decline [26].

Different studies report discrepancies regarding plasma levels of anti-A β antibodies in AD. Numerous

reports have demonstrated decreased [12, 13, 15, 27–31], elevated [16, 18, 32, 33], or equal [1, 34, 35] levels in AD patients compared to control subjects. Anti-A β_{42} antibodies levels in Down syndrome patients have been found to be higher in plasma compared to non-Down syndrome mentally retarded subjects with perinatal brain injury and to age-matched healthy controls [36]. Moreover, an autoimmune reaction against A β , mediated by anti-A β antibodies, has been implied to be directly involved in the pathogenesis of cerebral amyloid-related inflammation with increased levels of anti-A β antibodies in CSF [37, 38]. In the current study, instead of studying antibodies in plasma, we utilized the enzyme-linked immunospot (ELISpot) technique to investigate the presence of anti-A β antibody secreting cells in the blood. With this technique, first described by Czerkinsky et al. [39], secretion of anti-A β antibodies can be analyzed on the cellular level (Fig. 1) avoiding enzyme-linked immunosorbent assay (ELISA) associated problems, e.g. formation of immune complexes between circulating A β antibodies and native A β , when analyzing plasma samples. In addition, the ELISpot method enabled us to compare the antibody response toward monomeric and protofibrillar forms of A β . Here we have found that the number of B cells producing IgG antibodies towards A β_{42} protofibrils was significantly higher in AD patients compared to healthy individuals.

MATERIALS AND METHODS

Study material

Blood sampling from AD patients ($n = 50$) and age-matched healthy individuals ($n = 55$) was performed according to standardized procedures at the Memory Disorder Unit, Uppsala University Hospital, Uppsala, Sweden as approved by the Regional ethical committee in Uppsala (decision number 2009/097). Clinical AD

diagnosis was determined by the NINCDS-ADRDA criteria, after a comprehensive clinical diagnostic procedure including neuroimaging. Mini-Mental State Examination (MMSE, Folstein -75) was administered and scores were used as a rough measurement of cognitive function. Age-matched healthy individuals were recruited by local advertisement at the Memory Disorder Unit, Uppsala University. Inclusion of healthy individuals required absence of cognitive impairment or other dementia disorders. Both AD patients and age-matched healthy individuals volunteered to be part of the study and written informed consent was obtained from all subjects or their closest relative.

Preparation of biotinylated A β ₄₀ monomers and A β ₄₂ protofibrils

Synthetic A β ₄₀ monomers and A β ₄₂ protofibrils were prepared as previously described [18, 40, 41]. Synthetic A β ₄₀ (Polypeptide Laboratories AB, Limhamn, Sweden) dissolved in 10 mM NaOH, was diluted in 2 \times phosphate buffered saline (PBS) to 100 μ M. Synthetic A β ₄₂ (American Peptide Company Inc., Sunnyvale, CA, USA) dissolved in 10 mM NaOH, diluted in 10 \times PBS to 443 μ M (2 mg/ml), was incubated for 30 min at 37°C and centrifuged for 5 min at 17 900 \times g to remove any insoluble aggregates and then diluted with PBS to a final concentration of 100 μ M. Both A β ₄₀ monomers and A β ₄₂ protofibrils were biotinylated with Sulfo-NHS-LC-Biotin (Thermo Fischer Scientific Inc., Waltham, MA, USA) according to manufacturer's guidelines with an A β /biotin molar ratio of 1/20. The A β ₄₂ protofibril concentration is expressed in monomer concentration units.

A β protofibril ELISA

A β protofibril ELISA using the monoclonal antibody 158 (mAb158) was performed according to the protocol described by Englund et al. [42]. In short, 96-well EIA/RIA plates (Corning Inc., Corning, NY, USA) were coated with 200 ng/well of mAb158 antibody in 100 μ l PBS at 4°C overnight. Plates were blocked with 1% bovine serum albumin (BSA) in PBS with 0.15% Kathon. Standard series of synthetic A β ₄₂ protofibrils, either biotinylated or not, were diluted in ELISA incubation buffer (0.05% Tween, 0.1% BSA and 0.15% Kathon in PBS at pH 7.4) and added to the plates for incubation 2 h at room temperature (RT). After washing, the plates were incubated for 1 h at RT with 0.5 μ g/ml mAb158 conjugated to horseradish peroxidase. K-blue aqueous

TMB substrate (Neogen Corporation, Lansing, MI, USA) was used as horseradish peroxidase substrate, and the reaction was stopped with 1 M H₂SO₄. Absorbance was measured by Tecan Infinite M200 PRO (Tecan Group Ltd., Männedorf, Switzerland) spectrophotometer at 450 nm and analyzed with Magellan v7.0 software (Tecan Group Ltd.). Washing was performed by adding 250 μ l washing buffer (PBS with 0.1% Tween 20 and 0.15% Kathon) repeated three times between each step of the ELISA.

PBMC preparation

Whole blood, sampled in BD Vacutainer CPT™ Cell Preparation Tubes with sodium heparin (BD bioscience, Franklin Lakes, NJ, USA), was diluted 1:2 in PBS and gently layered over cold Ficoll-Paque PLUS (GE Healthcare, Little Chalfont, UK) and kept on ice before centrifuged for 20 min at 900 \times g without brake at 18–20°C. The cell layer on top of the Ficoll-Paque PLUS consisting of peripheral blood mononuclear cells (PBMCs) was collected and diluted in PBS. PBMCs were centrifuged again for 7 min at 450 \times g at 18–20°C. The supernatant was removed and cells were resuspended in 5 ml PBS and counted before they were centrifuged at 350 \times g for 7 min at 18–20°C. Finally, cells were resuspended in freezing medium (10% DMSO in heat inactivated fetal bovine serum (FBS) (Nordic Biolabs AB, Täby, Sweden) and placed at -70°C in a Mr. Frosty™ Freezing Container (Thermo Fisher) for at least 3 h before being transferred to liquid nitrogen for extended storage.

Flow cytometry

For each individual cell sample, 5 \times 10⁶ PBMCs were quickly thawed at 37°C and diluted in 40 ml cold wash buffer (PBS supplemented with 2.5% FBS (Life Technologies Ltd. Paisley, UK) and 0.1% sodium azide). The cell suspension was centrifuged at 250 \times g for 5 min and after discarding the supernatant, the pellet was resuspended in 400 μ l wash buffer. Each sample was stained for 1 h in a light-protected environment at 2°C with titrated amounts of anti CD19 labelled with Alexa Fluor 700 (BD Biosciences). Samples were analyzed using a BD LSR II Special Order System, controlled by the BD FACSDiva 6.0 software (BD Biosciences). A preliminary forward scatter (FSC) versus side scatter (SSC) gate was used to identify lymphocytes and, depending on sample size, a total of up to 100.000 in-gate events were recorded. All datasets were migrated to FlowJo 7.6.5 (Treestar Inc. Ashland, OR, USA) for further gating and analysis.

Activation of B cell antibody production

Five million PBMCs were thawed and diluted in complete medium (RPMI 1640 (Nordic Biolabs), supplemented with a mix of penicillin (100 U/ml) and streptomycin (100 μ g/ml) (Thermo Fisher), L-glutamine (2 mM) (Life Technologies), Hepes (10 mM) (Sigma-Aldrich, Saint Louis, MO, USA), and FBS (10%) (Nordic Biolabs). The PBMCs were washed twice in complete medium by centrifugation for 10 min at $200 \times g$ before incubation in complete medium at 37°C , 5% CO_2 for 1 h. After resuspension, any debris or cell aggregates were allowed to sediment and the cells were transferred to a new centrifuge vial. To activate the IgG production, cells were stimulated for 72 h at 37°C , 5% CO_2 in complete medium supplemented with the polyclonal activator R848 (1 μ g/ml) (Mabtech AB, Nacka Strand, Sweden) and recombinant human Interleukin-2 (10 ng/ml) (Mabtech AB).

ELISpot

EliSpot^{PLUS} kit for human IgG (Mabtech AB) was used to study the IgG producing B cells. First, ELISpot plates were activated with 70% Ethanol (50 μ l/well) during 1 min and washed with sterile H_2O and coated with mouse anti-human IgG mAbs MT91/145 (15 μ g/ml) overnight at 4°C . Plates were then washed five times with sterile PBS followed by 1 h incubation in complete medium. PBMCs were added in triplicates to the plates at a concentration of 500.000 cells/well for the analysis of anti-A β antibodies and 50.000 cells/well for measuring the total number of IgG secreting cells. After incubation for 24 h in 37°C , 5% CO_2 , cells were removed by washing four times in PBS in a Tecan Hydrospeed plate washer (Tecan Group Ltd.) followed by incubation with biotinylated A β_{42} protofibrils (500 nM) or biotinylated A β_{40} monomers (500 nM) for 2 h at RT to detect anti-A β antibodies and with biotinylated α -human IgG mAbs MT78/145 (1 μ g/ml) for determination of the total number of IgG producing cells. After another round of washing, the plates were incubated for 1 h at RT with the conjugate Streptavidin-Alkaline Phosphatase (Streptavidin-ALP) (1:1000 in PBS supplemented with 0.5% FBS). Finally, plates were washed and developed with filtered 5-bromo-4-chloro-3-indolyl-phosphate in conjunction with nitro blue tetrazolium (BCIP/NBT) substrate until spots were evident. After stopping the substrate reaction by washing extensively in tap water, plates were dried and analyzed with an ELISpot reader (AID, Strassberg, Germany).

Statistical analysis

GraphPad Prism 5 was used for all statistical analyses (GraphPad Software, Inc., La Jolla, USA). Unpaired *t* test with Welch's correction was used throughout the study and the results are presented in scattergrams or box plots with mean \pm standard deviation. For correlation, Spearman rank correlation was used with the 95% confidence interval, spearman *r* and *p* values included in the graph. Level of significance were set at **p* < 0.05, ***p* < 0.01, and ****p* < 0.001. The study was blinded until time of statistical analysis.

RESULTS

Blood from age-matched AD patients (*n* = 50, mean age 77.8 years) and healthy individuals (*n* = 55, mean age 74.3 years) were used for the preparation of PBMCs. For the analysis of cells secreting anti-A β antibodies, 500.000 cells stimulated for 72 h with the polyclonal activator R848 and recombinant IL-2 were added to each well of ELISpot plates coated with anti-IgG. As a positive control, cells (50,000 cells/well) were added in parallel wells but analyzed for the total number of IgG secreting cells. Samples with no or very few IgG producing cells in the positive control wells (AD patients (*n* = 7), healthy individuals (*n* = 10)) were excluded from the study. Similarly, samples (AD patients (*n* = 4), healthy individuals (*n* = 11)) with too low numbers of PBMCs already after extraction or after thawing were excluded. The remaining samples that were included in the study are displayed in Table 1. In general, more IgG-producing B cells were found in blood from individuals with AD compared to age matched controls (Fig. 2A). However, no differences in B cell numbers were detected when comparing AD patients to healthy individuals by flow cytometry (Fig. 2B).

To investigate the presence of B cells secreting anti-A β antibodies, two different biotinylated A β preparations were used; synthetic A β_{40} monomers and synthetic A β_{42} protofibrils. Synthetic A β_{42} protofibrils were here defined as soluble A β aggregates larger than 100 kDa, i.e., eluting in the void volume on a size exclusion Sephadex 75 column. The A β_{42} protofibrils were first run in an A β protofibril specific ELISA to control that the protofibril structure was intact after the biotinylation step. The biotinylated A β_{42} protofibrils were readily detected by a protofibril specific ELISA and there was no significant difference between A β_{42} protofibrils with or without biotin confirming conformational maintenance (Fig. 2C). In general, a

Table 1

AD patients and age-matched healthy individuals included in the study. The PBMCs used in the study were derived from AD patients and age-matched healthy controls. The table presents gender, age and ApoE allele frequencies for AD patients and healthy controls, respectively

	AD patients (n = 46)	Healthy individuals (n = 44)
Age (years)		
Mean \pm SD	78.0 \pm 6.5	74.8 \pm 7.0
Range	63–90	51–87
Gender		
Female/Male	20/26	24/20
MMSE score*		
Mean \pm SD	21.0 \pm 3.8	
Range	10–27	
ApoE allele**		
E2	11.1%	15.9%
E3	91.1%	90.9%
E4	62.2%	38.6%

*MMSE score from 1 patient was not available and was therefore not included in the calculations. **ApoE genotype from 1 AD patient was not available and was therefore not included in the calculations.

higher number of cells produced antibodies recognizing synthetic A β_{42} protofibrils than synthetic A β_{40} monomers ($p < 0.0001$) (Fig. 3A). While there was no significant difference between AD patients and healthy individuals with regard to the number of plasma B cells producing antibodies against monomeric A β_{40} (Fig. 3B), the number of cells secreting antibodies to A β_{42} protofibrils was significantly higher in AD individuals compared to healthy individuals ($p = 0.043$) (Fig. 3C). This difference was even more evident for ApoE4 allele homo- or heterozygous AD individuals ($p = 0.031$). Moreover, in AD patients the number of B cells producing antibodies to A β_{42} protofibrils

correlated weakly with the total number of IgG producing B cells (Spearman $r = 0.59$, $p < 0.001$) (Fig. 3D).

No statistically significant correlation was found between the frequencies of anti-A β antibody secreting cells in the AD group and Mini Mental Status (based on Mini Mental Test) (Fig. 4). Nor was there a correlation between the number of A β -specific cells and age or gender except for in the control group, where females had significantly higher frequencies of B cells producing antibodies towards A β_{42} protofibrils than males ($p = 0.025$).

DISCUSSION

Use of different methods, study setups and various forms of A β may explain the conflicting results regarding anti-A β antibodies in AD and healthy individuals. The vast majority of earlier publications reporting A β antibody levels in human serum/plasma have used ELISA [12, 13, 28, 31–35, 43–48]. With ELISA, the reliability of the measurements can be questioned as there is a risk of immune complex formation between circulating A β antibodies and native A β in the blood. Thus, antibodies may be prevented from binding A β in ELISA plates, leading to an underestimation of antibody levels. This problem can be overcome by measuring anti-A β antibodies after dissolving the complexes, typically through a brief treatment with acid. Gustaw et al. measured the levels using ELISA before and after dissociation and found that anti-A β antibody levels were increased in both AD patients and normal control subjects and that AD patients displayed higher levels of anti-A β antibodies compared to normal controls after treatment [29, 30]. Another

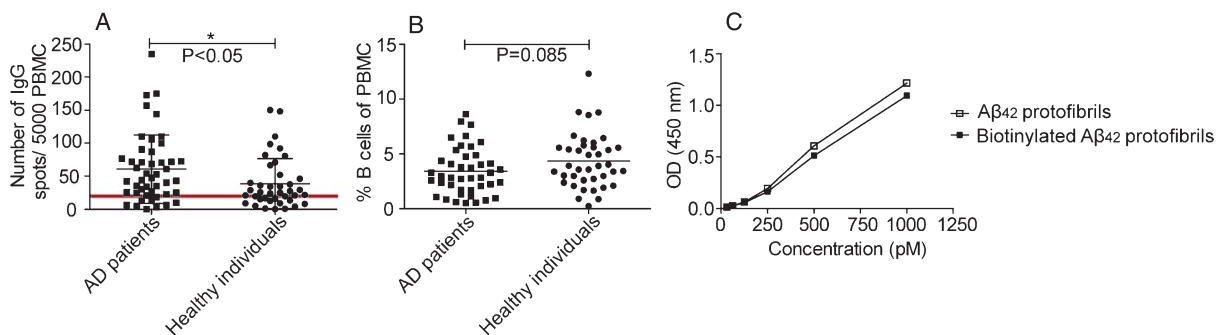


Fig. 2. Characterization of PBMCs and validation of the antigen. A) Total IgG production from activated B cells. Number of spots representing IgG producing cells detected in the ELISpot. AD individuals ($n = 39$) (■) had higher number of spots than healthy controls ($n = 30$) (●) ($p \leq 0.05$). PBMCs from individuals failing activation (spots < 20) (AD patients ($n = 7$), healthy controls ($n = 10$)) were excluded from the study (below horizontal line). B) B cell levels in AD patients and healthy individuals. The proportion of B cells in PBMCs was defined with flow cytometry by determining the number of CD19 $^{+}$ cells. There was no significant difference in the percentage of B cell in AD patients compared to healthy controls ($p = 0.085$). C) A β_{42} protofibril evaluation with protofibril specific ELISA. No considerable difference was noticed comparing biotinylated A β_{42} protofibrils with non-biotinylated A β_{42} protofibrils.

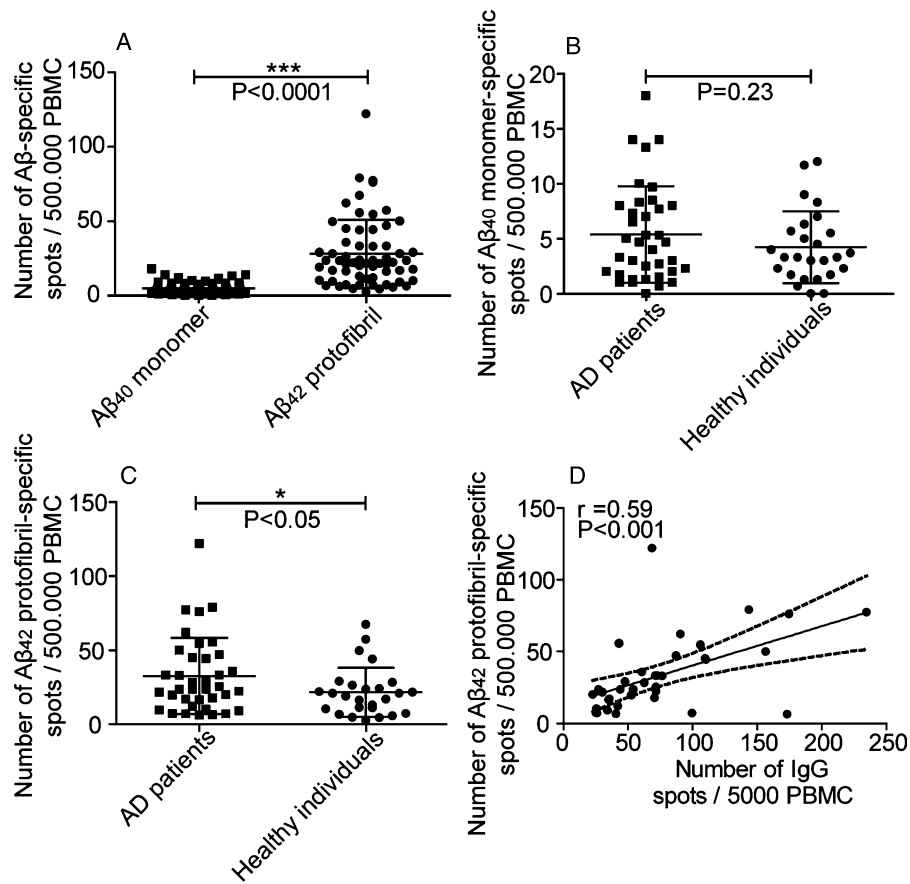


Fig. 3. B cell production of anti-A β_{40} monomer and anti-A β_{42} protofibril antibodies. A) Number of spots representing cells producing antibodies to A β_{40} monomers (■) and A β_{42} protofibrils (●). Looking at the whole study material ($n = 63$), antibodies detected A β_{42} protofibrils to a higher degree than A β_{40} monomers ($p < 0.0001$). B) B cell production of anti-A β_{40} monomer antibodies. Number of spots representing cells producing antibodies to A β_{40} monomers in AD patients ($n = 37$) (■) and healthy individuals ($n = 26$) (●). No significant difference was seen when comparing AD patients with healthy controls ($p < 0.23$). C) Number of spots representing cells producing antibodies detecting A β_{42} protofibrils in AD individuals ($n = 37$) (■) and healthy individuals ($n = 26$) (●). There was a significant difference in number of spots comparing AD patients with healthy individuals ($p < 0.05$). D) In AD patients, the number of spots representing B cells producing antibodies binding to A β_{42} protofibrils correlated weakly with the number of spots representing the total number of IgG producing B cells (Spearman $r = 0.59$, $p < 0.001$).

problem when using ELISA is that A β monomers may artificially adopt amyloidogenic epitopes due to surface adsorption [15], potentially causing misinterpretations regarding antibody recognition of specific A β forms. Therefore, to distinguish different forms of A β in immunoassays, A β should preferably be kept in solution. We used the ELISpot assay since this not only eliminates the problem of antibodies occurring in complexes but also allows the testing of A β in a soluble form. The ELISpot assay is also very sensitive and enables the detection of the very small populations typically seen in antigen-specific responses. This way ELISpot enabled us to accurately measure the presence of B cells producing anti-A β antibodies in AD patients and age-matched healthy individuals and to

compare the anti-A β antibody selectivity toward A β_{40} monomers and A β_{42} protofibrils. The choice of different A β peptides for generation of monomers and protofibrils was motivated by their different propensity to aggregate and thus maintain their conformation throughout the experiment. Since the C-terminus of aggregated A β is less accessible for antibody binding [49, 50], we do not expect the two extra C-terminal amino acids in the A β_{42} protofibril preparation to affect the results.

Before measuring the A β specific IgG production, flow cytometry was performed to estimate the total B cell numbers in the blood of both AD patients and healthy individuals. Earlier reports have found lower levels of B cells in AD compared to healthy individuals

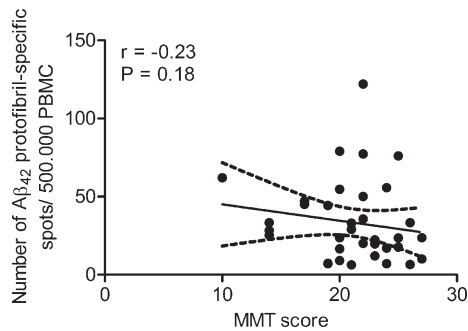


Fig. 4. Anti-A β_{42} protofibril antibody response correlated with mini mental state. No correlation was seen when comparing the levels of anti-A β_{42} protofibril selective antibody responses to the state of cognition in AD patients ($n = 22$).

[51–54]. However, Speciale et al. observed differences in IgG levels in serum between AD and healthy controls [52] and Pellicano et al. observed activation of B cells by A β_{42} by enhanced expression of the chemokine CCR5 [51]. Our data did not show any significant difference between AD and healthy individuals regarding the percentage of B cells (Fig. 2B). Surprisingly, ELISpot revealed the overall number of IgG producing B cells to be higher in the AD patients compared to the healthy individuals, suggesting a larger proportion of more easily activated B cells in AD patients.

Regarding antibodies binding to A β_{40} monomers, we only detected low numbers of B cells producing such antibodies in both AD patients and healthy individuals. As A β monomers represent the native form of the peptide in the human body, this was expected and has also been shown before [55]. In contrast, B cells producing antibodies specific for A β_{42} protofibrils were found in higher numbers and were increased in AD patients compared to the age-matched healthy controls. This result is likely to be a consequence of increased levels of A β protofibrils in AD patients, which would support their suggested importance in the pathology of the disease [7, 18]. We also found a weak correlation between the number of B cells producing A β_{42} protofibril specific antibodies and the total number of IgG producing cells. There is a growing recognition that immune responses in AD patients are dysregulated, something that may also play a role in AD [56]. The number of IgG producing B cells might be a consequence of enhanced activation of the immune response in AD. Our study did not investigate whether the antibodies recognizing A β_{42} protofibrils also recognized generic epitopes common to amyloid structures on other proteins.

As previously reported for anti-A β antibodies in plasma [27, 34], MMSE, age, or gender, except for women in the group of healthy individuals, did not show any correlation to the levels of B cells producing anti-A β antibodies, thereby abating the role of such antibodies as a potential biomarker for AD. Nevertheless, it would be interesting to follow AD patients over time to see if and how the number of B cells producing anti-A β antibodies correlates with the cognitive decline on an individual level. Conti et al. have previously described that AD patients receiving acetylcholinesterase inhibitors (AChEI) increased the anti-A β_{42} antibody levels in plasma compared to untreated AD patients [57]. AD patients show an increased production of interleukin-4 and monocyte chemoattractant protein-1, two positive regulators of Th2 differentiation, following AChEI treatment. Possibly, AChEI treatment can induce a Th2-mediated immune response leading to an increase of immunoglobulin production by B lymphocytes, including anti-A β_{42} antibodies [58]. However, as anti-A β_{42} antibodies levels were evaluated by ELISA, the differences between AChEI treated and untreated AD patients could reflect variations in A β /anti-A β_{42} antibody complexes rather than number of anti-A β_{42} antibodies. As all patients in the AD group except for four had been or were still on treatment with AChEI in our study, evaluation regarding AChEI influence on anti-A β antibody production from B cells among AD patients was not possible.

In conclusion, we report for the first time that ELISpot can be used to measure anti-A β antibodies from B cells in the blood. Low levels of B cells producing antibodies to A β_{40} monomers were seen in both AD and healthy individuals whereas the number of cells producing antibodies to A β_{42} protofibrils was significantly higher in AD compared to healthy individuals. Both IVIG and therapeutic anti-A β antibodies originally derived from healthy individuals are evaluated as AD therapies. Thus, the role of anti-A β antibodies in AD, as well as their physiological importance need to be further evaluated to learn if they could contribute to new therapeutic approaches for AD.

ACKNOWLEDGMENTS

We are grateful to Anne-Marie Ljungberg for her assistance during PBMC preparation and Käthe Ström for blood sampling. This work was supported by the Swedish Research Council (#2012-2172, LL), Alzheimerfonden (FEP, SS) and Uppsala Berzelii

Technology Centre for Neurodiagnostics (DS, SS, LL, FEP).

Authors' disclosures available online (<http://j-alz.com/manuscript-disclosures/15-0236r1>).

REFERENCES

- [1] Klyubin I, Cullen WK, Hu NW, Rowan MJ (2012) Alzheimer's disease A β assemblies mediating rapid disruption of synaptic plasticity and memory. *Mol Brain* **5**, 25.
- [2] Klyubin I, Walsh DM, Cullen WK, Fadeeva JV, Anwyl R, Selkoe DJ, Rowan MJ (2004) Soluble Arctic amyloid beta protein inhibits hippocampal long-term potentiation *in vivo*. *Eur J Neurosci* **19**, 2839-2846.
- [3] Walsh DM, Klyubin I, Fadeeva JV, Cullen WK, Anwyl R, Wolfe MS, Rowan MJ, Selkoe DJ (2002) Naturally secreted oligomers of amyloid beta protein potently inhibit hippocampal long-term potentiation *in vivo*. *Nature* **416**, 535-539.
- [4] Hartley DM, Walsh DM, Ye CP, Diehl T, Vasquez S, Vassilev PM, Teplow DB, Selkoe DJ (1999) Protofibrillar intermediates of amyloid beta-protein induce acute electrophysiological changes and progressive neurotoxicity in cortical neurons. *J Neurosci* **19**, 8876-8884.
- [5] Lacor PN, Buniel MC, Furlow PW, Clemente AS, Velasco PT, Wood M, Viola KL, Klein WL (2007) A β oligomer-induced aberrations in synapse composition, shape, and density provide a molecular basis for loss of connectivity in Alzheimer's disease. *J Neurosci* **27**, 796-807.
- [6] Nilsberth C, Westlind-Danielsson A, Eckman CB, Condron MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L (2001) The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced A β protofibril formation. *Nat Neurosci* **4**, 887-893.
- [7] Fukumoto H, Tokuda T, Kasai T, Ishigami N, Hidaka H, Kondo M, Allsop D, Nakagawa M (2010) High-molecular-weight beta-amyloid oligomers are elevated in cerebrospinal fluid of Alzheimer patients. *FASEB J* **24**, 2716-2726.
- [8] Santos AN, Ewers M, Minthon L, Simm A, Silber RE, Blennow K, Prvulovic D, Hansson O, Hampel H (2012) Amyloid-beta oligomers in cerebrospinal fluid are associated with cognitive decline in patients with Alzheimer's disease. *J Alzheimers Dis* **29**, 171-176.
- [9] Tucker S, Moller C, Tegerstedt K, Lord A, Laudon H, Sjobahl J, Soderberg L, Spens E, Sahlin C, Waara ER, Satlin A, Gellerfors P, Osswald G, Lannfelt L (2015) The murine version of BAN2401 (mAb158) selectively reduces amyloid-beta protofibrils in brain and cerebrospinal fluid of tg-ArcSwe mice. *J Alzheimers Dis* **43**, 575-588.
- [10] Savage MJ, Kalinina J, Wolfe A, Tugusheva K, Korn R, Cash-Mason T, Maxwell JW, Hatcher NG, Haugabook SJ, Wu G, Howell BJ, Renger JJ, Shughrue PJ, McCampbell A (2014) A sensitive A β oligomer assay discriminates Alzheimer's and aged control cerebrospinal fluid. *J Neurosci* **34**, 2884-2897.
- [11] Gold M, Pul R, Bach JP, Stangel M, Dodel R (2012) Pathogenic and physiological autoantibodies in the central nervous system. *Immunol Rev* **248**, 68-86.
- [12] Du Y, Dodel R, Hampel H, Buerger K, Lin S, Eastwood B, Bales K, Gao F, Moeller HJ, Oertel W, Farlow M, Paul S (2001) Reduced levels of amyloid beta-peptide antibody in Alzheimer disease. *Neurology* **57**, 801-805.
- [13] Weksler ME, Relkin N, Turkenich R, LaRusse S, Zhou L, Szabo P (2002) Patients with Alzheimer disease have lower levels of serum anti-amyloid peptide antibodies than healthy elderly individuals. *Exp Gerontol* **37**, 943-948.
- [14] Dodel R, Balakrishnan K, Keyvani K, Deuster O, Neff F, Andrei-Selmer LC, Roskam S, Stuer C, Al-Abed Y, Noelker C, Balzer-Geldsetzer M, Oertel W, Du Y, Bacher M (2011) Naturally occurring autoantibodies against beta-amyloid: Investigating their role in transgenic animal and *in vitro* models of Alzheimer's disease. *J Neurosci* **31**, 5847-5854.
- [15] O'Nuallain B, Acero L, Williams AD, Koeppen HP, Weber A, Schwarz HP, Wall JS, Weiss DT, Solomon A (2008) Human plasma contains cross-reactive A β conformer-specific IgG antibodies. *Biochemistry* **47**, 12254-12256.
- [16] Di Malta C, Fryer JD, Settembre C, Ballabio A (2012) Astrocyte dysfunction triggers neurodegeneration in a lysosomal storage disorder. *Proc Natl Acad Sci U S A* **109**, E2334-E2342.
- [17] Szabo P, Mujalli DM, Rotondi ML, Sharma R, Weber A, Schwarz HP, Weksler ME, Relkin N (2010) Measurement of anti-beta amyloid antibodies in human blood. *J Neuroimmunol* **227**, 167-174.
- [18] Sehlin D, Englund H, Simu B, Karlsson M, Ingelsson M, Nikolajeff F, Lannfelt L, Pettersson FE (2012) Large aggregates are the major soluble A β species in AD brain fractionated with density gradient ultracentrifugation. *PLoS One* **7**, e32014.
- [19] Du Y, Wei X, Dodel R, Sommer N, Hampel H, Gao F, Ma Z, Zhao L, Oertel WH, Farlow M (2003) Human anti-beta-amyloid antibodies block beta-amyloid fibril formation and prevent beta-amyloid-induced neurotoxicity. *Brain* **126**, 1935-1939.
- [20] St-Amour I, Pare I, Tremblay C, Coulombe K, Bazin R, Calon F (2014) IVIg protects the 3xTg-AD mouse model of Alzheimer's disease from memory deficit and A β pathology. *J Neuroinflammation* **11**, 54.
- [21] Morgan D (2010) Immunotherapy for Alzheimer's disease. *J Intern Med* **269**, 54-63.
- [22] Taguchi H, Planque S, Nishiyama Y, Symersky J, Boivin S, Szabo P, Friedland RP, Ramsland PA, Edmundson AB, Weksler ME, Paul S (2008) Autoantibody-catalyzed hydrolysis of amyloid beta peptide. *J Biol Chem* **283**, 4714-4722.
- [23] Relkin NR, Szabo P, Adamiak B, Burgut T, Monthe C, Lent RW, Younkin S, Younkin L, Schiff R, Weksler ME (2009) 18-Month study of intravenous immunoglobulin for treatment of mild Alzheimer disease. *Neurobiol Aging* **30**, 1728-1736.
- [24] Dodel R, Rominger A, Bartenstein P, Barkhof F, Blennow K, Forster S, Winter Y, Bach JP, Popp J, Alferink J, Wiltfang J, Buerger K, Otto M, Antuono P, Jacoby M, Richter R, Stevens J, Melamed I, Goldstein J, Haag S, Wietek S, Farlow M, Jessen F (2013) Intravenous immunoglobulin for treatment of mild-to-moderate Alzheimer's disease: A phase 2, randomised, double-blind, placebo-controlled, dose-finding trial. *Lancet Neurol* **12**, 233-243.
- [25] Relkin N (2014) Clinical trials of intravenous immunoglobulin for Alzheimer's disease. *J Clin Immunol* **34**(Suppl 1), S74-S79.
- [26] Alzheimer Research Forum, Drugs in Clinical Trials: Aducanumab, <http://www.alzforum.org/therapeutics/aducanumab>, Accessed April 4, 2015.
- [27] Brettschneider S, Morgenthaler NG, Teipel SJ, Fischer-Schulz C, Burger K, Dodel R, Du Y, Moller HJ, Bergmann A, Hampel H (2005) Decreased serum amyloid beta(1-42)

- autoantibody levels in Alzheimer's disease, determined by a newly developed immuno-precipitation assay with radiolabeled amyloid beta(1-42) peptide. *Biol Psychiatry* **57**, 813-816.
- [28] Moir RD, Tseitlin KA, Soscia S, Hyman BT, Irizarry MC, Tanzi RE (2005) Autoantibodies to redox-modified oligomeric A β are attenuated in the plasma of Alzheimer's disease patients. *J Biol Chem* **280**, 17458-17463.
- [29] Gustaw KA, Garrett MR, Lee HG, Castellani RJ, Zagorski MG, Prakasam A, Siedlak SL, Zhu X, Perry G, Petersen RB, Friedland RP, Smith MA (2008) Antigen-antibody dissociation in Alzheimer disease: A novel approach to diagnosis. *J Neurochem* **106**, 1350-1356.
- [30] Gustaw-Rothenberg KA, Siedlak SL, Bonda DJ, Lerner A, Tabaton M, Perry G, Smith MA (2010) Dissociated amyloid-beta antibody levels as a serum biomarker for the progression of Alzheimer's disease: A population-based study. *Exp Gerontol* **45**, 47-52.
- [31] Qu BX, Gong Y, Moore C, Fu M, German DC, Chang LY, Rosenberg R, Diaz-Arrastia R (2014) Beta-amyloid auto-antibodies are reduced in Alzheimer's disease. *J Neuroimmunol* **274**, 168-173.
- [32] Mruthinti S, Buccafusco JJ, Hill WD, Waller JL, Jackson TW, Zamrini EY, Schade RF (2004) Autoimmunity in Alzheimer's disease: Increased levels of circulating IgGs binding A β and RAGE peptides. *Neurobiol Aging* **25**, 1023-1032.
- [33] Storace D, Cammarata S, Borghi R, Sanguineti R, Giliberto L, Piccini A, Pollero V, Novello C, Caltagirone C, Smith MA, Bossu P, Perry G, Odetti P, Tabaton M (2010) Elevation of beta-amyloid 1-42 autoantibodies in the blood of amnesic patients with mild cognitive impairment. *Arch Neurol* **67**, 867-872.
- [34] Hyman BT, Smith C, Buldyrev I, Whelan C, Brown H, Tang MX, Mayeux R (2001) Autoantibodies to amyloid-beta and Alzheimer's disease. *Ann Neurol* **49**, 808-810.
- [35] Klaver AC, Coffey MP, Smith LM, Bennett DA, Finke JM, Dang L, Loeffler DA (2011) ELISA measurement of specific non-antigen-bound antibodies to A β 1-42 monomer and soluble oligomers in sera from Alzheimer's disease, mild cognitively impaired, and noncognitively impaired subjects. *J Neuroinflammation* **8**, 93.
- [36] Conti E, Galimberti G, Piazza F, Raggi ME, Ferrarese C (2010) Increased soluble APP α , A β 1-42, and anti-A β 1-42 antibodies in plasma from down syndrome patients. *Alzheimer Dis Assoc Disord* **24**, 96-100.
- [37] Boncoraglio GB, Piazza F, Savoirdo M, Farina L, DiFrancesco JC, Prioni S, Tagliavini F, Parati EA, Giaccone G (2015) Prodromal Alzheimer's disease presenting as cerebral amyloid angiopathy-related inflammation with spontaneous amyloid-related imaging abnormalities and high cerebrospinal fluid anti- β autoantibodies. *J Alzheimers Dis* **45**, 363-367.
- [38] Piazza F, Greenberg SM, Savoirdo M, Gardinetti M, Chiapparini L, Raicher I, Nitrini R, Sakaguchi H, Brioschi M, Billo G, Colombo A, Lanzani F, Piscosquito G, Carriero MR, Giaccone G, Tagliavini F, Ferrarese C, DiFrancesco JC (2013) Anti-amyloid beta autoantibodies in cerebral amyloid angiopathy-related inflammation: Implications for amyloid-modifying therapies. *Ann Neurol* **73**, 449-458.
- [39] Czerkinsky CC, Nilsson LA, Nygren H, Ouchterlony O, Tarkowski A (1983) A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. *J Immunol Methods* **65**, 109-121.
- [40] Sehlin D, Sollvander S, Paulie S, Brundin R, Ingelsson M, Lannfelt L, Pettersson FE, Englund H (2010) Interference from heterophilic antibodies in amyloid-beta oligomer ELISAs. *J Alzheimers Dis* **21**, 1295-1301.
- [41] Magnusson K, Sehlin D, Syvanen S, Svedberg MM, Philipson O, Soderberg L, Tegerstedt K, Holmquist M, Gellerfors P, Tolmachev V, Antoni G, Lannfelt L, Hall H, Nilsson LN (2013) Specific uptake of an amyloid-beta protofibril-binding antibody-tracer in A β PP transgenic mouse brain. *J Alzheimers Dis* **37**, 29-40.
- [42] Englund H, Sehlin D, Johansson AS, Nilsson LN, Gellerfors P, Paulie S, Lannfelt L, Pettersson FE (2007) Sensitive ELISA detection of amyloid-beta protofibrils in biological samples. *J Neurochem* **103**, 334-345.
- [43] Baril L, Nicolas L, Croisile B, Crozier P, Hessler C, Sassolas A, McCormick JB, Trannoy E (2004) Immune response to A β -peptides in peripheral blood from patients with Alzheimer's disease and control subjects. *Neurosci Lett* **355**, 226-230.
- [44] Gruden MA, Davudova TB, Malisaukas M, Zamotin VV, Sewell RD, Voskresenskaya NI, Kostanyan IA, Sherstnev VV, Morozova-Roche LA (2004) Autoimmune responses to amyloid structures of A β (25-35) peptide and human lysozyme in the serum of patients with progressive Alzheimer's disease. *Dement Geriatr Cogn Disord* **18**, 165-171.
- [45] Nath A, Hall E, Tuzova M, Dobbs M, Jons M, Anderson C, Woodward J, Guo Z, Fu W, Kryscio R, Wekstein D, Smith C, Markesbery WR, Mattson MP (2003) Autoantibodies to amyloid beta-peptide (A β) are increased in Alzheimer's disease patients and A β antibodies can enhance A β neurotoxicity: Implications for disease pathogenesis and vaccine development. *Neuromolecular Med* **3**, 29-39.
- [46] Sohn JH, So JO, Hong HJ, Kim JW, Na DR, Kim H, Kim H, Nam E, Ha HJ, Kim YH, Mook-Jung I (2009) Identification of autoantibody against beta-amyloid peptide in the serum of elderly. *Front Biosci* **14**, 3879-3883.
- [47] Song MS, Mook-Jung I, Lee HJ, Min JY, Park MH (2007) Serum anti-amyloid-beta antibodies and Alzheimer's disease in elderly Korean patients. *J Int Med Res* **35**, 301-306.
- [48] Jianping L, Zhibing Y, Wei Q, Zhikai C, Jie X, Jinbiao L (2006) Low avidity and level of serum anti-A β antibodies in Alzheimer disease. *Alzheimer Dis Assoc Disord* **20**, 127-132.
- [49] Stenh C, Englund H, Lord A, Johansson AS, Almeida CG, Gellerfors P, Greengard P, Gouras GK, Lannfelt L, Nilsson LN (2005) Amyloid-beta oligomers are inefficiently measured by enzyme-linked immunosorbent assay. *Ann Neurol* **58**, 147-150.
- [50] Englund H, Degerman Gunnarsson M, Brundin RM, Hedlund M, Kilander L, Lannfelt L, Pettersson FE (2009) Oligomerization partially explains the lowering of A β 42 in Alzheimer's disease cerebrospinal fluid. *Neurodegener Dis* **6**, 139-147.
- [51] Pellicano M, Bulati M, Buffa S, Barbagallo M, Di Prima A, Misiano G, Picone P, Di Carlo M, Nuzzo D, Candore G, Vasto S, Lio D, Caruso C, Colonna-Romano G (2010) Systemic immune responses in Alzheimer's disease: *In vitro* mononuclear cell activation and cytokine production. *J Alzheimers Dis* **21**, 181-192.
- [52] Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P (2007) Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. *Neurobiol Aging* **28**, 1163-1169.

- [53] Richartz-Salzbürger E, Batra A, Stransky E, Laske C, Kohler N, Bartels M, Buchkremer G, Schott K (2007) Altered lymphocyte distribution in Alzheimer's disease. *J Psychiatr Res* **41**, 174-178.
- [54] Xue SR, Xu DH, Yang XX, Dong WL (2009) Alterations in lymphocyte subset patterns and co-stimulatory molecules in patients with Alzheimer disease. *Chin Med J (Engl)* **122**, 1469-1472.
- [55] O'Nuallain B, Wetzel R (2002) Conformational Abs recognizing a generic amyloid fibril epitope. *Proc Natl Acad Sci U S A* **99**, 1485-1490.
- [56] Britschgi M, Wyss-Coray T (2007) Systemic and acquired immune responses in Alzheimer's disease. *Int Rev Neurobiol* **82**, 205-233.
- [57] Conti E, Galimberti G, Tremolizzo L, Masetto A, Cereda D, Zanchi C, Piazza F, Casati M, Isella V, Appollonio I, Ferrarese C (2010) Cholinesterase inhibitor use is associated with increased plasma levels of anti-Abeta 1-42 antibodies in Alzheimer's disease patients. *Neurosci Lett* **486**, 193-196.
- [58] Reale M, Iarlori C, Gambi F, Feliciani C, Isabella L, Gambi D (2006) The acetylcholinesterase inhibitor, Donepezil, regulates a Th2 bias in Alzheimer's disease patients. *Neuropharmacology* **50**, 606-613.