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Relationship Between Inflammatory Mediators, A β Levels and ApoE Genotype in Alzheimer Disease

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Abstract

Activation of inflammatory processes is observed within the brain as well as periphery of subjects with Alzheimer's disease (AD). Whether or not inflammation represents a possible cause of AD or occurs as a consequence of the disease process, or, alternatively, whether the inflammatory response might be beneficial to slow the disease progression remains to be elucidated. The cytokine IL-18 shares with IL-1 the same pro-inflammatory features. Consequent to these similarities, IL-18 and its endogenous inhibitor, IL-18BP, were investigated in the plasma of AD patients versus healthy controls (HC). An imbalance of IL-18 and IL-18BP was observed in AD, with an elevated IL-18/IL-18BP ratio that might be involved in disease pathogenesis. As part of the inflammatory response, altered levels of RANTES, MCP-1 and ICAM-1, molecules involved in cell recruitment to inflammatory sites, were observed in AD. Hence, correlations between IL-18 and other inflammatory plasma markers were analyzed. A negative correlation was observed between IL-18 and IL-18BP in both AD and HC groups. A positive correlation was observed between IL-18 and ICAM-1 in AD patients, whereas a negative correlation was evident in the HC group. IL-18 positively correlated with A β in both groups, and no significant correlations were observed between IL-18, RANTES and MCP-1. An important piece of evidence supporting a pathophysiologic role for inflammation in AD is the number of inflammatory mediators that have been found to be differentially regulated in AD patients, and specific ones may provide utility as part of a biomarker panel to not only aid early AD diagnosis, but follow its progression.

Keywords

IL-18; IL-18BP; ICAM-1; RANTES; MCP-1; THP-1; amyloid- β peptide; Alzheimer's disease; neuroinflammation

Introduction

Alzheimer's disease (AD) is clinically characterized by a progressive cognitive decline accompanied by the presence of amyloid plaques and neurofibrillary tangles within the brain. The major component of neuritic plaques is the amyloid- β ($A\beta$) protein. $A\beta$ peptides can be measured both within cerebrospinal fluid (CSF) and plasma; however, the source of the peptides remains unknown. $A\beta$ -Apolipoprotein (Apo)E and $A\beta$ -ApoJ complexes may cross the blood-brain barrier (BBB) [1] and plasma $A\beta$ may be incorporated into amyloid plaques. Thus, ApoE may represent a key regulator of brain $A\beta$ levels, and it is likely that processes that regulate ApoE activity will influence $A\beta$ deposition and clearance. The ApoE- ϵ 4 allele is known to be a risk factor for common late-onset familial and sporadic AD [2]. Protein interactions between ApoE and tau protein or $A\beta$ are proposed as mechanisms that could explain this genetic effect. As yet, however, the mechanisms controlling $A\beta$ deposition or clearance and the relationship between peripheral and brain $A\beta$ concentrations remain incompletely understood. Giri et al. have shown that $A\beta$ can induce the migration of monocytes across a monolayer of normal human brain endothelial cells that serves as a cellular model of the BBB [3], suggesting that the same may occur in vivo.

Depending on their activation status, T cells may cross-talk with neurons and microglia, and either mediate damage or protect neurons from stressful stimuli [4]. Inter-Cellular Adhesion Molecule 1 (ICAM-1) is involved in cell-cell interactions and leukocyte extravasation at inflammatory sites, and has been found not only in senile plaques containing $A\beta$ but also within structures containing low levels of $A\beta$. It is hence likely that ICAM may have a peripheral role in AD, as supported by a study showing increased plasma levels of ICAM-1 in individuals with AD compared to healthy controls (HC). An inflammatory state is invariably associated with AD, as evidenced by an increase of pro-inflammatory cytokines and chemokines (cyto-chemokines) around amyloid deposition. CC chemokines, such as RANTES (regulated on activation, normal T-cell expressed and secreted) and monocyte chemoattractant protein (MCP), are found on endothelial cells, glia and neurons throughout the brain, suggesting important functions for this inflammatory superfamily in the CNS [5,6]. RANTES and MCP-1 expression has been associated with a wide range of inflammatory disorders and some neurological disorders, including AD [7]. In these diseases, RANTES could be correlated with Th1 (helper T cell) responses, since it is thought to promote leukocyte infiltration in sites of inflammation and activate T cells [8,9]. MCP-1, however, promotes attraction and activation of monocytes, activates T cells, NK cells, and basophils, and plays a role in trans-endothelial migration of inflammatory cells. Recently, some authors have reported that MCP-1 and RANTES show opposing trends during healthy ageing, consequent to a complex remodelling of the cytokine network and altered T cell function [9]. Interleukin 18 (IL-18), a cytokine primarily recognized to be a modulator of immune functions, plays an important role in the deregulation of Th1-mediated diseases and exerts proinflammatory effects by inducing gene expression and synthesis of TNF- α , IL-1, chemokines [10], and adhesion molecules [11-12].

IL-18 is generated as a biologically inactive precursor molecule (pro-IL-18) that lacks a signal peptide. The processing and release of IL-18 from its precursor-form is induced by

inflammasomes. These multiprotein oligomers contain caspase-1 and other immune proteins (NALP and PYCARD, and occasionally caspase-5 or -11) cleave precursors of pro-inflammatory cytokines, such as IL-1 β and IL-18, to generate their mature and active forms. Interestingly, A β activates NALP-dependent inflammasomes, which can subsequently evoke the hallmarks of AD via caspase cascades and the release of inflammatory cytokines. In this regard, the expression of IL-18 appears to be significantly elevated in brain specimens from AD patients and also in A β treated neurons from transgenic Alzheimer mice [13]. IL-18 can additionally enhance amyloid plaque formation by inducing the expression of presenelin-1 (PS-1) and the A β cleaving enzyme, BACE-1. Likewise, IL-18 can elevate protein levels of Cdk5/p35 and GSK-3 kinases, leading to tau phosphorylation [13]. These results suggest that IL-18 may act as a signal that mediates communication between the nervous and immune systems. The activity of IL-18 can be regulated by IL-18-binding protein (IL-18BP), which is constitutively expressed and secreted in mononuclear cells, has a high and selective affinity for IL-18 and thus appears to be the natural inhibitor of IL-18 activity [14]. It is feasible that IL-18BP, localized in plaques may function as the binding site for IL-18, and this complex may thereby inhibit plaque uptake from the extracellular space to the glial cells. Thus, whereas IL-18 can enhance progression of AD, the IL-18BP neutralizing IL-18 activity can potentially slow the progression of AD.

Recent studies have suggested a possible involvement of systemic cyto-chemokines in AD, although debate continues as to whether brain cytokine overproduction might contribute to the pool of peripheral cytokines consequent to spillover from the central nervous system (CNS) or, alternatively, whether peripheral cytokines might affect human brain functions by crossing the BBB and interacting within the CNS. The presence of the ApoE- ϵ 4 allele, a major risk factor for sporadic AD, was also analyzed to verify the relationship between the inflammatory molecules and ApoE genotype and to determine their value in a panel of possible biomarkers in AD.

Materials and Methods

Subjects

Patients with probable AD were considered appropriate for enrolment (20 men and 18 women) aged 73.8 $\pm$ 5.5 years from the Department of Neurology, University of Chieti-Pescara, Chieti, Italy. The diagnosis of probable AD was based on the guidelines of the Diagnostic and Statistical Manual of Mental Disorders–fourth edition (DSM-IV- TR) [15] and the criteria of the National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) [16]. Cognitive and functional status was assessed by the Mini Mental State Examination (MMSE) [17]. A full history, including family history, was completed for each patient. Written informed consent was obtained from all subjects or their legal caregivers. All subjects were assessed in a uniform manner with identical instruments and procedures. Laboratory blood tests were performed to exclude metabolic causes of dementia (FT3, FT4, TSH, Vitamin B-12 and folic acid) and assessed the inflammatory status (erythrocyte sedimentation rate and C-reactive protein) of each recruited subject.

AD patients with concomitant neoplastic or hematological disorders, alcoholism, diabetes, recent infections, abnormal white blood cell count or surgery, severe hepatic or renal insufficiency, myocardial infarction or cranial trauma in the previous 6 months, or who had undergone antiplatelet, anti-inflammatory, antihypertensive, antineoplastic, corticosteroid or immunosuppressive drug treatments in the preceding 2 weeks were excluded. All patients were requested to have undergone a computed tomography or magnetic resonance imaging (MRI), to validate the diagnosis of AD. None of the participants were smokers or vegetarians, none were known to be taking vitamin B12 or folates (including multivitamins) or prescribed drugs known to affect circulating homocysteine or nitric oxide concentrations. Signs of malnutrition were excluded on the basis of physical examination and biochemical parameters (serum albumin, vitamin B-12 and folic acid).

We also included 39 healthy (18 men, 21 women, mean age 72.7 ± 4.8) age-frequency matched controls. All control subjects had no history of neurological or psychiatric disorders and underwent the same examinations and laboratory blood tests as the AD patients.

Genotyping

DNA was purified from 200 μ l whole blood with the QIAamp® DNA Blood Mini Kit (Qiagen, Milano, Italy) according to the manufacturer's Protocol, DNA binds specifically to the QIAamp silica-gel membrane while contaminants pass through, and pure and ready-to-use DNA is then eluted in water or low-salt buffer. The DNA concentration was measured by reading optical densities at 260 nm, and it then was stored at -20°C . until utilized. The fourth exon of the ApoE gene was amplified by the polymerase chain reaction (PCR), by using biotinylated primers, and the ApoE genotype determined by using a reverse DNA hybridization test, as recommended by INNO-LiPA ApoE Kit (Innogenetics S.r.l, Italy).

Plasma collection

Citrate treated (Sarstedt, Nümbrecht, Germany) plasma samples were obtained from AD patients and HC subjects with informed consent by peripheral vein puncture. Plasma was prepared by centrifugation (10 min at 2000 rpm), the samples were then aliquoted and stored in polypropylene tubes at -80°C until analysed. A consecutive code number was assigned to each sample to ensure that all assays were performed in a blinded manner.

Plasma A β

Plasma levels of A β , were determined with commercially available single-parameter ELISA kits (Biosource International, Camarillo, CA, U.S.A.). With each assay, the clinical samples, together with a blank (sample diluent), the prepared calibrator solutions and the appropriate controls, were tested strictly following the test instructions provided within the kit inserts. Absorbance was measured by Bio-Rad ELISA plate reader (Bio-Rad, Hercules, CA, USA) set at 450 nm and 550nm λ . To correct for optical imperfections in the microplate, 550 nm λ values were subtracted from 450 nm λ values. All samples were run in duplicate. If the intra-assay coefficient of variation (CV) was $>20\%$ (calculated as $\text{range} \times 100 / \text{average}$) or if concentrations obtained were out-of-range (OD values not between mean OD values of highest and lowest calibrator concentration), samples were retested (with appropriate extension of the calibrator concentration range in the case of any out-of-range

concentrations). The concentration range of the test kits, as described in the package inserts, is A β ₄₀: 7.1–1000pg/ml.

Quantification of cytokine levels

Concentrations of IL-18 and IL-18BP in the serum samples and culture supernatants were determined using commercial ELISA kits from Bender Medsystems (Burlingame, CA, USA) and R&D Systems (Minneapolis, MN, USA), respectively. The detection limits for these kits are 12 pg/mL and 60 pg/mL, respectively. Influence deriving from cross-reactivity and interference of circulating factors of the immune system was evaluated by spiking these proteins at physiologically relevant concentrations into a human IL-18 positive sample. No cross reactivity or interference was detectable, notably not with pro-IL-18. The IL-18BP kit is specific for measurement of the “a” isoform, which accounts for almost all the IL-18-binding activity in the human circulation. The inter- and intra-assay coefficients of variation were 8% and 5%, respectively. For each individual, free IL-18 was calculated from the individual's data on serum IL-18 and IL-18BP concentrations, in accordance with the law of mass action [18]. The calculation was based on the fact that IL-18BP binds IL-18 with a 1:1 stoichiometry and with a dissociation constant (K_d) of 0.4 nmol/L.

Chemokine assay

MCP-1 and RANTES release were evaluated by commercial ELISA kits (Endogen, Woburn, MA, USA), following the manufacturer's instructions. Before assay, samples were thawed at room temperature and diluted (1:10), with the sample diluents, in order to obtain values lower than the upper limit of the calibration range. The detection limit of the assay (described in the ENDOGEN Lab manual) was <10pg/ml for MCP-1 and <2pg/ml for RANTES. The range of analysis was between 51–2000 pg/ml for both MCP-1 and RANTES. The intra- and inter-assay reproducibility were >90%. Duplicate values that differed from the mean by greater than 10% were not considered for further analysis.

Soluble adhesion molecule ICAM-1

Plasma concentrations of ICAM-1 were measured with a commercially available ELISA, in accord with the manufacturer's protocol (Invitrogen, Life Technologies, Paisley, UK)

Isolation of PBMCs (peripheral blood mononuclear cells)

Separation and stimulation of PBMCs was performed as described elsewhere [19]. Briefly, 10 ml EDTA tubes (Terumo, Leuven, Belgium) were filled with venous blood drawn from AD patients and HC volunteers between 9 and 10 a.m. The PBMC fraction was obtained by density centrifugation of diluted blood (1 part blood to 1 part pyrogen-free saline) over Ficoll-Paque (GE Healthcare, EuroClone, Pero (MI) Italy). PBMCs were washed twice in saline and suspended in culture medium (RPMI-1640, Sigma) supplemented with 10% FBS, L-glutamine, and PES (Sigma-Aldrich Co., St. Louis, MO, USA). The cells were counted and the concentration was adjusted to 1×10^6 cells/ polypropylene round-bottom tube (Becton Dickinson, Franklin Lakes, NJ). The viability of cells, assessed by Trypan blue dye exclusion, was not significantly influenced by the cell culture conditions, and more than 98% of PBMCs were viable at the end of incubation. After a 24 hr incubation at 37°C, cell-

free supernatants were removed and stored at -80°C until assay, and pelleted cells were similarly kept until analysis. No solution contained detectable LPS, as determined by the Limulus amoebocyte lysate assay (sensitivity limit 12 pg/ml; Associates of Cape Cod, MA, USA), and mycoplasma contamination (General-probe II; General-probe Inc., San Diego, CA, USA) was found to be negative. The same batch of serum and medium were used in all experiments.

THP-1 maintenance and preparation

Cells were grown as a suspension culture and maintained in RPMI-1640 media with 10% fetal bovine serum and 200 ug/mL of penicillin and streptomycin at a cell density of 2×10^5 cells/mL (37°, 5% CO₂). The cells were split and the media changed three times a week. Before each experiment, cell viability was assessed by Trypan blue exclusion. Cell suspensions were harvested by centrifugation for 5 min at 4°, 300 *g*. Finally, cells together with cell-free supernatants were maintained at -80°C until analysis.

mRNA extraction and reverse transcription–polymerase chain reaction analysis

Total RNA was extracted from PBMC and THP-1 cell cultures using TRIzol reagent (Invitrogen, Life Technologies, Paisley, U.K.), according to the manufacturer's protocol. The RNA concentration was estimated by measuring the absorbance at 260 nm λ using a Bio-Photometer (Eppendorf AG, Hamburg, Germany), and RNA samples were kept frozen at -80 °C until use. Purified RNA was electrophoresed on a 1% agarose gel to assess the integrity of the purified RNA. One microgram of RNA was reverse transcribed into cDNA using a High Fidelity Superscript reverse transcriptase commercially available kit (Applied Biosystems, Foster City, CA, USA), in accord with the manufacturer's instructions. mRNA/cDNA specific cytokine primer pairs were designed and PCR was performed using the following primer pairs (20 pmol/ l each): IL-18 (product 342 bp), forward: 5' GCTTGAATCTAAATTATCAGTC 3' and reverse: 5' GAAGATTCAAATTGCATCTTAT 3'. IL18BP α (product 466 bp), forward: 5' TGGGAGATGTAGCCGACCTT 3' and reverse: 5' GCCCGACCTCTATTTC 3', 18S (product 199 bp) was used as a control, forward: 5' TTGCCATCACTGCCATTAAG 3' and reverse: 5' TTTCCATCCTTTACATCCTTCTG 3'. All polymerase chain reactions (PCRs) were performed in PCR-express cyclers (Hybaid, Heidelberg, Germany). The PCR amplification condition was as follows: 94 °C 1 min, 55 °C, 1 min, 72 °C 1 min 32 cycles; 94 °C 45 sec, 57 ° 45 sec, 72 °C 1 min, 32 cycles, 94 °C 1 min, 55 °C, 1 min, 72 °C 1 min 28 cycles. The program was terminated with a period of 10 min at 72 °C. To be within the exponential phase of the semi-quantitative PCR reaction, the appropriate number of cycles was pre-established for every set of samples. PCR products were separated by gel electrophoresis on 2% agarose gels and visualized by ethidium bromide staining. All gels were scanned and the normalized intensities of all reverse transcription (RT)-PCR products were determined by the BioRad gel documentation system (BioRad, Hercules, CA, USA). Mean $\pm$ SD intensities were calculated for all RT-PCR experiments.

Statistical analysis

All quantitative characteristics are summarized as the mean value $\pm$ standard deviation (SD) and all qualitative variables as frequency and percentage. The results are reported separately for AD patients (stratified in ApoE- ϵ 4, ApoE- ϵ 3 and ApoE- ϵ 2) and HC subject.

A Shapiro-Wilk test could not confirm that the data are distributed normally and, therefore, non-parametric statistical methods were used in their analysis. Box-whiskers plots were used for the presentation of cytokine levels and show median values (transverse lines in the box), the 25th and 75th percentile range (hinges of the box) and the range of the values that fall within the 1.5 inter-quartile range (whiskers). The points outside the ends of the whiskers are outliers or suspected outliers.

Differences in characteristics and inflammatory molecule levels of patients amongst HC and AD were tested by Mann-Whitney U test and Pearson chi-square test for continuous and categorical variables, respectively. Kruskal-Wallis H test was applied for assessing the comparison between the following four groups (ApoE- ϵ 4, ApoE- ϵ 3, ApoE- ϵ 2 and HC), such comparison between groups were then evaluated using a Kruskal-Wallis post-hoc test. The Spearman rho correlation coefficient was applied to evaluate the correlation between IL-18 and other inflammatory molecules. All statistical tests were evaluated at an alpha level of 0.05. Statistical analysis was performed using SPSS® Advanced Statistical 11.0 software (SPSS Inc., Chicago, Illinois, USA).

Results

Subjects

The demographics and clinical characteristics of the population studied are presented in Table 1. The two groups were similar with respect to age, gender and educational state. AD patients, as expected, had a significantly lower Mini-Mental Status Examination (MMSE) score compared to the HC group. The ApoE- ϵ 4 allele frequency was higher within the AD group. Within the AD ApoE genotype subgroups, there were no statistical differences between gender, age, education and MMSE.

Plasma concentration of the inflammatory molecules

Significant differences in plasma levels of IL-18 were observed between AD patients and controls. As IL-18 is neutralized by its circulating natural inhibitor, IL-18BP, a good estimation can be provided by the simultaneous detection of both IL-18 and IL-18BP. Dramatically higher plasma IL-18BP levels were observed in HC compared to AD subjects (3720.3 ± 663.3 vs 359.2 ± 142.5 pg/mL). In accord with lower circulating levels of IL-18BP, the mean free IL-18 plasma level in AD patients was elevated (247.8 ± 92.6 pg/mL (Table 2)), and all individual levels of IL-18 were above the highest observed level in control subjects by approximately 2-fold ($p < 0.001$ Mann-Whitney U test). Consequently, in AD patients the ratio of IL-18/IL-18BP (0.89 ± 0.68) was higher than in HC (0.03 ± 0.01). Analysis of IL-18BP and IL-18 levels demonstrated a significant negative correlation between these two proteins in each group ($\rho = -0.437$, $p < 0.01$ and $\rho = -0.395$; $p < 0.05$ for AD and HC, respectively). Unbound IL-18BP was in excess compared with total IL-18 in HC, whereas

within the AD group there was relatively little difference between IL-18 and IL-18BP levels (Table 2).

To assess whether the observed IL-18 elevation supports the hypothesis that activation of the inflammatory response in AD is Th1-dependent, the levels of RANTES, MCP-1 and sICAM-1 were quantified in the plasma of all AD and HC subjects. Shown in Table 2, approximately 3-fold higher levels of RANTES were evident in the plasma of AD compared to HC subjects, whereas lower plasma MCP-1 levels (0.73-fold) were apparent in AD. These results replicate the previously established finding that higher RANTES and lower MCP-1 levels are expressed and released from PBMCs isolated from peripheral blood of AD compared to those from HC [20]. Additionally, and in accord with data reported by Rentzos et al. [21], plasma levels of ICAM-1 were significantly elevated ($p=0.004$) in AD (188.5 ± 83.5 pg/mL) compared to HC (152.7 ± 15.8 pg/mL) (Table 2).

Correlation between IL-18 concentrations and IL-18BP, ICAM-1, RANTES and MCP-1

Correlations were studied between free IL-18 concentrations and various molecules detected in plasma of AD and HC subjects. As described above, a negative significant correlation was observed between IL-18 and IL-18BP in both groups. A positive correlation was observed between ICAM-1 and IL-18 in AD patients ($\rho=0.629$, $p<0.001$), whereas a negative correlation was observed in HC ($\rho=-0.192$, $p=0.256$). IL-18 was positively correlated with A β in both groups ($\rho=0.571$, $p<0.001$ and $\rho=0.052$, $p=0.761$, respectively). No significant correlations were observed between IL-18 and RANTES and MCP-1 (Fig. 1).

IL-18 and IL-18BP mRNA expression

The mRNA expression of IL-18 and IL-18BP was assessed in freshly isolated PBMCs from 10 AD and 10 HC subjects amongst those enrolled in the study, to define mechanisms responsible for the observed changes in the circulating levels of these proteins. Figure 2 shows that higher IL-18 mRNA expression was observed in PBMCs from AD patients compared to HC (1.26 ± 0.02 vs 1.02 ± 0.01), as was the case for IL-18BP mRNA (0.9 ± 0.3 vs 0.5 ± 0.01).

Interaction of ApoE genotype with biological markers

In order to elucidate the impact of ApoE genotype on the levels of analyzed inflammatory markers, AD patients were subdivided based on their ApoE- ϵ genotype. A significant influence of ApoE genotype on IL-18 and IL-18BP levels was observed. Specifically, AD patients with an ApoE- $\epsilon 4$ allele showed higher IL-18 plasma levels (304.6 ± 88.6 pg/ml) compared to patients with ApoE- $\epsilon 3$ (218.0 ± 87.8 pg/ml) and ApoE- $\epsilon 2$ (202.7 ± 59.5 pg/ml) ($p=0.009$ Kruskal-Wallis H test) (Fig. 3); however, ApoE genotype had no significant effect on IL-18 mRNA expression levels (Fig. 4). Conversely, AD patients with ApoE- $\epsilon 2$ and ApoE- $\epsilon 3$ possessed higher plasma IL-18BP levels (490.3 ± 120.9 pg/ml and 418.1 ± 90.7 pg/ml, respectively) than patients with ApoE- $\epsilon 4$ (221.6 ± 59.1 pg/ml) (Fig. 3). An analysis of ApoE genotype interaction on IL-18BP mRNA expression determined that IL-18BP mRNA levels were lower in ApoE- $\epsilon 4$ ($n=3$; 0.58 ± 0.01) compared to ApoE- $\epsilon 3$ ($n=3$; 0.61 ± 0.01) and ApoE- $\epsilon 2$ ($n=4$; 1.28 ± 0.02) (Fig. 4). The relative levels of plasma IL-18, expressed as the ratio IL-18/IL-18BP, were 1.5 ± 0.7 for ApoE- $\epsilon 4$, 0.5 ± 0.2 for ApoE- $\epsilon 3$ and 0.4 ± 0.2 for

ApoE-ε2. This lower IL-18/IL-18BP ratio in the plasma of AD patients with ApoE-ε2 supports the prospect of a differential regulation of IL-18BP production amongst ApoE genotypes.

The association between AD APOE genotype and plasma levels of RANTES, MCP-1 and ICAM-1 is shown in Table 3. Significantly higher levels of RANTES were detected in ApoE-ε4 compared to ApoE-ε3 carriers. In contrast, ApoE-ε2 AD patients possessed significantly higher MCP-1 plasma levels in respect to both ApoE-ε4 ($p<0.01$) and ApoE-ε3 ($p<0.05$). ICAM-1 plasma levels were not significantly different across genotypes, albeit there was a trend towards higher levels in ApoE-ε4 *versus* ApoE-ε3 *versus* ApoE-ε2 carriers, with the latter being within the realm of HC values.

Aβ and Aβ challenge

Plasma levels of Aβ₁₋₄₀ proved to be lower in our AD (83.06 ± 32.1 pg/ml) group than in HC (106.1 ± 19.8 pg/ml) subjects and, similar to Mehta et al. [22], showed no association with MMSE score. However, plasma Aβ levels were significantly influenced by ApoE genotype (ApoE-ε4 114.8 ± 21.7 ; ApoE-ε3 59.5 ± 18.8 and ApoE-ε2 69.5 ± 18.1 pg/ml; $p<0.001$ Kruskal-Wallis H test), a finding similarly described by Blennow et al. [23] and in accord with the reported differential ability of ApoE isoforms to facilitate the proteolytic degradation of soluble Aβ [24], with ApoE-ε4 showing little activity and hence being associated with higher Aβ levels.

To elucidate mechanisms, PBMCs from HC subjects and THP-1 monocytes were challenged with Aβ and the production and IL-18 and IL-18BP was evaluated. These cells respond to fibrillar forms of Aβ₁₋₄₂, Aβ₁₋₄₀ and Aβ₂₅₋₃₅ with similar efficiency. Both THP-1 and PBMC were treated with different concentrations of Aβ and cells treated with LPS alone were used as a positive control. IL-18BP mRNA was expressed at higher constitutive levels than IL-18 mRNA (Fig. 5). *Ex vivo* treatment with LPS has been described to induce a marked up-regulation of IL-18 and IL-18BP [25] and, as illustrated in Figure 5, our treatment of LPS-stimulated THP-1 monocytes with Aβ₁₋₄₀ (10 μM) significantly increased the mRNA expression of IL-18 and IL-18BP. The concentrations of IL-18 and IL-18BP measured in the supernatants of PBMC and THP-1 cultures confirmed the transcription data. IL-18 release was significantly increased (~8.9-fold and ~7.6-fold in THP-1 and PBMC, respectively) after incubation with LPS+Aβ, compared to LPS alone. IL-18BP release was, likewise, significantly elevated following incubation with LPS+Aβ (~6.3-fold and ~4.4-fold in THP-1 and PBMC, respectively), compared to LPS alone (Fig.6). We propose that this condition may, in part, emulate the increased inflammatory response that could occur during AD pathogenesis.

Discussion

Our interest focused on the levels of inflammatory molecules within the plasma of AD patients and their relation with the presence of the ε4 allele of ApoE, the major genetic susceptibility factor for late-onset AD. Interestingly, studies on AD have revealed that IL-1β and IL-18 are the key cytokines that display major up-regulation in AD patients, both in brain and plasma [26,13]. The role of IL-1β in AD has been extensively investigated [27],

and some studies have indicated a crucial role for IL-18 in the pathogenesis of AD [28,29]. In this study, a comparison of their plasma levels in AD patients with HC subjects assessed the feasibility of pro-inflammatory IL-18 and its endogenous inhibitor IL-18BP as biomarkers for AD. This evaluation expands our previous investigation that revealed higher expression and release of pro-inflammatory cytokines in PBMCs deriving from AD patients [30,31]. A primary finding within the current study is the relation between high plasma levels of pro-inflammatory cyto-chemokines and the ApoE genotype of AD patients. Specifically, AD patients with the $\epsilon 4$ allele appear to be more susceptible to a pro-inflammatory status than those without this allele. Hence *ex vivo* experiments were undertaken to assess the role of ApoE genotype on IL-18 and IL-18BP mRNA expression in PBMCs.

IL-18 is a potent pro-inflammatory cytokine of the IL-1 superfamily [32-34] that, like IL-1 β , is synthesized as a 23-kDa biologically inactive precursor peptide that is subsequently cleaved by caspase-1 to the active 18-kDa monomer. IL-18 acts in synergy with other cytokines and, via its receptor, activates Th1 cells and stimulates release of other pro-inflammatory cytokines, such as TNF- α , IL-1 β and IL-8, as well as nitric oxide and prostaglandins [35,36]. IL-18 not only induces IFN- γ production but also up-regulates Fas ligand (CD95), which is expressed on activated T cells and NK cells, and plays a central role in regulating the immune response by inducing apoptosis in activated lymphocytes. The principal protection against disease-promoting actions of IL-18 is provided through its natural counterpart, IL-18BP. Of relevance, this soluble binding protein has no transmembrane or cytoplasmic domain. Its genomic sequence lacks any coding exon for such domains, and thus IL-18BP does not derive from cleavage of a membrane-bound receptor. Rather, and like IL-18, IL-18BP is regulated at the level of its mRNA, that is constitutively expressed in peripheral blood lymphocytes and its induction occurs, at least in part, as a result of IL-18-mediated IFN- γ production. Hence, IL-18BP act as a signal for terminating the IL-18 mediated inflammatory response and, in general, a molar excess of IL-18BP to IL-18 exists, that is in the order of 30-fold in healthy humans [37]. In our AD patients, an IL-18/IL-18BP imbalance was observed, with concentrations of plasma IL-18 being elevated and levels of IL-18BP substantially decreased *versus* HC subjects (both $p < 0.001$). In line with this heightened plasma IL-18 level in AD, an increase in IL-18 mRNA was evident in PBMCs derived from AD patients *vs.* HC subjects (Fig. 2). However, contrasting with the IL-18BP plasma level reduction noted in our AD patients, mRNA IL-18BP expression was elevated in AD *vs.* HC PBMCs. It is hence possible that AD patients have a reduced ability to regulate plasma IL-18 that likely occurs at the level of IL-18BP production, which appears to be deficient. With an IL-18/IL-18BP ratio that is close to unity (0.86) in AD, the availability of plasma IL-18 remains both high and potentially sufficient to perpetuate the pathogenesis of AD. An examination of the relationship between ApoE genotype and IL-18BP mRNA expression (Fig. 4 in comparison to Fig. 2) indicates that expression levels of IL-18BP mRNA were higher ($p < 0.05$) than those of HC subjects only for AD ApoE- $\epsilon 2$ carriers, whereas APOE- $\epsilon 3$ and - $\epsilon 4$ carriers were not different from HC ($p > 0.05$). In contrast, ApoE genotype had little effect on IL-18 mRNA expression levels (Fig. 4). In synopsis, those AD subjects with ApoE- $\epsilon 2$ genotype showed a higher expression

and production of IL-18BP and lower IL-18 plasma levels, corroborating the importance of IL-18BP as the inhibitor of the pro-inflammatory activity of IL-18.

As IL-18 induces the synthesis of cell adhesion molecules, nitric oxide, chemokines and other proinflammatory cytokines [20,38,39], we therefore analyzed plasma sICAM-1 levels in our subjects. A significantly higher level of sICAM-1 was evident in AD compared to HC plasma (Table 2), and a correlation between IL-18 and sICAM-1 levels was determined (Fig. 1). These observations are in accord with studies showing that IL-18 enhances sICAM-1 expression and might play a potential role in immune-regulation by mediating adhesive interactions between endothelium and circulating cells during inflammatory reactions [40,41]. In addition, the IL-18 initiated cytokine cascade has been proposed to be dependent on the up-regulation of ICAM-1 on monocytes; indeed, anti-ICAM-1 antibody has been described to down-regulate IL-18-induced IL-12, TNF- α and IFN γ production and up-regulate IL-10 production in a concentration-dependent manner [42]. In primary rat astrocytes, the mRNA expression of IL-1 α , IL-1 β , and IL-6 is significantly up-regulated by ICAM-1 engagement, suggesting an ICAM-1-mediated inflammatory signal [43]. Hence, the concomitant determination of IL-18, IL-18BP and ICAM-1 could be used as a supplementary non-invasive procedure for monitoring immune reactions in AD.

The entry of activated leukocytes into the central nervous system is guided by leukocyte-secreted chemokines that act in an autocrine manner [44]. The opposing trends in MCP-1 and RANTES levels present in AD (Table 2) likely relates to a complex remodelling of the cytokine network and altered T cell function. In our previous studies we hypothesized that the observed levels of RANTES and MCP-1 in AD subjects, may reflect an alteration in the Th1/Th2 balance [45] and may regulate the selective migration of Th1/Tc1 and Th2/Tc2 cells to the target tissue and affect the type 1 and type 2 immune response [46,47].

Within the current study, we additionally evaluated whether or not the levels of RANTES, MCP-1 and ICAM-1 are correlated with ApoE genotype (Table 3). We hence divided AD patients on the basis of ApoE genotype, and higher levels of RANTES and sICAM-1 and reduced levels of MCP-1 were observed in those with ApoE- ϵ 4, compared to patients with ApoE- ϵ 2. A potential explanation for these results should take into account the role of IL-18. Interestingly, a positive correlation was observed between levels of IL-18, ICAM-1, RANTES and A β , whereas a negative correlation was observed between IL-18, IL-18BP and MCP-1. ApoE- ϵ 4 patients, furthermore, showed higher plasma A β levels that were sufficient to induce IL-18 and consequently to increase RANTES and ICAM-1, but insufficient to induce MCP-1 and IL-18BP. MCP-1 has been reported to play a crucial role in plaque clearance in animal models of AD [48], suggesting a potentially reparative role. Hence in subjects with lower levels of MCP-1, the up regulation of inflammatory molecules likely helps drive neurodegeneration. Of relevance, the promoter region of the ApoE gene contains inflammatory response transcription factor sites [49] that support the cross-directional regulation of ApoE- ϵ 4 expression by inflammatory proteins and vice versa [50,51].

Previous cross-sectional studies comparing plasma A β levels in patients with sporadic AD and controls have reported contradictory results. Elevations of plasma A β ₄₀ [52] or A β ₄₂ [53,54] have been described, whereas other studies found no differences between AD and

controls [55]. A non-significant correlation between the type of clinical diagnosis and serum A β intensity has been described, however, the presence of the ApoE ϵ 4 allele and certain ABCB1 genotypes may predict serum A β levels [56]. In general, it has been suggested that changes in blood A β levels may be an indicator of AD onset and progression [57,58], albeit further research in this area would be valuable, with the platelet likely contributing the largest source of A β peptide in human blood [59]. Platelets have the highest level of APP expression amongst all peripheral tissues and are able to produce all APP fragments found in neurons. Hence platelets are able to process APP *via* the amyloidogenic pathway (involving β -secretase and γ -secretase) to generate A β [60]. The existence of an A β -fingerprint in blood can thus partly be explained by the fact that γ -secretase has multiple (seven) potential cleavage sites [61] and it is likely active in platelets. The BBB has been described as critical for brain A β homeostasis [1], it is therefore plausible that BBB dysfunction alters the ability of brain A β to cross the BBB and enter in the periphery [62], which triggers sICAM-1 shedding from peripheral cells. One can then postulate that brain A β accumulation with AD onset and progression may elicit a response by endothelial cells at the BBB that results in an increase in plasma sICAM-1 levels.

Studies have shown that peripheral hemopoietic cells, such as monocytes, can cross the BBB and differentiate into microglial cells within the brain parenchyma [63]. Microglia and astrocytes have been identified as brain-derived sources of growth factors and inflammatory mediators. A well characterized peripheral model of cytokine production by cultured PBMCs, derived from AD and HC subjects, was hence utilized to elucidate the relative increase in inflammatory response that the brain could be subjected to from microglia and astrocyte exposure to from soluble A β and its accumulation in brain. Notably, IL-18 expression was markedly elevated upon exposure of PBMCs to A β , which is in accord with studies showing that interaction of A β with either microglial cells or THP-1 monocytes, pretreated with LPS, elicit similar responses and increase secretion of proinflammatory cytokines [64]. Hence it is generally considered that A β provokes the microglial-mediated inflammatory response, by stimulating glial cells to release inflammatory mediators, which contributes both to neuronal apoptosis [65], synaptic dysfunction and, ultimately, cognitive impairment that is characteristic of AD.

In summary, we demonstrate disturbance within the delicate cytokine/chemokine network in AD patients with up-regulation of specific cytokines/chemokines leading to imbalance of their normal homeostasis. A fuller understanding of the control of IL-18/IL-18BP expression and adaptive regulatory mechanisms in A β -stimulated peripheral mononuclear cells are important not only for maintaining an optimal balance of immune system function but also to allow modulation of the development of neuroinflammation in AD. A clear dys-equilibrium in IL-18/IL-18BP is evident in the inflammatory processes of AD and, consequent to the role of IL-18 early within the initiation cascade of severe inflammatory reactions, further elucidation of its part in the pathogenesis of AD could aid in rebalancing the complex actions of the altered cytokine/chemokine network. Hence a panel of biomarkers may prove more useful than an individual protein for assessment of early diagnosis and/or disease progression of AD. Such a panel that allows determination of changes in the ratio of cyto-chemokines known to be regulated and mechanistically linked, as epitomized by IL-18/IL-18BP, likely yields a greater potential to detect a statistically

relevant change, rather than assessment of IL-18 alone whose change may [13,26] or may not [66] always reach statistical significance. In a move towards this goal, in the current study we characterized plasma cyto-chemokine levels along with ICAM-1, A β and ApoE genotype to elucidate their suitability as part of a putative AD biomarker panel. The changes in key proteins within this panel clearly warrant further investigation both in plasma and CSF in HC, mild cognitive impairment, AD and other neuropsychiatric disorders.

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Figure 1.

Correlation coefficient between IL-18 concentration and inflammatory molecules analyzed in plasma of Alzheimer Disease (AD) patients and healthy controls (HC) subjects.

Figure 2.
Expression of IL-18 and IL-18BP mRNA in patients with Alzheimer Disease (AD) and in healthy controls (HC).

Figure 3.

Box-whiskers graphs of IL-18 and IL-18BP levels in AD patients, stratified in ApoE-e4, ApoE-e3 and ApoE-es2, and HC subject. Box-whiskers plots show the 25th and 75th percentile range (box) and median values (transverse lines within the box). Statistically significant differences were found between four groups (p-value of Kruskal-Wallis post hoc test).

Figure 4. mRNA levels of IL-18 and IL-18BP in AD patients with different ApoE-ε genotypes. PBMC mRNA levels were analyzed by semiquantitative RT-PCR using primers for hIL-18, hIL-18BP and β-actin.

Figure 5.

Fold-increase of A β -mediated mRNA expression of IL-18 and IL-18BP in THP-1 monocytes and PBMCs. Data are representative of three separate experiments. The intensities of bands for IL-18 and IL-18BP are normalized to the 18S signals. Quantification of three separate experiments was performed. The data are expressed as the fold increase relative to mRNA expression of each gene in LPS-stimulated THP-1 and PBMC cells. Data are representative of pooled experiments.

Figure 6. Secretion of IL-18 and IL-18BP in response to LPS+A β . PBMC and THP-1 cells were cultured in RPMI containing A β (10 μ g/ml) and were incubated for 18 hr in the presence or absence of LPS (10 μ g/ml). The data represent the fold-increase over the LPS-induced IL-18 and IL-18BP production.

Table 1

Characteristics of patients with Alzheimer's disease (AD) for different ApoE genotype and healthy control (HC) subjects.

Variable	AD						
	HC (n=39)	AD (n=38)	p-value ^a	ApoE-ε4 (n=14)	ApoE-ε3 (n=15)	ApoE-ε2 (n=9)	p-value ^c
Age (years), <i>mean</i> ± <i>SD</i>	72.7±4.8	73.8±5.5	0.171	72.9±6.2	75.9±5.1	72.0±4.5	0.702
Gender, <i>n</i> (%)			0.734 ^b				0.803 ^b
Male	18 (46.2)	20 (52.6)		6 (42.8)	8 (53.3)	6 (66.7)	
Female	21 (53.8)	18(47.4)		7 (57.2)	7 (46.7)	4(33.3)	
MMSE score, <i>mean</i> ± <i>SD</i>	25.7±3.2	19.2±3.9	<0.001	19.3±4.1	18.8±4.1	19.6±3.3	0.963
ADAS-Cog, <i>mean</i> ± <i>SD</i>	-	21.6±1.4		20.7±1.6	21.3±2.1	20.9±2.3	
Disease duration (months), <i>range</i>	-	8-24	-	8-24	9-24	9-20	
Education (years), <i>mean</i> ± <i>SD</i>	10.2±2.1	9.3±3.3	0.093	9.1 ±3.2	9.3 ±3.3	9.5 ±3.2	0.972

^aMann-Whitney U test;

^bChi-squared test AD patients vs HC subject.

^cKruskal-Wallis H test.

MMSE: higher scores correspond to better performance

ADAS-Cog: higher scores indicate greater cognitive impairment

Table 2

Mean $\pm$ Standard deviation of inflammatory molecule levels in patients with Alzheimer's disease (AD) and healthy control (HC) subjects.

Variable	HC (n=39)	AD (n=38)	p-value ^a
IL-18(pg/ml)	102.6 $\pm$ 11.4	247.8 $\pm$ 92.6	<0.001
IL-18BP(pg/ml)	3720.3 $\pm$ 663.3	359.2 $\pm$ 142.5	<0.001
IL-18/IL-18BP	0.03 $\pm$ 0.01	0.89 $\pm$ 0.68	<0.001
MCP-1 (pg/ml)	2196.9 $\pm$ 472.8	1599.3 $\pm$ 578.5	<0.001
RANTES (pg/ml)	1812.0 $\pm$ 583.5	5344.4 $\pm$ 2204.8	<0.001
ICAM-1 (pg/ml)	152,7 $\pm$ 15.8	188.5 $\pm$ 83.5	0.004

^aMann-Whitney U test

Table 3

Mean $\pm$ standard deviation of plasma RANTES, MCP-1 and ICAM-1 levels in different ApoE genotype AD patients.

Variable	ApoE- ϵ 4 (n=14)	ApoE- ϵ 3 (n=15)	ApoE- ϵ 2 (n=9)	Kruskal-Wallis H test p-value
RANTES (pg/ml)	6659.4 $\pm$ 2068.7	4047.7 $\pm$ 1923.9 ^{**}	5459.9 $\pm$ 1690.3	0.010
MCP-1 (pg/ml)	1388.8 $\pm$ 355.7	1486.8 $\pm$ 471.8	2114.1 $\pm$ 743.8 ^{**†}	0.018
ICAM-1 (pg/ml)	207.5 $\pm$ 48.9	184.3 $\pm$ 98.5	163.9 $\pm$ 102.9	0.053

*
p<0.05,

**
p<0.01 and

p<0.001 Kruskal-Wallis post hoc test vs ApoE- ϵ 4

†
p<0.05 Kruskal-Wallis post hoc test vs ApoE- ϵ 3