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Selective acetyl- and butyrylcholinesterase inhibitors reduce amyloid-β ex vivo activation of peripheral chemo-cytokines from Alzheimer's disease subjects: exploring the cholinergic anti-inflammatory pathway

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Abstract

Increasing evidence suggests that the early pathogenesis of Alzheimer's disease (AD) is driven by elevated production and/or reduced clearance of amyloid-β peptide (Aβ), which is derived from the larger Aβ precursor protein (APP). Aβ aggregates to form neurotoxic soluble oligomers that trigger a cascade of events leading to neuronal dysfunction, neurodegeneration and, ultimately, clinical dementia. Inflammation, both within the brain and systemically, together with a deficiency in the brain neurotransmitter acetylcholine, which underpinned the development of anticholinesterases for the symptomatic treatment of AD, are invariable hallmarks of the disease. The inter-relation between Aβ, inflammation and cholinergic signaling is complex, with each feeding back onto the others to drive disease progression. To elucidate these interactions plasma samples and peripheral blood mononuclear cells (PBMCs) were evaluated from healthy control (HC) subjects and AD patients. Plasma levels of acetyl- (AChE) and butyrylcholinesterase (BuChE) as well as Aβ were significantly elevated in AD *vs*. HC subjects, and acetylcholine showed a trend towards reduced levels. Aβ challenge of the AD and HC PBMCs resulted in greater release of inflammatory cytokines interleukin-1β (IL-1β), monocyte chemotactic protein-1

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(MCP-1) and tumor necrosis factor-alpha (TNF-α) from AD *vs.* HC subjects, with IL-10 expression being similarly affected. THP-1 monocytic cells, a cell culture counterpart of PBMCs and brain microglial cells, responded similarly to Aβ as well as to phytohaemagglutinin (PHA) challenge, to allow preliminary analysis of the cellular and molecular pathways that underpin Aβ-induced changes in cytokine expression. In light of prior studies demonstrating that APP expression was regulated by specific cytokines and anticholinesterase drugs, the latter were evaluated on Aβ- and PHA-induced chemo-cytokine expression. Co-incubation with selective inhibitors, such as the acetylcholinesterase (AChE)-inhibitor (−)-phenserine and the butyrylcholinesterase (BuChE)-inhibitor (−)-cymserine analogues mitigated the rise in cytokine levels, and suggest that augmentation of the cholinergic anti-inflammatory pathway may prove valuable in AD.

Keywords

Alzheimer's disease; inflammation; cytokines; amyloid-β peptide (Aβ); IL-1β; TNF-α; MCP-1; IL-6; IL-10; acetylcholinesterase (AChE); butryrylcholinesterase (BuChE); phenserine; cymserine; bisnorcymserine; phytohaemagglutinin (PHA) THP-1 cells; peripheral blood mononuclear cells (PBMCs); cholinesterase inhibitors

Introduction

Age-related dementia is rising in incidence in line with increasing life expectancy and afflicts in excess of 25 million people worldwide, of these 50 to 75% have Alzheimer disease (AD) [1,2]. The progressive declines in cognitive ability and functional capacity associated with AD are accompanied by the classical microscopic disease hallmarks of intracellular neurofibrillary tangles containing hyperphosphorylated tau protein and apolipoprotein E, and extracellular senile (neuritic) plaques that contain many proteins, but in particular amyloid-β peptide (Aβ) comprising 1–42 amino acids [3,4]. Aβ peptide is derived from the larger Aβ precursor protein (APP) by the action of β- and γ-secretases for the biogenesis of Aβ [3,4], which is detected normally in different cell types and tissues across evolutionary species. Aβ aggregation, in particular soluble oligomers, triggers a cascade of events that leads to neuronal dysfunction, neurodegeneration and ultimately to clinical dementia [5]. These Aβ aggregates may induce direct neurotoxic actions [6] but, additionally, may induce neurodegeneration indirectly by initiating a pro-inflammatory cascade that results in the release of inflammatory cytokines [7–9]. Hence, invariably present alongside the AD pathological features is the presence of neuroinflammation [10,11].

Increasing evidence supports close communication between the occurrence of systemic inflammation and that occurring within the central nervous system across a range of disorders, and particularly in AD [12–14]. Studies have proposed that AD presents systemic manifestations triggered by molecular and biophysical alterations that occur early during disease progression [15]. The systemic pathophysiologic view of AD is consistent with recent observations that amyloid and tau metabolic pathways are ubiquitous within the human body and manifest across a number of non-nervous system tissues, including blood, saliva and skin [16]. Aβ1–40 as well as 1–42 are generated in the brain as well as in the periphery, and it is thought that circulating levels of Aβ may impact Aβ deposits present

within the brain. Receptor mediated movement of soluble Aβ across the blood–brain barrier (BBB) is driven by transporters, such as the low-density lipoprotein receptor-related protein-1 (LRP-1) [17,18] for efflux, and the receptor for advanced glycation end products (RAGE) [19] for influx. Aβ-specific IgG is capable of binding Aβ peptide within the blood, and may encourage efflux of Aβ from the brain to the blood via the “peripheral sink” mechanism [20]. Previous studies [21] have shown that microglial cells in the brain derive from peripheral hemopoietic cells, such as monocytes, and share expression of many surface receptors and signalling proteins and the overlap of gene expressions related to AD. Notably, Aβ induces the migration of both monocytes and human monocytic THP-1 cells across a model of the blood-brain barrier (BBB) [22,23]. Exposure of human THP-1 monocytes to fibrillar forms of Aβ drives the activation of protein tyrosine kinases that initiates the activation of signalling pathways. In addition, exposure of these cells to Aβ stimulates the inflammatory response and, thereby, increases the expression of cytokines such as IL-1β, IL-6, TNF-α [22]. In vivo studies confirm that Aβ induces the activation and migration of monocytes across mesenteric blood vessels [22], suggesting that a similar phenomenon occurs in the brain vasculature.

The development of the “cholinergic hypothesis” in 1982 [24], linking the cholinergic deficit in AD brain to the hallmark cognitive decline, underpinned the later development of acetylcholinesterase (AChE) inhibitors that remain the mainstay of current AD symptomatic treatment. More recent research has united the cholinergic with the amyloid and tau hypotheses, defining numerous connections between each whereby Aβ can lower the synthesis and release of acetylcholine (ACh), cholinergic receptor expression and transduction mechanisms, and reciprocal changes in cholinergic signalling can modify the processing of APP and Aβ generation [25]. In particular, specific anticholinesterases have been shown to regulate the levels of APP and its metabolites via cholinergic [26–28] and non-cholinergic mechanisms [29]. Although Aβ is the principal constituent of senile plaques, other proteins co-localize, in particular AChE that may have a role in promoting Aβ aggregation to enhance its toxicity [30–32]. Indeed, Aβ-exposure enhances AChE expression in cell culture and in the intact brain of mouse models of AD [33]. In addition, the cholinergic system is present and functional in non-neuronal tissues, including immune cells [34–37]. In this regard, the activation of the T-cell receptor by phytohaemagglutinin (PHA) or by anti-CD11a antibodies triggers the expression of choline acetyltransferase (ChAT) and muscarinic M5 receptors and enhances the synthesis of ACh. ACh is additionally involved in the induction of CD4+ T-cell maturation as well as in the generation of cytolytic CD8+ T-lymphocytes under *in vitro* conditions [38]. Furthermore, ACh has been described to modulate the activity of immune cells via auto- and paracrine loops. The ligation of ACh to nicotinic receptors inhibits cytokine synthesis and release, and thus the ‘cholinergic anti-inflammatory pathway’ provides a physiological mechanism that effectively links ACh to the inhibition of inflammation.

In light of the interaction between ACh and inflammation, the aim of the current study was to assess the effects of newly available anticholinesterases that possess a selectivity between AChE and butyrylcholinesterase (BuChE) on cytokine production and the signalling pathways in peripheral blood mononuclear cells (PBMCs) and a macrophagic THP-1 cell line. Not only do the current clinically available anticholinesterases possess different

selectivity's between AChE and BuChE [39,40], but also recent studies have demonstrated that both enzyme subtypes co-regulate ACh activity [41–47]. This is particularly relevant in AD where brain levels of AChE are decreased and BuChE elevated [48,49]. The brain elevation of ACh levels augments both nicotinic and muscarinic receptor signalling, and the latter in particular has been widely reported to confer protection against a wide array of insults leading to neuronal dysfunction and death [50]. At low concentrations, nicotine can additionally improve memory functions and reduce amyloid plaque burden in transgenic mouse models of AD [51,52]. Furthermore, the α7 nicotinic ACh receptor (nAChR) has recently been identified as an anti-inflammatory target on macrophages [53,54], to allow nicotinic agonists and ACh to potentially elicit anti-inflammatory effects via the "immune cholinergic system" [55]. In vitro studies have demonstrated that nicotine can impact immune cells by inhibiting their production of IL-2 and TNF-α in human lymphocytes; whereas in mice, administration of α7nAChR agonists inhibits not only TNF-α but also IL-1, IL-6 and IL-8 [55,56]. Therefore, another aim of this study was to assess the molecular pathways that underpin these changes in cytokine expression, especially the mitogen-activated protein kinases (MAPKs) that are a family of serine/threonine kinases that comprise ERK (Extracellular signal–Regulated Kinase) and p38 MAPK.

Our results demonstrate that blood mononuclear cells and THP-1 provide important model systems to study how modulation of the non-neurological cholinergic system can impact immunological reactions that take part in the immune response of AD patients. In addition, we suggest that the cholinergic system and cytokine network not only represent therapeutic targets but may also serve as potential marker of disease progression and pharmacological action of anti-dementia compounds.

Materials and Methods

Cell culture and reagents

The THP-1 monocytic cell line was obtained from American Type Culture Collection (Manassas, VA, USA), and cultured in complete medium composed of RPMI 1640 containing 10% heat-inactivated FCS, 10 mM HEPES, 2 mM glutamine, 100 U/ml penicillin (all reagents were purchased by Sigma-Aldrich, St. Louis, MO, USA). Human peripheral blood mononuclear cells (PBMCs) were isolated, from blood collected in sodium citrate as the anticoagulant, by centrifugation with Ficoll-Paque Plus (GE Healthcare Life Sciences) and were washed twice in phosphate buffered saline (PBS). The viable cells (95–98% as assessed by trypan blue dye exclusion) were re-suspended at a concentration of 2×10^6/ml in complete RPMI 1640.

Lyophilized synthetic Aβ (1–42) peptide was obtained from Sigma-Aldrich and prepared before use as previously described [57]. Aβ was dissolved in dimethyl sulfoxide to be diluted next at a concentration of 1 mM in sterile double-distilled pyrogen-free water, after which it was aliquoted and stored at −20°C. THP-1 cells and PBMCs were stimulated by Aβ (10 μM/ml). Lipopolysaccharide (LPS *E. coli* 0111:B4; 10 μg/ml; Sigma-Aldrich) or PHA (3 μg/ml; Sigma-Aldrich) was used as a positive control for THP-1 and PBMC cytokine production, respectively. On the basis of our previous studies and those of others, this LPS

dose and the incubation time were shown to induce the maximal stimulation for the release of proinflammatory cytokines.

Pharmacological inhibitors (Calbiochem (Millipore Corporation, Billerica, MA, USA) used were PD98059 (specific MEK1 inhibitor used at 10 μM), LY294002 (specific PI-3K inhibitor 20 μM), SB202190 (specific p38 inhibitor 10 μM) and (−)-phenserine (**1**), (−)-phenethylcymserine (**2**), (−)-bisnorcymserine (**3**) and (−)-cymeserine (**4**) (each synthesized in the form of their tartrate salt to in excess of 99% chiral and optical purity [58,59]). Inhibitors were added to cells 30 min prior to stimulation with Aβ. At the end of incubation, PBMCs and THP-1 cells were removed by centrifugation at 500 × *g* for 10 min. The cell pellet and medium were collected and stored at −80°C until further use. In parallel studies, cells were pre-incubated for 30 min with nicotine or mecamylamine (10 μM, Sigma-Aldrich, Italy) to explore the involvement of the nAChR on Aβ-induced effects in THP-1 cells.

Subjects

PBMCs from patients affected by probable Alzheimer disease (AD) and healthy control (HC) subjects were studied (Table 1). The AD subjects were selected from the Neurological Clinic of Villa Serena Hospital, Città Sant'Angelo Pescara, Italy. Diagnosis of probable AD was according to standard clinical procedures and followed the NINCDS/ADRDA and DSM-III-R criteria [60,61]. Cognitive performance and alterations were evaluated according to the Mini Mental State Examination (MMSE) and the Global Deterioration Scale. AD patients included in the study did not present major co-morbidity, such as cancer, symptomatic (present or previous) cardiovascular diseases, and major inflammatory diseases such as autoimmunity and infections. All AD patients had undergone magnetic resonance or computed tomography within the previous 6 months. Samples from HCs were from unrelated individuals matched for gender and age with AD patients. These subjects had previously undergone complete neurological examinations and were judged to be in good health based on their clinical history. Systemic exclusion criteria included the following: vascular dementia inflammatory, autoimmune, hematologic, or neoplastic disease; diabetes mellitus, thyroid disease, alcohol addiction, acute or chronic infection; history of hepatic or renal failure, myocardial infarction, cranial trauma and surgery within the last 6 months; abnormal white blood cell counts; erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins, cholesterol, triglycerides; use of diuretic, anti-inflammatory, antineoplastic, corticosteroid, immunosuppressive, antidepressant, or anticholinergic drugs within the prior 2 months. Our study was approved by the Local Research Ethics Committee (document number 118/10.12.2012) and informed consent was obtained from all AD patients and HCs before their inclusion in the study. Written informed consent was obtained from all subjects or their legal caregivers. All data in this study were analyzed anonymously, and the samples were considered to be medical waste materials. All subjects were assessed in a uniform manner with identical instruments and procedures. In randomly selected AD patients recruited in this study, we analyzed some of the parameters obtained in AD subjects recruited from our previous studies, and the results were similar (data not showed).

Measurement of Aβ Levels

Plasma levels of Aβ1–40 were determined with commercially available single-parameter ELISA kits (Biosource International, Camarillo, CA, USA). 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 the 450 nm λ values. All samples were run in duplicate. In the event that either the intra-assay coefficient of variation (CV) was >20% (calculated as range×100/average) or concentrations obtained were out-of-range of the standard curve (i.e., 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 Aβ1–40: 7.1–1000pg/ml.

Measurement of ACh Levels

ACh was measured by commercial colorimetric/fluorimetric kit (Abcam, Cambridge, UK). Fifty microliter of the sample was mixed with 50 μL of reaction solution including choline assay buffer, choline probe (Ch), enzyme mix and AChE according to the manufacturer's instructions. The level of Ch/ACh (pmol/well) was calculated by plotting the fluorescence of each sample in relation to choline standard curve. The standard curve, according to the fluorimetric procedure as indicated by manufacturer's instructions, was obtained by diluting the Choline Standard to generate 0, 10, 20, 30, 40, 50, 100 and 200 pmol/well of the Choline Standard. Measurement of the fluorescence was by Glomax Multi Detection System (Promega) at λ Ex/Em 535/587 nm. In relation to the intensity of the fluorescence of the samples, the x values (corresponding to Ch/ACh concentration and reported as pmol/well) were calculated by the standard curve equation using GraphPad Prism 5.0. The limit of detection of Choline is defined by the analyte concentration resulting in a fluorescence higher than that of the dilution medium (0 dose of standard choline = Blank).

BuChE and AChE Activity assay

BuChE and AChE activity were determined by a modified microassay method of Ellman [62]. For AChE, the assay were performed with 1μM acetylthiocholine and 50μM tetraisopropyl pyrophoshoramide (iso-OMPA), a selective irreversible inhibitor of BuChE; while for BuChE, the assay were performed with 1mM butyrylthiocholine and 10 μM BW284c51. One milliunit (mU) of AChE and BuChE activity was defined as the number of nmoles of substrate (ACh or BuCh) hydrolysed per min at 22°C.

Quantification of cytokines

The supernatants of the cultured PBMCs and THP-1 cells were collected and the following cytokines and chemokines were evaluated using specific ELISA Kits (Pierce Endogen, Rockford, IL USA) according to the manufacturer's instructions. The detection limit for these kits is <1 pg/ml for IL-1β and IL-6, <2 pg/ml for TNF-α, <3 pg/ml for IL-10 and <10

pg/mL for MCP-1. The intra-and interassay CV was < 10%. All results are expressed in picograms per milliliter

Detection of proteins activated by phosphorylation in THP-1 cells and PBMCs

Protein phosphorylation was evaluated using the cell Fast Activated Cell-based ELISA (FACE™ In-Cell Western) colorimetric Kits (Active Motif, Vinci-Biochem, Vinci, Italy). At the end of incubation cells were fixed and then incubated with a primary antibody specific for the native inactive protein or activated protein of interest. Thereafter, they were subsequently incubated with a secondary HRP-conjugated antibody and developing solution that provides a quantitative colorimetric reaction. The phospho- and total-protein antibodies can be used on equivalent cell cultures to determine the effects of cell treatment on the ratio of phosphorylated to total protein. Total and phospho protein were each assayed in triplicate using the phospho and total protein antibodies. Absorbance was read on a spectrophotometer at 595 nm λ. Data was plotted after correction for cell number (determined using Crystal Violet).

mRNA Extraction and Reverse Transcription–Polymerase Chain Reaction Analysis

Total RNA was extracted from PBMCs and THP-1 cell cultures using TRIzol reagent (Invitrogen, Life Technologies, Paisley, UK), 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. Polymerase chain reaction (PCR) was performed using the mRNA/cDNA specific cytokine primer pairs (Table 2). All PCRs were performed in PCR-express cyclers (Hybaid, Heidelberg, Germany). The PCR amplification condition was as follows: 95°C 5 min, 95°C 30 sec, 60°C 30 sec, 72°C 30 sec, 72°C 10 min, 30 cycles. 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 ± SD intensities were calculated for all PCR experiments.

Statistical Analysis

Quantitative variables are summarized as the mean value and standard deviation (SD) and qualitative variables as frequency and percentage. The results are reported separately for AD patients and HC subjects. Statistical analysis was conducted according to the distribution of each variable, as assessed by the Shapiro-Wilk W test.

Differences in characteristics and in AChE, BuChE, ACh and A levels between HC and AD subjects were tested by t-test for unpaired data or Pearson chi-square test for continuous and categorical variables, respectively. The Spearman rho correlation coefficient (ρ) was applied to evaluate the correlation between Aβ, AChE, BuChE and ACh levels.

Four different Analysis of Variance (ANOVA) for repeated measurements were also used to evaluate the effect of each factor (group and Aβ) and their interaction (group*Aβ) on inflammatory cytokine levels. In all models a priori contrasts, were used to compare the mean of the different parameters between AD and HC subjects, between basal and Aβ conditions in either AD or HC groups. ANOVA was also used to evaluate the effect of each compound (**1**), (**2**), (**3**) and (**4**), on the production of IL-1β, MCP-1, TNF-α and IL-10 in PBMCs of AD and HC subjects. In all models a priori contrasts, were used to compare the mean of different parameters between AD and HC subject.

For comparing the generation of each cytokine between untreated and compound (**1**), (**2**), (**3**) and (**4**) treated cells, a Kruskal-Wallis H test was applied. A Mann-Whitney U test with p-value correction was applied for the pairwise comparisons in post-hoc analysis.

Statistical analysis was performed using SPSS® Advanced Statistical 11.0 software (SPSS Inc, Chicago, Illinois, USA).

Results

Plasma levels of AChE, BuChE, ACh and Aβ in AD and HC subjects

In our AD (n=20) and HC (n=20) subjects plasma levels of AChE, BuChE, ACh and Aβ were evaluated using commercial kits. Results indicated that ACh levels, although not significantly different, were lower in the plasma of AD (3.6±2.7 pmol/mL) in comparison to control subjects (5.1±3.7 pmol/mL), with some overlap in the values occurring between the two groups. In accord with immuno-chemical studies [63], higher BuChE compared to AChE plasma levels were detected in both AD patients and HC subjects. Notably, levels of AChE and BuChE were significantly elevated in the AD group with respect to the HC one ($p<0.001$) (Table 3). Mean plasma levels of Aβ in AD and HC subjects were 141.4±29.0 ng/ml and 78.5±20.4 ng/ml respectively. Hence, the first clear observation in this study was that the amount of Aβ in the AD patients was on average 2-fold higher than that of the control group ($p<0.001$), and the correlation between plasma AChE, BuChE, ACh, and Aβ levels was not statistically significant.

Effect of Aβ on the expression of cytokines and chemokines in THP-1 cells

Previous studies have documented that THP-1 cells acquire a microglia-like morphology when treated with LPS, and there is a consistent similarity in responsiveness to all Aβ peptides between primary microglia and THP-1 monocytes [64,65]. Thus, THP-1 was used as a model of primary human microglia. We performed a preliminary experiment to select an optimal and non-toxic Aβ concentration that activated THP-1 cells. Aβ peptide (10 μM) treatment robustly increased mRNA expression of IL-1β, TNF-α and MCP-1, and more weakly IL-10 in THP-1 monocytes (Figure 1). We then determined whether this Aβ-induced rise in mRNA expression in THP-1 cells translated to similar changes in cytokines and chemokines production. Indeed, protein levels of IL-1β, TNF-α, and MCP-1 were elevated by 4.3-, 5.2-, and 4.5-fold, respectively (data not show).

Effect of Aβ on cytokine and chemokine production in PBMCs

The baseline levels of cytokines in resting and PHA-stimulated PBMCs were comparable to data obtained in previous studies. The Aβ-induced rise in cytokine-levels from PBMCs peaked at 24 h and declined afterwards [66]. We therefore analyzed the production of inflammatory cytokines from PBMCs isolated from 20 AD and 20 HC subjects after 24 h of incubation with Aβ 10 μM. It is noteworthy that basal production of all cytokines analyzed in PBMCs from AD patients was significantly higher than those from HC subjects (Table 4). Levels of pro-inflammatory IL-1β, MCP-1, TNF-α and pleiotropic IL-6 were significantly elevated in PBMCs treated with Aβ, with a greater rise in levels of IL-1β, MCP-1 and TNF-α in AD patients (Table 4).

Role of pharmacological cholinesterase inhibitors on Aβ-induced cytokine production in THP-1 cells

To elucidate the role of AChE and BuChE in the production of inflammatory cytokines, we exposed THP-1 cells and PBMCs to (−)-phenserine (**1**), (−)-phenethylcymserine (**2**), (−)-bisnorcymserine (**3**) and (−)-cymeserine (**4**). As illustrated in Figure 2, all are close structural analogues of one another. However, the former, (−)-phenserine (**1**), has selectivity for AChE over BuChE, whereas all cymserine analogues, (**2**), (**3**) and (**4**), have preferences for BuChE inhibition over AChE, with (−)-phenethylcymserine (**2**) providing absolute selectivity for BuChE, (**3**) providing >100-fold selectivity and (**4**) mild selectivity (15-fold) [41,58,67]. Both AChE-selective (−)-phenserine (**1**) and BuChE selective (−)-bisnorcymserine (**3**) are in clinical development.

Comparing our data on the action of (−)-phenserine (**1**) *vs.* compounds (**2**), (**3**) and (**4**) on Aβ-mediated cytokine production, it appears that AChE preferential inhibition significantly ameliorated the TNF-α and IL-1β Aβ-induced elevations ($p<0.05$). Additionally, inhibition of BuChE, as assessed by (−)-phenethylcymserine (**2**), significantly mitigated the Aβ-induced rise in MCP-1 and TNF-α ($p<0.05$) Minimal effect was found on IL-6 Aβ-induced elevations; however, anti-inflammatory IL-10 levels, that were unaffected by Aβ alone, were significantly increased in the presence of (−)-phenserine (**1**) (Figure 2).

Effect of cholinesterase inhibitors on Aβ-induced cytokine production in PBMCs from AD and HC subjects

As illustrated in Figure 3, PBMCs obtained from AD patients were highly sensitive to Aβ challenge, which induced a heightened generation of the proinflammatory cytokines TNF-α, MCP-1 and IL-1β compared to similarly challenged PBMCs from HC subjects (($p<0.05$). By contrast, anti-inflammatory IL-10 levels were similar in Aβ challenged PBMCs from AD and HC subjects. The administration of (−)-phenserine (**1**) fully ameliorated the elevated production of IL-1β and significantly lowered the rise in TNF-α levels induced by Aβ in PBMCs from AD subjects ($p<0.05$). Additionally, slight reductions in both of these cytokines were apparent in Aβ challenged PBMCs from HC subjects. In contrast, no effect was achieved by (−)-phenserine (**1**) on levels of MCP-1 and IL-10.

In relation to the BuChE selective inhibitors (**2**), (**3**) and (**4**), Aβ-induced elevations in AD PBMCs in IL-1β, TNF-α, and MCP-1 were each significantly lowered ($p<0.05$). A small

reduction was apparent by (−)-phenethylcymserine (**2**) on TNF-α and MCP-1 levels in HC PBMCs; but of note, a dramatic elevation in IL-10 was found (Figure 3).

In light of reductions in IL-1β levels by both (−)-phenserine (**1**) and (−)-cymserine analogues (**3** and **4**), the concentration-dependence of these inhibitors was assessed in PBMCs derived from AD patients following a PHA challenge that, like Aβ, substantially elevated proinflammatory cytokine levels (not shown) and particularly IL-1β (Figure 4). Incubation with 0.1 to 10 μM of (−)-phenserine (**1**) and (−)-cymserine analogues (**3** and **4**) fully mitigated the PHA-induced IL-1β elevation.

Effect of signalling pathway inhibitors on Aβ-induced cytokine and chemokine expression

To determine which signalling pathways are essential for Aβ-mediated increased production of cytokines in our cell systems, we exposed PBMCs and THP-1 cells to Aβ for 30 min and the phosphorylation of P38MAPK, PI3K and ERK1/2 were, thereafter, quantified using FACE Kits, as described in 'Materials and Methods'. In accord with previous studies, PI3K/Akt, ERK and p38 MAPK phosphorylation in THP-1 cells and PBMCs were induced by Aβ (data not shown). Hence, in Table 5 mRNA levels are shown of IL-1β, MCP-1, TNF-α and IL-10 in THP-1 monocytes that were preincubated for 30 min with specific inhibitors. Use of the MEK1/2 inhibitor PD98059 (20 μM) significantly lowered Aβ induced up regulation of IL-1β and MCP-1 expression by 55% and 35%, respectively, and elevated IL-10 expression by 26% ($p<0.05$). Similarly to examine the role of PI3K in the Aβ-treated cells, PI3K activity was blocked with LY294002 (10 μM), which reduced Aβ-induced mRNA expression of IL-1β and MCP-1 by 30% and 36%, respectively. Next, the inhibitor SB203580 (10 μM) was used to define p38MAPK involvement, which reduced the expression of IL-1β and TNF-α by 35% and 50% respectively. The extent of inhibition of Aβ-induced mRNA expression and release of TNF-α and IL-1β by MAPK inhibitor (SB203580) and MEK1/2 inhibitor (PD98059) was similar (data not show).

These results suggest that Aβ up regulates MCP-1 and IL-1β mRNA expression via PI3K/Akt and ERK signalling pathways, and TNF-α by activation of p38MAPK. IL-10 production was reduced by the inhibition of PI3K but not by inhibition of either ERK or p38 MAPK. To investigate whether the effects of (−)-phenserine (**1**) and (−)-bisnorcymserine (**3**) on cytokine production are MEK1/2-, p38MAPK- or PI3K-mediated, THP-1 cells were incubated for 30 min with LY294002, SB203580 or PD98059 prior to addition of (**1**) or (**2**) and then were incubated with Aβ 10 μM for 24 hr. Preliminary results indicate that only co-treatment with PD98059 augmented the (−)-phenserine and (−)-bisnorcymserine-mediated attenuation of Aβ-induced IL-1β and MCP-1 production, and that simultaneous incubation with LY294002 and (−)-bisnorcymserine altered IL-1β and MCP-1 levels in PBMCs of only 3 of the 8 AD patients analyzed. Thus, additional studies and a larger number of patients are warranted to clarify the signalling pathways associated with the actions of the assessed AChE and BuChE inhibitors.

Effect of acetylcholine receptor antagonists and agonists on (−)-phenserine (1) and (−)-bisnorcymserine (2) down-regulation of Aβ-induced IL-1β and TNF-α

As THP-1 and mononuclear cells express nAChRs, we investigated the actions of the cholinergic agonist nicotine and antagonist mecamylamine on Aβ-induced cytokine expression. Neither the agonist nor antagonist, separately, altered the expression of cytokines; however, pre-treatment with nicotine (10 μM) significantly attenuated Aβ-induced IL-1β and TNF-α production. Thereafter, THP-1 cells were pre-incubated with (−)-phenserine **(1)** or (−)-bisnorcymserine (**3**), treated with nicotine and the production of IL-1β and TNF-α was quantified following Aβ challenge. As illustrated in Figure 5, treatment with (**1**) and (**3**) weakly augment the attenuation of IL-1β and TNF-α induced by nicotine. This effect was not attributable to a reduction of cellular viability, which was measured by MTT assay and was unchanged. No effect of nicotine, with or without (−)-phenserine **(1)** or (−)-bisnorcymserine (**3**), was observed on either MCP-1 or IL-10 production. Mecamylamine had no significant effect on any of the cytokines studied.

Discussion

Whereas cerebrospinal fluid (CSF) largely mirrors the composition of the brain extracellular space fluid, its collection by lumbar puncture is not a widespread procedure for the evaluation of AD. Apart from brain-specific changes, AD-attributed abnormalities apparent in many peripheral cell types, such as erythrocytes, lymphocytes, platelets and fibroblasts [68], have led to the concept that AD be considered as a systemic disorder whose most prominent pathology impacts cognitive functions within the central nervous system (CNS). In such a scenario, peripheral tissues offer a number of advantages in evaluating biochemical alterations in AD; clearly key among these is their ease of sampling. In this regard, potential blood biomarker molecules or metabolites would be more widely applicable and would reduce the need for invasive, expensive, and time-consuming sampling and testing. Hence in the current study, PBMCs and THP-1 cells were evaluated as they parallel responsiveness to Aβ as do primary microglia, and thus represent an applicable model to define relationships between neuroinflammation and cholinergic dysfunction.

The initial finding of our study is that plasma levels of both AChE and BuChE were significantly higher in AD patients compared to HC subjects. In line with this, levels of ACh tended to be lower in the plasma of AD patients. Furthermore, levels of Aβ were on average 2-fold higher in AD *vs.* HC plasma samples.

There is compelling evidence that Aβ is a key factor in the development of AD [3,4,25] and growing evidence that changes in plasma Aβ levels may provide an indicator of AD onset and progression [69,70], albeit clearer understanding of the relationships between brain, CSF and systemic levels requires to be determined. Aβ peptide found present in AD brain is considered primarily made locally [25]. Produced in soluble monomeric form, Aβ generates soluble oligomers that are believed to be the primary toxic species [5,71], which eventually create the aggregated Aβ found in brain amyloid plaques. Following the intravenous injection of synthetic Aβ small amounts have been detected in the brain of rodents [72] and primates [73], and, reciprocally, soluble systemic Aβ has been found in plasma after central administration [74]). Hence, soluble forms of Aβ circulating within the plasma appear to be

readily in contact with immune cells outside the brain, with the potential of exerting modulatory activities on such peripheral cells that could then ultimately contribute to AD processes both in brain and peripherally [75]. Aβ peptide has been shown to induce the expression of IL-1β, TNF-α, and IL-6 in astrocytes and microglia in culture [76]. Conversely, cytokines secreted by microglial cells and astrocytes can induce the synthesis and modify the processing of APP [77,78], and a correlation has been established between Aβ neurotoxicity and cytokine production [79].

A large body of evidence supports the involvement of humoral mechanisms in the immune-to-brain communication. The brain exerts strong modulatory effects on immune function by activation of the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic (sympathetic) nervous system (SNS), and pro-inflammatory cytokines trigger the HPA axis via these neural or humoral mechanisms [79]. As a consequence, we evaluated the *in vitro* effect of Aβ on humoral response of peripheral cells by analysing the production of key inflammatory cytokines.

Our results demonstrate that Aβ pro-inflammatory cytokines, such as IL-1β, TNF-α, pleiotropic IL-6 and the chemokine MCP-1 in challenged PBMCs and THP-1 cells, a human monocytic cell line that bears features of microglial cells [80]. These results emphasizes the role of Aβ in the induction of cell migration, and thus elevated Aβ levels within the brain can potentially favour the migration of monocytes from the blood and into the brain. Moreover, inflammatory cytokines generated and secreted by monocytes and microglial cells can play a key role in neuronal cell damage and death.

Further to the hallmark generation and deposition of Aβ and the invariable presence of neuroinflammation in AD brain, cholinergic dysfunction is an assured feature. In line with the loss of cholinergic neurons in the nucleus basalis and its wide projection areas within the cerebral cortex, hippocampus and thalamus in AD, there is a decline in ACh levels and a parallel loss of AChE [24,25,81]. In contrast, a rise in brain BuChE levels occurs [48,49,42,43], creating an imbalance in the normal ratio of BuChE to AChE of 0.2 in healthy brain to as high as 11 in AD brain [44,47]. This deregulation in the balance between AChE and BuChE that both efficiently metabolize ACh likely results in a mismatch between ACh synaptic release, that is already reduced in AD brain, and its cleavage, further perturbing physiological function.

Under routine conditions in a healthy brain the rate of ACh metabolism by AChE and BuChE is limited only by the rate of its diffusion into the internalized gorge of either enzyme where binding and cleavage of ACh occurs. A key difference in the K_m of AChE and BuChE towards the metabolism of ACh distinguishes the two enzymes and result in their differential efficiencies to hydrolyze ACh in a concentration-dependent manner [43,44,47]. The K_m of BuChE is set high, making it more efficient at a high substrate concentration. In contrast, the K_m of AChE is set low providing it greater catalytic efficiency at low ACh concentrations; however, substrate-induced inhibition of AChE can occur at high ACh concentrations [82,83]. These slight differences in enzyme activities and locations of AChE and BuChE in the brain have led to the suggestion that, in the normal brain, AChE is the primary enzyme responsible for ACh hydrolysis with BuChE playing a supportive role at

high ACh concentrations [47], as occurs within the locality of the synaptic cleft where ACh levels have been predicted to reach μM levels with the potential to overcome and induce substrate-mediated inhibition of AChE. The close spatial relationship between glial BuChE and normal synaptic AChE would support optimal hydrolysis across a range of conditions to maximize brain cholinergic function. Imbalance in this finely tuned relationship between AChE and BuChE could potentially impact not only synaptic levels of ACh but also the cholinergic anti-inflammatory pathway [55,79].

The cholinergic anti-inflammatory pathway provides the efferent/motor arm of the inflammatory reflex, which is a critical neural circuit that regulates the immune response to injury and invasion. On one hand, the afferent arc of the inflammatory reflex is activated by cytokines and, in particular, TNF-α during local injury and infection to initiate a cascade of mediator release and the recruitment of inflammatory cells to the site of infection/injury to contain it. On the other hand, the cholinergic anti-inflammatory pathway provides a braking mechanism on the innate immune response to importantly protect the body from damage that can arise should a localized inflammatory response extend outside the local tissue or remain activated for too long. Such action is provided by the vagus (10^{th} cranial) nerve that innervates the celiac ganglion, the site of origin of the splenic nerve [84]. Stimulation of the efferent pathway of the vagus nerve results in transmission of action potentials to the spleen, where a subset of specialized T cells is activated to secrete ACh [85,86].

The anti-inflammatory action of ACh to regulate the production of pro-inflammatory cytokines is mediated via the α7nAChR. In this regard, ACh has been described to inhibit LPS-induced production of proinflammatory cytokines, including IL-1β, TNF-α in microglia cultures [87] and to significantly and concentration-dependently decrease TNF-α, IL-1β, IL-6 and IL-18 production by endotoxin-stimulated human macrophage cultures via a post transcriptional mechanism [53]. The levels of ACh are continuously regulated by the hydrolytic cholinesterases to rapidly degrade excess. When levels of AChE and BuChE become elevated, a resulting decline of ACh levels may in large part be responsible for the rise of pro-inflammatory cytokines – with the easing off of the cholinergic anti-inflammatory braking mechanism. With the altered expression of AChE within AD patients, elevated levels of BuChE correlating positively with disease progression [40,43,49], and with elevated systemic BuChE levels occurring with and providing a marker chronic inflammation [88], there are multiple opportunities that the normal homeostasis provided by the inflammatory reflex is adversely changed and/or becomes dysregulated in AD. In accord with this and notable in the current study, plasma levels of AChE and BuChE were significantly higher in the AD than HC group ($p<0.001$) (Table 3).

A systematic review and meta-analysis of studies assessing BBB permeability in aging, cerebral microvascular disease and AD suggest that permeability increases occur in AD and may represent an important mechanism in disease onset and progression [89], although it is difficult to ascertain whether elevated BBB permeability is consequential or causal. Nevertheless, profound changes in permeability associated with AD may facilitate the movement of AChE and BuChE as well as immune markers between the brain and blood compartments and vice versa; potentially allowing peripherally produced inflammatory proteins to modulate Aβ generation and amplify the formation of cerebral amyloid deposits.

In a previous study of the *in vitro* production of cytokines, we reported a significantly higher production of inflammatory cytokines IL-1β, IL-6, TNF-α and interferon-γ in PHA-stimulated PBMCs from AD subjects [14,90,91], and Rao et al., [92] likewise reported elevated BuChE and AChE in the plasma and tissue in AD. Since PBMCs possess the components for a non-neuronal cholinergic system, with molecular forms similar to those found in brain tissue that may play a key role in the regulation of inflammation [35–37], we used PBMCs and THP-1 cells to study the relationship between inflammation and cholinergic dysfunction. In this regard, evaluation of cytokine-expression/release by PBMCs and THP-1 cells challenged with Aβ, showed that IL-1β, TNF-α, MCP-1 and IL-6, all of which are pro-inflammatory cytokines, are increased upon 24 hr exposure to A. In contrast, IL-10 with anti-inflammatory activities, in LPS-stimulated THP-1 cells, was weakly elevated, and was not affected by Aβ in PBMCs of either AD patients or HC subjects.

The molecular pathways that underpin these changes in cytokine expression are clearly of interest, especially the MAPKs that are a family of serine/threonine kinases that comprise ERK and p38 MAPK. The role of ERK activation in response to Aβ peptides in neurons remains largely unclear. Two groups have reported that Aβ, in mature hippocampal neurons, enhances ERK phosphorylation and that MEK1 inhibitors prevent Aβ-mediated tau phosphorylation and neurite degeneration [93,94]. In contrast, other studies have shown that, in cortical or hippocampal neurons, Aβ does not affect ERK phosphorylation [95]. A major signalling pathway that, in particular, contributes quantitatively to the up-regulation of cytokine production in peripheral inflammation is the p38 MAPK pathway, especially the key regulatory enzyme p38a MAPK. Increasing evidence suggests that the p38 MAPK signalling cascade additionally contributes to CNS cytokine overproduction and ensuing neurodegenerative dysfunction. Previous studies have suggested that Aβ activates, with a similar time course, ERK and p38MAPK in microglia and THP-1 cells. It is now widely recognized that the PI3K/AKT pathway participates in multiple cellular processes, including cell growth, survival, and notably inflammation. Whether or not the PI3K/AKT pathway participates in Aβ-induced inflammatory cytokine production, however, is not well understood. Others have shown that the PI3K/AKT pathway positively regulates LPS signaling in monocytes/macrophages [96,97]. Conversely, other studies have indicated that LPS signaling and gene expression in monocytic cells was negatively regulated by the PI3K/AKT pathway [98,99]. This dichotomy may be due, in part, to the nonspecific effects of PI3K inhibitors or other factors such as cell types, agonists, and the concentration of the inhibitor.

Our examination of the effects of Aβ on kinase pathways shows that in PBMCs and THP-1 cells Aβ activated p38 MAPK, ERK1/2 and Akt, and that this activation was necessary for Aβ-induced cytokine production. Specifically, the pretreatment of cells with PI3K inhibitor (LY294002), p38 inhibitor (SB203580) and ERK inhibitor (PD98059) reduced Aβ-mediated IL-1β, TNF-α and MCP-1 production. These observations suggest that Aβ-induced cytokine expression in THP-1 and PBMCs from AD patients and HC subjects result from an activation of multiple signalling pathways that include MAPKs p38 and ERK, and PI-3K pathways.

In light of the elevated levels of plasma AChE and BuChE evident in our AD patients vs. HC subjects, and bearing in mind that these enzymes derive from multiple origins (including potentially the brain) and together with systemic cytokines can likely access the brain, we sought to determine whether selective cholinesterase inhibitors could mitigate Aβ-induced cytokine expression. The recent development of inhibitors with high selectivity for AChE ((−)-phenserine **(1)**: 80-fold) and BuChE ((−)-cymserine **(4)**: 15-fold, (−)-bisnorcymserine **(3)**: 110-fold, (−)-phenethylcymserine **(2)**: >5000-fold) [47,58] provides the potential to start to define the role AChE, BuChE and their clinically relevant inhibitors may have on inflammatory mediators that promote AD. Our preliminary results indicate that (−)-phenserine **(1)** reduced IL-1β and TNF-α release from AD PBMCs following Aβ challenge (Figure 3), and a lesser reduction was similarly observed with (−)-phenethylcymserine, (−)-bisnorcymserine and (−)-cymserine **(2–4)** that additionally mildly lowered MCP-1 levels. There was no observable effect of any of the inhibitors on IL-10 production. Our studies of THP-1 cells challenged with Aβ (Figure 2) largely verified these findings, with (−)-phenserine-induced AChE inhibition playing a role in TNF-α and IL-1β production and cymserine analogue-induced inhibition of BuChE reducing MCP-1 and TNF-α, likewise with little impact on IL-10 levels.

To evaluate both translation across immunological challenges as well as concentration-dependence, PBMCs were challenged with PHA in the presence and absence of selective AChE and BuChE inhibitors and IL-1β levels were quantified, as generation of this cytokine was lowered by **1–4** in our Aβ studies. In accord with prior studies, PHA elevated IL-1β production in PBMCs and this action was ameliorated by both (−)-phenserine (**1**) and cymserine analogues (**3** and **4**) at 0.1 μM, a concentration that all compounds retain their selectivity between AChE and BuChE [47,58].

Prior studies have demonstrated that nAChR agonists provide protective effects against Aβ-induced neutotoxicity [100] and that the nAChR is present on the human monocytic THP-1 cell line and human PBMCs. The binding of nicotine to the α7 nAChR decreases some inflammatory cytokines [56], such as TNF-α, MIP2 and IL-6. In light of this we probed our cellular system with a nicotinic agonist and antagonist to assess potential involvement of nicotinic mechanisms in our observed AChE and BuChE inhibitor associated actions. Our data using THP-1 cells showed that nicotine induced a reduction of TNF-α and IL-1β production. We additionally observed an inhibitory effect of the AChE and BuChE inhibitors (−)-phenserine (**1**) and (−)-bisnorcymserine (**3**) on Aβ-induced cytokine production. Synergy between nicotine's nAChR agonist action and the actions or (**1**) and (**3**) to inhibit cytokines production was not evident; however, further studies across wider dose ranges and use of more specific nicotinic agonists are warranted to ascertain nicotinic involvement.

Our present work showing the effect of selective AChE and BuChE cholinesterase inhibitors on Aβ-induced cytokine production in PBMCs has significant implication from the following perspective. It has become increasingly clear that Aβ can similarly induce the elevated release of CNS proinflammatory cytokines [7–11,76–78], as we have herein described for systemic ones, to generate a chronic inflammatory state that potentially cycles back to augment the amyloidogenic processing of APP into Aβ to likely impact disease progression. Notably, cytokines have been described to participate in APP gene regulation

[101]. Moreover, TGF-β1 potentiates Aβ generation in astrocytes and in transgenic mice [102]. Interestingly, Aβ (1–42) fibrillar precursors have been reported to induce TNF-α production in THP-1 cells [103]. Such Aβ/THP-1 cell interactions to induce TNF-α have been described to involve a PKC-dependent process [104], with Aβ subfragments (Aβ (25–35)) ineffectively inducing TNF-α production [105]. Identification of the pathway(s) via which extracellular Aβ activates such intracellular PKC-dependent secretion of TNF-α may help in developing new therapeutic strategies for AD. Furthermore, the recently proposed role of Aβ as a putative transcription factor and characterization of amyloid-β peptide interacting domain (AβID) in the APP and BACE1 promoter sequences are significant [106]. This interaction implicates a role for Aβ in activating apoptotic genes and in amyloidogenesis [107]. In this context, our present work showing the ability of selective AChE- and BuChE-inhibitors to prevent heightened cytokine secretion, assumes significance for AD treatment.

In synopsis, over recent years it has become increasingly clear that anticholinesterases, which remain the primary therapy in AD, have potential actions beyond simply ameliorating hallmark cholinergic deficits in neurotransmission found in the AD brain. In much the same manner that Aβ can impact cholinergic receptor expression on neurons, reduce ACh synthesis and induce neuronal dysfunction and loss, changes in cholinergic input and cholinergic drugs (both anticholinesterases and direct agonists) can modify APP expression, its processing to generate Aβ, and Aβ-induced toxicity [25,108–112]. It has become increasingly clear that Aβ can also induce the elevated release of CNS and systemic proinflammatory cytokines to generate a chronic inflammatory state that, likewise, cycles back to augment the amyloidogenic processing of APP into Aβ to likely impact disease progression. Here too, the cholinergic system has a role, acting as an anti-inflammatory brake, and anticholinesterase drugs may have the potential to impact or reverse this process.

The precise mechanisms responsible for Aβ-induced cytokine gene expression remain to be fully elucidated, and the increasingly available knowledge relating to Aβ-driven signalling creates a rising need for application of a biological system approach to understand this complex network. Increasing evidence now points towards an anti-inflammatory role for cholinesterase inhibitors [113,110]; suppressing the release of cytokines from activated microglia and PBMCs within the brain and blood, but likely working on many other levels too. Elucidating the mechanisms underpinning these actions, whether cholinergically mediated or direct non-cholinergically mediated actions, will hopefully aid their optimization and translation to clinical studies to provide better therapy.

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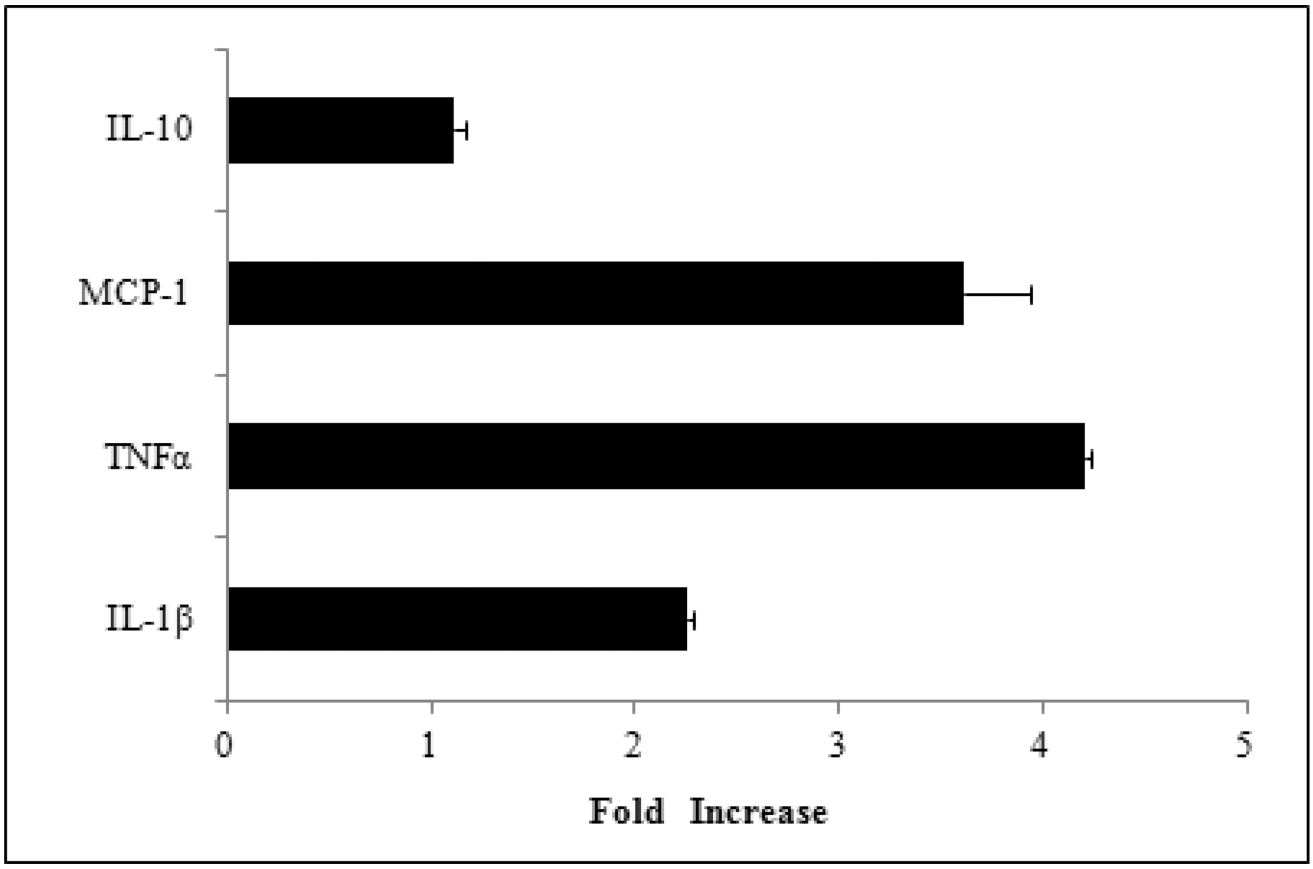

Figure 1.

Aβ-induced expression of IL-10, MCP-1, TNF-α and IL-1β in THP-1 cells. Data are expressed as the fold increase of mRNA expression of each cytokine in Aβ treated THP-1 cells relative to untreated cells. Data are representative of pooled experiments.

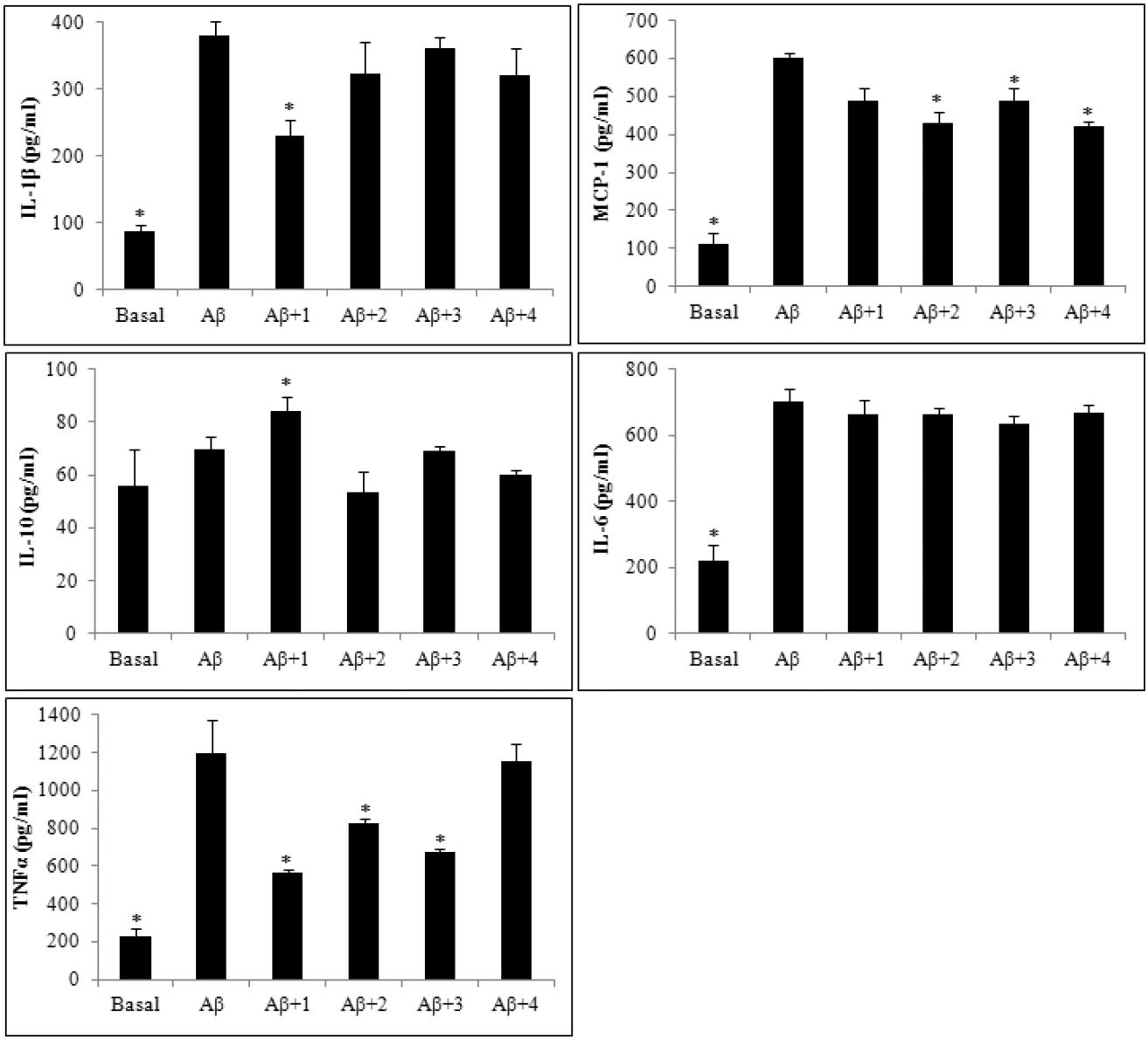

Figure 2.

Effects of pharmacological inhibitors (−)-phenserine (**1**), (−)-phenethylcymserine (**2**), (−)-bisnorcymserine (**3**) and (−)-cymserine (**4**) on Aβ-induced cytokines production in THP-1 cells. Cells were incubated with compound (**1**), (**2**), (**3**) or (**4**) all 10 uM, 24 hr before addition of 10 μM Aβ. ELISA analysis of cell-free supernatants was performed to quantify cytokine levels. Data are expressed as the mean and standard deviation of cytokine production. Kruskal Wallis H test p=0.016 for IL-1β, p=0.009 for MCP-1, p=0.025 for IL-10, p=0.044 for IL-6, p=0.006 for TNF-α. *p<0.05 post-hoc test *vs* Aβ

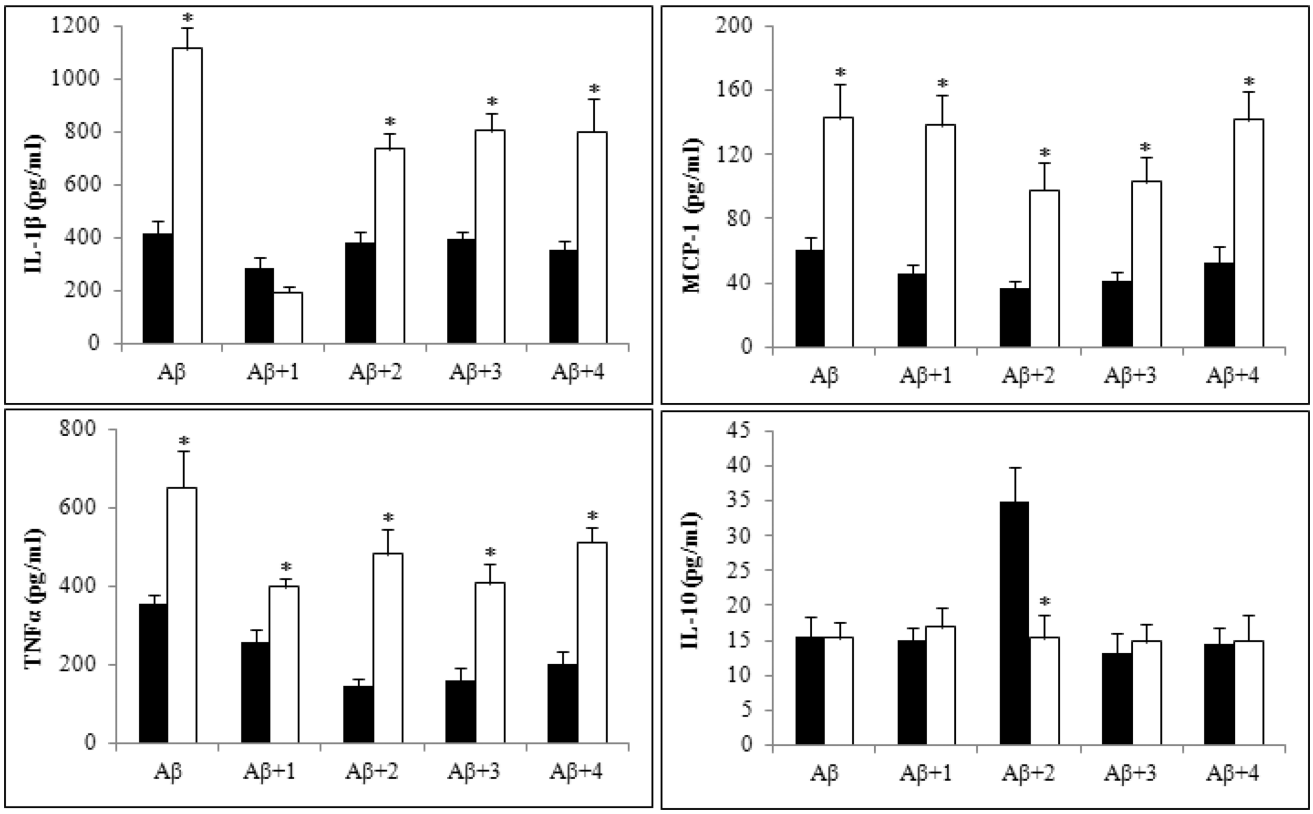

Figure 3.

Aβ-mediated secretion of IL-1β, MCP-1, TNF-α and IL-10 and their modulation by (−)-phenserine (**1**), (−)-phenethylcymserine (**2**), (−)-bisnorcymserine (**3**) and (−)-cymserine (**4**) (all 10 uM) in PBMCs from AD patients and HC subjects. At the end of incubation, cell-free supernatant was harvested, and the levels of released cytokines were determined by ELISA. Data are expressed as the mean and standard deviation of IL-1β, MCP-1, TNF-α and IL-10 production in PBMCs from AD (■) and HC (□) subjects. *p<0.05 post-hoc test *vs* HC levels.

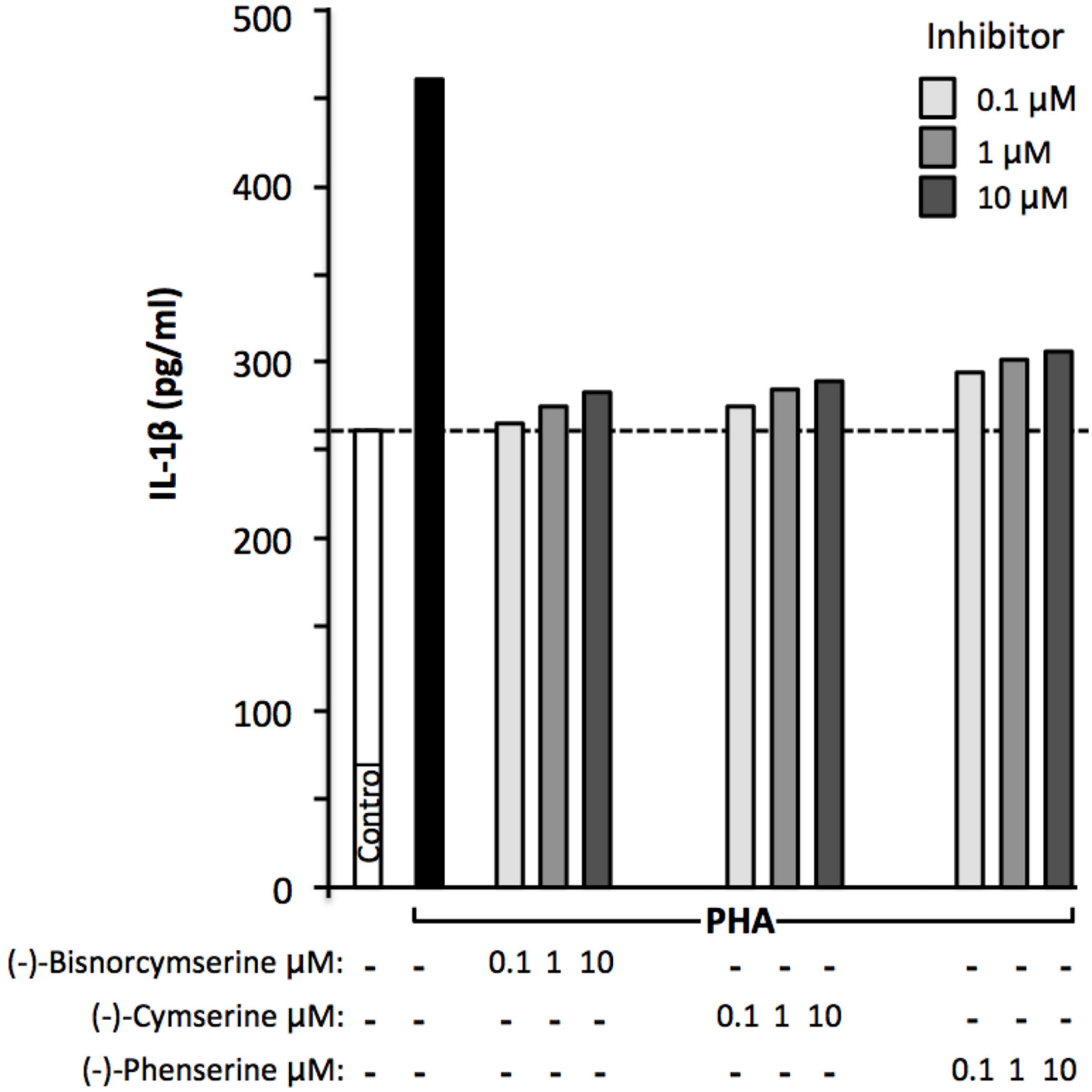

Figure 4.

PHA-induced elevated secretion of IL-1β and its concentration-dependent amelioration by (−)-phenserine (**1**), (−)-bisnorcymserine (**3**) and (−)-cymserine (**4**) (all 0.1 to 10 μM) in PBMCs from AD patients. At the end of incubation, cell-free supernatant was harvested, and the levels of released IL-1β, were determined by ELISA. Data are expressed as mean values.

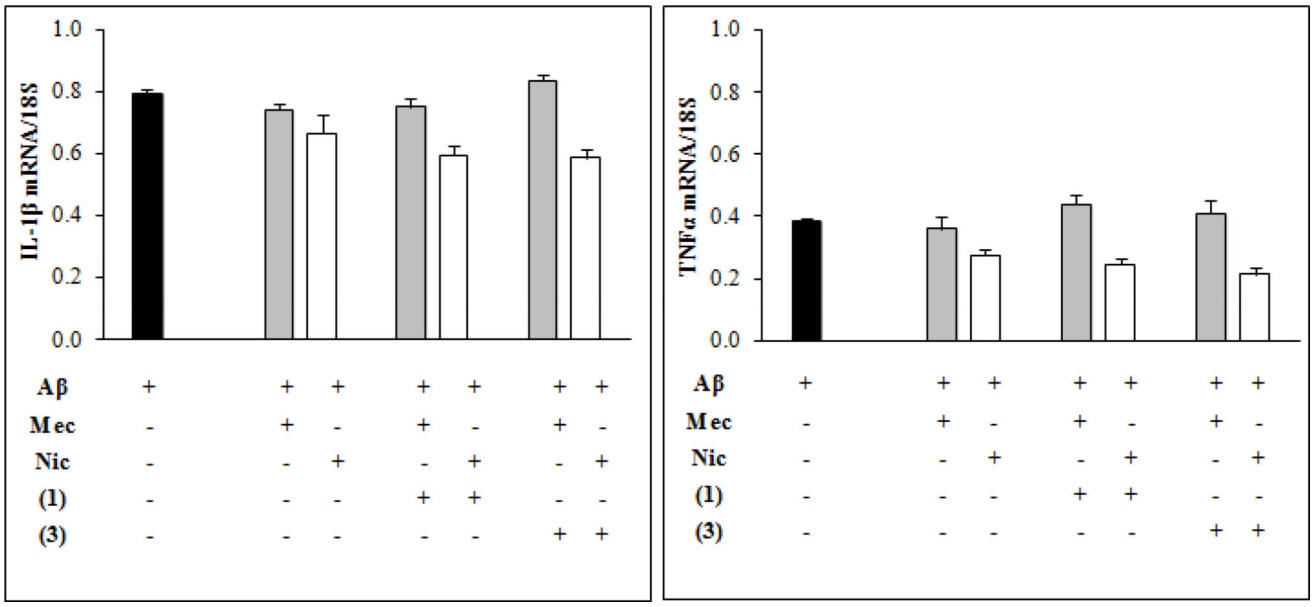

Figure 5.

Effect of pharmacological inhibitor (**1**) and (**3**) on Aβ-induced expression of IL-1β and TNF-α mRNA in THP-1 cells. THP-1 cells pre-incubated with 10 μM nicotine (Nic) or mecamylamine (Mec) for 30 min, were then treated with compound (**1**) and (**3**) (both 10 μM) for 24 h. After an additional 24 h of incubation with Aβ, levels of mRNA expression relative to that 18S, used as an internal control, were evaluated. Data are expressed as the mean and standard deviation of two independent experiments.

Table 1

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

Variable	HC (n=20)	AD (n=20)	p-value [a]
Age (years), *mean± SD*	73.3±5.1	75.6±5.5	*0.314*
Gender, *n (%)*			*0.744*[b]
Male	8 (40.0)	7 (35.0)	
Female	12 (60.0)	13 (65.0)	
MMSE score, *mean± SD*	26.2±3.1	18.5±2.3	*<0.001*
Disease duration (months), *range*	-	2–8	-
Education, *n (%)*			*0.687* [b]
<5 years	3 (15.0)	5 (25.0)	
5–13 years	8 (40.0)	8 (40.0)	
>13 years	9 (45.0)	7 (35.0)	

[a] t-test for unpaired data;

[b] Chi-squared test AD patients *vs.* HC subject.

Table 2

Sequences of the oligonucleotide primers utilized in the reverse transcriptase polymerase chain reaction.

Gene	Forward primer sequence (5'-3')	Revers primer sequence (5'-3')	product size
IL-1β	TGAGGATGACTTGTTCTTTGAAG	GTGGTGGTCGGAGATTCG	115 bp
IL 10	GAGAACCAAGACCCAGACATC	TCACTCATGGCTTTGTAGATGC	185 bp
MCP-1	AACTGAAGCTCGCACTCTCG	GAGTGAGTGTTCAAGTCTTCGG	338 bp
TNF-α	CCTTCCTGATCGTGGCAG	GCTTGAGGGTTTGCTACAAC	184 bp
18S	CTTTGCCATCACTGCCATTAAG	TCCATCCTTTACATCCTTCTGTC	199 bp

Table 3

Mean ± standard deviation of AChE, BuChE, ACh and Aβ in plasma of patients with Alzheimer's disease (AD) and healthy control (HC) subjects.

Variable	HC (n=20)	AD (n=20)	p-value [a]
AChE (mU/mL)	207.0±152.8	1244.3±259.2	*<0.001*
BuChE (mU/mL)	1031.8±99.5	3387.9±851.0	*<0.001*
ACh (pmol/mL)	5.1±3.7	3.6±2.7	*0.229*
Aβ (ng/mL)	78.5±20.4	141.4±29.0	*<0.001*

[a] t-test for unpaired data.

Table 4

Mean ± standard deviation of inflammatory cytokine production in PBMCs from patients with Alzheimer's disease (AD) and healthy control (HC) subjects.

Variable	HC (n=20)		AD (n=20)		p-value		
	Basal	Aβ	Basal	Aβ	$^{+}$ Aβ	$^{\#}$ group	† interaction
IL-1β (pg/ml)	60.2±23.1	418.4±212.4	175.9±90.7	1127.4±261.8	*<0.001*	*<0.001*	*<0.001*
MCP-1 (pg/ml)	24.5±5.6	58.9±14.5	56.9±21.6	143.4±38.3	*<0.001*	*<0.001*	*<0.001*
TNF-α (pg/ml)	82.7±29.8	350.4±167.2	124.4±35.9	649.9±257.0	*<0.001*	*<0.001*	*<0.001*
IL-6 (pg/ml)	277.7±139.7	2037.5±687.6	726.1±213.7	2953.5±868.9	*<0.001*	*<0.001*	*0.056*

Probability that effect on the addressed variable is influenced by:

$^{+}$Aβ, for each variable, the differences have been tested between the means of basal production of the two groups and the means of the Aβ-induced production of the two groups;

$^{\#}$groups, for each variable, the differences have been tested between the means of HC group in two conditions (basal and Aβ) and the means of the AD group in two conditions (basal and Aβ);

†probability that the effects are greater in one distinct group (interaction Aβ*group).

Table 5

Mean ± standard deviation of IL-1β, MCP-1, TNF-α and IL-10 production in THP-1 cells pre-incubated with LY294002, PD98059 or SB202190 and then incubated in the presence of Aβ for 24 h

	IL-1β	MCP-1	TNF-α	IL-10
Aβ	0.74±0.06	0.77±0.02	0.84±0.06	0.82±0.06
Aβ+LY294002	0.52±0.11*	0.49±0.11*	0.94±0.01	0.71±0.05
Aβ+PD98059	0.33±0.01*	0.50±0.01*	0.81±0.02	1.03±0.05*
Aβ+SB202190	0.47±0.18*	0.80±0.10	0.42±0.01*	0.93±0.12
p-value[a]	0.004	0.010	0.005	0.008

[a] Kruskal-Wallis H test.

* p<0.05 post hoc test *vs.* Aβ