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All-*trans*-Retinoic Acid Modulates Nitric Oxide and Interleukin-17A Production by Peripheral Blood Mononuclear Cells from Patients with Alzheimer's Disease

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Key Words
All-*trans*-retinoic acid · Alzheimer's disease · Inducible nitric oxide synthase · Immunomodulation · Interleukin-17A · Nitric oxide · Peripheral blood mononuclear cells

Abstract
Background: Alzheimer's disease (AD), the most common form of dementia in the elderly, is a neurodegenerative disorder associated with a complex pathophysiology. It is accepted that inflammation contributes to the pathogenesis of AD. All-*trans*-retinoic acid (ATRA) is a bioactive derivative of vitamin A that has shown immunomodulatory effects in many immune disorders. ***Objectives:*** In our study, we aimed to investigate in vitro immunomodulatory effects of ATRA on inducible nitric oxide synthase (iNOS) expression and interleukin-17A production during AD. ***Methods:*** Peripheral blood mononuclear cells (PBMCs) isolated from 30 Algerian AD patients and 14 age-matched nondemented controls were treated (or not) with ATRA. Production of NO and IL-17A in culture media was measured by the modified Griess method and enzyme-linked immunosorbent assay, respectively. Expression of iNOS in PBMCs was examined by fluorescence immunostaining. ***Results:*** Our results showed higher spontaneous in vitro production of NO related to overexpression of iNOS in AD patients compared to controls. Remarkably, ATRA treatment showed an important downregulatory effect on NO production and iNOS expression in patients. This effect was associated with a reduction in IL-17A production and increased IL-10 release. ***Conclusions:*** Taken together, our results indicate that ATRA exerts anti-inflammatory effects in AD. Furthermore, ATRA represents a promising tool for monitoring inflammatory responses associated with disease progression.

Introduction

Alzheimer's disease (AD) is the most common form of dementia in the elderly [1]. It is a progressive neurodegenerative disorder characterized by the insidious onset of memory loss and cognitive impairment [2]. AD pathophysiology is complex. However, the two major

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neuropathological hallmarks are senile plaques and neurofibrillary tangles [3, 4]. In addition to these lesions, a growing body of evidence shows that AD pathophysiology is closely associated with a chronic inflammatory response and an immune disorder within the brain that actively contributes to neurodegeneration [5]. Indeed, neuroinflammation in AD involves both residual immune cells that produce inflammatory mediators, including free radicals and proinflammatory cytokines [6, 7], and peripheral immune cells that infiltrate the brain [8, 9].

Nitric oxide (NO) is a free radical that is synthesized from L-arginine through the actions of the enzyme NO synthase (NOS) [10]. In addition to its various physiological functions in the brain, including neurotransmission and activities as a second messenger molecule [11], NO can be a potent neurotoxic effector when produced in excessive amounts [12]. There is increasing evidence for the potential involvement of NO in neurodegenerative diseases, including AD [13]. In a recent study, we showed the implication of NO pathways in AD progression [14]. The important NO release in the AD brain is mainly mediated by inducible NOS (iNOS) [15, 16], and iNOS expression is highly regulated by cytokines [17, 18].

Interleukin-17 (IL-17) is a proinflammatory cytokine that can be secreted by a newly described CD4+ helper T cell subset, Th17 cells [19]. In addition to its physiological role in bridging the immune system and hematopoiesis [20], Th17 cells serve a critical role in host defense against infection, autoimmunity, and inflammatory diseases [21, 22]. Several studies have shown that IL-17A upregulates iNOS expression [23]. In AD, few studies have characterized the Th17 pathway and its possible involvement in disease pathogenesis. IL-10 is an immunoregulatory cytokine that plays a crucial role in limiting inflammation by different mechanisms [24]. IL-10 has been shown to both suppress iNOS expression and reduce NO production in various cell types [25, 26].

Retinoic acids (RAs) are bioactive metabolites of retinoids (vitamin A derivatives) [27] that regulate a wide range of biological processes, including vision, cellular proliferation, differentiation, and embryogenesis [28, 29]. RAs have been traditionally considered to be antioxidant compounds [30] and have attracted the attention of researchers because of their additional anti-inflammatory and immunomodulatory functions [31]. Recent evidence has shown the ability of retinoids to affect several pathophysiological pathways related to AD through their various properties [32]. However, the mechanisms whereby RAs elicit these functions are not well understood.

Accordingly, and on the basis of our recent reports that support the importance of inflammation and involvement of NO in AD pathogenesis [14], this present study aims to investigate potential anti-inflammatory and immunomodulatory effects of a stereo-isomeric form of RA, all-*trans*-retinoic acid (ATRA), in AD by analyzing its actions on NO and cytokine production. Accordingly, we assessed the effects of ATRA treatment on in vitro NO, IL-17A, and IL-10 production, as well as iNOS expression using peripheral blood mononuclear cells (PBMCs) from Algerian patients with a diagnosis of AD.

Materials and Methods

Drug and Reagents

ATRA, RPMI-1640 medium, heat-inactivated fetal bovine serum, L-glutamine, penicillin, streptomycin, N-1-naphtyl-ethylene diamine (Griess A), and sulfanilamide (Griess B) were all purchased from Sigma-Aldrich (St. Louis, Mo., USA). The stock solution of ATRA was prepared in dimethyl sulfoxide (DMSO, Sigma-Aldrich) under subdued light at a concentration of 10^{-2} M, and then was stored in aliquots at –45°C until use. Anti-iNOS antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA) and goat antirabbit IgG secondary antibody conjugated to Alexa Fluor® 488 was obtained from Life Technologies (Carlsbad, Calif., USA). Human IL-17A and IL-10 enzyme-linked immunosorbent assay (ELISA) measurement kits were obtained from BioSource Europe S.A. (Nivelles, Belgium) and Invitrogen, respectively.

Subject Selection

A total of 30 probable Algerian AD patients (15 men and 15 women; mean age: 73.57 ± 11.00 years) who were followed at the Department of Neurology, Mustapha Pacha Hospital (Algiers, Algeria), were enrolled in this study. An AD diagnosis was made based on the DSM-IV (Diagnostic and Statistical Manual of Mental Disorders) and NINCDS-ADRDA (National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association) criteria [33, 34].

To exclude reversible causes of cognitive impairment, all patients underwent a complete clinical evaluation, including medical history, physical examination, laboratory analyses (complete blood count, thyroid function test, and serum levels of folic acid and vitamin B_{12}), and brain imaging. Cognitive performance evaluations were realized using a neuropsychological test battery including the Mini-Mental State Examination (MMSE) [35] and Clinical Dementia Rating scale (CDR) [36]. All patients with AD took acetylcholinesterase inhibitors and 6 patients took an additional antidepressant medication (selective serotonin reuptake inhibitors). Nonsteroidal anti-inflammatory and immunosuppressive drugs had not been administered for at least 2 weeks prior to sample collection. The control group included 14 age- and gender-matched volunteers without symptoms of cognitive impairment, as assessed using the MMSE. Characteristics of AD patients and controls are shown in table 1.

Table 1. Descriptive characteristics of Algerian AD patients and nondemented controls

Characteristic	AD Patients	Controls
Group size	n = 30	n = 14
Age, years	73.57±11.00	65.93±5.27
Gender, %		
Male	50	42.45
Female	50	57.55
Education, %		
Primary	76	71.42
Higher	24	28.58
MMSE score	14.33±5.74	28.50±1.29
Plasma nitrites levels, μM	24.69±6.57	19.10±4.47

Values are expressed as means ± SD.

Individuals with a history of a neurodegenerative disease other than AD, chronic liver dysfunction, renal failure, or infectious or inflammatory processes were excluded. All participants or their relatives provided written informed consent before admission to the study, which was approved by the ethics committee of the Algerian Thematic Research Agency in Health Sciences (ATRSS).

Blood Sample Collection

Whole blood samples from patients and control individuals were drawn by venipuncture into EDTA blood collection tubes (FL Medical, Torreglia, Italy) following an overnight fast. To avoid possible confounding effects of diurnal variation, samples were all collected at the same time of the day (between 8:30 and 10:30 a.m.). Plasma samples were separated by centrifugation at 2,800 rpm for 10 min, and then were stored at –45°C until analysis.

Separation of PBMCs and Cell Culture

Fresh peripheral blood samples from patients and control individuals were diluted in phosphate-buffered saline (PBS; 1:1, V/V) and PBMCs were separated by Ficoll-Hypaque (1.077 mg/ml) density gradient centrifugation (Sigma-Aldrich) at 2,800 rpm for 15 min. The resulting buffy coats were collected, washed twice with sterile ammonium chloride potassium solution (pH 7.4), and then washed with PBS at 4°C. Cells were resuspended in complete culture media consisting of RPMI-1640 medium supplemented with 5% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cell viability was estimated to be >96% using the Trypan blue dye exclusion test. PBMCs were transferred to U-bottom 96-well cell culture cluster plates at a 1×10^6 cells/ml final concentration and then were either untreated (baseline) or treated with 10^{-8} M ATRA. Working solutions of ATRA were freshly prepared by serial dilution of the stock solution in RPMI-1640. In parallel, a vehicle control (DMSO) dilution was made. ATRA and DMSO concentrations were confirmed to have no cytotoxic effects on cells. Further, PBMCs were incubated under standard conditions (37°C, 5% CO_2, and humidity) for 20 h, and then culture supernatants were collected and stored at –45°C for use in the NO and cytokine measurements.

Determination of Nitric Oxide Production

To assess NO production in plasma and PBMC culture media, levels of nitrites (stable derivatives of NO) were measured using a modified Griess method, as described by Touil-Boukoffa [37]. Briefly, Griess reagent composed of 0.5% N-1-naphtyl-ethylene diamine and 5% sulfanilamide prepared both in 20% HCl was added to samples. The mixture was incubated in a dark at room temperature for 20 min and optical densities were then determined using a spectrophotometer at λ = 543 nm. A standard curve generated using sodium nitrites ($NaNO_2$) was conducted for quantitation.

Fluorescence Immunostaining

After incubation, PBMCs that were cultured in the presence or absence of ATRA at 10^{-8} M were collected, washed twice in PBS, and then fixed on clean glass slides using 4% paraformaldehyde. After rinsing slides three times with 0.5% PBS-Tween, cells were permeabilized with 0.1% Triton-X100 for 30 min, and then were blocked using milk buffer (5% nonfat dry milk in PBS) for 2 h. Slides were drained and then incubated overnight with anti-iNOS primary antibody (sc-651; Santa Cruz Biotechnology) at 10 μg/ml in a humid chamber at 4°C. After rinsing the slides three times, secondary Alexa Fluor 488-conjugated antibody was added in the dark for 1 h. Slides were mounted with 90% glycerol in PBS and then were observed under a fluorescent microscope (Axioskop 2; Zeiss, Oberkochen, Germany). As a negative control, primary antibody was omitted; no staining was detected. Images were taken using a digital camera (Powershot A640; Canon, Tokyo, Japan).

Cytokine Measurements

Culture supernatants of PBMCs that were treated or not with ATRA at 10^{-8} M were collected. Levels of IL-17A and IL-10 were evaluated by ELISA according to the manufacturer's instructions (BioSource Europe S.A. and Invitrogen). Optical densities were measured at 540 nm with correction at 630 nm using a microplate reader (Labsystem, Vienna, Va., USA). Cytokine concentrations (pg/ml) were determined based on standard curves. The assay sensitivity was 2 pg/ml for IL-17A and <1 pg/ml for IL-10.

Statistical Analysis

For each data group, data were expressed as means ± standard error of the mean (SEM). As values were normally distributed, we analyzed data by unpaired or paired Student's t test using GraphPad Prism 6.01 software (GraphPad Inc., La Jolla, Calif., USA). Quantitative analysis of fluorescence was carried out using Image J. A threshold for statistically significant differences was set at $p \leq 0.05$.

Results

In vitro Spontaneous NO Production in PBMCs from AD Patients

Spontaneous NO production by PBMCs obtained from Algerian patients with an AD diagnosis (n = 30) and nondemented controls (n = 14) was assessed. Cells (10^6 cells/ml) were incubated in the absence of any inducer for 20 h, and then nitrite levels (μM) were measured by a

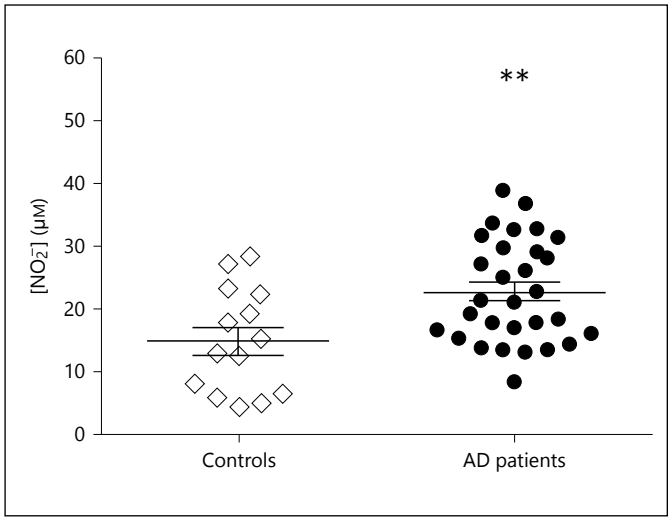

Fig. 1. Spontaneous in vitro NO production in AD patients and nondemented controls. PBMCs (10^6 cells/ml) from AD patients (n = 30) or nondemented controls (n = 14) were cultured under unstimulated conditions for 20 h. Nitrite levels were measured by a modified Griess method in culture supernatants. Data are presented as means ± SEM for independent experiments carried out in duplicate. In vitro spontaneous NO production was significantly higher in AD patients compared with controls (** $p \leq 0.01$; unpaired t test).

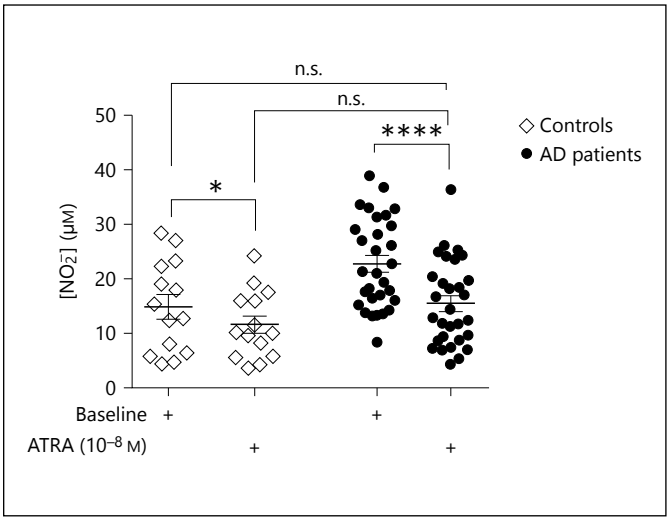

Fig. 2. Effects of ATRA treatment on in vitro NO production in AD patients and controls. PBMCs (10^6 cells/ml) from AD patients (n = 30) or controls (n = 14) who were treated or not (baseline) with ATRA at 10^{-8} M. After 20 h of incubation, culture supernatants were harvested and NO production assessed using a modified Griess method. The results of different experiments carried out in duplicate are presented as means ± SEM. ATRA treatment markedly reduced NO production by PBMCs from AD patients (**** $p \leq 0.0001$; paired t test). * $p \leq 0.05$.

modified Griess method in harvested culture supernatants. Data are represented in figure 1. Cells from patients showed significantly more spontaneous NO production ($[NO_2^-]_{AD\ patients} = 22.83 \pm 1.50$ μM) compared to controls ($[NO_2^-]_{controls} = 14.90 \pm 2.22$ μM; $p \leq 0.01$). Current data support our previous results that showed the involvement of NO in the pathogenesis of AD [14].

Effects of ATRA Treatment on in vitro NO Production in AD

To investigate the effects of ATRA treatment on in vitro NO production in AD, PBMCs were obtained from 30 AD patients and 14 nondemented controls that were treated or not (baseline) with ATRA at 10^{-8} M or DMSO. In a pilot study, this concentration of ATRA showed optimal results compared to other concentrations that we tested (10^{-7} and 10^{-9} M). After incubation for 20 h, culture supernatants were harvested and nitrite levels were measured as described above (fig. 2). We noted a significant reduction in NO production by PBMCs treated with ATRA in comparison to baseline levels. Interestingly, the reduction was extremely pronounced in AD patients ($[NO_2^-]_{ATRA}$ (10^{-8} M) = 15.54 ± 1.41 μM vs. $[NO_2^-]_{baseline} = 22.83 \pm 1.50$ μM; $p \leq 0.0001$) compared to controls ($[NO_2^-]_{ATRA}$ (10^{-8} M) = 11.66 ± 1.64 μM vs. $[NO_2^-]_{baseline} = 14.90 \pm 2.22$ μM; $p \leq 0.05$). No significant effect was observed in the DMSO-treated control cells.

Analysis of the Effects of ATRA on iNOS Expression in AD

Previously, iNOS has been reported to be highly expressed in PBMCs of AD patients compared to nondemented controls [14]. To investigate whether the downmodulatory effects of ATRA on in vitro NO production in AD are related to the regulation of iNOS expression, we used fluorescence immunostaining to analyze expression of this enzyme in patient and control PBMCs treated or not with ATRA or vehicle. As shown in figure 3, iNOS expression in untreated cells was significantly higher in AD patients compared with controls. The fluorescence intensity markedly decreased in AD cells treated with ATRA, indicating the downregulation of iNOS expression. A less pronounced regulatory effect of ATRA on iNOS expression was observed in control cells. Finally, no significant effect was observed for vehicle control-treated cells.

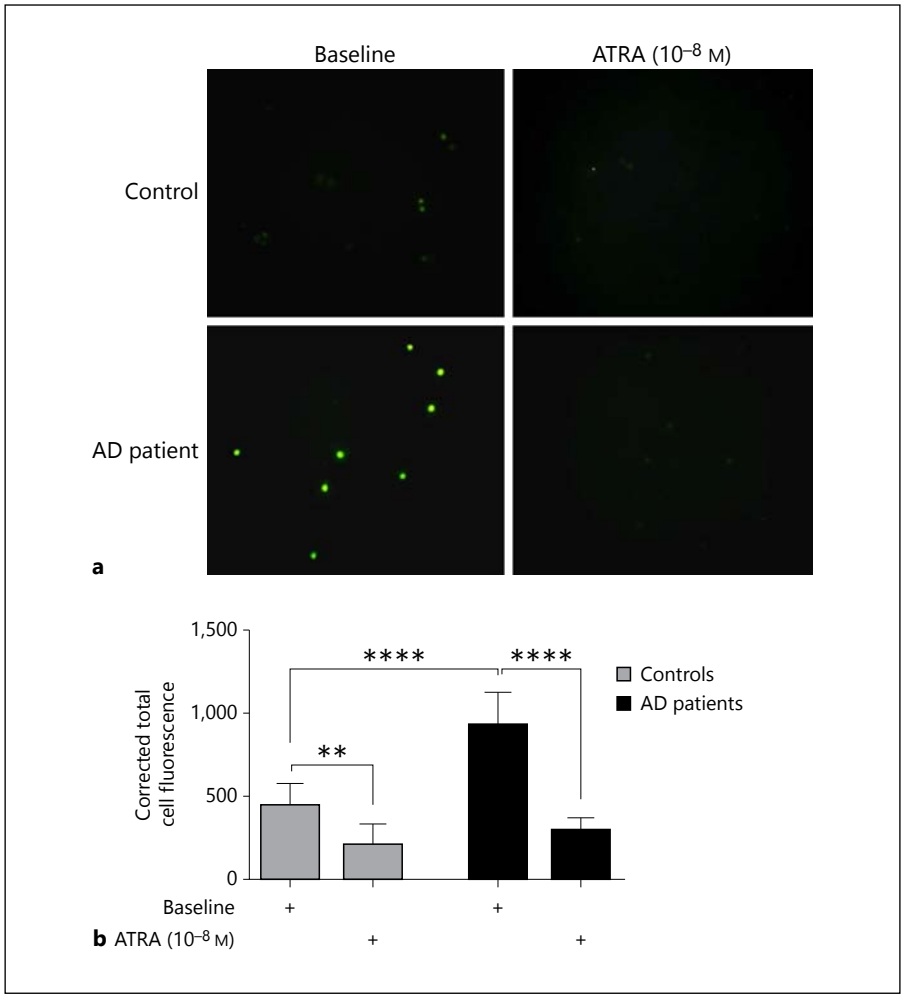

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Fig. 3. ATRA effects on iNOS expression by PBMCs from AD patients and controls. PBMCs isolated from AD patients (n = 8) and controls (n = 8) were cultured in the absence or presence of ATRA at 10^{-8} M under standard conditions. After incubation, cells were harvested and fixed, and then iNOS expression was analyzed by immunofluorescence as previously described. **a** Images represent arbitrarily selected areas (400× magnification) of experiments carried out in duplicate. **b** High expression of iNOS was detected in untreated cells of patients compared with controls (**** $p \leq 0.0001$, unpaired t test). ATRA treatment markedly downregulated iNOS expression in PBMCs from AD patients (**** $p \leq 0.0001$, paired t test) compared with control cells (** $p \leq 0.0001$, paired t test).

Effects of ATRA on in vitro IL-17A Release in AD

To better understand the mechanisms whereby ATRA downmodulates in vitro NO production in AD, we examined the effects of this drug on the in vitro release of IL-17A, a proinflammatory cytokine that can upregulate iNOS expression [23]. Amounts of IL-17A were measured by ELISA in supernatants of PBMC cultures that were treated or not with ATRA at 10^{-8} M or DMSO for 20 h under standard conditions. As shown in figure 4, we found that ATRA treatment extremely decreased in vitro IL-17A release in AD patient PBMCs ($[IL\text{-}17A]_{ATRA}$ (10^{-8} M) = 109.20 ± 4.38 pg/ml vs. $[IL\text{-}17A]_{baseline}$ = 119.40 ± 4.17 pg/ml; $p \leq 0.0001$) compared to controls in which the effect was less pronounced ($[IL\text{-}17A]_{ATRA}$ (10^{-8} M) = 61.36 ± 13.97 pg/ml vs. $[IL\text{-}17A]_{baseline}$ = 82.89 ± 9.62 pg/ml; $p \leq 0.001$). We did not observe a significant effect of the DMSO vehicle on cytokine production. More importantly, our results showed markedly higher baseline levels of IL-17A in AD patients ($[IL\text{-}17A]_{AD\ patients}$ = 119.40 ± 4.17 pg/ml) compared with controls ($[IL\text{-}17A]_{controls}$ = 82.89 ± 9.62 pg/ml; $p \leq 0.01$).

Effects of ATRA on in vitro IL-10 Production in AD

We were further interested to examine whether ATRA could affect the production of a cytokine that downregulates iNOS expression, such as IL-10 [25, 26]. IL-10 production was assessed by ELISA in supernatants of PBMCs from AD patients and controls after administration of ATRA at 10^{-8} M or vehicle (fig. 5). Interestingly, ATRA treatment significantly increased in vitro IL-10 production in AD patients ($[IL\text{-}10]_{ATRA}$ (10^{-8} M) = 10.83 ± 1.67 pg/ml vs. $[IL\text{-}10]_{baseline}$ = 5.47 ± 0.90 pg/ml; $p \leq 0.01$) compared with controls ($[IL\text{-}10]_{ATRA}$ (10^{-8} M) = 7.48 ± 0.70 pg/ml vs. $[IL\text{-}10]_{baseline}$ = 4.08 ± 0.31 pg/ml; $p \leq 0.05$). No significant effect was observed in the vehicle-treated cells. Our findings showed a statistically insignificant difference

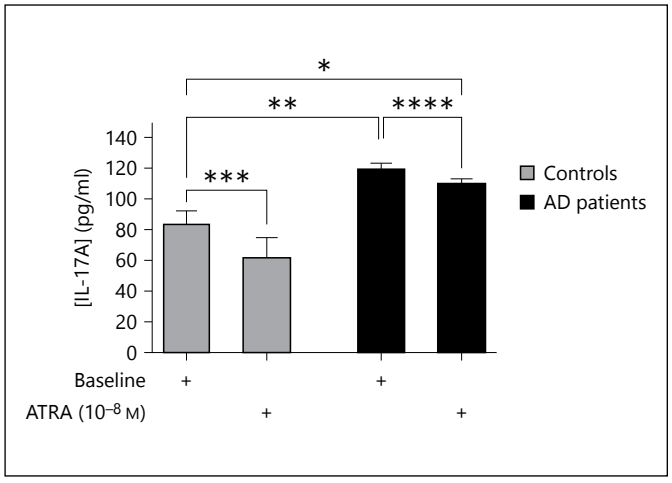

Fig. 4. Production of IL-17A by PBMCs from AD patients and controls following ATRA treatment. IL-17A levels were determined by ELISA in supernatants from ATRA treated and untreated PBMCs obtained from patients with an AD diagnosis (n = 15) and from nondemented controls (n = 10). Results are presented as means ± SEM. ATRA significantly reduced IL-17A release by PBMCs from AD patients (**** $p \leq 0.0001$; paired t-test). Baseline IL-17A amounts were significantly higher in AD patients compared with controls (** $p \leq 0.01$; unpaired t-test). * $p \leq 0.05$; *** $p \leq 0.001$.

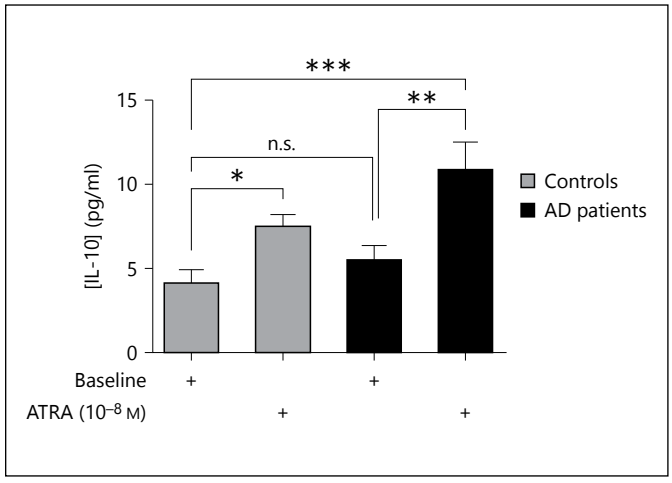

Fig. 5. Effects of ATRA administration on IL-10 release by PBMCs from AD patients and controls. IL-10 levels were measured by ELISA in supernatants from cultures of PBMCs from AD patients (n = 15) or controls (n = 10) in the presence or absence of ATRA at 10^{-8} M. Data are presented as means ± SEM. ATRA treatment significantly increased IL-10 production in AD patients (** $p \leq 0.001$; paired t test). An insignificant difference was observed in baseline IL-10 levels between AD patients and nondemented controls (n.s.; $p = 0.39$). * $p \leq 0.05$; *** $p \leq 0.001$.

in the in vitro baseline levels of IL-10 between AD patients ($[IL\text{-}10]_{AD\ patients} = 5.47 \pm 0.90$ pg/ml) and nondemented controls ($[IL\text{-}10]_{controls} = 4.08 \pm 0.31$ pg/ml; n.s., $p = 0.39$).

Discussion

Inflammation is well known to be an important factor in the pathogenesis of AD [5]. Previously, we reported the involvement of NO and proinflammatory cytokines (IFN-γ and TNF-α) in the progression of AD [14]. In this study, we aimed to evaluate the effects of ATRA (a vitamin A derivative) on NO production in AD and to investigate the mechanisms that might be involved.

Our data showed that increased amounts of NO were produced by unstimulated cells from AD patients compared to nondemented controls. This increased NO production appeared to be mediated by iNOS, which has been shown in our previous study [14] and in this present study to be highly expressed in PBMCs from AD patients. Our results are in accord with those of De Servi et al. [38] who showed increased activity of NOS in leukocytes from AD patients. Our data may reflect an activated state of cells that is related to ongoing pathological conditions in the patients. Indeed, several authors have reported alterations in systemic immune responses in AD along with other abnormalities that affect peripheral leukocytes [39, 40]. Pellicanò et al. [41] reported the overexpression of certain activation markers and chemokine receptors in unstimulated AD cells, which reflects the proinflammatory status of AD. Circulating proinflammatory cytokines could be related to the activation state of AD cells. Indeed, our previous data showed high systemic levels of IFN-γ and TNF-α in Algerian AD patients and demonstrated that these proinflammatory cytokines can upregulate in vitro NO production [14]. Moreover, it has been shown that peripheral blood cells from AD patients or in AD models can migrate from the periphery to the brain by crossing the blood brain barrier and then can colocalize with Aβ peptide, where they are naturally activated [8, 42].

Our data indicate important differences in plasma levels of nitrites in AD patients compared with controls (table 1), which are similar to our previous results supporting the involvement of NO in AD pathogenesis. Despite the cytotoxic effect played by NO when it is produced in excessive amounts, the protective effect of endothelial NOS-mediated NO in AD [43] should not be obscured.

Like many dietary antioxidant compounds, the possible role of vitamin A as a risk factor for AD has been studied. Indeed, reduced peripheral levels of vitamin A have

been detected in AD patients compared to nondemented subjects [44, 45]. Furthermore, higher systemic levels of β-carotene have been associated with better cognitive performance in older persons [46, 47]. In addition to their antioxidant properties, RAs can suppress inflammation and modulate immune responses in numerous inflammatory and autoimmune diseases [48, 49]. In AD, it has been reported that RAs can affect several mechanisms related to the disease [32]. In our present study, ATRA significantly downmodulated in vitro NO production in AD patients. Our data are consistent with the findings reported by Rafa et al. [48] and Djeraba et al. [49] in inflammatory bowel diseases and Behçet disease, respectively. Similar results have been shown by many studies conducted on rat microglia [50, 51]. Consistent with several findings from other groups [49, 52, 53], our results suggest that ATRA can inhibit NO production in AD cells by regulating iNOS expression. Other research groups have reported a relationship between anti-inflammatory effects of retinoids and inhibition of the NF-κB and JAK/STAT signaling pathways [49, 54, 55].

In our present study, ATRA significantly decreased in vitro IL-17A production, while enhancing IL-10 in AD patients. Our data are in accord with those reported by Wang et al. [56], who showed that RA increases IL-10 production in LPS-stimulated monocytes/macrophages. The immunomodulatory effects of ATRA on cytokine production could be the result of direct suppression or enhancement by the cytokine and/or as a consequence of effects on T cell differentiation. Indeed, RA is a key regulator of TGF-β-dependent immune responses, capable of inhibiting IL-6-driven induction of proinflammatory Th17 cells, and able to promote anti-inflammatory Treg cell differentiation [57–59]. Elias et al. [58] reported that RA could inhibit Th17 cell polarization and enhance Foxp3 expression through a Stat3/Stat5-independent signaling pathway. Similarly, the study of Xiao et al. [59] showed that ATRA can enhance TGF-β signaling by increasing the expression and phosphorylation of Smad3, thereby promoting increased Foxp3 expression. Likewise, it inhibits Th17 cell development by suppressing the expression of IL-6Rα, IRF-4, and IL-23R [59]. While several studies have shown that Th17 responses are suppressed by RA, a recent study showed that very low concentrations of RA can promote this response [60]. The mechanisms underlying the concentration-dependent effects of RA on T cell differentiation may depend on differences in the receptors through which RA signals [61].

IL-17 can be secreted by mononuclear and glial cells in the brain in response to some pathogens [62]. The potential involvement of this cytokine in the pathogenesis of AD is currently being explored. Our data indicate that significant in vitro baseline levels of IL-17A are present in AD patients, possibly implicating the Th17 cell subset in inflammatory responses associated with this disease. Our data are in accord with those of Pirker et al. [63] who showed upregulation of Th17 lymphocytes in PBMCs from AD patients. Similarly, Yin et al. [64] reported that IL-17A and Th17 lymphocytes might be involved in immune pathogenesis caused by the Aβ peptide in AD. Accordingly, we previously showed increased serum levels of IL-17A in Algerian AD patients compared with controls [65]. Similar results have been reported in a Chinese cohort of AD patients [66]. Furthermore, higher systemic levels of IL-17A have been noted in severe AD patients [65], suggesting the probable association of IL-17 with the chronicity of the disease.

We reported an insignificant difference between AD patients and controls in the in vitro spontaneous production of IL-10. These data are in favor of an uncontrolled inflammatory response in AD. Our data are similar to those of Lombardi et al. [67]. However, other studies have shown that resting concentrations of this anti-inflammatory cytokine produced by PBMCs from AD patients were elevated [68, 69]. Since unstimulated peripheral immune cells from AD patients appear to be activated, our results could be related to a probable negative modulatory effect on cytokine production. Indeed, studies have shown that IL-10 production by PBMCs from AD patients is reduced after Aβ stimulation [70, 71]. This observation was associated with an IL-10 gene polymorphism that is considered to be a risk factor for AD [71].

Although the cytokine signaling processes are similar in peripheral and local immune cells, further studies using appropriate cellular and animal models of AD are needed to clarify the mechanisms whereby ATRA can modulate immune-mediated disorders within the brain.

Conclusion

Overall, this present study strongly supports the involvement of NO in the pathophysiology of AD. Our findings showed the involvement of IL-17A in inflammatory responses associated with AD. We reported an important anti-inflammatory effect of ATRA in AD by downmodulating NO production. This effect is probably mediated by the regulation of iNOS expression and/or IL-17A and IL-10 production. Dietary or pharmacological administration of ATRA to AD patients, in combination

with current symptomatic treatment, might improve the inflammatory status of these patients and could represent a promising complementary therapeutic approach for the disease.

Disclosure Statement

The authors declare that they have no conflict of interest.

Acknowledgments

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References

1 Smith MA: Alzheimer disease. Int Rev Neurobiol 1998;42:1–54.
2 Friedland RP: Alzheimer's disease: clinical features and differential diagnosis. Neurology 1993;43:S45–S51.
3 Hardy J, Selkoe DJ: The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. Science 2002;297:353–356.
4 Delacourte A, Defossez A: Alzheimer's disease: Tau proteins, the promoting factors of microtubule assembly, are major components of paired helical filaments. J Neurol Sci 1986;76:173–186.
5 McGeer EG, McGeer PL: The importance of inflammatory mechanisms in Alzheimer disease. Exp Gerontol 1998;33:371–378.
6 Akiyama H, Arai T, Kondo H, Tano E, Haga C, Ikeda K: Cell mediators of inflammation in the Alzheimer disease brain. Alzheimer Dis Assoc Disord 2000;14:S47–S53.
7 Chao CC, Hu S, Sheng WS, Bu D, Burkinsky MI, Peterson PK: Cytokine-stimulated astrocytes damage human neurons via a nitric oxide mechanism. Glia 1996;19:276–284.
8 Togo T, Akiyama H, Iseki E, Kondo H, Ikeda K, Kato M, Oda T, Tsuchiya K, Kosaka K: Occurrence of T cells in the brain of Alzheimer's disease and other neurological diseases. J Neuroimmunol 2002;124:83–92.
9 Rezai-Zadeh K, Gate D, Town T: CNS infiltration of peripheral immune cells: D-Day for neurodegenerative disease. J Neuroimmune Pharmacol 2009;4:462–475.
10 Griffith OW, Stuehr DJ: Nitric oxide synthases: properties and catalytic mechanism. Annu Rev Physiol 1995;57:707–736.
11 Dawson TM, Snyder SH: Gases as biological messengers: nitric oxide and carbon monoxide in the brain. J Neurosci 1994;14:5147–5159.
12 Torreilles F, Salman-Tabcheh S, Guérin M, Torreilles J: Neurodegenerative disorders: the role of peroxynitrite. Brain Res Brain Res Rev 1999;30:153–163.
13 Togo T, Katsuse O, Iseki E: Nitric oxide pathways in Alzheimer's disease and other neurodegenerative dementias. Neurol Res 2004;26:563–566.
14 Belkhelfa M, Rafa H, Medjeber O, Arroul-Lammali A, Behairi N, Abada-Bendib M, Makrelouf M, Belarbi S, Masmoudi AN, Tazir M, Touil-Boukoffa C: IFN-γ and TNF-α are involved during Alzheimer disease progression and correlate with nitric oxide production: a study in Algerian patients. J Interferon Cytokine Res 2014;34:839–847.
15 Boje KM, Arora PK: Microglial-produced nitric oxide and reactive nitrogen oxides mediate neuronal cell death. Brain Res 1992;587:250–256.
16 Wallace MN, Geddes JG, Farquhar DA, Masson MR: Nitric oxide synthase in reactive astrocytes adjacent to beta-amyloid plaques. Exp Neurol 1997;144:266–272.
17 MacMicking J, Xie QW, Nathan C: Nitric oxide and macrophage function. Annu Rev Immunol 1997;15:323–350.
18 Possel H, Noack H, Putzke J, Wolf G, Sies H: Selective upregulation of inducible nitric oxide synthase (iNOS) by lipopolysaccharide (LPS) and cytokines in microglia: in vitro and in vivo studies. Glia 2000;32:51–59.
19 Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM, Weaver CT: Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005;6:1123–1132.
20 Schwarzenberger P, La Russa V, Miller A, Ye P, Huang W, Zieske A, Nelson S, Bagby GJ, Stoltz D, Mynatt RL, Spriggs M, Kolls JK: IL-17 stimulates granulopoiesis in mice: use of an alternate, novel gene therapy-derived method for in vivo evaluation of cytokines. J Immunol 1998;161:6383–6389.
21 Mezioug D, Touil-Boukoffa C: Interleukin-17A correlates with interleukin-6 production in human cystic echinococcosis: a possible involvement of IL-17A in immunoprotection against *Echinococcus granulosus* infection. Eur Cytokine Netw 2012;23:112–119.
22 Bettelli E, Oukka M, Kuchroo VK: T(H)-17 cells in the circle of immunity and autoimmunity. Nat Immunol 2007;8:345–350.
23 Trajkovic V, Stosic-Grujicic S, Samardzic T, Markovic M, Milijkovic D, Ramic Z, Mostarica Stojkovic M: Interleukin-17 stimulates inducible nitric oxide synthase activation in rodent astrocytes. J Neuroimmunol 2001;119:183–191.
24 Akdis CA, Blaser K: Mechanisms of interleukin-10-mediated immune suppression. Immunology 2001;103:131–136.
25 Arimoto T, Choi DY, Lu X, Liu M, Nguyen XV, Zheng N, Stewart CA, Kim HC, Bing G: Interleukin-10 protects against inflammation-mediated degeneration of dopaminergic neurons in substantia nigra. Neurobiol Aging 2007;28:894–906.
26 Belguendouz H, Messaoudène D, Lahmar K, Ahmedi L, Medjeber O, Hartani D, Lahlou-Boukoffa O, Touil-Boukoffa C: Interferon-γ and nitric oxide production during Behçet uveitis: immunomodulatory effect of interleukin-10. J Interferon Cytokine Res 2011;31:643–651.
27 Lerner AJ, Gustaw-Rothenberg K, Smyth S, Casadesus G: Retinoids for treatment of Alzheimer's disease. Biofactors 2012;38:84–89.
28 De Luca LM: Retinoids and their receptors in differentiation, embryogenesis, and neoplasia. FASEB J 1991;5:2924–2933.
29 Maden M: Retinoic acid in the development, regeneration and maintenance of the nervous system. Nat Rev Neurosci 2007;8:755–765.
30 Budhu AS, Noy N: Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. Mol Cell Biol 2002;22:2632–2641.
31 Manicassamy S, Pulendran B: Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol 2009;21:22–27.
32 Shudo K, Fukasawa H, Nakagomi M, Yamagata N: Towards retinoid therapy for Alzheimer's disease. Curr Alzheimer Res 2009;6:302–311.
33 American Psychiatric Association: Diagnostic and Statistical Manual of Mental Disorders, ed 4. Washington, American Psychiatric Association, 1994, p 866.
34 McKhan G, Drachman D, Folstein M, Katzman R, Price D, Stadlan EM: Clinical diagnosis of Alzheimer's disease: report of the NINCDS-ADRDA Work Group under the auspices of the Department of Health and Human Services Task Force on Alzheimer's disease. Neurology 1984;34:939–944.

35 Folstein MF, Folstein SE, McHugh PR: 'Mini-mental state'. A practical method for grading the cognitive state of patients for the clinician. J. Psychiatr Res 1975;12:189–198.
36 Hughes CP, Berg L, Danziger WL, Coben LA, Martin RL: A new clinical scale for the staging of dementia. Br J Psychiatry 1982;140:566–572.
37 Touil-Boukoffa C, Bauvois B, Sancéau J, Hamrioui B, Wietzerbin J: Production of nitric oxide (NO) in human hydatidosis: relationship between nitrite production and interferon-gamma levels. Biochimie 1998;80:739–744.
38 De Servi B, La Porta CA, Bontempelli M, Comolli R: Decrease of TGF-beta1 plasma levels and increase of nitric oxide synthase activity in leukocytes as potential biomarkers of Alzheimer's disease. Exp Gerontol 2002;37:813–821.
39 Britschgi M, Wyss-Coray T: Systemic and acquired immune responses in Alzheimer's disease. Int Rev Neurobiol 2007;82:205–233.
40 Reale M, Iarlori C, Feliciani C, Gambi D: Peripheral chemokine receptors, their ligands, cytokines and Alzheimer's disease. J Alzheimers Dis 2008;14:147–159.
41 Pellicanò M, Bulati M, Buffa S, Barbagallo M, Di Prima A, Misiano G, Picone P, Di Carlo M, Nuzzo D, Candore G, Vasto S, Lio D, Caruso C, Colonna-Romano G: Systemic immune responses in Alzheimer's disease: in vitro mononuclear cell activation and cytokine production. J Alzheimers Dis 2010;21:181–192.
42 Shalit F, Sredni B, Brodie C, Kott E: T lymphocyte subpopulations and activation markers correlate with severity of Alzheimer's disease. Clin Immunol Immunopathol 1995;75:246–250.
43 Austin SA, Santhanam AV, Hinton DJ, Choi DS, Katusic ZS: Endothelial nitric oxide deficiency promotes Alzheimer's disease pathology. J Neurochem 2013;127:691–700.
44 Zaman Z, Roche S, Fielden P, Frost PG, Niriella DC, Cayley AC: Plasma concentrations of vitamins A and E and carotenoids in Alzheimer's disease. Age Ageing 1992;21:91–94.
45 Jiménez-Jiménez FJ, Molina JA, de Bustos F, Ortí-Pareja M, Benito-León J, Tallón Barranco A, Gasalla T, Porta J, Arenas J: Serum levels of β-carotene, α-carotene and vitamin A in patients with Alzheimer's disease. Eur J Neurol 1999;6:495–497.
46 Jama JW, Launer LJ, Witteman JC, den Breeijen JH, Breteler MM, Grobbee DE, Hofman A: Dietary antioxidants and cognitive function in a population-based sample of older persons. The Rotterdam Study. Am J Epidemiol 1996;144:275–280.
47 Perrig WJ, Perrig P, Stähelin HB: The relation between antioxidants and memory performance in the old and very old. J Am Geriatr Soc 1997;45:718–724.
48 Rafa H, Saoula H, Belkhelfa M, Medjeber O, Soufli I, Toumi R, de Launoit Y, Moralès O, Nakmouche M, Delhem N, Touil-Boukoffa C: IL-23/IL-17A axis correlates with the nitric oxide pathway in inflammatory bowel disease: immunomodulatory effect of retinoic acid. J Interferon Cytokine Res 2013;33:355–368.
49 Djeraba Z, Boumedine K, Arroul-Lammali A, Otmani F, Belguendouz H, TouilBoukoffa C: Ex vivo immunomodulatory effect of all-trans-retinoic acid during Behçet's disease: a study in Algerian patients. Immunopharmacol Immunotoxicol 2014;36:78–86.
50 Dheen ST, Jun Y, Yan YZ, Tay SS, Ling EA: Retinoic acid inhibits expression of TNF-alpha and iNOS in activated rat microglia. Glia 2005;50:21–31.
51 Kaur C, Sivakumar V, Dheen ST, Ling EA: Insulin-like growth factor I and II expression and modulation in amoeboid microglial cells by lipopolysaccharide and retinoic acid. Neuroscience 2006;138:1233–1244.
52 Sirsjö A, Gidlöf AC, Olsson A, Törmä H, Ares M, Kleinert H, Förstermann U, Hansson GK: Retinoic acid inhibits nitric oxide synthase-2 expression through the retinoic acid receptor-alpha. Biochem Biophys Res Commun 2000;270:846–851.
53 Oh GS, Pae HO, Seo WG, Shin MK, Kim IK, Chai KY, Yun YG, Kwon YO, Lim JS, Chung HT: Inhibitory effect of retinoic acid on expression of inducible nitric oxide synthase gene in L929 cells. Immunopharmacol Immunotoxicol 2001;23:335–342.
54 Carratù MR, Marasco C, Signorile A, Scuderi C, Steardo L: Are retinoids a promise for Alzheimer's disease management. Curr Med Chem 2012;19:6119–6125.
55 Choi WH, Ji KA, Jeon SB, Yang MS, Kim H, Min KJ, Shong M, Jou I, Joe EH: Antiinflammatory roles of retinoic acid in rat brain astrocytes: suppression of interferon-gamma-induced JAK/STAT phosphorylation. Biochem Biophys Res Commun 2005;329:125–131.
56 Wang X, Allen C, Ballow M: Retinoic acid enhances the production of IL-10 while reducing the synthesis of IL-12 and TNF-alpha from LPS-stimulated monocytes/macrophages. J Clin Immunol 2007;27:193–200.
57 Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H: Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 2007;317:256–260.
58 Elias KM, Laurence A, Davidson TS, Stephens G, Kanno Y, Shevach EM, O'Shea JJ: Retinoic acid inhibits Th17 polarization and enhances FoxP3 expression through a Stat3/Stat-5 independent signaling pathway. Blood 2008;111:1013–1020.
59 Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK: Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. J Immunol 2008;181:2277–2284.
60 Uematsu S, Fujimoto K, Jang MH, Yang BG, Jung YJ, Nishiyama M, Sato S, Tsujimura T, Yamamoto M, Yokota Y, Kiyono H, Miyasaka M, Ishii KJ, Akira S: Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. Nat Immunol 2008;9:769–776.
61 Manicassamy S, Pulendran B: Retinoic acid-dependent regulation of immune responses by dendritic cells and macrophages. Semin Immunol 2009;21:22–27.
62 Li GZ, Zhong D, Yang LM, Sun B, Zhong ZH, Yin YH, Cheng J, Yan BB, Li HL: Expression of interleukin-17 in ischemic brain tissue. Scand J Immunol 2005;62:481–486.
63 Pirker A, Assem-Hilger E, Schmied M, Dal-Bianco P: Upregulation of T-helper subtype 17 lymphocytes in cultured peripheral blood mononuclear cells of patients with Alzheimer's disease. Alzheimers Dement 2010;6:S262.
64 Yin Y, Wen S, Li G, Wang D: Hypoxia enhances stimulating effect of amyloid beta peptide (25–35) for interleukin 17 and T helper lymphocyte subtype 17 upregulation in cultured peripheral blood mononuclear cells. Microbiol Immunol 2009;53:281–286.
65 Belkhelfa M, Rafa H, Medjeber O, Behairi N, Abada-Bendib M, Makrelouf M, Belarbi S, Masmoudi AN, Tazir M, Touil-Boukoffa C: IL-17 and nitric oxide pathways are involved in inflammatory response during Alzheimer's disease: a study in Algerian patients. Cytokine 2012;59:541.
66 Chen JM, Jiang GX, Li QW, Zhou ZM, Cheng Q: Increased serum levels of interleukin-18, -23 and -17 in Chinese patients with Alzheimer's disease. Dement Geriatr Cogn Disord 2014;38:321–329.
67 Lombardi VR, Garcia M, Rey L, Cacabelos R: Characterization of cytokine production, screening of lymphocyte subset patterns and in vitro apoptosis in healthy and Alzheimer's disease (AD) individuals. J. Neuroimmunol 1999;97:163–171.
68 Heneka MT, O'Banion MK: Inflammatory processes in Alzheimer's disease. J Neuroimmunol 2007;184:69–91.
69 Rocha NP, Teixeira AL, Coelho FM, Caramelli P, Guimarães HC, Barbosa IG, da Silva TA, Mukhamedyarov MA, Zefirov AL, Rizvanov AA, Kiyasov AP, Vieira LB, Janka Z, Palotás A, Reis HJ: Peripheral blood mono-nuclear cells derived from Alzheimer's disease patients show elevated baseline levels of secreted cytokines but resist stimulation with β-amyloid peptide. Mol Cell Neurosci 2012;49:77–84.
70 Speciale L, Calabrese E, Saresella M, Tinelli C, Mariani C, Sanvito L, Longhi R, Ferrante P: Lymphocyte subset patterns and cytokine production in Alzheimer's disease patients. Neurobiol Aging 2007;28:1163–1169.
71 Arosio B, Trabattoni D, Galimberti L, Bucciarelli P, Fasano F, Calabresi C, Cazzullo CL, Vergani C, Annoni G, Clerici M: Interleukin-10 and interleukin-6 gene polymorphisms as risk factors for Alzheimer's disease. Neurobiol Aging 2004;25:1009–1015.