Original Research

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Peripheral Expression of IL-6, TNF-α and TGF-β1 in Alzheimer's Disease Patients

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Abstract

Objective: Neuroinflammation is involved in the pathology of Alzheimer's disease (AD). Peripheral levels of various inflammatory cytokines are altered during AD pathogenesis. This study aimed to examine the role of peripheral inflammation in AD pathogenesis by measuring serum levels and gene expression of specific inflammatory cytokines in AD patients. Therefore, we analyzed serum interleukin-6 (IL-6) and transforming growth factor-beta 1 (TGF-β1) levels and mRNA expressions of IL-6 and tumor necrosis factor-alpha (TNF-α) in peripheral blood mononuclear cells of AD patients and controls.

Materials and Methods: We quantitatively assessed serum IL-6 and TGF-β1 levels in 25 AD patients and 33 age-matched controls using enzyme-linked immunosorbent assay. In addition, we evaluated the mRNA expressions of IL-6 and TNF-α in peripheral blood mononuclear cells from 31 AD patients and their respective controls (n=30) via quantitative real-time polymerase chain reaction.

Results: AD patients tended to have higher serum IL-6 and TGF-β1 levels compared to the controls, but the difference was not statistically significant. IL-6 and TNF-α mRNA expression was significantly downregulated in AD patients compared to the controls (p=0.000 and p=0.004; respectively). Serum IL-6 and TGF-β1 levels were negatively correlated with age in AD patients (p=0.025, r=-0.467 and p=0.035, r=-0.431; respectively).

Conclusion: The outcomes of this study suggest a disruption in the peripheral immune response in individuals with AD. The observed decreased expression of cytokine genes in peripheral leukocytes may indicate a contributory mechanism through which peripheral cytokines influence AD pathogenesis.

Keywords: Alzheimer's disease, cytokine, inflammation, peripheral blood cells, serum

Introduction

Alzheimer's disease (AD) is a clinically heterogeneous progressive neurodegenerative disease that results in impaired cognitive functions and decreased activities of daily living (1). Many factors, both environmental and genetic, play a role in the development of AD and influence the disease's onset and progression (2). Inflammation is one of the factors that play a major role in the pathogenesis of AD. The complex process of the inflammatory response in AD includes the involvement of peripheral immune cells, activation of multiple cellular signaling pathways, and release of inflammatory mediators (3). All these factors, individually or together, can lead to neuronal death in AD (3). Neuropathological, epidemiological, and genetic evidence indicate that neuroinflammation begins early in AD and proceeds continuously (4-6). Neuroinflammation seems to have multiple roles, being a neuroprotective component during an acute response while turning into a chronic inflammatory response in the presence of prolonged stimuli (7). Signaling molecules known as cytokines play crucial roles in the inflammatory response. While some cytokines, referred to as pro-inflammatory

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cytokines, stimulate inflammation, other cytokines, known as anti-inflammatory cytokines, inhibit the activity of pro-inflammatory cytokines (8).

Studies have demonstrated that the levels of both pro-inflammatory and anti-inflammatory cytokines are increased in the brain and cerebrospinal fluid (CSF) of AD patients (9). In recent years, increasing evidence has indicated that peripheral and central inflammation may work together in the etiology and development of AD (10,11). According to several studies, circulating immune cells and cytokines can cross the blood-brain barrier (12-15) and enter the brain, and affect neuroinflammation by modulating microglial activation (16,17). Furthermore, a number of studies have documented alterations in the profile of peripheral blood immune cells (18). In addition, cytokine secretion from stimulated blood cells was negatively related to disease severity (19). Many studies have reported altered peripheral cytokine levels in AD, but the findings are not entirely consistent. In most of the studies, peripheral levels of pro-inflammatory cytokines, including interleukin-6 (IL-6), interleukin-1 beta (IL-1β), and tumor necrosis factor alpha (TNF-α), were reported to be increased (20-23). Still, some studies found normal (24-30) or decreased levels (31). Additionally, transforming growth factor-beta 1 (TGF-β1) and other anti-inflammatory cytokines were found to be higher in the plasma and serum of AD patients than that of controls (32,33).

The main purpose of our study was to investigate the levels of serum IL-6 and TGF-β1 in patients with AD compared to age-matched controls without dementia, while also exploring the mRNA levels of IL-6 and TNF-α in peripheral blood leukocytes to evaluate their potential role in AD pathogenesis and assess their association with the disease. The study may also contribute to a better understanding of the inflammatory mechanisms underlying AD and provide insights into potential therapeutic targets.

Materials and Methods

Patients and Controls

The patients were subjected to extensive clinical and cognitive testing. The diagnosis of "probable AD" and the exclusion criteria have previously been described in detail (34). The participants' overall cognitive state was assessed using the mini-mental state examination (MMSE). The university's Ethics Committee approved the study protocol. Informed consent was obtained from individual participants or legal guardians for subjects unable to consent.

Serum Cytokine Levels

Participants' serum was collected by centrifuging peripheral blood samples at 1400 g for 10 minutes, after which serum samples were aliquots, promptly frozen, and kept at -80°C until used again. The enzyme linked immunosorbent assay (ELISA) was used to determine the concentrations of TGF-β1 and IL-6 in the serum.

The Human TGF-β1 Quantikine ELISA Kit (DB100B R&D Systems, Minneapolis, MN, USA; Standard range: 31.3-1000 pg/mL; Sensitivity: 1.7-15.4 pg/mL, Sample dilution factor: 40) and the Human IL-6 Quantikine HS ELISA Kit (HS600b R&D Systems, Minneapolis, MN, USA; standard range: 0.156-5 pg/mL; sensitivity: 0.016-0.110 pg/mL, sample dilution factor: 1) were used to assess serum IL-6 and TGF-β1 levels. All standards and samples were assayed in duplicate.

mRNA Expression Analysis

The Trizol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) was used to isolate total RNA from peripheral blood leukocytes. First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA). The mRNA levels of IL-6 and TNF-α were determined by quantitative real-time polymerase chain reaction (qRT-PCR) using iTaq Universal SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's instructions on a LightCycler 480 Instrument (Roche Diagnostics, Germany). The qPCR reactions were performed in triplicate. The mRNA expressions were normalized to the expression of a reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and analyzed using the $2^{-\Delta\Delta Ct}$ method. The RT-qPCR primers for the IL-6, TNF-α and GAPDH are given in Table 1.

Statistical Analysis

SPSS version 24 (IBM Corp., USA) was used for all statistical analyses. To compare categorical and continuous variables sequentially, Fisher's exact test and Student's t-test were used. Pearson's chi-square test was used to compare the allelic distribution of APOE ε4 across groups. The Mann-Whitney U test was used to evaluate cytokine expression levels between the groups. The Spearman correlation test was used to examine the correlations. The significance level was set at $p<0.05$.

Results

Patient Demographics

A total of 66 subjects were enrolled in the study including 32 patients with AD (24 female/8 male, mean age of 58.2 ± 7.3 years) and 34 healthy controls (20 female/14 male, mean age of 55.3 ± 17.3 years). Table 2 shows that APOE ε4 carriers were significantly more common ($p=0.014$) in the AD group. Furthermore, as predicted, the MMSE score was considerably lower in the AD group ($p<0.001$).

Serum Cytokine Levels

Serum cytokine levels were available for 25 AD patients and 33 controls. As shown in Figure 1A, AD patients (median 3.08 pg/mL; range 1.51-6.39 pg/mL; n=23) tended to have higher serum IL-6 levels than the controls (median 2.62 pg/mL; range 0.79-7.75 pg/mL; n=30), although the difference was not statistically significant (p=0.129). Likewise, there was a statistically insignificant increase (p=0.674) in serum TGF-β1 levels (median 45891.9 pg/mL; range 22119.63-75892.97 pg/mL; n=24) compared to that of control group (median 41815.09 pg/mL; range 18520.45-63383.94 pg/mL; n=33) (Figure 1B). Serum IL-6 and TGF-β1 levels did not significantly differ in the two gender groups. Furthermore, IL-6 and TGF-β1 levels remained unchanged in the acetylcholinesterase inhibitor-treated and acetylcholinesterase inhibitor + NMDA receptor antagonist-treated subgroups.

mRNA Expression Levels

The mRNA expression levels of IL-6 and TNF-α were analyzed in 31 AD patients and 30 controls. Leukocyte mRNA expression levels of IL-6 were significantly decreased in AD patients (0.553 ± 0.503; n=30) compared to that of control group (1.423 ± 1.105; n=30) (p<0.001; Figure 2A). Similarly, AD patients (0.686 ± 0.681; n=31) had significantly lower TNF-α mRNA levels than that of control group (1.503 ± 1.354; n=29) (p=0.004; Figure 2B). Female patients had significantly lower IL-6 (0.386 ± 0.297; n=22) (p<0.001) and TNF-α mRNA levels (0.419 ± 0.709; n=23) (p≤0.001) compared to that of control group (1.441 ± 1.131; n=17 for IL-6 and 1.895 ± 1.521; n=18 for TNF-α).

The effects of different medication treatments on IL-6 and TNF-α mRNA expression are shown in Table 3. As shown in the table, IL-6 and TNF-α levels have been found to significantly decreased in the acetylcholinesterase inhibitor-treated subgroup compared to the controls (p=0.018 and 0.021; respectively). In the acetylcholinesterase inhibitor + NMDA receptor antagonist-treated subgroup, IL-6 and TNF-α levels were significantly reduced compared to the controls (p<0.001 and 0.006; respectively).

Table 1. IL-6, TNF-α and GAPDH qRT-PCR primers

Gene	Primer sequence (5'-3')
IL-6	AACATGTGTGAAAGCAGCA
	CAGCTCTGGCTTGTTCCT
TNF-α	GAGCACTGAAAGCATGATCC
	CAGGAAGGAGAAGAGGCTGA
GAPDH	ATCTTCCAGGAGCGAGATC
	CAGGAGGCATTGCTGATGA

IL: Interleukin, TNF: Tumor necrosis factor, qRT-PCR: Quantitative real-time polymerase chain reaction, GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

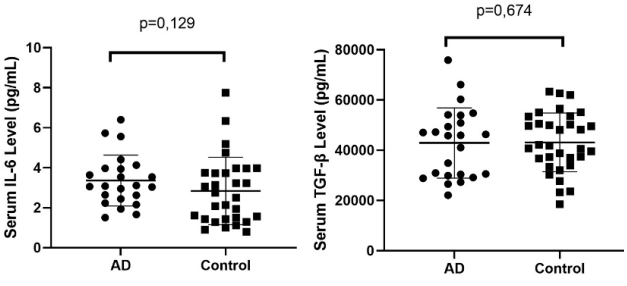

Figure 1A-B. Serum cytokine levels in AD patients and controls. The mean is indicated together with the standard deviation for IL-6 (a) and TGF-β1 (b).
AD: Alzheimer's disease group. IL: Interleukin, TGF: Transforming growth factor

Correlation Analysis

The Spearman correlation analysis was used to investigate the association between cytokine expression levels and MMSE, age, and age of onset. Increased serum IL-6 levels were found to be correlated with decreased age in AD patients (r=-0.467, p=0.025; Figure 3A). Both the age and age of AD onset were inversely correlated with serum TGF-β1 levels (r=-0.431, p=0.035 and r=-0.493, p=0.014; respectively; Figure 3B and C). Furthermore, the age of controls was inversely correlated with serum TGF-β1 levels (r=-0.515, p=0.002; Figure 3D). However, no significant correlations were found between MMSE scores, age, age of onset, and cytokine mRNA levels in either group.

Discussion

The present study evaluated the importance and possible contributions of inflammatory cytokines TGF-β1, TNF-α, and IL-6 in AD. Our results indicated that AD patients tended to have higher serum IL-6 and TGF-β1 levels than the controls, but this difference was insignificant. Conversely, the mRNA expression of TNF-α and IL-6 was decreased in the blood leukocytes of AD patients.

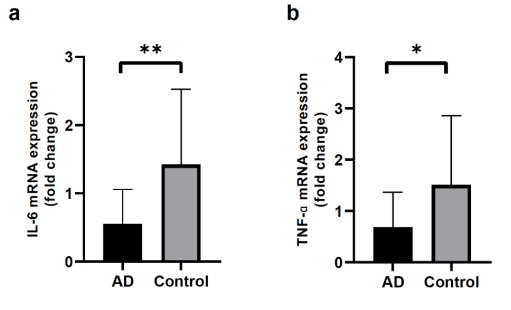

Figure 2A-B. Leukocyte mRNA levels of cytokines in AD patients and controls. The mean is indicated together with the standard deviation for IL-6 (a) and TNF-α (b).
*AD: Alzheimer's disease group. * p<0.05; ** p<0.001, IL: Interleukin, TNF: Tumor necrosis factor*

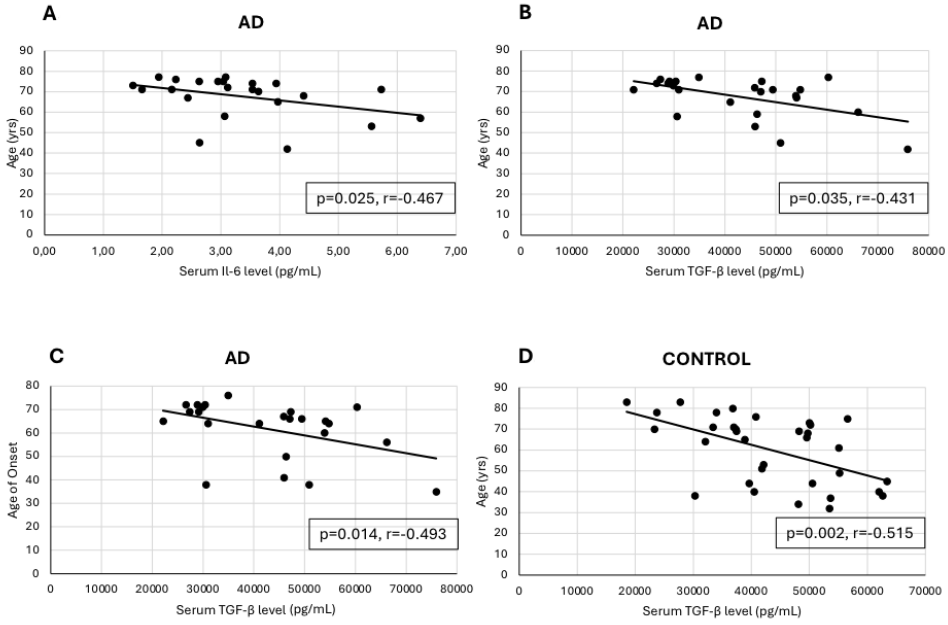

Figure 3A-D. Correlations of serum IL-6 and TGF-β1 levels with age and age at onset.
IL: Interleukin, TGF: Transforming growth factor, AD: Alzheimer's disease

Table 2. Descriptive characteristics of patients and control subjects

	Patients (n=32)	Controls (n=34)	p-value
Gender (male/female)	8/24	14/20	0.198
Age, (years)	58.2 ± 7.3 (42-78)	55.3 ± 17.3 (27-83)	0.377
Age of onset, (years)	51.8 ± 6.8 (35-64)	-	
MMSE score	16.2 ± 7.3 (n=22)	29.0 ± 1.3 (n=28)	**<0.001**
Family history, n (%)	24 (75)		
Education, years	8.63 ± 4.31	17.42 ± 8.06	**<0.001**
APOE ε4 carrier, n (%)	14 (43.8)	5 (14.7)	**0.014**
Medical Comorbidities, n (%)			
Hypertension	4 (18.2)	0 (0)	0.274
Diabetes	4 (15.4)	1 (7.1)	0.640
Smoking	8 (32)	2 (13.3)	0.269
Medications, n (%)			
Acetylcholinesterase inhibitors	18 (72)		
NMDA receptor antagonist	10 (40)		
Antipsychotic	6 (24)		
SSRI	13 (52)		

A t-test was used to compare means and Fisher's exact test for percentages. AD: Alzheimer's disease, MMSE: Mini-mental state examination, n: number of individuals.

Table 3. IL-6 and TNF-α mRNA expression levels in the different medication treatment-groups of AD patients

	Control (n=30)	AChE inhibitor (n=7)	p-value	AChE inhibitor +NMDAR antagonist (n=10)	p-value
IL-6	1.423 ± 1.105	0.484 ± 0.458	**0.018**	0.348 ± 0.212	**0.000**
TNF-α	1.503 ± 1.354	0.443 ± 0.293	**0.02**	0.484 ± 0.357	**0.006**

The values are represented as mean ± standard deviation. p-value as compared to controls, IL: Interleukin, TNF: Tumor necrosis factor, AD: Alzheimer's disease

IL-6 is a cytokine that is critical for the development and survival of neurons and is produced primarily by active microglia and astrocytes in the brain (35,36). IL-6 increases the production of amyloid beta precursor protein (APP) and induces tau phosphorylation and neurofibrillary tangles (37). The reports on peripheral IL-6 levels in AD are conflicting; some groups found decreased levels (31), whereas others did not see any changes (24-30). However, in most investigations, IL-6 levels in plasma or serum are elevated, therefore it is regarded to have a key role in late-onset AD neuroinflammation (21,22,38-46). Although AD patients' serum IL-6 levels were higher than those of the controls in our study, the difference in levels was not statistically significant. Moreover, our findings showed an inverse relationship between age and serum IL-6 levels in AD patients. This result agrees with research by Dursun et al. (21), who reported that age and serum IL-6 levels in Turkish patients with late-onset AD were negatively correlated. The exact source and mechanisms leading to the IL-6 increase in peripheral fluids remain unclear. Because the central administration of amyloid beta 1-42 in mice induced an increase in IL-6 levels in the plasma and brain (47), it is possible that the elevated IL-6 levels in the periphery may result from amyloid beta deposition in the brain. IL-6 overexpression may be indicative of its neuroprotective effect in the beginning phases of the disease (48).

TGF-β is a cytokine with anti-apoptotic functions that promote neuronal survival. TGF-β1 plays a role in many AD-related processes, including neuroinflammatory response, microglial activation, amyloid deposition and regional distribution, inhibition of cell death, and control of established or possible risk factors for AD (49). TGF-β1 up-regulates the transcription of the *APP* gene and contributes to amyloid beta deposition in human astrocytes (50). Plasma, serum, and CSF levels of AD patients were higher than those of controls, indicating its possible role in AD pathogenesis (32,33). Although our findings did not show any significant differences in serum TGF-β1 levels, AD patients have higher levels of TGF-β1, which is consistent with other studies that show higher levels of TGF-β1 in AD patients (33,51,52). Additionally, TGF-β1 levels were also inversely related to age in AD patients. Nonetheless, our healthy subjects exhibited a similar correlation between TGF-β1 and age. To elaborate, TGF-β1 functions as an inhibitor of pro-inflammatory cytokines such as IL-6. Studies suggest that there is crosstalk between the TGF-β1 and IL-6 signaling pathways, and IL-6 production is negatively regulated by TGF-β1 (53,54). Therefore, it could be suggested that in AD, increased levels of IL-6 might induce TGF-β1 production to neutralize the pro-inflammatory actions of IL-6.

Studies of cytokine levels in plasma and serum are challenging because of their short half-lives, necessitating measurement in peripheral blood mononuclear cells or affected tissue. While quantifying cytokines at the protein level is more predictive of their effects, several factors may mask their detection by ELISA. Thus, cytokine mRNA quantification is widely used to investigate cytokine profiles. To this end, in our study we examined *IL-6* and *TNF-α* gene expression in leukocytes of AD patients and found that mRNA expression of these cytokines was downregulated in AD patients. TNF-α expression is low in the healthy brain (55), but high levels of TNF-α are secreted from activated microglia in AD patients in response to the Aβ40 and Aβ42 peptides (56). Numerous investigations have indicated that TNF-α and IL-6 act mostly together and affect each other (57-59). IL-6 expression was demonstrated to be induced by TNF-α in cultured cortical neurons (59) and astrocytes (60). Decreased IL-6 and TNF-α mRNA expression found in our study could suggest that these cytokines may together contribute to the inflammatory response in AD. However, it should be considered that cytokine mRNA expression does not directly reflect corresponding cytokine levels but may provide insight into the upstream regulators of gene expression (61).

Study Limitations

As limitations, it is crucial to first recognize that its cross-sectional nature limits the determination of changes in blood cytokine levels with disease progression. Causality will not be confirmed until further long-term prospective studies are undertaken. Second, our study had a relatively small number of participants. Third, there is a lack of information regarding important confounding factors such as, participants' dietary intake, physical activity, body weight, smoking status, excessive alcohol consumption, medication use, and underlying medical condition. Finally, cytokine polymorphisms that could affect gene expression were not investigated.

Conclusion

Overall, the results of our study and those of previous studies support peripheral inflammatory dysregulation in AD. This peripheral immune response in AD may be independent or secondary to the inflammatory reaction in the brain. Numerous investigations have highlighted that bidirectional communication occurs between the brain and periphery via several mechanisms, including the diffusion of cytokines or infiltration of peripheral immune cells through the impaired blood-brain barrier. Therefore, although the actual mechanism is not clearly understood, it is clear that there is crosstalk between the central and peripheral immune systems. Additional research ought to explore at the immune system's role in the etiology of AD.

Ethics

Ethics Committee Approval: The İstanbul University, İstanbul Faculty of Medicine Clinical Research Ethics Committee approved the study (approval number: 2014/1185, date: 08.06.2021).

Informed Consent: Informed consent was obtained from individual participants or legal guardians for subjects unable to consent.

Authorship Contributions

Surgical and Medical Practices: P.K., E.L., B.S., E.Ş., B.B., H.A.H., H.G., N.E.Ü., Concept: G.G., B.S., E.Ş., B.B., H.A.H., H.G., N.E.Ü., Design: G.G., N.E.Ü., Data Collection or Processing: G.G., P.K., E.L., B.S., E.Ş., B.B., H.A.H., H.G., Analysis or Interpretation: G.G., P.K., E.L., N.E.Ü., Literature Search: G.G., P.K., Writing: G.G.

Conflict of Interest: No conflict of interest was declared by the authors.

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