

T-Cells Show Increased Production of Cytokines and Activation Markers in Alzheimer's Disease

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Abstract

In Alzheimer's disease (AD), there is growing evidence of the presence of both systemic and local inflammatory processes, which might be either causative or a reaction to pathology. To characterize the systemic inflammatory profile of AD, we investigated peripheral blood lymphocyte subsets of AD patients and age-matched Healthy Controls (HC), and their production of the pro-inflammatory cytokines IL-6, IL-17, TNF α , and IFN γ , together with surface expression of lymphocyte-activation-markers.

Peripheral Blood Mononuclear Cells (PBMCs) were stained *ex vivo* for activation markers. Intracellular cytokine staining was done after activation *in vitro* with CD3/CD28 for four days. Cells were analyzed using flow cytometry.

In AD patients we found significantly increased frequencies of both CD4 $^{+}$ and CD8 $^{+}$ cells producing IL-6 and IL-17, respectively. In addition we detected a significantly increased percentage of IFN γ producing CD4 $^{+}$ cells and CD4 $^{+}$ cells expressing the activation markers CCR5 and HLA-DR. Taken together, our data supports the existence of a systemic inflammatory process involving also Th17-cells in AD.

Keywords: Alzheimer's disease; Cytokines; Inflammation; T-Helper-Cells

Introduction

Alzheimer's disease is a progressive neurodegenerative disorder characterized by irreversible impairment of cognitive and memory functions and physical deterioration. The etiology of AD is still largely unclear and thought to be a multifactorial process. Typically depositions of amyloid protein plaques between and neurofibrillary tangles within nerve cells can be detected. These processes stimulate microglia to produce immunological mediators, including pro-inflammatory cytokines and cytotoxic substances [1]. It has been shown that the inflammatory process is not restricted to the Central Nervous System (CNS), since also in peripheral blood an increase of activated T-cells together with elevated levels of the pro-inflammatory cytokines IL-6, IL-1, and TNF- α , respectively have been detected previously [2,3]. Therefore we aimed to investigate the production of the pro-inflammatory cytokines IFN γ , IL-17, IL-6, and TNF α as well as the expression of various activation markers on CD4 $^{+}$ and CD8 $^{+}$ T cells in peripheral blood of AD-patients compared to age- matched healthy controls (HC).

Methods

Patients and healthy controls

The study was approved by the local ethics committee of the Medical University of Vienna, Austria and informed consent of all participants was obtained according to the declaration of Helsinki. Twenty four elderly individuals were enrolled in the study: 14 patients had a diagnosis of mild to moderate AD (Mini Mental State Examination (MMSE) 26-12), 10 individuals served as healthy controls. The clinical diagnosis of AD was performed at the neurology department of the Medical University of Vienna, Austria according to the NINCDS-ADRDA Alzheimer's criteria. All patients underwent complete medical and neurological workup including laboratory analysis and CT or MRI scan to exclude reversible causes of dementia. Individuals with other neurological disorders, history of malignancies, inflammatory diseases or immunomodulatory medication were not enrolled in the study. Complete blood count, C-reactive protein and

erythrocyte sedimentation rate was within the normal range in all individuals during the time of the study. Antidementia medication (Acetylcholinesterase inhibitors or Memantin) had to be stable over a period of three months prior to inclusion.

Blood sample collection, cell isolation and staining

Whole blood was collected in vacutainer-tubes containing EDTA and 3 ml were subsequently used for extracellular staining. Whole blood was incubated at 4°C with fluorochrome labeled monoclonal antibodies in a 1:10 dilution (CD4 FITC Mouse Anti- IgG1 Clone RPA-T4 and CD 4 APC Mouse Anti-Human IgG1 Clone L200, CD8 PE-Cy5 Mouse anti-Human IgG2a Clone 3B5, CD62L FITC Mouse Anti-Human IgG1 Clone DREG-56, CD27 FITC Mouse Anti-Human IgG1 Clone M-T271, CCR6 APC Mouse Anti-Human IgG1 Clone 11A9, CCR7 PE Rat Anti-Human IgG2a Clone 3D12, CD25 PE-Cy5 Mouse Anti-Human IgG1 Clone M-A251, CCR5 PE Mouse Anti-Human IgG2a, Clone 2D7/CCR5, CXCR3 PE-Cy5 Mouse Anti-Human IgG 1, Clone 1C6/CXR3, HLA-DR PE-Cy5 Mouse Anti-Human IgG2a Clone G46-6, and matching isotype controls for colour compensation, all obtained from Becton Dickinson biosciences, Europe) for 20 min in Falcon round bottom tubes (Becton Dickinson biosciences, Europe). Subsequently erythrocyte-lysis was performed for 20 min using BD FACS lysing solution (Beckton Dickinson biosciences, Europe) according to the manufacturers protocol. Then cells were washed twice in PBS and analyzed immediately.

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For intracellular cytokine staining 18 ml whole blood was collected in heparin coated vacutainer-tubes (vacutainer tubes: Becton Dickinson & Co., Rutherford, NJ, USA). Peripheral blood monocytes (PBMCs) were separated by density centrifugation at 4°C using Ficoll Hypaque solution (Sigma-Aldrich, St. Luis, Missouri). Cells were washed twice in phosphate buffered saline (PBS), then number of viable leukocytes was determined by trypan blue exclusion staining. To expand T-cells PBMCs were distributed in 96well round bottom plates (Becton Dickinson & Co., Rutherford, NJ, USA) at a density of about 1.5×10^5 cells/well and incubated for four days in unspecific t-cell polarizing conditions using RPMI-1640 medium (Invitrogen, GIBCO®) containing 1 ng/ml CD3 and 1 ng/ml CD28, 5% each of HEPES, glutamate, non-essential amino-acids, pyruvate, and heat-inactivated 10% human serum at 37°C in a humidified 5% CO₂ atmosphere. We identified a stimulation time of 96 hours as being optimal for the reliable detection of IL-17-producing T cells. At day 4, cells were re-stimulated with 10 ng/ml PMA, 1 ng/ml Ionomycin (both Sigma Aldrich, St. Luis, Missouri) and 5 ng/ml GolgiPlug (Beckton Dickinson biosciences, Europe) for 5 hours. Cells were then harvested into 1.5 ml FACS tubes, fixed and permeabilized (BD Cytofix/Cytoperm kit, Beckton Dickinson Biosciences, Europe), according to manufacturers protocol. To avoid unspecific staining, cells were then blocked with beriglobin 3 mg/ml (CSL Behring, Austria).

Then cells were stained at 4°C for 30 min for CD4 APC Mouse Anti-Human IgG1 Clone L200, CD8 PE-Cy5.5 Mouse Anti-Human IgG2 Clone 3B5, TNF-alpha PE Mouse Anti-Human IgG2 Clone MAb11, IL-6 PE Rat-Anti-Human IgG 2a Clone MQ2-6A3, IFN γ PE Mouse Anti-Human IgG1 Clone B27 (all Beckton Dickinson bioscience, Europe), and IL-17 FTIC Mouse Anti-Human IgG1 Clone eBio64DEC17 (eBioscience, Austria) as well as matched isotype-controls. All antibodies were used at a dilution of 1:10. Cells were washed twice at 4°C in PBS and analyzed immediately by flow cytometry [4-6].

Flow cytometric analyses

Analyses of ten-thousand cells per acquisition were performed using a 4-colour Beckton Dickinson FACS Calibur cytometer running on CellQuest Pro software. Isotype controls and single stained preparations were first run for color compensation. Linear amplifiers were used for forward and side scatters and logarithmic amplifiers for fluorescence labeled channels. Lymphocytes were identified in the forward and side scatter first and gated on either CD4 or CD8, and further gated on extracellular activation markers or cytokines. Results are given as proportion of cells expressing the activation marker or cytokine within the CD4 or CD8 subset as well as Mean Fluorescence Intensity (MFI).

Statistical methods

Normal distribution of each variable was tested using Kolmogorov-Smirnov-test. Data are expressed as mean \pm standard deviation. Group comparisons between AD and HC were done using Student's t-test for normally distributed variables and Mann-Whitney-U-test for not normally distributed variables. Correlations were assessed using Spearman's correlation-coefficient. A p-value <0.05 was considered statistically significant. Logistic regression analysis was done including AD as dependent variable, CCR5, HLA-DR, CD4+IL6, CD8+IL6, CD4+Ifn-g, CD4+IL-17 and CD8+IL17 as variables influencing AD, and age and sex as dummy variables to identify possible confounders. Data analysis was performed using SPSS version 15 .0 (SPSS, Inc. Chicago, Illinois, USA).

Results

We included 14 AD (7 female, mean age 70y \pm 4.5) and 10 HC (5 female, mean age 73y \pm 5.3). Mean MMSE was 21 (12-26) in AD and 30 in HC. All AD were on stable dosage of one of the following cholinesterase inhibitors: galantamine, rivastigmine and donepezil and one patient had additional memantine medication (Table 1). Absolute lymphocyte count was obtained from routine laboratory examination and did not differ between AD and HC. The CD4+ and CD8+ fractions were assessed by flow-cytometric analyses and a CD4/CD8 ratio was calculated. No differences between AD and HC and no correlations with MMSE or age were observed regarding the CD4/CD8 ratio. Surface activation markers and chemokine-receptors of CD4+ and CD8+ cells were assessed *ex vivo* by flow-cytometry. Within the CD4+ subset cells of AD expressed significantly more often HLA-DR (26.7% \pm 8.7 vs. 14.5% \pm 6.2, Student's t-test, p=0.008). Amongst the chemokine-receptors CCR5, CCR6, and CCR7, only the frequency of cells expressing CCR5 was significantly increased in AD (33.2% \pm 14.1 in AD vs. 19.6 \pm 10.3 in HC, MWU, p=0.022). CD69 was also increased on CD4+ in AD, but missed statistical significance (5.7% \pm 5.1 in AD vs. 2.5% \pm 1.5 in HC, Student's t-test, p=0.088). Expression of the other activation markers tested CD25, CD27, CD62L, and CXCR3 did not differ between AD and HC. Amongst the CD8+ subset the activation markers and chemokine-receptors CCR5, CCR7, CD27, CD58, and CXCR3 did not differ significantly between AD and HC (Figure 1). Cytokine-production of TNF α , IL-6, IFN γ , and IL-17 in CD4+ and CD8+ cells was then assessed by flow-cytometry after 4 day stimulation with CD3/CD28.

The percentage of CD4+ and CD8+ cells producing IL-17 was higher in AD than in HC (CD4+ 6.2% \pm 2.7 vs. 1.5% \pm 0.6, Student's t-test, p<0.001 and CD8+ 9.6% \pm 4.7 vs. 2.6% \pm 1.3, Student's t-test,

Age/Sex	AD/HC	MMSE	CRP	WBC
60/m	HC	30	<1mg/dl	<10 G/l
85/f	HC	30	<1mg/dl	<10 G/l
81/m	HC	30	<1mg/dl	<10 G/l
71/f	HC	30	<1mg/dl	<10 G/l
77/m	HC	30	<1mg/dl	<10 G/l
60/f	HC	30	<1mg/dl	<10 G/l
58/f	HC	30	<1mg/dl	<10 G/l
77/m	HC	30	<1mg/dl	<10 G/l
79/f	HC	30	<1mg/dl	<10 G/l
73/m	HC	30	<1mg/dl	<10 G/l
71/m	AD	19	<1mg/dl	<10 G/l
63/m	AD	15	<1mg/dl	<10 G/l
63/m	AD	20	<1mg/dl	<10 G/l
63/m	AD	26	<1mg/dl	<10 G/l
67/f	AD	18	<1mg/dl	<10 G/l
69/m	AD	23	<1mg/dl	<10 G/l
74/f	AD	23	<1mg/dl	<10 G/l
75/f	AD	22	<1mg/dl	<10 G/l
68/f	AD	12	<1mg/dl	<10 G/l
75/f	AD	21	<1mg/dl	<10 G/l
70/m	AD	21	<1mg/dl	<10 G/l
68/m	AD	12	<1mg/dl	<10 G/l
--/m	AD	22	<1mg/dl	<10 G/l
70/f	AD	26	<1mg/dl	<10 G/l

Table 1: Baseline characteristics

$p < 0.001$). The same result could be obtained regarding IL-6 producing CD4+ and CD8+ cells (CD4+ $4.7\% \pm 1.7$ vs. $2.4\% \pm 0.7$, Student's t-test, $p < 0.001$ and CD8+ $6.2\% \pm 3$ vs. $2.6\% \pm 1.4$, Student's t-test, $p = 0.005$). The percentage of cells producing IFN γ was increased in AD only in the CD4+ subset ($8.4\% \pm 3.6$ vs. $4.7\% \pm 2.3$ Student's t-test, $p = 0.001$). Percentage of cells producing TNF α did not differ between AD and HC neither in the CD4+ nor in the CD8+ subset. The MFI of the above mentioned cytokines did not differ between AD and HC in the CD4+ and CD8+ subsets (Figure 2).

Age and sex did not correlate with percentage of the cytokine producing CD4+ and CD8+ cells. Regarding MMSE only percentage of CD4+IL-17+ cells correlated with MMSE when testing all subjects included in the study (Spearman, $p = 0.023$), but not in AD alone.

Notably, the frequency of cells expressing HLA-DR correlated with the percentage of CD4+ cells producing IFN γ , IL-6, and IL-17 (Spearman, $p = 0.002$, $p = 0.049$, and $p = 0.036$).

When performing logistic regression modelling including CD4+IFN γ +, CD4+IL6+, CD8+IL6+, CD4+IL17+, CD8+IL17+, CD4+CCR5+, and CD4+HLA-DR+, CD4+IL17+ (all increased in AD) as well as age and sex as control variables, only CD4+IL17+ remained significantly associated with AD ($p = 0.009$).

Taken together, these results suggests a pro-inflammatory disposition in AD consisting of an increased expression of the activation markers HLA-DR and CCR5 on CD4+ cells and an increased proportion of lymphocytes able to produce IL-17, IL-6 and IFN-g.

Discussion

There is an emerging concept that alterations of both innate and acquired branches of the immune system [7] contribute to pathogenesis of AD. However, to date, there is no general consensus regarding alterations of lymphocyte subsets in AD patients. We investigated activation markers and cytokine production of PBMCs in AD and HC. Interestingly we detected a characteristic pro-inflammatory profile in AD consisting of an increase of activated CD4+ lymphocytes with surface-expression of HLA-DR and CCR5 together with increased percentages of circulating lymphocytes producing IL-17, IL-6, and IFN γ , while total amounts of CD4+ and CD8+ cells were not altered in AD.

Lymphocyte activation markers in AD

CCR5 was increased on CD4+ cells in AD ex vivo both in our investigation as well as previously shown after stimulation with A β [3,8]. This fits to the concept of a pro-inflammatory process accompanying AD. T-cells are able to respond to chemokines produced in the brain and by HBMECs via CCR5. Increased expression of CCR5 might explain their migration into the brain in AD [9] as the CCR5-ligand CCL3 is expressed on astrocytes and microglia in AD [10]. Furthermore the pro-inflammatory cytokines TNF α , IL-1 and IFN γ have been found to be increased in peripheral blood of AD patients. They can further induce the expression of the CCR5-ligands CCL3, CCL4 and CCL5, respectively in vitro on human brain microvascular endothelial cells (HBMECs) [11]. In mouse-mesangial cells, IL-17 elicited the expression of the CCR5 ligand CCL3 [12], however this has not yet been investigated for HBMECs.

The other activation marker found to be increased on CD4+ cells in our investigation was HLA-DR. HLA-DR initiates immune reactions by binding antigens to T-cells. The increased number of CD4+ cells expressing HLA-DR in AD is consistent with previous reports, where the expression of HLA-DR on circulating T-cells in AD as well as post mortem in brains of AD was associated with disease severity [2,13-15]. We found no correlation between expression of HLA-DR and MMSE, which might be due to the relatively small sample size.

Pro-inflammatory cytokines

We found IL-17 to be significantly increased in CD4+ cells of AD patients vs. HC. Amongst all pro-inflammatory cytokines increased in AD, IL-17 is of special interest, as it bears a manifold of biological roles well compatible with what is currently known about inflammation in AD. The relevance of IL-17 in peripheral blood might differ from intracerebral IL-17, as it was shown to be reduced in cerebrospinal fluid in AD and other neurodegenerative disorders [16].

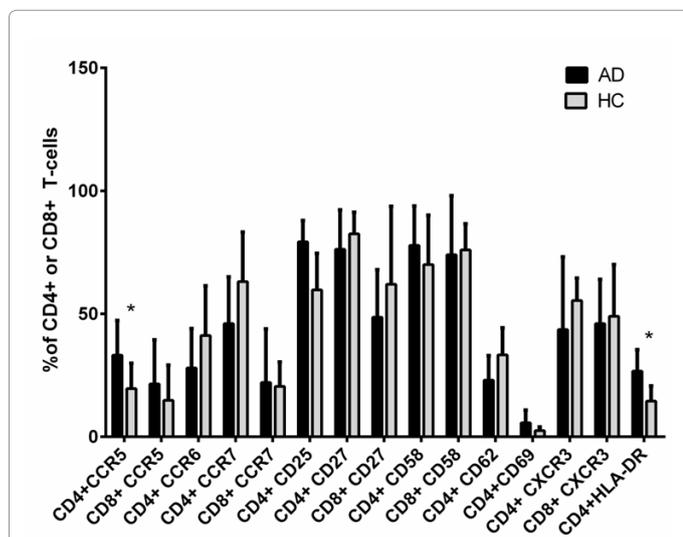


Figure 1: Amount of circulating CD4+ and CD8+ T-cells with surface expression of activation markers and chemokine-receptors in Alzheimer's disease (black columns) and healthy controls (grey columns) given as percentages of either CD4+ or CD8+ cells (mean), whiskers indicate standard deviation, * marks significant differences ($p < 0.05$).

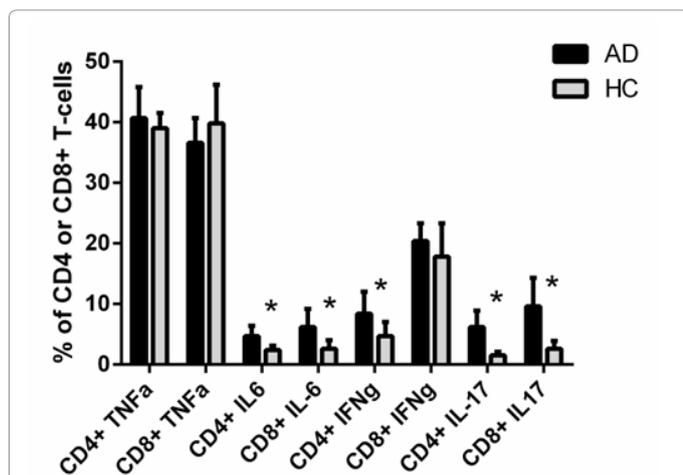


Figure 2: Frequency of cytokine producing CD4+ and CD8+ T-cells in Alzheimer's disease (black columns) and healthy controls (grey columns), given as percentage of CD4+ or CD8+ T-cells (mean), whiskers indicate standard deviation, * marks significant difference ($p < 0.05$).

Peripheral blood IL-17 can weaken tight junctions of the blood-brain-barrier to facilitate leukocyte entry into the CNS, and it induces production of the pro-inflammatory cytokines IL-6, TNF α , and IL-1 - all previously shown to be increased in peripheral blood of AD [11,17].

In AD stimulation of PBMCs with A β lead to an increased production of the Th17-cytokines IL-21 and IL-22 (but not IL-17) in CD4+ cells and to an increased production of IL-6 and IL-23 in macrophages/monocytes, both cytokines important for differentiation and stabilization of Th17 cells [18]. In contrast, we found an increased percentage of CD4+ cells producing IL-17. This discrepancy requires further investigation, but it might be at least partially explained by patient selection and methodological differences, as we used only unspecific stimulation with CD3/CD28. Still, our findings together with existing literature show an increased underlying proportion of cells able to produce Th17-related cytokines in AD suggesting that Th17 cells might be of some relevance in the inflammatory process in AD.

Less is known about the biological function of CD8+ Tc17 cells, except their appearance in various diseases (e.g. psoriasis, malignancies [19]). CD8+ cells were not altered in our investigation in general, but the relative increase of the CD8+ Tc17-cells needs further investigation. The increased amount of CD4+ and CD8+ T-cells producing IL-6 and IFN γ , even without antigen-specific stimulation, is consistent with previous data and further supports the hypothesis of inflammation being involved at some stage in the natural disease course of AD [3].

Nevertheless, data about alterations of the immune-system in AD are complex and controversial, and it remains to be shown, if systemic inflammation is an underlying pathogenic mechanism or rather an epiphenomenon due to release of intracerebral A β 42 and cytokines in AD. Although our study is an observational one with a limited number of subjects, it revealed significant results supporting the existence and importance of a systemic inflammatory process, characterized by an increased expression of HLA-DR and CCR5 and increased percentages of T-cells capable of production of IL-17, IFN γ and IL-6. How increased inflammatory activity might reflect disease progression and/or disease severity is an interesting question and requires future investigations. Characterizing AD using inflammatory-profiling might help to identify selected patient groups for clinical trials and immune-modulatory therapeutic strategies. Embedded in this context our investigation may serve as an impulse for longitudinal studies including a focus on Th17-cells.

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