

Systemic Immune Responses in Alzheimer's Disease: In Vitro Mononuclear Cell Activation and Cytokine Production

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Abstract. To investigate the systemic signs of immune-inflammatory responses in Alzheimer's disease (AD), in the present study we have analyzed blood lymphocyte subsets and the expression of activation markers on peripheral blood mononuclear cells (PBMCs) from AD patients and age-matched healthy controls (HC) activated *in vitro* by recombinant amyloid- β peptide (rA β_{42}). Our study of AD lymphocyte subpopulations confirms the already described decrease of the absolute number and percentage of B cells when compared to HC lymphocytes, whereas the other subsets are not significantly different in patients and controls. We report the increased expression of the activation marker CD69 and of the chemokine receptors CCR2 and CCR5 on T cells but no changes of CD25 after activation. B cells are also activated by rA β_{42} as demonstrated by the enhanced expression of CCR5. Moreover, rA β_{42} induces an increased expression of the scavenger receptor CD36 on monocytes. Some activation markers and chemokine receptors are overexpressed in unstimulated AD cells when compared to controls. This is evidence of the pro-inflammatory status of AD. Stimulation by rA β_{42} also induces the production of the pro-inflammatory cytokines IL-1 β , IL-6, IFN- γ , and TNF- α , and of the anti-inflammatory cytokines IL-10 and IL-1Ra. The chemokines RANTES, MIP-1 β , and eotaxin as well as some growth factors (GM-CSF, G-CSF) are also overproduced by AD-derived PBMC activated by rA β_{42} . These results support the involvement of systemic immunity in AD patients. However, our study is an observational one so we cannot draw a conclusion about its contribution to the pathophysiology of the disease.

Keywords: Alzheimer's disease, chemokine, cytokine, PBMC, rA β_{42}

INTRODUCTION

Alzheimer's disease (AD) is a heterogeneous and progressive neurodegenerative disease that in Western

societies accounts for the majority of clinical senile dementia. Since no early peripheral and reasonable signs of the disease have been identified so far, diagnosis can be made with firmness only upon brain autopsy. Moreover, no treatment is yet available to end or turn round the disease; existing drugs are only able to relieve symptoms for some time [1,2].

Pathological changes in the AD brain include neuronal loss, senile plaques, and neurofibrillary tangles. Together with the presence of intraneural tangles, con-

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sisting of phosphorylated tau-proteins, senile plaques are the hallmark of the pathological diagnosis of AD. However, the increased production of amyloid- β ($A\beta$) from the amyloid- β protein precursor and its brain accumulation and deposit in the senile plaques lead to inflammation and neuronal damage [3]. Depending on whether the 40th or the 42nd amino acid in C-terminus is the last of the $A\beta$ protein, it consists of two major forms, $A\beta_{40}$ and $A\beta_{42}$. The latter tends to cluster into oligomers, forming $A\beta$ fibrils that deposit as microstructures, amyloid plaques, and it is the predominant form that accumulates in the brain parenchyma of AD patients. Senile plaques result from the accumulation of several other proteins and an inflammatory reaction around deposits of amyloid. In addition, plaques contain dystrophic neurites, activated microglia, and reactive astrocytes. Aggregated amyloid fibrils and inflammatory mediators secreted by microglial and astrocytic cells contribute to neuronal dystrophy [3,4].

Inflammation clearly occurs in pathologically vulnerable regions of the AD brain, and it does so with the full complexity of local peripheral inflammatory responses [5]. The microglia activation can be due to local or systemic inflammation. In fact, a strong local inflammatory stimulus such as a previous head trauma is a risk factor for AD and several epidemiological studies clearly show that blood elevations of acute phase proteins, markers of systemic inflammatory stimuli, may be risk factors for cognitive decline and dementia. Furthermore, in experimental animals chronic systemic inflammatory response induced by lipopolysaccharide administration also induces glial activation. After activation, the microglia cells modify their morphology and become tissue macrophages producing inflammatory mediators [3,6]. Accordingly, case-control studies clearly demonstrate the role of inflammatory genes in AD [3,7,8]. In particular, two recent meta-analyses by our group have underscored the role of cytokine polymorphisms in AD susceptibility, hence indicating the role of immune-inflammatory responses in AD [9,10].

$A\beta$ induces a local immune response involving glial cells and astrocytes. The innate immunity tries to clear $A\beta$ and induces the production of inflammatory proteins such as complement factors, acute-phase proteins, pro-inflammatory cytokines, and chemokines that will be chronically produced and can induce neurotoxicity [5,11].

Although much evidence suggests the involvement of a systemic immune response in AD, it is poorly characterized [12]. Indeed, blood derived cells seem to accumulate in the AD brain [13], while other studies

have shown changes in the distribution and reactivity of immune cells in the blood [12,14–16]. As reviewed by Britschgi and Wyss-Coray [12], many studies have shown that there is communication between central and systemic immune responses. In particular neuroinflammation induces the efflux of central nervous system (CNS) proteins, such as $A\beta$, or inflammatory mediators across the blood-brain-barrier (BBB); this may cause systemic immune reaction and recruitment of myeloid or lymphocytic cells into the CNS [12]. Thus, communication between the CNS and immune system in AD could influence both the lymphocyte distribution in the blood and the production of immune mediators [12]. Indeed peripheral blood mononuclear cells (PBMCs) from AD patients produce higher levels of some cytokines, such as interleukin (IL)-1 β and IL-6 compared to PBMC from control subjects [17]. Other studies have shown that $A\beta$ stimulates macrophage inflammatory protein (MIP)-1 α overexpression on peripheral T cells and its receptor CCR5 expression on brain endothelial cells for T cells crossing BBB [18].

In addition, the production of Regulated on Activation, Normal T Expressed and Secreted (RANTES) by AD PBMCs seems to increase after stimulation by $A\beta$ [19]. Moreover, an immune dysregulation was recently documented as dramatic alterations on CD4+ subsets in patients with mild AD have been reported. In particular, decreased percentages of naïve cells and an increase of memory cells, an increased number of CD4+ lymphocytes that lack the co-stimulatory molecule CD28, and a reduction of CD4+CD25^{high} T regulatory cells (Treg) have been observed [20].

These data suggest a significant involvement of both the innate and acquired immunity in AD patients, although there are not enough data to determine if these are the causes or the consequences of the disease. To gain insight into this topic, we have investigated some basic immune parameters in patients with AD, and we have performed *in vitro* activation studies of AD PBMCs with recombinant $A\beta$ (r $A\beta_{42}$) to evaluate cellular reactivity to the peptide and the cytokine and chemokine production.

MATERIALS AND METHODS

Subjects

Diagnosis of probable AD was according to standard clinical procedures and followed the NINCDS/ADRDA and DSM-III-R criteria [21,22]. Cognitive perfor-

mance and alterations were measured according to the Mini Mental State Examination (MMSE) and the Global Deterioration Scale. All AD cases were defined as sporadic because their family history did not mention any first-degree relative with dementia. The population of AD consisted of 40 patients from Sicily (27 women and 13 men; age range: 64–86 years; mean age: 78.4 ± 7.6). 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. Eighteen of them were under treatment for hypertension and/or diabetes with drugs not known to affect the immune system. According to MMSE, 16 patients were affected by severe dementia (< 17), whereas the remaining 24 were affected by moderate grade of dementia ($> 17 < 24$). Patients with vascular dementia were not included in the study. Since treatment with acetyl-cholinesterase inhibitors may modulate cytokine expression [23], patients were included before starting that therapy. Healthy controls (HC) were 25 unrelated individuals (15 women and 10 men; age range: 63–85; mean age 77.5 ± 7.2) randomly selected from a retirement home. These subjects had complete neurological examinations and were judged to be in good health based on their clinical history and blood tests (complete blood cell count, erythrocyte sedimentation rate, glucose, urea nitrogen, creatinine, electrolytes, C reactive protein, liver function tests, iron, proteins, cholesterol, triglycerides). The controls were collected from the same population as the patient cohort. The University Hospital Ethics Committee approved the study, and informed consent was obtained from all guardians of patients and controls according to Italian law.

Whole blood was collected by venopuncture in vacutainer tubes containing ethylenediamine tetraacetic acid. The samples were kept at room temperature and used within 2 h for the various experiments. Lymphocyte subsets were analyzed in 40 AD and 25 HC. Cell cultures for activation and cytokine production were performed on PBMCs from 18 AD and 15 HC randomly selected, taking into account gender, age range, and the ratio between severe and moderate AD. In fact, we studied 11 patients affected by moderate AD (4 men and 7 women, mean age 79.6 ± 1.3), 7 patients affected by severe AD (2 men and 5 women, mean age 81.0 ± 1.5), and 15 HC (6 men and 9 women, mean age 78.5 ± 1.2).

Preparation and characterization of rA β_{42}

Isolation and purification of rA β_{42} were performed as described by Carrotta et al. [24]. To obtain small

oligomers, rA β_{42} was dissolved in double-distilled H₂O (ddH₂O) to a final concentration of 1.4 mM and incubated at 37°C for 96 h. Immediately after dissolution (time 0), and after incubation, aliquots were taken and stained adding thioflavin-T (ThT) at a final concentration of 70 μ M and applied to microscope slides [25]. The presence of aggregates with dimensions in the range of micrometers was visualized with the fluorescence optics of an Axioscop 2 microscope (Zeiss, USA). The images were captured using an Axio-cam digital camera interfaced with a computer. Moreover, rA β_{42} at time 0, and after incubation at 37°C for 96 h, was analyzed by Western blot. Analysis of the samples was carried out using SDS-PAGE, and after running, the gel was transferred to nitrocellulose and the filter was incubated with anti-Histidine (anti-His) 1:5000 (Pierce, Rockford, IL, USA) [24].

Cell cultures

PBMCs were separated by centrifugation with Ficoll-Lympholyte (Cedarlane Laboratories Limited, Ontario, Canada) and washed twice in PBS. Viability was assessed by trypan blue dye exclusion. The viable cells (95–98% of the preparation) were re-suspended at the concentration of $1 \times 10^6/\mu$ l in complete medium composed of RPMI 1640 (Euroclone, Devon, UK) supplemented with 10% heat-inactivated fetal calf serum (Euroclone), 1% penicillin/streptomycin, 10 mM HEPES, and 1 mM L-glutamine.

Cells were cultured in 24-well flat-bottom plates at 1.5×10^6 cells per well in RPMI medium. The PBMCs were either unstimulated or stimulated by oligomeric rA β_{42} (10 μ g/ml) obtained as described above. For the chemokine receptor expression study, the PBMCs were either unstimulated or stimulated by phytohaemagglutinin (PHA) (3 μ g/ml), anti-CD3 (α -CD3) (1 μ g/ml), rA β_{42} (10 μ g/ml), for 48 h at 37°C in a humidified 5% CO₂ atmosphere. The cells were then collected and used for flow cytometry.

Flow cytometry

In order to evaluate the T lymphocyte subsets in both AD patients and HC, fresh samples were stained using the following monoclonal antibodies: anti-CD3, anti-CD4, anti-CD8, anti-CD16, anti-CD19, anti-TCR $\gamma\delta$.

In order to evaluate the activation of PBMC obtained from AD patients and HC, the *in vitro* cultured cells were stained as follows: anti-CD25_{FITC}/anti-CD3_{PE}/anti-CD19_{PECy5}, anti-CD69_{FITC}/anti-CD3_{PE}/anti-

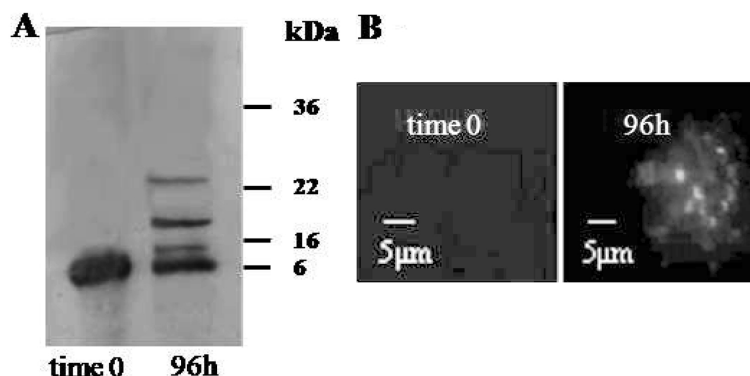


Fig. 1. Characterization of $rA\beta_{42}$. A) Western blot of $rA\beta_{42}$ incubated with anti-His, immediately after dissolution in ddH₂O (time 0) and after incubation for 96 h at 37°C. On the right, molecular weight standards are indicated. B) Staining with ThT. Fluorescence images of representative areas of the observation field at time 0 and at 96 h.

CD19_{PE-Cy5}, anti-HLADR_{FITC}/anti-CD19_{PE}/anti-CD80_{PE-Cy5} and anti-CD36_{FITC}/anti-CD14_{PE}/CD80_{PE-Cy5}. The activation-induced expression of chemokine receptors was evaluated by staining cultured cells with the following monoclonal antibodies: anti-CD192 (CCR2)_{AlexaFluor®647}, anti-CD193 (CCR3)_{PE} and anti-CD195 (CCR5)_{PE-Cy5} on B (CD19⁺) and T (CD3⁺) cells. All monoclonal antibodies were directly coupled either to fluorescein isothiocyanate (FITC), R-phycoerythrin (R-PE), phycoerythrin-Cyanine 5 (PE-Cy5), or Alexa Fluor® 647 (Pharmingen, BD Bioscience, Mountain View, CA, USA).

All the measurements were made with a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA) with the same instrument setting. At least 10⁴ cells were analyzed using CellQuestPro (Becton Dickinson, San Jose, CA, USA) software.

Cytokine production assay

The supernatants of the cultured PBMCs were collected and the following cytokines and chemokines were evaluated using a Bio-Plex kit (BioRad, Munich, Germany): IL-1 β , IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, basic fibroblast growth factor (BFGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon(IFN)- γ , IFN- γ -inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, MIP-1 α , MIP-1 β , platelet-derived growth factor-bb (PDGF-bb), RANTES, tumor necrosis factor (TNF- α), and vascular endothelial growth factor (VEGF). 50 μ l per sample were analyzed on the Luminex 100 (BioRad) according to manufacturer's instructions.

Statistical analysis

Values (percentage or MFI), given as the mean \pm SEM, were compared using one-way analysis of variance (ANOVA). Differences were considered significant when a p value < 0.05 was obtained by comparison of the two different groups.

RESULTS

Characterization of $rA\beta_{42}$

To confirm that $rA\beta_{42}$, prepared as previously described, forms oligomers *in vitro*, an aliquot of the samples at time 0 and at 96 h obtained as described in the previous section was incubated with anti-His. As shown in Fig. 1A, on the basis of the molecular weight of the detected bands, we established that only monomers were present in the sample at time 0, and different small oligomers (ranging from 12 to 24 kDa) in the sample at 96 h. Furthermore, after staining with ThT no visible structures in the sample at time 0 were detectable, whereas, small structures of dimensions up to 2 μ m in the 96 h sample were present (Fig. 1B). Therefore, the preparations used in our experiments were made up of oligomers.

Lymphocyte subsets

Table 1 shows the percentage and the absolute number of the main lymphocyte subpopulations evaluated in 25 HC and 40 AD patients. As shown, and as previously reported by others [26–28], we report a significant decrease in the percentage and absolute number

Table 1
Lymphocyte subpopulations in 40 AD subjects and 25 HC. Data are expressed as Mean ± SD of the percentage and as absolute number

Subpopulations	HC % (Mean ± SD)	HC (Absolute number/ μ L)	AD % (Mean ± SD)	AD (Absolute number/ μ L)
CD3 ⁺	67.3 ± 11.8	1196 ± 444	68.4 ± 9.2	1260 ± 420
CD4 ⁺	44.3 ± 9.6	801 ± 310	45.7 ± 8.9	845.6 ± 340
CD8 ⁺	20.0 ± 10.5	373 ± 214	21.0 ± 8.9	361 ± 160
CD19 ⁺	9.8 ± 5.3	184 ± 128	7.0 ± 3.7*	110 ± 54*
CD3 ⁻ CD16 ⁺	17.2 ± 9	350 ± 195	15.7 ± 6.7	319 ± 165
$\gamma\delta$ ⁺	3.0 ± 3.3	45 ± 55	2.5 ± 1.8	54 ± 39

Significance has been evaluated by ANOVA test. AD vs. HC * $p = 0.01$.

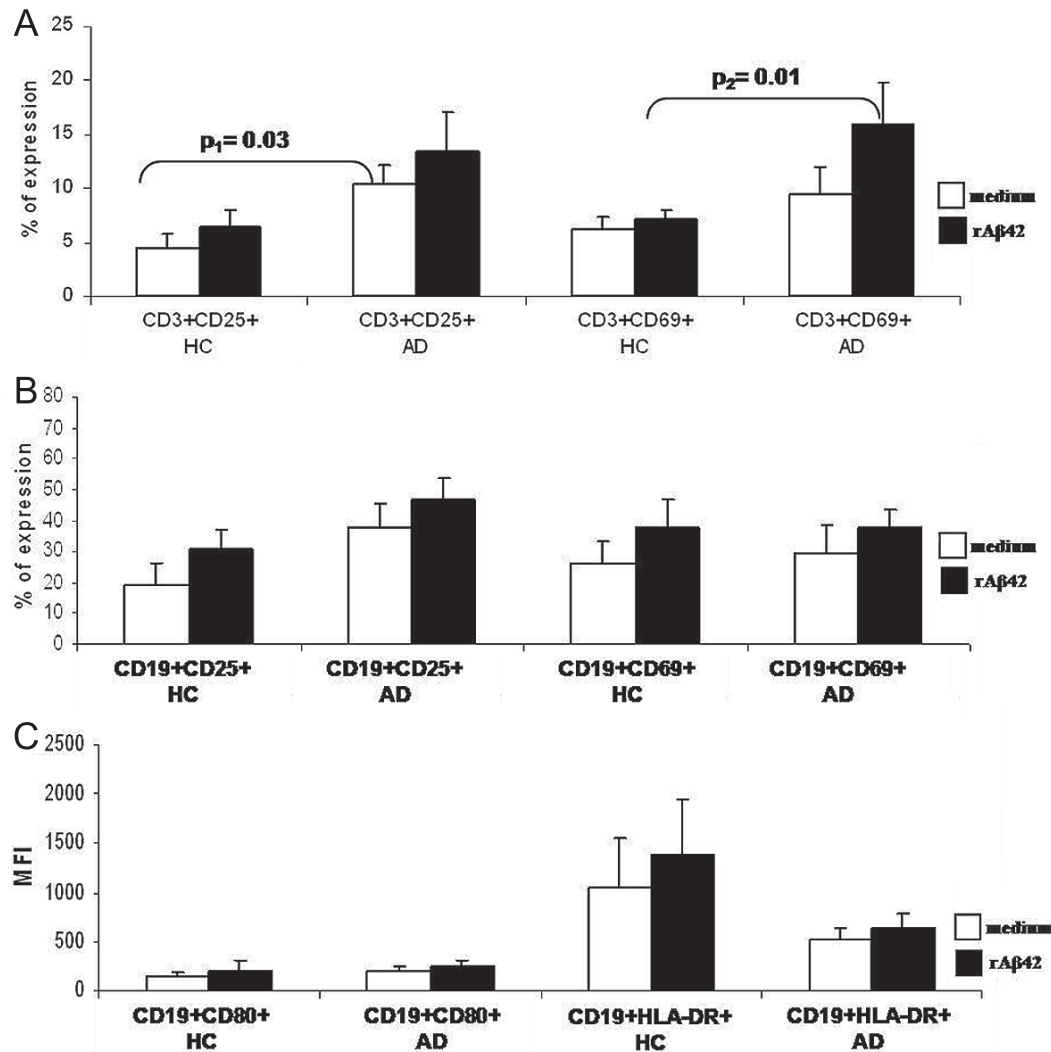


Fig. 2. Percentage (Mean ± SEM) of expression of early activation markers CD25 and CD69 on T (CD3⁺) (panel A) and B (CD19⁺) (panel B) cells of HC ($n = 15$) and AD subjects ($n = 18$) cultured in medium (white) or with rAβ₄₂ (black). Panel C shows the MFI (Mean ± SEM) of expression of CD80 and HLA-DR on B cells of HC ($n = 15$) and AD subjects ($n = 18$) cultured in medium (white) or with rAβ₄₂ (black). Statistical analysis was performed by ANOVA test. Significant values are indicated, $p_1 =$ CD25 expression on unstimulated (medium) AD cells vs. unstimulated HC cells; $p_2 =$ CD69 expression on rAβ₄₂-stimulated cells from AD vs. rAβ₄₂-stimulated cells from HC. Concerning B cells, no significant differences were observed for CD25 expression on rAβ₄₂-stimulated cells from AD vs. rAβ₄₂-stimulated cells from HC, for CD69 expression on unstimulated cells (medium) AD vs. unstimulated HC cells, nor for both CD80 and HLA-DR between the groups studied.

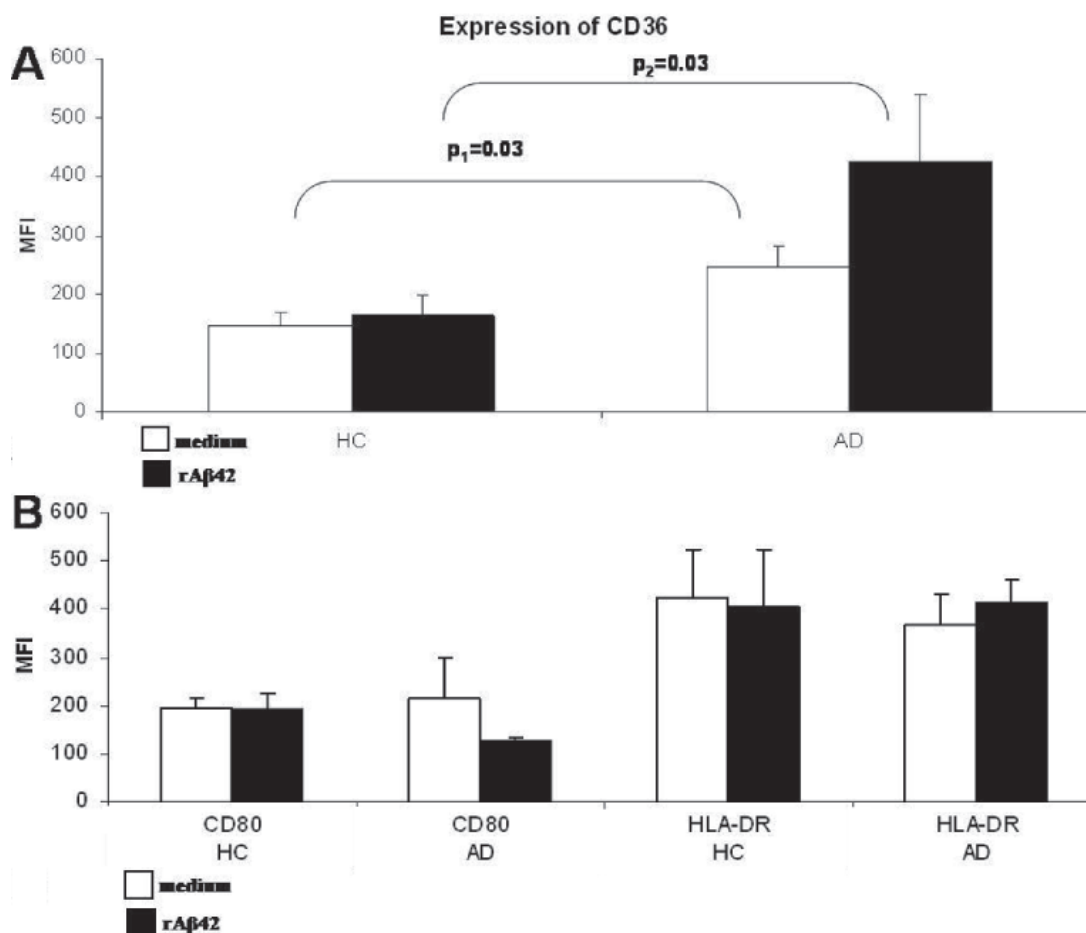


Fig. 3. MFI (Mean \pm SEM) of expression of CD36 (panel A) and CD80 and HLA-DR (panel B) on monocytes (CD14+) of HC ($n = 15$) and AD ($n = 18$) subjects cultured in medium (white) or with rA β_{42} (black). Statistical analysis was performed by ANOVA test. Significant values are indicated, $p_1 =$ unstimulated (medium) AD cells vs. unstimulated HC cells, $p_2 =$ rA β_{42} -stimulated cells from AD vs. rA β_{42} -stimulated cells from HC. No significant differences were observed for CD36 expression on unstimulated cells (medium) HC vs. stimulated HC cells, and on unstimulated (medium) AD cells vs. stimulated AD cells nor for the expression of CD80 and HLA-DR in the groups studied.

of circulating B lymphocytes of AD patients compared to HC. No significant differences have been instead observed between the two groups concerning the other lymphocyte subsets.

PBMC activation

To study the response to A β , we stimulated PBMCs from 18 AD and 15 HC (see Materials and Methods) with the oligomeric form of rA β_{42} and analyzed the expression of the activation markers CD25 and CD69 on T and B lymphocytes, and of CD80 and HLA-DR on B lymphocytes.

We report (Fig. 2a) a significantly higher expression of CD25 on unstimulated T cells from AD patients compared to unstimulated T cells from HC, whereas

the stimulation by rA β_{42} does not induce significant differences between the two groups. On the contrary, CD69 is not significantly higher in unstimulated T cells from AD patients and is significantly overexpressed after stimulation by rA β_{42} . The expression of CD25, CD69, HLA-DR, and CD80 on B cells showed no significant differences, after stimulation, between the two groups studied. We again observed that lymphocytes of AD patients showed a basically higher, but not significant, expression of some of these activation markers (Fig. 2b, c).

We have also evaluated the activation of monocytes after stimulation by rA β_{42} . We have observed a higher basal expression of CD36, a class B scavenger receptor that interacts with fibrillar A β [25,29], and a significant different expression of this receptor after stimula-

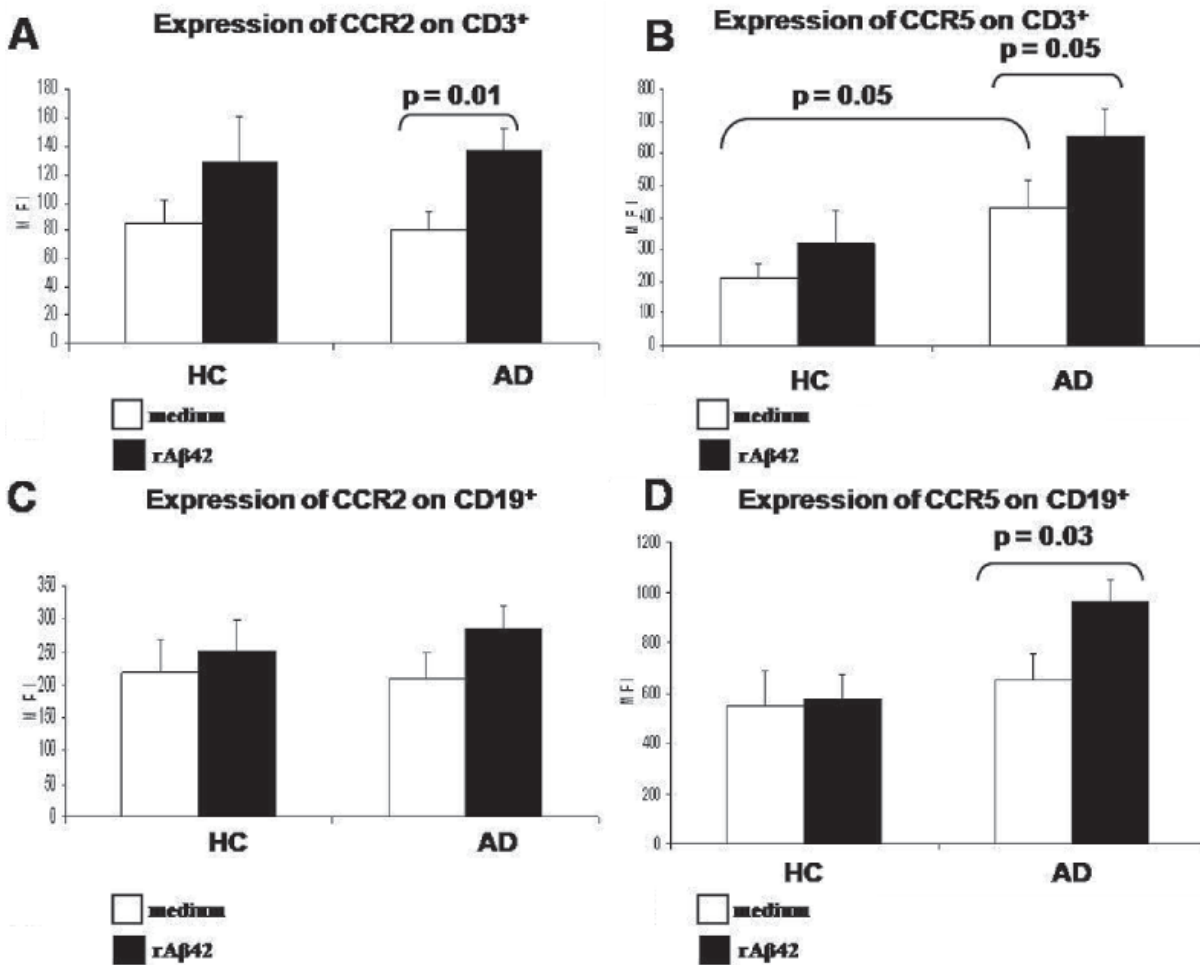


Fig. 4. MFI (Mean \pm SEM) expression of CCR2 and CCR5 on CD3⁺ T cells (panels a, b) and CD19⁺ B (panels c, d) peripheral lymphocytes obtained from 15 HC subjects and 18 AD patients and cultured for 48 h in presence (black) or absence (white) of rA β ₄₂. Statistical analysis was performed by ANOVA test. P values are calculated comparing the MFI \pm SEM of positive cells obtained from unstimulated and rA β ₄₂-stimulated cells. Comparing unstimulated vs. stimulated cells we obtain significant values in: a) CCR2 expression on T cells from AD patients, (unstimulated vs. stimulated $p = 0.01$); b) CCR5 expression on T cells from AD patients, (unstimulated vs. stimulated $p = 0.05$); c) CCR2 expression on B cells from AD patients, (unstimulated vs. stimulated not significant); d) CCR5 expression on B cells from AD patients, (unstimulated vs. stimulated $p = 0.03$). Concerning the unstimulated PBMCs significant difference are observed comparing CCR5 expression on T cells from HC and AD. Indeed there is a higher basal expression of CCR5 on AD patients (HC vs. AD $p = 0.05$).

tion by rA β ₄₂ between patients and controls (Fig. 3a). In contrast, analyzing the expression of CD80 and of HLA-DR on monocytes, we have not seen any variation between the groups studied (Fig. 3b).

In a further set of experiments, we have evaluated the expression of CCR2 (MCP-1 receptor), CCR3 (RANTES and eotaxin receptor) and of CCR5 (MIP-1 α , MIP-1 β , and RANTES receptor) on T (CD3⁺) and B (CD19⁺) lymphocytes.

As shown (Fig. 4a-d), CCR2 (Fig. 4a) and CCR5 (Fig. 4b) are overexpressed on T cells stimulated by rA β ₄₂, in both groups, although the significance was attained only in AD patients. In addition, the basal

expression of CCR5 on the CD3⁺ cells is significantly higher in AD patients. The evaluation of the same receptors on B cells, after activation by rA β ₄₂, shows a significantly greater expression of CCR5 in AD patients, and not in controls. No differences were observed for CCR2 expression on CD19⁺ cells (Fig. 4c, d) as well as for CCR3 expression on T and B cells (data not shown).

The stimulation of cells from HC and AD by PHA or α -CD3, used as further control, does not induce the expression of these chemokine receptors either in HC or AD patients. Figure 5 shows a typical experiment that compares the response of cells from AD pa-

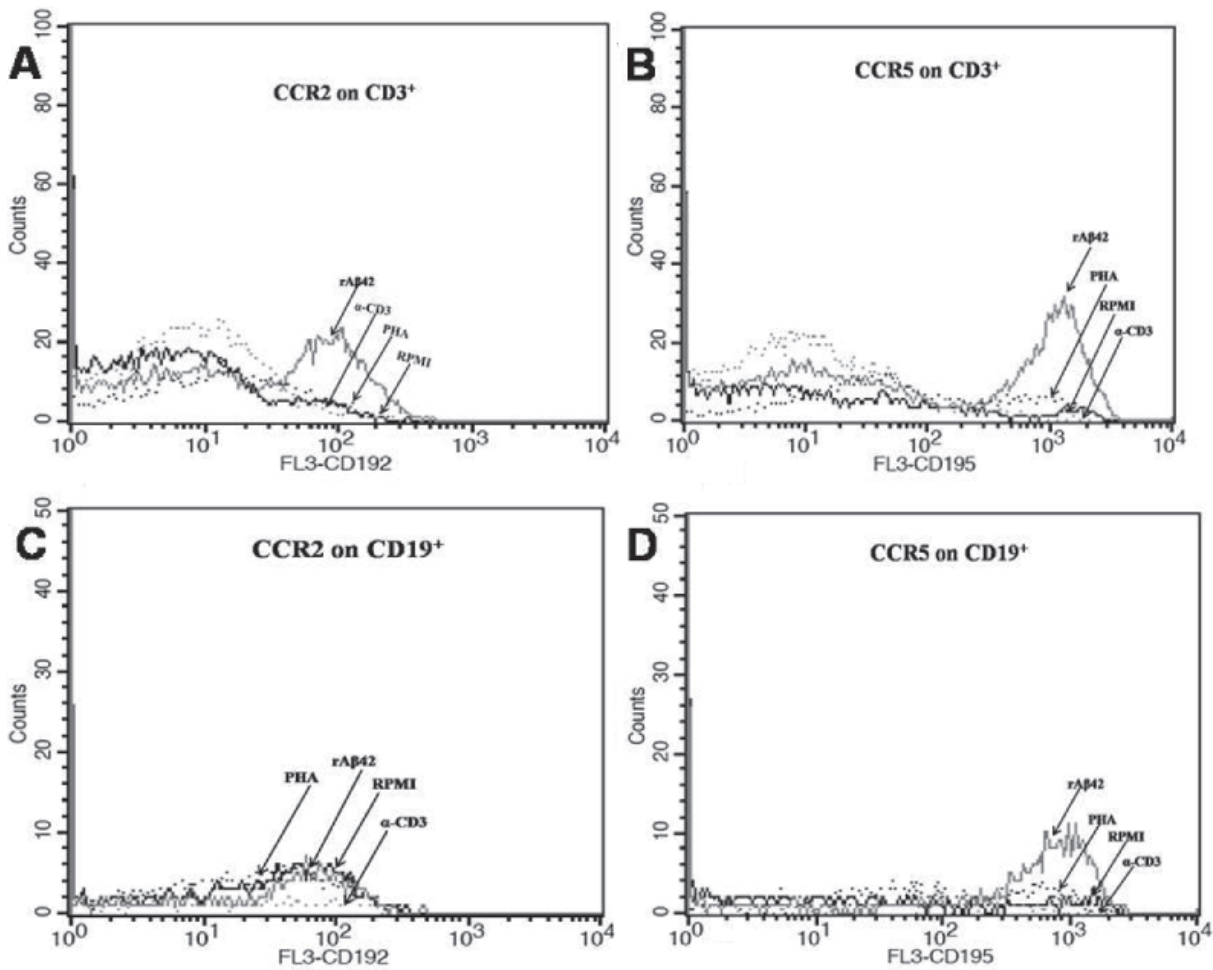


Fig. 5. Representative expression of CCR2 and CCR5 on CD3⁺ T cells (panels a, b) and CD19⁺ B cells (panels c, d) obtained from one AD patient, and cultured for 48 h in presence of PHA (.....), α -CD3 (.....), rA β ₄₂ (—), or none (RPMI) (—).

tients to the different stimuli used (PHA, α -CD3, and rA β ₄₂). As shown (Fig. 5a, b), CD3 cells overexpress CCR2 (panel A) and CCR5 (panel B) when stimulated by rA β ₄₂, whereas the stimulation by either PHA or α -CD3 gives a fluorescence intensity like that of unstimulated T cells. Panel C and panel D depict the results obtained stimulating CD19 B cells. As shown, no changes are observed in the expression of CCR2, using rA β ₄₂, PHA or α -CD3. Activation by rA β ₄₂ causes an increased expression of CCR5 on B cells, whereas the stimulation by either PHA or α -CD3 gives a fluorescence intensity like that of unstimulated B cells.

AD patients ($N = 18$) of this subgroup showed the same changes in lymphocyte populations (i.e., B lymphocyte decrease) as well as in the initial group (7.0 ± 2.2 versus 7.0 ± 3.7). None of these results were af-

ected by the gender and the MMSE of AD patients; in particular no differences have been observed comparing data obtained from subjects with severe (< 17) versus moderate AD.

Cytokine production

We have analyzed the production of cytokines, growth factors, and chemokines in supernatants obtained from AD- and HC-PBMC cultured for 48 h in the presence/absence of the oligomeric form of rA β ₄₂. As shown in Table 2, after stimulation by rA β ₄₂, PBMC obtained from AD patients significantly increased the production of both pro-inflammatory cytokines (IL-1 β , IL-6, IFN- γ , TNF- α) and anti-inflammatory cytokines (IL-10, IL-1ra) as well as some growth factors (GM-

Table 2

Percent of increase of cytokines, growth factors, and chemokines secretion by PBMCs of 15 HC and 18 AD patients in presence/absence of $rA\beta_{42}$. The percentage was calculated using the following formula: stimulated-unstimulated/unstimulated $\times 100$. In bracket % of increase of p25–p75 values. Significance has been evaluated by ANOVA test. n.s. = not significant

Cytokines	$rA\beta_{42}$ HC vs. medium HC Increase (%) (median and p-25–p75 values)	$rA\beta_{42}$ AD vs. medium AD Increase (%) (median and p-25–p75 values)	p ₁	p ₂
IL-1 β	4 (0–22)	211 (133–285)	n.s.	0.01
IL-1ra	66 (0–258)	202 (152–288)	n.s.	0.006
IL-6	0 (0–151)	410 (280–532)	n.s.	0.01
IFN- γ	0 (0–52)	106 (84–134)	n.s.	0.002
TNF- α	0 (0–168)	63 (35–104)	n.s.	0.02
IL-10	0 (0–0)	236 (100–542)	n.s.	0.03
Growth Factors				
GM-CSF	0 (0–90)	77 (62–100)	n.s.	0.003
VEGF	0 (0–322)	32 (10–82)	n.s.	n.s.
PDGF bb	57 (0–283)	14 (0–26)	n.s.	n.s.
G-CSF	(0–280)	547 (367–1367)	n.s.	0.01
Chemokines				
Eotaxin	0 (0–128)	114 (98–141)	n.s.	0.003
MIP-1 α	188 (0–577)	1710 (163–5032)	n.s.	n.s.
MIP-1 β	74 (0–377)	465 (152–639)	n.s.	0.02
RANTES	275 (0–955)	123 (57–206)	n.s.	0.05
MCP-1(MCAF)	0 (0–0)	0 (0–0)	n.s.	n.s.

p₁ = Significance of differences between Medium and $rA\beta_{42}$ of HC; p₂ = Significance of differences between Medium and $rA\beta_{42}$ of AD.

CSF, G-CSF) and some chemokines (eotaxin, MIP-1 β , RANTES) when compared to the cells cultured in medium. On the contrary, cells obtained from HC do not show any significant increase in the production of these cytokines.

By analyzing data according both to gender and to AD severity, no differences were observed. As previously stated, AD patients of this subgroup showed the same changes in lymphocyte populations (i.e., B lymphocyte decrease) as well as in the initial group.

DISCUSSION

Many studies have reported alterations of the immune system in AD and the involvement of both the innate and acquired branches of the immune system [3, 12]. In this paper, we report data obtained studying cells and factors involved in immune response in AD patients.

The “peripheral lymphocytes” topic has been examined by different groups with conflicting results [12, 20, 26–28], and currently there is no general consensus on the modifications of lymphocyte subsets in AD patients [12]. Our study of AD lymphocyte subpopulations confirms the already described decrease of the absolute number and percentage of B cells when compared to HC lymphocytes [26–28], whereas the other subsets are not significantly different in patients and

controls. This decreased number of B cells is already present in elderly when compared to young people [30]. Therefore, it suggests an exacerbation in AD of this feature linked to a reduced output of B cells from bone marrow [30]. This might be due to the well known systemic pro-inflammatory status of AD patients [3, 6–10, 31] and present results. On the T cell side, the results reported in different studies are discordant with each other. In fact, whereas our data are in agreement with Speciale et al. [26], Xue et al. [28] show a significant reduction of CD3+, CD4+ and CD8+; Richartz-Salzbürger et al. [29] confirm the decrease of CD3+ T cells and CD8+ T cells, but showed a slightly increase of CD4+. On the other hand, as previously stated, dramatic alterations on CD4+ subsets in patients with mild AD have been reported by Larbi et al. [20]. These differences might be the result of methodological differences among the different studies, including inclusion criteria of both AD patients and HC.

However, the finding that the B cell compartment is modified in AD patients compared to HC, together with the increased expression of the chemokine receptor CCR5 on B cells, after stimulation by $rA\beta_{42}$, suggests the involvement of B cells in the complex cellular interactions active in AD patients. On the other hand, $rA\beta_{42}$ stimulation does not change the expression of the activation markers CD25 and CD69 on B cells: it is an unexpected result since B cells can produce antibodies against A β [32].

On the other hand, we report a good response of T cells to $A\beta$; in fact the CD69 activation marker is overexpressed in $rA\beta_{42}$ -stimulated AD cells when compared to HC stimulated cells. Furthermore, the chemokine receptors CCR2 and CCR5 are overexpressed in AD cells after *in vitro* stimulation by $rA\beta_{42}$. This is an interesting result since the reactivity and ability of T cells to respond to the chemokines that can be produced locally in the brain might explain the migration of T cells across the brain microvascular endothelial cells occurring in AD [12]. It is noteworthy that some activation markers and chemokine receptors are overexpressed in unstimulated AD cells when compared to controls. This is evidence of the pro-inflammatory status of AD [3,6–10,31]. The increase of CD25+ T lymphocytes in cells from AD patients cultured in medium only might suggest an increase of Treg (CD4+CD25^{high}). However, using as marker of Treg FOXP3, Rosenkranz and colleagues [33] did not report their increase in PBMC just collected from AD patients when compared to elderly donors. On the contrary, Larbi and coworkers [20] show that CD4+CD25^{high} are reduced in PBMC just collected from AD when compared with both young and old donors. Our experimental conditions are different as we observe an increase of basal expression of CD25 on total CD3 cultured for 48 h with medium only. So, in our opinion, the increase of CD3+CD25+ cells in AD patients might be the consequence of the basal inflammatory milieu of AD patients [3,6–10,31].

The increased expression of the scavenger receptor CD36 on monocytes from AD subjects in unstimulated and stimulated cultures is also an intriguing result as circulating monocytes might efficiently bind plasmatic $A\beta$. That causes the production of cytokines, chemokines, and reactive oxygen species, hence activating the signaling cascade useful for cellular migration, adhesion, and phagocytosis. In addition, the engagement of monocytes might render these cells more efficient in T cell activation [34].

Our data show that, in addition to expressing chemokine receptors, PBMC *in vitro* stimulated by $rA\beta_{42}$ are able to produce different chemokines and cytokines, rendering these cells active players in the inflammatory response in AD patients. The study of *in vitro* production of cytokines shows a significantly higher production of inflammatory cytokines IL-1 β , IL-6, and TNF- α and of IFN- γ by AD *in vitro* stimulated PBMC. This is not a surprising result as inflammation is a characteristic of AD [3] and a high responder cytokine profile is associated with AD [9,10,32]. We

also report an increase of anti-inflammatory cytokines IL-10 and IL-1ra, and we hypothesize that this increase *in vitro* production should balance the higher *in vitro* production of pro-inflammatory cytokines. However, as previously stated, there is an efflux of amyloid from CNS that can prime lymphocytes. Some authors have demonstrated a reduction of both pro- and anti-inflammatory cytokines, hence assuming a general impairment of immune functions in AD patients, whereas others have demonstrated a decrease of IL-10, an increase of MIP1- α and an increase of IFN- γ , respectively [12,18,19,26]. Methodological differences (mitogen or $A\beta$ stimulation) among the different studies, including inclusion criteria of both AD patients and healthy controls, might explain the great variability of data. Since monocytes are the main source of IL-6 and TNF- α and they possibly bind efficiently $A\beta_{42}$ via CD36, the pattern of cytokine production observed in the present paper is that to be expected.

It has been demonstrated that peripheral T lymphocytes of AD patients produce higher MIP-1 α levels than age-matched controls [18]. This observation together with the expression of the MIP-1 α receptor CCR5 on the human brain microvascular endothelial cells (HBMEC), might explain the migration of T cells across the blood-brain barrier. Microglial cells also produce MIP-1 α . It has been demonstrated that MCP-1 via CCR2 expressed on brain endothelial cells contribute to increased brain endothelial permeability [12,18,35–37]. Here we show a higher expression of CCR5 on T and B lymphocytes, and of CCR2 on T cells stimulated *in vitro* by $rA\beta_{42}$, participating in a vicious circle in the brain.

In contrast to these data, in our system we do not observe any significant overproduction of MIP-1 α in PBMC *in vitro* stimulated by $rA\beta_{42}$. This discrepancy might be due to the different experimental system since the production-binding of MIP-1 α “*in vivo*” or “*in vitro*” was assessed using HBMEC [18]. As previously described by others [38], our AD patients show increased production of RANTES binding CCR5 also, when compared to unstimulated cells of the same donors. Finally, the results of MCP-1 production are very unpredictable in the different individuals of the two subject cohorts, so it is impossible to draw any conclusion.

A higher level of chemokines after stimulation with PHA has been demonstrated by other authors [38]. We have not evaluated the production of cytokines and chemokines from cells stimulated with α CD3 or PHA, but in our system the stimulation with mitogens does

not change the expression of CCR5 and CCR2 on T and B cells.

Finally, cytokine, growth factor, and chemokine production was obtained in a bulk culture preparation as we are interested in an *ex-vivo* model of interaction between different cells and factors. Furthermore, no proliferation assay was performed, because in our system no changes in IL-2 production were detectable after stimulation by rA β ₄₂ in both AD and HC (data not shown).

On the whole, our data demonstrate that immune-inflammatory parameters are modified in PBMC obtained from AD patients and stimulated *in vitro* by rA β ₄₂. Data on involvement of the immune system in AD are controversial and difficult to fully understand. The results obtained in our study can be the mirror of T and microglial abilities in AD patients. Therefore, these data on the activation of peripheral lymphocytes by rA β ₄₂ support the occurrence in AD of the systemic activation of immune-inflammation [31,39,40], and, on the other hand demonstrated by the high level of basal activation. We know that the brain is not an immunologically privileged site since peripheral immune cells can influence local reactions and can be influenced by local immune-inflammatory responses [12].

We contribute new information about soluble factors and cellular ligands that might be involved in the pathogenesis or in the tissue damage observed in AD patients. However, our study is an observational one; hence we cannot draw a conclusion on its contribution to the pathophysiology of the disease. The knowledge that senile plaques, a hallmark of AD (together with neurofibrillary tangles) are formed by A β , has suggested the design of immunotherapies with the aim of removing or reducing the senile plaques from the brain. Unfortunately clinical trials of A β vaccination of AD patients show an unacceptable rate of meningoencephalitis [41]. On the other hand, intravenous immunoglobulin (IVIg) has been proposed as a potential agent for AD immunotherapy because it contains antibodies against A β . Accordingly, a retrospective case-control analysis demonstrated that previous treatment with IVIg is associated with a reduced risk of developing AD [42]. Thus, this kind of study might be useful to obtain biomarkers of AD for monitoring the effectiveness of therapeutic interventions [43].

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