

# Increased Pro-Inflammatory Response by Dendritic Cells from Patients with Alzheimer's Disease

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**Abstract.** Alzheimer's disease (AD) is characterized by abnormal accumulation of amyloid- $\beta$  peptide ( $A\beta$ ) into extracellular fibrillar deposits, paralleled by chronic neuroinflammatory processes. Although  $A\beta$  seems to be causative in AD brain damage, the role of the immune system and its mechanisms still remain to be clarified. We have recently shown that normal monocyte-derived dendritic cells (MDDCs), when differentiated in the presence of  $A\beta_{1-42}$ , acquire an inflammatory phenotype and a reduced antigen presenting ability. Here we studied MDDCs derived from AD patients in comparison with MDDCs obtained from healthy control subjects (HC). MDDCs from AD patients, at variance with HC-derived cells, were characterized by an augmented cell recovery, a consistent increase in the expression of the pro-inflammatory ICAM-1 molecule, a decrease in the expression of the co-stimulatory CD40 molecule, and an impaired ability to induce T cell proliferation. Furthermore, MDDCs from AD produced higher amounts of IL-6 than HC-derived cells, confirming the more pronounced pro-inflammatory features of these cells in AD patients. Consistent results have been also obtained with monocytes, the MDDC precursors. In fact, while unstimulated monocytes do not appear to be different in AD and HC, after stimulation with lipopolysaccharide, AD monocytes overexpressed ICAM-1 with respect to controls, suggesting that common pathways of monocyte activation and MDDC differentiation are altered in AD. Overall, these findings show that AD-linked dysregulated immune mechanisms exist, which lead to dendritic cell-mediated over-activation of inflammation and impaired antigen presentation, thus supporting the view that immune cell activation could play an important role in AD pathogenesis.

Keywords: Alzheimer's disease, innate immune cells, inflammation, monocyte-derived dendritic cells, monocytes

## INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disease that leads to progressive neuronal loss and cognitive impairment. Principal hallmarks of the disease are brain extracellular deposits named senile plaques, mainly constituted of amyloid- $\beta$  peptides ( $A\beta$ ), and intracellular neurofibrillary tangles of hy-

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perphosphorylated tau protein [1]. Moreover, cerebral inflammatory processes may play a pivotal role in AD. In fact, the brains of AD patients exhibit a prominent activation of innate immune responses characterized by elevated levels of inflammatory mediators. This local inflammatory response, likely triggered by abnormal  $A\beta$  deposits, is mainly orchestrated by resident cells surrounding the senile plaques, such as activated microglia [2,3], but a possible contribution has been also recently proposed for blood-derived cells which have been suggested to participate in modulating senile plaque formation [4]. The role of activated microglia and their involvement in the pathogenesis of AD is, to date, not well defined. Notably, microglia are the immunocompetent cells of the brain, resembling other phagocytic immune cells, such as macrophages and dendritic cells (DCs). In particular, DCs are the most efficient antigen-presenting cells (APC) of the human body, with the unique capacity to prime, sustain, and control immune responses [5]. Thus, DCs stand at the crossroads between innate and adaptive immunity and their dysregulation has become apparent in the immunopathogenesis of several chronic neuroinflammatory diseases [6]. In AD brain, the existence of a microglia subset with DC-like features and strong ability to influence the disease has been hypothesized [7]. Furthermore, DC-like cells have been extensively characterized in the central nervous system (CNS). They could be found as resident cells of the CNS [8] developing from brain microglia [9]; alternatively, they could infiltrate into the CNS from the blood [10], although the mechanism is yet to be determined. Contrary to other neurodegenerative diseases such as multiple sclerosis or prion diseases, where the involvement of DCs is widely documented [11,12], in AD no data so far have been reported about DC participation in the disease. In a previous study, we suggested a role for DCs in the neuroinflammatory processes in AD by demonstrating that normal monocyte-derived dendritic cells (MDDCs) obtained from healthy donors, when differentiated in the presence of  $A\beta_{1-42}$ , show a reduction of HLA expression molecules with inefficient presenting antigen ability and acquire a pronounced inflammatory profile [13]. In the present study, we analyzed MDDCs obtained from AD patients in comparison with the same cells derived from matched healthy control subjects (HC). We further analyzed the precursors of MDDCs, the monocytes, both in their circulating unstimulated form and after *in vitro* lipopolysaccharide (LPS)-mediated activation in both groups of subjects. Cell recovery, phenotype, and functional characteristics of

both MDDCs and monocytes from AD and HC were evaluated in order to identify potential dysregulation in innate immune cells, which could reflect the chronic inflammatory response underlying the brain damage in AD.

## MATERIALS AND METHODS

### Subjects

Twenty patients with a diagnosis of probable AD consistent with NINCDS-ADRDA criteria were included. These subjects were drug free and underwent the first clinical examination for the diagnosis of AD. All subjects were assessed with the Mental Deterioration Battery (MDB) [14] to investigate performances in cognitive domains (neuropsychological battery is described in the experimental procedures section). Exclusion criteria included: i) major medical illnesses and autoimmune-inflammatory diseases; ii) comorbidity of primary psychiatric or neurological disorders; iii) known suspected history of alcoholism or drug abuse; or iv) MRI evidence of focal parenchymal abnormalities.

Twenty subjects were included as healthy controls. These control subjects were neither related to one another nor to the AD patients, and their inclusion criteria were: i) vision and hearing sufficient for compliance with testing procedures; ii) laboratory values within the appropriate normal reference intervals; and iii) neuropsychological domain scores above the cutoff scores, corrected for age and educational level, identifying normal cognitive level in the Italian population. Exclusion criteria were: i) major medical illnesses, known or suspected history of alcoholism or drug dependence and abuse during lifetime; ii) mental disorders (i.e., schizophrenia, mood disorders, anxiety disorders, personality disorders, and any other significant mental disorder) according with DSM-IV criteria [15] assessed by the Structured Clinical Interviews for DSM-IV Axis I (SCID-I) [16] and Axis II (SCID-II) [17], and/or neurologic disorders diagnosed by an accurate clinical neurological examination; iii) dementia diagnosis, according with DSM-IV criteria [18] or mild cognitive impairment according with Petersen criteria [19], and confirmed by the administration of the MDB; iv) Mini Mental State Examination (MMSE) score  $< 26$  [20]; or v) presence of vascular brain lesions, white matter lesions, brain tumor and/or cortical and subcortical atrophy, even of mild level, on MRI scan. Two expert neu-

Table 1  
Demographic and clinical characteristics of subjects

	HC (n = 20)	AD (n = 20)
Gender (M/F)	5/15	5/15
Age (y)	73.1 ± 1.6	73.5 ± 1.5
Education (y)	10.5 ± 1.0	6.3 ± 0.9*
MMSE score	28.6 ± 0.3	20.7 ± 1.1**
Disease duration (y)	–	1.8 ± 0.3

Data are expressed as mean ± SEM. \*AD versus HC,  $p = 0.004$ ; \*\*AD versus HC,  $p = 0.0001$ .

roradiologists examined all MRIs to exclude potential brain abnormalities. Both of them were blind to participant identities. White matter lesions were considered present if they were hyperintense on both FLAIR and T2 weighted images. We included only subjects who, in the opinion of both observers, did not have any lesion. Thus, also one visible lesion of any dimension was considered as an exclusion criterion. These volunteers were examined using a 3T Allegra MR Imager.

The study was performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki, reviewed and approved by the local ethical committee. All subjects gave their informed consent prior to their inclusion in the study. Demographic and clinical characteristics of enrolled subjects are summarized in Table 1.

### Cognitive evaluation

A trained neuropsychologist conducted the cognitive evaluations. To obtain a global index of cognitive level, the MMSE was administered. To assess each single cognitive domains, the MDB was used. The MDB is a standardized and validated neuropsychological instrument, comprising cognitive tests pertaining to the elaboration of verbal and visuospatial materials [21].

### Cells

After collecting 30 ml of blood by venipuncture, erythrocytes were removed by FACS lysing solution following manufacturer's indication (BD Pharmingen, San Diego, CA) and cells were stained to characterize circulating monocytes by flow cytometry, as described hereafter. In alternative, monocytes were analyzed following LPS stimulation of peripheral blood mononuclear cells (PBMCs). As previously described [22], PBMCs were freshly isolated from heparinized venous blood by centrifugation on density gradient (Lympholyte-H, Cederlane, Hornby, Canada). Then  $2 \times 10^6$  PBMCs, suspended in 0.5 ml of complete

medium (RPMI 1640 medium, Invitrogen Life Technologies, supplemented with 10% fetal bovine serum, Hyclone, Logan, UT), were plated in 48-well plates and incubated at +37°C for 18 h with or without LPS (200 ng/ml; *E. coli* strain O55:B5, Sigma-Aldrich, St. Louis, MO). After incubation, PBMCs were collected and stained to analyze membrane marker expression by flow cytometry. The percentages (mean ± SEM) of CD14<sup>+</sup> monocytes recovered by PBMC cultures were similar between AD patients and HC, both in untreated (AD = 13.5 ± 1.8; HC = 11.4 ± 1.8) and LPS-stimulated cells (AD = 15.4 ± 1.6; HC = 15.3 ± 2.1).

MDDCs were generated from CD14<sup>+</sup> circulating monocytes, which in turn were separated from PBMCs by using anti-CD14 mAb-coupled magnetic beads followed by MACS column separation (Miltenyi Biotec, Bologna, Italy) according to the manufacturer's protocol. Purified CD14<sup>+</sup> cells (purity > 98%) were cultured for 8 days at the final density of  $1.5 \times 10^6$  cells/ml in 24-well plates (Costar, Cambridge, MA), in fresh complete medium supplemented with 50 ng/ml GM-CSF and 10 ng/ml IL-4 (Euroclone, Milan, Italy). Such cell populations have been indicated throughout the study as immature MDDCs. In order to induce DC maturation, 200 ng/ml of LPS was added to immature MDDCs for the last 48 h of culture (LPS-MDDCs). On day 8, both immature and mature MDDCs were collected and analyzed. Cell count and viability were determined by trypan blue (Sigma) exclusion and confirmed by flow cytometry analysis.

### Flow cytometry and mAbs

The following fluorescent dye-labeled mAbs (all from BD) were used for flow-activated cell sorting (FACS) analysis: anti-HLA-DR (clone L243), anti-HLA-ABC (clone G46-2.6), anti-CD80 (clone L307.4), anti-CD86 (clone 2331/FUN-1), anti-CD83 (clone HB15e), anti-CD14 (clone M5E2), anti-CD1a (clone HI149), anti-CD11c (clone B-ly6), anti-CD40 (clone 5C3), anti-ICAM-1 (clone HA58); negative controls were isotype-matched mAbs. To determine membrane phenotype, cells were washed in assay buffer (PBS, 0.5% BSA, and 0.1% sodium azide), incubated with fluorescent mAbs for 15 min at +4°C, washed and then fluorescence emission of cell suspension was analyzed by flow cytometry. Samples were acquired with a FACSCalibur flow cytometer (BD Immunocytometry Systems, San Jose, CA). Data were analyzed using CellQuest software (BD; version 3.2.1) and expressed as mean fluorescence intensity (MFI).

### Endocytosis

Endocytic activity of MDDCs was assessed by measuring uptake of FITC-dextran (Sigma) as previously described [13]. Briefly, cells were incubated for 1 h with 1 mg/ml FITC-dextran at either +37°C, or +4°C as control, then washed three times with PBS. Ten thousand cells of each sample were analyzed by flow cytometry as above described. Finally, dextran endocytosis was quantified as MFI values.

### Cytokine assays

Recommended pairs of specific antibodies (coating and detecting) and standards for TNF- $\alpha$ , IL-1 $\beta$ , IL-6, IL-10, and IL-12 (p70) were purchased from Endogen (Woburn, MA) and used according to the manufacturer's instructions. IL-18 was determined by ELISA using coating antibody (clone 125-2H), detecting antibody (clone 159-12B), and standard human recombinant IL-18 all from MBL (Nagoya, Japan). The detection limit was 15 pg/ml for all the cytokines tested.

### Mixed leukocyte reaction

The ability of MDDCs to stimulate allogeneic T cell responses was analyzed by mixed leukocyte reaction (MLR). PBMCs were isolated, as previously described, from peripheral blood of healthy donors, then naïve CD4<sup>+</sup>/CD45RA<sup>+</sup> T cells were negatively selected by naïve CD4<sup>+</sup> T cell isolation kits (Miltenyi Biotec). Immature and mature MDDCs derived from both AD and HC subjects were washed three times with PBS, suspended in fresh complete medium, then co-cultured at different cell numbers with  $1 \times 10^5$  allogeneic CD4<sup>+</sup> T cells in 96-well, U-bottom culture plates for 4 days. T cell proliferation was measured by adding 1  $\mu$ Ci/well of <sup>3</sup>H-thymidine (Amersham Pharmacia, Aylesbury, UK) for the last 16 h of culture. Incorporation of <sup>3</sup>H-thymidine was quantified using a  $\beta$ -counter (Microbeta, PerkinElmer, Boston, MA).

### Statistical analysis

Analysis was performed using the Prism version 4 (GraphPad software, San Diego, CA). Statistical significance was assessed using the Mann-Whitney test. Two-way analysis of variance (ANOVA) followed by the Bonferroni's *post hoc* test was used to evaluate the effect of the AD diagnosis on T cell proliferation induced by MDDCs. Differences were considered significant at  $p < 0.05$ .

## RESULTS

### Increased MDDC recovery in AD versus HC

In order to analyze possible DC alteration in AD patients, human monocytes from both AD and HC subjects were purified from PBMCs and differentiated *in vitro* to generate MDDCs. At the end of the differentiation procedure, the recovery of MDDCs was evaluated in terms of living cell numbers. As reported in Fig. 1, although the starting number of monocytes used to generate DCs was the same in all tested conditions, the cell yield after the 8 day-culture was significantly higher when MDDCs were generated from AD patients, compared to HC subjects. In fact, a statistically significant increase in cell recovery was observed in AD-derived versus HC-derived MDDCs at both their immature ( $p = 0.0001$ ) and mature ( $p = 0.0002$ ) stages.

### Comparable antigen internalization ability by MDDCs between AD and HC

MDDCs derived from AD and HC subjects were further analyzed for their functional properties. Since MDDCs, particularly in their immature form, are very efficient at internalizing soluble antigens, we evaluated their antigen uptake capability by measuring FITC-dextran cell internalization. The amount of endocytosis of immature MDDCs, reported as MFI values  $\pm$  SEM, were  $38 \pm 10$  in AD, and  $47 \pm 8$  in HC. No statistically significant differences in endocytosis ability between AD- and HC-derived immature MDDCs were evidenced. As expected, mature MDDCs from both subject groups completely lose their ability to uptake antigens (not shown).

### Increased ICAM-1 and decreased CD40 expression by MDDCs in AD versus HC

In order to assess whether MDDC phenotype was altered in AD patients in comparison to HC subjects, cell surface expression of selected markers on both immature and mature MDDCs was analyzed by flow cytometry. In Fig. 2, a characteristic panel of surface molecule expression is reported in terms of fluorescence histograms for MDDCs derived from two donors, each of them representative for HC or AD subject group. No substantial differences were observed between the two subjects groups. The same results are summarized in Table 2, where the phenotypic data of MDDCs derived from all subjects are shown in terms of mean MFI val-

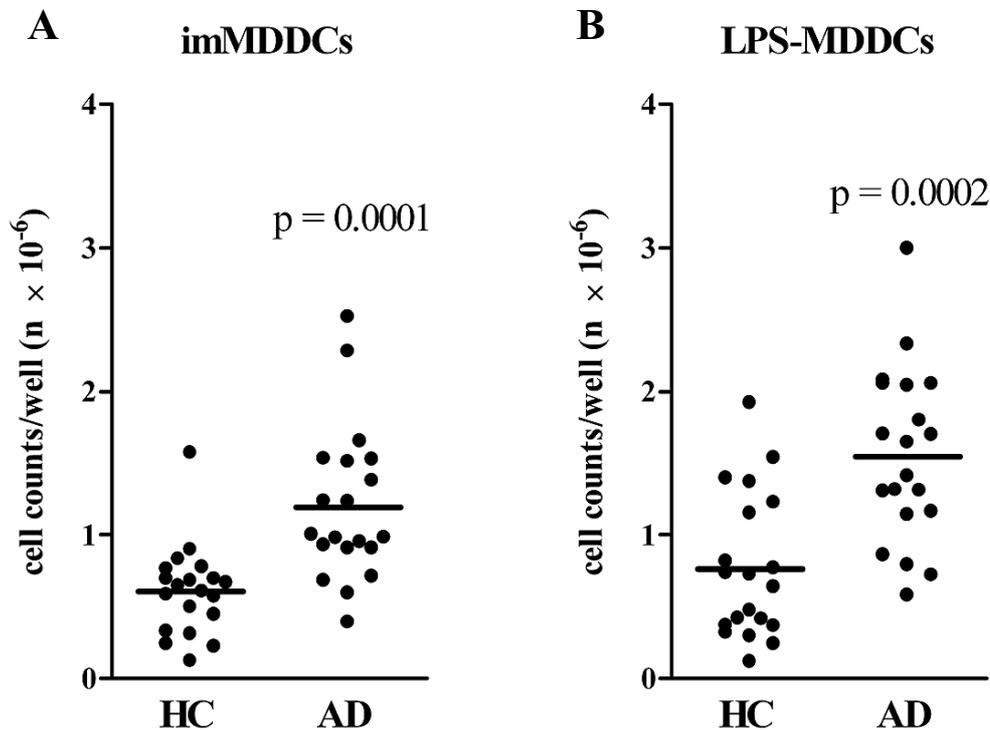


Fig. 1. Increased cell recovery of MDDCs obtained from AD patients. AD- and HC-derived MDDCs were counted by trypan blue exclusion at the end of the 8 day-culture, at immature stage (A) or following stimulation with LPS (B). Single results from 20 AD patients and 20 HC subjects were reported, bars = medians. Significant difference AD-derived versus HC-derived in immature ( $p = 0.0001$ ) and mature ( $p = 0.0002$ ) MDDCs.

ues. More in detail, CD1a and CD11c were expressed, as expected, on all MDDCs at both their immature and mature stage. Moreover, the presentation molecules HLA-ABC and HLA-DR, similar to the co-stimulatory molecules CD80 and CD86, were partially expressed in immature MDDCs and upregulated in all mature cells. Finally, the maturation molecule CD83 was expressed specifically on mature MDDCs. Taken together, these data indicate that the DCs we differentiated and matured from both HC and AD subjects display a typical immunophenotype of *in vitro* cultured MDDCs.

Interestingly, a difference between AD and HC subjects was found in some other specific cell surface markers. In particular, the pro-inflammatory cell adhesion molecule ICAM-1 was increased in AD- versus HC-derived MDDCs, as shown in Fig. 3. In fact, as reported both in the representative histogram plots referred to a single individual of each subject group (Fig. 3A) and in the scatter plots referred to all subjects (Fig. 3B and 3C), MDDCs derived from AD patients expressed higher levels of ICAM-1 than HC subjects both at immature ( $p = 0.009$ ) and mature ( $p = 0.04$ ) cell stages.

Another significant difference in cell surface marker expression between AD and HC was monitored regarding the co-stimulatory molecule CD40 (Fig. 4). The level of expression is shown both in terms of fluorescence histograms of MDDCs derived from two subjects, each representative of HC or AD group (Fig. 4A), and as scatter plot from all HC and AD subjects (Fig. 4B and 4C). As expected, CD40 was upregulated by LPS in both HC donors (immature versus mature MDDCs,  $p = 0.001$ ) and AD patients (immature versus mature MDDCs,  $p = 0.003$ ), but no difference between the two groups of subjects was observed in immature cells. On the contrary, the level of CD40 expression on LPS-MDDCs was significantly lower in AD with respect to HC subjects ( $p = 0.02$ ). Taken together, these data suggest a possible alteration of AD-derived MDDCs both in terms of a general increase in pro-inflammatory cell features and, specifically in mature cells, a reduction of co-stimulatory ability.

#### Decreased APC ability by MDDCs in AD versus HC

Since a peculiar function of MDDCs is their capacity, particularly strong after maturation, to present the

Table 2  
Surface molecule expression in MDDCs derived from AD and HC subjects

cells	group	CD1a	CD11c	HLA-ABC	HLA-DR	CD83	CD80	CD86
imMDDCs	HC	36.3 ± 5.8	43.9 ± 5.5	12.6 ± 1.9	33.5 ± 7.6	4.4 ± 0.4	19.6 ± 2.7	14.7 ± 2.4
	AD	49.9 ± 9.4	54.0 ± 6.4	13.5 ± 2.7	36.6 ± 5.1	4.3 ± 0.7	17.3 ± 1.5	11.0 ± 1.3
LPS-MDDCs	HC	63.2 ± 8.9	98.5 ± 10.2	24.2 ± 2.5	59.7 ± 5.4	15.2 ± 2.1	115.3 ± 16.2	108.9 ± 19.8
	AD	52.4 ± 6.4	80.0 ± 7.2	24.7 ± 2.7	67.4 ± 10.0	12.2 ± 1.0	96.1 ± 10.1	90.9 ± 12.2

Data are expressed as mean ± SEM of MFI values (HC and AD,  $n = 20$ ).

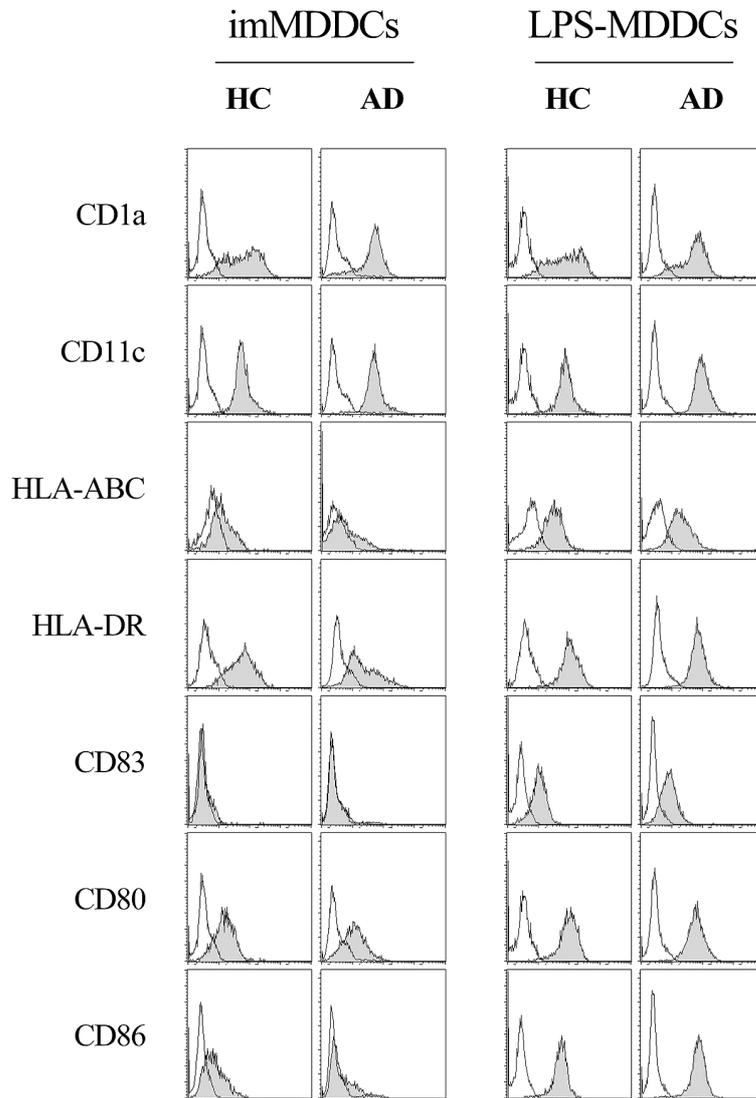


Fig. 2. Comparable expression of phenotype markers in MDDCs derived from AD patients and HC subjects. Flow cytometric analysis of CD1a, CD11c, HLA-ABC, HLA-DR, CD83, CD80, and CD86 molecule expression on immature (imMDDCs) and mature (LPS-MDDCs) MDDCs from two subjects, each of them representative of HC or AD subject group. Fluorescence histograms for each surface molecule (filled histograms) in comparison with isotype controls (empty histograms) are reported. Data are from a single subject/group, representative of HC ( $n = 20$ ) and AD ( $n = 20$ ) subjects, all with similar results.

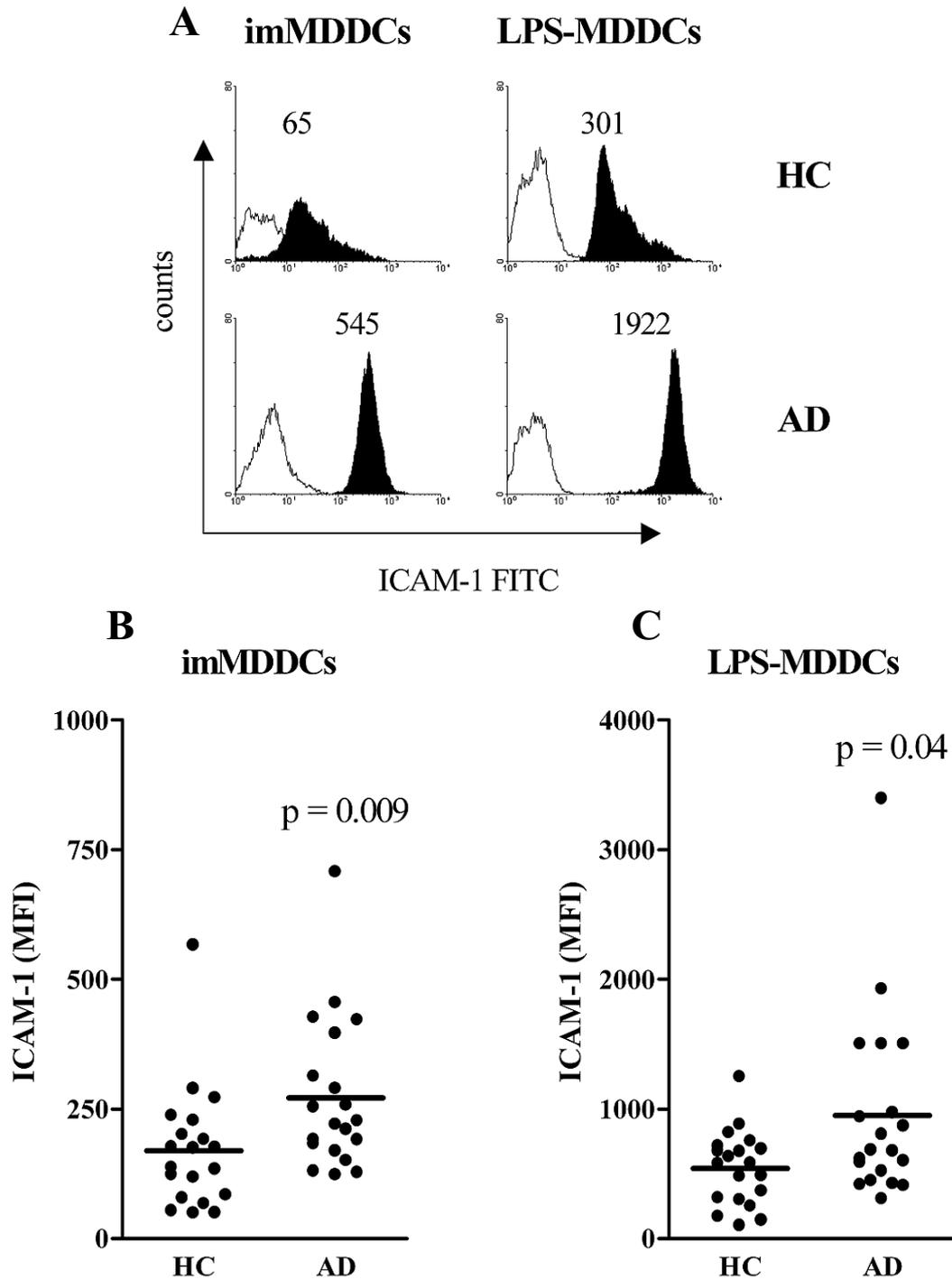


Fig. 3. Increased membrane expression of ICAM-1 both in immature and mature MDDCs derived from AD patients. (A) Flow cytometric analysis of ICAM-1 molecule expression from immature and mature MDDCs from two subjects, each of them representative of HC or AD subject group. Fluorescence histograms for ICAM-1 (filled histograms) in comparison with isotype-matched control (empty histograms) are reported. MFI values are indicated in correspondence of the fluorescent peak in each panel. Data from 20 HC subjects and 20 AD patients were shown for immature MDDCs (B) and LPS-matured MDDCs (C), bars = medians. Significant difference AD-derived versus HC-derived immature ( $p = 0.009$ ) and mature ( $p = 0.04$ ) MDDCs.

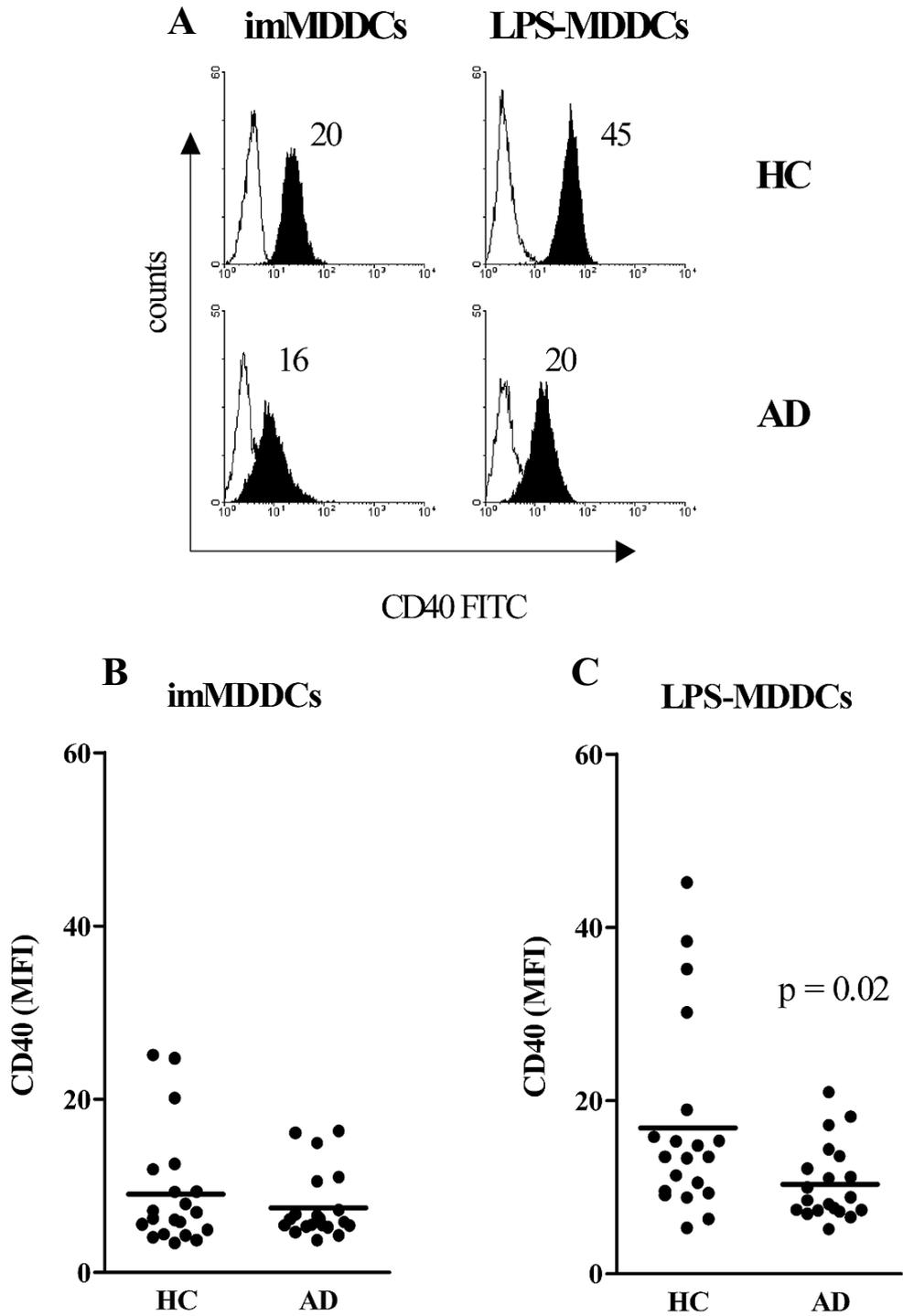


Fig. 4. Decreased membrane expression of CD40 in mature MDDCs derived from AD patients. (A) Flow cytometric analysis of CD40 molecule expression from immature and mature MDDCs from two subjects, each of them representative of HC or AD subject group. Fluorescence histograms for CD40 (filled histograms) in comparison with isotype-matched control (empty histograms) are reported. MFI values are indicated in correspondence of the fluorescent peak in each panel. Data from 20 HC subjects and 20 AD patients were shown for immature MDDCs (B) and LPS-matured MDDCs (C), bars = medians. Significant difference AD-derived versus HC-derived mature ( $p = 0.02$ ) MDDCs.

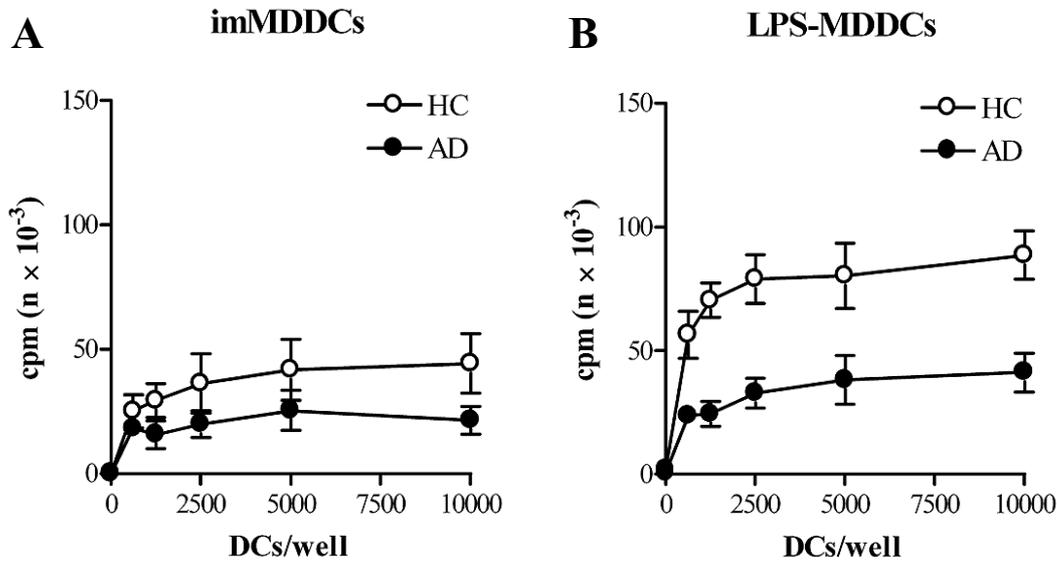


Fig. 5. Decreased ability to stimulate allogeneic T cell proliferation in mature MDDCs derived from AD patients. MDDCs derived from AD patients and HC donors, immature (A) or matured with LPS (B), were cultured at different cell numbers with  $1 \times 10^5$  allogeneic CD4<sup>+</sup>/CD45RA<sup>+</sup> T cells. Proliferation was determined on day 4 of co-culture by addition of <sup>3</sup>H-thymidine for the last 16 h. Data are shown as mean  $\pm$  SEM from independent experiments performed in triplicates on 3 AD patients and 3 HC subjects. Significant difference AD-derived vs. HC-derived LPS-MDDCs ( $p = 0.01$ , two-way ANOVA).

antigen and prime naïve T cells, a MLR was performed to investigate a possible alteration of APC ability in AD. Thus, allogeneic naïve CD4<sup>+</sup>/CD45RA<sup>+</sup> T cells were co-cultured with both immature and mature MDDCs originated from the two groups of subjects and T cell proliferation was eventually measured. As shown in Fig. 5A, immature MDDCs from both AD and HC slightly induced T cell proliferation without any significant difference between the two groups of subjects. Interestingly, when LPS-matured MDDCs from AD and HC were compared (Fig. 5B), we observed a significantly reduced ability to prime T cell proliferation by AD cells (two-way ANOVA,  $p = 0.01$ ). In fact, whereas LPS-MDDCs from HC donors induced significant higher levels of T cell proliferation with respect to the same cells at their immature stage (two-way ANOVA,  $p = 0.01$ ), immature and LPS-MDDCs from AD patients showed a fully comparable ability to induce T cell proliferation. In conclusion, as also previously indicated by the reduced expression of the co-stimulatory CD40 molecule, MDDCs obtained from AD patients have an impaired APC ability.

#### Increased IL-6 production by MDDCs in AD versus HC

Since environmental conditions and, in particular, the cytokine secretion profile are crucial for the driv-

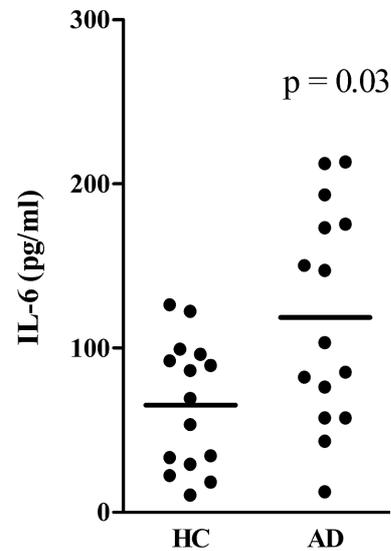


Fig. 6. Increased IL-6 production by immature MDDCs derived from AD patients. Cell supernatants derived from both immature and LPS-treated MDDCs were collected at the end of the 8 day-culture and cytokines measured by ELISA. Scatter graph represents IL-6 levels expressed as pg/ml produced by immature MDDCs obtained from 15 AD patients and 15 HC donors. Significant difference AD-derived versus HC-derived immature MDDCs ( $p = 0.03$ ).

ing strength and characteristics of the immune responses, we measured the amounts of some pro- and anti-inflammatory cytokines released by immature and LPS-

Table 3  
Cytokine production by MDDCs derived from AD and HC subjects

cells	group	IL-6	IL-1 $\beta$	TNF- $\alpha$	IL-18	IL-12	IL-10
imMDDCs	HC	61 $\pm$ 10	< 15	< 15	20.1 $\pm$ 6.7	< 15	18 $\pm$ 0.3
	AD	119 $\pm$ 17*	< 15	< 15	23.8 $\pm$ 9.8	< 15	18 $\pm$ 0.3
LPS-MDDCs	HC	13938 $\pm$ 2164	43.0 $\pm$ 13.5	1794 $\pm$ 361	25.9 $\pm$ 3.6	1672 $\pm$ 335	147 $\pm$ 52
	AD	15658 $\pm$ 2416	36.5 $\pm$ 17.7	1835 $\pm$ 313	36.8 $\pm$ 15.7	2482 $\pm$ 779	219 $\pm$ 80

Data are expressed as mean  $\pm$  SEM of pg/ml (HC and AD,  $n = 15$ ). \*AD versus HC,  $p = 0.03$ .

matured MDDCs from AD and HC subjects. As summarized in Table 3, taking into consideration the immature MDDCs, no substantial differences were observed in all cytokines between AD and HC, with the only exception being IL-6, which was significantly higher in AD as compared to HC cells. Furthermore, although a mild increase in the levels of both pro- and anti-inflammatory mediators (IL-6, TNF- $\alpha$ , IL-18, IL-12, and IL-10) was observed in mature cells derived from AD as compared to HC, the differences between the two subject groups were not statistically significant in all mature MDDC cultures. In Fig. 6, the scatter plot of IL-6 production in immature MDDCs from the two groups of subjects is reported, showing that this cytokine is significantly higher in immature MDDCs derived from AD as compared to HC ( $p = 0.03$ ). This data set further confirms that AD-derived MDDCs have increased pro-inflammatory features as compared to HC-derived cells.

#### *Increased ICAM-1 and HLA-DR expression by stimulated monocytes in AD versus HC*

In order to investigate whether the defects observed in AD-derived MDDCs were also present in their precursor cells, we analyzed circulating monocytes. We observed that the percentage (mean values  $\pm$  SEM) of blood CD14<sup>+</sup> cells with monocyte morphology, as evaluated by flow cytometry, was comparable in AD patients (5.0  $\pm$  0.3) versus HC subjects (5.8  $\pm$  0.4). Similarly, the phenotype analysis performed on HLA-DR, CD11c, and ICAM-1 molecule expression confirmed the resemblance between AD and HC unstimulated circulating monocytes (data not shown). On the other hand, when PBMC were cultured for 18 h in the presence of LPS and subsequently monocytes analyzed, some striking differences between AD and HC were detected. In particular, the expression of ICAM-1 and HLA-DR, which was comparable between AD and HC unstimulated monocytes, was instead significantly increased following LPS exposure in AD compared to HC monocytes (Fig. 7). Monocyte recovery from HC and AD donors was the same in both unstimulated and LPS-stimulated conditions (data not shown).

## DISCUSSION

Our previous study demonstrated that MDDCs obtained from healthy donors, when differentiated *in vitro* in the presence of the amyloid peptide A $\beta_{1-42}$ , showed an increased recovery, a reduced expression of both HLA molecules and APC ability and, interestingly, acquired an inflammatory phenotype [13]. Those data suggested that the pathogenic properties of A $\beta$  peptides could implicate a skewing of DC functions toward inflammatory features. The current study shows that MDDCs from AD patients are different from cells obtained from age-matched control subjects, since they have increased recovery, decreased expression of the costimulatory CD40 molecule, impaired ability to prime T cells, and increased expression of the pro-inflammatory molecules ICAM-1 and IL-6. Such results are in full agreement with our previous data.

We initially observed that MDDCs obtained from AD patients were different from HC-derived MDDCs in terms of cell recovery. In fact, in AD-derived MDDCs the increased cell number was already evident at the DC immature stage and maintained after the *in vitro* maturation period. On the contrary, no difference between AD and HC subjects was observed in the number of circulating monocytes, the cells from which MDDCs originate. Thus, our findings suggest that AD-derived MDDCs are specifically altered in their differentiation program. It is well known that the differentiation of monocytes toward immature DCs is regulated by a multiplicity of environmental factors, principally cytokines, that influence cell homeostasis and viability. Since inflammatory processes are increasingly implicated in AD [3], it is possible that the inflammatory status observed in AD brains could interfere with the physiological processes leading to the differentiation of immune cell precursors and could also have an effect in the periphery. However, further investigation is required to characterize the molecular mechanism involved in the differentiation pathway dysregulation of myeloid cells in AD patients.

The second important observation we reported in this study is that MDDCs from AD patients have an im-

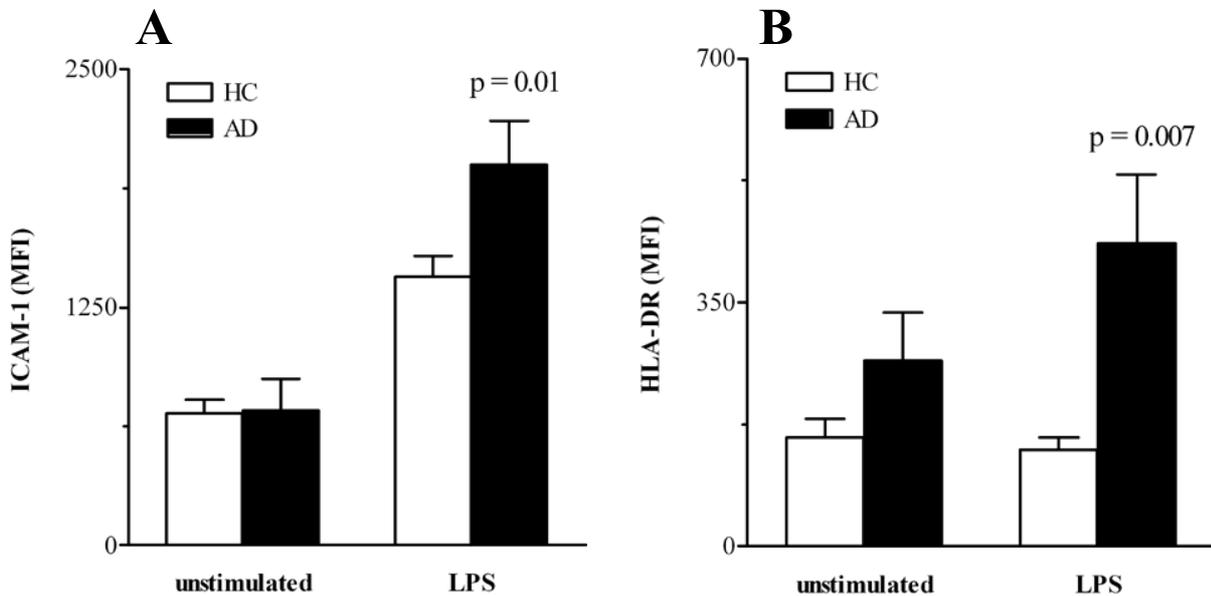


Fig. 7. Increased membrane expression of ICAM-1 and HLA-DR on LPS-stimulated monocytes derived from AD patients. Flow cytometric analysis of ICAM-1 (A) and HLA-DR (B) expression from AD- and HC-derived PBMCs cultured in the absence or in the presence of LPS (200 ng/ml) for 18 h. Mean MFI  $\pm$  SEM from 7 AD patients and 15 HC donors are reported. Significant difference AD- versus HC-derived LPS-stimulated monocytes (ICAM-1,  $p = 0.01$  and HLA-DR,  $p = 0.007$ ).

paired ability to function as APC and to activate T cells. Regarding the ability of MDDCs to uptake the antigen, as reported in this study, we did not observe any difference between AD and HC cells and these data were further confirmed by using  $A\beta_{1-42}$ -FITC as specific phagocytic stimulus (Ciarabella et al., personal observation). Different results were instead obtained when the co-stimulatory ability of MDDCs was taken into consideration. In fact, although the phenotype analysis of cellular molecules involved in DC differentiation did not show striking differences between AD and HC cells, a general reduction of co-stimulatory molecule expression was observed in AD-derived DCs, especially after their maturation. This trend became statistically significant with respect to CD40 molecules, suggesting that a defective maturation of DCs, selectively related to their ability to activate specific immune response, may also occur in AD. Indeed, CD40 signaling involvement has been largely suggested in  $A\beta$ -mediated pathogenesis of AD and, accordingly, it has been proposed to be involved in the conversion of activated microglia cells from the phagocytic to the APC phenotype [23]. Interestingly, this phenotype of AD-derived MDDCs characterized by reduced CD40 expression is further confirmed by the MLR data reported in this study, which clearly demonstrated that DCs obtained from AD patients have a reduced APC ability.

The third and major result of this study is that the expression of ICAM-1 on cell membrane of AD-derived DCs was higher than controls, both in immature and mature cells. Consistently, an increased expression of this molecule was also observed in LPS-stimulated monocytes of AD patients as compared to HC. The ICAM-1 molecule is expressed on DC surface and mediates DC-lymphocyte interaction. Its engagement promotes Th1 polarization with a consequent activation of an inflammatory immune response. Interestingly, ICAM-1 expression on DCs is strikingly upregulated by pro-inflammatory stimuli such as IL-18 [24], a cytokine which appears to be significantly increased in AD patients both at brain [25] and peripheral blood cell levels [22]. In general, ICAM-1 is considered a pro-inflammatory molecule and since neurodegenerative diseases, like multiple sclerosis or Parkinson's disease, are associated with a considerable degree of neuroinflammation, it is not surprising that ICAM-1 is aberrantly hyperexpressed by glial cells of patients affected by these diseases [26,27]. Regarding AD, it was observed that reactive astrocytes at the periphery of senile plaques and microglia stimulated with  $A\beta_{1-42}$  express high levels of ICAM-1 [28,29]. The above reported observations show a link between ICAM-1 expression and AD, both *in vivo* and *in vitro*. Interestingly, the increased expression of ICAM-1 is not lim-

ited to the brain, but it has also been shown in the periphery of AD patients [30,31]. More recently, soluble ICAM-1 was identified as one of the specific markers of AD and as a predictor of progression from mild cognitive impairment, the pre-clinical form of the disease, to AD [32]. Our data indicate that a selective and consistent overexpression of ICAM-1 occurs in MDDCs and activated monocytes obtained from AD patients, and this is especially true in response to inflammatory stimuli, such as LPS, which are able to activate signal transduction pathways common to those triggered by  $A\beta_{1-42}$  [33]. Thus, these results support the idea that ICAM-1 might be important in  $A\beta$ -mediated inflammatory pathways of AD, where an increased trafficking of leukocytes, likely including myeloid DCs and their precursors, may play a role. More generally, these results confirm that ICAM-1 overexpression is involved in neurodegenerative diseases.

The last outcome of this study is the observation that immature MDDCs from AD patients produce significantly increased amounts of IL-6 than MDDCs from HC donors. The involvement of IL-6 in AD has been widely demonstrated [2]. In fact, it was described that IL-6 is overexpressed in  $A\beta$  plaques [34] and, in the periphery, plasma IL-6 is significantly higher in AD patients than controls [35]. Moreover, a recent study demonstrated an increased production of IL-6, together with IL-8 and IL-10, in individuals with mild cognitive impairment suggesting that the alteration of immune parameters are early events in AD [36]. Furthermore, an interesting amplification mechanism between ICAM-1 expression and IL-6 production exists. In fact, upon proper stimulation (e.g., infection), DCs produce IL-6 which in turn induces ICAM-1 expression. At the same time, the ICAM-1 engagement can induce production of pro-inflammatory cytokines as IL-6. Concerning the CNS, it was described that ICAM-1 triggering upregulates IL-6 production in primary rat astrocytes [37] and that  $\alpha$ -synuclein strongly induces expression of both IL-6 and ICAM-1 in human astrocytes [38]. Therefore, although in this study we did not directly investigate the link between ICAM-1 and IL-6 expression, we cannot exclude a possible relationship between these two molecules, which we found simultaneously overexpressed by MDDCs derived from AD patients.

Circulating monocytes, the precursors of MDDCs, did not appear intrinsically abnormal in their unstimulated state in AD patients but, similarly to MDDCs, they were significantly hyper-responsive to inflammatory stimulation, with respect to HC monocytes, sup-

porting the notion that cells of the mononuclear phagocyte lineage are "primed" in chronic neurodegenerative diseases like AD [39]. Indeed our data are in agreement with previous studies showing that cultured AD monocytes, when compared to monocytes obtained from control subjects, produce higher amounts of cytokines and are defective in  $A\beta$  phagocytosis, in particular following stimulation [40,41].

In conclusion, the results of the present study indicate for the first time that MDDCs are functionally altered in AD patients. In fact, in agreement with a number of previous studies that reported an upregulation of inflammatory features in AD, our data indicate that cells of the monocyte lineage, in particular MDDCs, show an impaired APC ability paralleled by a greater propensity to respond to inflammatory stimuli when derived from blood of AD patients, as compared to the same cells obtained from healthy individuals. In line with our previous study demonstrating that human monocytes continuously treated with  $A\beta$  during all their *in vitro* differentiation process become dysregulated MDDCs, MDDCs obtained from AD patients have an impaired ability to prime a specific immune response and become highly efficient in amplifying inflammation, probably as a consequence of the long lasting *in vivo* stimulation due to the pathogenic  $A\beta$  persistence. Indeed, we observed that a 24-48 h *in vitro* stimulation of MDDCs with  $A\beta$  is not sufficient to modify the differentiation program already undertaken by cells. This observation is true both for MDDCs from AD as well as from HC donors, both young and elderly (Ciaramella et al., personal observations). It is thus tempting to speculate that subjects suffering from AD, possibly as a result of the inefficient attempt to eliminate  $A\beta$  elevation and because of its consequent accumulation, have dysregulated DCs, which ultimately may become responsible for sustaining and amplifying the persistent inflammatory condition leading to AD chronic brain damage. Therefore, these observations indicate DCs as players in orchestrating AD neuroinflammation, opening new avenues for a better understanding of the role of innate immune cells in the development of AD and suggesting DCs as potential cell targets for innovative therapeutic approaches aimed to limit or even prevent neuronal loss caused by neurodegenerative diseases.

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