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Systemic immune system alterations in early stages of Alzheimer's disease

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Abstract

Immune activation and inflammation play significant roles in the pathogenesis of Alzheimer's disease (AD). To test whether AD patients showed systemic manifestations of inflammation, blood from 41 patients with early stages of AD and 31 aged-match elderly controls were evaluated. Cellular markers for monocyte/macrophage (MO) activation and CD8 T lymphocyte were increased in early AD patients. Expression of monocyte CCR2, the receptor for monocyte chemoattractant protein-1 (MCP-1), was decreased; however, plasma MCP-1 levels were significantly increased and were related to degree of MO activation in AD. These findings suggest that AD pathogenesis may be influenced by systemic immunologic dysfunction and provides potential immunologic targets for therapeutic intervention.

Keywords

Alzheimer's disease; systemic immune activation/inflammation; monocyte/macrophage (MO); monocyte chemoattractant protein-1 (MCP-1)

1. Introduction

Alzheimer's disease (AD) is an age-related neurodegenerative disorder characterized by progressive impairment of memory and cognitive function leading to dementia. There are currently more than 5 million Americans afflicted with this disease, which has also become the seventh leading cause of death in this country (Galluzzi et al., 2010). In AD, brains are characterized by the presence of neurofibrillary tangles, prominent activation of a local inflammatory response and accumulation of β-amyloid into amyloid plaques. Apart from brain-specific changes, an increasing number of studies in AD have reported alterations in systemic immune responses including changes in lymphocyte and macrophage distribution and activation, the presence of autoantibodies, or abnormal inflammatory factors and cytokine production (Kusdra et al., 2000, Galimberti et al., 2006, Mruthinti et al., 2006, Speciale et al., 2007, Pellicano et al., 2010, Hochstrasser et al., 2011, Kim et al., 2011,

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Parker et al., 2013, Pellicano et al., 2012). Earlier studies on blood specimens from patients with AD also found elevated levels of plasma lipopolysaccharide (LPS), a potent inflammatory stimulus, and the degree of this elevation was directly related to levels of abnormally activated blood monocyte/macrophages (MO) in AD patients (Zhang et al., 2009). Most recently, Zhang *et al.* reported increased expression of Toll-like receptor 2 (TLR2) and TLR4 on peripheral blood mononuclear cells from AD patients (Zhang et al., 2012); both TLRs play a key role in inflammatory neurodegeneration binding the highly hydrophobic amyloid peptides or LPS in AD (Tahara et al., 2006, Walter et al., 2007, Udan et al., 2008). Together these data have led to the speculation that AD might be a systemic inflammatory disorder resulting in cognitive dysfunction associated changes in the CNS.

Although many studies implicate inflammation and systemic immune dysfunction in AD, little is known about how systemic immune abnormalities relate to AD pathogenesis. The present study was undertaken to determine the extent of cellular and plasma immunologic abnormalities in patients with early stages of AD. Immunophenotypic analyses and measurements of plasma cytokine and immunoglobulin levels were used to identify degree of immune activation in T-cell and monocyte subsets as well as in plasma from patients with early AD as compared to elderly controls. We report here evidence for significant immunologic abnormalities in the blood of patients with early AD.

2. Materials and Methods

2.1. Subjects

Forty-one patients diagnosed with AD (24 females and 17 males; age range: 58–91 years, mean 77.9 ± 7.7) at the Forbes Norris MDA/ALS Research Center (San Francisco, California, USA) had blood drawn in accordance with the CPMC (California Pacific Medical Center, San Francisco, California, USA) and UCSF committees on human research guidelines, coordinated by the UCSF AIDS and Cancer Specimen Resource (ACSR) program. Diagnosis of probable AD was achieved following the guidelines of the National Institute of Neurological and Communicative Disorders and Stroke- Alzheimer's Disease and Related Disorders (NINCDS-ADRDA criteria) (McKhann et al., 1984). Cognitive status was assessed using the Mini Mental State Examination (MMSE) and the global deterioration scale. AD patients were classified as mild AD, as evidenced by MMSE scores ranging from 21 to 28 out of 30 (mean 24.5 ± 2.1). Subjects with MMSE scores below 21 were excluded from the study due to issues related to informed consent.

Thirty-one age-matched elderly controls without any neurological signs and symptoms of dementia (15 females and 16 males; age range: 61–91 years, mean 75.4 ± 9.5) were from the San Francisco bay area and met criteria similar to that required for standard blood donation. All elderly control blood samples were processed in a similar manner to the AD patient blood specimens.

2.2. Flow Cytometry

10 ml of peripheral blood was drawn from each patient and elderly control into heparinized tubes and transferred to the laboratory at room temperature for same day immunologic studies. Cellular immunologic activation was evaluated by quantitating levels of CD38 on T-cell subsets and MHC antigen class II, HLA-DR, on CD14 cells. CD16 (Fc gamma III receptor) expression on CD14 cells was used as another marker for monocyte differentiation and has been an antigen associated with cytokine expression patterns characteristic of tissue macrophages (Ziegler-Heitbrock et al., 1993, Frankenberger et al., 1996). The monocyte granularity associated with its differentiation was measured by CD14-associated "backgating" on side light-scatter characteristics (SSC). Whole blood was stained with anti-CD14-fluorescein isothiocyanate (FITC), anti-CD16-phycoerythrin (PE) (DAKO,

Carpinteria, CA, USA), anti-CCR2-PE (R&D Systems Inc., Minneapolis, MN, USA), anti-HLA-DR-PE, anti-CD8-FITC, anti-CD38-PE, and anti-CD4-peridinin chlorophyll protein (PerCP) (Becton-Dickinson, San Jose, CA, USA) for 30 minutes at room temperature. Negative controls consisted of aliquots stained with isotype IgG-FITC, IgG-PE, and IgG-PerCP; all staining was performed as per manufacturer's specifications. Samples were then lysed with FACS Lysing Solution (Becton-Dickinson) for 10 minutes at room temperature followed by phosphate-buffered solution (PBS, $Ca^{++}Mg^{++}$ free) wash (UCSF cell culture facility, San Francisco, CA, USA). The stained cells were then resuspended in 1 ml of fixing solution (1% paraformaldehyde in PBS, with 0.1% sodium azide). Analysis was accomplished by acquisition of data on a FACScan flow cytometer (Becton-Dickinson) with Cellquest Pro software where at least 20,000 cells were counted per analysis.

2.3. Detection of plasma MCP-1

Plasma from AD patient and elderly control blood was obtained by Percoll gradient centrifugation, and was frozen at −70°C until assayed. Plasma levels of monocyte chemoattractant protein-1 (MCP-1) were quantified by ELISA (R&D Systems Inc.) according to the manufacturer's instructions, and read at a wavelength of 450 nm (reference wavelength 562 nm) with a BIO-RAD Model 2550 EIA Reader (BIO-RAD Laboratories, Richmond, CA, USA). Mean optical densities of duplicates were calculated and converted in chemokine concentrations, using calibration curves generated in each experiment.

2.4. Detection of plasma IgG and IgM

Plasma from AD patient blood was obtained by Percoll gradient centrifugation, and was frozen at −70°C until use. Standard ELISA for determination of plasma antibody: Anti-Human IgG Fab or anti-Human IgM (Sigma, St. Louis, MO, USA) were coated (100 μl/well) into 96-well ELISA plates (Nunc, Roskilde, Denmark) by incubation for at least one hour at 37°C. The plates were washed one time with TBS (150mM NaCl, 20mM Tris-HCl, pH7.4), then blocked for 30 minutes by addition of 150 μl/well of BLOTTO (TBS plus 0.1% Tween-20, 2.5% normal goat serum, 2.5% non-fat dry milk) at room temperature, with gentle rocking. ELISA plates were subsequently washed once (1X) with TBS. Serial dilutions of serum were added to coated plates (duplicate wells each dilution, 100 μl/well) and allowed to react for 90 minutes, room temperature. A standard calibration series (0 to 5 μg/ml) for IgG and IgM (Sigma) was prepared, added to ELISA wells, and incubated in parallel. BLOTTO was used in all dilutions. Following the 90-minute incubation, all fluids were removed by aspiration, then all plates were washed 3X with TBS. Bound IgG antibodies were detected by adding 100 μl/well of anti-Human IgG alkaline phosphatase-conjugate (Promega Corp., Madison, WI, USA) diluted 1:10000 in BLOTTO. Bound IgM antibodies were detected by adding 100 μl/well of anti-Human IgM alkaline phosphatase-conjugate (Kirkegaard & Perry, Gaithersburg, MD, USA) diluted 1:5000 in BLOTTO. Antibody conjugates were incubated for one hour at room temperature with gentle agitation. Conjugates were removed by aspiration and plates washed 4X with TBS. Development of color reaction was effected by addition of 100 μl of PNPP substrate (Sigma) to each well, followed by incubation for 20 minutes at room temperature. The optical density in each well was read at 405nm. Any plasma with exceptionally low or high values was re-tested.

2.5. Statistical analysis

Cut-off values for defining cell activation as "positive" and "negative" for AD patients were determined by comparison with values from AD-negative, elderly healthy donors. Results are expressed as the mean ± SD. Statistical analysis of group differences, linear regressions and Pearson correlations were performed by GraphPad Prism 5.0 program (GraphPad Software, San Diego, CA, USA). Distribution of groups was analyzed by D'Agostino and

Pearson omnibus normality test. For all analyses, a value of $p < 0.05$ was considered significant.

3. Results

A cross-sectional study of immune activation was performed on blood from 41 patients with early stages of AD as compared to 31 elderly controls. Relative immunologic activation for this study was evaluated by measuring levels of activation antigen CD38 on T-cell subsets, HLA-DR, CD16, and chemokine receptor 2 (CCR2) on CD14 monocytes. Plasma levels of MCP-1, IgG and IgM were determined by ELISA quantitation. Table 1 summarizes the results of this study. Within the T-cell population, patients with early AD had a significant proportional increase in levels of the CD4 T lymphocyte subset ($p = 0.0095$), and a slight decrease in the percentage of CD8 T lymphocytes. This resulted in an increase in the CD4/CD8 ratio in AD patients. No evidence of CD4 T-cell expression of CD38 antigen above controls was observed in patients with early AD. However, the group of AD patients showed elevated levels of CD8/CD38 reactivity compared to elderly controls ($p = 0.0219$). The overall status of humoral immunity was evaluated by quantitating levels of plasma-IgG and -IgM in patients with early stages of AD and controls. As shown in Table 1, no significant differences in levels of plasma-IgG and - IgM were found between AD patients and controls.

Analysis of monocyte/macrophage markers showed that CD14+ monocytes from patients with early stages of AD expressed significantly higher than control levels of HLA-DR ($p < 0.0001$). Almost half of the CD14 cells in AD blood had characteristics of tissue macrophages, expressing significantly higher levels of the CD16 antigen ($p < 0.0001$) The aberrant monocytic phenotype defined by higher expression of HLA-DR and CD16 was associated with significant differences in CD14-associated SSC (measure of granularity and differentiation) between AD patients and elderly controls. Compared with elderly controls, monocytes from AD patients had statistically increased granularity (higher SSC values) ($p < 0.0001$). Lower levels of the absolute percent of CD14 cells within the total white blood cell count were found in AD patient blood compared to controls ($p = 0.0051$).

Compared to the elderly control population, significantly elevated levels of plasma MCP-1, a peripheral monocyte-related inflammatory marker, were observed in early AD patients ($p = 0.0264$), and expression of MCP-1 receptor CCR2 was lower on AD CD14+ monocytes ($p = 0.0001$). There was a direct and significant relationship between CD16 monocyte expression and plasma MCP-1 levels in AD ($r = 0.5006$, $p = 0.0127$, $n = 24$; Fig. 1). And degree of AD monocyte CD16 expression was positively correlated with CD14+ monocyte granularity, as shown in Fig. 2 ($r = 0.7042$, $p < 0.0001$, $n = 41$). No relationship was found between CD16 monocyte expression and either plasma MCP-1 levels or monocyte granularity in the elderly control population.

To evaluate whether systemic immune activation would be related to disease status, immune activation parameters from Table 1 were compared with the clinical disease status in AD. Degree of T-cell and monocyte activation both were independent of severity of disease as defined by MMSE score in AD. Similarly, no relationship was observed between plasma MCP-1 levels and AD disease status.

4. Discussion

It is clear now that interactions between the brain and the immune system do occur on a continuing basis (Ransohoff et al., 2003). During a variety of pathological conditions in the CNS, exogenous leukocytes are recruited to the diseased brain to respond to injury, to protect and regenerate the CNS (Britschgi and Wyss-Coray, 2007, Yong and Rivest, 2009, Malm et al., 2010). Blood-derived monocyte/macrophages play a key role in

neuroinflammatory processes seen in AD. In response to inflammatory stimuli blood-derived monocyte/macrophages migrate across a compromised blood-brain barrier (BBB) and express chemokine receptors to guide immune cells to inflammatory sites in AD brains (Fiala et al., 2002, Malm et al., 2005, Simard et al., 2006, Malm et al., 2008, Gate et al., 2010, Rezai-Zadeh et al., 2011). Several studies also reported increased numbers of T cells in AD brains (Itagaki et al., 1988, Rogers et al., 1988, Togo et al., 2002). Moreover, the genetic, cellular, and molecular changes associated with AD provide ever-stronger support for an activation of immune and inflammatory processes in the disease (Wyss-Coray and Rogers, 2012). However, the systemic immune alternations accompanying the development of cognitive decline and knowledge on the involvement of the immune system as a mechanism for initiating or exacerbating AD have not been well characterized.

In the current study, we performed immunophenotypic analyses and humoral immunity assessment of blood from patients with early stages of AD as compared to age-matched elderly controls. In concordance with previous findings of increased activation of microglia/macrophages colocalized with the area of heavy β-amyloid concentration in the CNS of AD patients (Edison et al., 2008), persistently activated monocyte/macrophages were observed in the blood of patients with early AD in the current and in our previous studies (Zhang et al., 2005, Zhang et al., 2009). The high levels of HLA-DR on AD CD14 cells were coupled with an elevation in the proportion of CD14 cells co-expressing the differentiation/tissue macrophage marker, CD16. Evidence accumulating over the past two decades indicates that these CD16 monocytes with higher level expression of HLA-DR appear to be more mature and tissue macrophage-characterized, and were labeled proinflammatory based on higher expression of proinflammatory cytokines and higher potency in antigen presentation (Ziegler-Heitbrock et al., 1993, Frankenberger et al., 1996, Weber et al., 2000, Ancuta et al., 2006, Ziegler-Heitbrock, 2007, Abeles et al., 2012). Our earlier report also showed that abnormal monocyte activation in AD patient blood and degree of this activation was related to elevated levels of plasma LPS (Zhang et al., 2009). As a systemic monocyte/macrophage activator, LPS induces its effects through stimulation of CD14-bearing inflammatory cells. LPS associated toxicity is mediated through systemic monocyte/macrophage and endothelial cell activation, and release of inflammatory cytokines.

The increase in levels of plasma MCP-1 observed in the current study is in keeping with the previous reports of higher MCP-1 levels in the serum and/or the cerebrospinal fluid (CSF) in AD patients (Galimberti et al., 2003, Westin et al., 2012). As one of the most potent chemotactic factors for monocytes, MCP-1 may regulate BBB permeability to facilitate leukocyte transmigration into the CNS (Stamatovic et al., 2003, Stamatovic et al., 2005). MCP-1 secreted into the perivascular space of the BBB not only attracts leukocytes, but also has a role in ‘opening’ the BBB during leukocyte extravasations. Furthermore, MCP-1 has been shown to be a likely candidate to initiate the contact between immune cells and neurons (Flugel et al., 2001). With characteristics of tissue macrophages, the circulating activated CD14+/CD16+ monocytes in patients with neurodegenerative diseases enter the CNS under constitutive and inflammatory conditions (Fischer-Smith et al., 2001, Ancuta et al., 2004, Ancuta et al., 2006), and expose neural cells to neuro-toxic factors similar to those released by activated macrophage reported to cause neural-cell damage in vitro (Pulliam et al., 1997). Not previously observed was the finding that the higher MCP-1 levels observed in plasma from early AD patients were directly related to degree of CD16 monocyte expression. MCP-1 expression induced in the periphery with associated monocyte activation/differentiation may target immune cells (for example, CD14+CD16+) bearing/releasing harmful agents, such as TNF-α, MCP-1, and IL-6 (Frankenberger et al., 1996, Belge et al., 2002, Ancuta et al., 2004, Ancuta et al., 2006), into the CNS in the absence of direct CNS lesions. Severity of neurological disorders such as AD may be due in part to neuro-toxic factors released by these activated monocyte/macrophages when crossing the

BBB and migrating into the CNS (Fischer-Smith et al., 2001, Belge et al., 2002, Minagar et al., 2002, Ancuta et al., 2004, Ancuta et al., 2006). Elevated levels of MCP-1 and abnormally high HLA-DR expression on CD16 expressing monocytes in AD suggests an ongoing inflammatory stimulus driving these cells to traffic into the CNS, which may explain the lower levels of the percentage of CD14 monocytes seen in AD patients.

Compared to elderly controls, the expression of CCR2, a critical monocyte chemokine receptor for MCP-1, was markedly decreased on AD blood monocytes. There is strong evidence that MCP-1/CCR2 signaling is implicated in the recruitment of monocyte/macrophages and activated lymphocytes into the brain in neuropathological states (Mennicken et al., 1999, Stamatovic et al., 2005, D'Mello et al., 2009). Overexpression of MCP-1 in the brain appears to desensitize/down modulate CCR2 on microglia, making them nonresponsive to injury (Huang et al., 2005). Hence, increased plasma MCP-1 and abnormally activated monocyte/macrophages in AD blood may lead to desensitization of circulating monocyte/macrophage CCR2, essentially creating a CCR2-deficient status and preventing activated monocyte/macrophages from migrating across the BBB, similar to the protective reaction of host immune response to monocyte/macrophage mediated damage observed in other CNS diseases (Izikson et al., 2000, Huang et al., 2001, Zhang et al., 2006).

In accordance with the results observed by others (Elovaara et al., 1987, Kay et al., 1987, Giometto et al., 1988), the levels of plasma-IgG and -IgM were similar between patients with early stages of AD and controls in the current study. However, conflicting results regarding peripheral lymphocyte phenotypes in AD have been reported from different groups, and currently there is no general consensus on the modifications of lymphocyte subsets in AD patients (Britschgi and Wyss-Coray, 2007). In the current study, early stage AD patients showed a significant increase in the percentage of T-cells expressing CD4, and the percentage of CD8+ T-cells was found to be slightly decreased as compared with elderly controls. These data confirm previous findings by other investigators (Lombardi et al., 1999, Richartz-Salzburger et al., 2007, Schindowski et al., 2007), but are not in complete agreement with the results from Larbi et al, who used frozen peripheral blood mononuclear cells for the flow cytometric analysis and showed a significant reduction of CD4+ T-cells in mild AD patients (Larbi et al., 2009).

In the study of T-cell activation markers, CD38 expression was significantly increased on CD8+ T-cells in the blood of early AD patients as compared to controls. By contrast, the CD4/CD38 reactivity remained within the range of elderly controls. This observation may be consistent with findings that CD8+ T cells appear to be potentially involved in AD pathogenesis (Schindowski et al., 2007, Speciale et al., 2007). CD38 expression on CD8+ T cells is linked to immune system activation and is associated with cytotoxic effector function (Savarino et al., 2000). The capability of T-cells to cross the BBB and enter into the CNS appears to be primarily dependent upon the activation state of the lymphocytes (Hickey, 1991, Engelhardt and Ransohoff, 2005). Activated T-cells are capable of trafficking into the CNS. Activated CD8+ lymphocytes found in early AD patients may be related to host response to β-amyloid and may be the reason that the CD8 immunoreactivity is dominant in AD brains compared to CD4 T-cells (Itagaki et al., 1988, Rogers and Mufson, 1990), however, both CD4 and CD8 T-cell subsets have been found in AD brains (McGeer et al., 1989, Fiala et al., 2002). Together these data deliver strong support for active roles of systemic activation/inflammation and immune responses in pathogenesis of AD and the ability to monitor this process by an evaluation of blood cell and plasma markers.

In summary, this study demonstrates systemic immune activation/inflammation occurring persistently even in early stages of AD. Our findings provide further evidence for the presence of inflammatory and immune-related markers and processes in the systemic

immune system in AD patients. The parameters described in this study may be useful in both clinical evaluation and research efforts to further characterize immune dysfunction during disease progression and lead to a better understanding of immunopathologic processes associated with AD pathogenesis.

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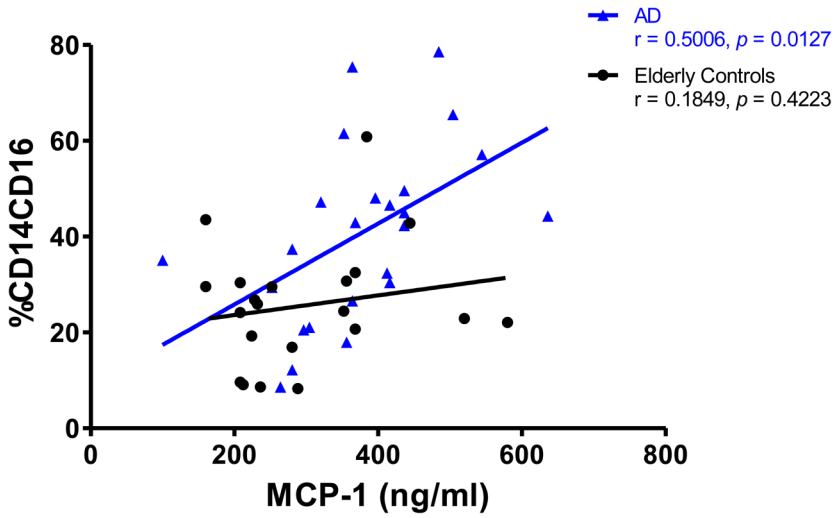

Figure 1.

Relationship of plasma MCP-1 levels to macrophage activation/differentiation defined by CD14 co-expression of CD16 in elderly controls and patients with early stages of AD. Positive correlation of plasma MCP-1 levels with degree of CD16 expression on CD14+ monocyte in early AD (r = 0.5006, $p = 0.0127$, n = 24).

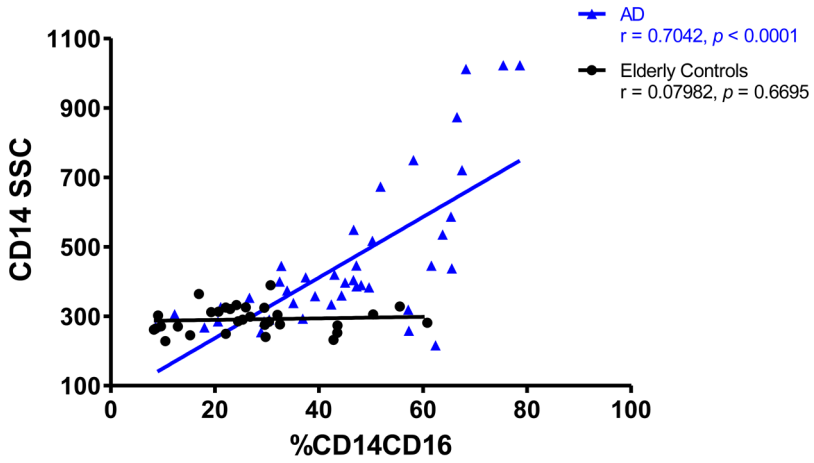

Figure 2.

Relationship between monocyte CD16 expression and CD14+ monocyte granularity in elderly controls and patients with early stages of AD. Positive correlation of monocyte CD16 expression with CD14+ monocyte granularity in early AD (r = 0.7042, $p < 0.0001$, n = 41).

Table 1

Comparative analysis of humoral immunity and differentiation antigen expression in blood of early AD patients and elderly controls

Parameters	AD patients (n = 41)	Controls (n = 31)	*p* Value
CD4/CD8	3.39 ± 2.50	2.56± 1.84	NS
%CD4	47.22 ± 10.84	40.42 ± 10.52	0.0095
%CD8	19.60 ± 11.30	21.25 ± 9.57	NS
%CD4CD38	25.70 ± 11.60	23.51 ± 10.10	NS
%CD8CD38	16.01 ± 11.65	9.80 ± 5.89	0.0219
%CD14	3.10 ± 1.37	4.04 ± 1.36	0.0051
Mean CD14HLA-DR[a]	1032.84 ± 445.17	640.71 ± 249.29	< 0.0001
%CD14CD16	45.18 ± 17.29	26.96 ± 13.68	< 0.0001
CD14SSC[b]	456.5 ± 214.2	291.0 ± 37.7	< 0.0001
MFI CD14CCR2[c]	5.59 ± 6.65 (n = 23)	20.34 ± 14.17 (n = 22)	0.0001
MCP-1 (pg/ml)	376 ± 110 (n = 24)	299 ± 115 (n = 21)	0.0264
Plasma-IgG (mg/ml)	10.83 ± 7.61 (n = 25)	11.22 ± 4.98 (n = 50)	NS
Plasma-IgM (mg/ml)	1.54 ± 1.31 (n = 25)	1.72 ± 1.16 (n = 50)	NS

[a] Mean HLA-DR fluorescence expressed on CD14+ monocyte.

[b] CD14-associated side light-scatter characteristics.

[c] Median fluorescence intensity of CCR2 on CD14+ monocyte