

Altered features of monocytes in adult onset leukoencephalopathy with axonal spheroids and pigmented glia: A clue to the pathomechanism of microglial dyshomeostasis

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ABSTRACT

Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) is an autosomal-dominant type of leukoencephalopathy caused by gene mutation of *colony stimulating factor 1 receptor*, which is expressed mainly on monocyte lineage cells such as monocytes in the peripheral blood and microglia in the brain. Hence, microglial dysfunction is regarded as critical in the pathogenesis of ALSP. However, functional changes in these cells have not been elucidated. In this study, we report the phenotypic and functional alterations of monocytes in four patients with ALSP. Flow cytometric analysis revealed altered expression of antigen presentation- and migration-related molecules, an inflammatory shift in cytokine production and phagocytic impairment in ALSP monocytes. We speculate that the observed altered features of monocytes are mostly shared by microglial cells, leading to the clinical history and pathological characteristics of ALSP. Our analysis of PB monocytes provides novel insights into the pathogenesis of ALSP.

1. Introduction

Adult-onset leukoencephalopathy with axonal spheroids and pigmented glia (ALSP) is an autosomal-dominant adult-onset leukoencephalopathy with various progressive neurological symptoms including cognitive and behavioral dysfunction. Pathological studies have revealed characteristic features in the central nervous system (CNS): prominent axon and myelin loss accompanied by giant neuroaxonal swelling (spheroids), microglial pigmentation and abnormal morphology, and an uneven distribution of microglial cells (Konno et al.,

2017). Recently, *colony stimulating factor 1 receptor* (CSF1R) was identified as a causative gene of ALSP. CSF1R is a cell-surface receptor for macrophage colony-stimulating factor (M-CSF) and interleukin (IL) 34 and is crucial for regulating the survival, proliferation, differentiation, and function of monocyte lineage cells (Rademakers et al., 2011). CSF1R is mainly expressed by monocytes in the peripheral blood (PB) and microglia in the CNS. Hence, ALSP is regarded as a primary microglial disorder (Tada et al., 2016). However, the functional changes that are induced by mutation of CSF1R in monocyte lineage cells are unknown.

Abbreviations: ALSP, adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; APC, allophycocyanin; CNS, central nervous system; Cy7, cyanine 7; CSF1R, colony stimulating factor 1 receptor; FcγR, a receptor for the Fc portion of IgG; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; HS, healthy subjects; IgG, immunoglobulin G; IL, interleukin; LPS, lipopolysaccharide; M-CSF, macrophage CSF; MFI, mean fluorescence intensity; PE, phycoerythrin; PerCP/Cy5.5, peridinin chlorophyll protein-cyanine 5.5; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; SEM, standard error of mean; SR-A, scavenger receptor A; TGF, transforming growth factor; TNF, tumor necrosis factor

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Table 1
Summary of information of all study participants.

	Gender	Age, y (at onset)	Gene mutation	mRS	Monocyte (/μl) (% in WBC)	WBC (/μl)	Initial symptoms
Patients with ALSP (n = 4)							
I ^a	Male	46 (41)	c.2375C > A ^b	4	289 (4.5%)	6420	Apathy, acalculia, offensive to family members
II ^a	Female	44 (40)		4	471 (16.3%)	2890	Apathy, decline in verbal fluency
III	Female	40 (UN)	c.1765G > A ^b	4	262 (4.6%) ^c	5700 ^c	Right spastic hemiplegia
IV	Female	26 (20)	c.2381 T > C ^b	5	353 (6.2%) ^c	5700 ^c	Stuttering, decline in verbal fluency, disturbance of attention
Controls							
Patient with AARS2-L (n = 1)							
	Female	41 (30)	c.2255 + 1G > A ^d c.1145C > A ^d	4	318 (6.7%)	4750	Indifference to surroundings, cognitive decline, dystonia (left hands)
HS (n = 6)							
		Age, y Mean ± SD (range)					
1 male, 5 female		42 ± 3.4 (39–49)					

Abbreviations: ALSP = adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; HS = healthy subjects; mRS = modified Rankin scale; UN = unknown; y = years; WBC = white blood cells.

^a Siblings.

^b Colony stimulating factor 1 receptor gene.

^c Most recent data.

^d Mitochondrial alanyl-transfer RNA synthetase gene.

In this study, we analyzed phenotypic and functional changes in chemotaxis, antigen presentation, cytokine production, and phagocytosis in PB monocytes from patients with ALSP because these cells are the most easily accessible materials for functional analysis among representative monocyte lineage cells. Our analysis of PB monocytes provides novel insights into the pathogenesis of ALSP.

2. Materials and methods

2.1. Participants

A total of four ALSP patients were enrolled in this study, comprising one man and three women aged 39 ± 7.8 years (mean ± standard deviation, range: 26–46) (Table 1). Patient I, II, and III were wheelchair-bound at sampling, and Patient IV with the youngest age at onset was bed-ridden. Cognitive decline or apathy was noted as the initial symptom in Patient I, II, and III, but motor impairment was preceded by cognitive decline in Patient IV. There were no clinical histories or laboratory tests suggestive of systemic immunological abnormality. Brain MRI and CT of Patient I are shown in Fig. 1 and the radiological findings were shared by other patients. Bi-allelic mutations in *mitochondrial alanyl-transfer RNA synthetase* (AARS2) were recently reported to cause rare leukodystrophy that is clinically, radiologically, and pathologically indistinguishable from ALSP (Dallabona et al., 2014; Lakshmanan et al., 2017; Lynch et al., 2016; Taglia et al., 2018). Therefore, comparison of monocyte function between ALSP and AARS2-related leukodystrophy (AARS2-L) provided crucial information on how specific monocyte function is related to brain disorders in ALSP. Thus, one patient with AARS2-L was additionally recruited (Table 1). Age- and sex-matched healthy subjects (HS) were also enrolled.

2.2. Standard protocol approvals, registrations, and patient consents

The study was approved by the Medical Ethics Committee of Kyoto University School of Medicine and was conducted in accordance with the principles contained within the Declaration of Helsinki. All participants provided informed consent.

2.3. Sample collection and gating strategy

PB mononuclear cells (PBMCs) were collected as previously described with the same adjustments (Okada et al., 2018). For surface molecule examination, untouched monocytes were enriched from

PBMCs using minimal labeling of unwanted cells by magnetic cell separation (Pan Monocyte Isolation Kit, human; Miltenyi Biotec, Auburn, CA, USA) and stained with anti-human CD14 (clone 63D3), CD16 (clone 3G8), CD64 (clone 10.1), CD80 (clone 2D10), CD86 (clone IT2.2), CD62L (clone DREG-56), CX3CR1 (clone 2A9–1), and CCR2 (clone K036C2) antibodies conjugated to peridinin chlorophyll protein–cyanine 5.5 (PerCP/Cy5.5), phycoerythrin (PE), allophycocyanin (APC), fluorescein isothiocyanate (FITC), Pacific Blue, APC-cyanine7 (Cy7), PE/Cy7, and APC, respectively (all purchased from BioLegend, San Diego, CA, USA) according to the manufacturer's protocol.

Data were acquired using a FACS Canto II flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed with FlowJo software (TreeStar, Ashland, OR, USA). Mean fluorescence intensity (MFI) was calculated for quantification of protein expression.

Monocytes were gated according to forward scatter, side scatter, and expression of CD14 and CD16 after exclusion of doublets (Fig. S1A). After incubation in the presence of each stimulus described below, CD14-positive cells were analyzed after gating in the forward scatter/side scatter plot, because CD16 expression was reduced after incubation.

2.4. Cell culture and stimulation

To examine responses to M-CSF and granulocyte-macrophage colony-stimulating factor (GM-CSF), monocytes were cultured in 96-well U-bottom plates at a concentration of 8×10^4 /well for 6 days in 200 μl/well Macrophage-SFM (Thermo Fisher Scientific, Tokyo, Japan) supplemented with 50 units/ml penicillin G and 50 μg/ml streptomycin (Penstrep; Thermo Fisher Scientific) and containing either 100 ng/ml human recombinant M-CSF (BioLegend) or 10 ng/ml human recombinant GM-CSF at 37 °C in 5% CO₂. On day 3, half the medium was replaced with fresh medium containing each CSF, and after 6 days of incubation, surface molecules were analyzed with flow cytometry.

2.5. Analysis of intracellular cytokines

PBMCs were cultured at a density of 2×10^5 /well in Macrophage-SFM (Thermo Fisher Scientific) in 96-well U-bottom plates and stimulated for 4 h with 10 μg/ml lipopolysaccharide (LPS) (Enzo Life Sciences, Farmingdale, NY, USA) and Brefeldin A solution (eBioscience, Hatfield, UK) at 37 °C in 5% CO₂. Stimulated PBMCs were harvested, washed, and stained with anti-human CD14 PerCP/Cy5.5 (clone 63D3) antibody (BioLegend). For intracellular staining, cells were washed

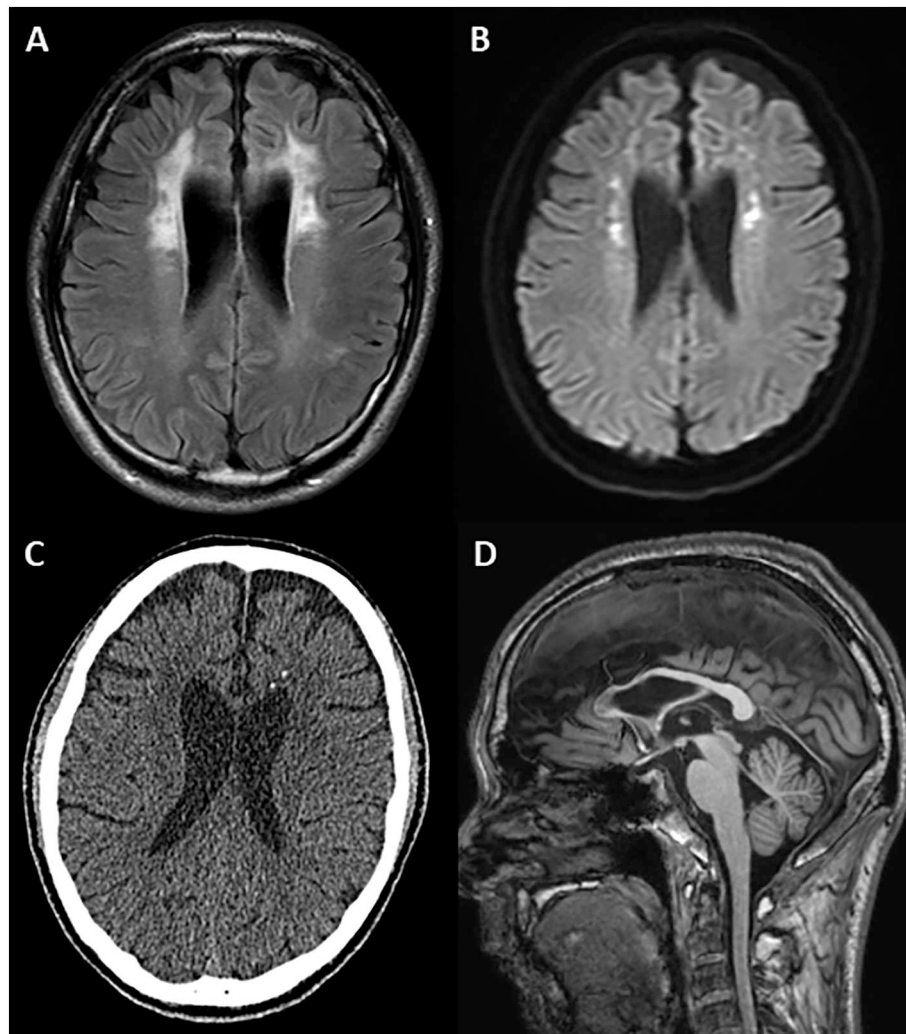


Fig. 1. Neuroimaging studies of Patient I at the age of 43.

Brain MRI of the patient showed increased FLAIR signal intensity within the deep white matter dominant in frontal lobe accompanied with scattered or spotty diffusion restriction in the areas of FLAIR signal abnormalities (A, B). Fine calcifications within white matter lesions were observed in brain CT (C). Sagittal MRI view revealed thinned corpus callosum (D).

CT = computed tomography; FLAIR = fluid-attenuated inversion recovery; MRI = magnetic resonance imaging.

again, fixed, permeabilized, and stained with anti-human antibodies. Anti-human antibodies included anti-IL-10 Alexa Fluor (AF) 647 (JES3-9D7) antibody, anti-tumor necrosis factor (TNF) α AF488 (Mab11) antibody, anti-IL-6 PE/Cy7 (MQ2-13A5) antibody, anti-transforming growth factor (TGF) β Brilliant Violet 421 (TW4-2F8) antibody (all purchased from BioLegend), and anti-IL-12p70 PE (20C2) antibody (BD).

2.6. Phagocytosis assay

Phagocytosis analysis was performed according to the manufacturer's instructions. Briefly, PBMCs were incubated with FITC-labeled bare latex beads or beads coated with rabbit immunoglobulin G (IgG) (Phagocytosis Assay Kit FITC; Cayman Chemical, Ann Arbor, MI, USA) for 2 h. Cells were stained with anti-human CD14 PerCP/Cy5.5 (clone 63D3) (BioLegend), and the percentage of CD14-positive cells that ingested beads was determined as FITC-positive cells (Fig. S1B).

2.7. Statistical analysis

Data are the mean $\pm$ standard error of mean (SEM). For statistical analysis, we used the non-parametric Mann-Whitney *U* test for

comparisons of two unpaired groups. All data sets were analyzed using GraphPad Prism software (GraphPad, San Diego, CA, USA). A *p*-value $< .05$ was considered statistically significant. Representative results of flow cytometric analysis are indicated in the histogram in Fig. S1C.

3. Results

3.1. Absolute counts and subsets of monocytes

Routine blood tests repeatedly revealed that the absolute count of monocytes per microliter of blood was within the normal value. Generally, human monocytes are subdivided according to CD14 and CD16 expression into classical (CD14 $^{++}$ CD16 $^{-}$), intermediate (CD14 $^{++}$ CD16 $^{+}$), and non-classical (CD14 $^{+}$ CD16 $^{++}$) subsets, each of which represents functional characteristics for motility, antigen presentation, cytokine production, or phagocytosis (Wong et al., 2011; Ziegler-Heitbrock, 2015). We observed a distinct reduction in non-classical monocytes compared with HS in Patient I, II and III and this was consistent with a previous report (Hofer et al., 2015) (Fig. 2A, B), whereas the most advanced case, Patient IV, showed a strong increase in this subset (indicated with the open dot in Fig. 2B). The values

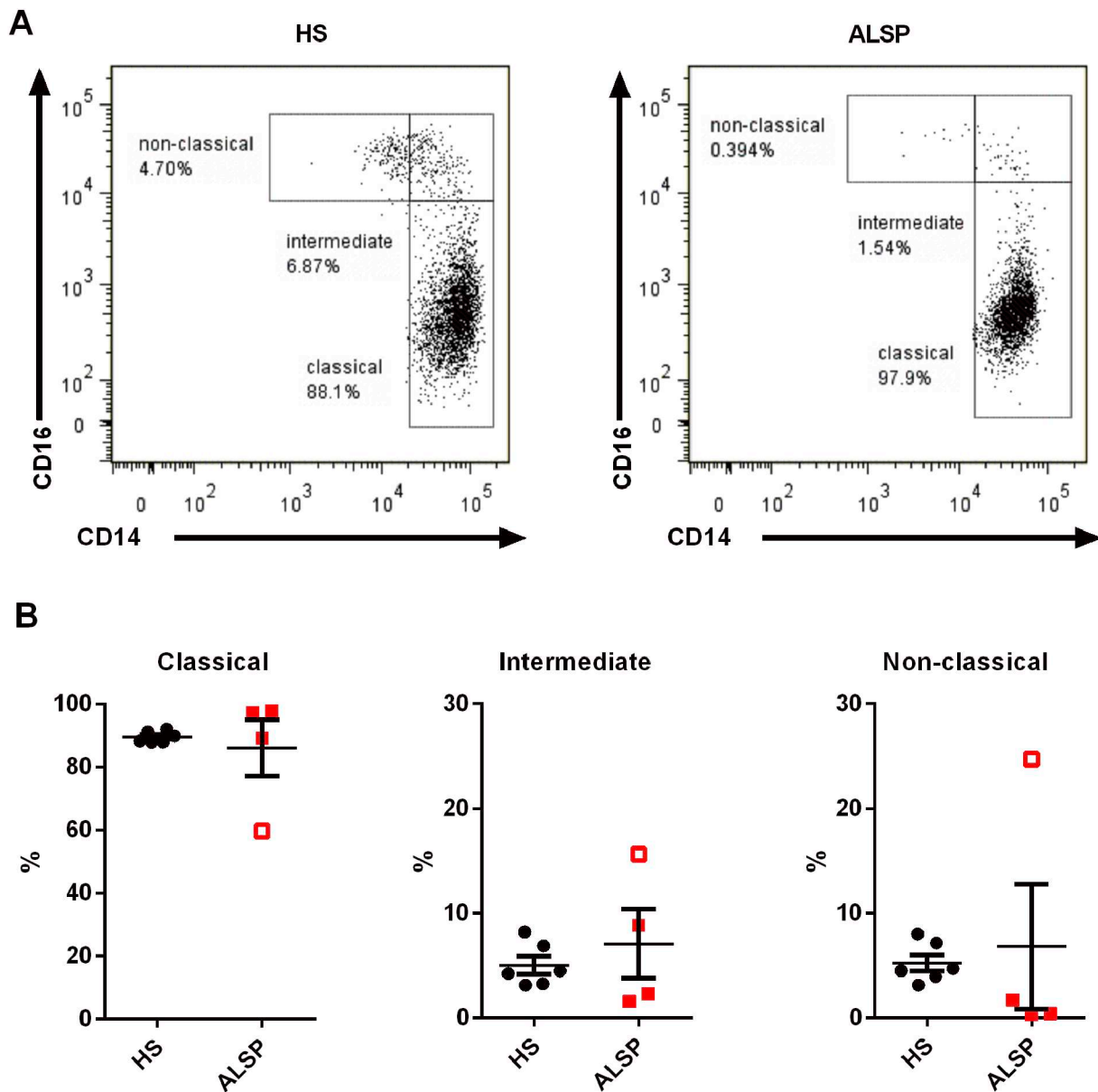


Fig. 2. Identification of peripheral monocyte subsets.

A. Monocyte subsets were subdivided into classical, intermediate, and non-classical monocytes based on expression of CD14 and CD16. Representative data are shown (right panel: HS, left panel: ALSP). B. The frequency of each subset is shown. The values for Patient IV are indicated as open dots. Results are expressed as the mean $\pm$ SEM.

ALSP = adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; HS = healthy subjects.

recorded for Patient IV are shown as open dots in subsequent figures.

3.2. Increased expression of migration- and adhesion-related molecules on ALSP monocytes

Analysis of PB monocytes from patients with ALSP demonstrated an increase in the expression of the chemokine receptor, CCR2 ($p = .0095$), and a similar tendency in CX3CR1 expression (Fig. 3A). Upregulated expression of CD62L, an adhesion molecule, was observed in ALSP monocytes compared with HS ($p = .0095$) (Fig. 3A).

3.3. Altered expression of co-stimulatory molecules on ALSP monocytes

The expression level of CD86 was higher on PB monocytes from patients with ALSP compared to HS ($p = .0095$), and expression of CD80 exhibited a similar tendency but without significance (Fig. 3B). In

contrast, expression of CD80 and CD86 became lower on monocytes from ALSP patients compared to HS after 6 days in culture with M-CSF, indicating impaired upregulation of these molecules in response to M-CSF (Fig. 3C). Exposure to GM-CSF similarly induced insufficient upregulation of CD80 and CD86 on ALSP monocytes although the difference was not statistically significant probably because of the limited number of participants (Fig. 3D). Of note, isotype control MFI increased in cells cultured for 6 days probably because the culture induced larger cells with a complex surface membrane.

3.4. Pro-inflammatory shift in cytokine production by ALSP monocytes

To investigate additional functional characteristics of monocyte lineage cells from ALSP, we investigated cytokine production by monocytes. We observed a significant reduction in IL-10 production by ALSP monocytes ($p = .0048$) and a trend toward an increase in TNF- α

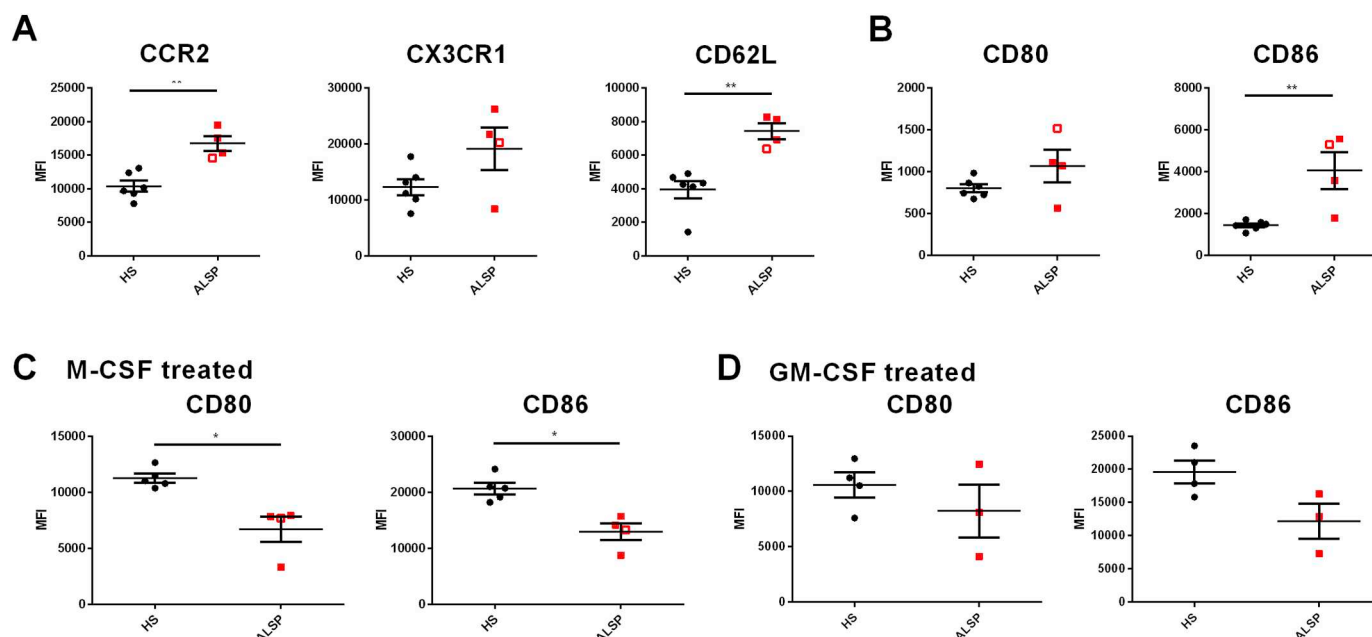


Fig. 3. Expression levels of migration- and adhesion-related molecules and of antigen presentation-related molecules.

Untouched monocytes were isolated by magnetic cell separation, and the surface expression of CCR2, CX3CR1, and CD62L (A) and of CD80 and CD86 (B) was examined with flow cytometry. To study the expression levels of CD80 and CD86, monocytes were cultured for 6 days in the presence of M-CSF (C) or GM-CSF (D) before flow cytometric analysis. Dot plots portray the MFI for each protein in the total monocytes. The values for Patient IV are indicated as open dots. Bars indicate the mean $\pm$ SEM. * $p < .05$, ** $p < .01$.

ALSP = adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; CSF = colony stimulating factor; HS = healthy subjects; MFI = mean fluorescence intensity;

secretion in response to 4-h stimulation with LPS (Fig. 4). We also measured intracellular production of IL-6, IL-12p70, and TGF- β , but observed no significant differences (Fig. 4). Note that we only analyzed the frequency of cytokine-positive cells, not the total amount of cytokine.

3.5. Impaired IgG-mediated phagocytosis in ALSP monocytes

Phagocytosis of pathogens, apoptotic cells and debris for host defense and tissue homeostasis is a key feature of monocyte lineage cells. After 2-h culture in medium containing beads, the uptake of IgG-conjugated beads by monocytes from patients with ALSP was reduced in comparison with HS whereas we observed no difference in the frequency of monocytes that had ingested IgG-unconjugated beads (Fig. 5A). The expression of CD64 (a receptor for the Fc portion of IgG (Fc γ R) I), a high-affinity activating receptor on monocyte lineage cells that strongly induces phagocytosis of IgG-coated antigens, was not significantly different between HS and ALSP patients (Fig. 5B).

In addition to the phagocytosis assay, we examined the expression of scavenger receptors: CD163, a hemoglobin scavenger receptor (Etzerodt and Moestrup, 2013), and CD204, a macrophage scavenger receptor (scavenger receptor A: SR-A) (Kelley et al., 2014). Except for the Patient IV with the earliest onset and rapid progression, expression of CD163 on monocytes was higher in patients with ALSP than any HS (Fig. 5B). When excluding Patient IV, a significant difference was recognized between the two groups ($p = .036$) although no statistical difference was seen among all patients. CD204 expression tended to be reduced on monocytes from all patients (Fig. 5B).

3.6. Monocytes in AARS2-related leukodystrophy

A series of examinations revealed that monocyte phenotypes in AARS2-L were similar to those of HS, but not to ALSP although there were two exceptional features. The expression of CD80 was as high as that in ALSP monocytes, and bare bead uptake was accelerated

compared with both HS and ALSP (Table S1).

4. Discussion

In this study, we revealed the phenotypic and functional changes in PB monocytes derived from patients with ALSP.

We determined monocyte subsets according to CD14 and CD16 expression. Focusing on the three patients shared by clinical stages of previous reports (Konno et al., 2017), namely with the exception of Patient IV, non-classical monocytes were distinctly reduced in frequency compared with HS ($p = .024$). On the other hand, we observed an exceptional case (Patient IV) who showed by far the highest frequency of non-classical monocytes among all participants even including HS. The reason for this exception remains unknown. However, the relevant case was clinically distinct from the other patients with ALSP in that she showed the youngest symptomatic onset with motor impairment and was in the most advanced clinical stage compared with any patient including the previous report (Hofer et al., 2015) (Table 1). We described expression of surface markers on total monocytes in the Results and in the below Discussion, but not separately for each subset of monocytes. This is because we found no particular depending difference on subsets of monocytes regarding surface expression including the atypical Patient IV (Fig. S2).

The presence of the chemokine receptors, CCR2 and CX3CR1, is critical for myeloid cell trafficking along inflamed vessels and subsequent accumulation in the brain in both physiological and pathological conditions (El Khoury et al., 2007; Guedes et al., 2018; Prinz and Mildner, 2011; Prinz and Priller, 2010). The distinct expression of CCR2 and CX3CR1 on PB monocytes from patients with ALSP suggests altered mobility of monocyte lineage cells, which may explain the uneven or restricted distribution of microglia in ALSP brain. As shown in other neurodegenerative diseases, imbalance or dysregulation of the CX3CR1 chemokine-chemokine receptor axis likely causes initiation or exacerbation of neuronal inflammation and injury in ALSP because CX3CR1 plays an important role in the housekeeping functions of microglia and

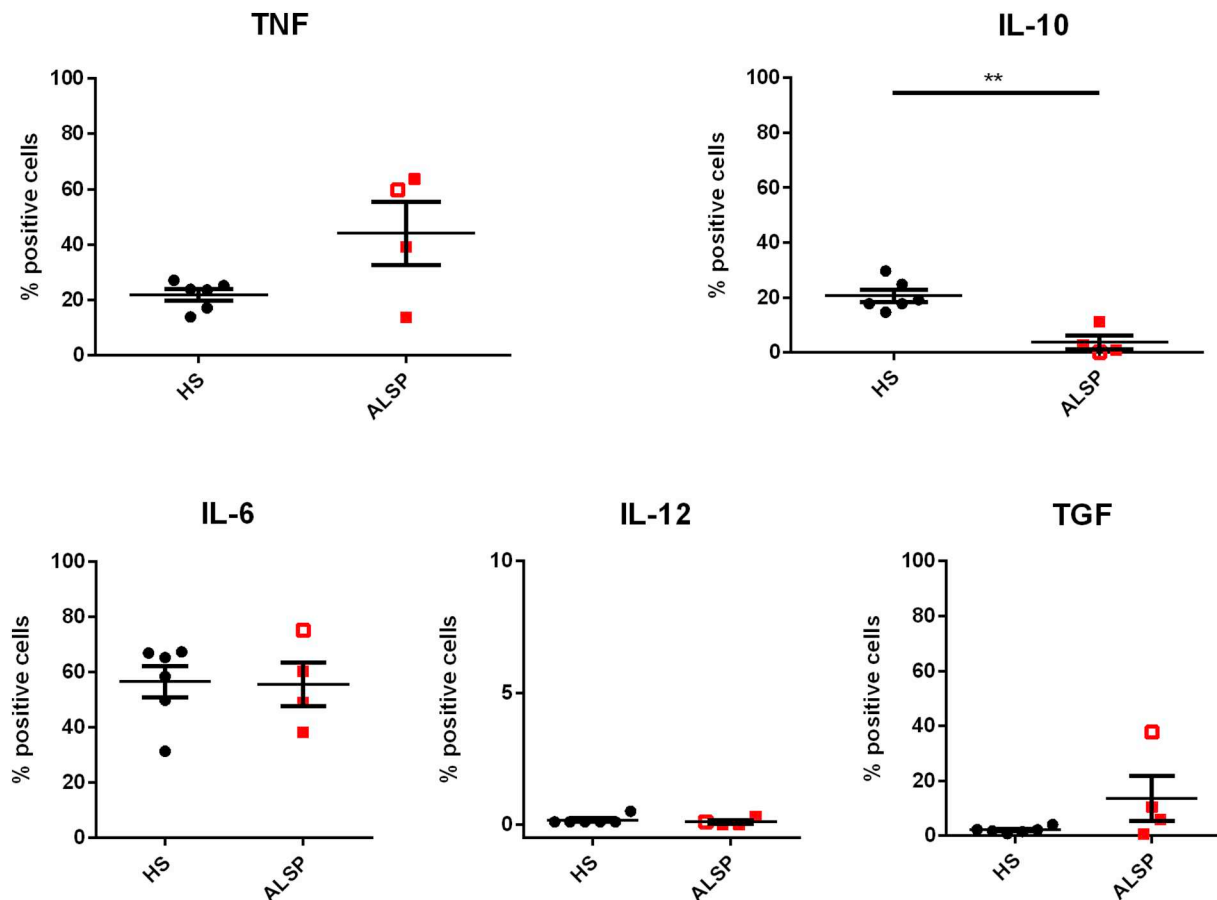


Fig. 4. Cytokine productions by monocytes.

To study the production of TNF- α and IL-10 by monocytes in response to LPS stimulation, cytokines were intracellularly stained and analyzed by flow cytometry. Monocytes were gated according to forward scatter, side scatter, and expression of CD14. The values for Patient IV are indicated as open dots. Frequency of cytokine-positive monocytes is indicated in dot plots (bars: mean $\pm$ SEM). ** $p < .01$.

ALSP = adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; HS = healthy subjects; IL = interleukin; LPS = lipopolysaccharide; TNF = tumor necrosis factor; TGF = transforming growth factor.

contributes to neuronal well-being or homeostasis (Hickman et al., 2018; Mecca et al., 2018). CD62L, crucial for recruitment of leukocytes to sites of inflammation (Grewal et al., 2001; McCreedy et al., 2018), was upregulated on monocytes from patients with ALSP. The reason remains unclear. We suggest two possibilities. One is that CD62 upregulation is associated with CNS inflammation. The other is that CD62 upregulation results from compensation for altered mobility of monocytes.

Both M-CSF and GM-CSF activate monocytes and upregulate the expression of CD80 and CD86, potentiating their capacity for antigen presentation (Dewar et al., 2005; Samaniego et al., 2014; Zarif et al., 2016). Chronic higher expression of these molecules and poor response to both CSFs, not specifically to M-CSF, suggests aberrant activation of monocyte lineage cells in ALSP.

Activated microglia are a main source of cytokines in the brain. Cytokine production by monocytes (increased TNF- α and decreased IL-10) in response to LPS suggests that ALSP monocytes are likely to skew the pro-inflammatory state. Hence, the inflammatory cascade resulting from repeated exposure to stimuli may play important roles in accumulation of damage in the ALSP brain, and be associated with the late onset of neurologic manifestations in the fourth or fifth decade (i.e., long after birth) and the relatively rapid progression after onset.

Microglial cells are the major professional phagocytes in the brain (Fu et al., 2014; Hickman et al., 2018; Janda et al., 2018; Salter and Stevens, 2017). A study of *Csf1r* haplo-insufficient mice, an animal model of ALSP (Chitu et al., 2015; Chitu et al., 2016), suggested an

altered phagocytic function by microglia in ALSP. In addition, IgG-mediated phagocytosis of opsonized antigens is implicated in the pathogenesis of several neurodegenerative diseases, e.g., Alzheimer's disease and Parkinson's disease (Cao et al., 2012; Fu et al., 2014; Marsh et al., 2016). All these observations are compatible with the altered phagocytic ability of PB monocytes observed in this study.

Fc γ Rs play important roles in the initiation of IgG-mediated phagocytosis in the CNS (Cao et al., 2012; Derecki et al., 2014; Fu et al., 2014; Marsh et al., 2016). However, the expression of the major activating Fc γ R, CD64, was not significantly different between monocytes from HS and patients with ALSP in our results, and its contribution to phagocytic impairment is unclear.

An elaborate neuropathological study indicated that the expression of some activation markers and scavenger receptors including CD163 was upregulated before axonal loss in ALSP brain and downregulated later (Oyanagi et al., 2017). Our examination revealed higher expression of CD163 on monocytes in all ALSP patients compared to HS, except for one patient in the most advanced disease stage (Patient IV). The distinct expression of CD163 on monocytes may come from different disease stages. Decreased expression of CD204 on ALSP monocytes was also observed in this study. Several observations indicate that a decrease in scavenger receptor activity such as CD204 contributes to the accumulation of abnormal proteins and to the progression of neurodegeneration in Alzheimer's disease (Kelley et al., 2014).

Finally, we observed that monocytes from a patient with AARS2-L showed a similar phenotype to HS rather than to ALSP. Although

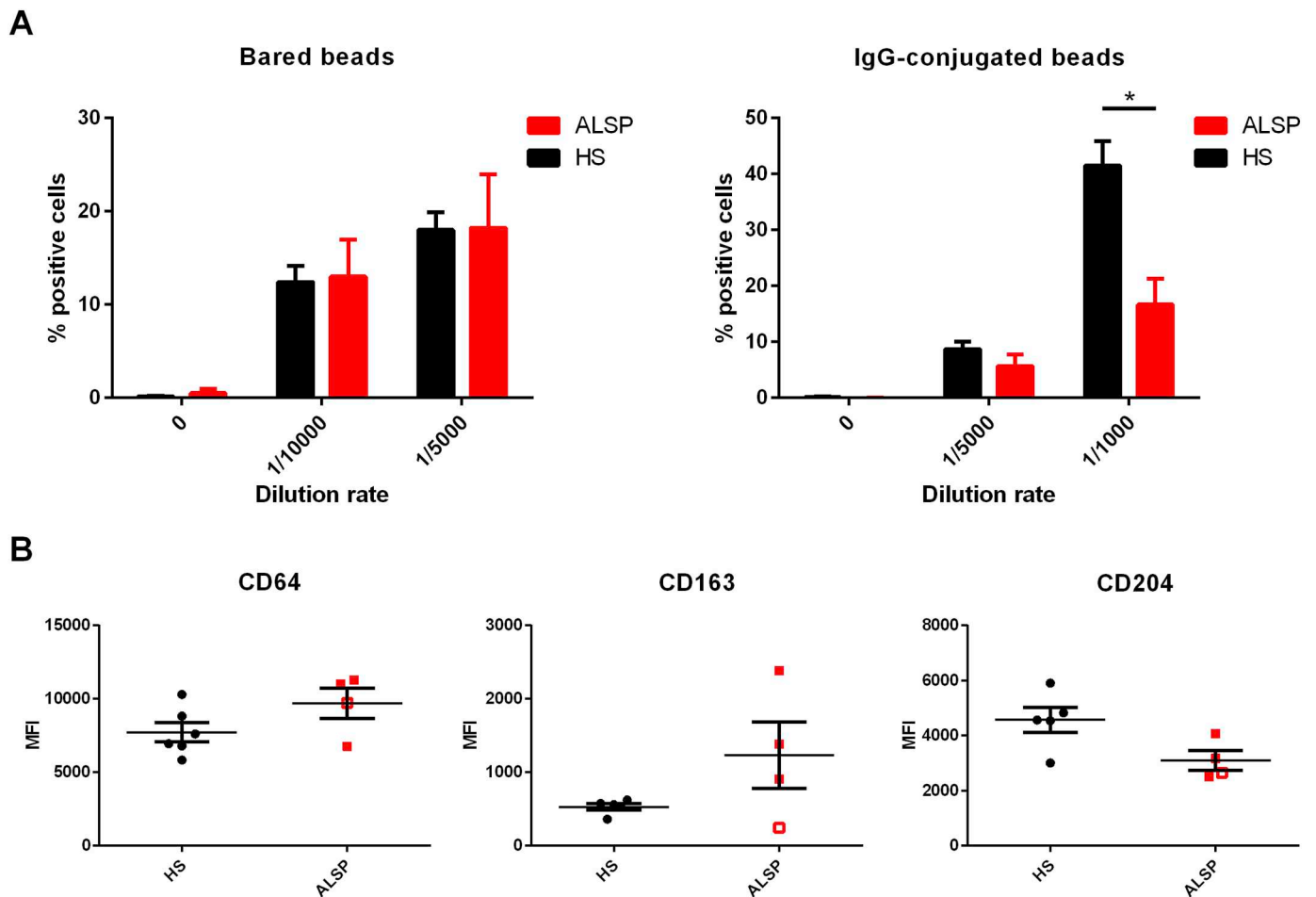


Fig. 5. Phagocytosis assay and expression of Fc receptor and scavenger receptors.

A. PBMCs were incubated with FITC-labeled bare latex beads or beads coated with rabbit Ig for 2 h before cytometric analysis. The frequency of CD14-positive cells that ingested beads was determined as FITC-positive cells. B. The MFI for CD64, CD163, and CD204 of untreated sorted monocytes is shown. The values for Patient IV are indicated as open dots. Data are presented as the mean $\pm$ SEM. $*p < .05$.

ALSP = adult-onset leukoencephalopathy with axonal spheroids and pigmented glia; HS = healthy subjects; FITC = fluorescein isothiocyanate; Ig = immunoglobulin G.

mutations in AARS2 lead to a clinical, radiologic and pathologic phenotype indistinguishable from ALSP, no association with dysfunction of monocyte lineage cells in AARS2-L was shown in this study (Lakshmanan et al., 2017; Lynch et al., 2016; Taglia et al., 2018). Even though the small number of patients limited the significance, our results suggest that monocytic alteration associated with brain disorders is specific to ALSP.

Despite our results, however, there were no clinical histories suggestive of systemic monocytic dysfunction. Further studies are needed to reveal why an altered function of monocyte-lineage cells that does not lead to apparent clinical immunological disorders in the periphery cause dyshomeostasis of the brain in ALSP patients.

5. Conclusion

This is the first report on PB monocytes from patients with ALSP. We speculate that the altered features of PB monocytes observed in this study mostly, if may not fully, represent those of microglial cells, leading to the clinical history and pathological characteristics of ALSP. Our study will likely facilitate further studies to confirm our observations and to overcome the limitation of our small sample size and to unravel the disease mechanism, leading to the development of treatment strategies.

Supplementary data to this article can be found online at <https://>

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Disclosure statement

All authors reports no disclosures.

Declaration of Competing Interest

None.

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