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cells

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Abstract

Microglia are believed to be the only resident immune cells in the central

nervous system (CNS), originating from hematopoietic-derived myeloid cells and

invading the CNS during development.   However, the detailed mechanisms of

differentiation and transformation of microglial cells are not fully understood.

Here, we demonstrate that murine microglial cells show 2 morphological forms in

vitro, namely, small round cells expressing CD11b, Iba1, triggering receptor

expressing on myeloid cells-2 (TREM2), and weakly expressing major

histocompatibility complex (MHC) class II and large flat cells expressing only

CD11b and Iba1.   Moreover, lineage-negative bone marrow (LN) cells cultured

with primary mixed glial culture cells could differentiate into only the small round

microglia-like cells despite the absence of CCR2 and Gr-1 expression.   Addition

of macrophage colony stimulating factor (M-CSF) to LN cell culture allowed the

proliferation and expression of TREM2 in LN cells, and the addition of

neutralizing anti-M-CSF antibodies suppressed the proliferation of LN cells

despite the expression of TREM2.   When LN cells were cultured with M-CSF,

the number of small round cells in the culture was considerably low, indicating

that the small round morphology of the immature cells is not maintained in the

presence of only M-CSF.　On the other hand, when LN cells were grown in the

presence of astrocytes, the small round cells were maintained at a concentration of

approximately 30% of the total population.　Therefore, cell-cell contact with

glial cells, especially astrocytes, may be necessary to maintain the small round

shape of the immature cells expressing TREM2.

Introduction

Microglia are believed to be the only resident immune cells in the central

nervous system (CNS); they develop from hematopoietic-derived myeloid cells

and invade the CNS during development (Ling & Wong, 1993).   Microglial cells

are recognized to play an important role not only in neuroinflammatory and

neurodegenerative diseases such as multiple sclerosis and Alzheimer's disease but

also in neuroprotective and anti-neuroinflammatory processes (Akiyama &

McGeer, 2004; Sanders & De Keyser, 2007; Takahashi et al., 2007).   In a recent

study, it has been reported that physiological microglial phagocytosis induced the

efficient removal of apoptotic cells and cellular debris without inflammatory

processes; this process is expected to be a novel, attractive target for protection

from neuroinflammation or neurodegeneration (Takahashi et al., 2005, 2007;

Neumann & Takahashi, 2007).   The detailed mechanisms underlying the

differentiation and transformation of microglial cells, however, are not fully

understood.   Even in adulthood, hematopoietic-derived cells develop into

resident perivascular macrophages and microglia (Hickey & Kimura, 1988; Priller

et al., 2001; Simard & Rivest, 2004).   Although the exact cellular subtype of

myeloid precursors that develop into microglia is unknown, it has been reported

that only Ly-6C^{high}CCR2+ monocytes can invade and differentiate into perivascular microglia (Mildner et al., 2007).

The bone marrow produces new blood cells, including all cell types of the myeloid lineage, some of which may differentiate into microglia. Lineage-negative bone marrow (LN) cells are defined by the absence of surface markers such as CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1, and TER-119 and are considered to comprise many hematopoietic precursors, including microglia precursors.   Thus, LN cells might represent microglial precursors and may serve as a natural vehicle for CNS cells in gene therapy.

In this study, we show that murine microglial cells are present in vitro in 2 morphological forms, namely, as small round cells expressing CD11b, Iba1, triggering receptor expressing on myeloid cells-2 (TREM2), and weakly expressing major histocompatibility complex (MHC) class II and as large flat cells expressing only CD11b and Iba1.   We found that LN cells could differentiate into the small round-type but not the large flat-type microglia-like cells. Moreover, we concluded that not only macrophage colony-stimulating factor (M-CSF) but also cell-cell contacts with astrocytes play an important role in microglial differentiation.

Materials and Methods

Isolation of LN cells from adult GFP mice bone marrow

Bone marrow cells were collected from 8- to 10-week-old C57BL/6 mice (Charles River, Japan) or green fluorescence protein (GFP) transgenic mice on a C57BL/6 mice background that were kindly provided by Dr. Masaru Okabe (Osaka University, Japan) by flushing out the femora and tibiae of the hind limb. Erythrocyte removal was performed by lysis with the Mouse Erythrocyte Lysing Kit (R&D, Minneapolis, MN). For eliminating lineage marker-positive cells via negative selection, bone marrow cells were incubated at 4°C for 30 min with 8 types of rat monoclonal antibodies against mice lineage markers (CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1, and TER-119 (R&D)). The cells were then washed and incubated with immunomagnetic beads (Invitrogen, Tokyo, Japan) at 4°C for 30 min. Finally, LN cells were collected by the removal of lineage-positive bone marrow cells by using a magnet stand that attracted the lineage marker-positive cells attached to the antibodies.

All experiments were approved by the Ethics Committee of Kanazawa University and performed in accordance with the guidelines of the local animal care and use committee of Kanazawa University.

Primary mixed glial cell culture

Microglial cells were prepared from the brains of postnatal days 3–5 (P3–P5) of C57BL/6 mice as previously described (Takahashi et al., 2005). Briefly, meninges were removed mechanically, and the cells were dissociated by trituration and cultured in basal medium Eagle (BME; Invitrogen), 10% fetal calf serum (FCS; Invitrogen), 1% glucose (Sigma, Tokyo, Japan), 1% L-glutamine (Invitrogen), and 1% penicillin/streptomycin (Invitrogen) for 14 d to form a confluent glial monolayer.   LN bone marrow cells obtained from GFP mice were added onto the confluent glial monolayer.

Treatment of LN cell culture with mixed glial cell culture supernatant, neutralizing antibodies, or cytokines

LN cells were cultured with the mixed glial cell culture supernatant obtained from the day-14 primary mixed glial culture.   LN cells were also cultured with a culture medium containing M-CSF (10 ng/ml, Peprotech, Rocky Hill, NJ), tumor necrosis factor-α (TNF-α; 10 ng/ml, Peprotech), or vascular endothelial growth factor (VEGF; 50 ng/ml, Peprotech) for 7 d.   For the neutralizing assay,

anti-M-CSF (2 μg/ml, R&D), anti-TNF-α (2 μg/ml, R&D), or anti-VEGF (1 μg/ml, R&D) antibodies were added in the LN cell culture for 7 d.

Immunohistochemistry

Mixed glial cell cultures with or without GFP-positive (GFP+) LN cells were fixed in 4% paraformaldehyde for 1 h, blocked by Protein Block (Dako, Denmark) for 2 h, and then immunostained with monoclonal rat antibodies directed against CD11b (Serotec, Oxford, UK) and a secondary fluorescence rhodamine-conjugated antibody directed against rat IgG (1:200, Millipore, Billerica, MA).　To identify the cell type, cells were double-labeled with a purified polyclonal sheep antibody directed against TREM2 (1:50, R&D), monoclonal rabbit antibodies directed against Iba1 (Wako, Kanagawa, Japan), and glial fibrillary acidic protein (GFAP; Dako) and monoclonal mouse antibodies directed against IAb (BD Pharmingen, Tokyo, Japan), followed by a secondary fluorescein isothiocyanate (FITC)-conjugated antibody directed against mouse IgG.　Images were collected by fluorescence microscopy with a 20× objective (Olympus, Tokyo, Japan).　A confocal microscope with a 40× objective (Zeiss, Jena, Germany) was used to obtain z-stack images, and series of optical sections

(512×512 pixels, pixel size: 440 nm) were collected at intervals of 380 nm.

Images were analyzed using the Zeiss LSM Image browser (Zeiss).

To quantify the number of cells, 10 fields under the 20× objective were

randomly selected and photographed by fluorescence microscopy using a digital

camera (Olympus) for each experiment.　Total cells, GFP-positive cells, and

positively immunostained cells were classified and counted according to their

morphology.

Isolation of microglia and splenic macrophages

Microglial cells and GFP+ LN cells-derived microglial cells in primary

mixed glial culture were obtained by shaking the flasks at 150 rpm for 2 h.

Adult CNS microglia and splenic macrophages were obtained from GFP mice.

The cortex and spinal cord of GFP mice were isolated and homogenized.

Homogenates were incubated with 0.3 Wunsch units/ml Liberase Blendzyme 3

(Roche, Tokyo, Japan) and 0.1 mg/ml DNaseI (Roche) in RPMI 1640 medium at

37°C for 30 min.　Microglia were separated through a density gradient.　The

cells were suspended in 27% Percoll (GE Healthcare, Tokyo, Japan) and overlaid

with a 72% gradient.　The density gradient was centrifuged at 2,800 rpm for 30

min at 4°C.   Myelin collected in the 27% Percoll layer was removed.   The

majority of microglia were found in the interface of the 27% and 72% Percoll

layers.   Cells were obtained from this interface and washed from the Percoll with

phosphate buffered saline (PBS).   The spleen was isolated and cut into small

fragments.   Cells were incubated with 0.3 Wunsch units/ml Liberase Blendzyme

3 (Roche) and 0.1 mg/ml DNaseI (Roche) in RPMI 1640 medium at 37°C for 45

min.   Erythrocyte removal was performed using ACK solution.

Flow cytometry analysis of LN cells, microglia, and splenic macrophages

For flow cytometry analysis, cells were first incubated for Fc-receptor

blockade by CD16/CD32 antibody (BD Pharmingen) and then stained with

PE-conjugated anti-IAb antibody and anti-CD45 antibody (BD Pharmingen),

PerCP-Cy5.5-conjugated anti-CD11b antibody (BD Pharmingen),

APC-conjugated anti-TREM2 antibody (R&D) and anti-F4/80 antibody

(eBioscience, San Diego, CA), or purified anti-CCR2 antibody (Abcam, Tokyo,

Japan) followed by rhodamine-conjugated anti-Goat IgG (Millipore, Billerica,

MA).   Analysis was performed with a FACSCalibur flow cytometer (BD

Biosciences, Tokyo, Japan).

Proliferation assay

Microglial cells and GFP+ LN cells-derived microglia-like cells in primary mixed glial culture, adult CNS microglia, and splenic macrophages were cultured with various concentrations of M-CSF (0–100 ng/ml), and cultures were pulsed with 1 mCi of [³H]TdR (MP Biomedicals, Tokyo, Japan) for the last 16 h of the incubation.   Cell incorporation of [³H]TdR was counted with a Topcount (Perkin Elmer, Boston, MA).   The mean cpm of triplicate cultures was calculated.

Stimulation by anti-TREM2 antibody

Small round cells were added to culture dishes coated with the anti-TREM2 monoclonal antibody (R&D) or control antibody and centrifuged at 1500 rpm for 5 min.   After 60 min, the cells were fixed at a final concentration of 4% formaldehyde for 10 min at 37 °C.   Following centrifugation, the supernatant was removed, and the cells were resuspended in 90% ice-cold methanol and incubated for 30 min at 4°C.   Cells were washed and stained with Alexa 647-conjugated anti-phospho-ERK1/2 antibody (Cell Signaling Technology, Tokyo, Japan) for 30 min.   Analysis was performed using a

FACSCalibur flow cytometer.

Bio-Plex Cytokine Assay System

Culture supernatant samples were analyzed simultaneously for 17 different

cytokines and chemokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ,

TNF-α, IL-15, IL-18, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF-BB, and

VEGF) using the Bio-Plex Cytokine Assay System (Bio-Rad Laboratories,

Hercules, CA) according to the manufacturer's instructions.   Briefly, 50 μl of

each sample and standard (Bio-Rad) were added to 50 μl of antibody-conjugated

beads (Bio-Rad) in a 96-well filter plate (Millipore).   After 30-min incubation,

the plate was washed, and 25 μl of a biotinylated antibody solution (Bio-Rad) was

added to each well, followed by 30-min incubation again.   The plate was washed,

and 50 μl of streptavidin-conjugated phycoerythrin (PE; Bio-Rad) was added to

each well and incubated for 10 min.   After a final wash, the contents of each well

were resuspended in 125 μl of assay buffer (Bio-Rad) and analyzed using a

Bio-Plex Array Reader (Bio-Rad).   The lower detection limit for each cytokine

or chemokine was 2 pg/ml.

Statistical analysis

Data are presented as mean ± S.D. of at least three independent experiments.   Data were analyzed by the Mann-Whitney U test to determine significant differences.

Results

Characterization of microglia in the primary mixed glial culture

To characterize microglial cells in the mixed glial culture, we stained several

microglia markers such as Iba1, CD11b, TREM2, and IAb (MHC class II

molecule of C57/BL6 mice) on the cultured microglial cells (Fig. 1A).

CD11b-positive (CD11b+) cells in the primary mixed glial culture showed two

major morphological forms, namely, small round-shaped cells (32.8 ± 6.9% S.D.

of CD11b+ cells) expressing Iba1, TREM2, and IAb (Iba1+ cells, 91.9 ± 10.2%

S.D.; TREM2+ cells, 97.5 ± 3.5% S.D.; IAb+ cells; 88.9 ± 19.2% S.D.) and large

flat cells (67.2 ± 6.9% S.D. of CD11b+ cells) expressing only Iba1 (Iba1+ cells,

81.1 ± 2.6% S.D.; TREM2+ cells, 3.3 ± 1.1% S.D.; IAb+ cells, 6.4 ± 2.8% S.D.;

Iba1 vs. TREM2, *P* = 0.0495; Iba1 vs. IAb, *P* = 0.0495) (Fig. 1B, C).

Z-sectioned scans by confocal microscopy revealed that small, spherically shaped

cells lay above the astrocytes, and large flat cells lay under the astrocytes (Fig.

1D).   Moreover, the spherical cells appeared to have extended processes toward

the astrocytes (Fig. 1E).

To investigate the time point at which both the forms of the microglial cells

appeared in the primary mixed glial culture, we performed a kinetic study.   At

day 1 after the culture of mixed glial cells, CD11b+ cells showed a small

amoeboid shape (Fig. 1F).   At day 3, the CD11b+ cells assumed a larger flat

shape.   Small round CD11b+ cells were very few in number until day 5 (day 3,

1.9 ± 1.1% S.D.; day 5, 2.7 ± 0.9% S.D.) but increased greatly after day 7 (day 7,

10.9 ± 1.1% S.D., **vs. day 3, *P* = 0.0495;** day 14, 32.8 ± 6.9% S.D., **vs. day 3, *P* =**

0.0495) (Fig. 1F, G).

Characteristics of LN cells

LN cells were isolated by negative selection using magnet beads.   The

purity of LN cells after negative selection by flow cytometry was consistently

above 90% on several examinations (Fig. 2A).   LN cells expressed neither the

microglia marker TREM2 nor CD11b, which is one of the lineage markers for

negative selection (Fig. 2B).   No expression of CCR2 or Gr-1 (lineage markers

for negative selection) was detected on the LN cells, which have previously been

described as markers of microglial precursors (Fig. 2B).   IAb, which is an MHC

class II antigen, was negative on the surface of the LN cells.

Differentiation of LN cells into microglia-like cells

LN cells obtained from GFP mice were co-cultured with primary mixed glial

cells for 2 weeks.   Two weeks after the co-culture, the cells were stained by

anti-TREM2, anti-Iba1, anti-MHC class II, and anti-CD11b antibodies, followed

by rhodamine- or Cy3-conjugated secondary antibodies (Fig. 3A).   GFP+ cells

showed 2 major morphologies, i.e., small and round cells with bright nuclei (27.7

± 7.9% S.D. in GFP+ cells) and flat cells with dark nuclei (69.9 ± 6.9% S.D. in

GFP+ cells), similar to the microglia in the primary mixed glial cells seen in Fig.

1.   Most of the small round cells were TREM2-, Iba1-, CD11b-, and IAb-positive

(TREM2+ cells, 92.7 ± 1.9% S.D.; Iba1+ cells, 93.6 ± 1.6% S.D.; CD11b+ cells,

96.5 ± 3.1% S.D.; IAb+ cells, 84.6 ± 13.4% S.D.) (Fig. 3B) and were spherically

shaped along the z-axis (Fig. 3C).   On the other hand, flat cells expressed none

of the following: CD11b, Iba1, TREM2, or IAb (TREM2+ cells, 6.5 ± 3.9% S.D.;

Iba1+ cells, 3.5 ± 1.0% S.D.; CD11b+ cells, 9.3 ± 3.7% S.D.; IAb+ cells, 3.9 ±

1.9% S.D.).   **Since TREM2/DAP12 signaling is known to induce ERK**

phosphorylation in immature dendritic cells, we analyzed the

phosphorylation of ERK using flow cytometry after cross-linking stimulation

of LN cell-derived small round cells in order to determine whether the

TREM2 on small round cells was functional.   Stimulation of TREM2 of the

LN cell-derived small round cells induced phosphorylation of ERK as demonstrated by a specific antibody recognizing the phosphorylated form of ERK (Fig. 3D).

In the kinetic study, GFP+ LN cells were immunostained at days 1, 7, and 14 after co-culturing with primary mixed glial cells with anti-TREM2 and anti-Iba1 antibodies followed by secondary antibodies. At day 1, LN cells had already differentiated into 2 morphological groups—small round cells expressing Iba1 and TREM2 and flat amoeboid cells (Fig. 3**E**). Interestingly, the ratio of LN cell-derived small round and flat cells remained identical at day 1 and day 7 but reduced at day 14 (day 1, 45.7 ± 2.5% S.D.; day 7, 41.7 ± 1.9% S.D., **vs. day 1, P = 0.0495**; day 14, 29.5 ± 6.4% S.D., **vs. day 1, P = 0.0495**) (Fig. 3**F**).

Analysis of surface cell markers and proliferative capacity of LN cell-derived small round cells

In many publications, not only the expression of CD11b, Iba1, and F4/80 but also low expressions of CD45 and MHC class II have been used as microglial markers. Among them, low expressions of CD45 and MHC class II are one of the most important resting microglial markers. We measured the quantitative

expression of CD45 and MHC class II on brain microglia, cultured microglia, and

LN cell-derived small round cells.   As shown in Figure 4A, LN cell-derived

small round cells showed low expressions of CD45 and MHC class II, which was

identical to results for cultured and brain microglia as compared to those for

spleen-derived macrophages.   LN cell-derived small round cells were also F4/80

positive, which is known to be another microglial marker.

Since microglia can continue to proliferate and differentiate to macrophages,

we investigated the proliferative capacity of brain microglia and LN cell-derived

small round cells with various concentrations of M-CSF, and compared this

capacity to that of splenic macrophages.   LN cell-derived small round cells

showed increased incorporation of [^{3}H] thymidine similar to that by brain

microglia, but [^{3}H] thymidine incorporation by splenic macrophages did not

increase in a low concentration of M-CSF (1 ng/ml) (**LN cell-derived small**

round cells vs. splenic macrophages, *P* = 0.0495; brain microglia vs. splenic

macrophages, *P* = 0.0495) (Fig. 4B).

These results indicate that small round cells and microglia are similar in

terms of their cell surface molecules and the proliferative capacity.

Proliferation and differentiation of LN cells in the presence of M-CSF

To examine the humoral factors necessary for the differentiation of LN cells

into microglia, we cultured LN cells with the supernatant of the primary mixed

glial culture for 7 d.　Most of the LN cells cultured with this supernatant

(LN-Sup cells) were flat in shape and expressed CD11b, Iba1, TREM2, and IAb

(Fig. 5A).

Next, we measured the concentrations of cytokines in the supernatant of the

primary mixed glial culture using the Bio-Plex Cytokine Assay System.　Among

the assayed cytokines, the concentrations of M-CSF, VEGF, and TNF-α were

remarkably high (614.2 ± 121.5 S.D. pg/ml of M-CSF, 2349.4 ± 845.9 S.D. pg/ml

of VEGF, and 585.5 ± 278.2 S.D. pg/ml of TNF-α) compared with those of the

other cytokines (Fig. 5B).　IL-2, IL-4, IL-5, and GM-CSF were not detected, and

the concentrations of IL-1β, IL-10, IFN-γ, IL-15, FGF-basic, and MIG were very

low (24.8 ± 10.7 S.D. pg/ml of IL-1β, 12.4 ± 6.9 S.D. pg/ml of IL-10, 18.0 ± 13.0

S.D. pg/ml of IFN-γ, 34.4 ± 12.5 S.D. pg/ml of IL-15, and 21.6 ± 11.9 S.D. pg/ml

of FGF-basic).　The concentrations of IL-18, LIF, MIP-2, and PDGF-BB were at

an intermediate level (61.9 ± 23.3 S.D. pg/ml of IL-18, 92.5 ± 23.8 S.D. pg/ml of

LIF, 34.7 ± 11.6 S.D. pg/ml of MIG, 57.0 ± 31.6 S.D. pg/ml of MIP-2, and 115.7 ±

36.1 S.D. pg/ml of PDGF-BB).

To investigate the effect of each cytokine on the differentiation of LN cells,

we cultured LN cells with M-CSF, VEGF, or TNF-α for 7 d.   All the LN cells

cultured with M-CSF (LN-MCSF cells) were CD11b- and TREM2-positive (Fig.

5C).   Morphologically, most LN-MCSF cells showed flat shapes on confocal

microscopy (Fig. 5D).   The number of LN cells cultured with TNF-α (LN-TNF

cells) or with VEGF (LN-VEGF cells) were very few, although these cells

expressed CD11b and TREM2 (Fig. 5C).

Next, LN cells were cultured with the mixed glial culture supernatant

containing neutralizing antibodies of M-CSF, TNF-α, or VEGF.   Addition of the

anti-VEGF antibody or anti-TNF-α antibody resulted in no change in the

morphology or surface markers as compared with the culture containing the

control antibodies; however, the addition of anti-M-CSF antibodies remarkably

reduced the cell number despite the expression of TREM2 (Fig. 5C).

Cell-to-cell contact between microglia and astrocytes

In order to reveal the role of cell-to-cell contact between microglia and other glial cells, especially astrocytes, in the differentiation of LN cells to microglia, we performed immunostaining of the mixed glial culture with LN cells by anti-GFAP antibody followed by secondary rhodamine-conjugated antibody against rabbit-IgG. Analysis by confocal microscopy revealed that LN cell-derived small round cells were positioned above the GFAP-positive astrocytes (Fig. 6). On the other hand, large flat cells lay immediately beneath the GFAP-positive astrocytes.

Discussion

Microglia, the immune cells of the CNS, exist in 3 distinct forms—amoeboid, ramified, and reactive microglia. Ramified microglia are present in the brain parenchyma and constitute approximately 10–20% of the total population of glial cells in an adult (Vaughan & Peters, 1974; Banati, 2003). Ramified microglia are small round cells comprising branching processes and are considered to be functionally inactive. Further, they are known to express TREM2 in vivo (Schmid et al., 2002; Sessa et al., 2004) but not MHC class II (Santambrogio et al., 2001; Servet-Delprat et al., 2002). Recently, HSP60 has been identified as the ligand of TREM2, and its interaction with microglia has been demonstrated to stimulate microglial phagocytosis (Stefano et al., 2009). This result indicates that TREM2 might play an important role in physiological phagocytosis as one of the microglia-specific functions. TREM2 expression on small round cells **in primary mixed glial culture** might indicate that small round cells have greater mobility as microglia than do large flat cells in patrolling the brain environment in order to identify HSP60-expressing cells. Moreover, as shown in Figure 4A, small round cells weakly expressed MHC class II molecules similar to brain microglia. These results support our hypothesis that small round microglia in

vitro differentiated to a greater extent than did large flat cells.   Consistent with

the results of the current study, previous studies have reported 2 different

morphological shapes of microglia in primary mixed glial culture cells derived

from mice (Saura et al., 2003) and rats (Tanaka et al., 1999; Kuwabara et al.,

2003).   In another study, the kinetic analysis of rat primary mixed glial culture

demonstrated that "amoeboid" or "round" cells appeared during the early stages

of the culture and that the majority of ramified microglia were formed after the

complete formation of the astrocyte monolayer (Tanaka et al., 1999).   We

demonstrated that LN cells directly differentiated into small round microglia-like

cells without the intermediary formation of large flat precursors.   Although

Ly-6C^{high} Gr-1+CCR2+ monocytes in the peripheral blood have been reported to

be the precursors of adult murine microglia (Mildner et al., 2007; Getts et al.,

2008), the LN cells in our study did not express CCR2 or Gr-1 (the anti-Gr-1

antibodies used in this study also reacted with Ly-6C).   The role of CCR2, a

crucial chemokine receptor for the chemotactic attraction of monocytes or

macrophages during CNS inflammation, has been investigated in animal models

of multiple sclerosis, such as experimental autoimmune encephalomyelitis (Fife et

al., 2000; Izikson et al., 2000).   However, the physiological development and

functions of microglia are independent of CCR2 expression (Mildner et al., 2007).

Since the absence of CCR2 expression is highly related to the Ly-6C^{high}

subpopulation (Mildner et al., 2007), Ly-6C may play a more important role than

CCR2 in microglial differentiation.   It is unclear why the LN cells differentiated

into small round microglia-like cells despite the lack of Ly-6C and Gr-1

expression, but we hypothesize that the blood-brain barrier plays a key role in the

differentiation of LN cells.   Indeed, Ly-6C is known to regulate endothelial

adhesion and the homing of CD8+T cells by activating integrin-dependent

adhesion pathways (Hänninen et al., 1997).

On the other hand, the LN cells in the current study did not differentiate

into flat cells as the precursors of small round microglia.   Since they were first

described by del Rio-Hortega (1932), the developmental origin of microglia has

not been completely elucidated.   It is widely hypothesized that microglia are

derived from myeloid lineage precursors and/or hematopoietic precursors during

CNS development.   The fact that the formation of microglia occurs before the

onset of vascularization in the developing brain (Wang et al., 1996) suggests that

hematopoietic stem cells, which act as precursors to microglia during

development, may be more primitive than precursor LN cells.   Therefore, it is

likely that the LN cells adopt a different pathway to differentiate into small round microglia-like cells.

The supernatant of the primary mixed glial cultures derived from mice contains many cytokines such as M-CSF, VEGF, and TNF-α.   Our study showed that M-CSF plays an important role in the differentiation and proliferation of LN cells.   TREM2 is a unique molecule that is only expressed on microglia, osteoclasts, and immature dendritic cells (Colonna, 2003), while other microglia-specific markers such as Iba1 and CD11b are also expressed on macrophages and monocytes.   Since TREM2 was not expressed on the mature dendritic cells or the activated microglia (macrophages), it is likely that TREM2 expression may be restricted to the immature stages of myeloid lineage cells.   In our study, M-CSF induced the proliferation of LN cells and the expression of not only CD11b and Iba1 but also TREM2 on LN cells; this indicates that M-CSF determines the differentiation of LN cells into the myeloid lineage, but not into fully differentiated cells.   Although the addition of anti-M-CSF antibodies appears not to suppress the expression of TREM2 on the LN cells, these TREM2+ cells may have originated from more mature precursors that are independent of M-CSF.   These immature LN cells would eventually be fully differentiated under

physiological conditions.   When LN cells were cultured with M-CSF, the number

of small round cells in the culture was considerably low, indicating that the small

round morphology of the immature cells is not maintained in the presence of only

M-CSF.   On the other hand, when LN cells were grown in the presence of other

glial cells (especially astrocytes), the small round cells were maintained at a

concentration of approximately 30% of the total population.   Therefore, cell-cell

contact with glial cells, especially astrocytes, may be necessary to maintain the

small round shape of immature cells expressing TREM2.

Acknowledgments

Abbreviations

BME, basal medium Eagle; CNS, central nervous system; DAPI,

4′,6-diamidino-2-phenylindole; FCS, fetal calf serum; FITC, fluorescein

isothiocyanate; GFAP, glial fibrillary acidic protein; GFP, green fluorescence

protein; LN cells, lineage-negative bone marrow cells; M-CSF, macrophage

colony stimulating factor; MHC, major histocompatibility complex; PE,

phycoerythrin; TNF-α, Tumor Necrosis Factor-α; TREM2, triggering receptor

expressing on myeloid cells-2; VEGF, vascular endothelial growth factor.

References

Akiyama, H. & McGeer, P. L. (2004) Specificity of mechanisms for plaque

removal after A beta immunotherapy for Alzheimer disease. *Nat. Med.*, **10**,

117–118; author reply 118–119.

Banati, R. B. (2003) Neuropathological imaging: in vivo detection of glial

activation as a measure of disease and adaptive change in the brain. *Br. Med.*

Bull., **65**, 121–131.

Colonna, M. (2003) TREMs in the immune system and beyond. *Nat. Rev.*

Immunol., **3**, 445–453.

del Rio-Hortega, P. (1932) Microglia. In Penfield, W. (ed.), *Cytology and Cellular*

Pathology of the Nervous System. vol. 2, Hoeber, New York, pp. 481–534.

Fife, B. T., Huffnagle, G. B., Kuziel, W. A. & Karpus, W. J. (2000) CC

chemokine receptor 2 is critical for induction of experimental autoimmune

encephalomyelitis. *J. Exp. Med.*, **192**, 899–905.

Getts, D. R., Terry, R. L., Getts, M. T., Müller, M., Rana, S., Shrestha, B.,

Radford, J., Van Rooijen, N., Campbell, I. L. & King, N. J. C. (2008) Ly6c+

"inflammatory monocytes" are microglial precursors recruited in a

pathogenic manner in West Nile virus encephalitis. *J. Exp. Med.*, **205**,

2319–2337.

Hänninen, A., Jaakkola, I., Salmi, M., Simell, O. & Jalkanen, S. (1997) Ly-6C

regulates endothelial adhesion and homing of CD8(+) T cells by activating

integrin-dependent adhesion pathways. *Proc. Natl. Acad. Sci. U. S. A.*, **94**,

6898–6903.

Hickey, W. F. & Kimura, H. (1988) Perivascular microglial cells of the CNS are

bone marrow-derived and present antigen in vivo. *Science*, **239**, 290–292.

Izikson, L., Klein, R. S., Charo, I. F., Weiner, H. L. & Luster, A. D. (2000)

Resistance to experimental autoimmune encephalomyelitis in mice lacking

the CC chemokine receptor (CCR)2. *J. Exp. Med.*, **192**, 1075–1080.

Kuwabara, Y., Yokoyama, A., Yang, L., Toku, K., Mori, K., Takeda, I.,

Shigekawa, T., Zhang, B., Maeda, N., Sakanaka, M. & Tanaka, J. (2003)

Two populations of microglial cells isolated from rat primary mixed glial

cultures. *J. Neurosci. Res.*, **73**, 22–30.

Ling, E. A. & Wong, W. C. (1993) The origin and nature of ramified and

amoeboid microglia: A historical review and current concepts. *Glia*, **7**, 9–18.

Mildner, A., Schmidt, H., Nitsche, M., Merkler, D., Hanisch, U. K., Mack, M.,

Heikenwalder, M., Brück, W., Priller, J. & Prinz, M. (2007) Microglia in the

adult brain arise from Ly-6ChiCCR2+ monocytes only under defined host

conditions. *Nat. Neurosci.*, **10**, 1544–1553.

Neumann, H. & Takahashi, K. (2007) Essential role of the microglial triggering

receptor expressed on myeloid cells-2 (TREM2) for central nervous tissue

immune homeostasis. *J. Neuroimmunol.*, **184**: 92–99.

Priller, J., Flügel, A., Wehner, T., Boentert, M., Haas, C. A., Prinz, M.,

Fernández-Klett, F., Prass, K., Bechmann, I., de Boer, B. A., Frotscher, M.,

Kreutzberg, G. W., Persons, D. A. & Dirnagl, U. (2001) Targeting

gene-modified hematopoietic cells to the central nervous system: use of

green fluorescent protein uncovers microglial engraftment. *Nat. Med.*, **7**,

1356–1361.

Sanders, P. & De Keyser, J. (2007) Janus faces of microglia in multiple sclerosis.

Brain. Res. Rev., **54**, 274–285.

Santambrogio, L., Belyanskaya, S. L., Fischer, F. R., Cipriani, B., Brosnan, C. F.,

Ricciardi-Castagnoli, P., Stern, L. J., Strominger, J. L. & Riese, R. (2001)

Developmental plasticity of CNS microglia. *Proc. Natl. Acad. Sci. U. S. A.*,

98, 6295–6300.

Saura, J., Tusell, J. M. & Serratosa, J. (2003) High-yield isolation of murine

microglia by mild trypsinization. *Glia.*, **44**, 183–189.

Schmid, C. D., Sautkulis, L. N., Danielson, P. E., Cooper, J., Hasel, K. W.,

Hilbush, B. S., Sutcliffe, J. G. & Carson, M. J. (2002) Heterogeneous

expression of the triggering receptor expressed on myeloid cells-2 on adult

murine microglia. *J. Neurochem.*, **83**, 1309–1320.

Servet-Delprat, C., Arnaud, S., Jurdic, P., Nataf, S., Grasset, M. F., Soulas, C.

Domenget, C., Destaing, O., Rivollier, A., Perret, M., Dumontel, C., Hanau,

D., Gilmore, G., Belin, M. F., Rabourdin-Combe, C. & Mouchiroud, G.

(2002) Flt3+ macrophage precursors commit sequentially to osteoclasts,

dendritic cells and microglia. *BMC Immunol.*, **3**, 15.

Sessa, G., Podini, P., Mariani, M., Meroni, A., Spreafico, R., Sinigaglia, F.,

Colonna, M., Panina, P. & Meldolesi, J. (2004) Distribution and signaling of

TREM2/DAP12, the receptor system mutated in human polycystic

lipomembraneous osteodysplasia with sclerosing leukoencephalopathy

dementia. *Eur. J. Neurosci.*, **20**, 2617–2628.

Simard, A. R., & Rivest, S. (2004) Bone marrow stem cells have the ability to

populate the entire central nervous system into fully differentiated

parenchymal microglia. *FASEB J.*, **18**, 998–1000.

Stefano, L., Racchetti, G., Bianco, F., Passini, N., Gupta, R. S., Bordignon, P. P. & Meldolesi, J. (2009) The surface-exposed chaperone, Hsp60, is an agonist of the microglial TREM2 receptor. *J. Neurochem.*, **110**, 284–294.

Takahashi, K., Rochford, C. D. P. & Neumann, H. (2005) Clearance of apoptotic neurons without inflammation by microglial triggering receptor expressed on myeloid cells-2. *J. Exp. Med.*, **201**, 647–657.

Takahashi, K., Prinz, M., Stagi, M., Chechneva, O. & Neumann, H. (2007) TREM2-transduced myeloid precursors mediate nervous tissue debris clearance and facilitate recovery in an animal model of multiple sclerosis. *PLoS Med.*, **4**, e124.

Tanaka, J., Toku, K., Sakanaka, M. & Maeda N. (1999) Morphological differentiation of microglial cells in culture: involvement of insoluble factors derived from astrocytes. *Neurosci. Res.*, **34**, 207–215.

Vaughan, D. W. & Peters, A. (1974) Neuroglial cells in the cerebral cortex of rats from young adulthood to old age: an electron microscope study. *J. Neurocytol.*, **3**, 405–429.

Wang, C. C., Wu, C. H., Shieh, J. Y., Wen, C. Y. & Ling, E. A. (1996) Immunohistochemical study of amoeboid microglial cells in fetal rat brain. *J.*

Anat., **189** (Pt 3), 567–574.

Figure legends

Figure 1.

Morphology of microglia in primary mixed glial culture.   *A*, Immunocytology of

primary mixed glial culture stained with anti-Iba1, anti-TREM2, anti-IAb,

anti-CD11b, and 4′,6-diamidino-2-phenylindole (DAPI).   Arrowheads indicate

small round cells.   Arrows indicate large flat cells.   Bar: 50 μm.   *B*, The

percentage of round cells or flat cells among staining-positive cells was quantified

by microscopic analysis.   Data are presented as mean ± standard deviation (S.D.).

C, The percentage of Iba-1-, TREM2-, or IAb-positive cells among

CD11b-positive (CD11b+) round or flat cells was quantified by microscopic

analysis.   Data are presented as mean ± S.D.   *D*, Z-stack immunofluorescence

confocal microscopy of primary mixed glial culture stained with anti-CD11b (red),

anti-GFAP (green), and DAPI (blue).   Bar: 10 μm.   *E*, Z-stack

immunofluorescence confocal microscopy of primary mixed glial culture stained

with anti-CD11b (red), anti-GFAP (green), and DAPI (blue).   Bar: 10 μm.   *F*,

Immunocytology of primary mixed glial culture.   Cultures were fixed at days 1,

3, 5, and 7 of the culture and stained with primary anti-CD11b and anti-GFAP

antibodies followed by rhodamine- or FITC-conjugated secondary antibody and

DAPI.   Arrowheads indicate small round cells.   Bar: 50 μm.   *G*, The

percentage of CD11b+ round or flat cells was quantified by microscopic analysis.

Data are presented as mean ± S.D. ***, P < 0.05***.   Data are representative of three

independent experiments.

Figure 2.

Flow cytometry analysis of lineage-negative bone marrow (LN) cells.   *A*, Flow

cytometry analysis of LN cells (filled histogram) and bone marrow cells (open

histogram) stained with rat monoclonal antibodies against mice lineage markers

(CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1, and TER-119).

Numbers above the lines indicate the percentage of LN cells.   *B*, Flow cytometry

analysis of LN cells.   Filled histograms, staining with antibodies to markers

below plots; Open histograms, isotype-matched control antibody.   Data are

representative of three independent experiments.

Figure 3.

Lineage-negative bone marrow (LN) cells co-cultured with primary mixed glial

culture.   *A*, Immunocytology of LN cells derived from GFP mice co-cultured

with primary mixed glial culture.   Cultures were stained with primary anti-Iba1, anti-TREM2, anti-IAb, and anti-CD11b antibodies followed by rhodamine- or Cy3-conjugated secondary antibody and DAPI.   Arrows indicate double-positive cells.   White arrowheads indicate GFP-negative rhodamine- or Cy3-positive cells.   Red arrowheads indicate GFP-positive (GFP+) rhodamine- or Cy3-negative cells.   Bar: 50 μm.   *B*, Percentage of round or flat cells among GFP+ cells or double-positive cells.   Data are presented as mean ± S.D.   *C*, Z-stack immunofluorescence confocal microscopy of LN cells derived from GFP mice co-cultured with primary mixed glial culture stained with anti-TREM2 (red) and DAPI (blue).   GFP and TREM2 double-positive cells show a spherical shape.   Bar: 10 μm.   ***D*, Phosphorylation of ERK after cross-linking stimulation of LN cell derived small round cells by flow cytometry.   Red line histograms, stimulated with anti-TREM2 antibody; Blue line histograms, stimulated with control antibody; Filled histograms, stained with isotype-matched control antibody.   Data are representative of three independent experiments.**   *E*, Immunocytology of LN cells derived from GFP mice co-cultured with primary mixed glial culture.   Cells were fixed at days 1, 7, and 14 after co-culture and stained with primary anti-TREM2 or anti-Iba1 antibodies followed by

rhodamine-conjugated secondary antibody and DAPI.   Arrows indicate GFP and

TREM2/Iba1 double-positive cells.   Arrowheads indicate GFP+ and

rhodamine-negative cells.   Bar: 50 μm.   *F*, Percentage of round or

amoeboid/flat cells among GFP+ cells was quantified by microscopic analysis.

The proportion of small round and amoeboid/flat cells of GFP+ cells showed no

changes at days 1 and 7, but the number of small round cells reduced at day 14.

Data are presented as mean ± S.D. **, *P* < 0.05**.   Data are representative of three

independent experiments.

Figure 4.

Surface cell markers and proliferative capacity of lineage-negative bone marrow

(LN) cell-derived small round cells.   *A*, Flow cytometry analysis of cultured

microglia, LN cell-derived small round cells, brain microglia, and splenic

macrophages.   Open histograms, staining with antibodies to markers below plots;

Filled histograms, isotype-matched control antibody.   Data are representative of

three independent experiments.   B, Proliferative capacity of cultured microglia,

LN cell-derived small round cells, brain microglia, and splenic macrophages.

Proliferation was measured by thymidine incorporation.   Data are presented as

mean ± S.D. **_*, P < 0.05._** Data are representative of three independent experiments.

Figure 5.

Proliferation and expression of TREM2 on lineage-negative bone marrow (LN) cells in the presence of M-CSF. *A*, Immunocytology of LN cells cultured with supernatant of primary mixed glial culture. Cultures were stained with anti-Iba1, anti-TREM2, anti-IAb, anti-CD11b, and DAPI. Bar: 50 μm. *B*, Analysis of concentration of cytokines (IL-1b, IL-2, IL-4, IL-5, IL-10, GM-CSF, IFN-γ, TNF-α, IL-15, IL-18, FGF-basic, LIF, M-CSF, MIG, MIP-2, PDGF-BB, and VEGF) in the supernatant of primary mixed glial culture by using the Bio-Plex Cytokine Assay System. ND; not detectable. Data are presented as mean ± S.D. of 12 independent experiments. *C*, Immunocytology of LN cells cultured with M-CSF-, VEGF-, or TNF-α-containing medium and anti-MCSF, anti-VEGF, or anti TNF-α antibody-containing supernatant of mixed glial culture. The cells were stained with anti-TREM2 antibodies, CD11b antibodies, and DAPI. LN cells cultured with M-CSF showed no change in their morphology or surface markers as compared with LN cells cultured with the supernatant of primary

mixed glial culture; however, the addition of anti-M-CSF antibodies remarkably

reduced the cell number.   Bar: 50 μm.   *D*, The percentage of spherical or flat

cells was quantified by confocal microscopy.   Data are presented as mean ± S.D.

_*, P < 0.05_.   Data are representative of three independent experiments.

Figure 6.

Z-stack immunofluorescence confocal microscopy of lineage-negative bone

marrow cells derived from GFP mice co-cultured with primary mixed glial culture

stained with anti-GFAP (red) and DAPI (blue).   Bar: 10 μm.

A

Iba1/CD11b   TREM2 /CD11b   TREM2/IAb

B

Percentage of round or flat cells (%)

- round cell
- flat cell

CD11b   Iba-1   TREM2   IAb

C

Percentage of staining positive cells (%)

- round cell
- flat cell

Iba1   TREM2   IAb

D

top

bottom

z-stacks image

CD11b   GFAP   CD11b GFAP DAPI

E

top

bottom

z-stacks image

CD11b   GFAP   CD11b GFAP DAPI

F

day1

day3

day5

day7

CD11b   GFAP   CD11b GFAP DAPI

G

Percentage of round or flat cells (%)

- round cell
- flat cell

day3   day5   day7   day14

A

GFP | Iba1
DAPI | merge
Iba1

GFP | CD11b
DAPI | merge
CD11b

GFP | TREM2
DAPI | merge
TREM2

GFP | IAb
DAPI | merge
IAb

B

■ round cell   □ flat cell

Percentage of round or flat cells (%)

LN | Iba1+ | CD11b+ | TREM2+ | Iab+

C

top
bottom
z-stacks image

GFP | TREM2 | GFP TREM2 DAPI

D

— Anti-TREM2 antibody
— Control antibody

LN cell-derived small round cells

Counts

Phospho-ERK 1/2

E

day1
day7
day14

GFP | TREM2 | merge   GFP | Iba1 | merge

F

■ round cell   □ ameboid/flat cell

Percentage of round or ameboid/flat cells (%)

day1 | day7 | day14

A

Cultured microglia

LN cell-derived small round cell
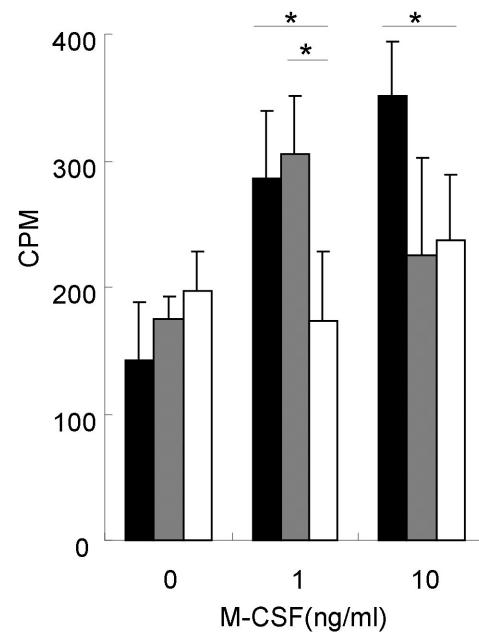
Brain microglia

Splenic macrophage

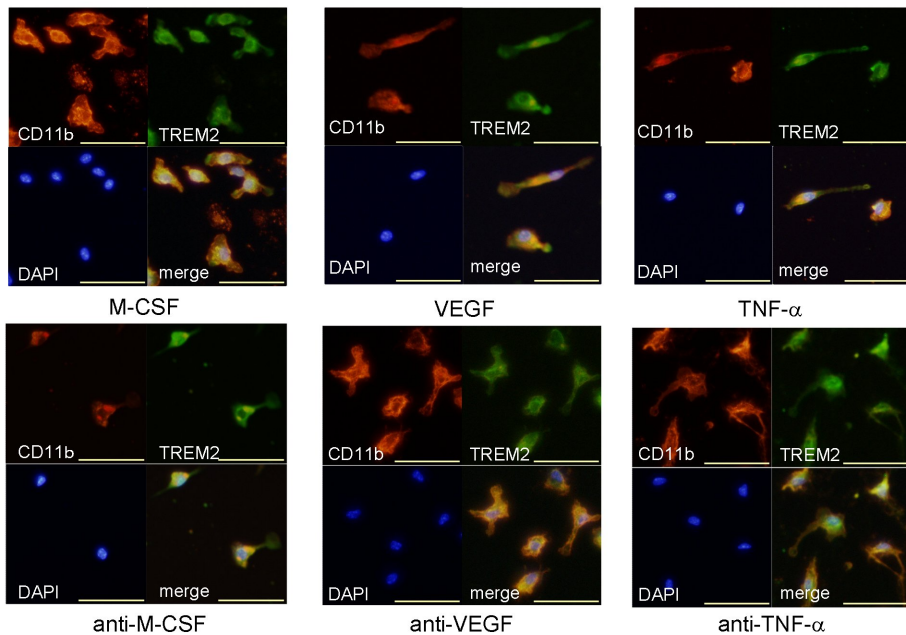CD45   CD11b   F4/80   IAb

B

- LN cell-derived small round cell
- Brain microglia
- Splenic macrophage

CPM

M-CSF(ng/ml)

A — Mixed glial culture supernatant

B

C — M-CSF, VEGF, TNF-α, anti-M-CSF, anti-VEGF, anti-TNF-α

D

top

bottom

z-stacks
image

| GFP | GFAP | GFP GFAP DAPI |