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Development of a culture system to induce microglia-like cells from haematopoietic cells

D. Noto*†[1], H. Sakuma*‡[1], K. Takahashi†§, R. Saika*, R. Saga*, M. Yamada†, T. Yamamura* and S. Miyake*¶

**Department of Immunology, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Tokyo, †Department of Neurology and Neurobiology of Aging, Kanazawa University Graduate School of Medical Science, Kanazawa, ‡Department of Brain Development and Neural Regeneration, Tokyo Metropolitan Institute of Medical Science, Tokyo, §Department of Neurology, National Hospital Organization Iou National Hospital, Kanazawa, and ¶Department of Immunology, Juntendo University Graduate School of Medicine, Tokyo, Japan*

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Aims: Microglia are the resident immune cells in the central nervous system, originating from haematopoietic-derived myeloid cells. A microglial cell is a double-edged sword, which has both pro-inflammatory and anti-inflammatory functions. Although understanding the role of microglia in pathological conditions has become increasingly important, histopathology has been the only way to investigate microglia in human diseases. **Methods:** To enable the study of microglial cells *in vitro*, we here establish a culture system to induce microglia-like cells from haematopoietic cells by coculture with astrocytes. The characteristics of microglia-like cells were analysed by flow cytometry and functional assay. **Results:** We show that triggering receptor expressing on myeloid cells-2-expressing microglia-like cells could be induced from lineage negative cells or monocytes by coculture with astrocytes. Microglia-like cells exhibited lower expression of CD45 and MHC class II than macrophages, a characteristic similar to brain microglia. When introduced into brain slice cultures, these microglia-like cells changed their morphology to a ramified shape on the first day of the culture. Moreover, we demonstrated that microglia-like cells could be induced from human monocytes by coculture with astrocytes. Finally, we showed that interleukin 34 was an important factor in the induction of microglia-like cells from haematopoietic cells in addition to cell–cell contact with astrocytes. Purified microglia-like cells were suitable for further culture and functional analyses. **Conclusion:** Development of *in vitro* induction system for microglia will further promote the study of human microglial cells under pathological conditions as well as aid in the screening of drugs to target microglial cells.

Keywords: astrocytes, haematopoietic cells, interleukin 34, microglia, monocytes, triggering receptor expressing on myeloid cells-2 (TREM2)

Introduction

Microglial cells are the only resident immune cells in the central nervous system (CNS). Microglial cells play an important role not only in inflammation and neurodegenerative processes such as multiple sclerosis and Alzheimer's disease but also in anti-neuroinflammatory

Correspondence: Sachiko Miyake, Section Head, Department of Immunology, National Institute of Neuroscience, 4-1-1 Ogawahigashi, Kodaira, Tokyo 187-8502, Japan. Tel: 042-341-2711 (Ext: 5244); Fax: 042 346 1753; E-mail: miyake@ncnp.go.jp

[1]Daisuke Noto and Hiroshi Sakuma contributed equally to this work. Authors have no conflict of interest to declare.

and neuroprotective processes [1–3]. Microglial cells develop from haematopoietic-derived myeloid cells and invade the CNS during development [4,5]. A recent report demonstrates that microglia arise early in mouse development and derive from primitive macrophages in the yolk sac [6]. In adulthood, haematopoietic-derived cells develop into resident perivascular macrophages and microglia [7–10]. Although the detailed mechanisms underlying the differentiation of microglial cells are not fully understood, the peripheral monocyte is thought to be a candidate for the microglial precursor under pathological conditions.

Previously, we reported that lineage-negative bone marrow cells (LN^- cells) cocultured with primary mixed glial cells could differentiate into 'triggering receptor expressing on myeloid cells-2-' (TREM2) expressing microglial cells [11]. TREM2 is a unique molecule that is predominantly expressed on microglia, osteoclasts and immature dendritic cells [12]. Microglia expressing TREM2 perform physiological microglial phagocytosis and efficient removal of apoptotic cells and cellular debris without inducing inflammatory processes. Furthermore, the injection of TREM2-expressing myeloid cells into experimental autoimmune encephalomyelitis (EAE) mice induced amelioration of clinical symptoms [3,13]. Moreover it has been recently reported that TREM2 is highly expressed in amyloid plaque-associated microglia in mouse models of Alzheimer's disease and that TREM2-expressing microglia may evoke neuroprotective immune responses [14]. Therefore, the TREM2-associated microglia phagocytic process is expected to be a novel, attractive target for protection from neuroinflammatory or neurodegenerative disorders [3,13,15]. Although the need to understand the function of microglial cells has increased, the tools with which to investigate microglial cells are limited. Therefore, it is important to establish a procedure for inducing microglial cells *in vitro*.

The key differentiation factor(s) in microglial commitment is still unknown. Colony stimulating factor-1 receptor (CSF-1R) is widely expressed in mononuclear phagocytes including microglia, and macrophage colony stimulating factor (M-CSF), a CSF-1R ligand, induces the proliferation of these cells. Interleukin 34 (IL-34) shares CSF-1R with M-CSF and also acts as a proliferating factor for macrophages [16]. IL-34 has been demonstrated to be produced by neuronal cells and promote microglial proliferation [17]. However, the contribution of IL-34 to the differentiation of microglia remains to be elucidated.

In this study, we show that TREM2-expressing microglia-like (ML) cells could be induced from LN^- cells and even from monocytes when cocultured with astrocytes. These ML cells expressed low levels of CD45 and MHC class II (MHC II) and, like microglial cells, exhibited a ramified shape in brain slice cultures. Furthermore, ML cells could also be induced from monocytes obtained from human peripheral blood by coculture with astrocytes. Finally, we determined that IL-34 is an important factor in the induction of ML cells from haematopoietic cells.

Materials and methods

Mice

All experimental procedures were approved by the Ethics Committee for the Treatment of Laboratory Animals of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, approval ID: 21-25 and 22-25. Seven-week-old female C57BL/6J (B6) and SJL/L mice were purchased from CLEA Laboratory Animal Corporation (Tokyo, Japan) and Charles River Laboratories (Yokohama, Japan) respectively. Green fluorescence protein (GFP) transgenic mice on a B6 mice background, in which enhanced GFP was expressed under the control of beta-actin promoter and cytomegalovirus enhancer, were kindly provided by Dr Masaru Okabe (Osaka University, Osaka, Japan). Animals were maintained in specific pathogen-free conditions and all care and use were in accordance with institutional guidelines.

Antibodies

Antibodies (Abs) against mouse CD3, CD4, CD5, CD8α, CD11b, B220, Gr-1, TER-119, TREM2 and IL-34 (for neutralization) were obtained from R&D Systems (Minneapolis, MN, USA). Anti-IAb (Murine MHC II), anti-CD16/CD32, PerCP-CY5.5-anti-Gr-1, allophycocyanin (APC)-anti-CD11c, phycoerythrin (PE)-anti-CD45R, PE-anti-CD49b, PE-anti-NK1.1, PE-anti-Ly6G, biotin-anti-Ly6C Abs (all for mouse antigens) were purchased from BD Bioscience (Franklin Lakes, NJ, USA). We obtained anti-mouse CD11b, PE-anti-CD90.2, APC-anti-IAb Abs and APC-Cy7-streptoavidin, anti-CD45.1, anti-CD45.2 from Biolegend (San Diego, CA, USA), APC-anti-F4/80 Ab and eFluor 615-anti-glial fibrillary acidic protein (GFAP) Ab from eBioscience (San Diego, CA, USA) and unconjugated

anti-GFAP Ab from Dako (Glostrup, Denmark). FITC-anti-human CD14, APC-anti-human CD16, FITC-anti-CD11b and PC5-anti-HLA-DR Abs were obtained from Beckman Coulter (Tokyo, Japan). Anti-mouse interleukin 34 Ab (for immunocytochemistry) was obtained from Abcam (Cambridge, UK). Rhodamine-conjugated anti-rat IgG, anti-sheep IgG and anti-rabbit-IgG were obtained from Jackson ImmunoResearch (West Grove, PA, USA). Cy5-conjugated anti-rat IgG was obtained from Invitrogen (Carlsbad, CA, USA). Anti-Iba1 Ab was obtained from Wako Pure Chemical (Osaka, Japan).

Isolation of murine LN$^-$ cells, monocytes, peritoneal macrophages, splenic monocytes and macrophages

To isolate LN$^-$ cells, bone marrow cells were collected from B6 mice, GFP mice or SJL/J mice by flushing the femora and tibiae of the hind limb and erythrocytes were lysed using the a Mouse Erythrocyte Lysing Kit (R&D Systems, Minneapolis, MN, USA). LN$^-$ cells were negatively selected using Abs against lineage markers (CD3, CD4, CD5, CD8α, CD11b/MAC-1α, B220, Gr-1 and TER-119) and immunomagnetic beads (Invitrogen, Tokyo, Japan).

Mouse mononuclear cells were isolated from the blood of GFP mice by Lymphosepar II buffer (Immuno-Biological Laboratories, Fujioka, Japan), and stained with PerCP-Cy5.5-anti-Gr-1 Ab and APC-anti-CD11b Ab. CD11b^{hi} Gr-1$^+$ and Gr-1$^-$ monocytes were sorted using a FACSAria or FACSAria II (BD Bioscience, Tokyo, Japan). Both Gr-1$^+$ and Gr-1$^-$ populations contain a negligible number of neutrophil because more than 95% of cells stained positive for F4/80 (data not shown).

To prepare murine peritoneal macrophages, B6 mice were injected intraperitoneally with 10 ml of Dulbecco's Modified Eagle's Medium (DMEM) and cells were collected by lavaging peritoneal cavity. Harvested cells were cultured on plate for 2 h and adherent cells were used as peritoneal macrophages.

To isolate murine splenic monocytes, splenocytes 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 45 min and then CD11b$^+$ CD45R$^-$ CD49b$^-$ NK1.1$^-$ Ly6G$^-$ CD90.2$^-$ CD11c$^-$ F4/80$^-$ IAb$^-$ splenic monocytes [18] and CD11b$^+$ F4/80^{hi} CD11c$^-$ splenic macrophages were sorted using a FACSAria or FACSAria II (BD Bioscience).

Isolation of microglia from the CNS

To isolate microglia from the CNS of adult mice, cortexes and spinal cords were isolated and homogenized. Homogenates were incubated with Liberase Blendzyme 3 and DNaseI and then were separated through a density gradient. Cells were obtained from the interface between the 27% and 72% Percoll (GE Healthcare, Tokyo, Japan) layers [3].

Induction of murine ML cells

Primary mixed glial cell cultures were prepared from the brains of postnatal day 3–5 (P3–P5) B6 mice as previously described [13]. Briefly, meninges were removed mechanically, and the cells were dissociated by trituration. Cells were plated in 75 cm^2 tissue culture flask at a density of 7.5×10^6 cells per flask and cultured in basal medium Eagle containing 10% foetal calf serum (FCS), 1% glucose, 1% L-glutamine and 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 10% CO_2 and 90% air for 14 days to form a confluent glial monolayer. Microglial cells were obtained by shaking the flasks at 150 r.p.m. for 2 h and their purity was >98%. Astrocytes were prepared after removal of CD11b$^+$ cells using Dynabeads conjugated with anti-CD11b Ab (Invitrogen, Carlsbad, CA, USA). The purity of astrocytes was >96% determined by anti-GFAP and anti-CD11b immunofluorescence. Astrocytes were then seeded into culture flasks or Lab-tek II eight-well chamber slides (Thermo Fisher Scientific, Yokohama, Japan) at a density of 7.5×10^6 cells per flask or 1.0×10^5 cells per chamber slide well, and cultured in the medium described above for 10 days to form a confluent monolayer. Mouse LN$^-$ cells or monocytes were seeded on astrocytes at a density of 1.5×10^6 cells per flask or 1.0×10^4 cells per chamber slide well and cultured for 7 days. To test the effect of colony stimulating factors on the differentiation of ML cells, 1.0×10^4 LN$^-$ cells or monocytes were cultured per chamber slide well on astrocytes in the presence of recombinant mouse M-CSF (Peprotech, Rocky Hill, NJ, USA) or mouse IL-34 (R&D Systems).

Brain slice culture

Brain slices were prepared from postnatal day 5–7 B6 mice. After decapitation, the forebrain was removed and transversely sliced to 350 mm thickness with a McIlwain

tissue chopper (The Mickle Laboratory Engineering, Guildford, UK). The brain slices were transferred onto a porous translucent membrane (Millicell-CM: PICM03050, Millipore, Billerica, MA, USA) and were cultured at the culture medium interface for 1 week. For quantitative analysis of microglial cells or macrophages cocultured with brain slice cultures, the cocultures were fixed in 4% paraformaldehyde for 1 h, and mounted with VECTASHIELD HardSet Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired using an FV1000-D microscope (Olympus, Tokyo, Japan). For each group, 25 fields were randomly selected and photographed by fluorescence microscopy using a digital camera. For quantification, the ramification index (RI) was calculated using the following formula: RI = cell area/convex area, where 'convex area' is the area of a polygonal object that is defined by the cells' most prominent projections as described previously [19,20].

Human astrocyte and monocyte culture

Human astrocytes were purchased from Lonza (Basel, Switzerland) and were cultured in Astrocyte Basal Medium supplemented with AGM SingleQuots (Lonza) at a density of 4.0×10^5 cells per 75-cm^2 tissue culture flask. When cells reached confluence, the cells were harvested and replated at the same density in flask or plated on chamber slide at 5.0×10^4 cells per well. Astrocytes at passage 2 or 3 were used for experiments. These astrocytes were all positive for GFAP and did not contained CD11b$^+$ microglia determined by immunocytochemistry (data not shown). Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood by Ficoll-Paque (GE Healthcare) separation and then were stained with FITC-anti-CD14Ab and APC-anti-CD16 Ab. CD14^{hi}CD16^{lo} and CD14^{dull}CD16^{hi} monocytes were sorted using a FACSAria II (BD Bioscience).

Isolation of murine ML cells

To obtain ML cells for isolated culture, 1.5×10^6 LN$^-$ cells from SJL/J mice were cultured on 7.5×10^6 astrocytes monolayer in 75 cm^2 culture flasks for 7 days. ML cells were isolated by shaking the flasks at 150 r.p.m. for 2 h and contaminated microglia in feeder astrocytes were removed using immunomagnetic beads (Invitrogen) conjugated with anti-CD45.2 Ab. More than 98% of these cells stained positive for both CD11b and CD45.1 determined by flow cytometry.

Culture of murine microglia, ML cells and peritoneal macrophages

Microglia and ML cells detached by shaking-off methods as well as peritoneal macrophages were plated onto 48-well plates at a density of 5.0×10^4 cells per well and cultured in DMEM supplemented by 10% FCS, 1% glucose, 1% L-glutamine, and antibiotics at 37°C with 5% humidified CO_2. For morphological analysis, 5.0×10^4 cells were incubated on eight-well chamber slide for 7 days. For cytokine analysis, cells at day 3 were treated by 20 ng/ml lipopolysaccharide (LPS from *Escherichia coli 0111:B4*, Invivogen, San Diego, CA, USA). The levels of tumour necrosis factor α (TNFα) and IL-6 in culture supernatants were measured using OptEIA ELISA kits as described (BD Bioscience, Tokyo, Japan). For phagocytosis assay, 5.0×10^4 cells on eight-well chamber slide were incubated with 2.0×10^5 Alexa Fluor 594-conjugated Zymosan A BioParticles (Molecular Probes, Eugene, OR, USA) for 6 h. Cells were stained with DAPI/CD11b and the percentage of CD11b$^+$ cells engulfing particles were quantified using fluorescence microscopy.

Immunohistochemistry

Monocytes or LN$^-$ cells isolated from GFP mice and cultured on murine astrocytes were fixed in 4% paraformaldehyde. After blocking with Protein Block (Dako), samples were immunostained with anti-CD11b, followed by rhodamine- or Cy5-anti-rat IgG, anti-TREM2 Ab followed by rhodamine-anti-sheep IgG, anti-GFAP followed by rhodamine-anti-rabbit IgG or anti-IAb followed by rhodamine-anti-mouse IgG Ab. Images were acquired using an FV1000-D microscope (Olympus, Tokyo, Japan). To obtain Z-stack images, series of optical sections (512 × 512 pixels, pixel size: 440 nm) were collected at intervals of 330 nm. To quantify the number of cells, 10 fields under a 20× objective were randomly selected and immunostained cells were counted. For intracellular staining of IL-34, murine primary mixed glia were stained using anti-mouse IL-34 followed by FITC-anti-rabbit IgG Ab and eFluor 615-anti-GFAP Ab.

Flow cytometry analysis

For flow cytometry analysis, mouse brain microglia, spleen macrophages or LN^- cell- or monocyte-derived ML cells were first incubated with Fc-receptor-blocking CD16/CD32 Ab and then were stained with PE-anti-IAb, anti-CD45, PerCP-Cy5.5-anti-CD11b and anti-F4/80 Ab. Human monocyte-derived ML cells and monocytes were stained with FITC-anti-CD11b, PC5-anti-HLA-DR Abs and anti-TREM2 Ab followed by APC-anti-goat IgG Ab or biotin-anti-CD45 Ab followed by APC-Cy7-streptavidin. Analysis was performed with a FACSCalibur flow cytometer (BD Bioscience, Tokyo, Japan).

Statistical analysis

Data are presented as mean ± SD of at least three independent experiments. Data were analysed by the Mann–Whitney *U*-test to determine significant differences.

Results

ML cells are induced from LN^- cells by coculture with astrocytes

We previously demonstrated that mouse LN^- cells changed to ML cells expressing TREM2 and low levels of CD45 and MHC II when cocultured with mixed glial culture cells [11]. We also found that ML cells are induced only when they are positioned on astrocytes, suggesting the importance of cell–cell contact with astrocytes. We therefore first tested whether mouse LN^- cells differentiated to ML cells when cocultured with murine astrocytes. We isolated LN^- cells from the bone marrow of GFP mice and cultured them with astrocytes purified from mixed glial cultures. One week later, GFP-positive (GFP^+) LN^- cell-derived cells showed two morphological forms, namely, small round-shaped cells (34.0 ± 3.8% SD of GFP^+ cells) and large flat cells (66.0 ± 3.8% SD of GFP^+ cells) (Figure 1**A**,**B**) similar to when cocultured with mixed glial cell cultures. Almost all small round cells expressed CD11b, TREM2 and MHC II (IAb) ($CD11b^+$ cells, 98.8 ± 0.8% SD; $TREM2^+$ cells, 97.4 ± 4.2% SD; MHC II^+ cells; 94.8 ± 3.4% SD), whereas large flat cells expressed only CD11b and not TREM2 or MHC II ($CD11b^+$ cells, 93.6 ± 4.6% SD; $TREM2^+$ cells, 10.5 ± 5.0% SD; MHC II^+ cells; 13.1 ± 6.2% SD) (Figure 1**C**). Based on their expression of TREM2, we concluded that LN^- cell-derived small round cells were ML cells, similar to the observation that LN^- cell-derived small round cells expressed TREM2 and CD11b when positioned above mixed cell culture. Analyses by confocal microscopy revealed that the LN^- cell-derived-ML cells were in contact with GFAP-positive astrocytes and were positioned above the astrocytes. On the other hand, flat cells were attached to the bottom of culture chamber slide (Figure 1**D**). These results indicate that LN^- cells differentiate into ML cells when cultured on astrocytes.

Induction of ML cells from mouse monocytes by coculture with astrocytes

It has been reported that upon damage to the CNS from various insults including irradiation or encephalitis, monocytes are recruited from peripheral blood and serve as precursors for microglia. We therefore next examined whether mouse monocytes differentiate into ML cells when cocultured with murine astrocytes. As $Gr\text{-}1^+$ monocytes invade the CNS and become microglia [21,22], we sorted $Gr\text{-}1^+$ and $Gr\text{-}1^-$ monocytes from the peripheral blood of GFP mice, cocultured these cells with astrocytes for 2 weeks, and then evaluated the number of GFP^+ cells (Figure 2**A**). The number of GFP^+ $Gr\text{-}1^+$ monocyte-derived cells was significantly larger than that of GFP^+ $Gr\text{-}1^-$ monocyte-derived cells ($Gr\text{-}1^+$ monocytes, 84.9 ± 26.0/mm^2; $Gr\text{-}1^-$ monocytes, 25.7 ± 9.3/mm^2, $P = 0.009$), and was slightly smaller than that of LN^- cell-derived cells (LN^- cells, 127.5 ± 36.6/mm^2; *vs.* $Gr\text{-}1^+$ monocytes, $P = 0.04$) (Figure 2**B**).

Both $Gr\text{-}1^+$ and $Gr\text{-}1^-$ monocyte-derived GFP^+ cells showed either a small round shape ($Gr\text{-}1^+$ monocytes, 34.6 ± 6.4% SD; $Gr\text{-}1^-$ monocytes, 36.0 ± 5.2% SD) or a large flat shape ($Gr\text{-}1^+$ monocytes, 65.4 ± 6.4% SD; $Gr\text{-}1^-$ monocytes, 63.9 ± 5.2% SD) similar to LN^- cells cocultured with astrocytes (Figure 2**D**). Most of the small round cells expressed CD11b, TREM2 and MHC II ($Gr\text{-}1^+$ cells: CD11b+ cells, 96.5 ± 3.1% SD; $TREM2^+$ cells, 99.4 ± 0.8% SD; MHC II^+ cells, 92.9 ± % SD, $Gr\text{-}1^-$ cells: $CD11b^+$ cells, 96.5 ± 3.1% SD; $TREM2^+$ cells, 93.5 ± 9.1% SD; MHC II^+ cells, 97.4 ± % SD). On the other hand, a large population of flat cells expressed only CD11b but not TREM2 or MHC II ($Gr\text{-}1^+$ cells: $CD11b^+$ cells, 92.8 ± 1.2% SD; $TREM2^+$ cells, 15.5 ± 6.1% SD; MHC II^+ cells, 6.5 ± % SD, $Gr\text{-}1^-$ cells: $CD11b^+$ cells, 97.1 ± 1.5% SD; $TREM2^+$ cells, 15.6 ± 3.4% SD; MHC II^+ cells, 11.4 ± % SD), indicating that small round cells but not flat cells were

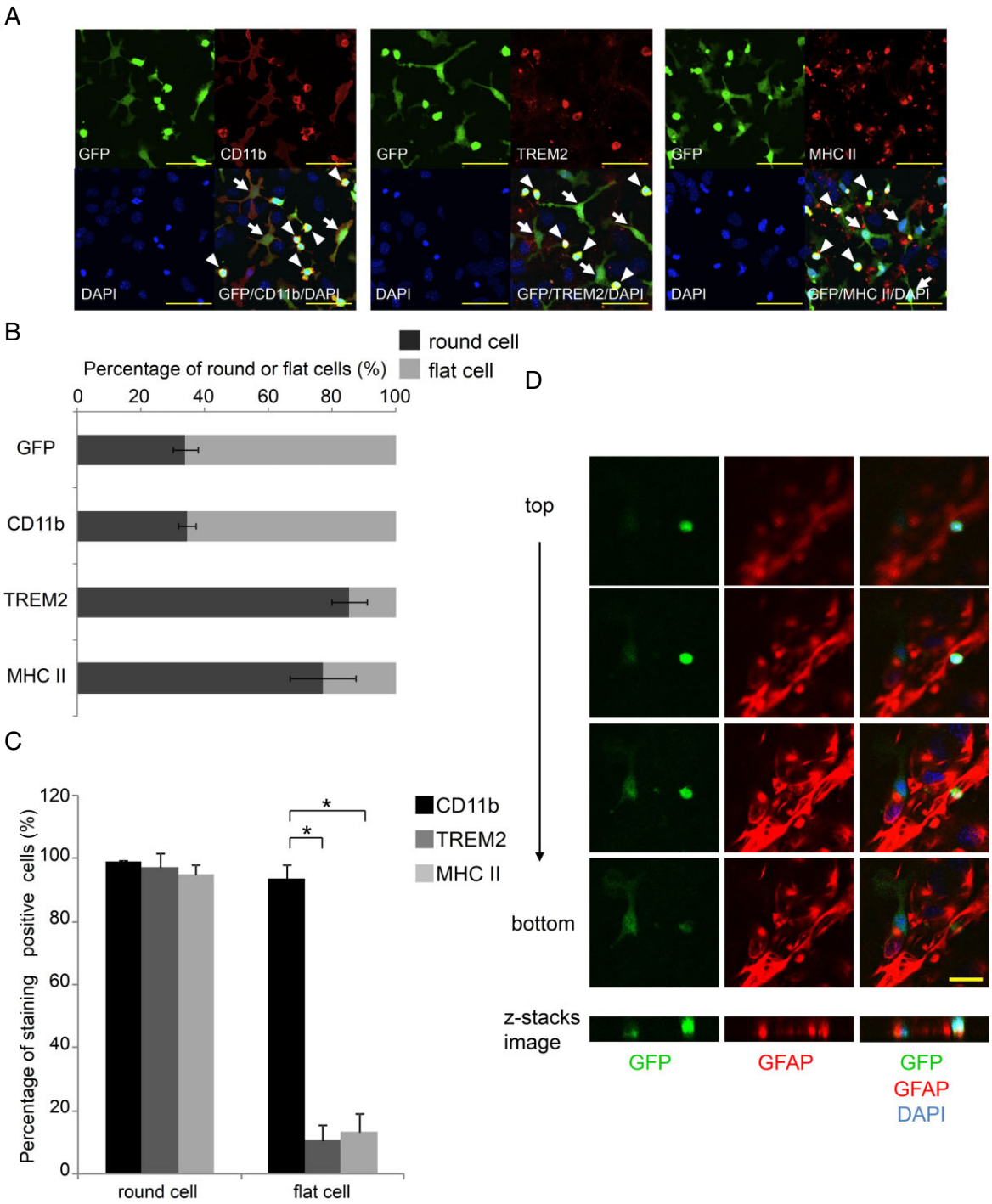

Figure 1. LN⁻ cells cocultured with astrocytes. (**A**) Immunohistology of LN⁻ cells derived from GFP mice cocultured with astrocytes. Cultures were stained with primary anti-CD11b, anti-TREM2 and anti-MHC II (IAb) antibodies followed by rhodamine-conjugated secondary antibody and DAPI. Arrows indicate GFP-positive large flat cells. Arrowheads indicate GFP-positive small round cells. Bar: 50 µm. (**B**) The percentage of round cells or flat cells among GFP⁺ or staining-positive cells was quantified by microscopic analysis. Data are presented as mean ± standard deviation (SD). (**C**) The percentage of CD11b-, TREM2- or MHC II-positive cells among GFP⁺ round or flat cells was quantified by microscopic analysis. Data are presented as mean ± SD. (**D**) Z-stack immunofluorescence confocal microscopy of coculture of GFP-positive LN⁻ cells and astrocytes stained with anti-GFAP (red) and DAPI (blue). Bar: 10 µm. Data are presented as mean ± SD. *$P < 0.05$. Data are representative of three independent experiments.

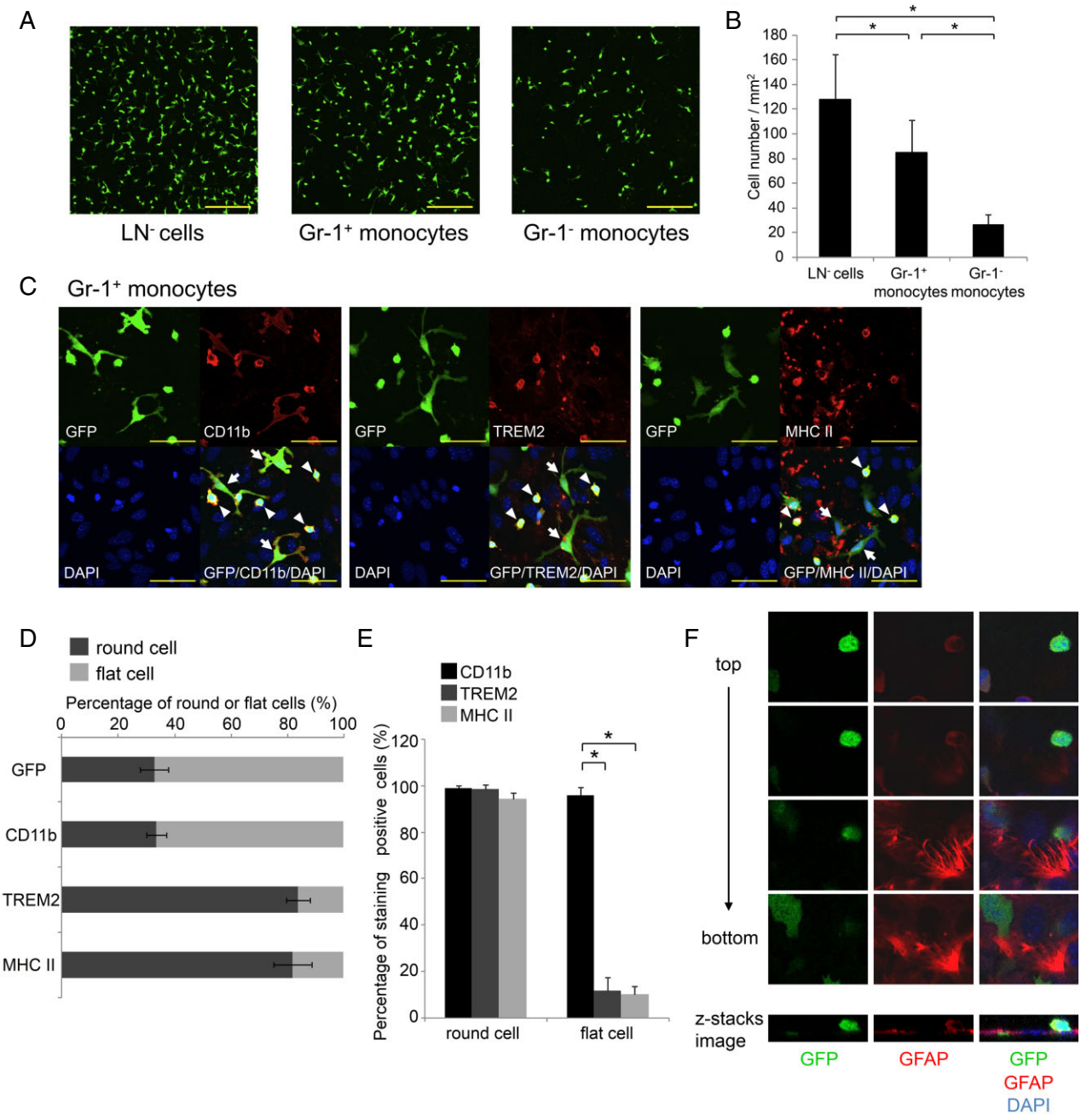

Figure 2. Murine monocytes cocultured with astrocytes. (**A**) Fluorescent microscopic images of GFP⁺ LN⁻ cells, GR-1⁺ monocytes or Gr-1⁻ monocytes cocultured with astrocytes. Bar: 200 µm. (**B**) The numbers of GFP⁺ cells in **A** were quantified by microscopic analysis. Data are presented as mean ± standard deviation (SD). (**C**) Immunocytology of Gr-1⁺ monocytes derived from GFP mice cocultured with astrocytes. Cultures were stained with primary anti-CD11b, anti-TREM2 and anti-MHC II antibodies followed by rhodamine-conjugated secondary antibody and DAPI. Arrows indicate GFP⁺ large flat cells. Arrowheads indicate GFP⁺ small round cells. Bar: 50 µm. (**D**) The percentage of round cells or flat cells among GFP⁺ or staining-positive cells was quantified by microscopic analysis. Data are presented as mean ± SD. (**E**) The percentage of CD11b, TREM2- or MHC II-positive cells among GFP-positive round or flat cells was quantified by microscopic analysis. Data are presented as mean ± SD (**F**) Z-stack immunofluorescence confocal microscopy of coculture of GFP⁺ Gr-1⁺ monocytes and astrocytes stained with anti-GFAP (red) and DAPI (blue). Bar: 10 µm. Data are presented as mean ± SD. $^*P < 0.05$. Data are representative of three independent experiments.

TREM2-positive ML cells similar to LN$^-$ cell-derived ML cells (Figure 2**E**). We again confirmed that the small round cells were in contact with GFAP-positive astrocytes and positioned above astrocytes by analysis using confocal microscopy (Figure 2**F**). These data indicate that ML cells were induced from both Gr-1$^+$ and Gr-1$^-$ monocytes when cocultured on astrocytes, although Gr-1$^+$ monocytes could differentiate into ML cells more efficiently than Gr-1$^-$ monocytes.

Characterization of cell surface markers on monocyte-derived ML cells

As a single marker to identify microglia has not been identified, a combination of low levels of expression of multiple macrophage antigens such as CD45 and MHC II are commonly used as markers of resting microglial cells. To quantify the level of expression of CD45 and MHC II on monocyte-derived ML cells, we obtained monocytes from the spleens of GFP mice as the spleen serves as a reservoir of peripheral monocytes [18] due to the difficulty to obtain sufficient numbers of monocytes from peripheral blood. We first confirmed that ML cells were induced from splenic monocytes when cocultured with astrocytes in the same manner as peripheral blood monocytes (Figure 3**A**). In flow cytometric analysis, LN$^-$ cell-derived ML cells and spleen monocyte-derived ML cells, like brain microglia, showed lower expression levels of CD45 and MHC II compared with spleen-derived macrophages (Figure 3**B**). These results indicated that ML cells induced from monocytes are phenotypically similar to LN$^-$ cell-derived ML cells and brain microglia.

Morphological analysis of monocyte-derived ML cells in brain slice culture

In addition to the low expression of macrophage antigens, a ramified morphology is characteristic of resting microglia in brain tissue and we therefore next investigated the morphology of ML cells in brain tissue. We prepared brain slice cultures from C57BL/6 mouse pups (P4–6) and, 1 week later, added LN$^-$ cell-derived ML cells, splenic monocyte-derived ML cells, cultured microglia, LN$^-$ cells, splenic monocytes or spleen macrophages onto the brain slice cultures. The cocultures were fixed after 1 day or 4 days and were analysed by confocal microscopy. Cultured microglia exhibit a ramified shape with long processes after 1 day in culture. Similarly, monocyte-derived ML cells as well as LN$^-$ cell-derived ML cells showed a ramified shape 1 day after starting the culture. In contrast, freshly prepared LN$^-$ cells or spleen monocytes showed a round shape (Figure 4**A**). The number of spleen macrophages remaining was very low, and these cells did not show a ramified shape. After 4 days coculture with brain slices, ML cells, cultured microglia, LN$^-$ cells and monocytes all showed a ramified shape. For quantitative analysis of cell morphology, we calculated a ramification index as described previously [19,20]. Lower value of ramified index indicates more ramified shape. The ramification indexes of LN$^-$ cells and monocytes cocultured with brain slices for 1 day were significantly higher than these of ML cells and cultured microglia, indicating LN$^-$ cells and monocyte showed more round shape than ML cells at day 1 (LN$^-$ cells: 0.808 ± 0.175, LN$^-$ cell-derived ML cells: 0.518 ± 0.145, $P < 0.0001$, splenic monocytes: 0.879 ± 0.131, splenic monocyte-derived ML cells: 0.526 ± 0.148, $P < 0.0001$, cultured microglia: 0.567 ± 0.175, *vs.* LN$^-$ cells: $P < 0.0001$, *vs.* monocytes: $P < 0.0001$) (Figure 4**B**, Table 1). The ramification indexes of LN$^-$ cells and monocytes after 4 days culture with brain slices were significantly lower than those of the 1-day cocultures (LN$^-$ cells: 0.453 ± 0.195, $P < 0.0001$, monocytes: 0379 ± 0.129, $P < 0.0001$). These data indicate that ML cells acquire a microglial phenotype in terms of morphology.

ML cells induced from human monocytes

To develop a culture system of human microglia would be an important advancement. Therefore we next examined whether ML cells could be induced from human monocytes when cultured with astrocytes. It has been suggested that Gr-1$^-$ monocytes lose the ability to differentiate into microglia during the process of maturation. Human CD14$^+$CD16$^-$ monocytes and CD14$^+$CD16$^+$ monocytes correspond to mouse Gr-1$^+$ monocytes and Gr-1$^-$ monocytes, respectively, although the functions of human CD16$^-$ monocytes are not identical to those of murine Gr-1$^+$ monocytes [23]. We therefore prepared monocytes from the peripheral blood of healthy volunteers, and sorted CD14$^+$CD16$^-$ and CD14^{dull}CD16$^+$ monocytes for coculture with foetal human astrocytes. The number of cells derived from the CD14$^+$CD16$^-$ and CD14^{dull}CD16$^+$ monocytes were similar and cells derived from both populations expressed CD11b and TREM2, similar to murine monocytes cocultured with astrocytes (Figure 5**A**,**B**). Flow cytometric analysis revealed that the

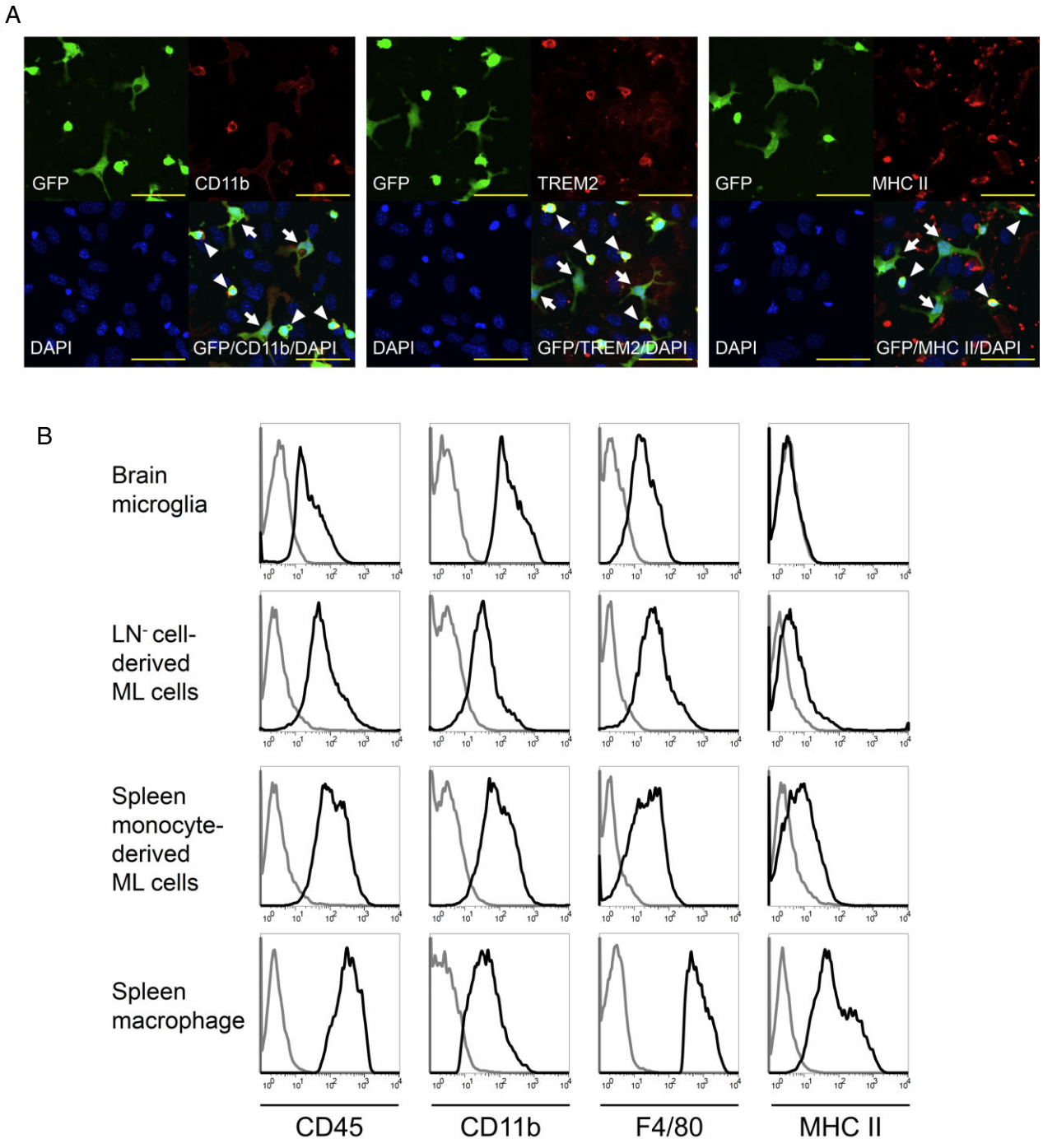

Figure 3. Cell surface markers of spleen monocyte-derived ML cells. (A) Immunocytology of spleen monocytes derived from GFP mice cocultured with astrocytes. Cultures were stained with primary anti-CD11b, anti-TREM2 and anti-MHC II antibodies followed by rhodamine-conjugated secondary antibody and DAPI. Arrows indicate GFP^+ large flat cells. Arrowheads indicate GFP^+ small round cells. Bar: 50 μm. (B) Flow cytometry analysis of brain microglia, LN^- cell-derived ML cells, spleen monocyte-derived ML cells and splenic macrophages. Black histograms, staining with antibodies to markers below plots; grey histograms, isotype-matched control antibody. Data are representative of three independent experiments.

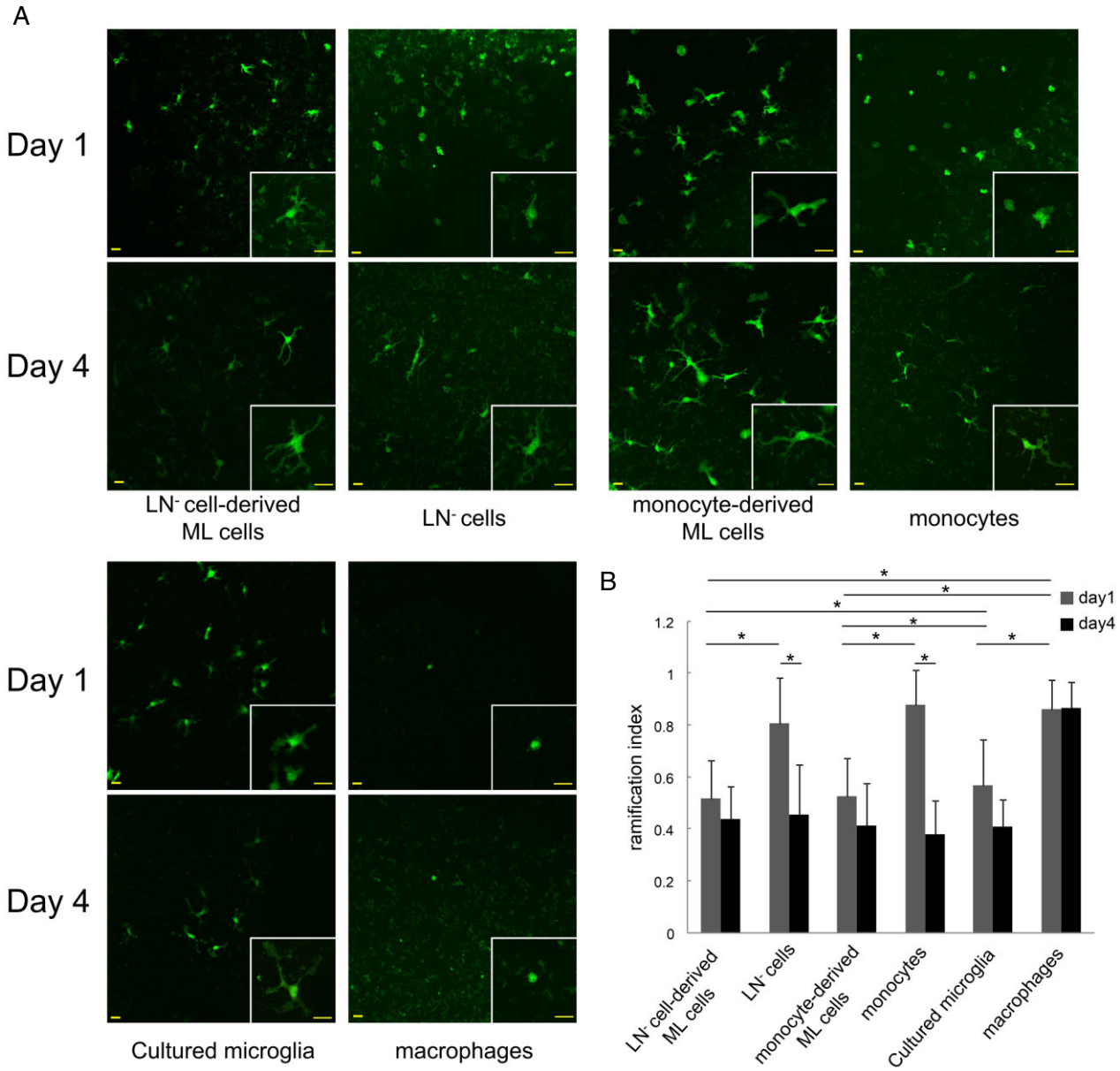

Figure 4. Morphological analysis of ML cells in brain slice cultures. (**A**) Confocal microscopic images of LN⁻ cell-derived ML cells, LN⁻ cells, monocyte-derived ML cells, monocytes, cultured microglia or spleen macrophages induced in brain slice cultures. Bar: 200 μm. (**B**) The ramification indexes of brain slice culture-induced cells were quantified by confocal microscopic analysis. Data are presented as mean ± SD. $^{*}P < 0.05$.

Table 1. Ramification indexes of brain slice culture-induced cells

	LN⁻ cell-derived ML cells	*LN⁻ cells*	*Monocyte-derived ML cells*	*Monocytes*	*Cultured microglia*	*Macrophages*
Day 1	0.518 ± 0.145	0.808 ± 0.175	0.526 ± 0.148	0.879 ± 0.131	0.567 ± 0.175	0.861 ± 0.114
Day 4	0.436 ± 0.129	0.453 ± 0.195	0.412 ± 0.164	0379 ± 0.129	0.406 ± 0.106	0.865 ± 0.102

Each data are presented as mean ± standard deviation (SD). LN⁻ cells, lineage-negative bone marrow cells; ML cells, microglia-like cells.

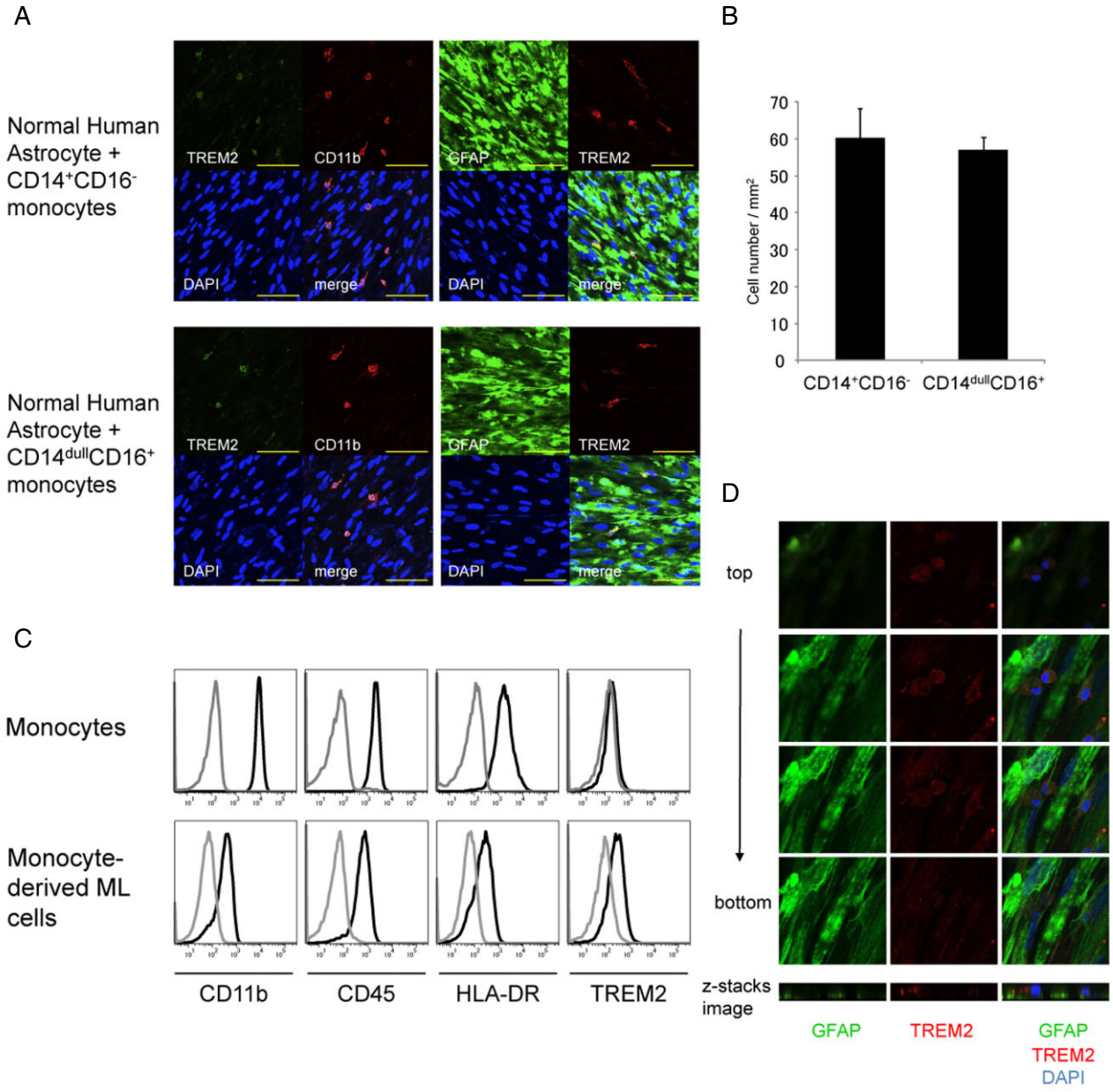

Figure 5. Human monocytes cocultured with astrocytes. (**A**) Immunocytology of human monocytes cocultured with astrocytes. Cultures were stained with primary anti-CD11b, anti-TREM2 and anti-GFAP antibodies followed by FITC- or rhodamine-conjugated secondary antibody and DAPI. Bar: 50 μm. (**B**) The number of monocyte-derived cells was quantified by microscopic analysis. Data are presented as mean ± standard deviation (SD). (**C**) Flow cytometry analysis of monocytes and monocyte-derived ML cells. Black histograms, staining with antibodies to markers below plots; grey histograms, isotype-matched control antibody. Data are representative of three independent experiments. (**D**) Z-stack immunofluorescence confocal microscopy of coculture of monocytes and astrocytes stained with anti-GFAP (green), anti-TREM2 (red) and DAPI (blue). Data are representative of three independent experiments. Bar: 10 μm.

down-modulation of CD45 and HLA-DR on monocyte-derived cells compared of those on monocytes (Figure 5**C**). Analysis with confocal microscopy showed that all monocyte-derived cells were located on the GFAP-positive astrocytes in contrast to murine monocytes, which were located on both astrocytes and the plastic surface (Figure 5**D**). These data indicate that ML cells could be induced from human monocytes derived from peripheral blood when cocultured on astrocytes.

Murine IL-34 promotes differentiation of haematopoietic cells into ML cells

We previously demonstrated a tropic effect of M-CSF on LN^- cells cocultured with mixed glial cells. When cocultured with murine astrocytes, mouse LN^- cells proliferated significantly in the presence of M-CSF or IL-34 [medium, 75.2 ± 34.9 cells/mm^2; M-CSF (50 ng/ml), 142.3 ± 34.2 cells/mm^2, $P < 0.005$; IL-34 (50 ng/ml), 99.9 ± 36.4 cells/mm^2, $P < 0.05$] (Figure 6**A**). Both M-CSF and IL-34 promoted the proliferation of LN^- cells in dose-dependent manner (Figure 6**B**). More than 61.8% of LN^- cells changed to flat-shaped cells in the presence of M-CSF (medium, 42.6 ± 18.4 cells/mm^2; M-CSF, 87.9 ± 18.4 cells/mm^2; $P < 0.00005$), whereas 60.2% of LN^- cells changed to round-shaped cells grown in the presence of IL-34 (medium, 32.5 ± 16.5 cells/mm^2; IL-34, 54.4 ± 15.8 cells/mm^2; $P < 0.005$) (Figure 6**A**,**C**). Small round-shaped cells expanded when cultured with IL-34, but not large flat cells cultured with M-CSF expressed TREM-2, indicating that round-shaped cells are ML cells (Figure 6**D**). M-CSF and IL-34 had similar effects on peripheral monocytes cocultured with astrocytes, although the effect on proliferation was less compared with their effect on LN^- cells. More than 60.4% of monocytes changed to flat-shaped cells in the presence of M-CSF (medium, 45.6 ± 5.8 cells/mm^2; M-CSF, 60.8 ± 10.2 cells/mm^2, $P < 0.005$), whereas 52.6% of monocytes changed to round-shaped cells in the presence of IL-34 (medium, 22.0 ± 5.7 cells/mm^2 SD; IL-34, 60.6 ± 6.6 cells/mm^2 SD, $P < 0.00005$) (Figure 6**E**,**F**). Treatment with anti-mouse IL-34 blocking Ab significantly inhibited the proliferation of round-shaped cells (medium, 38.3 ± 10.0 cells/mm^2; control IgG, 34.2 ± 8.3 cells/mm^2, anti-IL-34 Ab, 16.3 ± 4.9 cells/mm^2, $P < 0.0005$) (Figure 6**G**,**H**). In order to clarify which cells produce IL-34 in the culture system, we performed immunocytochemical analysis of primary mixed glial cells. Approximately 12% of astrocytes produced IL-34 (Figure 6**I**). These results suggest that IL-34 is an important factor in the induction of ML cells from LN^- cells and monocytes *in vitro*.

Human IL-34 promotes differentiation of human monocytes into ML cells

We then examined whether human IL-34 enhanced induction of ML cells in human monocyte-astrocyte coculture. Both human M-CSF and IL-34 increased the proliferation of $CD14^+CD16^-$ monocytes cultured on astrocyte in a dose-dependent manner (Figure S1**A**,**B**). Interestingly, treatment with high concentration of IL-34 (>20 ng/ml) resulted in an increase in the number of cells with a spindle-like morphology. These spindle-shaped cells as well as round-shaped cells expressed CD11b, Iba1 and TREM2 (Figure S1**C**), indicating that these cells are similar to ML cells rather than flat-shaped cells. These results indicated that human IL-34 facilitated differentiation from human monocytes to ML cells.

Isolated ML cells are viable and reactive

Finally, we harvested ML cells from astrocyte coculture and assessed their viability and function. We induced ML cells from LN^- cells from SJL/J mice ($CD45.1^+$) on astrocytes from C57BL/6 mice ($CD45.2^+$) and depleted $CD45.2^+$ cells by negative selection using immunomagnetic beads. Using this method, we obtained approximately 1.0×10^5 ML cells from 1.5×10^6 LN^- cells. These cells survived in the absence of feeder astrocytes for 7 days and morphologically mimicked cultured microglia (Figure S2**A**). All cells were positive for CD11b, Iba1 and TREM-2 (Figure S2**B**). Isolated ML cells produced TNFα and IL-6 in response to LPS and phagocytosed zymosan-coated particles (Figure S2**C**,**D**). These data indicated that isolated murine ML cells are suitable for further culture and functional analyses.

Discussion

Microglial cells are the resident immune cells in the CNS. Morphologically, a ramified shape is characteristic of resting microglia [24,25]. Except for this morphological feature, it is not easy to distinguish microglia from other types of cells, especially macrophages, because a single specific marker to identify microglial cells is still unknown. It has been reported that one of the important features of

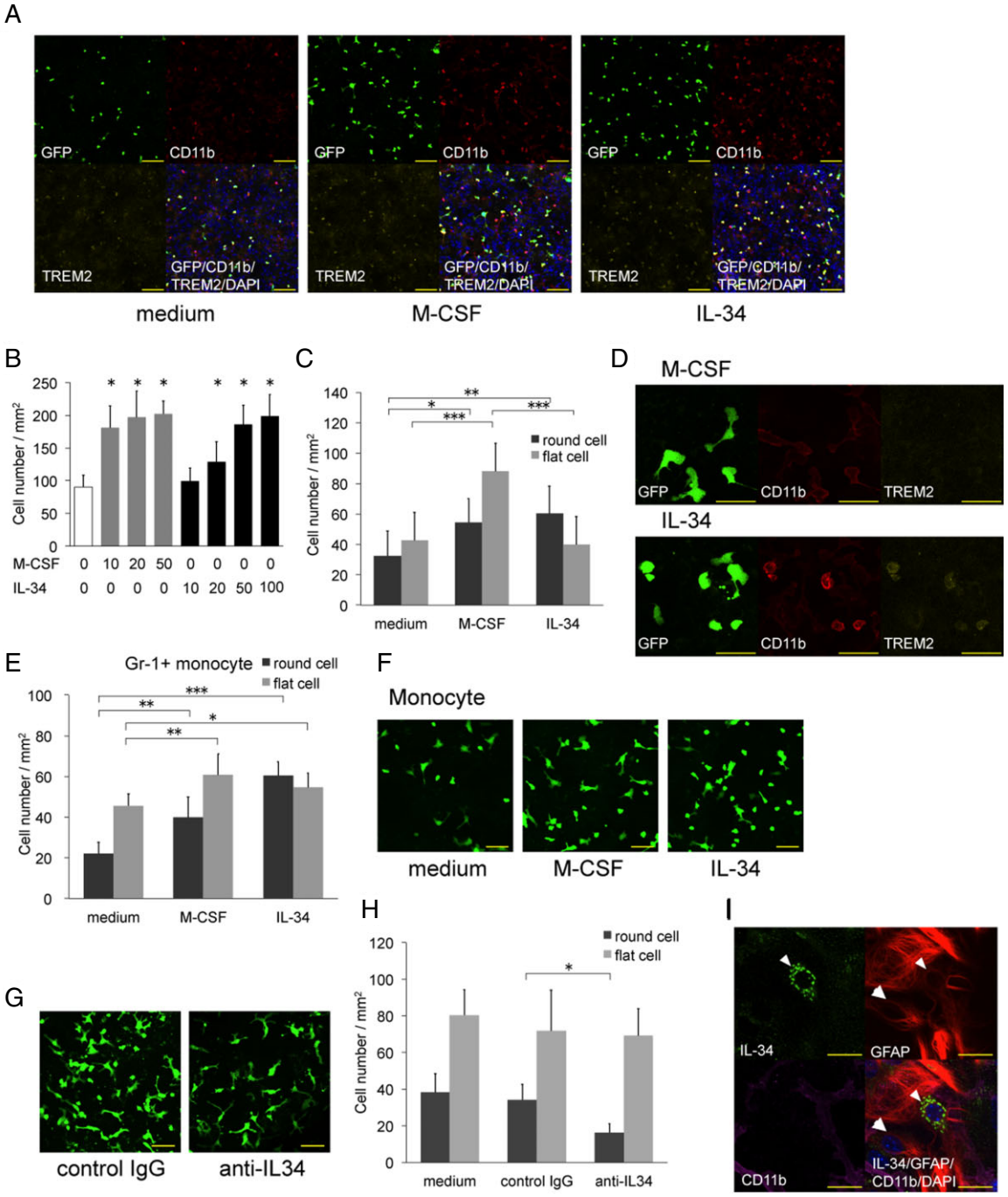

Figure 6. Differentiation assays of LN⁻ cells. (**A**) Immunocytology of mouse LN⁻ cells cultured on murine astrocytes with or without M-CSF or IL-34. Bar: 50 μm. (**B**) Dose–response relationship between M-CSF/IL-34 and the number of ML cells differentiated from LN⁻ cells (cell number per mm^2). *$P < 0.05$ as compared with untreated cells. (**C**) Quantitative analysis of small round-shaped cells and large flat cells differentiated from LN⁻ cells (cell number per mm^2). (**D**) Morphological characteristics of LN⁻ cells differentiated with M-CSF or IL-34. Bar: 10 μm. (**E**, **F**) Quantitative analysis (**E**) and immunocytology (**F**) of mouse monocytes cultured on murine astrocytes in the absence or presence of M-CSF or IL-34, Bar: 50 μm. (**G**, **H**) Fluorescent microscopic images (**G**) and quantitative analysis (**H**) of LN⁻ cells cultured on astrocytes with or without anti-IL-34 antibody. IL-34 neutralization resulted in the significant reduction of round cells. *$P < 0.05$ as compared with cells treated by control IgG. Bar: 50 μm. (**I**) Intracellular staining of IL-34 of mouse primary mixed glial cells. Some GFAP-positive astrocytes showed granular staining in cytoplasm (arrowhead), whereas other astrocytes were not stained with anti-IL-34 Ab (arrow), Bar: 20 μm. Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.005$, ***$P < 0.00005$.

microglial cells is down-modulation of CD45 compared with other CNS macrophages in rodents [26,27] and humans [28]. As reported previously, ML cells derived from LN^- cells showed lower expression levels of CD45 and MHC II than spleen macrophages and expressed TREM2, indicating ML cells exhibit a phenotype similar to that of brain microglia [29–32]. In this study, ML cells induced from monocytes also expressed lower levels of CD45 and MHC II and expressed TREM2 similar to brain microglia and LN^- cell-derived ML cells. Moreover, ML cells introduced into brain slice culture immediately presented ramified shapes on the first day of culture in contrast to LN^- cells and monocytes, which changed to a ramified shape in brain slice culture several days after starting the culture. These results suggest that ML cells gain a microglial phenotype including the capability of developing a ramified shape in three-dimensional circumstances, although identification of a specific marker for microglia would be required to conclude that ML cells are identical to brain microglia.

It has been reported that overexpression of TREM2 in microglia enhances microglial phagocytic capacity and reduces production of inflammatory cytokines [13]. Recently, Heat shock protein 60 was reported to be a TREM2 ligand, suggesting that TREM2 may be associated with the removal of apoptotic debris under physiological conditions [33]. Furthermore, it has been suggested that TREM2-expressing cells have a neuroprotective role in neuroinflammatory or neurodegenerative disease models [3,15]. Thus, identification of a factor(s) to induce microglia differentiation and TREM2 expression may provide a novel therapeutic target(s) for neuroinflammatory or neurodegenerative diseases.

In the present study, we demonstrated that murine LN^- cells and monocytes cocultured with astrocyte monolayers changed into two morphologically distinct cell types, small round cells (ML cells) and flat cells. The flat cells were negative for TREM2 and MHC II in immunohistochemical analysis and could be distinguished from ML cells in terms of surface expression markers. Analysis with confocal microscopy revealed that the positional relationship between astrocytes and ML cells was different from that of flat cells. ML cells were in contact with the GFAP-positive astrocytes and positioned above astrocytes. On the other hand, flat cells attached to the bottom of culture chamber slide. These data indicate that cell–cell contact and the positional relationship with astrocytes may be important for the differentiation and maintenance of TREM2-positive ML cells. In contrast to murine cells, all human monocytes cocultured with astrocytes were positioned on the cell layer and were not attached to the culture chamber slide. Consistent with this finding, all human monocytes cocultured with astrocytes express TREM2 and therefore are not divided into two populations, namely $TREM2^+$ and $TREM2^-$ cells. Astrocytes in the CNS interact with various types of cells including other astrocytes, neurones, cells of the blood-brain barrier or microglia. Therefore, cell polarity is a required, asymmetrical function of astrocytes [34] and the cell polarity of astrocytes is affected by inflammatory conditions or extracellular matrices [35,36]. *In vitro*, astrocytes may possess polarity and express different proteins on their upper *vs.* lower surface. In the normal CNS, microglia contact astrocytes, but $CD45^{hi}$ perivascular macrophages are separated by the basal membrane and have no contact with astrocytes. Acquiring a microglial phenotype including the down-regulation of CD45 expression levels might be affected by cell–cell contact with astrocytes. Although flat cells were negative for TREM2 and MHC II, it is still possible that both round cells and flat cells are two different subpopulations of the microglial continuum. Characterization of flat cells in comparison with round cells was hampered by difficulty in obtaining pure flat cells population.

We showed that not only M-CSF but also IL-34 supported the proliferation of LN^- cells and monocytes when cocultured with astrocytes. Importantly, IL-34 predominantly induced ML cells expressing TREM2, whereas M-CSF promoted the proliferation of macrophage-like large flat cells. Moreover, IL-34 induced ML cells more efficiently from LN^- cells than from monocytes. Ab-mediated blocking of IL-34 significantly abrogated ML cells induction. Previous reports demonstrated IL-34 production from both neuronal cells and astrocytes [17,37], and we clearly demonstrated that some astrocytes in the primary mixed glial cells actually produced IL-34. Interestingly, IL-34 is expressed in the E11.5 murine embryo [38] in which microglia arise from primitive myeloid progenitors [6]. These findings suggest that IL-34 has a unique activity on immature progenitor cells. Although M-CSF and IL-34 share CSF-1R as their common receptor, M-CSF and IL-34 share no homology in their amino-acid sequences and differ in their biological activities and signalling [39]. Recently, IL-34 has been reported to direct the differentiation of myeloid cells into microglia in the CNS [40]. Our finding that IL-34 is more efficient in inducing ML cells compared with M-CSF provide a further support for their different activities in microglial cell development.

There has been controversy as to whether bone marrow derived haematopoietic cells reach the brain through the bloodstream and populate as microglia. In a murine bone marrow transplantation model, haematopoietic cells entered the CNS and differentiated into microglia when blood brain barrier was disrupted by irradiation and premature bone marrow cells were mobilized to circulation, but monocyte entry was not observed when the brain was shielded from irradiation [8]. Moreover, when chimeric mice obtained by parabiosis were used, there was no evidence of microglia progenitor recruitment from the circulation [41]. *In vivo* lineage tracking study also revealed that postnatal haematopoietic progenitors do not significantly contribute to microglia homeostasis in the adult brain [6]. Therefore, microglia are not replenished by blood-borne cells under physiological conditions. These findings, however, do not rule out the possibility that haematopoietic cells have a potential to differentiate into microglia *in vitro*. Our data indicate that ML cells derived from bone marrow LN^- cells or peripheral monocytes serve as a substitute for microglia.

Several methods have been described for the induction of microglia from haematopoietic cells *in vitro*. For example, murine bone marrow cells cultured in astrocyte-conditioned medium and granulocyte-monocyte colony stimulating factor were reported to differentiate towards microglia [42]. However, it was difficult for us to induce small round ML cells cultured with astrocyte-conditioned medium as mentioned in our previous paper [11]. Another group reported that murine bone marrow-derived macrophages acquired a deactivated ML phenotype when cocultured with either an astroglial or a neuronal cell line [43].

In summary, we developed a culture system for the induction of ML cells by coculture with astrocytes. Moreover, we identified IL-34 as a potent inducer of ML cells *in vitro* in addition to cell–cell contact with astrocytes. *In vitro* culture systems for the induction of ML cells will not only help in the investigation of the function of microglial cells in patients with various diseases including neurodegenerative and psychiatry disorders but will also serve as an important tool in the screening for new therapeutic reagents to target microglial cells.

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Contribution of listed authors

S. Miyake, D. Noto and H. Sakuma designed, supervised and evaluated experiments. D. Noto, H. Sakuma, R. Saika and R. Saga performed and evaluated experiments. K. Takahashi, M. Yamada and T. Yamamura evaluated experiments. S. Miyake, D. Noto and H. Sakuma prepared the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Effect of human IL-34 on the differentiation of human ML cells. (**A**) Fluorescent microscopic images of human monocytes cocultured with human astrocytes in the presence or absence of M-CSF (50 ng/ml) or IL-34 (50 ng/ml). Data are representative of three independent experiments. Bar: 100 μm. (**B**) Dose–response relationship between M-CSF/IL-34 and the number of ML cells differentiated from monocytes (cell number per mm^2). Data are presented as mean ± SD. $^*P < 0.05$ as compared with untreated cells. (**C**) Immunofluorescence of human ML cells. Human monocytes culture with human IL-34 (50 ng/ml) mainly showed spindle-like morphology (arrow) and all cells were positive for TREM2. Data are representative of three independent experiments. Bar: 50 μm.

Figure S2. Functional characterization of isolated ML cells. (**A**) Morphology of peritoneal macrophages, microglia isolated from mixed glial culture, and murine ML cells isolated from astrocyte coculture. Data are representative of three independent experiments. Bar: 50 μm. (**B**) Immunofluorescence of isolated murine ML cells. All cells were positively stained with Iba1, CD11b, TREM2 and MHC II. Data are representative of three independent experiments. Bar: 100 μm. (**C**) Phagocytosis assay: percentage of peritoneal macrophages, microglia and murine ML cells phagocytosing zymosan-coated particles. Data are presented as mean ± SD. (**D**) Cytokine production after LPS treatment. Cells were treated with or without LPS (20 ng/ml) and concentration of TNFα and IL-6 in culture supernatants were determined by ELISA. Incubation periods were 6 h for TNFα and 24 h for IL-6. Data are presented as mean ± standard error (SE).

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