Biochemical and Biophysical Research Communications 463 (2015) 109–115

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

MafB antagonizes phenotypic alteration induced by GM-CSF in microglia

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ARTICLE INFO

Article history:
Received 5 May 2015
Available online 19 May 2015

Keywords:
Microglia
GM-CSF
MafB
Membrane protrusion
RhoA

ABSTRACT

Microglia are tissue-resident macrophages which are distributed throughout the central nervous system (CNS). Recent studies suggest that microglia are a unique myeloid population distinct from peripheral macrophages in terms of origin and gene expression signature. Granulocyte-macrophage colony-stimulating factor (GM-CSF), a pleiotropic cytokine regulating myeloid development, has been shown to stimulate proliferation and alter phenotype of microglia *in vitro*. However, how its signaling is modulated in microglia is poorly characterized. MafB, a bZip transcriptional factor, is highly expressed in monocyte-macrophage lineage cells including microglia, although its role in microglia is largely unknown. We investigated the crosstalk between GM-CSF signaling and MafB by analyzing primary microglia. We found that *Mafb*-deficient microglia grew more rapidly than wild-type microglia in response to GM-CSF. Moreover, the expression of genes associated with microglial differentiation was more downregulated in *Mafb*-deficient microglia cultured with GM-CSF. Notably, such differences between the genotypes were not observed in the presence of M-CSF. In addition, we found that *Mafb*-deficient microglia cultured with GM-CSF barely extended their membrane protrusions, probably due to abnormal activation of RhoA, a key regulator of cytoskeletal remodeling. Altogether, our study reveals that MafB is a negative regulator of GM-CSF signaling in microglia. These findings could provide new insight into the modulation of cytokine signaling by transcription factors in microglia.

1. Introduction

Microglia are tissue-resident macrophages distributed throughout the central nervous system (CNS), and continuously survey their environment [1]. Microglia derive from c-kit$^+$ uncommitted erythromyeloid progenitors (EMPs) in the yolk sac [2,3]. After colonization in the CNS, microglia are maintained by self-renewal, that is, independently of hematopoietic stem cells (HSCs) [4,5]. Microglia have a unique molecular requirement for their development: they are dependent on transcription factors PU.1 and IRF8 [3], but not on transcription factor Myb, which is necessary for bone-marrow-derived macrophages [3,6]. In addition, signaling from colony-stimulating factor 1 receptor (CSF-1R), which is a receptor for macrophage colony-stimulating factor (M-CSF) and interleukin-34 (IL-34), is required not only for the development [2,7], but also for the homeostasis of microglia [8]. These findings suggest that signaling networks and transcriptional programs in microglia are distinct from those in other tissue macrophages.

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is a pleiotropic cytokine which is able to promote differentiation into dendritic cells [9]. It has been shown that GM-CSF contributes to the progression of some CNS diseases including experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis [10]. Several *in vitro* studies have shown that GM-CSF stimulates proliferation of microglia [11] and converts them into dendritic cell-like phenotypes [12]. Although GM-CSF has potent biological effects on microglia, how its signaling is modulated in microglia is poorly characterized.

MafB is a bZip transcription factor which is highly expressed in the monocyte-macrophage lineage [13]. MafB promotes differentiation into macrophages [14], whereas it inhibits differentiation into dendritic cells [15]. Although MafB is expressed in mature microglia as well [3,16], its role in microglia is largely unknown. Here we show that *Mafb* deficiency potentiates the effect of GM-CSF on microglia. Moreover, *Mafb*-deficient microglia display rounded morphology in response to GM-CSF through abnormal

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http://dx.doi.org/10.1016/j.bbrc.2015.05.036
0006-291X/

activation of RhoA. Our study reveals that MafB is a negative regulator of GM-CSF signaling in microglia.

2. Materials and methods

2.1. Mice

Mafb-deficient mice in which the GFP gene was inserted into the endogenous *Mafb* locus were described previously [13]. The day when a vaginal plug was found after mating was defined as the embryonic day 0.5 (E0.5). Mice were maintained in specific pathogen-free conditions in a Laboratory Animal Resource Center. All experiments were performed according to the Guide for the Care and Use of Laboratory Animals at the University of Tsukuba.

2.2. Mixed glial culture and isolation of primary microglia

Mixed glial culture was prepared from P1–P3 or E18.5 brains as previously described [17]. Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 1% Penicillin-Streptomycin (Life technologies). 10 ng/ml GM-CSF (R & D systems) or 10 ng/ml M-CSF (R & D systems) were added to the culture medium when needed. As M-CSF poorly stimulates proliferation of microglia *in vitro*, whole suspended cells were seeded onto poly-D-lysine (Sigma)-coated 75 cm^2 flasks. When cultured with GM-CSF, suspended cells were seeded onto poly-D-lysine-coated 6-well plates at a density of 2×10^4 cells/cm^2. Cells were grown in the incubator at 37 °C with 5% CO_2. Culture media were changed every 3–4 days. At 7–10 days *in vitro* (DIV), microglia were detached from the astroglial layer by shaking or vigorous pipetting and used for the subsequent experiments. The cell number was counted with a hemocytometer after trypan blue staining. Isolated microglia were plated and immunostained with anti-Iba-1 antibody, showing that the purity was >95%.

2.3. MTS assay

Mixed glial culture was treated with different concentrations of GM-CSF (0, 0.01, 0.1, 1.0, 10 ng/ml). At DIV10, microglia were isolated and viable cell numbers were measured with an MTS assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega) according to the manufacturer's instructions. Data are expressed as absorbance at 490 nm.

2.4. Flow cytometry

Freshly dissociated cells from E18.5 brains or primary microglia at DIV 8–10 were used. Cells were labeled with PE- or APC-conjugated antibodies against CD11b (M1/70, Biolegend) or F4/80 (BM8, eBioscience). Data were acquired on an LCR (BD Bioscience) and analyzed with FlowJo software (Tree Star). For freshly dissociated cells, viable cells were gated using forward and side scatter as previously described [18].

2.5. Immunohistochemistry

Brains were fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C, cryoprotected in 30% sucrose and embedded in Tissue-Tek OCT compound (Sakura Finetek). Frozen sections were prepared at 10 μm thickness using a cryostat. Sections were blocked with 10% normal goat serum plus 0.3% Triton X-100 in PBS for 1 h at room temperature. For detection of microglia, sections were incubated with anti-Iba-1 antibody (1:2000; Wako) overnight at 4 °C. After washing, slides were incubated with Alexa Fluor 594-conjugated secondary antibody (Life technologies) for 1 h at room temperature. Fluorescent images were acquired using a BIOREVO BZ-9000 fluorescence microscope (KEYENCE).

2.6. Quantitative RT-PCR

Total RNA was extracted from primary microglia with a Nucleospin RNA II kit (Macherey Nagel) and cDNA was synthesized with a QuantiTect Reverse Transcription kit (QIAGEN), each according to manufacturer's instructions. Real-time PCR was run on a Thermal Cycler Dice Real Time System Single (Takara) with SYBR Green PCR master mix (Takara). Primer sequences used were as follows; *Mafb* forward, 5′-CATCACCATCATCACCAAGC-3′; *Mafb* reverse, 5′-AGAAGCGGTCCTCCACACTA-3′; *Ccl2* forward, 5′-GGTCCCTGTCATGCTTCTGG-3′; *Ccl2* reverse, 5′-TTGGGATCATCTTGCTGGTG-3′, *P2ry12* forward, 5′-CGCCTGCCTTGATCCATTCA-3′; *P2ry12* reverse, 5′-CTCTTCGCTTGGTTCGCCAC-3′; *Cx3cr1* forward, 5′-AGTTCCCTTCCCATCTGCTC-3′; *Cx3cr1* reverse; 5′-AATGTCGCCCAAATAACAGG-3′; *Hprt* forward, 5′-TTGTTGTTGGATATGCCCTTGACTA-3′; *Hprt* reverse, 5′-AGGCAGATGGCCACAGGACTA-3′. All PCR was performed in duplicates. Amplification products were quantified by the standard curve method. mRNA levels of each gene were normalized to that of *Hprt*.

2.7. Quantification of spreading cells

Primary microglia were plated in 6-well plates at a density of $2–4 \times 10^5$ cells/well. After 30 min, cells were observed with a phase-contrast microscope at 200× magnification and pictures were taken. Spreading cells were counted from 4 to 5 fields that were randomly selected. For inhibition of the RhoA/ROCK pathway, ROCK inhibitor Y27632 (10 μM, Wako) was added just before plating.

2.8. Immunocytochemistry

Primary microglia were plated on coverslips. After 30 min, cells were fixed with 4% PFA in PBS for 20 min at room temperature. Cells were washed with PBS and blocked with 3% normal goat serum plus 0.1% Triton X-100 in PBS. Cells were incubated with anti-RhoA antibody (1:1000, Cytoskeleton) overnight at 4 °C. After washing, cells were incubated with Alexa Fluor 647-conjugated secondary antibody and Alexa Fluor 546 phalloidin (1:40, Life technologies) for 1 h at room temperature. Fluorescent images were acquired on a Leica TSC SP5 confocal laser scanning microscope (Leica Microsystems) using a 63× oil immersion objective.

2.9. Pull-down assay for active RhoA

The amount of GTP-bound form of RhoA protein was measured with the RhoA Activation Assay kit (Cytoskeleton) according to the manufacturer's instructions. Primary microglia were lysed 30 min after plating. Samples were pulled-down with GST-fusion rhotekin-RBD, electrophoresed by 12% SDS-PAGE, and immunoblotted with anti-RhoA antibody (1:1000, Cytoskeleton). Band intensity was quantified with Image J software (National Institutes of Health). Three independent experiments were performed. Data are represented as a ratio of GTP-bound RhoA to total RhoA.

2.10. Statistical analysis

All data are expressed as mean ± SEM. The difference between the two groups was analyzed with Student's *t*-test. For multiple comparison, Bonferroni correction was used. All statistical analyses were performed with R software (http://www.r-project.org). $p < 0.05$ was considered significant.

3. Results

3.1. GM-CSF treatment stimulates proliferation and alters phenotype of microglia in vitro more potently than M-CSF

M-CSF and GM-CSF are well-known cytokines which play an important role in the development of myeloid cell lineage [19]. To compare the biological effects of M-CSF and GM-CSF on microglia, we prepared mixed glial culture from P1–P3 mouse brains and treated it with M-CSF (10 ng/ml) or GM-CSF (10 ng/ml). As previously reported [20], microglia appeared as round cells on an astrocyte layer at 7–9 days *in vitro* (DIV). Notably, microglia cultured with GM-CSF grew rapidly, whereas M-CSF treatment did not stimulate their proliferation compared to FBS alone (Fig. 1A). When mixed glial culture was treated with different concentrations of GM-CSF (0, 0.01, 0.1, 1.0, 10 ng/ml), microglia grew in a dose dependent manner (Fig. 1B). Given GM-CSF alters the phenotype of microglia [12], we examine the surface expression of CD11b, a differentiation marker of monocyte-macrophage lineage by flow cytometry. GM-CSF treatment drastically diminished the surface expression of CD11b on microglia compared to FBS alone. In contrast, M-CSF treatment resulted in only a subtle reduction in the expression of CD11b (Fig. 1C).

3.2. MafB expression is upregulated as microglia become mature, but downregulated by GM-CSF

To monitor MafB expression in microglia, we analyzed the brains from *Mafb*$^{GFP/+}$ mice in which GFP expression faithfully reproduces MafB expression [13]. Immunostaining for Iba-1, a widely-used marker for microglia, showed that GFP expression was detected at low levels in the immature microglia at E18.5 and P7. At P30, microglia displayed mature ramified form along with strong GFP signal (Fig. 2A). These results indicate that MafB expression in microglia is associated with their maturity.

To determine whether MafB expression is regulated by extrinsic cytokines, we examined GFP fluorescence of primary microglia from *Mafb*$^{GFP/+}$ mice by flow cytometry. Notably, GM-CSF treatment drastically reduced GFP fluorescence compared to FBS alone and M-CSF treatment (Fig. 2B). Consistent with the data from flow cytometry, the *Mafb* mRNA level in microglia cultured with GM-CSF was reduced to approximately 10% of that in microglia cultured with M-CSF (Fig. 2C).

3.3. Mafb deficiency potentiates the effect of GM-CSF on microglia in vitro

To explore the role of MafB in microglia, we analyzed primary microglia from embryonic brains of *Mafb*-deficient (*Mafb*$^{GFP/GFP}$ = *Mafb*$^{-/-}$) mice, which die from central apnea immediately after birth [13,21]. Flow cytometric analysis of freshly dissociated cells from E18.5 brains showed that the proportion of *Mafb*$^{-/-}$ microglia (CD11b^{+} F4/80^{+}) was unaffected (Fig. 3A). Given previous studies suggesting that MafB decreases the responsiveness to M-CSF in macrophages [22] and HSCs [23], we tested whether *Mafb* deficiency affects the responsiveness of microglia to M-CSF or GM-CSF *in vitro*. Unlike macrophages and HSCs, in the presence of M-CSF, the cell number of *Mafb*$^{-/-}$ microglia was unchanged compared to *Mafb*$^{+/+}$ microglia (Fig. 3B). In contrast, *Mafb*$^{-/-}$ microglia grew more rapidly than *Mafb*$^{+/+}$ microglia in the presence of GM-CSF (Fig. 3C). In addition, the surface expression of CD11b was more downregulated in *Mafb*$^{-/-}$ microglia in the presence of GM-CSF despite there was no difference between the genotypes in the presence of M-CSF (Fig. 3D). Moreover, we examined *P2ry12* mRNA

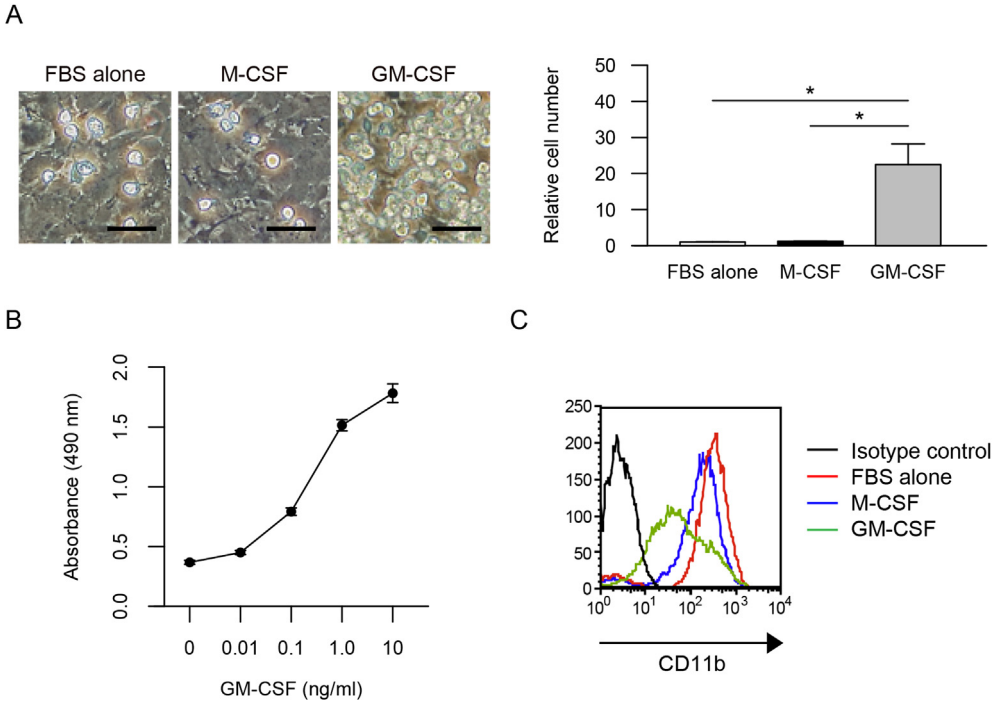

Fig. 1. GM-CSF stimulates proliferation and alters phenotypes of microglia *in vitro* more potently than M-CSF. (A) Mixed glial culture was prepared from P1–3 brains and treated with M-CSF (10 ng/ml) or GM-CSF (10 ng/ml) for 8 days. Left, representative images of mixed glial culture. Round and loosely-attached cells are microglia. Scale bars represent 50 μm. Right, quantification of the number of primary microglia isolated from mixed glial culture. The cell number is expressed relative to FBS alone. n = 3 per group. *$p < 0.05$. (B) Dose-dependent effect of GM-CSF on proliferation of primary microglia. GM-CSF was added at different concentrations (0, 0.01, 0.1, 1.0, 10 ng/ml). At DIV10, the proliferation of primary microglia was measured with an MTS assay. Data are expressed as absorbance at 490 nm. n = 4 per group. (C) Flow cytometric analysis for CD11b on primary microglia cultured with M-CSF (10 ng/ml) or GM-CSF (10 ng/ml). Data are representative of three or more replicates.

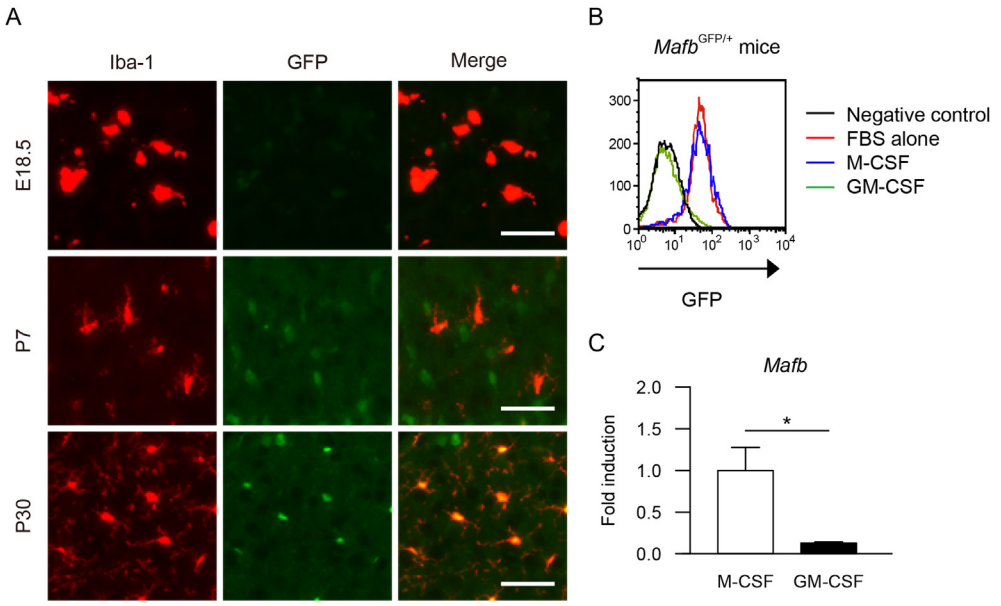

Fig. 2. MafB expression is upregulated as microglia become mature, but downregulated by GM-CSF. (A) Immunohistochemical images of cerebral cortex from *Mafb*$^{GFP/+}$ mice at E18.5, P7, or P30. Iba-1 positive cells (red) indicate microglia. Scale bars represent 50 μm. (B) Flow cytometric analysis for GFP fluorescence of *Mafb*$^{GFP/+}$ microglia cultured with M-CSF (10 ng/ml) or GM-CSF (10 ng/ml). Wild-type microglia were used as a negative control. Data are representative of three or more replicates. (C) Quantitative RT-PCR for *Mafb* mRNA of primary microglia cultured with M-CSF (10 ng/ml) or GM-CSF (10 ng/ml). n = 4 per group. $^*p < 0.05$.

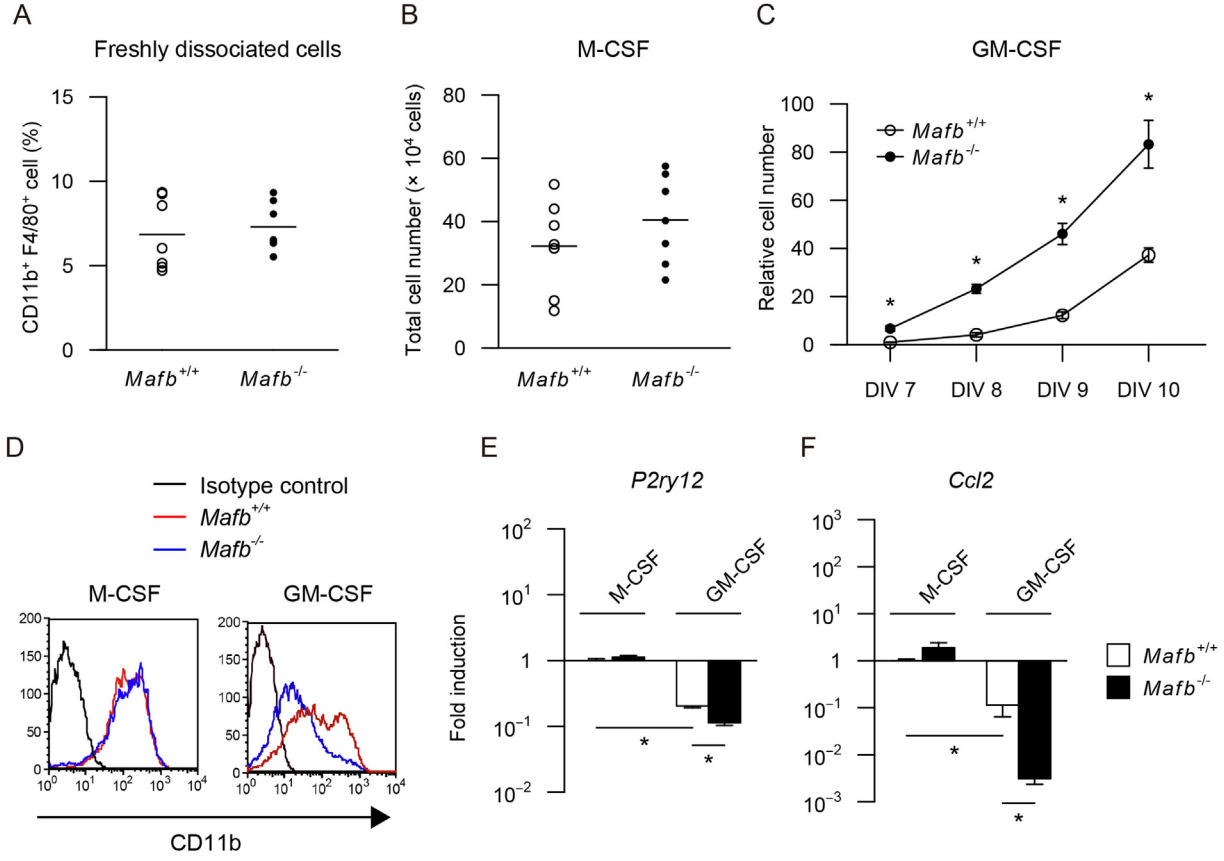

Fig. 3. *Mafb* deficiency potentiates the effect of GM-CSF on microglia *in vitro*. (A) Percentage of microglia, which are identified as CD11b$^+$ F4/80$^+$ cells by flow cytometry, in freshly dissociated cells from *Mafb*$^{+/+}$ or *Mafb*$^{-/-}$ brains at E18.5. n = 7 per group. No significant difference is observed. (B) *Mafb*$^{-/-}$ microglia cultured with M-CSF do not show a significant difference in the cell number. n = 6 per group. (C) *Mafb*$^{-/-}$ microglia grow more rapidly than *Mafb*$^{+/+}$ microglia in the presence of GM-CSF. n = 7 per group. $^*P < 0.05$. (D) Flow cytometric analysis for CD11b expression on primary microglia from *Mafb*$^{+/+}$ or *Mafb*$^{-/-}$ brains cultured with M-CSF or GM-CSF. Data are representative of three or more replicates. (E, F) Quantitative RT-PCR for *P2ry12* (E) and *Ccl2* mRNA (F). n = 4–6 per group. $^*p < 0.05$.

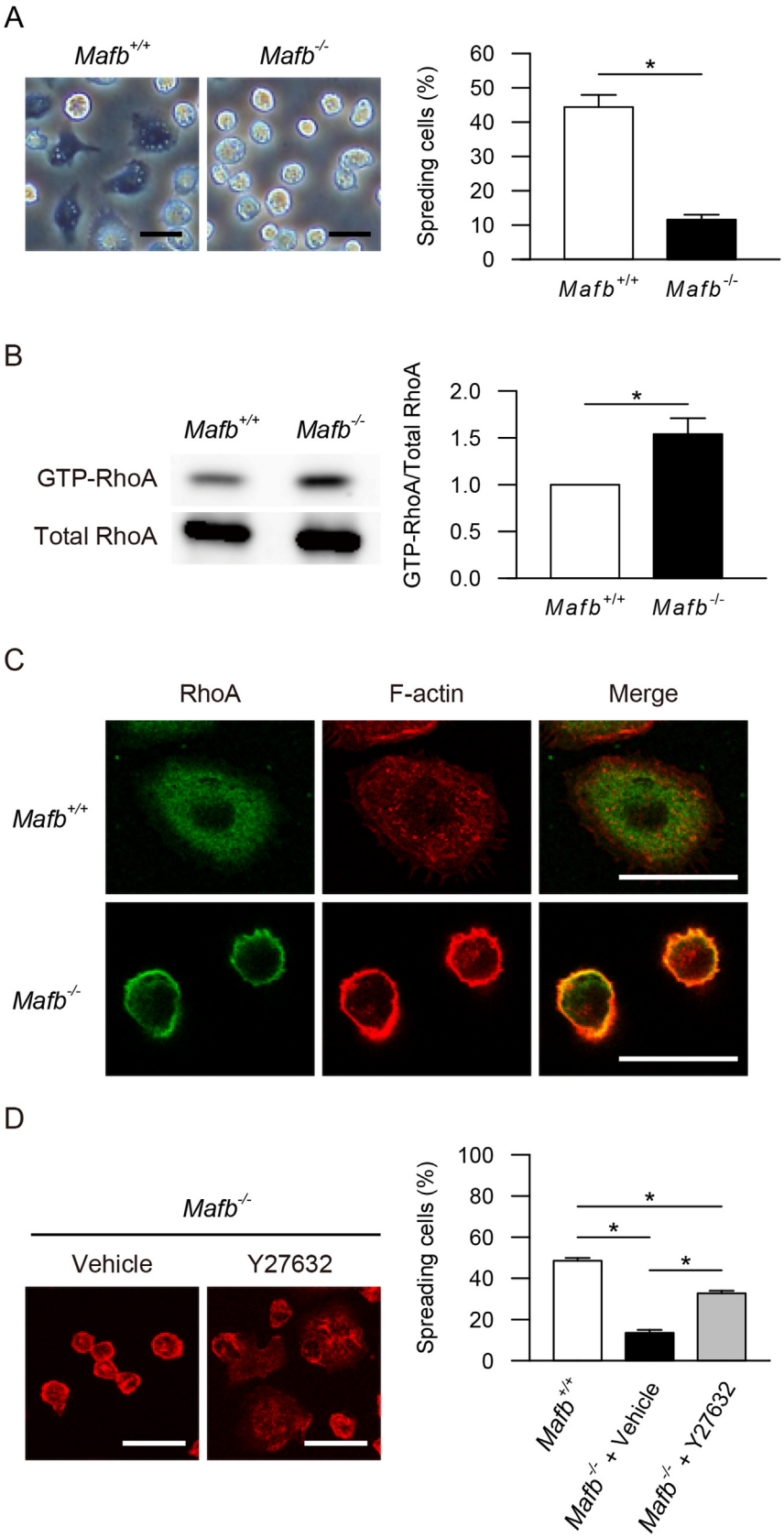

Fig. 4. *Mafb*-deficient microglia cultured with GM-CSF display rounded morphology probably due to abnormal activation of RhoA. (A) Left, representative images of primary microglia cultured with GM-CSF from $Mafb^{+/+}$ or $Mafb^{-/-}$ mice 30 min after plating. Scale bars represent 25 μm. Right, percentage of spreading cells 30 min after plating. n = 5 per group. $^{*}p < 0.05$. (B) Pull-down assay for GTP-bound RhoA. Cells were lysed 30 min after plating. Three independent experiments were performed and the intensity was quantified with densitometry. Data are expressed as a ratio of GTP-RhoA to total RhoA. $^{*}p < 0.05$. (C) Confocal images of isolated microglia showing the localization of RhoA (green) and F-actin (red). Scale bars represent 25 μm. (D) Treatment with ROCK inhibitor Y27632 (10 μM) partially rescues the spreading of $Mafb^{-/-}$ microglia. Left, representative confocal images of $Mafb^{-/-}$ microglia treated with vehicle or Y27632. Cells were stained with Alexa Fluor 546 phalloidin. Scale bars represent 25 μm. Right, quantification of the number of spreading cells. n = 3 per group. $^{*}p < 0.05$.

which are associated with the maturity of microglia and downregulated by GM-CSF [16]. As expected, *Mafb*$^{-/-}$ microglia showed further downregulation of *P2ry12* mRNA compared to *Mafb*$^{+/+}$ microglia in the presence of GM-CSF (Fig. 3E).

A recent study has shown that the amount of *Ccl2* expression represents the extent of macrophage polarization induced by M-CSF and that blockade of CCL2 upregulates the expression of a set of genes induced by GM-CSF [24]. *Mafb*$^{-/-}$ microglia showed further downregulation of *Ccl2* expression in the presence of GM-CSF (Fig. 3F). Taken together, these results suggest that *Mafb* deficiency potentiates GM-CSF signaling in microglia *in vitro*, which might be attributable to further downregulation of *Ccl2*.

3.4. *Mafb*-deficient microglia cultured with GM-CSF display rounded morphology probably due to abnormal activation of RhoA

A previous study has shown that *Mafb*-deficient macrophages display multiple and frequently branched formations in response to M-CSF, suggesting that MafB is involved in the regulation of cell morphology [25]. Having shown that *Mafb* deficiency potentiates the effect of GM-CSF on microglia, we tested whether *Mafb*$^{-/-}$ microglia display different morphology in response to GM-CSF. As shown in Fig. 4A, *Mafb*$^{-/-}$ microglia cultured with GM-CSF barely extended their membrane protrusions after plating, despite approximately half of the *Mafb*$^{+/+}$ microglia being spread on the plate (Fig. 4A). It is well known that Rho GTPases including RhoA, Rac1, and Cdc42 are key regulators for cytoskeletal remodeling [26]. Stimulation with GM-CSF is reported to activate RhoA and subsequent proliferation of macrophages [27]. In addition, too much activation of RhoA inhibits the membrane protrusion formation, resulting in rounded morphology [28,29]. These findings led us to the hypothesis that the rounded morphology of *Mafb*$^{-/-}$ microglia is caused by abnormal activation of RhoA. To test this hypothesis, we examined the amount of GTP-bound (active) RhoA with a pull-down assay. Although total amount of RhoA is comparable between the genotypes, GTP-RhoA was significantly increased in *Mafb*$^{-/-}$ microglia (Fig. 4B). In addition, immunocytochemical analysis showed that RhoA was localized on the plasma membrane in *Mafb*$^{-/-}$ microglia (Fig. 4C), which is a hallmark of activation of RhoA [28,29]. ROCK is known as a main downstream effector of RhoA [26]. As expected, treatment with ROCK inhibitor Y27632 (10 μM) resulted in an increased number of spreading cells in *Mafb*$^{-/-}$ microglia (Fig. 4D). Considering all this, it is plausible that abnormal activation of RhoA is the cause of the rounded morphology of *Mafb*$^{-/-}$ microglia cultured with GM-CSF.

4. Discussion

The main findings of the present study are, first, that the expression of MafB is upregulated as microglia become mature, but downregulated by GM-CSF stimulation. Second, *Mafb* deficiency enhances the effect of GM-CSF on microglia, that is, stimulation of proliferation and alteration of the phenotype. Third, *Mafb*$^{-/-}$ microglia displays rounded morphology in response to GM-CSF through RhoA activation. These findings reveal that MafB is a negative regulator of GM-CSF signaling in microglia. To our knowledge, we show for the first time the crosstalk between GM-CSF and MafB.

MafB is bZip transcription factor which is highly expressed in monocyte-macrophage lineage [13]. Although MafB has been shown to be expressed in mature microglia [3,16], its function is largely unknown. In the present study, we confirmed that MafB is expressed at low levels in embryonic and neonatal microglia, but at high levels in adult microglia. Our observation, together with the previous studies, raises the possibility that MafB is involved in the maturation of microglia. At present, however, it is quite difficult to analyze the function of MafB in adult microglia *in vivo* because *Mafb*-deficient mice die immediately after birth [13,21]. Generation of microglia specific *Mafb* knockout mice will be required for future studies.

GM-CSF is well-known cytokine which play an important role in the development of myeloid cell lineage [9]. Although GM-CSF has potent biological effects on microglia *in vitro* including stimulation of their proliferation [11] and alteration of their phenotypes [12], how GM-CSF signaling is modulated in microglia is poorly characterized. We found that MafB expression is downregulated by GM-CSF. Moreover, *Mafb*$^{-/-}$ microglia proliferate more rapidly accompanied by further downregulation of differentiation-associated genes in response to GM-CSF compared to *Mafb*$^{+/+}$ microglia. Our findings indicate that GM-CSF signaling and MafB mutually antagonize in microglia. On the other hand, previous studies have shown that *Mafb* deficiency leads to higher responsiveness to M-CSF in macrophages [22] and HSCs [23]. According to these studies, MafB does not affect their responsiveness to GM-CSF. In contrast, we found that *Mafb*$^{-/-}$ microglia show higher responsiveness to GM-CSF, but not to M-CSF. This discrepancy might be explained by the uniqueness of microglia. A comprehensive expression analysis revealed that microglia have a gene expression signature distinct from that of other macrophages [16]. Such comprehensive data sets might be useful to account for the discrepancy.

In addition, we found that *Mafb*$^{-/-}$ microglia cultured with GM-CSF display a rounded morphology accompanied by abnormal activation of RhoA, which is a key regulator of actomyosin contraction [26]. A previous study suggested that the RhoA/ROCK pathway regulates the motility of activated microglia [30]. Although we did not investigate it in the present study, it can be expected that *Mafb*$^{-/-}$ microglia cultured with GM-CSF display abnormal motility in response to some stimuli.

In the present study, however, molecular mechanisms linking MafB and GM-CSF signaling remains unsolved. First, there is a possibility that MafB induces the expression of genes encoding suppressors of GM-CSF signaling. We found that *Ccl2* is more downregulated in *Mafb*$^{-/-}$ microglia than in *Mafb*$^{+/+}$ microglia in the presence of GM-CSF. It was recently suggested that CCL2, a well-known chemokine to attract monocytes/macrophages, might suppress the expression of a set of genes induced by GM-CSF in macrophages [24]. In addition, a previous study has shown that MafB directly binds to the Maf recognition site (MARE) in the promoter region of the *Ccl2* gene [31]. Thus, it is possible that MafB antagonizes GM-CSF signaling through the induction of the *Ccl2* gene. Interestingly, a previous study has shown that MafB antagonizes type I interferon signaling through directly interacting with IRF3 [32]. Thus, another possibility should be considered that MafB directly interacts with the downstream effectors of GM-CSF such as STAT5 [9] or IRF5 [33].

In conclusion, our study reveals that MafB antagonizes the effect of GM-CSF on microglia *in vitro*. Since the molecular mechanisms underlying the function of MafB are still unclear, the identification of its targets would provide new insights into modulation of cytokine signaling by transcription factors in microglia.

Acknowledgments

We thank Masami Ojima and Akiko Fujii for technical assistance, and Brian Purdue for grammatical revision of the manuscript. This work was supported by a Grant-in-Aid for Scientific Research (21220009) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT). R.K. is a recipient of a Japan Society for the Promotion of Science Research Fellowship for Young Scientists (13J00759).

Conflict of interest

The authors declare no competing financial interests.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.05.036.

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