Astrocyte Stimulates Microglial Proliferation and M2 Polarization in vitro through Cross-Talk between Astrocyte and Microglia

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Abstract: Microglia are resident immune cells of the central nervous system such as brain-specific macrophages and also known to regulate the innate immune functions of astrocytes through secretory molecules. This conversation plays an important role in brain functions and homeostasis as well as in neuropathologic disease. In this study, we aimed to elucidate whether astrocytes and microglia can cross-talk to induce microglial polarization and proliferation, which can be further regulated under the brain stroke-mimic microenvironment. Microglia in mixed glial culture increased their survival and proliferation and altered to the M2 microglia, whose role was provided by CD11b+GFAP+ astrocytes by showing approximately tenfold increase in microglia cell proliferation after the astrocyte reconstitution. Furthermore, GM-CSF stimulated microglial proliferation approximately tenfold and induced to CCR7+ M1 microglia, whose phenotype could be suppressed by anti-inflammatory cytokines such as IL-4, IL-10, and Substance-P. Also, astrocyte in the microglia co-culture revealed A2 phenotype, which could be activated to A1 astrocyte by TNFα and IFNγ under the stroke-mimic condition. Altogether, astrocyte in the mixed glial culture stimulated the microglia proliferation and M2 polarization possibly through its acquisition of A2 phenotype, both of which could be converted to M1 microglia and A1 astrocytes under the inflammatory stroke-mimic environment. This study demonstrated that microglia and astrocyte can be polarized to M2 microglia and A2 astrocytes respectively through the cross-talk in vitro and provided a system to explore how microglia and astrocyte may behave in the inflammatory disease milieu after in vivo transplantation.

Keywords: Microglia; M1/M2 polarization; astrocyte; microglia and astrocyte cross-talk

1. Introduction

Glial cells are non-neuronal cells in the central and peripheral nervous systems. Glial cells in the central nervous system (CNS) include oligodendrocytes, astrocytes, ependymal cells, and microglia [1]. In this study, we are focusing on microglia and astrocytes. Microglia are resident immune cells of the CNS, such as brain-specific macrophages. In a healthy brain, microglia show ramified morphology as resting state and patrol the brain to regulate brain homeostasis [2]. Microglia become activated after brain injury, either classically activated or alternatively activated, and are referred to as M1 and M2 types. The activated microglia show morphological change from ramified shape to rounded or elongated spindle shape and migrate to the injury site [3]. At the injury site, two types of
activated microglia play an opposite role. M1 microglia play a pro-inflammatory role under brain injury by releasing harmful cytokines, which induce neuronal death. While M2 microglia release anti-inflammatory cytokines, clean up the debris in the injury site by phagocytosis and induce tissue repair by secretion of neuroprotective growth factors [4,5].

In the case of stroke, the M2 microglia/macrophages are increased within 5 days after injury. However, after the M2 microglia reaches the peak, they are decreasing, while M1 microglia/macrophages show steady enhancement until 14 days after injury [6]. Although it is unclear that an increase of M1 microglia means increasing M1 polarization of resting microglia or skewing of M2 microglia to M1 microglia, increase of M2 polarization or inhibit the M1 skewing will be the key factors to inhibit the aggravation of injury and induce tissue repair. There are many previous studies about the regulation of microglia M1/M2 polarization. Interleukin-4 (IL-4) and Interleukin-10 (IL-10) are the well-known cytokines that induce M2 microglia, and tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) are the representative inducers of M1 microglia [4,7,8]. Substance-P (SP) is a neurotransmitter composed of 11 amino acids regulating immune modulation [9]. In a previous study, SP injection into a rat spinal cord injury model increased the number of M2 microglia/macrophages at the injury site [10]. Also, SP induced the M2 polarization of rat bone marrow macrophage and skewed the M1 macrophages to M2 macrophages [11]. The microglia and macrophages play a similar role at the tissue level and share many markers. Nevertheless, the SP effect in microglia is unclear.

Astrocytes are the most abundant cells in the brain, and they maintain CNS homeostasis, provide structural support, and regulate ion, nutrient, and gas concentration [12,13]. Astrocytes become reactive astrocytes after injury. Reactive astrocytes act as a dual-edged sword because they play a dual role in CNS disease. Reactive astrocytes enhance damage by releasing pro-inflammatory cytokines, forming the glial scar, alleviating the inflammation, and boosting tissue regeneration [14,15]. Just like microglia, recently reactive astrocytes are also distinguished as two different types; A1 astrocytes play a harmful role, while A2 astrocytes are neuroprotective [16,17]. However, the role of A1/A2 astrocytes is well-known. The study about the inducer of A1/A2 astrocytes and the makers of both reactive astrocytes to be more done. Furthermore, cross-talk between astrocytes and neurons and microglia and neurons were vigorously studied [18,19], whereas cross-talk between astrocytes and microglia is relatively less studied. During CNS disease, astrocytes are activated later than microglia. Thus, cross-talk between microglia and astrocyte is focused on astrocytes, while cross-talk between these two cells focused on microglia and the correlation of both A1/A2 astrocytes and microglia need to be studied.

In this study, we aimed to elucidate whether astrocytes and microglia can cross-talk especially focused on the microglial phenotypic change and microglia polarization via SP. We found that microglia co-cultured with mixed glial cells for 14 days was polarized to the M2 phenotype, and the microglial proliferation was increased. Moreover, to further demonstrate the key cells that induced these microglial changes among the mixed glial cells, we performed microglia and astrocyte co-culture. Microglia-astrocyte co-culture showed more proliferating cells, as confirmed by BrdU incorporation assay. Also, to increase the microglial proliferation, we treated various cytokines to the microglia and demonstrated that GM-CSF increased the proliferation of microglia and induced M1 polarization. This polarization was inhibited with sequential treatment of SP, IL-4, or IL-10 after GM-CSF treatment. Furthermore, we demonstrate that the reactive astrocyte of co-culture was skewed to A2 type by confirming A1/A2 specific marker expression. In conclusion, cross-talk between microglia and astrocyte increases microglial proliferation and M2-polarization, and SP can suppress the M1 polarization of microglia.

2. Results

2.1 Astrocytes in whole brain cell isolates stimulate microglial survival and proliferation in the mixed culture in vitro
To study the regulation of microglial phenotype by other cells in the brain in vitro, microglia was cultured immediately after MACS cell isolation of whole brain cells with microglial/macrophage-specific marker CD11b antibody or CD11b+ microglia was isolated after two weeks culture of whole brain cell isolates (Figure 1A). Only 0.3% of total brain cell isolates was CD11b+ microglia immediately after the cell isolation (Figure 1B), most of which died around 5 to 7 days during the cell culture (data not shown). After two weeks culture of whole brain cell isolates, CD11b+ microglia increased to approximately 5.7% of total cells (Figure 1B), similarly to the previous report [20]. With two weeks mixed culture of whole brain cell isolates, microglia at day 0 increased approximately three folds at day 14 (Figure 1C). Also, the level of CD11b expression increased from the low expresser (CD11blo) at day 0 to the high (CD11bhi) at day 14 as shown in FACS analysis (Figure 1D). This supports the concept that other cells in whole brain cell isolates may positively aid the survival and proliferation of CD11b+ microglia.

Figure 1. CD11b+/GFAP+ astrocyte from whole brain culture induced microglial proliferation and activation. (A) The experimental scheme of microglia isolation by MACS. Day 0 microglia (MG d0) were isolated immediately from whole brain cell isolates by CD11b+ MACS and day 14 microglia (MG d14) were isolated from the 2 weeks mixed glial cell culture. (B) The CD11b+ cells yield of MG d0 and MG d14 (n= 11). (C) The number of CD11b+ cells per brain for MG d0 and MG d14 (n=11). (D) Flow cytometry analysis of MG d0 and MG d14 with the microglia specific marker CD11b. Fluorescent intensity of CD11b was enhanced in MG d14 compared to that of MG d0. (E) Fluorescence images of CD11b+ cells sorted from the 2 weeks mixed glial cell culture stained with microglia specific marker iba-1 and astrocyte specific marker GFAP. (F) Bright field image of MG d14. Yellow arrowhead; ramified microglia, black arrowhead; bipolar-shape microglia, white arrow; round-shape microglia (G) Fluorescence images of MG d14 stained with microglia specific markers CD11b and iba-1 and astrocyte specific marker GFAP. ***P<0.001; Scale bar = 100 μm.

In order to find out cellular identity of approximately 94% other cells remained in the two weeks mixed culture of whole brain isolates, both CD11b− and CD11b+ cells were collected and immuno-stained with antibodies to astrocytes markers or microglia (Figure 1E-G). Almost all CD11b+ cells were iba-1 negative GFAP expressing activated astrocytes. In contrast, most CD11b+ sorted cells at day 14 showed bipolar and round morphologies in the activated state of microglia but only some showed the ramified morphology of the resting microglia (Figure 1F). Approximately 99% of CD11b+ cells were positive for microglia specific marker CD11b and Iba-1 and only 1% of CD11b+ sorted cells were positive for astrocyte specific marker GFAP (Figure 1G). Thus, astrocytes are main cellular components in the mixed culture of whole brain cell isolates, which may provide favorable microenvironment for microglia survival and proliferation.
2.2 Astrocytes in the mixed culture preferentially induce M2 type microglia in vitro

The phenotypic change of microglia during two weeks ex vivo culture of whole brain cell isolates was determined (Figure 2). CD11b+ sorted cells were FACS-analyzed using antibodies of CD68 as activated microglia and CD206/CD163 as M2 microglia markers (Figure 2A). CD68+CD206+ microglia increased from 71.9% at day 0 to 99.5% at day 14 and CD206+CD163+ microglia also increased from 55.9% at day 0 to 98.6% at day 14. To confirm the M2 polarization of microglia at day 14, microglia was stained with antibodies to CD68, CD206, CD163 as activated M2 microglia markers and CCR7 as M1 microglia marker (Figure 2B). Almost all microglia were positive in CD68 and CD206 expression and approximately 80±5.20% microglia were positive in CD163, whereas CCR7+ M1 microglia was only 26.6±4.52% (Figure 2C). Probably, CCR7+ microglia seem to overlap with some of CD68 and CD206 expressing microglia.

Figure 2. Phenotypic identity of MG d14 as M2 microglia. (A) Flow cytometry analysis of MG d0 and MG d14 with CD206, CD68, and CD163 antibodies. CD206+CD68+ cells and CD206+CD163+ cells increased in MG d14. (B) Fluorescence image of CD68, CCR7, CD206, and CD163 in MG d14. Left white box; magnified image of each marker positive cells. (C) Quantitative analysis of CD68+, CCR7+, CD206+, and CD163+ cells among CD11b+ cells. (n=5) (D) Fluorescence image of the phagocytosis assay of MG d14. Left white box; magnified image of E.coli particle positive cells. Arrowhead; actin ruffles. (E) Quantitative analysis of E.coli particle engulfing cells among MG d14 (n=5). ***P<0.001; Scale bar = 100 μm

Since microglia is a tissue resident immune cell, it is expected to clean-up the cell debris or foreign particle by phagocytosis [21]. To examine phagocytic function of microglia at day 14 culture, fluorescence-labeled E.coli-particles were treated to the microglia culture and E.coli-engulfing microglia was visualized by actin cytoskeletal staining (Figure 2D). Many of actively phagocytic microglia with actin ruffles was detected. Approximately, 73% of microglia was shown to have phagocytic function (Figure 2E). Taken together, microglia derived from neonatal rat brain can proliferate in the mixed culture of whole brain isolates and preferentially transform to M2 type microglia with phagocytic function.

2.3 Reconstitution of astrocyte in the microglia culture enhances microglial proliferation

Microglial survival and proliferation increased in the mixed culture of whole brain cell isolates, which were mainly GAFP+ activated astrocytes (Figure 1). We hypothesized
that astrocytes may play an important positive role in microglial survival and proliferation by direct contact and/or by their secretomes. After CD11b+ cell sorting, microglia culture was reconstituted with astrocytes or astrocytes conditioned medium and their effect on cell proliferation was measured by BrdU incorporation assay (Figure 3A). Since initial ratio of CD11b+/CD11b- cells in 2 weeks mixed glial culture was approximately 1/15 (Figure 1B), CD11b+ microglia were re-plated in a 1:10 ratio of microglia: astrocytes pretreated with Cytarabine (Ara-C) or without astrocytes and Iba1-BrdU incorporated cells, representing proliferating microglia, were counted (Figure 3B-C). The BrdU-Iba1+ microglia were almost ten times higher in the astrocyte co-culture comparing to that in microglial single culture, which was repeated at different cell density. This result suggests that astrocytes stimulate initial microglial proliferation through direct cell contact.

To examine the effect of astrocyte secretome on microglial proliferation, astrocyte conditioned medium (ACM) was treated to the microglia culture in a ratio of 1:1 or 1:5 with microglia culture medium (MCM) and BrdU incorporation assay was performed (Figure 3D). BrdU-incorporated microglia were 2.5 folds higher at 1:1 ratio of ACM: MCM than that in MCM only, which got lower at 1:5 ratio of ACM:MCM. Therefore, astrocytes clearly secrete some factors stimulating microglial proliferation but direct cellular contact with astrocytes further stimulate microglial proliferation.

2.4 Cytokine profiles of microglia, astrocytes, and their co-culture

In order to explore the difference in cytokine profiles secreted from astrocytes and/or microglia, conditioned media of microglia only, astrocytes only, and astrocyte/microglia co-culture at different cell ratio were collected for 1 day and analyzed by rat cytokine array (Figure 4A-D). Most cytokines; Activin A, Agrin, β-NGF, CINC-3, CNTF, Fractalkine, GM-
CSF, ICAM-1, IFN-γ, IL-1α, IL-1β, IL-1R6, IL-2, IL-4, IL-6, IL-10, IL-13, Leptin, L-Selectin, MIP-3α, MMP-8, PDGF-AA, Prolactin R, RAGE, Thymus chemokine, were similarly expressed in both microglia and astrocytes. However, cytokines such as CINC-1 (CXCL-1, neutrophil chemoattractant), CINC-2α, LIX, TNF-α, MCP-1 (CCL2 chemokines), TIMP-1, and VEGF were expressed higher in astrocyte than microglia. Among them, CINC-1, MCP-1, TIMP-1, and VEGF were expressed in astrocytes two folds higher than in microglia. Cytokines such as β-NGF, CINC-1, CINC-2α, CINC-3, CNTF, GM-CSF, ICAM-1,

| A.                     | b | c | d | e | f | g | h | i | j | k | l |
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| 7                      |   |   |   |   |   |   |   |   |   |   |   |   |
| 8                      |   |   |   |   |   |   |   |   |   |   |   |   |

Figure 4. The secretome analysis of single or co-culture of microglia and astrocyte with rat cytokine array. (A) Cytokine array map. The array can detect 34 soluble mediators. Pos; positive control, Ne; negative control. (B) The cytokine arrays were performed with conditioned medium of microglia, astrocyte single culture, or microglia-astrocyte co-culture in various ratios (C) The quantitative data of the fold increase of array spots between microglia, astrocyte single culture and microglia-astrocyte co-culture. *p < 0.05, **p < 0.01, and ***p < 0.001

IFN-γ, IL-10, Leptin, LIX, MCP-1, MIP-3α, MMP-8, PDGF-AA, Prolactin R, RAGE, Thymus chemokine-1, TIMP-1, VEGF were elevated in the microglia co-cultured with astrocytes, which are known to be involved in inflammation, neuronal migration and proliferation, and vascular repair and remodeling. Among them, only TNF-α was reduced in the co-culture. Several cytokines secreted from the microglia and astrocytes in the co-culture may coordinate act on the microglia proliferation and induction of M2 phenotype of microglia as well as on astrocyte phenotypes.

2.5 TNF-α and IFN-γ further activated M2 microglia to iNOS- and IL-1β-expressing M1 microglia, which was partially inhibited by SP

It was explored whether M2 microglia in the mixed glia culture can be further converted to M1 phenotype upon exposure of pro-inflammatory cytokines such as TNF-α and IFN-γ, which can be also modulated by co-existence of a neuropeptide SP, also known
as M2 cytokine [11]. TNF-α and IFN-γ altered the morphology of primary microglia from round amoeboid and short bipolar morphology to the bipolar morphology with elongated process (Figure 5A, B). SP itself did not alter morphological change significantly but partially inhibited the TNF-α and IFN-γ mediated process elongation, which

Figure 5. M1 polarization of MG d14 by treatment of TNF-α and IFN-γ. (A) Bright field images of microglia cultured with TNF-α and IFN-γ, cultured with TNF-α, IFN-γ, and SP, and microglia cultured with TNF-α, IFN-γ, SP and RP67580 (RP, NK1 receptor antagonist) (B) Quantitative data of elongated process of microglia under various cytokine treatment. (C, D) Quantitative data of mRNA expression of TGF β, IGF1, iNOS, and IL-1β in 3h after treatment (C) and 1d after treatment (D). (n=3). (E) Quantitative data of NO production in various cytokine combination at different time points. (n=3) (F) The western blot analysis of Bcl-xL, IκB, and BAD in microglia cultured with TNF-α, IFN-γ, SP and RP67580. (G) Quantitative data of Bcl-xL, IκB, and BAD expression (n=3). *p < 0.05, **p < 0.01, and ***p < 0.001; Scale bar = 100 μm

was nullified by co-treatment with NK-1 receptor antagonist RP67580. To define the phenotype of TNF-α and IFN-γ activated microglia, qPCR of TGF β and IGF1 for M2 microglia and iNOS and IL-1β for M1 microglia was performed (Figure 5C, D). TNF-α and IFN-γ did not affect the expression of TGF β and IGF1 significantly but markedly increased the expression of iNOS and IL-1β at 1 day post treatment. Along with iNOS induction, elevated NO production was observed in TNF-α and IFN-γ treated microglia (Figure 5E). Accordingly, TNF-α and IFN-γ activated microglia revealed M1 phenotype microglia. Furthermore, TNF-α and IFN-γ increased Bcl-xL expression, suggesting self-defense mechanism from NO-induced apoptosis [22], which was inhibited by SP co-treatment (Figure 5 F, G). Microglia in the mixed glia culture skewed M2 phenotype, which can be further polarized to M1 phenotype upon the exposure of pro-inflammatory cytokines TNF-α and IFN-γ.
2.6 GM-CSF stimulated microglial proliferation and CCR7\(^+\) M1 phenotype in vitro, which was subsequently suppressed by anti-inflammatory cytokines such as IL-4, IL-10, and SP

Since microglia population in the brain is very low, it is very important to increase microglial proliferation and reversibly convert M1 and M2 phenotypes in vitro for adoptive cell therapy. In our previous study, bone marrow monocyte/macrophages can be activated and proliferated by GM-CSF treatment, which can be induced to polarize into either M1 or M2 phenotype by anti-inflammatory cytokines such as IL-4/13, IL-10 or pro-inflammatory cytokines such as TNF-\(\alpha\) and IFN-\(\gamma\), respectively, where SP, known as a neurotransmitter or neurohormone, was identified as one of a novel anti-inflammatory cytokine [11]. To explore whether GM-CSF can stimulate microglia proliferation as well as its phenotypic conversion, microglia with M2 phenotype was incubated with different cytokines and BrdU-incorporated cells were counted (Figure 6A-F). GM-CSF and IL-4
stimulated microglia proliferation approximately ten folds and six folds higher, respectively than that of non-treated microglia, even though only marginal stimulation was observed in SP, TNF-α, and IFN-γ treatment (Figure 6 A-C). Then, it was explored whether GM-CSF-treated microglia can be further converted to M1/M2 phenotype upon treatment of anti-inflammatory cytokines or pro-inflammatory cytokines (Figure 6D-F). Most microglia treated with GM-CSF were brightly expressing M1 microglia marker CCR7 and activated microglia marker CD68, which were fairly maintained by subsequent -α and IFN-γ treatment even though their cell proliferation was rather regressed than sustained GM-CSF treatment. However, CCR7 expression in GM-CSF-treated microglia was markedly reduced by subsequent removal of GM-CSF and treatment of anti-inflammatory cytokines such as IL-4, IL-10, and SP. Thus, microglia can be markedly expanded by GM-CSF treatment, whose M1 microglia phenotype can be repressed by subsequent treatment of anti-inflammatory cytokines.

2.7 Reactive astrocytes in the co-culture were skewed to A2 astrocyte, which could be induced to A1 astrocytes upon TNF-α and IFN-γ treatment

Just like microglia, recently reactive astrocytes are also distinguished as two different types; A1 astrocytes play a harmful role, while A2 astrocytes are neuroprotective [16,17]. In order to examine phenotypic alteration of astrocyte in the co-culture with microglia, GFAP+ astrocytes were exposed to oxygen glucose deprivation (OGD) condition, similar to the stroke microenvironment in combination with inflammatory cytokines TNF-α and IFN-γ (Figure 7A). Based on qPCR analysis, OGD did increase the expression of CXCL10, a neutrophil chemo-attractant, which was markedly elevated by TNF-α and IFN-γ exposure, approximately 400 and 6,000 folds respectively. In addition, TNF-α and IFN-γ markedly elevated expression of A1 astrocytes markers such as Amigo 2, and Serping 1, where IFN-γ seems to work more potently as an A1 inducer than a known A1 inducer TNF-α. In contrast, A2 astrocyte markers such as CD109 and Emp1 were reduced by TNF-α and IFN-γ treatment. This result suggests that astrocytes in microglia co-culture were most likely skewed to A2 astrocyte and could be induced to A1 astrocyte phenotype by TNF-α and IFN-γ in the OGD condition, among which IFN-γ may be most potent A1 inducer in the stroke environment.

Then, modulation of A1/A2 phenotype by pro-inflammatory or anti-inflammatory cytokines was determined (Figure 7B-C). TNF-α markedly increased CINC-1 and CINC-2, which was major cytokines in the astrocyte but not VEGF based on ELIZA assay and CINC-2 gene expression by real time PCR analysis. Anti-inflammatory cytokines such as IL-4, IL-10, and SP did not affect CINC 1/2 expression and both cytokines did not affect TGF β expression. Thus, astrocytes in microglia co-culture could be very susceptibly induced to A1 astrocyte phenotype by IFN-γ or TNF-α and CINC-1/2 expression could be applied as a whole marker for TNF-α-inducible A1 astrocyte.
3. Discussion

In this study, we investigated the cross-talk between microglia and astrocyte and their phenotypic conversion in vitro. Microglia could be isolated from neonatal rat brains by CD11b+ MACS isolation. At fourteen days of the mixed glial cell culture, the number of CD11b+ microglia increased approximately three folds (Figure 1B-C), which usually died within a week without the co-culture (data not shown). Also, during the two weeks co-culture, CD11b+ cells become brighter in CD11b expression and mostly converted to the CD206 and CD163 positive M2 phenotype microglia from ~56% at day 0 to ~99% at day 14 (Figure 2A-B). Considering that microglia at day 0 is the naive microglia of in vivo brain, the cells in the mixed glial culture affect the M2 polarization of microglia. To characterize non-microglia cell population in mixed glial cell culture, CD11b+ cells after CD11b+ MACS isolation was stained with microglia and astrocyte specific markers. Almost 99% of CD11b+ cells showed GFAP positive astrocyte (Figure 1F). In other words, astrocytes, a major cell population in the mixed glial culture, stimulated the proliferation and M2 polarization of microglia, which was confirmed by reconstitution of astrocyte to the CD11b+ MACS-sorted microglia culture for direct cell-cell contact and also by astrocyte conditioned medium (Figure 3B-D). However, microglia cell proliferation was significantly higher in direct cell-cell contact in the microglia-astrocyte co-culture than its conditioned medium. Additionally, in vitro cytokine arrays of microglia, astrocyte, microglia and astrocyte co-culture in a different cell ratio showed that both cells secrete many cytokines involved in immune response, neurotrophic response, tissue repair, and tissue remodeling (Figure 4), which may work coordinately in the microglia proliferation as well as M2 polarization. Especially, astrocyte in the mixed glia culture also seem to acquire A2 phenotype astrocyte during the mixed glial cell culture. Finally, it was shown that M2 microglia and A2 astrocyte can be further converted to M1 microglia and A1 astrocyte under inflammatory microenvironment and stroke-mimic environment (Figure 5). This study demonstrated the cross-talk between microglia and astrocyte to facilitate M2 microglia and A2 astrocytes, which can be further regulated under a variety of cytokine microenvironment, expecting their behaviour in the in vivo environment after transplantation as adoptive cell therapy.

During ischemic injury, microglia are firstly activated and polarized to the M2 phenotype. The activated microglia then migrate to the injury site. However, this M2 microglia phenotype is only transiently retained and is reduced 5 days after injury. In contrast, M1 phenotype microglia are steadily increased during injury [6]. It is still unclear whether the M2 microglia are skewed to the M1 microglia or resting microglia polarize to the M1 microglia and astrocyte-derived factors directly affects these phenotypic changes. In this study, we demonstrated that microglia may proliferate and adopt M2 microglia in the presence of A2 astrocyte, where both cells may bidirectionally communicate through direct cell-cell contact as well as secreted molecules. In addition, M2 microglia are not terminally differentiated but can proliferate and polarize to M1 microglia by astrocyte-secreting cytokine such as GM-CSF, whose phenotype also can be further modulated by anti-inflammatory cytokines. Therefore, M1/M2 polarization may be specifically interpreted in the context of cytokine milieu in the vicinity. Under the brain microenvironment after ischemic injury, many inflammatory cytokines can activate the M1 microglia polarization [23,24]. As shown in our data, M2 microglia can be polarized to M1 microglia upon the exposure to inflammatory cytokines such as TNF-α and IFN-γ (Figure 5). The M2 microglia play an anti-inflammatory role in CNS injury by the secretion of many growth factors and cytokines that promote tissue repair. However, applying M2 microglia as a
cell therapy is quite challenging because a microglial cell source such as the brain is difficult to isolate from humans, the number of microglia in the brain is too low and the injury environment can polarize the injected M2 microglia to M1 microglia. Thus, it may be technically important for adoptive cell therapy of M2 microglia to treat a variety of neurodegenerative diseases with chronic inflammatory background to increase M2 microglia cell population by in vitro astrocyte co-culture, GM-CSF priming, and subsequent induction of M2 polarization by IL-4, IL-10, and SP. However, the therapeutic effect of M2 microglia injection to the stroke model and maintenance of their phenotypes has to be further demonstrated.

In our previous study, intravenous injection of SP in a rat spinal cord injury model increased the M2 microglia/macrophage at the injury site [10]. Also, SP induces the M2 polarization of in vitro macrophages [11] but the role of SP in microglia polarization was still unclear. In this study, it was demonstrated that SP may not markedly inhibit TNF-α and IFN-γ-induced M1 polarization but do suppress the TNF-α and IFN-γ-induced inhibition of M1 microglial apoptosis by reducing the Bcl-xL level (Figure 5F-G) and also SP can suppress M1 polarization after GM-CSF removal (Figure 6E-F). Still, it needs more researches on whether SP can skew M1 microglia to M2 microglia or not in conjunction with different cytokines.

Recently, astrocytes are also classified into two types with opposite functions. The A1 astrocyte is known as a ‘harmful’ astrocyte induced by inflammatory microglia and the A2 astrocyte is known as a ‘protective’ astrocyte possibly by anti-inflammatory microglia. However, the biomarkers except mRNA level to classify astrocyte phenotype and the factor(s) which induce(s) the A2 astrocyte is unknown [16,17]. According to this study, the astrocytes co-cultured with microglia seem to be skewed to A2 phenotype since TNF-α and IFN-γ under the OGD condition markedly stimulated expressions of CXCL10, a neutrophil chemoattractant, approximately 400 and 6,000 folds respectively as well as A1 astrocytes-specific genes such as Amigo 2 and Serping 1 but suppressed A2 astrocyte-specific genes such as CD109 and Emp 1 (Figure 7). Also, CINC-1 and CINC-2, which was shown to be major cytokines released from astrocyte based on cytokine array (Figure 4), may be considered to be a novel marker of A1 astrocyte since TNF-α, a known A1-inducing cytokine, strongly stimulated CINC-1/2 secretion and expression (Figure 7B). Therefore, this microglia-astrocyte co-culture system can be applied further to elucidate the phenotypic conversion of the A1/A2 astrocytes and the bidirectional communication between microglia and A1 and A2 reactive astrocytes in a variety of neuropathological-mimic microenvironment.

In conclusion, microglia and astrocyte can be polarized to M2 microglia and A2 astrocytes respectively through the cross-talk in vitro, both of which could be further converted to M1 microglia and A1 astrocytes under the inflammatory stroke-mimic environment. This study provides a system to expand M2 microglia and A2 astrocytes for adoptive cell therapy aiming a variety of neuroinflammatory neurodegenerative diseases such as Alzheimer disease, Parkinson disease, and Autism and also to expect how microglia and astrocyte may behave in the inflammatory disease milieu after in vivo transplantation under brain injury.

4. Materials and Methods

4.1 Animals

All animal experiments were approved by the Animal Studies Committee of Kyung Hee University (KHUASP #16-012 approved on July 1st, 2016) in Yong In, Korea, and performed under the Institutional Animal Care and Use Committee (IACUC) guidelines.

4.2 Mixed glial cell culture and isolation of microglia

Mixed glial cell culture was prepared from the brain of postnatal day (P) 1-3 inbred lewis rats (Orientbio, Sungnam, Korea), modified as previously described [25]. Briefly, the meninges and the blood vessels of the whole-brain were removed under the dissecting
microscope. Then, the brain was minced into small pieces in serum-free Dulbecco’s modified Eagle’s medium/nutrient mixture F12 mixture (1:1) (DMEM/F12, Gibco, Grand Island, NY, USA) and centrifuged. The pellet was re-suspended in DPBS (Welgene, Daegu, Korea). After washing, the cells were seeded in 75-cm² culture flasks in 15ml of DMEM/F12 (1:1) containing 10% FBS (Gibco), 2 mM L-glutamine (Welgene), and 50 U/ml penicillin and 50 mg/ml streptomycin (Welgene). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂, 95% air. The media was changed every 3 days, and the culture was maintained for 14 days.

To harvest microglia, microglia were isolated from mixed glial cell culture on day 14 using magnetic-activated cell sorting (MACS). Total glial cells were detached using 0.25% Trypsin-EDTA and neutralized with complete culture medium. Then, the cells were washed with PBS and re-suspended to a cell density of 1×10⁷ cells/ml. The cells were incubated with CD11b antibody (1:100, BD Bioscience, San Jose, CA, USA) for 5 min at 4°C and anti-mouse IgG microbeads (1:5, Miltenyi, Bergisch Gladbach, Germany) for 15 min at 4°C. The cells were washed twice with PBS. During washing, the MACS column was equilibrated by applying 10ml of autoMACS rinsing solution (Miltenyi). The cell suspension was applied to the LS column (Miltenyi) and allowed to pass through the resin by gravity flow. The column was washed twice with autoMACS rinsing solution. Then, the column was taken off from the magnet and the CD11b+ microglia eluded. The microglia were seeded into 6 well plates at a cell density of 1×10⁵ cells/ml or seeded into the 24 well plates at a cell density of 5×10⁴ cells/ml and incubated with DMEM/F12 (1:1) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Welgene), and 50 U/ml penicillin and 50 mg/ml streptomycin (Welgene) at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

4.3 Phenotype conversion of M1 and M2 microglia in vitro

To investigate the phenotypical change of microglia, MG d14 were pre-treated with 10 ng/ml GMCSF (R&D systems, Minneapolis, MN, USA) for 5 days, then incubated with the following cytokines for 3 days: 10 ng/ml GMCSF (R&D systems), 100nM Substance-P (SP, Sigma, ST. Louis, MO, USA), 10 ng/ml rat IL-4 (R&D systems), 10 ng/ml rat IL-10 (R&D systems), 10 ng/ml TNF-α (Sigma), 20ng/ml IFN-γ (R&D systems).

4.4 Astrocyte culture and OGD in vitro

CD11b astrocytes were collected from CD11b MACS. Cells were incubated with DMEM/F12 (1:1) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Welgene), and 50 U/ml penicillin and 50 mg/ml streptomycin (Welgene) at 37°C in a humidified atmosphere of 5% CO₂, 95% air. The culture was maintained for 14 days.

To mimic the in vivo stroke environment, CD11b astrocytes isolated by CD11b MACS were seeded into 6 well plates at a cell density of 1×10⁶ cells/ml and incubated with no glucose DMEM (Gibco) at 37°C in a humidified atmosphere of 1% O₂, 5% CO₂ air for 24h. Then, 10ng/ml TNF-α (Sigma) and 10ng/ml IFN-γ (R&D systems) were treated for 4h at 37°C in a humidified atmosphere of 5% CO₂, 95% air mimicking the reperfusion of ischemic stroke.

4.5 Phagocytosis assay of microglia

On day 1, the MG d14 were incubated with 25μg/ml E.coli BioParticle (Thermo, Waltham, MA, USA) for 30 min and fixed with 3.7% formaldehyde. The cells were permeabilized with 0.2% Triton X-100 for 5 min and incubated with TRITC-conjugated phalloidin (Sigma, 1:1000) for 30 min at RT. Then, the cells were mounted with Vectashield mounting medium containing DAPI (Vector, Burlingame, CA, USA) and immunofluorescence images were obtained using the Leica DMI 4000 B fluorescence microscope (Leica Microsystems, Wetzlar, Germany).

4.6 Reconstitution of astrocyte feeder on microglia culture
Astrocytes were cultured as described above. When the cells reached confluence, 10μM Ara-C (Sigma) was added and washed with PBS. Then, the MG d14 isolated from mixed glial cell culture were added on the top of the astrocyte feeder and incubated for 24 h in DMEM/F12 (1:1) supplemented with 10% FBS (Gibco), 2 mM L-glutamine (Welgene), and 50 U/ml penicillin and 50 mg/ml streptomycin (Welgene) at 37°C in a humidified atmosphere of 5% CO₂, 95% air.

4.7 Flow cytometry

To harvest MG d0, the brain of P1-3 inbred lewis rats (Orientbio) was minced and the CD11b⁺ cells isolated using MACS without mixed glial cell culture. The MG d14 were isolated from mixed glial cell culture and trypsinized. Both cells were blocked with 1% bovine serum albumin (BSA, Sigma) and incubated with primary antibody for 30 min at 4°C: CD11b (BD Bioscience, 1:100), CD206 (Abcam, Cambridge, MA, USA, 1:200), CD163 (AbD Serotec, Oxford, UK, 1:100), and CD68 (Millipore Burlington, MA, USA, 1:200). After washing the cells with 0.1% BSA solution, the cells were incubated with secondary antibody for 30 min at 4°C: APC donkey anti-rabbit (Jackson ImmunoResearch, W Baltimore Pike, PA, USA, 1:500) and FITC goat anti-mouse (Abcam, 1:500). The data was acquired with BD FACSDiva 7.0 software.

4.8 Immunofluorescence staining

The microglia and astrocytes were fixed with 3.7% formaldehyde (Sigma) and permeabilized by treating 0.2% Triton X-100 in PBS for 5 min. Then, the samples were blocked by 20% normal goat serum (Vector) for 1 h at RT. For immunofluorescence staining, primary antibodies were treated at 4°C overnight; CD11b (BD Bioscience, 1:100), ionized calcium-binding adapter molecule-1 (Iba-1, Sigma, 2:200), CD163 (AbD Serotec, 1:100), CD206 (Abcam, 1:200), CD68 (Millipore, 1:500), CCR7 (Abcam, 1:500), and GFAP (Abcam, 1:500). The secondary antibodies were treated for 1 h at RT; Alexa 488 goat anti-mouse (Invitrogen, Waltham, MA, USA, 1:1000), Alexa 488 goat anti-rabbit (Jackson ImmunoResearch, 1:1000), Cy3 goat anti-mouse (Jackson ImmunoResearch, 1:1000), and Cy3 goat anti-rabbit (Jackson ImmunoResearch, 1:1000). The cells were mounted with Vectashield mounting medium containing DAPI (Vector) and immunofluorescence images were obtained using the Leica DMI 4000 B fluorescence microscope (Leica Microsystems). The cell counting was done at 200x magnification by using Adobe Photoshop CS6 software.

4.9 BrdU incorporation assay

The ACM was collected from CD11b⁺ astrocyte culture and mixed with fresh MCM at two different ratios (1:1 and 1:5). The diluted conditioned medium was treated to microglia and incubated for 24 h. At 15 h after seeding, 20μM BrdU (Sigma) was added to the culture medium and incubated for 9 h. The microglia were fixed with ice-cold 100% absolute methanol (Merck, Darmstadt, Germany) for 5 min. Then the microglia were incubated with anti-BrdU antibody (Roche, Basel Schweiz, 1:25) overnight at 4°C and with Alexa 488 goat anti-mouse antibody (Invitrogen, 1:1000) for 1 h at RT. The nucleus was stained with propidium iodide (PI) (Sigma, 1:1000) for 30 min at RT. The sample was mounted with Vectashield mounting medium (Vector) and immunofluorescence images were obtained using the Leica DMI 4000 B fluorescence microscope (Leica Microsystems). BrdU positive cells were counted at 100x magnification by using Adobe Photoshop CS6 software.

The MG d14 were incubated with the following cytokines for 5 days: 10 ng/ml GMCSF (R&D systems), 100nM SP (Sigma), 10 ng/ml IL-4 (R&D systems), 10 ng/ml IL-10 (R&D systems), 10 ng/ml TNF-α (Sigma), 20ng/ml IFN-γ (R&D systems), 20μM BrdU was added 24 h before cell fixation. The staining procedure was the same as described above.
4.10 Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. The template cDNAs were synthesized using a Superscript III RT-PCR kit (Invitrogen). Then, the qPCR was performed by using TB Green® Premix Ex Taq™ II (Takara Bio.Inc., Shiga, Japan) with 1 μg of total RNA and the following primer sequences for RT-PCR: rat TGF-β (Forward: CACTCCGTGGCTTCTAGTG, Reverse: CTGGCCAGCCTTAGTTTGA), rat IGF2 (Forward: AGCTTCTGCTCAACGCCATT, Reverse: CTTGTTACCTTTCCAGCCT), rat IL-1β (Forward: TGGCAACTGAGGCGTTC, Reverse: CCAAGTCAAGGGCCTTTGGA), rat CXCL10 (Forward: CTCGCAAGTCTATCCTGGCG, Reverse: CCCTGTGTGCTCAATGGGAC), rat Amigo2 (Forward: TGCTCTAGCTCAACCCAG, Reverse: TGGACAGGAGGTGCTTGG), rat Serping1 (Forward: TGCTGTAGCTGGGATAGA, Reverse: TCGTAAGCAGGCGTCAACA), rat CD109 (Forward: GAGCTCAGTGGAGATCGGAC), rat Emp1 (Forward: TCTTACGATTCAATTGCACTCTCAAGCATGAA, Reverse: TAGCTCAGAGCGGTGTCACA), rat β2 (Forward: AAGCCTTCAGGGGACACTGTG, Reverse: GGCTATGACTTCTGCGTGG).
4.14 ELISA

The astrocytes were incubated with followed cytokines: 100nM SP, 10ng/ml IL-4, 10ng/ml IL-10, 10ng/ml TNF-α, or 20ng/ml IFN-γ, and the conditioned medium was collected 24h after cytokine treatment. CINC-1, CINC-2 and VEGF levels were measured using rat CINC-1, CINC-2 and VEGF ELISA kits (R&D systems). Data from three independent experiments were statistically analyzed.

4.15 Statistics

All data are presented as the mean ± SEM values. Statistical analysis of all data was carried out by GraphPad Prism software. The unpaired, two-tailed Student’s t-test was used to determine statistically significant differences (*P-value < 0.05).

Supplementary Materials:

Author Contributions: YS; conceived idea, supervised the study, designed the experiments, interpreted the data, and wrote the manuscript, SK; designed the experiments, conducted the experiments, interpreted the data, and wrote the manuscript.

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