Bipolar/rod-shaped microglia are proliferating microglia with distinct M1/M2 phenotypes

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Microglia are considered the resident immune cells in the central nervous system (CNS) that regulate the primary events of neuroinflammatory responses. Microglia also play key roles in repair and neurodegeneration of the CNS after injury. Recent studies showed that trains of bipolar/rod-shaped microglia align end-to-end along the CNS injury site during the initial recovery phase. However, the cellular characteristics of bipolar/rod-shaped microglia remain largely unknown. Here, we established a highly reproducible in vitro culture model system to enrich and characterize bipolar/rod-shaped microglia by simply generating multiple scratches on a poly-d-lysine/laminin-coated culture dish. Trains of bipolar/rod-shaped microglia formed and aligned along the scratches in a manner that morphologically resembled microglial trains observed in injured brain. These bipolar/rod-shaped microglia were highly proliferative and expressed various M1/M2 markers. Further analysis revealed that these bipolar/rod-shaped microglia quickly transformed into amoeboid microglia within 30 minutes of lipopolysaccharide treatment, leading to the upregulation of pro-inflammatory cytokine gene expression and the activation of Jak/Stat. In summary, our culture system provides a model to further characterize this highly dynamic cell type. We suggest that bipolar/rod-shaped microglia are crucial for repairing the damaged CNS and that the molecular mechanisms underlying their morphological changes may serve as therapeutic biomarkers.

Microglia are mononuclear phagocytes located in the CNS, and their functions include antigen presentation, cytokine and chemokine production, neurogenesis-promoting neurotrophic factor secretion1,2 and phagocytosis to remove dead cells and pathogens as a component of innate immune responses3. Microglial activation is associated with many neurological conditions, such as inflammatory brain diseases and brain injuries4,5. The activation states of microglia can be loosely categorized into “classical activation” (M1) and “alternative activation” (M2) as commonly described for macrophages6. The microglial M1/M2 classification paradigm, which was originally adopted for macrophages, remains controversial in part due to the partially overlapping expression of macrophage M1 and M2 markers in microglia under physiological or pathological conditions7–10 (see Discussion for more details). In general, an M1 phenotype represents pro-inflammatory activity, including the production of pro-inflammatory cytokines, such as tumor necrosis factor (Tnf) and interleukin-1b (Il-1b). M2 microglia exhibit an anti-inflammatory phenotype by expressing Il-10 and transforming growth factor-β (Tgf-β), which facilitate wound healing8,11,12.

Microglia are morphologically dynamic cells whose morphological changes are closely associated with their functional activities13–15. Most previous studies focused on ramified and amoeboid microglia largely based on the finding that they are the most common forms of microglia found in the brain at different developmental stages5,15. Ramified microglia act as surveying cells by actively sensing the surrounding environment via dynamic processes13,16,17. Amoeboid microglia are highly motile and participate in phagocytosis14,15. The availability of a well-defined and reproducible culture system to examine ramified and amoeboid microglia would greatly enhance the understanding of these forms of microglia. The ramified or amoeboid morphology can be maintained by culturing microglia on fibronectin-coated and laminin-coated surfaces, respectively18. However, bipolar/rod-shaped microglia remain very poorly understood and characterized, primarily due to the lack of a well-defined culture model system and the difficulties in imaging these highly dynamic live microglia in deep brain tissues19,20. Recent studies have shown that bipolar/rod-shaped microglia transiently form trains of cells aligned end-to-end at the damaged site after brain injury19–21. Bipolar/rod-shaped microglia are also found in the cerebral cortex of...
patients with neural disorders such as Alzheimer’s disease, subacute sclerosing panencephalitis, lead encephalopathy and viral encephalitis\(^{22-24}\). Despite progress in characterizing the functional role of bipolar/rod-shaped microglia, their fundamental molecular and cellular properties remain largely unknown.

In this study, we established a simple and highly reproducible cell culture system to examine bipolar/rod-shaped microglia. We found that trains of bipolar/rod-shaped microglia aligned end-to-end along the scratched area of a poly-d-lysine (PDL)- and laminin-coated surface in close resemblance to the microglial alignment observed after brain injury in vivo\(^{19-21}\). Our time-lapse live cell imaging study showed that the scratched areas attracted colonies of microglia. These highly proliferative (proliferating cell nuclear antigen [PCNA]-positive) bipolar/rod-shaped microglia displayed differential expression of M1 and M2 markers, suggesting that these microglia exhibited distinct M1/M2 phenotypes. Following treatment with the classic M1 stimulus lipopolysaccharide (LPS), bipolar/rod-shaped microglia were readily transformed into the amoeboid form, including the upregulation of Tnf and Il-1b expression in approximately 30 minutes. Therefore, we propose that our culture system serves as a valuable tool for the further characterization and functional analysis of bipolar/rod-shaped microglia.

**Results**

Bipolar/rod-shaped microglia align in parallel with the direction of the scratch. Mouse primary microglia were seeded on PDL/laminin-coated culture chambers with or without scratches on the surface. Interestingly, we found that most of the microglia in the scratched area extended bipolar processes in random directions rather than the proliferation of colonized microglia in the scratched area, there was no significant difference in cell density between 1 DIV (26.4 ± 3.40 cells/mm\(^2\)) and 6 DIV (36.9 ± 6.12 cells/mm\(^2\)) in the non-scratched area (Fig. 2b). This finding suggested that bipolar/rod-shaped microglia exhibit a higher proliferative capacity than amoeboid microglia. Therefore, we examined the expression of the proliferating cell marker PCNA in the microglial cell cultures. The majority of prominent PCNA-positive immunoreactivity was localized to the nuclei of bipolar/rod-shaped microglia in the scratched area (Fig. 2c), suggesting that these bipolar/rod-shaped microglia were highly proliferative.

The scratched area promotes microglial colonization. A higher cell density was observed at 1 DIV in the scratched area (68.4 ± 11.79 cells/mm\(^2\)) than in the non-scratched area (26.4 ± 3.40 cells/mm\(^2\)). It is very likely that the microglia migrated toward scratches as opposed to the proliferation of colonized microglia in the scratched area, as it took five days for the number of microglia to double (Fig. 2b). To test this hypothesis, we performed time-lapse imaging of live microglia, which illustrated that the microglia migrated toward scratches during the initial 14 hours (Fig. 3 and Supplementary Video S1 and S2). The observed clustering of microglia in the scratched area beginning at 1 DIV occurred due to the active migration of microglia from the non-scratched area (Fig. 3) rather than the proliferation of colonized microglia in the scratched area. These results demonstrated that the scratches damaged the extracellular matrix (laminin) on the coating surface, which attracted the colonization of microglia, further suggesting that our in vitro system mimicked the in vivo brain injury conditions.

Differential expression of M1 and M2 markers in bipolar/rod-enriched microglia. To determine the activation state of the bipolar/rod-shaped microglia, we performed qPCR to assess the mRNA levels of genes associated with M1 or M2 activation. We compared the gene expression of microglia in non-scratched (amoeboid-enriched) and scratched (bipolar/rod-enriched) PDL/laminin-coated culture dishes (Fig. 4a). Amoeboid microglia are known to produce pro-inflammatory cytokines and, thus, are assumed to be M1-activated\(^{28-31}\). At 2 DIV, we found that four

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**Figure 1** | Bipolar/rod-shaped microglia aligned along the scratch of the coated surface in vitro. (a) Bipolar/rod-shaped microglia colonized in the scratched area of a PDL/laminin-coated surface. The cell processes were randomly aligned at 1 DIV and then displayed a more synchronized alignment along the scratch at 6 DIV. (b) Both bipolar/rod-shaped (arrows) and amoeboid microglia expressed the microglial markers Iba1 (red) and CD11b (green). The dotted line indicates the boundary between the scratched (left to the line) and non-scratched areas. Scale bars: A: 100 \(\mu\)m; B: 50 \(\mu\)m.
out of the five analyzed M1 markers were significantly downregulated, including Tnf (0.73-fold), Il-1b (0.59-fold), Cd32 (0.66-fold) and Cd86 (0.66-fold), in bipolar/rod-enriched cultures compared with amoeboid microglia (Fig. 4b). Interestingly, two out of the four examined M2 markers, Il-10 (0.57-fold) and Tgf-β (0.80-fold), were also significantly down-regulated (Fig. 4b). At 6 DIV, three out of the four M1 examined markers remained down-regulated, with the exception of Tnf (Fig. 4c). In contrast, the expression of all M2 markers was restored to the levels in amoeboid microglia (Fig. 4c). Taken together, these data indicated that bipolar/rod-shaped microglia exhibit reduced expression of M1 and M2 markers during early stages of train formation, and M2 marker expression increased after stable microglia alignment occurred.

**M1 activation of bipolar/rod-shaped microglia by LPS.** Based on our qPCR analysis, we found that bipolar/rod-shaped microglia exhibited reduced expression of M1 markers, suggesting that these cells may be quiescent with respect to pro-inflammatory cytokine production. Therefore, we examined whether 1) bipolar/rod-shaped microglia are susceptible to M1 activation by LPS, which is a widely used M1 stimulus that is known to trigger pro-inflammatory cytokine production11,32–37, and 2) bipolar/rod-shaped microglia must change morphologically before they can be activated, as...
bipolar/rod-shaped microglia have been suggested to represent a transitional stage between the activated and deactivated states.

The bipolar/rod-shaped microglia converted into the amoeboid form within 30 minutes in the presence of LPS, and their amoeboid morphology was maintained for at least 5 hours (Fig. 5a). In contrast, no morphological change was observed in the microglial cultures treated with the vehicle control PBS (Fig. 5a). Next, we performed qPCR to analyze the expression of the pro-inflammatory cytokines \( \text{Tnf} \) and \( \text{Il-1b} \) at 30 minutes and 6 hours post-LPS treatment. Remarkably, \( \text{Il-1b} \) expression was increased by 7-fold at 30 minutes and by greater than 1,100-fold at 6 hours compared with the corresponding controls; alternatively, \( \text{Tnf} \) was up-regulated by 56-fold and greater than 300-fold, respectively (Fig. 5b). We also examined the expression of \( \text{Jak1} \) and \( \text{Stat3} \) because the \( \text{Jak1}/\text{Stat3} \) pathway is a well-known modulator of pro-inflammatory cytokine expression in microglia. The expression of both \( \text{Jak1} \) and \( \text{Stat3} \) was up-regulated by approximately 1.6-fold at 30 minutes. After 6 hours, \( \text{Jak1} \) and \( \text{Stat3} \) expression was significantly increased by 1.8-fold and 3.6-fold, respectively (Fig. 5c). Moreover, we examined the response of amoeboid microglia to LPS as a positive control. The amoeboid microglia maintained their morphology after LPS stimulation or PBS treatment (Supplementary Figure S1a). Consistent with other studies, qPCR analysis indicated that both \( \text{Tnf} \) and \( \text{Il-1b} \) were significantly up-regulated after 30 minutes and 6 hours of LPS stimulation compared with PBS treatment (Supplementary Figure S1b). Taken together, our results showed that bipolar/rod-shaped microglia rapidly responded to LPS stimulation by transforming into the amoeboid form and by inducing the gene expression of key pro-inflammatory cytokines via the up-regulation of \( \text{Jak1} \) and \( \text{Stat3} \).

### Discussion

In the current study, we developed a simple, cost-effective, and highly reproducible culture method for the enrichment of bipolar/rod-shaped microglia by simply generating multiple scratches on a PDL/laminin-coated surface, resulting in the formation of trains of bipolar/rod-shaped microglia exclusively in the scratched area. Using this culture method, we have characterized these bipolar/rod-shaped microglia exclusively in the scratched area. This method has been utilized to propagate bipolar/rod-shaped microglia in vitro, allowing for the study of their morphological and functional properties.

Using this culture method, we have characterized these bipolar/rod-shaped microglia exclusively in the scratched area. This method has been utilized to propagate bipolar/rod-shaped microglia in vitro, allowing for the study of their morphological and functional properties. Bipolar/rod-shaped microglia were first documented nearly a century ago. However, little information is available regarding this microglia type. Several studies provided further insights into bipolar/rod-shaped microglia, especially their association with neuropathologies and brain injuries. Transient accumulation of bipolar/rod-shaped microglia trains has been observed at the site of injury during the early phase of brain damage, which may be crucial for minimizing further damage and facilitating repair, as reducing the number of proliferating microglia results in more severe damage to the cortex after ischemic insult. More importantly, the bipolar/rod-shaped microglia trains primarily colocalized with neurons and axons but not with other glia after brain injury. Our study is consistent with other in vivo studies showing prominent proliferation of bipolar/rod-shaped microglia, suggesting that the formation of highly proliferative bipolar/rod-shaped microglia trains may represent a critical step for increasing the number of microglia at the site of injury. Subsequently, bipolar/rod-shaped microglia rapidly transform into an amoeboid morphology and exhibit phagocytic activity, further enhancing the phagocytosis of dead or dying neurons to minimize brain damage.

Persistent production of pro-inflammatory cytokines by microglia results in chronic neuroinflammation and is associated with the development of neurodegenerative diseases such as Parkinson’s disease and Alzheimer’s disease. Our data showed that bipolar/rod-shaped microglia expressed lower levels of the pro-inflammatory cytokines \( \text{Tnf} \) and \( \text{Il-1b} \), implying that bipolar/rod-shaped microglia do not contribute to inflammation-induced neurodegeneration. However, the expression of the anti-inflammatory cytokines \( \text{Il-10} \) and \( \text{Tgf-\beta} \) in bipolar/rod-shaped microglia increased from 2 DIV to 6 DIV. Il-10 is known to inhibit pro-inflammatory cytokine production by microglia. Taken together, bipolar/rod-shaped microglia might exert neuroprotective effects by producing greater amounts of anti-inflammatory cytokines than pro-inflammatory cytokines at the site of brain injury. The balance between pro-inflammatory and anti-inflammatory cytokine expression determines the extent of recovery from brain injury and neurodegenerative disease progression.

Microglia and macrophages exhibit distinct expression profiles of M1 and M2 markers, possibly due to their different origins. Accumulating evidence suggests that the concept of microglial classification into M1 and M2, which was originally adopted for macrophages, is becoming increasingly controversial. Studies have shown that some M1 or M2 markers are expressed only in macrophages and not in microglia. For instance, human M2-activated microglia did not express M2 macrophage markers, such as Arg1, ChI3L, CD23, CD163, and CD206. During postnatal brain development, microglia express both M1 and M2 markers, suggesting that immature microglia do not commit to either the M1 or M2 phenotype. CCL2, a chemo-attractant protein that triggers CNS inflammation, induces the expression of both pro-inflammatory and anti-inflammatory responses in microglia, thereby exhibiting neither an M1- nor an M2-skewed phenotype. This finding agrees well with our data showing that naive bipolar/rod-shaped microglia did not exclusively express M1 or M2 markers but could be readily transformed into an M1 phenotype shortly after activation using...
LPS. Surprisingly, the detection of both LPS-induced morphological changes and the up-regulation of pro-inflammatory cytokine/Jak1/Stat3 gene expression occurred within approximately 30 minutes. Therefore, we suggest that bipolar/rod-shaped microglia do not participate in pro-inflammatory cytokine production but can rapidly transform into an active form and produce high levels of pro-inflammatory cytokines upon LPS stimulation.

As summarized in Fig. 6, we demonstrated that scratching PDL/laminin-coated culture dishes enriched the formation of bipolar/rod-shaped microglia, which were highly proliferative and committed to neither an M1 nor an M2 phenotype. Upon LPS treatment, bipolar/rod-shaped microglia rapidly transformed into an amoeboid form and became M1-activated. We propose that our in vitro culture system can be used for gene expression studies and further functional characterization of M1/M2 markers in bipolar/rod-shaped microglia, which may exhibit therapeutic potential for neurological diseases.

Methods

Animals. The present study was performed in strict accordance with the American Veterinary Medical Association (AVMA) Guidelines on Euthanasia by exposing animals to carbon dioxide. All experimental protocols were approved by the Animal Research Ethics Committee at City University of Hong Kong (Ref. A-0017). We made the best efforts possible to reduce the number of animals used.

Primary microglia cultures. Primary microglia were isolated (≥99% purity) as previously described. Briefly, cerebral cortices dissected from postnatal day 1 to 3 C57BL/6 mice were trypsinized, mechanically dissociated and seeded on PDL (10 μg/ml)-coated T-75 flasks. The cultures were maintained in DMEM/10% FBS supplemented with macrophage colony stimulating factor (MCSF, 5 ng/ml, Peprotech #315-02). Then, the suspended microglia were retrieved from the culture medium for subsequent experiments.

Amoeboid-enriched and bipolar/rod-enriched microglia cultures. The 35 mm culture dishes or 8-well chamber slides were coated with PDL (10 μg/ml) followed by laminin (10 μg/ml). The laminin coating maintained the microglia in the amoeboid

Figure 5 | Bipolar/rod-shaped microglia rapidly responded to M1 activation using LPS. (a) Bipolar/rod-shaped microglia converted to an amoeboid morphology in response to LPS stimulation, but not PBS treatment, in 30 minutes. (b) qPCR showed significant up-regulation of Il-1b and Tnf after 30 minutes and 6 hours of LPS stimulation compared with PBS treatment (n=4). (c) The qPCR results showed up-regulation of Jak1 and Stat3 shortly after 30 minutes that increased significantly after 6 hours of LPS treatment compared with PBS treatment (n=4). *P < 0.05 based on Student’s t-test.

Figure 6 | Schematic diagram illustrating the characterization of bipolar/rod-shaped microglia. Bipolar/rod-shaped microglia are highly proliferative and expressed low levels of M1 and M2 markers. Upon LPS treatment, bipolar/rod-shaped microglia rapidly transformed into an amoeboid morphology and became M1-activated.
form⁴⁻⁷; thereafter, we refer to these cultures as "amoeboid-enriched" cultures. For microglia were seeded on the scratched culture dishes or chamber slides and were normalized. The primer sequences that were used are listed in Table 1.

Confocal Image (Leica) as described previously⁵. Images were captured at 5-minute intervals for more than 14 hours.

Time-lapse live cell imaging. Microglia were seeded on a scratched PDL/laminin-coated 35 mm culture dish. Time-lapse imaging was performed using an SP5 Strain Synthesis SuperMix (Invitrogen). Triplicate qPCR reactions for each sample were performed using the KAPA SYBR Fast qPCR Kit (KAPA) in an ABI 7500 FAST Real-Time PCR system. Ct-values were recorded to calculate the relative fold-change in expression according to the formula 2⁻DDCT.

Cell counting. Cell counting was performed using the ImageJ Cell Counter Plugin. Cells were counted in both the scratch Edem and non-scratched regions and then expressed relative to the area examined (i.e., cells/mm²).

Time-lapse live cell imaging. Microglia were seeded on a scratched PDL/laminin-coated 35 mm culture dish. Time-lapse imaging was performed using an SP5 confocal microscope (Leica) as described previously⁵. Images were captured at 5-minute intervals for more than 14 hours.

LPS treatment. LPS (1 mg/ml in PBS, Sigma L4516) was added to the culture medium at a final concentration of 10 μg/ml and then applied to the microglia cultures for 30 minutes or 6 hours. Then, total RNA was extracted from the microglia for qPCR analysis.

Immunostaining. Microglia were fixed using 4% paraformaldehyde at room temperature for 15 minutes. After blocking, the cells were incubated in primary antibodies at 4°C overnight. The primary antibodies used in this study were against Iba1 (Wako, #019-19741), CD11b (Serotec, MCA74G) and PCNA (Cell Signaling, #2586). Then, the cells were incubated in the corresponding secondary antibodies conjugated to Alexa Fluor® (Invitrogen) at room temperature for 1 hour. The nuclei were counter-stained with DAPI.

RNA extraction, reverse transcription and qPCR. RNA was extracted using Trizol reagent (Invitrogen). Reverse transcription was performed using Superscript III First Strand Synthesis SuperMix (Invitrogen). Triplicate qPCR reactions for each sample were performed using the KAPA SYBR Fast qPCR Kit (KAPA) in an ABI 7500 FAST Real-Time PCR system. Ct-values were recorded to calculate the relative fold-change in expression according to the formula 2⁻DDCT. Gapdh was used as the standard for normalization. The primer sequences that were used are listed in Table 1.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 5.0 software. All data are presented as the means ± SEM. Student’s t-test or one-way ANOVA was used for comparisons; P < 0.05 was considered to be significant.

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**Acknowledgments**

This work was supported in part by The Health and Medical Research Fund (HMRF), Food and Health Bureau, Hong Kong Special Administrative Region Government (Ref. No: 01112016, 01122026) and ECS/GRF grants from the Research Grant Council of the Hong Kong Special Administrative Region Government (CityU 161212 and CityU 160813).

**Author contributions**

W.Y.T. performed the experiments and data analysis. W.Y.T. and C.H.E.M. designed the experiments and wrote the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports.

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Tam, W. Y. & Ma, C. H. E. Bipolar/rod-shaped microglia are proliferating microglia with distinct M1/M2 phenotypes. *Sci. Rep.* **4**, 7279; DOI:10.1038/srep07279 (2014).