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

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Microglia are generally 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 secretion<sup>1,2</sup> and phagocytosis to remove dead cells and pathogens as a component of innate immune responses<sup>3</sup>. Microglial activation is associated with many neurological conditions, such as inflammatory brain diseases and brain injuries<sup>4,5</sup>. The activation states of microglia can be loosely categorized into “classical activation” (M1) and “alternative activation” (M2) as commonly described for macrophages<sup>6</sup>. 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 conditions<sup>7–10</sup> (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- $\beta$  (Tgf- $\beta$ ), which facilitate wound healing<sup>8,11,12</sup>.

Microglia are morphologically dynamic cells whose morphological changes are closely associated with their functional activities<sup>13–15</sup>. 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 stages<sup>5,15</sup>. Ramified microglia act as surveying cells by actively sensing the surrounding environment via dynamic processes<sup>3,13,16,17</sup>. Amoeboid microglia are highly motile and participate in phagocytosis<sup>14,15</sup>. 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, respectively<sup>18</sup>. 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 tissues<sup>19,20</sup>. Recent studies have shown that bipolar/rod-shaped microglia transiently form trains of cells aligned end-to-end at the damaged site after brain injury<sup>19–21</sup>. 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<sup>22–24</sup>. 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*<sup>19–21</sup>. 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

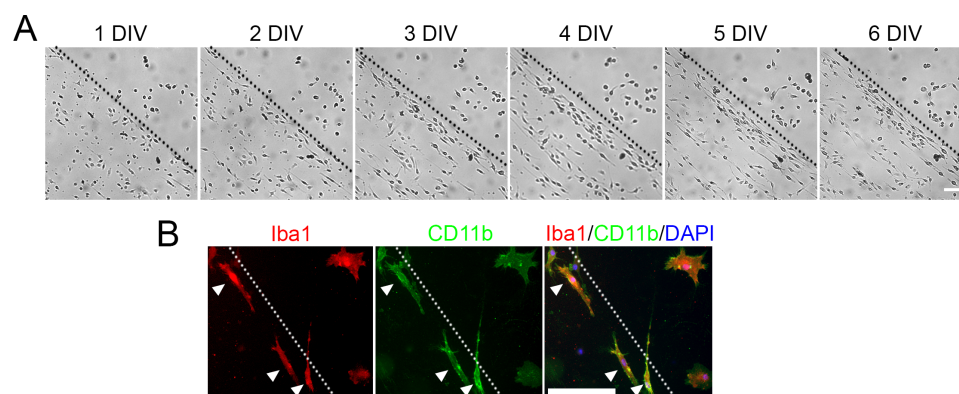
**Bipolar/rod-shaped microglia align in parallel with the direction of the scratch.** Mouse primary microglia were seeded on PDL/laminin-coated culture chambers with or without scratches on the surface. Interestingly, we found that most of the microglia in the scratched area extended bipolar processes in random directions beginning at 1 day *in vitro* (DIV) (Fig. 1a). Then, the microglia became densely packed, and their processes aligned in parallel with the direction of the scratch until a stable alignment was attained at 6 DIV. In the non-scratched area, most, if not all, of the microglia adopted an amoeboid morphology (Fig. 1a), as described previously<sup>18</sup>. To confirm that the bipolar/rod-shaped microglia maintained their microglial identity on the scratched surface, we performed immunostaining for the classical microglial markers Iba1<sup>25</sup> and CD11b<sup>26,27</sup>. The bipolar/rod-shaped and amoeboid microglia expressed similar levels of Iba1 and CD11b (Fig. 1b). Our data indicated that scratching the PDL/laminin-coated surface promoted the formation of bipolar/rod-shaped microglia without affecting their specificity, whereas the non-scratched surface supported the growth of amoeboid microglia.

**Bipolar/rod-shaped microglia are highly proliferative.** Recent studies showed that trains of bipolar/rod-shaped microglia formed

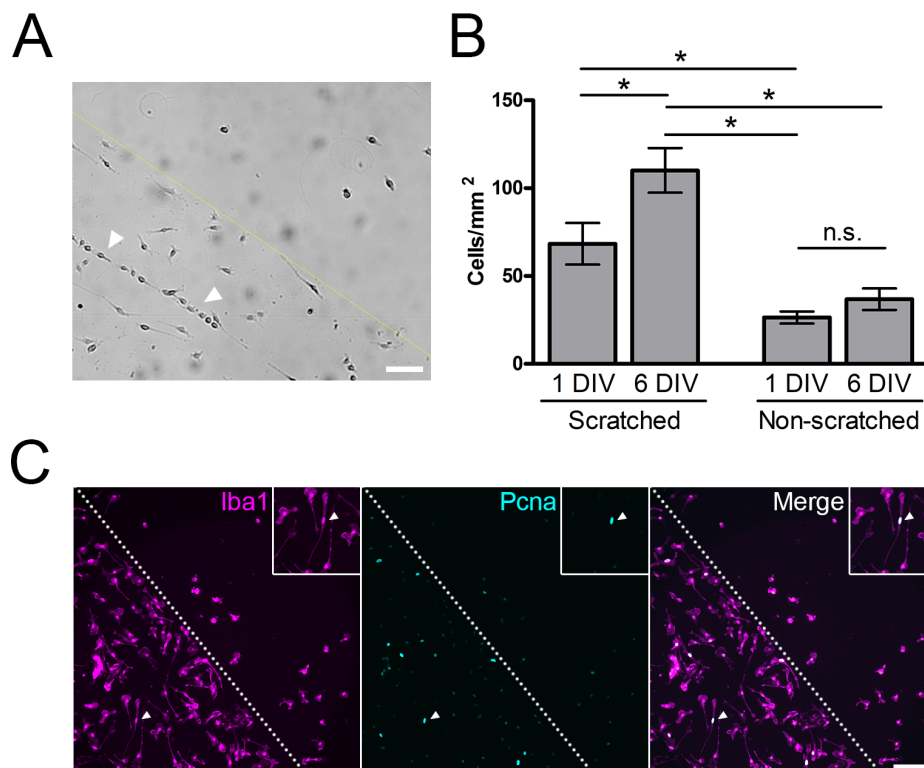
within 24 hours and peaked at 7 days at the site of injury after brain damage<sup>19,20</sup>. Here, in our *in vitro* system, we also observed trains of microglial colonies along the scratches within 24 hours after cell seeding (Fig. 2a). The aligned bipolar/rod-shaped microglia became densely packed in the scratched area compared to the non-scratched area at 6 DIV (Fig. 1a). In the scratched area, the cell density nearly doubled from  $68.4 \pm 11.79$  cells/mm<sup>2</sup> at 1 DIV to  $110.1 \pm 12.70$  cells/mm<sup>2</sup> at 6 DIV ( $p < 0.05$ ) (Fig. 2b). In contrast to the scratched area, there was no significant difference in cell density between 1 DIV ( $26.4 \pm 3.40$  cells/mm<sup>2</sup>) and 6 DIV ( $36.9 \pm 6.12$  cells/mm<sup>2</sup>) 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 ( $68.4 \pm 11.79$  cells/mm<sup>2</sup>) than in the non-scratched area ( $26.4 \pm 3.40$  cells/mm<sup>2</sup>). 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<sup>8,28–31</sup>. At 2 DIV, we found that four



**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.

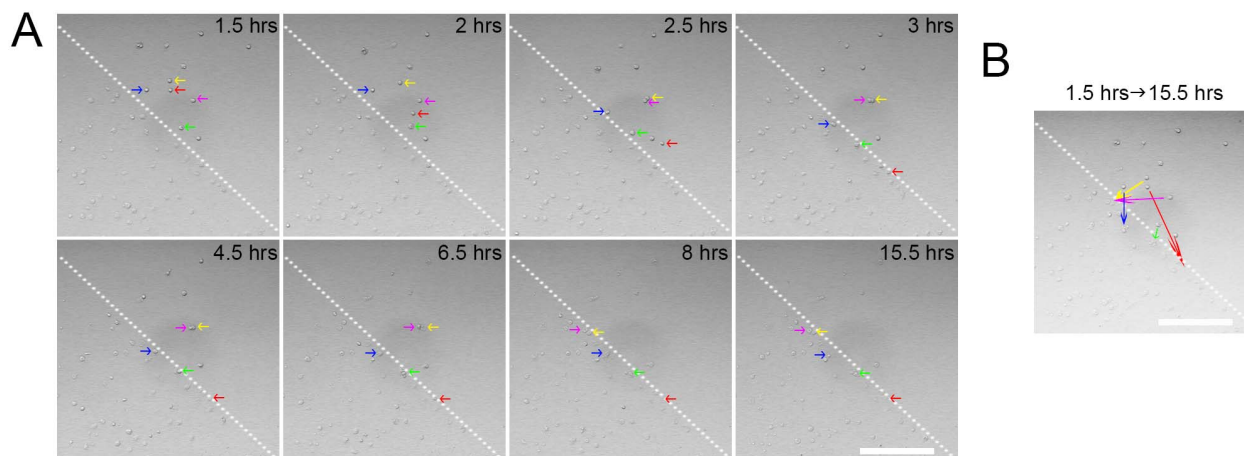


**Figure 2 | Bipolar/rod-shaped microglia were highly proliferative.** (a) Trains of microglia formed along the scratch (left of the dotted line). (b) The density of the microglia significantly increased in the scratched area from 1 DIV to 6 DIV ( $*P < 0.05$  by ANOVA) but not in the non-scratched area ( $n = 3$ ). (c) Strong signal of the proliferation marker PCNA was localized to the nucleus of the microglia primarily in the scratched area (left of the dotted line). Most of the PCNA<sup>+</sup> cells were bipolar/rod-shaped (inset). Scale bar: 100  $\mu\text{m}$ .

out of the five analyzed M1 markers were significantly down-regulated, 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- $\beta$*  (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

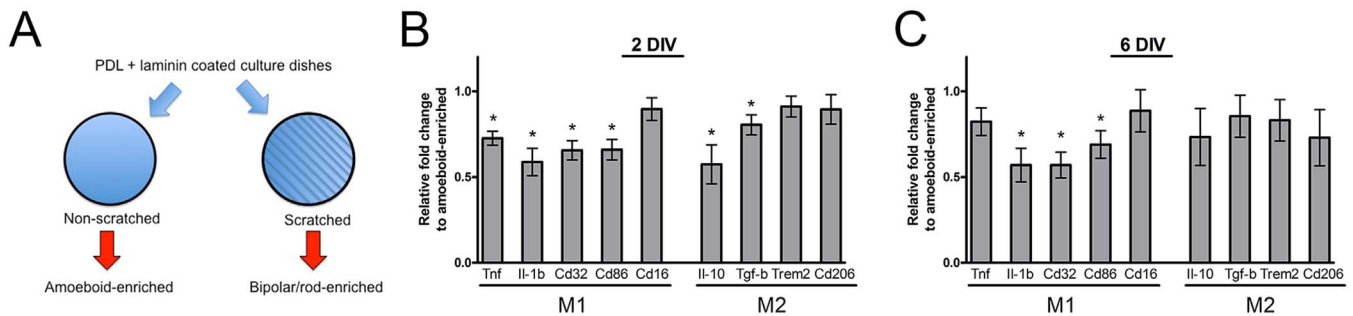
marker expression increased after stable microglia alignment occurred.

**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 production<sup>11,32–37</sup>, and 2) bipolar/rod-shaped microglia must change morphologically before they can be activated, as



**Figure 3 | Scratched area attracted colonies of microglia.** (a) Time-lapse live cell imaging showed the migration of microglia (depicted by arrows with different colors) from the non-scratched area to the scratched area. (b) Simplified migratory paths of the microglia from 1.5 hours to 15.5 hours after cell seeding. Scale bars: 250  $\mu\text{m}$ .





**Figure 4 | Reduced expression of M1 and M2 markers in bipolar/rod-shaped microglia.** (a) Schematic diagram illustrating the amoeboid-enriched and bipolar/rod-shaped enriched cultures. (b) Reduced expression of selected M1 and M2 markers in the bipolar/rod-enriched microglia cultures compared with the amoeboid-enriched cultures at 2 DIV ( $n = 5$  to 6). (c) This reduced M1 marker expression was generally maintained, whereas M2 marker expression increased at 6 DIV ( $n=5$  to 6). \* $P < 0.05$  based on Student's  $t$ -test.

bipolar/rod-shaped microglia have been suggested to represent a transitional stage between the activated and deactivated states<sup>38</sup>.

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 *Tnf* and *Il-1b* at 30 minutes and 6 hours post-LPS treatment. Remarkably, *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, *Tnf* was up-regulated by 56-fold and greater than 300-fold, respectively (Fig. 5b). We also examined the expression of *Jak1* and *Stat3* because the Jak1/Stat3 pathway is a well-known modulator of pro-inflammatory cytokine expression in microglia<sup>39–42</sup>. The expression of both *Jak1* and *Stat3* was up-regulated by approximately 1.6-fold at 30 minutes. After 6 hours, *Jak1* and *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 *Tnf* and *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 *Jak1* and *Stat3*.

## Discussion

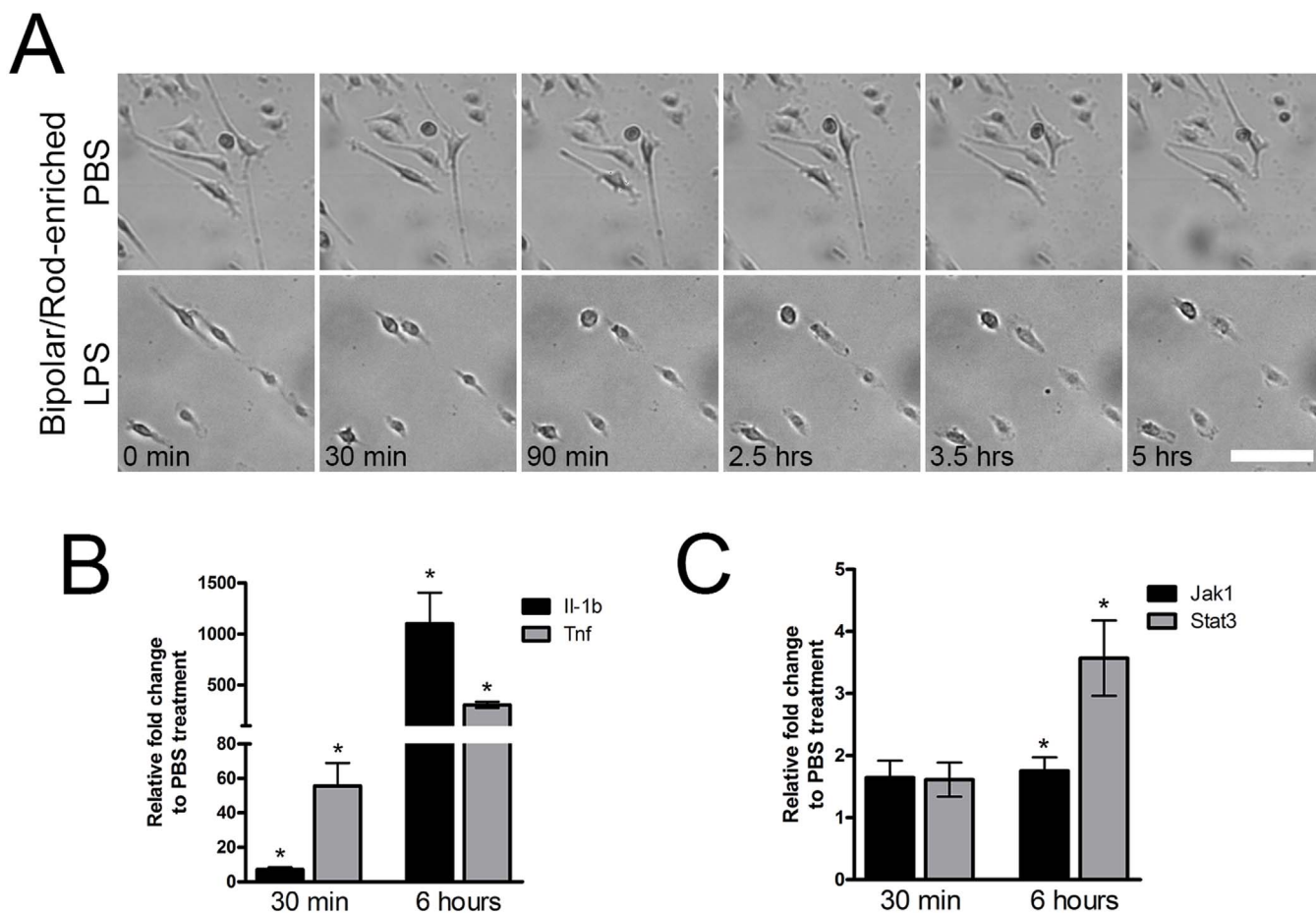
In the current study, we developed a simple, cost-effective, and highly reproducible culture method for the enrichment of bipolar/rod-shaped microglia by simply generating multiple scratches on a PDL/laminin-coated surface, resulting in the formation of trains of bipolar/rod-shaped microglia exclusively in the scratched area. Using this culture method, we have characterized these bipolar/rod-shaped microglia and revealed that they (1) are highly proliferative, (2) express reduced mRNA levels of pro-inflammatory M1 and anti-inflammatory M2 markers compared with amoeboid microglia, and (3) transform into the amoeboid form, including increased production of pro-inflammatory cytokines *Tnf* and *Il-1b*, via the up-regulation of *Jak1* and *Stat3* expression.

Bipolar/rod-shaped microglia were first documented nearly a century ago<sup>19,43</sup>. 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<sup>19,20,22,24</sup>. Transient accumulation of bipolar/rod-shaped microglia trains has been observed at the site of injury

during the early phase of brain damage<sup>19–21</sup>, 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<sup>44,45</sup>. More importantly, the bipolar/rod-shaped microglia trains primarily colocalized with neurons and axons but not with other glia after brain injury<sup>19,20</sup>. Our study is consistent with other *in vivo* studies showing prominent proliferation of bipolar/rod-shaped microglia<sup>14,24,46</sup>, 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<sup>47–49</sup>. Our data showed that bipolar/rod-shaped microglia expressed lower levels of the pro-inflammatory cytokines *Tnf* and *Il-1b*, implying that bipolar/rod-shaped microglia do not contribute to inflammation-induced neurodegeneration. However, the expression of the anti-inflammatory cytokines *Il-10* and *Tgf-β* in bipolar/rod-shaped microglia increased from 2 DIV to 6 DIV. *Il-10* is known to inhibit pro-inflammatory cytokine production by microglia<sup>50</sup>. 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<sup>51,52</sup>.

Microglia and macrophages exhibit distinct expression profiles of M1 and M2 markers, possibly due to their different origins<sup>12</sup>. Accumulating evidence suggests that the concept of microglial classification into M1 and M2, which was originally adopted for macrophages, is becoming increasingly controversial<sup>6,7,12</sup>. 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*, *Chi3l3*, *CD23*, *CD163* and *CD206*<sup>7,9</sup>. 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<sup>8</sup>. *CCL2*, a chemoattractant 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<sup>10</sup>. 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



**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.

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.

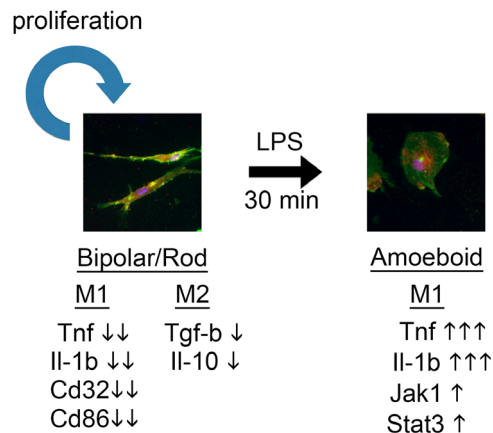
## Methods

**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 ( $\geq 99\%$  purity) as previously described<sup>27</sup>. Briefly, cerebral cortices dissected from postnatal day 1 to 3 C57BL/6 mice were trypsinized, mechanically dissociated and seeded on PDL ( $10 \mu\text{g/ml}$ )-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 ( $10 \mu\text{g/ml}$ ) followed by laminin ( $10 \mu\text{g/ml}$ ). The laminin coating maintained the microglia in the amoeboid



**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.



Table 1 | qPCR primers used in this study

Gene	Primer sequence (5' to 3')
Gapdh	Forward: CATGGCCTCCGTGTTCTCA Reverse: CCTGCTTACCACCTTCTTGAT
<b>M1 markers</b>	
Tnf	Forward: ACCACGCTCTTCTGTCTACT Reverse: GTTGTGAGTGTGAGGGTCTG
Il-1b	Forward: GAGGACATGAGCACCTTCTTT Reverse: GCCTGTAGTGCAGTTGTCTAA
CD32	Forward: AATCCTGCCGTTCTACTGATC Reverse: GTGTCACCGTGTCTTCCITGAG
CD86	Forward: GACCGTGTGTGTGTTCTGG Reverse: GATGAGCAGCATCACAAGGA
CD16	Forward: TTTGGACACCCAGATGTTTCAG Reverse: GTCTTCTTGAGCACCTGGATC
<b>M2 markers</b>	
Il-10	Forward: GCCTTATCGGAAATGATCCA Reverse: TCTCACCCAGGGAATCAAA
Tgf-β	Forward: TGCGCTTGCAGAGATTAATA Reverse: CGTCAAAGACAGCCACTCA
Trem2	Forward: CAGTTTCTCCTGCTGCTGAT Reverse: CAGTGCTTCAAGGCGTCATA
CD206	Forward: CAAGGAAGTGGCATTGT Reverse: CCTTTCAGTCTTTGCAAGC
Jak1	Forward: CTCTCTGTACACCTCTTCGC Reverse: TTGGTAAAGTAGAACCTCATGCG
Stat3	Forward: CACCTTGGATTGAGAGTCAAGAC Reverse: AGGAATCGGCTATATTGCTGGT

form<sup>18</sup>; thereafter, we refer to these cultures as “amoeboid-enriched” cultures. For bipolar/rod-enriched microglia cultures, we scratched the PDL/laminin-coated culture dishes or chamber slides using a P200 pipette tip. Then, purified primary microglia were seeded on the scratched culture dishes or chamber slides and were cultured in DMEM/10% FBS at 37°C in a 5% CO<sub>2</sub> incubator. Scratches generated by different laboratory members produced identical results.

**Cell counting.** Cell counting was performed using the ImageJ Cell Counter Plugin. Cells were counted in both the scratched and non-scratched regions and then expressed relative to the area examined (i.e., cells/mm<sup>2</sup>).

**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 previously<sup>53</sup>. 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<sup>-ΔΔCt</sup>; Gapdh was used as the standard for normalization. The primer sequences that were used are listed in Table 1.

**Statistical analyses.** 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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## 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

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