Stroke induces rapid activation and expansion of microglia, but the main source of microgliosis is controversial. Here we investigated the formation of microgliosis and infiltration of circulating cells in a photothrombosis stroke model by taking advantage of parabiosis and two-photon microscopy. We found that a small population of blood-derived CX3CR1<sup>GFP</sup><sup>/+</sup> cells infiltrated the cerebral parenchyma, but these cells did not proliferate and were phenotypically distinguishable from resident microglia. CX3CR1<sup>GFP</sup><sup>/+</sup> infiltrating cells also displayed different kinetics from reactive microglia. The number of CX3CR1<sup>GFP</sup><sup>/+</sup> infiltrating cells peaked on Day 5 after stroke and then decreased. The decline of these infiltrating cells was associated with an active apoptotic process. In contrast, reactive microglia were recruited to the ischaemic area continuously during the first week after stroke induction. Immunohistology and in vivo two-photon imaging revealed that cells involved in the process of microgliosis were mainly derived from proliferating resident microglia. Expansion of microglia exhibited a consistent pattern and our in vivo data demonstrated for the first time that microglia underwent active division in regions surrounding the ischaemic core. Together, these results indicated that CX3CR1<sup>GFP</sup><sup>/+</sup> infiltrating cells and reactive microglia represented two distinct populations of cells with different functions and therapeutic potentials for the treatment of stroke.

Keywords: stroke; microgliosis; infiltrating cells; microglia; parabiosis

Abbreviations: BBB = blood–brain barrier; BrdU = bromodeoxyuridine; GFP = green fluorescent protein; IBA1 = ionized calcium-binding adaptor molecule 1

Introduction

Microglia account for ~20% of glial cells in the CNS and exist throughout the brain, spinal cord and retina (Sanchez-Lopez et al., 2004; Saijo and Glass, 2011). As the immunocompetent cells in CNS, microglia have important roles in the surveillance, support, protection and restoration of tissue integrity (Davalos et al., 2005; Neumann et al., 2009; Schafer et al., 2012). In the normal brain, microglia exhibit ramified morphology and their processes undergo rapid extension and retraction to monitor the microenvironment (Davalos et al., 2005; Nimmerjahn et al., 2005; Wake et al., 2009). When the rigorously regulated CNS homeostasis is disturbed by pathological insults, microglia alter their morphology and gene expression, and their numbers increase markedly at the site of injury. This process of microglial activation, proliferation and scar formation has been known as ‘microgliosis’ (Calvo and Bennett, 2012).

It has been shown that precursors of microglia are of haematopoietic/myeloid origin and colonize the CNS during embryonic and early postnatal periods (Chan et al., 2007; Ginhoux et al., 2010;
Schulz et al., 2012; Kierdorf et al., 2013). In response to pathogens or damage, microglia are rapidly activated and accumulate in and around the lesion site (Davalos et al., 2005; Graeber, 2010). The precise origin of these accumulated microglia is still in debate. A major question has been whether circulating monocytes or myeloid progenitors can contribute to the resident microglial pool under pathological conditions.

Several studies using bone marrow chimeras with lethally irradiated recipients favor the view that microglia are of hematopoietic origin in the adult CNS (Kennedy and Abkowitz, 1997; Flugel et al., 2001; Priller et al., 2001). These studies show that donor-derived cells replace a considerable percentage of microglia in the acceptors over time (Tanaka et al., 2003; Kokovay and Cunningham, 2005; Priller et al., 2006). These findings suggest that mobilized bone-marrow cells are able to pass through the blood brain barrier (BBB) and differentiate into parenchymal microglia (Brazelton et al., 2000; Priller et al., 2001; Simard and Rivest, 2004; Dukic et al., 2006). However, there are concerns about the physiological significance of the findings obtained from irradiation bone marrow chimerism. Irradiation may cause apoptosis of endothelial cells and impact the integrity of BBB (Li et al., 2004; Ransohoff and Perry, 2009). In addition, transplantation of bone marrow cells may lead to artificial persistence of hematopoietic stem cells in the circulation (Ransohoff, 2007). To avoid the potential influence of the bone marrow-irradiation model itself, an alternative strategy using parabiotic mice was adopted. Without irradiation and transplantation, data from parabiosis models support an opposing view that little or no circulating cells may differentiate into microglia in the parenchyma (Ajami et al., 2007; Saijo and Glass, 2011), raising the possibility that circulating cells may not cross the intact BBB under certain pathological conditions. Accordingly, microglia are considered to expand as a result of proliferation of CNS-resident cells in response to injuries (Solomon et al., 2006; Ajami et al., 2007; Denes et al., 2007). These apparent contradictory views are further complicated by observations that circulating monocytes can enter the CNS but fail to differentiate into microglia (Ajami et al., 2011).

In this work, we took advantage of a photothrombosis stroke model, which is characterized by pathological disruption of BBB permeability without intervention of irradiation or transplantation, to address whether circulating cells could contribute to microgliosis. Parabiotic animals and in vivo two-photon imaging were used to assess the infiltration of circulating cells and the kinetics of microglial activation and proliferation in the living mouse cortex. Our data demonstrate that proliferation of local resident microglia rather than recruitment of circulating myeloid cells is the main source of microgliosis after ischemic stroke. Division of microglia was observed in real time in regions surrounding the ischemic core. We also found a small population of CX3CR1\textsuperscript{GFP/\textsuperscript{+}} circulating cells infiltrated brain parenchyma in parabiotic chimeras. These cells exhibited a phenotype different from activated microglia and had different kinetics during microgliosis. Although these circulating cells infiltrated into the ischemic zone, they did not undergo division or differentiate into microglia. Together, our data suggest that CX3CR1\textsuperscript{GFP/\textsuperscript{+}} infiltrating cells and resident microglia may have distinct functions in the pathological process of ischemic stroke.

### Materials and methods

#### Animals

Transgenic mice expressing green fluorescent protein (GFP) in monocytes, dendritic cells, natural killer cells, and microglia in the brain were purchased from the Jackson Laboratory and bred in the Laboratory Centre for Medical Sciences, Lanzhou University. In this mouse strain, Cx3cr1 gene was replaced by a GFP reporter gene, and all CX3CR1-expressing cells are expected to be GFP-positive (Jung et al., 2000). Animals aged 8–10 weeks and weighing 18–24 g were used throughout the study. All animals were bred in-house and maintained in an aseptic environment supplied with clean water and rodent chow ad libitum. All experimental procedures and protocols in the study were approved by the Ethics Committee of Lanzhou University, China.

#### Parabiosis

A modified parabiotic surgery was conducted following the procedure as previously described (Wright et al., 2001; Rossi et al., 2005). Pairs of weight-matched wild-type C57BL/6 and heterozygous CX3CR1\textsuperscript{GFP/\textsuperscript{+}} mice were subjected to parabiotic surgery. In brief, animals were anaesthetized by intraperitoneal injection of 20 mg/ml ketamine and 2 mg/ml xylazine. The flanks of mice were shaved and sterilized with betadine solution. An incision running from behind the ear to the hip was generated on the opposite sides of two mice to be paired. Opposing posterior muscles were joined with 5-0 chronic gut. The scapulas of each animal were fixed together and the dorsal and ventral skin edges were sutured with 4-0 silk. Mice were kept warm until completely recovered from anaesthesia and fed with normal water and food in a purpose-made container. During the first 7 days after surgery, Tylenol was mixed in food to alleviate the pain as needed. We have optimized the process and the survival rate after surgery was ~75%.

#### Flow cytometry

Chimerism of parabionts was determined by calculating percentages of GFP-positive cells in blood monocytes. Fourteen days or 17 days (3 days post-stroke induction) after surgery, the blood sample from both mice of parabiont were collected into tubes containing anticoagulant (acid-citrate-dextrose solution). After lysis of the red blood cells, the samples were assayed with flow cytometry (BD, LSRFortessa\textsuperscript{TM}) to measure the frequency of GFP-positive monocytes within the entire monocytes in the GFP-negative mice of the parabionts.

#### Rose bengal photothrombosis model

To induce acute ischemic stroke, we applied the rose bengal photothermolysis model as described previously (Watson et al., 1985; Zhang et al., 2005). Briefly, mice were deeply anaesthetized with ketamine-xylazine. A round cranial window (2 mm diameter) was thinned over the right somatosensory cortex at coordinates of −1.5 mm from bregma and −1.5 mm lateral on each mouse. The skull thickness was reduced to 50\textmu m by a high-speed drill. We injected rose bengal (0.03 mg/g mouse; Sigma) into the tail vein, and then exposed the cranial window under green light (535 ± 25 nm) for 2 min to activate the dye in a 1 mm\textsuperscript{2} area to form positional acute ischemia.
**Immunohistochemistry**

The mice were anaesthetized with an overdose of urethane and then transcardially perfused with 0.1 M PBS followed by 4% paraformaldehyde. The brains were dissected out and were post-fixed within 4% paraformaldehyde for 24 h at 4°C. Mouse brains were sectioned into 30 μm coronal sections on a vibrating microtome. Primary antibody staining was performed overnight at 4°C. For primary antibodies, we used rabbit antibodies to ionized calcium-binding adaptor molecule 1 (IBA1; 1:500; Wako Pure Chemical Industries), C-C chemokine receptor type 2 (CCR2; 1:100; Bioss), and activated caspase-3 (1:250; Bioss). Secondary antibodies included rhodamine-conjugated goat anti-rabbit (1:200; ZSGB-BIO) and Alexa Fluor® 350 donkey anti-rabbit (1:200; California, Invitrogen). All sections were then examined under an epifluorescence microscope or a confocal microscope.

To label newly synthesized DNA, animals received intraperitoneal injection of bromodeoxyuridine (BrdU, 50 mg/kg; Sigma) twice daily for a maximum of 7 days. The first injection was done 12 h after stroke induction. Mice were anaesthetized 3, 5 and 7 days after stroke induction and then the brains were harvested and sectioned. For BrdU staining, brain sections were rinsed for 10 min in double distilled water and heated at 37°C for 30 min in 2 M HCl. Sections were then neutralized with 0.1 M borate buffer (pH 8.5) for 3 x 15 min and incubated overnight with a rat monoclonal antibody against BrdU (1:500; AbD Serotec) followed by a rhodamine-conjugated goat anti-rat secondary antibody (1:200; ZSGB-BIO) for 1 h at room temperature. To examine whether engulfment of BrdU-positive dead cells could affect the evaluation of microglial proliferation, BrdU-labelled brain sections were stained with Hoechst 33258 (1:10 000; Sigma) for 10 min.

**Confocal microscopy and in vivo two-photon imaging**

Images of fluorescently stained tissue samples were captured sequentially on an Olympus FluoView FV1000 confocal laser scanning microscope (Olympus). Slices were visualized with UPlan Sapo ×20/0.75 or ×40/0.95 objectives. Stacks of 20 to 40 confocal optical sections (1024 x 1024 pixel arrays) were collected at 1 μm intervals in the z dimension.

The procedure of in vivo two-photon imaging was performed as described previously (Grutzendler et al., 2002; Zhang et al., 2005). Briefly, skulls of anaesthetized mice were exposed and were glued to a custom-made stainless steel plate with crazy glue. Then the skulls were thinned to ~25 μm in thickness at the location where photothermotic stroke model had been made. To image blood vessels, 50 μl 70 kDa-Texas Red dextran (10 mg/ml; Invitrogen) was injected from tail-veins to label blood plasma. After that, animals were fitted into a two-photon microscope and a ×25 water-immersion objective (×25/1.05; Olympus) was used to acquire images with laser of 890 nm to excite both GFP and Texas Red dextran.

**Data analysis**

To calculate the volume of reactive microglia, brains were sectioned into 30 μm coronal slices and every third slice was collected and photographed. Digital microphotographs of all brain sections were obtained at fixed image acquisition parameters. The area of each slice was measured using ImageJ software (http://rsb.info.nih.gov/ij/). The volume of reactive microglia was calculated by summing the area of reactive microgliosis in each section and multiplying by the distance between sampled sections (90 μm). To quantify the expansion of reactive microglia in CX3CR1 mice, three coronal slices were randomly selected from each brain and the density and the width of reactive microglia in different zones of the ischaemic area were calculated. To quantitate the number of infiltrating cells in GFP-negative mice of the parabionts, we counted all the infiltrating cells in each sampled slice. The number of infiltrating cells in each animal was calculated by multiplying the total number of infiltrating cells in all the sampled sections by the sampling interval (3). Statistical comparison among three or more groups were done by one-way ANOVA. Comparison of two groups was performed by unpaired t-test (two tailed). All data are presented as the mean ± the standard error of the mean. *P < 0.05, **P < 0.01.

**Results**

**Activation and expansion of microglia in the subacute phase of ischaemic stroke**

We first established blood chimerism in parabiotic mice. Two female mice were conjoined subcutaneously, one of which was wild-type and the other expressed GFP in microglia (Fig. 1A). Blood chimerism of the parabionts was confirmed before stroke induction. On average, 42.0 ± 2.1% (n = 6) CX3CR1GFP/− cells were verified in the blood of the GFP-negative partner 13–14 days after parabiotic operation when an efficient anastomotic circulation was established (Supplementary Fig. 1A), indicating complete blood sharing had been established between parabionts. Ischaemia was induced in a 1 mm² area in both mice in the somatosensory cortex by photothermosis. There was no significant change in blood chimerism before and after stroke (Supplementary Fig. 1B). To determine the dynamic response of microglia to ischaemic stroke, we harvested and examined brain slices from CX3CR1GFP/+ mice of parabionts every day for 1 week after stroke induction. In control animals, resident microglia displayed ramified processes and low expression of GFP under physiological conditions (Fig. 1B), and all the parenchymal microglia in a healthy brain are IBA1 positive (Supplementary Fig. 2). Consistent with others reports (Schilling et al., 2003; Neumann et al., 2009), we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Cells with swollen soma, played ramified processes and low expression of GFP under physiological conditions (Fig. 1B), and all the parenchymal microglia in a healthy brain are IBA1 positive (Supplementary Fig. 2). Consistent with others reports (Schilling et al., 2003; Neumann et al., 2009), we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Cells with swollen soma, played ramified processes, as well as an increase in cell number in the vicinity of the ischaemic site (Fig. 1C and D). Phenotypically, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Cells with swollen soma, played ramified processes, as well as an increase in cell number in the vicinity of the ischaemic site (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D). Consistent with others reports, we observed a rapid formation of microgliosis after stroke induction (Fig. 1C and D).

To examine the progression of microgliosis, we measured the volume of reactive microglia in the subacute phase of ischaemic stroke (Fig. 2A). We found that the total volume of reactive microglia increased gradually and reached a peak volume of 4.99 ± 0.44 mm³ on the fourth day after stroke. After that, the volume of reactive microglia decreased (Fig. 2A and Supplementary Table 1). The area of reactive microglia exhibited a consistent pattern and could be divided into three zones based on the difference in cell density and GFP fluorescent intensity: core zone,
accumulation zone, and marginal zone (Fig. 2B). The core zone was delimited as the ischaemic core region where only sparse reactive microglia were found in the first few days after ischaemia. However, more reactive microglia infiltrated the core zone at a later stage. The accumulation zone was identified as a band with the highest density and GFP intensity of microglia surrounding the ischaemic core. Microglia in the accumulation zone had hypertrophic cell body and lacked ramified processes. The region between the accumulation zone and outside non-ischaemic area was the marginal zone, where reactive microglia had fewer short and stout processes than resting microglia (Fig. 2B).

To investigate the kinetics of microglial activation and expansion, we measured changes in cell density of reactive microglia in these three distinct zones and the width of the accumulation zone from Day 1 to Day 7 after stroke induction. Cell densities in all three zones increased continuously within the first week after stroke, with the accumulation zone having the highest cell density and the fastest growth rate (Fig. 2C and Supplementary Table 1). Consistent with our findings on the volume changes of reactive microglia, the width of the accumulation zone broadened continuously during the first 4 days after stroke induction, and then decreased over the subsequent 3 days (Fig. 2D and Supplementary Table 1).

**CX3CR1<sup>GFP</sup>/+ circulating cells infiltrated into central nervous system through impaired blood–brain barrier after stroke**

To determine whether circulating cells have the ability to populate the CNS parenchyma and contribute to microgliosis after ischaemic stroke, we took advantage of peripheral chimerism with parabionts to distinguish circulating cells from resident microglia. We collected brains from wild-type mice of parabiotic pairs from the...
first day to the seventh day after stroke, and sectioned them into 30 μm slices on a vibrating microtome. In contrast to studies using models characterized by a diffuse or focal neurodegeneration in the absence of BBB dysfunction (Ajami et al., 2007; Mildner et al., 2007), infiltration of partner-derived CX3CR1 GFP/+ cells was readily identified in the ischaemic area in the ipsilateral hemisphere of the wild-type mice after stroke (Fig. 3A–F).

In vivo two-photon imaging indicated that CX3CR1 GFP/+ infiltrating cells were located in brain parenchyma. There was a significant leakage of plasma containing Texas Red dextran in the ischaemic area (Fig. 3G), implying disruption of the BBB in the ischaemic zone. The majority of CX3CR1GFP/+ infiltrating cells were located in the accumulation zone (Fig. 3A–C). However, the density of CX3CR1GFP/+ infiltrating cells in the accumulation zone of wild-type mice was much lower than the density of CX3CR1GFP/+ cells in the same region of their fluorescent partners (43.6 ± 3.5/mm² for CX3CR1GFP/+ infiltrating cells versus 1308.6 ± 40.7/mm² for microglia at Day 5 after stroke when the infiltrating cells had the highest density, \( P = 7.06 \times 10^{-11} \), Figs 2C and 3H). In addition to inducing ischaemia in both mice of parabionts, we also induced photothrombosis only in the wild-type mouse of the parabionts. A similar pattern of recruitment of CX3CR1GFP/+ infiltrating cells in response to ischaemic stroke was observed in the ischaemic zone of the wild-type mouse (Supplementary Fig. 3). We found there was no significant difference in the number of CX3CR1GFP/+ infiltrating cells between parabiotic (GFP) animals in which only the wild-type mouse or both of the parabionts were subjected to cerebral ischaemia (1910.3 ± 243.7 cells for ischaemia in both mice at Day 7 after stroke, \( P = 0.841 \)). These data suggested that ischaemia in wild-type mouse alone can mobilize and recruit partner-derived cells.

We also performed sham-operated controls without stroke induction in parabionts, and analysed sections spanning 1 to 4 months of parabionts. Consistent with results reported by using parabiosis (Ajami et al., 2007, 2011) and in contrast to data obtained by using bone marrow transplantation and irradiation (Mildner et al., 2007), no GFP-positive cells from their fluorescent partners were observed in parenchyma of the wild-type mice, supporting the concept that blood-derived cells don’t cross intact BBB under physiological conditions. Nevertheless, there were a small number of GFP-positive perivascular and meningeal cells in the wild-type mice of the parabionts (Supplementary Fig. 4).

These results are consistent with reports that both perivascular cells and meningeal macrophages are renewed, to some extent, by bone marrow-derived cells (Hickey and Kimura, 1988; Corti et al., 2002; Schilling et al., 2003; Hess et al., 2004).

**Phenotype and kinetics of CX3CR1GFP/+ infiltrating cells in response to ischaemic stroke**

We used IBA1 as microglia and mature macrophage marker to investigate the phenotypic feature of CX3CR1GFP/+ infiltrating...
Figure 3  CX3CR1<sup>GFP</sup> + circulating cells are recruited into the brain parenchyma after acute stroke. (A–C) Confocal images of brain sections stained with IBA1 antibody from GFP-negative mice of parabionts at 5 days after stroke induction. A small number of partner-derived cells (GFP-positive) infiltrated into the brain parenchyma. Note that the density of IBA1-positive cells (red) is much higher than that of the infiltrating GFP-positive cells. Scale bar = 200 μm. (D–F) Magnified views of the white box region in (A–C). Note only a small number of CX3CR1<sup>GFP</sup>+ infiltrating cells derived from circulation (GFP-positive) were IBA1-positive (arrow). Scale bar = 50 μm. (G) Two-photon image of CX3CR1<sup>GFP</sup> + infiltrating cells (green) and vasculature (red) 3 days after stroke. Partner-derived CX3CR1<sup>GFP</sup> + cells infiltrated into CNS parenchyma. Extravasation (leakage) of blood plasma labelled with Texas Red (TR) dextran was identified as the red fluorescence outside the vessels. Scale bar = 50 μm. (H) Changes in the number of CX3CR1<sup>GFP</sup> + infiltrating cells at different days after stroke in wild-type mouse of the parabiotic pairs (n ≥ 5). a–c indicate significant differences between the two groups [one-way ANOVA F(6,32) = 20.117, P < 0.001].
cells. We found that only 36.3 ± 3.0% of the CX3CR1GFP+/+ infiltrating cells were IBA1-positive in wild-type mice (Fig. 3D–F), in contrast with 97.3 ± 0.5% of GFP-positive cells that were IBA1-positive in the CX3CR1GFP+/+ transgenic partners (Fig. 1E–G). To further delineate the phenotype of these CX3CR1GFP+/+ infiltrating cells, brain sections were stained with CCR2 antibody. CCR2 is reported to express at high levels in inflammatory monocytes but not in microglia (Mizutani et al., 2012), and is needed for monocytes to enter into the CNS (Prinz and Priller, 2010; Ajami et al., 2011). Consistent with these reports, all CX3CR1GFP+/+ cells in CX3CR1GFP+/+ mice of parabionts were CCR2 negative (Supplementary Fig. 5A–C and G–I). Furthermore, although CCR2-positive cells were found in the ischaemic area of the wild-type mice (Supplementary Fig. 5D–F and J–L), none of the CCR2-positive cells were found in the ischaemic area of the wild-type mice also displayed different kinetics of microglial proliferation by measuring the incorporation of BrdU microgliosis in normal conditions, but they are able to infiltrate the CNS through impaired BBB induced by ischaemic stroke. These results are consistent with reports that blood-derived cells are recruited into the CNS through impaired BBB, but do not have the ability to

**Proliferation of local resident cells rather than recruitment of CX3CR1GFP+/+ circulating cells is the main source of microgliosis**

To investigate whether microglia can self-renew, we evaluated microglial proliferation by measuring the incorporation of BrdU in microglia of CX3CR1GFP+/+ mice after stroke. BrdU-labelled microglia (BrdU-positive, GFP-positive) were observed in the brains of CX3CR1GFP+/+ mice after stroke, mostly in the accumulation zone (Fig. 5A–D). Triple staining of GFP-positive cell with Hoechst 33258 and BrdU antibodies indicated that most BrdU signals in GFP-positive cells were located in the nuclei (Supplementary Fig. 8E–H), suggesting that BrdU signals mainly signify the mitotic event. Consistent with a continued increase in cell density of reactive microglia, we found that there was a significant increase in the frequency of proliferating microglia (BrdU-positive, GFP-positive) between 3 and 7 days after stroke (20.4 ± 2.2% to 30.3 ± 1.0%, P = 0.003) (Fig. 5E and Supplementary Fig. 8I–P). These data suggest that resident microglia proliferate and provide a sustainable source for microgliosis.

To directly monitor the formation of microgliosis, we examined microglial transformation and proliferation after ischaemic stroke by taking advantage of two-photon microscopic imaging of GFP-labelled microglia in living animals. Microglial cells in the ischaemic area retracted their motile processes gradually within hours, shifting from ramified to amoeboid morphology (Fig. 5F–I). In line with our data from BrdU immunohistochemistry, we found that microglia could undergo active division and we captured the entire process of microglial proliferation in the accumulation zone of microgliosis at Day 2 after stroke induction (Fig. 5J–Q and Supplementary Video 1). Cell division occurred in microglia with nearly spherical shape. The division was symmetric and microglia divided into two smaller daughter cells. The entire time course of microglial cell division took ~1 h.

**Discussion**

Our data indicate that circulating cells do not enter the CNS under normal conditions, but they are able to infiltrate the CNS through compromised BBB induced by ischaemic stroke. These results are consistent with reports that blood-derived cells are recruited into the CNS through impaired BBB, but do not have the ability to
populate the CNS with intact BBB (Kennedy and Abkowitz, 1997; Ajami et al., 2007, 2011; Mildner et al., 2007). In contrast to previous reports using irradiation and transplantation (Ajami et al., 2007, 2011; Mildner et al., 2007), the recruitment of CX3CR1GFP/+ circulating cells in our study occurred without experimental manipulation of bone marrow or haematopoietic cells, which may artificially mobilize myeloid precursors or introduce exogenous haematopoietic stem cells into circulation. We also detected infiltration of CX3CR1GFP/+ cells in parabionts where only the wild-type of the pair is lesioned. Thus, infiltration of CX3CR1GFP/+ cells from circulation was mainly dependent on the integrity of the BBB. Presumably, blood-derived signalling may be involved in mobilization and recruitment of CX3CR1GFP/+ circulating cells. CCR2 has been proposed to regulate the infiltration of monocytes into the CNS (Prinz and Priller, 2010; Ajami et al., 2011). However, we found that all the CX3CR1GFP/+ infiltrating cells in wild-type mice of the parabionts were CCR2-negative at both Day 2 and Day 7 after stroke. These results indicated that CCR2 may not be required for the recruitment of CX3CR1GFP/+ cells after ischaemia.

Although CX3CR1GFP/+ circulating cells infiltrated parenchyma, they neither proliferate nor differentiate into microglia. CX3CR1GFP/+ infiltrating cells are eventually lost in the ischaemic area. These findings suggest that infiltration of CX3CR1GFP/+ circulating cells and proliferation of parenchymal microglia were two independent events under ischaemic stroke. Our data indicated that expansion of CX3CR1GFP/+ infiltrating cells and reactive microglia had different kinetics. The number of CX3CR1GFP/+ infiltrating cells peaked on Day 5 after stroke and then decreased. Caspase-3 staining suggests that an active apoptotic process contributed to the decline of CX3CR1GFP/+ infiltrating cells. In addition, it is possible that the recovery of BBB may also limit the infiltration of circulating cells into brain parenchyma.

In contrast, reactive microglia increased continuously during the first week after stroke induction. Importantly, the number of CX3CR1GFP/+ infiltrating cells in wild-type mice was much less than the number of reactive microglia in their CX3CR1GFP/+ partners. Although the actual number of CX3CR1GFP/+ infiltrating cells derived from circulation to the CNS could be more than twice the number we showed considering the limitation of the parabiotic model (Ransohoff, 2007; Soulet and Rivest, 2008), we do not expect that those uncounted cells can reconcile the large difference (~30-fold) in density between these two cell populations. In addition, the distinction between the two cell populations was also evident in their immunological profile. Less than 40% of CX3CR1GFP/+ infiltrating cells expressed IBA1, whereas >97% of CX3CR1GFP/+ cells involved in microgliosis were IBA1-positive. A small percentage of IBA1-negative cells in CX3CR1GFP/+ mice of the parabionts may represent GFP-positive infiltrating cells from their own circulation. Taken together, our observations imply that, although CX3CR1GFP/+ circulating cells infiltrated CNS parenchyma after ischaemic stroke, these infiltrating cells did not proliferate and were different from resident microglia.

Consistent with several other studies, another major conclusion from this study was that microglial expansion at the lesion site was mainly dependent on the proliferation of CNS resident cells (Ladeby et al., 2005; Solomon et al., 2006; Ajami et al., 2007, 2011; Denes et al., 2007). Our data of BrdU-staining indicated that microglia can proliferate and we observed that 20–30% of reactive microglia were BrdU-positive. Although we cannot rule...
Figure 5  Transformation and proliferation of microglia after acute stroke. (A–C) Representative image of brain sections from CX3CR1<sup>GFP/+</sup> mice at 3 days after stroke, stained for BrdU. The presence of proliferative microglia (BrdU-positive and GFP-positive) was significant at this time and they distributed mostly in the accumulation zone surrounding the ischaemic core. BrdU is shown in red, and GFP in green. White arrows indicate BrdU-positive microglia and cyan arrow indicates a BrdU<sup>+</sup>/GFP<sup>−</sup> cell. Scale bar = 50 μm. (A–C) is the magnified view of the white box region in (D). Scale bar = 400 μm. (E) Percentage of proliferating microglia (GFP-positive, BrdU-positive) at 3, 5 and 7 days after stroke (n = 5, **P < 0.01). (F–I) In vivo two-photon time-lapse imaging showing morphological transformation of resident microglia (arrowhead) after ischaemic stroke induction. Microglia retracted their ramified processes in response to ischaemia. Scale bar = 20 μm. (J–Q) Time-lapse imaging showing the entire process of microglial division at Day 2 after stroke. A microglial cell (white asterisk) was divided into two smaller daughter cells (yellow asterisk and red asterisk) within hours. Scale bar = 20 μm.
Conclusion

In summary, we demonstrated that a small population of CX3CR1GFP+/− cells were recruited from circulation into the CNS after ischaemic stroke. These infiltrating cells displayed distinct properties from resident microglia and did not proliferate. Further studies are warranted to identify the precise cell lineage of these blood-derived cells. We also examined the detail response of reactive microglia and directly imaged the time course of microglial transformation and proliferation in vivo. Our data suggest that cells involved in the process of microglisation in the subacute phase of ischaemic stroke were mainly derived from local expansion of resident microglia. CX3CR1GFP+/− infiltrating cells and reactive microglia had different expansion kinetics and properties in response to ischaemic stroke and they may represent two distinct populations of cells with different functions and therapeutic potentials for the treatment of stroke.

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Supplementary material

Supplementary material is available at Brain online.

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