



# Group 2 innate lymphoid cells resolve neuroinflammation following cerebral ischaemia

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## ABSTRACT

**Background** Acute brain ischaemia elicits pronounced inflammation, which aggravates neural injury. However, the mechanisms governing the resolution of acute neuroinflammation remain poorly understood. In contrast to regulatory T and B cells, group 2 innate lymphoid cells (ILC2s) are immunoregulatory cells that can be swiftly mobilised without antigen presentation; whether and how these ILC2s participate in central nervous system inflammation following brain ischaemia is still unknown.

**Methods** Leveraging brain tissues from patients who had an ischaemic stroke and a mouse model of focal ischaemia, we characterised the presence and cytokine release of brain-infiltrating ILC2s. The impact of ILC2s on neural injury was evaluated through antibody depletion and ILC2 adoptive transfer experiments. Using Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice receiving passive transfer of IL-4<sup>-/-</sup> ILC2s, we further assessed the contribution of interleukin (IL)-4, produced by ILC2s, in ischaemic brain injury.

**Results** We demonstrate that ILC2s accumulate in the areas surrounding the infarct in brain tissues of patients with cerebral ischaemia, as well as in mice subjected to focal cerebral ischaemia. Oligodendrocytes were a major source of IL-33, which contributed to ILC2s mobilisation. Adoptive transfer and expansion of ILC2s reduced brain infarction. Importantly, brain-infiltrating ILC2s reduced the magnitude of stroke injury severity through the production of IL-4.

**Conclusions** Our findings revealed that brain ischaemia mobilises ILC2s to curb neuroinflammation and brain injury, expanding the current understanding of inflammatory networks following stroke.

## INTRODUCTION

Inflammation and immune responses are the main events in the pathological process following strokes.<sup>1,2</sup> The cessation of cerebral blood supply induces irreversible primary tissue damage; subsequent excitotoxicity and oxidative stress extends this primary damage to the partially preserved peri-infarct area in the following hours to days.<sup>3</sup> The first immune cells that respond to these events are brain-intrinsic microglia and are subsequently accompanied by infiltrating leucocytes from the periphery. Immediately after ischaemia, neutrophils

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Brain ischaemia elicits pronounced inflammation, which aggravates neural injury. However, the mechanisms governing the resolution of neuroinflammation remain unclear. As an innate immunoregulatory cell that can be swiftly mobilised without antigen priming, brain homing group 2 innate lymphoid cells (ILC2) may serve as a candidate element in tuning down neuroinflammation.

## WHAT THIS STUDY ADDS

⇒ We have identified the ILC2s migrated to the ischaemic brain. ILC2s activity is orchestrated by interleukin (IL)-33, predominantly produced by oligodendrocytes, and acts mostly via the release of IL-4. Our work provides novel evidence that ILC2s infiltrate the ischaemic brain during the early stage of stroke and protect against neural injury.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides novel evidence that ILC2s contribute to resolution of neuroinflammation and in turn reduce ischaemic brain injury. Therefore, immune therapies targeting ILC2s may be regarded as a treatment option, or a conjunctive method to current reperfusion therapies, to benefit patients who had an acute ischaemic stroke.

migrate into the injured brain, followed by monocytes, natural killer cells, T cells and B cells.<sup>4</sup> The entry of these cells into the brain is, in part, guided by alarmins or danger signals released by dying neural structures. On arrival in the brain, these peripheral leucocytes accelerate brain infarction by conditioning the focal inflammatory milieu and promoting microvascular dysfunction.<sup>5</sup> The detrimental role of these infiltrating leucocytes and immunomodulatory factors (interleukin (IL)-1, IL-17 and matrix metalloprotein 9 (MMP-9)) has been recognised, especially during the acute stage of stroke.<sup>2,4</sup>

Although numerous studies have addressed the kinetics and deleterious

aspects of leucocytes in brain ischaemia, the elements governing the resolution of brain inflammation in this setting have been less characterised.<sup>6</sup> An immune avenue in the resolution of acute neuroinflammation may be through the endogenous immune regulatory system, which involves regulatory T and B cells.<sup>7</sup> However, considering their limited number in the ischaemic brain,<sup>8</sup> their requirement of antigen presentation for activation, and partially divergent findings in various stroke models, uncertainty remains about the pathophysiological function of regulatory lymphocytes in stroke.<sup>9</sup> Thus, the need for an increased understanding of endogenous immune modulators post stroke continues.

Group 2 innate lymphoid cells (ILC2s) are specialised innate lymphocytes that lack special antigen receptors. In humans, ILC2s subset was identified as Lin<sup>-</sup>CD127<sup>+</sup> ILC population with Th2 marker CRTH2 expression; the corresponding murine ILC2s population was defined as CD45<sup>high</sup>CD90.2<sup>+</sup> ST2<sup>+</sup> lymphoid cells negative for lineage markers (CD3e, CD45R, CD11b, Ter119, Ly-6G, CD11c, TCR- $\beta$  and TCR- $\gamma\delta$ ), as reported previously. ILC2s are potent responders to alarmins, such as IL-33.<sup>10 11</sup> Once engaged with alarmins, ILC2s produce IL-4, IL-13 to modulate the inflammatory response after tissue injury.<sup>12</sup> However, it is still unclear if and how ILC2s are involved in central nervous system (CNS) inflammation following brain ischaemia. To clarify this question, we used brain tissues of patients who had an ischaemic stroke to characterise the presence and activity of brain-infiltrating ILC2s. Thereafter, we examined the impact of ILC2s on neural injury following brain ischaemia.

## MATERIALS AND METHODS

### Human brain sections

Brain tissues were collected within 4 hours after death. Among the 16 cases studied here, 7 cases were from patients who had an ischaemic stroke and died within 24 hours (4 men and 3 women) (online supplemental table S1). The locations of stroke lesions were within the cortical areas supplied by the middle cerebral artery. In this study, the nine control cases screened out were all patients without a history of neurological or neuropsychiatric diseases who died of non-neurological disease, of which five cases were men and four cases were women, and the selected tissue section position matched the position of stroke cases. All study patients had no heart failure or any infection at the time of death. There was no obvious difference in age at death between patients who had a stroke and controls (stroke: 71.3 $\pm$ 7.6 years of age; control: 67.5 $\pm$ 8.9 years of age; mean $\pm$ SEM;  $p$ >0.05; unpaired t-test).

### Mice

Mice were purchased from SPF Biotechnology Co (Beijing, China). The male and female

C57BL/6-H4tm1Nnt/J mice were from the Jackson Laboratory (Bar Harbor, Maine, USA). All animal experiments were completed according to the Animal Research: Reporting in vivo Experiments guidelines and approved by the Institutional Animal Care and Use Committee of Beijing Tiantan Hospital, Capital Medical University. In this study, the male and female C57BL/6 mice, 9–12 weeks of age, were adopted. Mice husbandry and care are faithfully adhered to international standards.

### Middle cerebral artery occlusion procedure

Male and female mice, 8–12 weeks of age, underwent focal cerebral ischaemia. The procedure for middle cerebral artery occlusion (MCAO) is shown in online supplemental materials. Mice were excluded if cerebral blood flow (CBF) was not satisfactory during occlusion or 10 min after reperfusion. If the relative CBF increases more than 50% of the blood flow before ischaemia, subjects were used for subsequent experiments (online supplemental figure S1).

### Physiological parameters monitoring in mice

We monitored the physiological parameters, including body weight, heart rate, oxygen saturation, body temperature, blood pressure and pH values in sham and MCAO mice. A physiological pressure transducer coupled with a data acquisition system (BP2010AUL, Softron Biotechnology Co, Beijing, China) was used to record the blood pressure and heart rate. A probe was placed inside the rectum of mice using a multichannel intelligent temperature analyser (SENDAE-9T4, SUN-GUN, Guangzhou, China) to measure their body temperature. The blood gas analysis (GME.Premier3000, Instrumentation Laboratory, California, USA) was used to detect blood oxygen and acidification.

### Neurological assessment

Neurological functional assessment is carried out according to the established process.<sup>13</sup> The method is shown in online supplemental materials. In addition, neural severity score, which covers the evaluation of such dimensions as motion, sensation and reflex, was also conducted. The results of corner turning test were carried out to evaluate the asymmetry of sensory movement and posture. As previously discussed, this paper uses forelimb placement test to obtain the reaction ability of forelimb forward movement of animals.<sup>14</sup>

### Neuroimaging

Infarct volume was evaluated with a 7T animal MRI (BioClinScan, Bruker, Germany) with a 30 cm horizontal-bore magnet and BioSpec Avance III spectrometer with a 72 mm linear transmitter coil and a mouse surface receiver coil for mouse brain imaging, according to described.<sup>5 15</sup> During the scanning process, the experimental animals were placed on a

blanket (Bruker Daltonics) to control body temperature at 37.0°C. Axial 2D multislice T2-weighted images of the brain were acquired with the following condition (Repetition Time (TR)=3080.0 ms; Echo Time (TE)=41 ms; number of averages=1; Field of view (FOV)=24 mm×30 mm; matrix size=192×320; and slice thickness=0.5 mm). The MRI result was treated using ImageJ package (Bethesda, Maryland, USA).

### Positron emission tomography (PET) scan

The cerebral glucose metabolism with a PET-CT scanner (InliView-3000B, Invitrogen, Carlsbad, California, USA) was acquired at day 3 after MCAO. Following the MRI-T2 scan, the mice were injected with 18F-fluorodeoxyglucos (18F-FDG). The micro-PET scanner acquired images within a 15 min scan. The three-dimensional ordered-subsets expectation-maximization (3-D OSEM) iterative method was applied to reconstruct images, and the PET reconstruction matrix was 140×140×0.5 mm.

The standard brain template was used to calibrate the PET-CT and MRI. MRI as 'reference and matching' marks the lesion core and border area, loads input PET-CT images and compares the difference in the intensity of PET images between the lesion core and border area. To reduce differences in metabolic levels among mice, the intensity of the 18F-FDG signal was normalised to that of the contralateral side. Signal intensity was calculated based on the PMODE Image treatment software.<sup>16</sup>

### Immunofluorescence

Frozen brain samples were blocked on 5% donkey serum, 5% Bovine Serum Albumin (BSA) and 0.3% Triton X-100 for 1 hour, and then cultured with primary antibodies against CRTH2 (PA5-20332, Invitrogen, Carlsbad, California, USA), CD127 (A7R34, 14-1271-82, eBioscience, San Diego, California), CD3e (17A2, 13-0032-82, eBioscience, San Diego, California, USA), ST2 (DIH9, 145302, Biolegend, San Diego, California), GFAP (ab7260, Abcam, Cambridge, Massachusetts, USA), NEUN (ab177487, Abcam, Massachusetts, USA), OSP (ab7474, Abcam, Cambridge, Massachusetts, USA) and IL-33 (AF3626, R&D Systems, Minnesota, USA), at 4°C for 12 hours. Subsequently, sections were cultured with the fluorochrome-conjugated secondary antibodies at 23°C for 1 hour: donkey anti-mouse 488 (A32766, Invitrogen, Carlsbad, California, USA), donkey anti-rabbit 594 (A32754, Invitrogen, Carlsbad, California, USA) and donkey anti-goat 488 (A32814, Invitrogen, Carlsbad, California, USA). Images were acquired using a Pathology Imaging System (Hopkinton, Massachusetts, USA).

### Flow cytometry

Single-cell suspensions were prepared from mouse blood, spleen, lung or brain tissues as previously

described.<sup>15 17–19</sup> Cells were incubated with FcγR blocker and the fluorochrome-labelled or their corresponding isotype controls antibodies were used to stain. The antibodies used to characterise ILC2s are shown in online supplemental materials. The cells were incubated for 4 hours with phorbol-12-myristate-13-acetate, ionomycin and GolgiStop, then fixed and permeabilised based on relevant permeabilisation kit (BD Biosciences, San Diego, California, USA). The intracellular staining antibodies are shown in online supplemental materials. During the whole experiment process, cells were stained by the live/dead fixable dye (Molecular Probes) to allow gating on viable cells. Flow cytometric data were analysed on an FACS Aria III flow cytometer (BD Bioscience). The gating was set using fluorescence Minus One (FMO) controls and were carried out based on Flow Jo V.10 (FlowJo.com) to analysis and are shown in online supplemental figure S2.

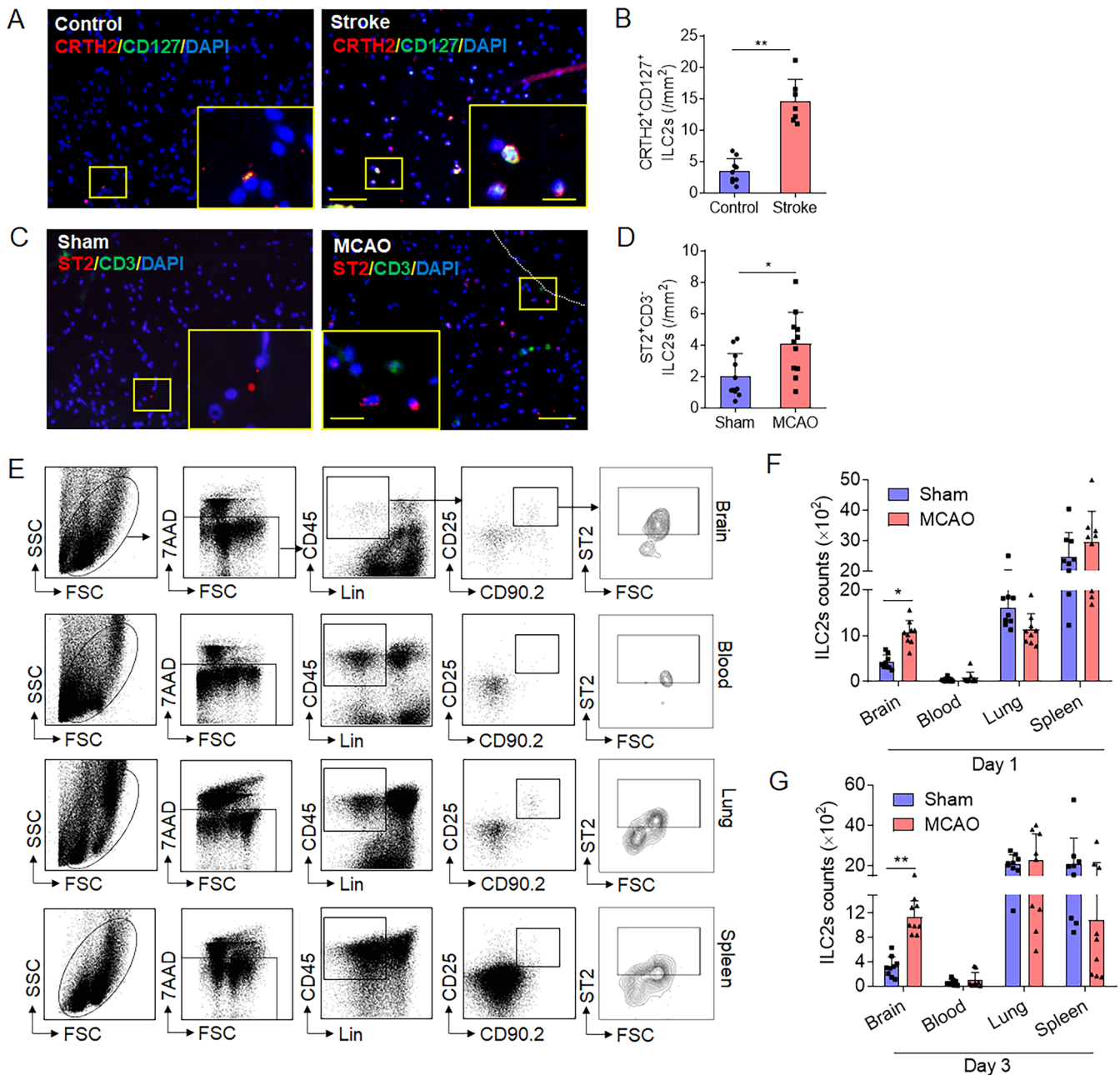
### Antibody and cytokine administration

Anti-CD90.2 antibody is particularly useful for removal of T lymphocytes from cell populations by complement-mediated cytotoxicity. In monoclonal antibody treatments, anti-CD90.2 mAb (30H12, 105315, Biolegend, San Diego, CA, USA) was purchased from BioXCell (West Lebanon, New Hampshire, USA). The mAb was administered by intravenous injection every 2 days with a dose of 300 µg/mouse and initiated 2 days prior to model induction. Control animals were treated with a rat IgG2b antibody as an isotype control immunoglobulin.<sup>20 21</sup> The efficiency of ILC2s depletion was verified by flow cytometry (online supplemental figure S3). The recombinant mouse IL-33 (580508, BioLegend, San Diego, CA, USA) was administered by intraperitoneal injection every 2 days (600 ng/mouse) for a total of 6 days. Control mice were treated with phosphate buffer saline (PBS) as sham group.<sup>20</sup>

### ILC2 isolation, culture and passive transfer

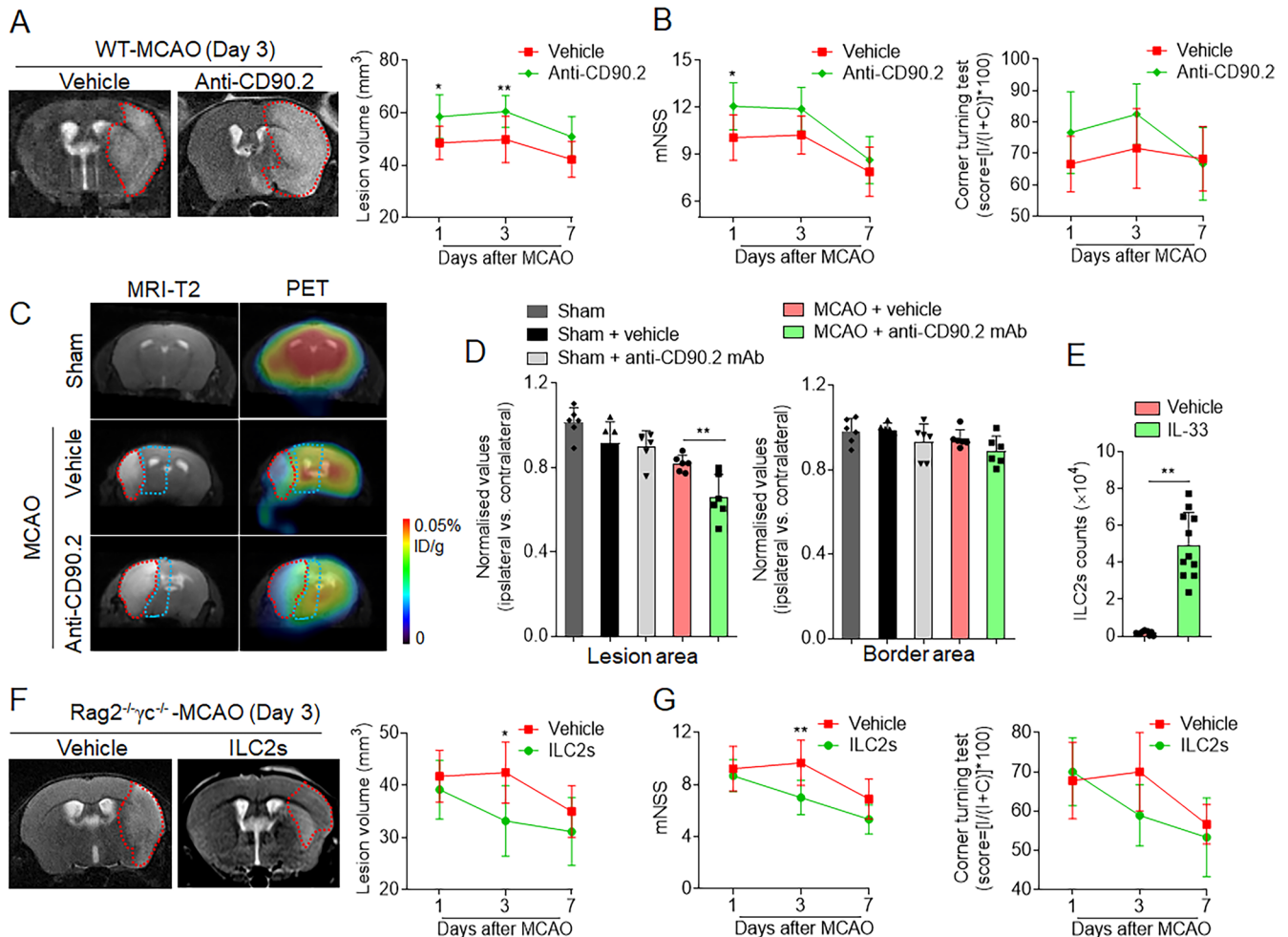
To induce sufficient ILC2s in vivo, naïve C57BL/6 or IL-4<sup>-/-</sup> mice were challenged by intraperitoneal injection of recombinant IL-33 (580508, BioLegend, San Diego, CA, USA) with same injection conditions for a total of 6 days. ILC2s were isolated from pooled splenocytes of wild-type C57BL/6 or IL-4<sup>-/-</sup> mice. The spleen tissues were separated and placed on a 70 µm cell strainer and subsequently homogenised based on a syringe plunger. Then washed by erythrocyte lysis buffer (349202, BD FACS, San Jose, California, USA), and the eluted cells were incubated for 5 min at 23°C. ILC2s were defined as CD45<sup>high</sup>CD90.2<sup>+</sup> ST2<sup>+</sup> lymphoid cells (CD3e, CD45R, CD11b, Ter119, Ly-6G, CD11c, TCR-β and TCR-γδ) as reported previously.<sup>22</sup>

For cell culture, sorted ILC2s were incubated by dulbecco's modified eagle medium (DMEM) high glucose (Invitrogen, Carlsbad, California, USA)



containing 10% fetal calf serum (FCS), 1 mM sodium pyruvate (Carlsbad, California, USA), non-essential amino acids (Carlsbad, California, USA) and 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (pH 7.4). For passive transfer, ILC2s were

purified via two rounds of cell sorting selection with flow cytometer. Purity of sorted ILC2s was verified by flow cytometry before transfer. Then highly purified (>95%) naïve or IL-4<sup>-/-</sup> ILC2s (1×10<sup>6</sup>) were injected into Rag2<sup>-/-</sup>γc<sup>-/-</sup> recipient mice 24 hours prior to



**Figure 2** Effects of group 2 innate lymphoid cells (ILC2s) on brain infarction and neurological deficits following cerebral ischaemia. (A,B) For antibody depletion, anti-CD90.2 mAb was treated by intravenous injection with a dose of 300 µg/mouse. Mice were treated with rat IgG2b as an isotype control immunoglobulin. The 7T-MRI and summarised results show the effects of antibody depletion of ILC2s on infarct volume (A) and neurological deficits (B) at the indicated time points (days 1, 3 and 7) after middle cerebral artery occlusion in wild-type mice (WT-MCAO).  $n=12$ . \* $p<0.05$  and \*\* $p<0.01$  according to analysis of variance (ANOVA) test. (C,D) At day 3 after MCAO, metabolic variation in lesion core and border area of MCAO mice receiving antibody depletion of ILC2 were detected based on positron emission tomography (PET) scans. PET/CT images show [18F]-FDG activity. The radioactivity in the unaffected tissue was applied as the standard to normalise results from lesion core and border area of ipsilateral hemisphere.  $n=6$ . \*\* $p<0.01$  according to ANOVA test for multiple comparisons at indicated time points. (E) To induce sufficient ILC2s, naïve C57BL/6 mice were challenged by intraperitoneal injection of recombinant IL-33 with condition of 600 ng/mouse for a total of 6 days. Spleens were collected to determine the IL-33-induced ILC2s number.  $n=12$ . \*\* $p<0.01$  according to independent t-test. (F,G) A total of  $1 \times 10^6$  purified ILC2s were isolated from splenocytes following in vivo IL-33 stimulation.  $Rag2^{-/-}\gamma c^{-/-}$  mice received intravenous injection of ILC2s following MCAO and reperfusion. The 7T-MRI and summarised results show the effects of passive transfer of ILC2s on infarct volume and neurological deficits at days 1, 3 and 7 after MCAO.  $n=9$ . \* $p<0.05$  and \*\* $p<0.01$  according to two-way ANOVA tests at indicated time points. Data are presented as the mean  $\pm$  SD.

MCAO or sham operations. Subsequently, the experimental animals were treated with IL-33 at 30 min and 24 hours after cell transfer.

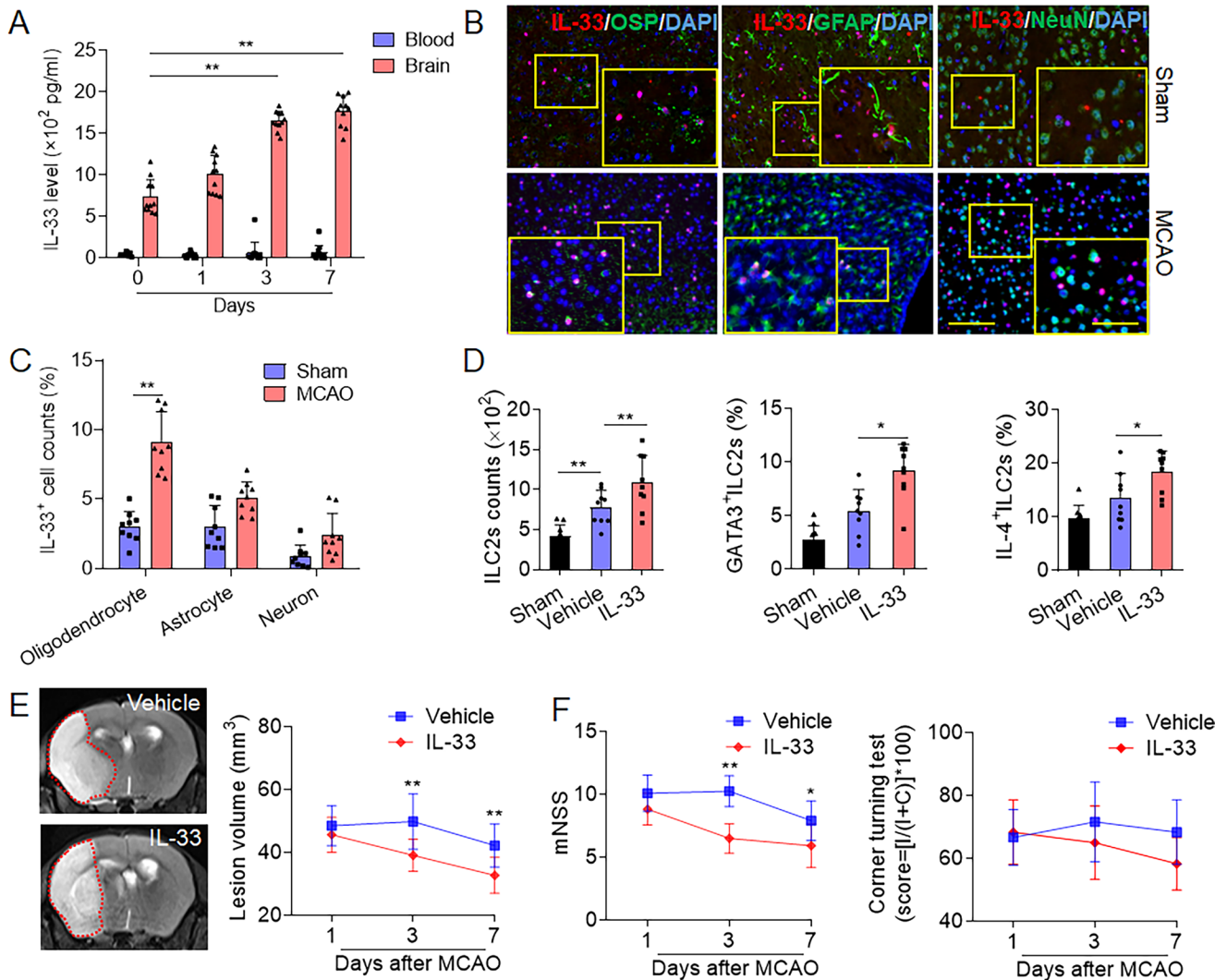
#### Enzyme-linked immunosorbent assay

Brain homogenates and blood were prepared from mice 1, 3 and 7 days after MCAO. For brain tissues, the brains were removed and immediately frozen in liquid nitrogen, then were homogenised in Radio Immunoprecipitation Assay (RIPA) buffer. The acid Protein Assay kit (Invitrogen, Carlsbad, California, USA) was applied to detect the protein content.

The total protein content kept at 1 mg/mL protein extract. Measurement of IL-33 in brain homogenates and blood were conducted by an ELISA kit (M3300; R&D Systems) based on the relevant instruction.

#### Statistical analysis

The experimental design was in accordance with the previous publications.<sup>1,5</sup> In order to better determine the sample size, this paper adopted  $\alpha=0.05$  and 80% power indicators are analysed, then the statistical differences of the results are judged to provide support for subsequent quantitative analysis. Power



**Figure 3** Oligodendrocytes express interleukin (IL-33) that expands group 2 innate lymphoid cells (ILC2s) and ameliorates ischaemic brain injury. (A) Brain tissues samples were collected from mice treated with sham operation or middle cerebral artery occlusion (MCAO) and reperfusion from 1 to 7 days. Measurement of IL-33 in brain homogenates and blood was conducted by an ELISA kit.  $n=11$ . \* $p<0.05$  and \*\* $p<0.01$  according to independent t-test. (B) Immunostaining shows the indicated cell subsets (GFAP<sup>+</sup>, astrocytes; NeuN<sup>+</sup>, neurons; OSP<sup>+</sup>, oligodendrocytes) expressing IL-33 at day 1 after sham surgery. Scale bar=100  $\mu$ m (inset=50  $\mu$ m). (C) Quantification of immunostaining results shows IL-33-producing cells in brain tissues at day 1 after sham operation or MCAO. CD45<sup>-</sup>OSP<sup>+</sup>, oligodendrocytes; CD45<sup>-</sup>GFAP<sup>+</sup>, astrocytes; CD45<sup>-</sup>NeuN<sup>+</sup>, neurons; CD45<sup>int</sup>CD11b<sup>+</sup>, microglia; CD45<sup>+</sup>CD31<sup>+</sup>, endothelial cells.  $n=9$ . \*\* $p<0.01$  according to Mann-Whitney test. (D) Brain tissues were collected from MCAO mice receiving intraperitoneal injection of IL-33. Flow cytometry analysis shows the number and activity of brain-infiltrating ILC2s in the indicated MCAO mice receiving IL-33 or vehicle (phosphate buffer saline, PBS).  $n=9$ . \* $p<0.05$ , \*\* $p<0.01$  according to one-way analysis of variance (ANOVA) test for multiple comparisons. (E) The 7T-MRI and summarised results show the effects of IL-33 injection on infarct volume at the indicated time points (days 1, 3 and 7) after MCAO. (F) The summarised results show the effects of IL-33 injection on neurological deficits after MCAO.  $n=12$ . \*\* $p<0.01$  according to ANOVA test for multiple comparisons at indicated time points. Data are described by manner of mean $\pm$ SD.

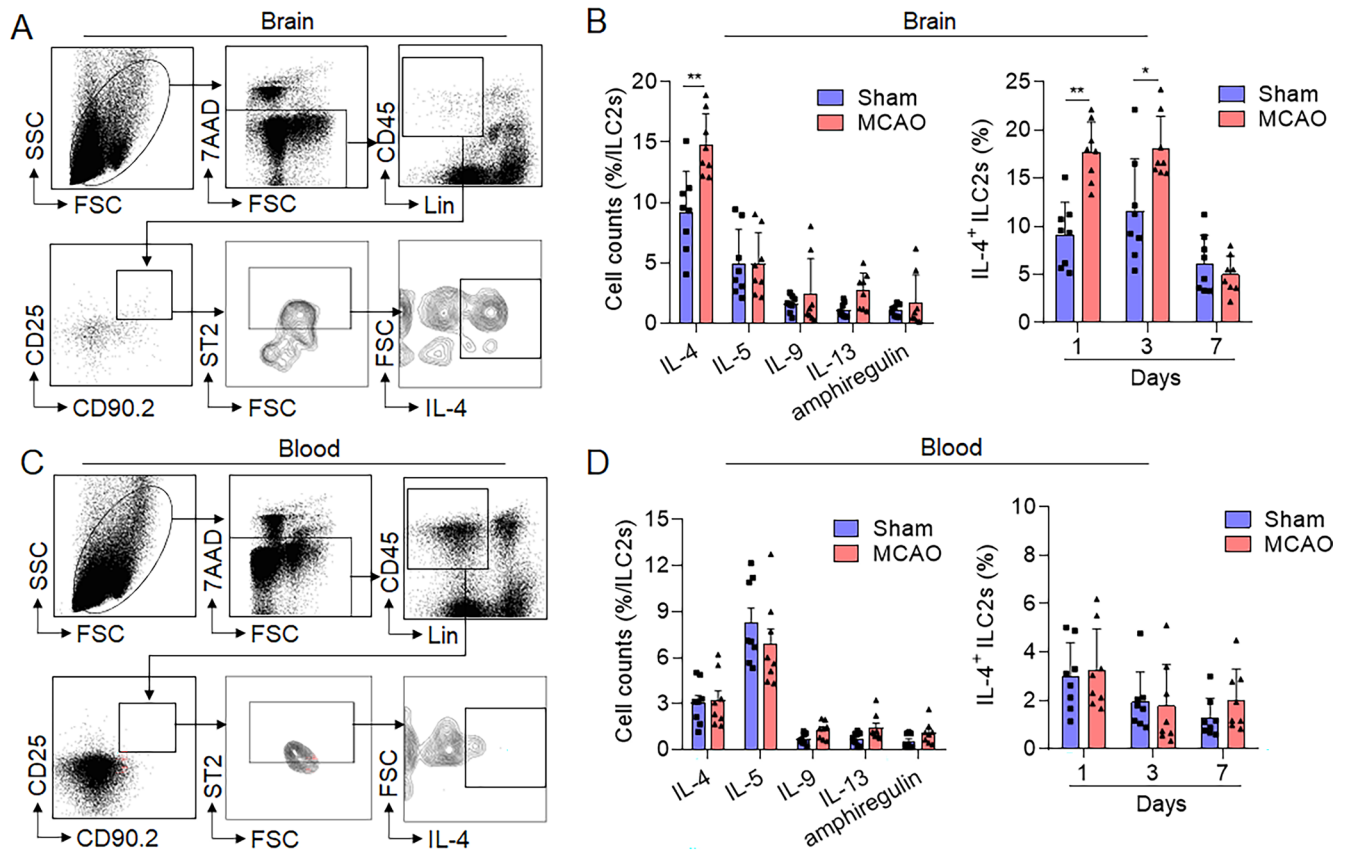
analysis and sample size calculation were performed with G\*Power (V.3.1) software using the Wilcoxon-Mann-Whitney test for two groups. Normality test was performed by Shapiro-Wilk test ( $\alpha=0.05$ ). Statistical significance was determined based on an independent t-test in univariate analysis. Then analysis of variance (ANOVA) and relevant post-hoc test were used for multiple groups test, two-way ANOVA were used to assess the entire time course variation comparisons. All analyses were conducted based on

Prism V.7.0. Data are described as mean $\pm$ SD.  $P<0.05$  is regarded as significant.

## RESULTS

### Brain infiltration of ILC2s in patients who had an acute ischaemic stroke and mice subjected to focal ischaemia

We first assessed the population of ILC2s in brain sections from postmortem brain tissues of deceased patients who had an acute ischaemic stroke; brain



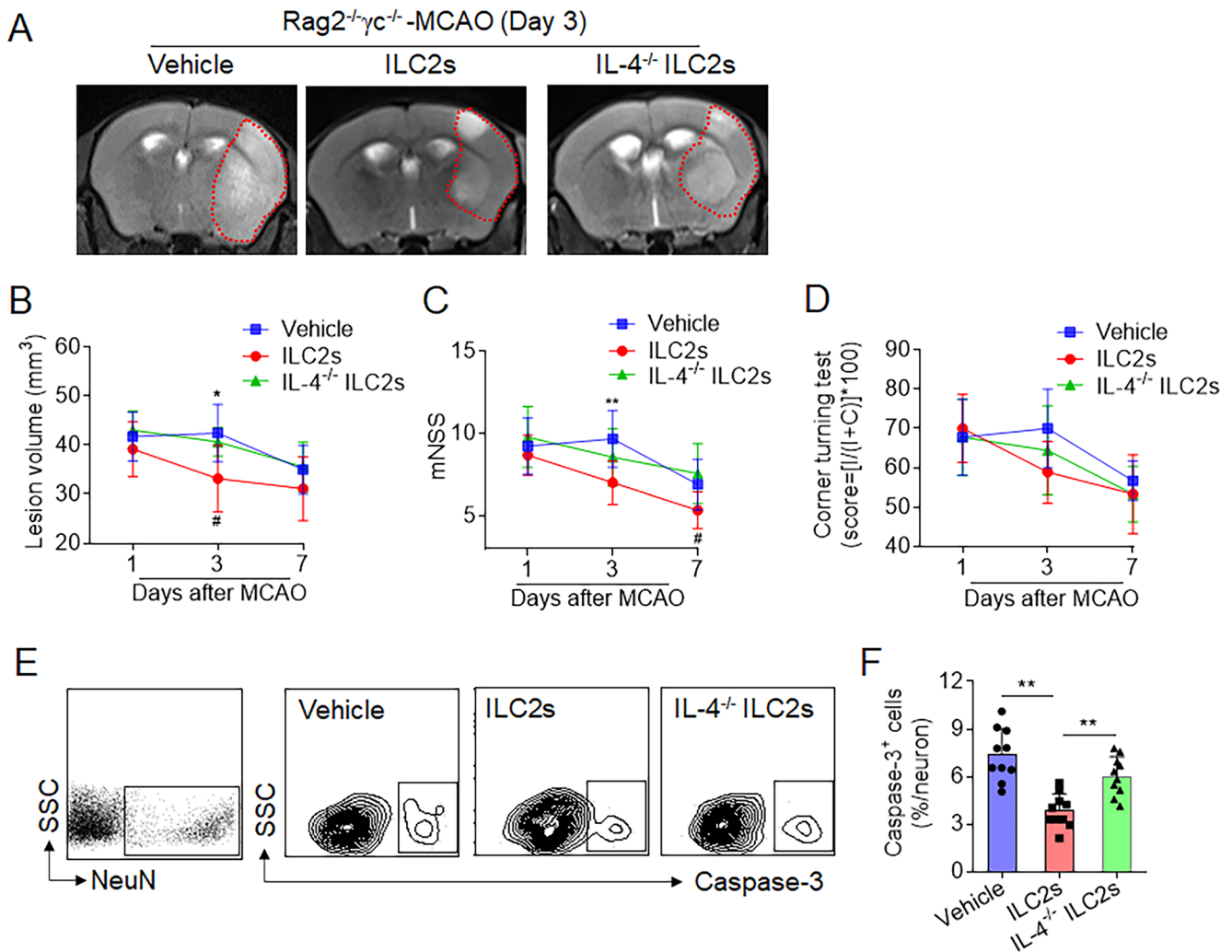
**Figure 4** Group 2 innate lymphoid cells (ILC2s)-released cytokines in the brain and periphery blood following brain ischaemia. Male and female C57BL/6 (B6) mice were treated with sham surgery or middle cerebral artery occlusion (MCAO). (A) Representative flow cytometry gating strategy showing interleukin (IL)-4-producing ILC2s in the mouse brain at day 1 after MCAO. Mouse ILC2s were identified as Lin<sup>-</sup>CD45<sup>high</sup>CD90.2<sup>+</sup>ST2<sup>+</sup>. (B) Left panel, summarised results depict the production of IL-4, IL-5, IL-9, IL-13 and amphiregulin in ILC2s obtained from experimental animal brains at day 1 after MCAO or sham. Right panel, IL-4-producing ILC2s in the brain from day 1 to day 7 after MCAO or sham. n=8. \*p<0.05, \*\*p<0.01 according to Mann-Whitney test. (C) Typical flow cytometry gating strategy showing IL-4-producing ILC2s in mouse blood after MCAO surgery at day 1. Mouse ILC2s were identified as Lin<sup>-</sup>CD45<sup>high</sup>CD90.2<sup>+</sup>ST2<sup>+</sup>. (D) Left panel, summarised results show the production of IL-4, IL-5, IL-9, IL-13 and amphiregulin in ILC2s obtained from mouse blood at day 1 post MCAO or sham. Right panel, IL-4-producing ILC2s in blood from day 1 to day 7 after MCAO or sham. n=8. Data are expressed as the mean±SD.

samples from non-neurological diseases died patients were applied as controls. The human Lin<sup>-</sup>CD127<sup>+</sup> ILC population with Th2 marker CRTH2 expression (chemoattractant receptor-homologous molecule expressed on Th2 lymphocytes) has been reported as a human equivalent of type 2 ILCs.<sup>23,24</sup> We identified an increasing count of CHRT2<sup>+</sup>CD127<sup>+</sup> cells in brain sections in the peri-infarct areas of the ischaemic stroke group versus those of control (figure 1A,B). Using a mouse model induced by 1 hour MCAO and reperfusion, a corresponding increased presence of CD3<sup>-</sup>ST2<sup>+</sup> ILC2s were found proximal to the infarct lesion (figure 1C,D).<sup>25</sup> We next evaluated the number of ILC2s in the periphery and the brain, using flow cytometry, in which ILC2s were identified as Lin<sup>-</sup>CD45<sup>high</sup>ST2<sup>+</sup>CD90.2<sup>+</sup> cells (Lin=CD3e, CD45R, CD11b, Ter119, Ly-6G, CD11c, NK1.1, CD4, CD5, CD8a, TCR-β and TCR-γδ). We observed an increased count of ILC2s in the ischaemic brain at day 1 persisting until day 3 after MCAO (figure 1E-G). In contrast,

there was no significant change in the number of ILC2s in peripheral organs (figure 1E-G).

#### ILC2s depletion enlarges ischaemic lesions in stroked mice

To investigate the influence of ILC2s on stroke severity, the anti-CD90.2 mAb antibody was applied to decrease ILC2s in vivo. Anti-CD90.2 mAb treatment did not impact the physiological parameters in sham or MCAO mice, including body weight, body temperature, heart rate, blood pressure and oxygen saturation during surgery (online supplemental table S2). Administration of anti-CD90.2 mAb resulted in augmented infarct volumes and worsened neurological deficits in MCAO mice (figure 2A,B). The technology of PET targeting in brain metabolism was helpful in assessing the brain's pathological changes after ischaemic brain injury.<sup>26</sup> Through in vivo brain 18F-FDG PET scan, we analysed the influence of antibody depletion of ILC2s following MCAO and found that ablation



**Figure 5** Effects of interleukin (IL)-4-producing group 2 innate lymphoid cells (ILC2s) on brain injury following cerebral ischaemia. ILC2s were isolated from pooled splenocytes of wild-type C57BL/6 or IL-4<sup>-/-</sup> mice. Groups of Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice were received intravenous injection of vehicle (phosphate buffer saline, PBS), wild-type ILC2s or IL-4<sup>-/-</sup> ILC2s followed by middle cerebral artery occlusion (MCAO). The 7T-MRI and summarised results show infarct volume (A,B) and neurological function (C,D) in groups of Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice (cell culture medium), wild-type ILC2s or IL-4<sup>-/-</sup> ILC2s at day 1, 3 and 7 after MCAO. n=9. \*p<0.05, \*\*p<0.01 according to analysis of variance (ANOVA) for the comparison of vehicle versus ILC2s at indicated time points; #p<0.05 according to ANOVA for the comparison of ILC2s versus IL-4<sup>-/-</sup> ILC2s at indicated time points. (E,F) Representative flow cytometry gating strategy and quantification showing Caspase-3 expression in neuronal cells, in groups of Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice receiving vehicle (cell culture medium), wild-type ILC2s or IL-4<sup>-/-</sup> ILC2s at the indicated time points after MCAO. n=12. \*\*p<0.01 according to two-tailed unpaired Student's t-test. Data are presented as the mean±SD.

of ILC2s obviously decreased glucose metabolism in ipsilateral hemisphere following MCAO, specifically in infarct lesion area (figure 2C,D). Because the anti-CD90.2 mAb also depleted T lymphocytes, we adopted Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice to evaluate the effects of passively transferred ILC2s on stroke severity. Exogenous IL-33 induced robust expansion and mobilisation of ILC2s for adoptive transfer assay (figure 2E). Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice receiving intravenous injection of ILC2s prior to MCAO induction exhibited reduced brain infarct volumes and improvement of neurological deficits (figure 2F,G).

### Oligodendrocytes are the main source of IL-33 that expand ILC2s following cerebral ischaemia

Following acute brain insults, injured brain cells release alarmins, such as IL-33, which play a central role in ILC2s activation and proliferation.<sup>27</sup> We detected the robust release of IL-33 in ischaemic brain in a time-dependent manner, but not in peripheral blood (figure 3A). To further determine the cellular source of IL-33 in ischaemic brain, we quantified the IL-33 level in neurons and oligodendrocytes. Among these cell types, we found that IL-33 was primarily produced by oligodendrocytes in the ischaemic brain (figure 3B,C).



### Expansion of ILC2s ameliorates ischaemic brain injury

After a series of experimental studies, we found that IL-33 rapidly expands ILC2s proportions and we measured the effects of ILC2s expansion on stroke severity. In MCAO mice receiving recombinant IL-33, we found an increased number of brain-infiltrating ILC2s and the high expression of GATA3 and IL-4 (figure 3D). Notably, IL-33 treatment reduced brain infarction and neurological deficits in MCAO mice (figure 3E,F). These results demonstrated that oligodendrocytes maybe a major source of IL-33, which serves as an alarmin that drives ILC2s response on ischaemic tissue injury.

### Brain-infiltrating ILC2s predominantly produce IL-4 following cerebral ischaemia

To characterise the activity of ILC2s in the brain and periphery following ischaemia, ILC2s-released cytokines in the blood and brain were assessed day 1 post MCAO. This result shows that ILC2s preferentially expressed IL-4 and IL-5 in the brain rather than other cytokines, specifically, IL-13, IL-9 and amphiregulin (figure 4A–D). Contrasting the higher frequency of IL-5 released by ILC2s in blood, brain-infiltrating ILC2s mainly expressed IL-4, which was significantly increased after brain ischaemia from 1 to 3 days, suggesting that IL-4 may contribute to ILC2s effects in stroke (figure 4B,D).

### The beneficial effect of ILC2s on ischaemic brain injury requires IL-4

To understand the effects of ILC2s-derived IL-4 on ischaemic brain injury, we sorted IL-4<sup>-/-</sup> ILC2s from IL-4<sup>-/-</sup> transgenic mice. We assessed infarct volume and neurological deficits in Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice receiving passive transfer of IL-4<sup>-/-</sup> ILC2s prior to MCAO induction. Infarct volumes were not altered in mice receiving ILC2s devoid of IL-4 (figure 5A,B). In addition, neurological deficits (figure 5C,D) and decreased neuronal apoptosis (figure 5E,F) in ILC2s-transferred Rag2<sup>-/-</sup>γc<sup>-/-</sup> mice were reversed in MCAO mice receiving IL-4<sup>-/-</sup> ILC2s. The result suggests that IL-4 is required for the beneficial effect of ILC2s in ischaemic brain injury.

## DISCUSSION

Mechanisms governing the resolution of neuroinflammation post stroke remain a mystery. Previous studies have suggested that regulatory T and B cells contribute to tuning immune reactivities in this setting.<sup>28–30</sup> Yshii *et al* reported that brain-resident regulatory T cells protect against pathological neuroinflammation.<sup>31</sup> Bodhankar *et al* reported that IL-10-producing B cells reduced infarct volume and limit CNS inflammation in stroke.<sup>30</sup> The limited number of regulatory T and B cells in the early phase of brain ischaemia and the time-consuming requisite antigen presentation in the activation of these cells belies a more complicated

picture of brain inflammation. Therefore, suppressor lymphocytes that may act during the early stage of stroke to limit the immune activities of the infarcted brain is a possibility. In this regard, the identification of ILC2s from the innate immune system increases our understanding of the endogenous regulatory machinery which restricts and resolves acute neuroinflammation in the ischaemic brain.

Previous studies have examined the impact of ILC2 on immune reactivities in the periphery. The ability of ILC2s to invade brain in various pathologies<sup>19 25 32–34</sup> suggests their potential action in the ischaemic brain. In adult murine models, studies have characterised ILC2s as a protective innate immune cell type that responds to brain injury, neuroinflammation or age-related cognitive decline.<sup>32 33</sup> ILC2s are activated by IL-33 to release IL-5 and IL-13 to partially improve recovery following spinal cord injury.<sup>33</sup> Moreover, ILC2s in the aged brain can produce abundant IL-5 and IL-13 in response to IL-33, improving neurogenesis and cognitive function in that context.<sup>32</sup> Here, we identified a robust expression of IL-4 in brain-infiltrating ILC2s. Research in human tissues demonstrates that IL-4 is as a potent immune factor that mediates type 2 immunity and is a key player that suppresses excessive inflammatory tissue injury after stroke.<sup>12 35 36</sup> Our present study demonstrates the mobilisation and capacity of brain ILC2s in producing anti-inflammatory factors, especially IL-4, to exercise immune suppressive functions in the ischaemic brain. Future studies are required to reveal whether, and to what extent, other ILC2s-derived factors, such as IL-5, may also contribute to the inhibitory effects of ILC2s on brain infarction in stroke. Innate lymphoid cells, that is, ILC2s, mainly exist in the brain parenchyma and meninges of brain.<sup>32 33</sup> The diverse tissue-associated ILC2 functions have been explored in adipose, lung and meninges.<sup>32 37 38</sup> The accumulation of ILC2s in the brain suggests that these ILC2s sense brain injury. ILC2s are potent responders to IL-33<sup>17 35 36</sup>; this notion is confirmed in the context of ischaemic brain injury in the result that exogenous IL-33-caused expansion of ILC2s to suppress injury. The robust expression of IL-33 by glial cells, particularly by oligodendrocytes in the ischaemic brain, shows how these glia cells are major players in mobilising and recruiting peripheral ILC2s into the brain. Fate mapping and parabiosis approaches are warranted to illustrate the origin of ILC2s in the context of acute brain ischaemia.

The study has several limitations. First, age different neurodeficit scores and comorbid influences on stroke outcome in models we adopted have not been sufficiently examined, this slightly precludes the translation of this work. Second, due to the low frequency of ILC2 population in brain, sample sizes of several experiments should be expanded to eliminate potential bias of cell phenotyping experiments.

Third, expansion of the ILC2s by exogenous IL-33 may not completely reflect the ILC2s feature in ischaemic brain. Fourth, the application of Rag2<sup>-/-</sup> mice as recipients in the adoptive transfer experiments may have indirect influence of other cell compartments.<sup>39 40</sup> Fifth, the present work focusses on the role of ILC2s at the acute phase of stroke, long-term impact of ILC2s remains unclear. Last, to manipulation ILC2 frequency for functional studies, reagents, that is, anti-CD90.2 mAb were given prior to MCAO. These limitations prompt further research questions to thoroughly understand the impact of innate immune cell ILC2s on the evolution of brain inflammation and neurological outcomes.

In conclusion, the present study provides novel evidence that ILC2s reduces ischemic brain injury. Therefore, immune therapies targeting ILC2s may serve as a potential treatment option, or at least a conjunctive approach to current reperfusion therapies, to benefit patients with acute ischemic stroke.

**Contributors** W-NJ, F-DS and QL formulated the study concept. PZ, YX, ZC, YL, NW, XZ and BZ performed the experiments. PZ, W-NJ and ML analysed the data and interpreted the results. YX, ZC and MY assisted in the revision of the manuscript. W-NJ, PZ and QL wrote the paper. W-NJ accepts full responsibility for the work and the conduct of the study, had access to the data, and controlled the decision to publish.

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