

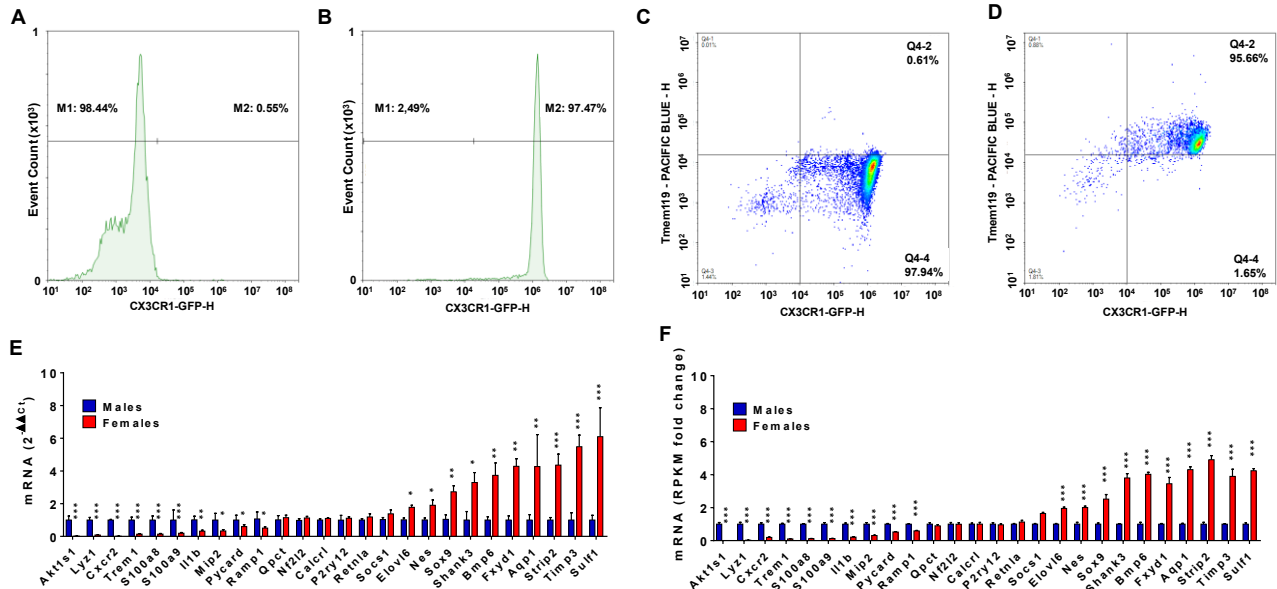
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## **Supplemental Information**

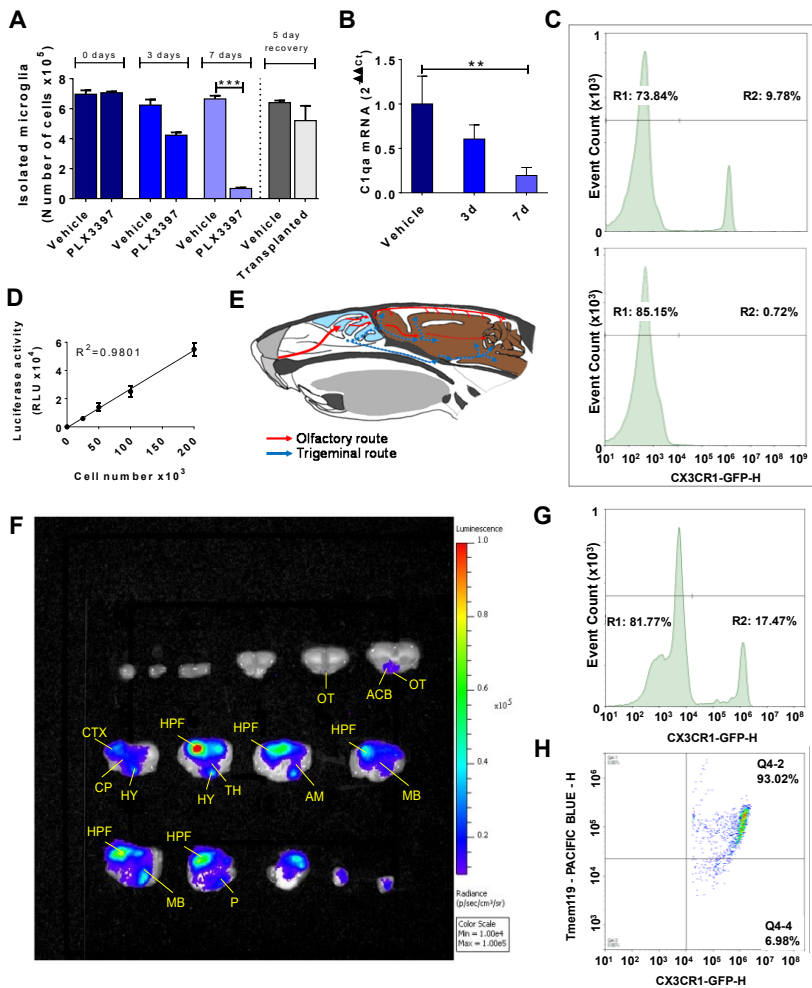
### **Sex-Specific Features of Microglia from Adult Mice**

**Alessandro Villa, Paolo Gelosa, Laura Castiglioni, Mauro Cimino, Nicoletta Rizzi, Giovanna Pepe, Federica Lolli, Elena Marcello, Luigi Sironi, Elisabetta Vegeto, and Adriana Maggi**

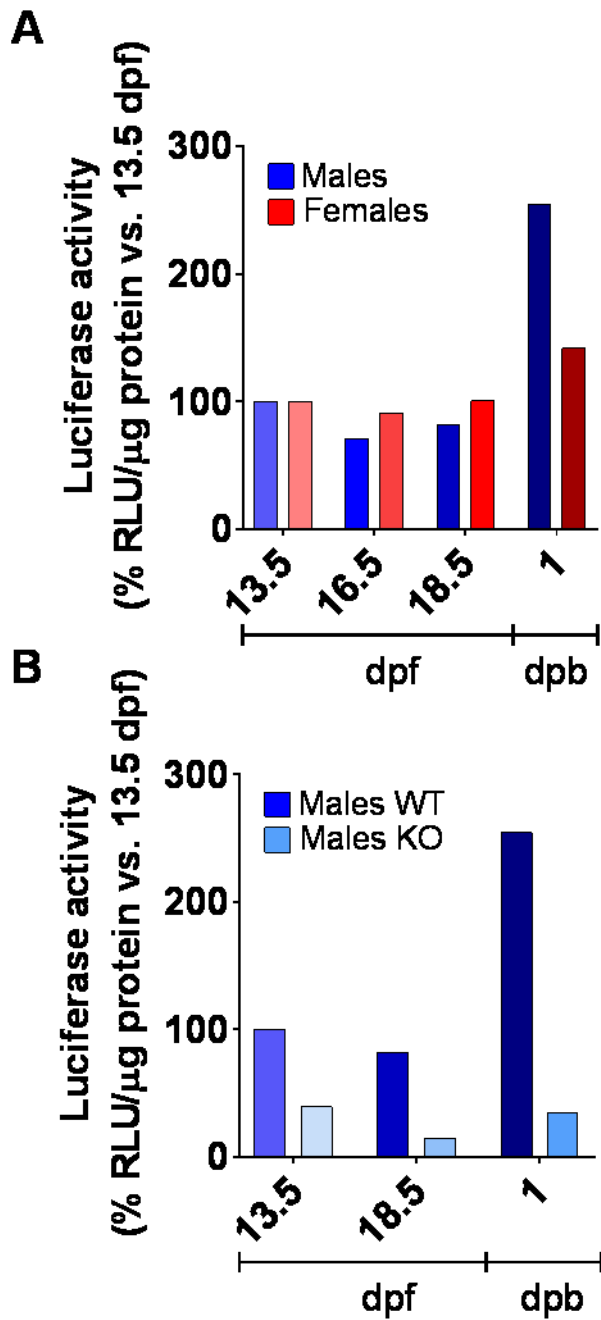
## Supplemental Figures and Legends



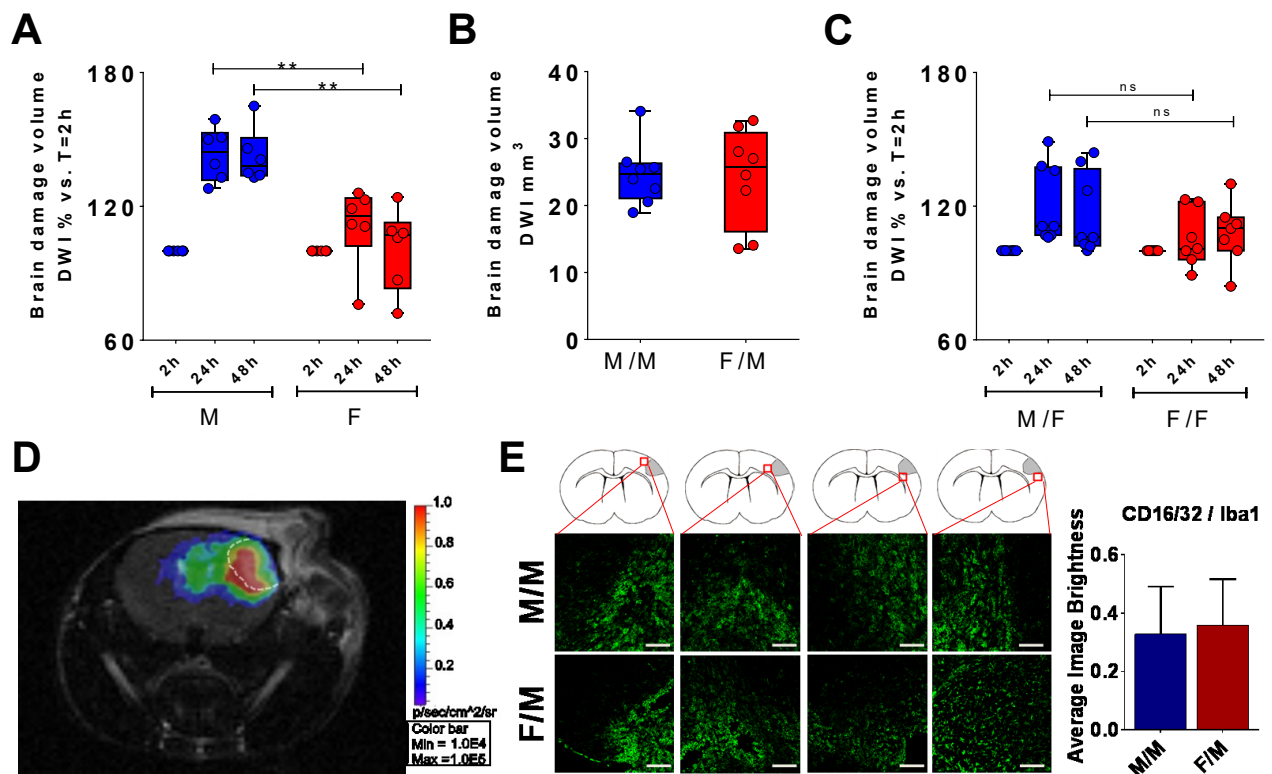
**Figure S1. Validation of the methodologies. Related to Figure 1.** Assessment of microglial purity after CD11b+ magnetic bead sorting by flow cytometry of GFP fluorescence in microglia isolated from the brain of wild type (A) or CX3CR1-GFP reporter (B) mice shows ~98% of GFP positive cells after microglia isolation. Density scatterplots of CX3CR1-GFP microglia incubated with isotype controls (C) or Tmem19-PacificBlue (D) show that GFP positive cells express the microglia specific marker Tmem19. Data are representative of three independent measures on n=3 individual animals/group. (E) qPCR validation: we selected a total of 26 genes (10 more expressed in males, 6 equally expressed and 10 more expressed in females). Data are expressed as  $2^{-\Delta\Delta C_t}$  using the 36B4 transcript as an internal reference standard. Columns represent the mean  $\pm$  s.e.m. of 6 animals per experimental group measured in triplicates. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  by one-way ANOVA and Tukey's method for multiple comparisons *versus* expression in male microglia (blue columns). The expression analyses made on the selected genes gave results superimposable with the results obtained by RNAseq (F).



**Figure S2. Female microglia retain their sex after transplantation in male brain. Related to Figure 3.** Effect on microglia of PLX3397 administration in adult C57BL/6 mice was evaluated (A) by microglia isolation and counting; (B) by qPCR analyses of C1qa gene. Data are expressed as  $2^{-\Delta\Delta C_t}$  using the 36B4 transcript as an internal reference standard. Columns represent the mean  $\pm$  s.e.m. of 3 animals measured in triplicates. \*\*,  $P < 0.01$  by two-way; \*\*\*,  $P < 0.001$  by two-way ANOVA versus expression in microglia isolated from vehicle-treated animals; (C) by flow cytometry (representative histogram of three independent measures on  $n=6$  individual animals/group) of GFP fluorescence in the brain of CX3CR1-GFP reporter mice treated for 7 days with vehicle (upper panel) or PLX3397 (lower panel). (D) Luciferase activity measured in extracts of microglia isolated from male G4063 mice and expressed as relative luciferase units (RLU). Dots represent the mean  $\pm$  s.e.m. of 3 independent measures on  $n=6$  individual animals/group. *Brain colonization by transplanted microglia.* (E) Schematic drawing of two routes of transnasal delivery of cells to the brain. After crossing the cribriform plate, the olfactory route (red arrows) divides into two branches: (1) the CSF branch and (2) the parenchymal branch. Dashed arrows represent possible hypothetical routes of cell delivery. The hypothetical trigeminal route (blue arrows) consists of at least two branches one of which crosses the cribriform plate into the parenchyma, where it diverges to the rostral and caudal parts of the brain. The second branch projects from the nasal mucosa to the trigeminal ganglion, where the exogenously applied cells are further distributed to the forebrain and caudal brain areas including the brainstem and the cerebellum. (F) Identification of brain areas mainly enriched by transplanted bioluminescent microglia, according to Allen Mouse Brain Reference Atlas classification. Bioluminescence-based optical imaging of brain slices of WT male mice 5 days after transnasal administration of 400,000 bioluminescent microglial cells isolated from female G4063 mice; pseudocolors represent the intensity of light emission (p/s/cm<sup>2</sup>/sr). Brain areas have been identified according to Allen Mouse Brain Reference Atlas classification (<http://www.brain-map.org/>); OT: Olfactory Tubercle; ACB: Nucleus Accumbens; CTX: Cortex; CP: Caudoputamen; HY: Hypothalamus; Th: Thalamus; HPF: Hippocampal Formation; AM: Anteromedial Nucleus; MB: Midbrain; P: Pons. (G) Flow cytometry analyses of GFP fluorescence in microglia isolated from the brain of mice transplanted with CX3CR1-GFP microglia show ~18% of GFP positive cells (transplanted cells) after microglia isolation (histogram is representative of  $n=6$  independent analyses). R2-gated density scatterplots of CX3CR1-GFP microglia incubated with Tmem119-PacificBlue (H) show that GFP positive cells still express the microglia specific marker Tmem119. Histogram and scatterplot are representative of three independent measures on  $n=6$  individual animals/group).



**Figure S3. Luciferase expression in ERE-Luc mice shows that perinatal activation of ER $\alpha$  is restricted to male mice. Related to Figure 3.** The ERE-Luc mouse was shown to selectively express luciferase under the control of intracellular estrogen receptors. (A) Quantitative analysis of luciferase expression in the brain of male and female ERE-Luc mice, shows that at postnatal day 1 (dpb) luciferase expression is significantly increased in the male, but not in the female brain; (B) quantitative analysis of luciferase in brain homogenates of male wild type (WT) or ER $\alpha$  KO (KO) ERE-Luc mice shows that the ER $\alpha$  is mainly responsible for the reporter expression. Each bar corresponds to relative luciferase units (RLU) representing the estrogen receptor activity vs. RLU measured at 13.5 dpf a) in male or female groups, or b) in male WT group.



**Figure S4. Protective action of female microglia in ischemia. Related to Figure 4.** Scatter plot showing the quantitative analysis of the brain damage volume determined by DWI measurements and expressed as percent change relative to the initial 2 hours' value (**B**) set to 100%, in control (**A**) and transplanted female recipient (**C**) mice. Solid lines represent mean  $\pm$  s.e.m. for  $n=6$  (**A**),  $n=8$  (**C**, M/F),  $n=7$  (**C**, F/F). \*\*,  $P < 0.01$ ; by two-way ANOVA and Sidak's method for multiple comparisons. ns= not statistically significant. (**D**) Representative image depicting the localization of transnasally-administered bioluminescent microglia 48h following pMCAO. The image was generated by the superimposition of bioluminescence imaging (in pseudocolors, representative of radiance (p/s/cm<sup>2</sup>/sr)) and T2-weighted MRI collected in the same animal at a distance of -2.30 mm from the bregma. (**E**) Immunofluorescence analysis of coronal sections of brains excised from adult mice that had undergone pMCAO, stained for CD16/32. M/M: male recipient transplanted with male microglia; F/M: male recipient transplanted with female microglia. Images are representative of  $n=3$  mice per experimental group, and report the observed fluorescence acquired in the areas indicated by red squares in the upper schematics. Scale bar: 10  $\mu$ m. Graph columns show the mean  $\pm$  s.e.m. of fluorescence brightness for CD16/32 (green channel) in Iba1 positive cells, measured in a double-blind manner in 20 fields per experimental group.

## **Supplemental Experimental Procedures**

### ***Animals and surgery***

Animals were housed in the animal care facility of the Department of Pharmacological and Biomolecular Sciences at the University of Milan. To avoid the variability related to rearing, environment, or diets affecting the metabolism and the microbiome, both well-known regulators of the brain immune system (Hooper et al., 2012) care was taken to match the mice in terms of age, and to take the same numbers of males and females from each of the litters utilized in the study. To limit any circadian influence and facilitate the analysis of the phase of the cycle, in all experiments animals were euthanized at the same hours (between 2:00-4:00 p.m.); intact females were at metestrus, a phase with low circulating estrogens. The phase of the reproductive cycle in female mice was assessed by blind analysis of vaginal smears mounted on glass microscope slides and stained with May-Grünwald-Giemsa method (MGG Quick Stain Kit; Bio-Optica, Milan, Italy) according to the manufacturer's protocol. Animal groups for the RNA sequencing experiment were given an estrogen-free diet (AIN93M, Mucedola, Settimo Milanese, Italy) 2 weeks before and throughout the experiment. Ovariectomy (ovx) or sham surgery was performed under mild anesthesia obtained by *s.c.* injection of 50µl solution of ketamine (93.6 mg/kg, Ketavet 100; Intervet, Milan, Italy) and xylazine (7.2 mg/kg, Rompun; Bayer, Milan, Italy). At definite time points, animals were euthanized by intraperitoneal (*i.p.*) injection of lethal ketamine and xylazine solution (150 and 12 mg/kg, respectively).

### ***Microglia sorting***

Adult mice were transcardially perfused with ice-cold phosphate-buffer saline (PBS), brains were dissected and washed in Hank's Balanced Salt Solution (HBSS; Life Technologies); after removing the meninges, brains from six mice were pooled as a single experimental group. Enzymatic cell dissociation was performed using Neural Tissue Dissociation Kit P (Miltenyi Biotec, Bologna, Italy), following a modified version of the protocol supplied by the manufacturer. Briefly, after

enzymatic digestion with papain, samples were dissociated mechanically, homogenized, and filtered through a 40- $\mu$ m cell strainer. After extensive washes in HBSS, myelin was removed by centrifuging the dissociated brain cells, which had previously been suspended in 10 ml of cold 0.9 M sucrose solution, at 850 g and 4°C for 10 min without braking. Floating myelin and the supernatant were discarded, and cells were processed for microglia magnetic sorting by incubating with CD11b MicroBeads (diluted 1:10 in PBS + 0.05% BSA; Miltenyi Biotec) for 15 min at 4°C; after washings, cells were suspended in 500  $\mu$ l of PBS + 0.05% BSA and applied to a magnetic column to purify CD11b<sup>+</sup> cells, namely microglia. Cell counting was performed using a TC20 Automated Cell Counter (BIORAD), according to the manufacturer's protocol. Immediately after isolation of microglia, cells were processed for subsequent analyses.

### ***PLX3397 administration***

For transnasal administration mice were placed in supine position, and a heated pad was inserted under the dorsal neck to induce a hyperextension of the head back. A total of 12  $\mu$ l PLX3397 (100  $\mu$ g) or vehicle solution (5% DMSO + 45% PEG300 + ddH<sub>2</sub>O) was given as nose drops (3 $\mu$ l/drop in each nostril corresponding to 6  $\mu$ l/administration), alternating between the left and right nostrils for two times, at intervals of 2 min. Subsequent doses were given each 12 h, for 1 week.

### ***RNA preparation***

RNA was purified using RNeasy minikit protocol (QIAGEN, Milan, Italy), according to the manufacturer's instructions, including a step with deoxyribonuclease incubation. RNA Quality Control was performed on all RNA samples with an electrophoretic run on a Bioanalyzer instrument using the RNA 6000 Nano Kit (Agilent, Santa Clara, CA). RNA Integrity Number was determined for every sample and all the samples were considered suitable for processing based on the RNA integrity (RIN > 8). RNA concentration was estimated through spectrophotometric measurement using a Nanoquant Infinite M200 instrument (Tecan, Austria). Sequencing libraries

were prepared using the TruSeq™ RNA Sample Preparation Kit (Illumina, San Diego, CA) using 1.8 µg of total RNA as input. Polyadenylated transcripts were purified using poly-T oligo-attached magnetic beads. PolyA RNA was fragmented at 94°C per 8 minutes and retrotranscribed using random hexamers. Multiple indexing adapters were ligated to the ends of the ds cDNA and the amount of DNA in the library was amplified with 10 PCR cycles. Final libraries were validated and quantified with the DNA1000 kit on the Agilent Bioanalyzer Instrument. Pooled libraries were sequenced on the Illumina Genome Analyzer II<sub>x</sub> producing an average of 13 M reads per library.

### ***Real time PCR***

One µg RNA were used for cDNA preparation using 8 U/µl of Moloney murine leukemia virus reverse transcriptase (Promega, Milan, Italy) in a final volume of 25 µl; the reaction was performed at 37°C for 1 h, and the enzyme inactivated at 75°C for 5 min. Control reactions without the addition of the reverse transcription enzyme were performed (data not shown). A 1:4 cDNA dilution was amplified using GoTaq®qPCR Master Mix technology (Promega) according to the manufacturer's protocol. The PCR was carried out in triplicate on a 96-well plate using 7900HT fast real time PCR system (Applied Biosystems, Life Technologies) with the following thermal profile: 2 min at 95°C; 40 cycles, 15 sec at 95°C, 1 min at 60°C. Data were analyzed using the  $2^{-\Delta\Delta C_t}$  method.

### ***Bioinformatic analysis***

BaseCall files were converted to FastQ files using Casava 1.8.2. Sequencing reads were aligned to the mouse genome (mm10) using TopHat v.2.0.9. Transcripts were reconstructed and quantified using Cufflinks v2.1.1 and differential expression analysis was performed using CuffDiff Trapnell, 2012)<sup>31</sup>. Cuffdiff uses the test statistics  $T = E[\log(y)]/\text{Var}[\log(y)]$ , where  $y$  is the ratio of the normalized counts between two conditions. Overrepresentation analysis (ORA) on DEG lists was performed using the Functional Annotation Tool in DAVID website (<https://david.ncifcrf.gov/>) and



Enrichr for enrichment analysis. The mouse genome was used as background list. Biological processes, molecular functions and KEGG pathways were investigated focusing on enriched terms with a Benjamini adjusted p-value less than 0.05. Transcription Factors potentially involved in the regulation of DEG were identified using the “ENCODE and ChEA Consensus TFs from ChIP-X” database. Heatmaps and hierarchical clustering have been obtained using Genesis software.

### **Primer list**

All mouse: *Xist* forward 5'- CTATCGCCCCAGGTCACATC-3'; *Xist* reverse, 5'- CCAGTGCAGAGGTTTTTGGC-3'; *Clqa* forward 5'-GACCACGGAGGCAGGGACAC-3'; *Clqa* reverse 5'-CTTCCCGTTGGGTGCTCGGC-3'; *Ki67* forward 5'-AGAGCTAACTTGCGCTGACT-3'; *Ki67* reverse 5'-GTTGTTCCCTGAGCAACTG-3'; *Cdk3* forward 5'- TGCTCGCACTTGGCTTCAAA-3'; *Cdk3* reverse 5'-GGTATGGTAGATCCCGGCTTATT-3'; *Akt1s1* forward 5'- GGACGAGCCCACTGAAACA-3'; *Akt1s1* reverse, 5'- CTGCCGTCGTCTGTGCTCT-3'; *Trem1* forward 5'- GTGTATCCAGCCCTCACAAC-3'; *Trem1* reverse 5'-GAAGCCAAGGTGAGGAGTCC-3'; *S100a9* forward 5'- AGTGTCCCTCAGTTTGTGCAGAATA-3'; *S100a9* reverse 5'-TGAGATGCCACACCCACTTT-3'; *Cxcl2* forward 5'- TGAACAAAGGCAAGGCTAACTGACC-3'; *Cxcl2* reverse 5'- ACGATCCAGGCTTCCCGGGTG-3'; *Shank3* forward 5'- GTGACCAGGAAACCCGAGG-3'; *Shank3* reverse, 5'-AACTTCTCCCCTTTTCTTCTCCG-3'; *Fxyd1* forward 5'- GGGGTCCAAAGTGCTTAGCT-3'; *Fxyd1* reverse 5'-CGCAGGGTGTGGTAATCGTA-3'; *Aqp1* forward 5'- CCTGCTGGCGATTGACTACA-3'; *Aqp1* reverse 5'-TGGTTTGAGAAGTTGCGGGT-3'; *Timp3* forward 5'- GGCCTCAATTACCGCTACCA-3'; *Timp3* reverse 5'- ATGCAGGCGTAGTGTGTTGGA-3'; *Il1β* forward 5'- TGCCACCTTTTGACAGTGATG-3'; *Il1β* reverse 5'-GCTGCGAGATTTGAAGCTGG-3'; *Ibal* forward 5'- ACCCAGCGGACAGACTGCCA-3'; *Ibal* reverse 5'-TTTCCTCCCTGCAAATCCCTGCT-3'; *36b4* forward 5'- GGCGACCTGGAAGTCCAAC-3'; *36b4* reverse 5'-

CCATCAGCACACAGCCTTC-3'. Amplification efficiencies for each target gene are comparable.

### ***Ex vivo imaging***

Mice were injected i.p. with 80 mg/kg of luciferin (Beetle Luciferin Potassium Salt; Promega, Madison, WI, USA) 15 min prior euthanasia and subjected to *ex vivo* imaging immediately after death. Brains were rapidly dissected and sectioned by means of a brain matrix (adult mouse, coronal and, 1 mm spacing; Ted Pella, Redding, CA). Imaging analysis was carried out with a CCD-camera (IVIS Lumina II Quantitative Fluorescent and Bioluminescent Imaging; PerkinElmer, Waltham, MA, USA) using 5 min exposures. Photon emission was quantified with the Living Image Software (PerkinElmer).

### ***Luciferase enzymatic assay***

Organs or cells were homogenized in Reporter Lysis Buffer (Promega, Italy) and lysates were subjected to three cycles of freezing and thawing. Proteins were separated from DNA and lysosomes by centrifugation ( $13000 \times g$  for 30min), after having measured the protein concentration of the extract by the Bradford assays, the biochemical assay of Luciferase activity was carried out with a luciferase assay buffer (470  $\mu$ M luciferine, 20 mM Tricine, 0.1 mM EDTA, 1.07 mM  $(\text{MgCO}_3)_4 \cdot \text{Mg}(\text{OH})_2 \times 5\text{H}_2\text{O}$ ; 2.67 mM  $\text{MgSO}_4 \times 7\text{H}_2\text{O}$  in  $\text{H}_2\text{O}$ , pH 7.8, with 33.3 mM DTT and 530  $\mu$ M ATP) by measuring luminescence emission with a luminometer. The relative luminescence units (RLU) determined during a measurement of 10 s time was expressed as RLU per microgram protein. As background, RLU were measured in the same organs or cells derived from WT mice. The RLU measured in WT mice were routinely subtracted from the RLU measured in the TG mice.

### ***Magnetic Resonance Imaging Analysis***

Mice were anesthetized with isoflurane (2% for induction, 1% for maintenance) in 1 L/min of O<sub>2</sub>, fixed on the holder and placed into the 6.4 cm diameter birdcage coil. A 3-orthogonal-plane, gradient echo scout acted as a geometric reference for locating the olfactory bulb. Three different DWIs were acquired using 3 orthogonal diffusion gradient directions; the reference images were identical but without diffusion gradients. Acquisition parameters were: repetition time (TR) = 1500 ms, effective echo time (TE) = 40 ms, number of averages (NA) = 8, matrix resolution = 128 x 128, slice thickness = 0.8 mm, interslice distance = 1 mm and number of contiguous slices = 10.

One week after MCAO, T2-Weighted images were obtained with a RARE sequence with the following parameters: TE = 80 ms, a TR = 3 sec, refocusing flip angle = 180°, FOV = 4.0x4.0 cm<sup>2</sup>, matrix resolution = 128x128, slice thickness = 0.8 mm, interslice distance = 1 mm and number of contiguous slices = 16. Twelve signal averages were recorded for a total scan time of 4 min and 48 sec. Lesions were identified as areas of high signal intensity.

### ***Immunocytochemistry***

Cells were fixed with 4% Paraformaldehyde (PFA)-4% sucrose in PBS solution at 4 °C and washed several times with PBS. Cells were permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature and then blocked with 5% BSA in PBS for 30 min at room temperature. Cells were then labelled with anti-Iba1 rabbit antibody (1:1000; Wako) overnight at 4 °C. Cells were washed and then incubated with goat anti-rabbit secondary antibody conjugated with Alexa Fluor 594 (1:200; Life Technologies, Italy) for 1 h at room temperature. Cells were then washed in PBS and mounted on glass slides with Fluoromount mounting medium (Sigma Aldrich).

### ***Immunofluorescence analysis***

One week after MCAO, transplanted mice were perfused with phosphate-buffered saline (PBS) and then 4% paraformaldehyde in PBS for at least 25 minutes. Brains were then removed, post-fixed 1 hour in 4% paraformaldehyde and cryoprotected in 30% sucrose solution until precipitation at 4°C.

Briefly, coronal sections of 20- $\mu\text{m}$  were incubated overnight at 4°C with the primary rabbit antibody anti-Iba1 (1:30,000; Biocare Medical., USA) in PBS plus 5% normal goat serum (Dako, Denmark) and 0.1% Triton-X 100. The signal intensity was enhanced using the High Sensitivity Tyramide Signal Amplification kit and fluorescent SA-Cyanine 5 (TSA PLUS Biotin KIT; Perkin-Elmer, Italy) and Alexa Fluor 633-conjugated streptavidin (1:2,000; Life Technologies, Italy), following the manufacturer's instructions. The sections were then incubated overnight at 4°C with the primary rabbit antibody anti-YM1 (1:100; StemCell Technologies, USA) and exposed for 2 hours at room temperature to goat anti-rabbit secondary antibody conjugated with Alexa Fluor 555 (1:600; Life Technologies, Italy). Nuclei were labeled with Hoechst 33258 (0.3  $\mu\text{g}/\text{ml}$ ; Life Technologies, Italy). For the quantitative analysis, we selected four fields of view (FOVs; 562.50  $\mu\text{m}$  x 562.50  $\mu\text{m}$ ) surrounding the ischemic lesion, as illustrated in the drawing (Fig. 4C). Images were acquired using a confocal microscope at 20x magnification (merge of 8- $\mu\text{m}$  z-stack at 2- $\mu\text{m}$  intervals) or 100x magnification (merge of 12- $\mu\text{m}$  z-stack at 0.5- $\mu\text{m}$  intervals, LSM510 META, Zeiss, Germany). Mean gray values of each channel were measured using ImageJ in a double-blind manner, in order to obtain a semi-quantitative evaluation of the expression of each marker.

### ***FACS cytometry***

Cell sorting experiments were performed on at least 500,000 cells for each sample by using MoFlo Astrios instrument (Beckman Coulter) equipped with 488, 546 and 640 nm lasers. For Tmem119 detection, cells were incubated 30 minutes at 4°C with Anti-TMEM119 rabbit antibody (1:500, Abcam), washed and incubated 30 minutes at 4°C with goat anti-rabbit secondary antibody conjugated with Pacific Blue (1:500, Life Technologies, Italy). Fluorescence pulses were detected using a band pass filter 531/26 nm for GFP, and 450/50 nm for Pacific Blue. Results were analyzed using Kaluza software (Beckman Coulter).